DEDICATION – in honour of Dr. David W. Robertson

During 2003, our close friend and colleague, Dr. David W. Robertson, passed away suddenly. David had served as the section editor of Central Nervous System section of Annual Reports in Medicinal Chemistry for 9 years (Vol. 30–38) and prior to that, for the Cardiovascular and Pulmonary Agents section for 5 years (Vol. 25–29). He made a tremendous contribution over these 14 years to this book. All who knew David could not forget his dedication, his knowledge of our industry, his voice of reason and his humour. Within the Pharmaceutical industry he was a formidable force, having been responsible for leading teams to discover medicines in many therapeutic areas. We, as colleagues and friends, miss him intensely but no more so that his family, as a devoted father to Andrew and Cassie, his children are his legacy. I would like to dedicate this volume of Annual Reports to David and his memory. David himself wrote about his core values and his dedication to the discovery of medicines "*I live in one of the golden eras of science and the pharmaceutical industry. I have chosen and been chosen for – a glorious pursuit, lofty calling, and have been honored to have at my disposal massive resources, and wonderful, intellectually gifted and highly productive colleagues. With these resources and co-workers, I recognize my awesome responsibility, and work intensely in our quest to make medicines that matter, and deliver hope to those who suffer from disease… We engineer medicines that matter, medicines that my mentors and I could only dream of when I began my career in the pharmaceutical industry. Our mission and my purpose as a leader, as a scientist and as a person, demands the very best that I can offer. We make a difference.*"

We are all incredibly honored to have had the opportunity to know David and to work with him.

Annette M. Doherty
Sandwich, UK
June 2004

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Section Editor: Annette M. Doherty, Pfizer Global Research & Development, Sandwich Laboratories, Sandwich, United Kingdom

PREFACE

Annual Reports in Medicinal Chemistry continues to focus on providing timely and critical reviews of important topics in medicinal chemistry together with an emphasis on emerging topics in the biological sciences, which are expected to provide the basis for entirely new future therapies.

Volume 39 mostly retains the familiar format of previous volumes, this year with 25 chapters. Sections I–IV are disease-oriented and generally report on specific medicinal agents with updates from Volume 38. As in past volumes, annual updates have been limited only to the most active areas of research in favor of specifically focussed and mechanistically oriented chapters, where the objective is to provide the reader with the most important new results in a particular field.

Sections V and VI continue to emphasize important topics in medicinal chemistry, biology, and drug design as well as the critical interfaces among these disciplines. Included in Section V, Topics in Biology, are chapters on the Pleiotropic effects of statins and Protein structure modeling. Chapters in Section VI, Topics in Drug Design and Discovery include drug transporters, the hit-to-lead discovery process, prodrug strategies in the design of antivirals, and pre-clinical assessment of drug-induced QT interval prolongation.

Volume 39 concludes with To Market, To Market – a chapter on NCE and NBE introductions worldwide in 2003. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in Volumes 1–39 of this series.

Volume 39 *of Annual Reports in Medicinal Chemistry* was assembled with the superb editorial assistance of Hannah Young and Lyn Hill and I would like to thank them for their hard work and enduring support. I have continued to work with innovative and enthusiastic section editors and my sincere thanks go to them again this year. I hope that you the reader will enjoy and profit from reading this volume.

Annette M. Doherty
Sandwich, UK
June 2004

Section 1
Central Nervous System Diseases

Editor: David Wustrow
Pfizer Global Research and Development
Ann Arbor
MI 48105

Attention Deficit Hyperactivity Disorder: Pathophysiology and Design of New Treatments

Shelly A. Glase and David J. Dooley

Pfizer Global Research and Development, Ann Arbor, MI 48105, USA

Contents

1. INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a relatively common psychiatric disorder in children and adolescents, occurring with a prevalence of 3–8% [1]. This disorder can also continue into and be diagnosed in adulthood with a prevalence of 2–4% [2]. The biological basis for ADHD is not well understood, yet is considered to reflect several heterogeneous variables including genetic polymorphisms, changes in neuroanatomy and neurochemistry, and environmental factors.

2. CURRENT PHARMACOLOGIC TREATMENTS

For several decades, the psychostimulants methylphenidate (**1**) and amphetamine (**2**) have been the mainstays in the treatment of ADHD, effectively controlling the core symptoms of impulsivity, hyperactivity, and inattention [3,4]. This efficacy is demonstrated as reductions in hyperarousal, impulsivity, motor restlessness, aggressiveness and antisocial behavior, and improvements in concentration, school and work performance. Compliance issues and concerns as regards multiple daily doses of these drugs to maintain effect has largely been eliminated by the introductions of longer acting formulations (e.g., Adderall XR®, Concerta®). These drugs act by blocking the reuptake of dopamine (DA) and norepinephrine (NE) and/or enhancing the release of these two neurotransmitters. Although they are often considered the ‘gold standards’ in ADHD

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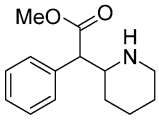
treatment, there are several issues that limit their widespread use inside and outside the US. The psychostimulants can produce side effects including insomnia and sleep disturbances, rebound phenomenon, appetite suppression and possible retardation of growth, tics, and exacerbation of comorbid disorders [2]. Moreover, these agents may not be effective or tolerated in all patients. Additionally, the psychostimulants are scheduled drugs (restricting prescribing/dispensing) often associated with negative cultural perceptions, and have the potential for abuse and diversion.



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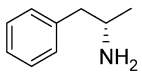

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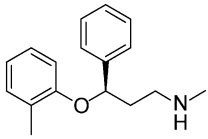

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The launch of the non-stimulant atomoxetine (Strattera®, **3**) in early 2003 for ADHD was the first instance in over 30 years of a new drug being approved for both child and adult ADHD [5]. In contrast to the psychostimulants, atomoxetine acts by selectively blocking the reuptake of NE, in a manner similar to the older tricyclic antidepressant drugs desipramine and nortryptiline. As of the first quarter of 2004, atomoxetine had garnered ~16% of the US ADHD market [6], providing physicians and psychiatrists with an alternative to the psychostimulants. Since atomoxetine is not scheduled or abused, it has significant advantages to the older agents. There are ongoing studies addressing the relative efficacy and tolerability of atomoxetine versus amphetamine and methylphenidate.

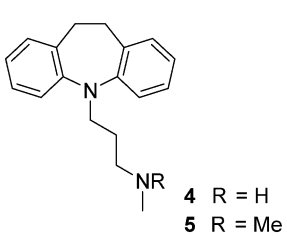
To compete successfully against the psychostimulants and atomoxetine, newer agents may need to demonstrate improvements in efficacy, safety and tolerability, and at the same time not be subject to scheduling or have the potential for abuse.

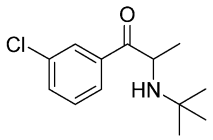
3. NEW THERAPEUTIC APPROACHES

3.1. Monoamine reuptake inhibitors

There is strong evidence that DA and NE play an important role in the pathophysiology of ADHD. The widely used psychostimulants, methylphenidate and amphetamine, and the non-stimulants desipramine (**4**), imipramine (**5**), bupropion (**6**) and atomoxetine act through the common mechanism of enhancing monoaminergic neurotransmission [7,8]. The therapeutic effects of the stimulants have been attributed to their ability to block reuptake and facilitate release (amphetamine) of DA in the striatum. The antidepressant bupropion and GW320659 (**7**, structurally similar to the active hydroxyl metabolite of bupropion) inhibit both DA and NE reuptake [9]. Bupropion has been shown to be an effective treatment for ADHD especially in the presence of co-morbid depression and bipolar disorder [10]. An initial study indicates that GW320659 may have clinically relevant efficacy in pediatric ADHD [11]. The tricyclic antidepressants (TCAs)

desipramine and imipramine, and the non-TCA atomoxetine are efficacious ADHD treatments that act through selective inhibition of NE reuptake [12,13]. Atomoxetine increases extracellular levels of NE and DA in the prefrontal cortex but does not affect DA levels in the striatum or nucleus accumbens [14,15].





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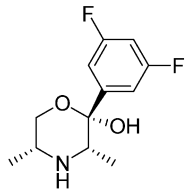

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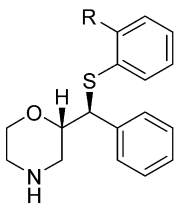
A number of selective NE reuptake inhibitors have been reported recently, these include the thio (**8**) and carbon (**9**) analogs of (S,S) reboxetine [16,17] and a series of 4-phenyl substituted tetrahydrofuro- and tetrahydropyrroloisoquinolines (**10**) which are described as having K_i ratios of ≥ 2 for the DA/NE transporters and K_i ratios of ≥ 5 for the 5-HT/NE transporters [18].



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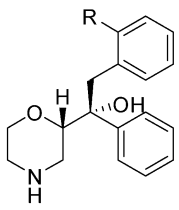

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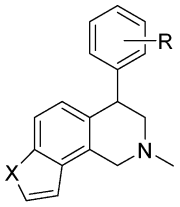

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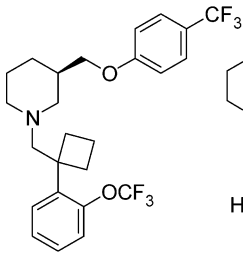
Several nonselective reuptake inhibitors have also been described. Structures **11**–**14** are piperidine analogs of desmethylsibutramine, which was shown to improve attention in healthy volunteers [19]. The NE and DA uptake IC_{50} values are < 10 nM for examples **11** and **12** and < 100 nM for example **13** [20–22]. Compound **14** inhibited both NE and DA uptake at 97% at 1 μM [23].



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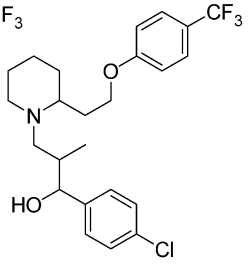

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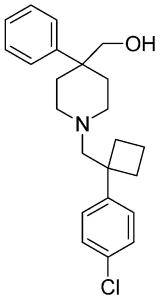

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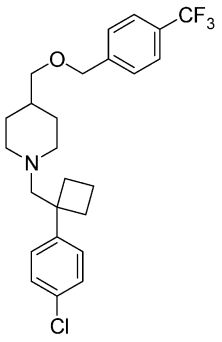

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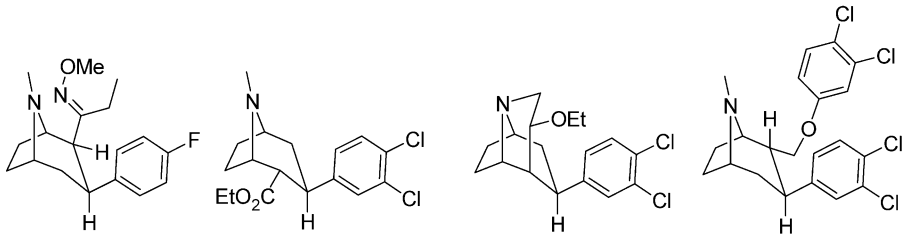
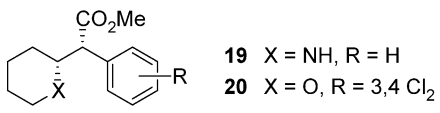
Tropane analogs (**15**–**18**) were also claimed as monoamine reuptake inhibitors [24–27]. Compound **18** had IC_{50} values of 15, 32 and 20 nM for DA, NE and 5-HT uptake, respectively [28].



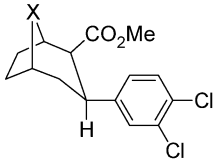
15 **16** **17** **18**

Conventional monoamine transporter ligand design has been driven by the assumption that a basic amine is necessary for the formation of an ionic bond with an aspartate residue on the DA, NE and 5-HT transporters. This notion has been challenged by the identification of several potent non-nitrogen containing DA and 5-HT transporter ligands that are highly selective against a wide range of receptors [29]. The high affinity usually attributed to the formation of an ionic bond in a nitrogen-containing compound can be compensated for by halogenation of the phenyl rings in the non-nitrogen counterparts. This suggests the existence of different binding domains on monoamine transporters, which raises the possibility of a new class of compounds with novel therapeutic profiles.

The (R,R) isomers of methylphenidate (**19**) and the oxy analog (**20**) both had IC_{50} values of 17 nM at the DA transporter. The oxy analog (**22**) is also equipotent to tropane (**21**) with IC_{50} values of 1 and 3 nM at the DA transporter and 2.5 and 6.5 nM at the 5-HT transporter, respectively. The carbon analog (**23**) is slightly weaker (DA; $IC_{50} = 11$ nM and 5-HT; $IC_{50} = 214$ nM).



19 X = NH, R = H
20 X = O, R = 3,4 Cl_2



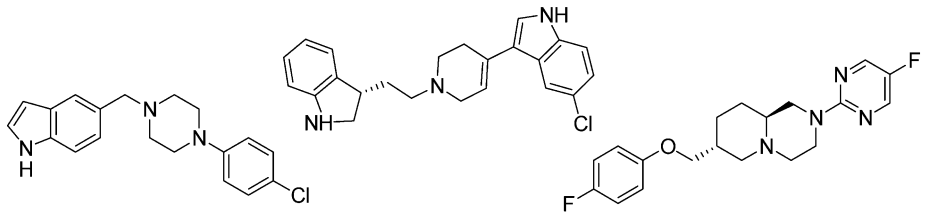
21 X = NMe
22 X = O
23 X = CH_2

3.2. Dopamine D_4 antagonists

ADHD is a highly heritable disorder, influenced by multiple genes. There is considerable documentation supporting the association between the 7-repeat allele of the dopamine D_4 (DA D_4) receptor and the personality trait of novelty seeking and ADHD, especially the inattention subtype [30–34]. It has been shown that motor hyperactivity in 6-hydroxydopamine lesioned juvenile rats can be dose-dependently inhibited by stimulants used to treat ADHD and selective antagonists for the dopamine D_4 but not D_2 receptors [35–38].

Several 3- and 5-substituted indole (**24**) and dihydroindole (**25**) dopamine D_4 ligands have been disclosed in recent patents. Compounds **24** and **25** were reported to have IC_{50}

values of 7.5 and 1.3 nM, respectively, at the dopamine $D_{4.2}$ receptors [39–43]. A series of octahydropyridopyrazine analogs (**26**) have also been described as dopamine D_4 ligands [44].

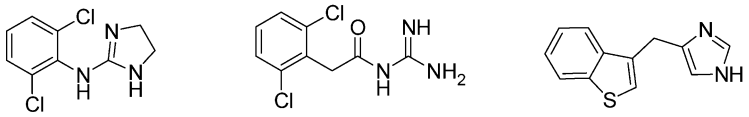


24 **25** **26**

3.3. α_2-Adrenergic agonists

The α_2-adrenergic agonists clonidine (**27**) and guanfacine (**28**) have been used in the treatment of ADHD [45,46] especially where co-morbid tic disorders can be exacerbated by stimulants [47,48]. Since there are no highly selective α_{2A}, α_{2B} or α_{2C} agonists, studies of cognitive function were carried out in α_{2A} [49] and α_{2C} [50] knock-out mice. The results demonstrated that the beneficial cognitive enhancing effects of α_2 agonists could be attributed to the α_{2A} subtype. Several genes are likely to be responsible for the heritability of ADHD. Genes within the dopaminergic system have received the most attention, but more recently the α_{2A} adrenergic receptor gene (ADRA2A) has been suggested to play a small role in the susceptibility or severity of ADHD [51].

A number of imidazobenzthiophenes and thiophenes were shown to have high affinity for the α_{2D} adrenergic receptors. The K_i value for **29** was 0.09 nM [52,53].



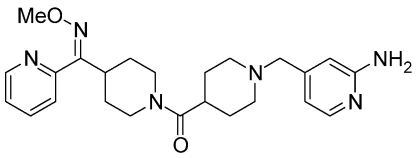
27 **28** **29**

3.4. Histamine H_3 antagonists

In addition to controlling the synthesis and release of histamine, H_3 receptors are also heteroreceptors that modulate the release of a number of other neurotransmitters that are implicated in the learning and motor disturbances in ADHD, these include DA, NE and acetylcholine [54]. H_3 antagonists have been shown to enhance attention and cognition in multiple animal models [55].

A recent thorough review summarizes advances in imidazole and non-imidazole H_3/H_4 receptor ligands [56]. The chemical structures of the non-imidazole ligands vary considerably but all contain an aliphatic tertiary amino group. Examples of these ligands are **30**, with an H_3 receptor binding $K_i = 0.83$ nM, and analogs where the pyridinyl oxime is replaced by substituted indoles and benzimidazolones [57–60].

The benzofuranylethylpyrrolidine (**31**) and the imidazolephenylmethanone (**32**) have K_i values of 0.6 and 1.3 nM, respectively, at the H^3 receptor [61,62].



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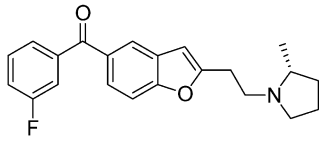

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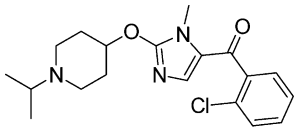

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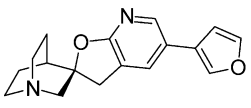
3.5. Nicotinic receptor agonists

Evidence indicates that abnormal regulation of nicotine acetylcholine receptors (nAChRs) may contribute to the inattention and cognitive impairments associated with ADHD. Presynaptic nAChRs modulate the release of NE, DA, 5-HT, acetylcholine, glutamate and γ-aminobutyric acid, neurotransmitters important to learning and memory [63]. nAChRs are comprised of a combination of 5α and β subunits which confer distinct pharmacologic properties. $\alpha_4\beta_2$ and α_7 antagonists were shown to disrupt working memory in rats [64]. In clinical trials, nicotine skin patches and the nicotine agoinist ABT-418 were shown to be effective in alleviating inattention in adults with ADHD [65,66]. The significantly higher occurrence of cigarette smoking (nearly twice the general population) in adolescents and adults with ADHD may represent a form of self-medication [67].

Most nicotinic ligands share a common structural motif that is characterized by a bicyclicamine attached to a variety of aromatic and heteroaromatic moieties. Numerous examples of 1-azabicyclo-[2.2.2]octane α_7 nAChR ligands can be found in the recent patent literature. Three representative examples are the spirofuropyridine (**33**), the octene (**34**) and heterocyclic amides such as (**35**). The K_i values are 0.033, 58, and 5 nM, respectively [68–77]. There are also many analogs of **35** with 1-azabicyclo-[2.2.1]heptane, -[3.2.1]octane, -[3.2.2]nonane and 7-azabicyclo-[2.2.1]heptane [78–89].



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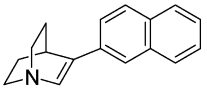

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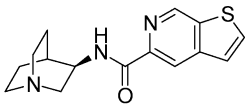

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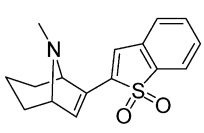
Additional examples of α_7 nAChR ligands are 8-azabicyclo-[3.2.1]octene (**36**) with an affinity of 18 nM and the 4-ester substituted and 4-keto substituted 1,4-diazabicyclo-[3.2.2.]nonanes (**37**) and (**38**) [90–93].



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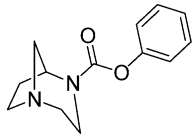

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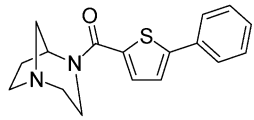

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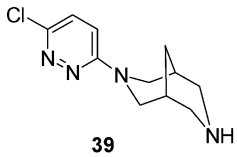
The pyridazinyl-3,7-diazabicyclo-[3.3.1]nonane (**39**) is an example of an $\alpha_4\beta_2$ nAChR ligand with an IC_{50} value of 3 nM [94]. Ureas such as (**40**) are described as positive allosteric modulators that enhance the efficacy of agonists at the α_7 nAChR by minimizing receptor desensitization [95].



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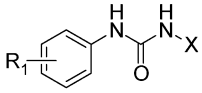

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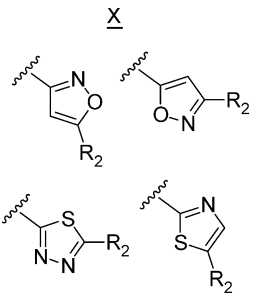

4. CONCLUSION

It is becoming apparent that for many individuals, ADHD is a lifelong impairment that affects their ability to function in academic, occupational and personal situations. Advances in understanding the biological basis of this disorder and the availability of novel compounds from a variety of mechanisms of action, offer the promise of new therapeutic options, with the opportunity to improve the number of successfully treated patients.

REFERENCES

[1] F. X. Castellanos and R. Tannock, *Nat. Rev. Neurosci.*, 2002, **3**, 617.
[2] J. K. Buitelaar, S. A. Montgomery and B. J. van Zwieten-Boot, *Eur. Neuropsychopharmacol.*, 2003, **13**, 297.
[3] D. E. Greydanus, H. D. Pratt, M. A. Sloane and M. D. Rappley, *Pediatr. Clin. N. Am.*, 2003, **50**, 1049.
[4] T. E. Wilens, *Drugs*, 2003, **63**, 2395.
[5] D. Simpson and G. L. Plosker, *Drugs*, 2004, **64**, 205.
[6] IMS Health, May 2004.

[7] S. R. Pliszka, J. T. McCracken and J. W. Maas, *J. Am. Acad. Child Adolesc. Psychiatry*, 1996, **35**, 264.
[8] S. R. Pliszka, *Stimulant Drugs and ADHD*, 2001, 332.
[9] J. L. Kelley, D. L. Musso, G. E. Boswell, F. E. Soroko and B. R. Cooper, *J. Med. Chem.*, 1996, **39**, 347.
[10] W. B. Daviss, R. J. Pan and J. M. Perel, *Expert Rev. Neurotherap.*, 2003, **3**, 9.
[11] J. DeVeaugh-Geiss, C. K. Conners, E. H. Sarkis, P. K. Winner, L. D. Ginsberg, J. M. Hemphill, A. Laurenza, C. F. Barrows, C. J. Webster, C. J. Stotka and M. Asgharnejad, *J. Am. Acad. Child Adolesc. Psychiatry*, 2002, **41**, 914.
[12] J. Biederman, D. R. Gastfriend and M. S. Jellinek, *J. Clin. Psychopharmacol.*, 1986, **6**, 359.
[13] J. L. Rapoport, P. O. Quinn, G. Bradbard, K. D. Riddle and E. Brooks, *Arch. Gen. Psychiatry*, 1974, **30**, 789.
[14] F. P. Bymaster, J. S. Katner, D. L. Nelson, S. K. Hemrick-Luecke, P. G. Threlkeld, J. H. Heiligenstein, S. M. Morin, D. R. Gehlert and K. W. Perry, *Neuropsychopharmacology*, 2002, **27**, 699.
[15] (a) S. M. Stahl, *J. Clin. Psychiatry*, 2003, **64** (1), 4; (b) S. M. Stahl, *J. Clin. Psychiatry*, 2003, **64** (2), 110; (c) S. M. Stahl, *J. Clin. Psychiatry*, 2003, **64** (3), 5.
[16] B. P. Clark, P. T. Gallagher and H. L. Haughton, *PCT Patent Appl.* WO 04018440, 2004.
[17] M. J. Cases-Thomas, H. L. Haughton, C. Lamas-Peteira, S. Ouwerkerk-Mahadevan, J. J. Masters, R. G. Simmonds, H. C. E. Rudyk and M. W. Walter, *PCT Patent Appl.* WO 04018441, 2004.
[18] J. P. Beck, A. D. Pechulis and A. E. Harms, *PCT Patent Appl.* US 2002091134, 2002.
[19] K. A. Wesnes, C. Garratt, M. Wickens, A. Gudgeon and S. Oliver, *Br. J. Clin. Pharmacol.*, 2000, **49**, 110.
[20] B. M. Aquila, T. D. Bannister, G. D. Cuny, J. R. Hauske, J. M. Holland, P. E. Persons, H. Radeke, F. Wang and L. Shao, *PCT Patent Appl.* US 2003050309, 2003.
[21] J. R. Hauske and B. M. Aquila, *PCT Patent Appl.* US 2003073681, 2003.
[22] M. Z. Hoemann, *PCT Patent Appl.* US 2003177607, 2003.
[23] H. Radeke and L. Shao, *PCT Patent Appl.* US 2003236283, 2003.
[24] P. Blundell, P. C. Meltzer and B. K. Madras, *PCT Patent Appl.* US 2003202819, 2003.
[25] A. H. Gouliaev, B. H. Dahl and D. Peters, *PCT Patent Appl.* WO 02102801, 2002.
[26] B. H. Dahl and A. H. Gouliaev, *PCT Patent Appl.* WO 02094827, 2002.
[27] F. Waetjen and A. H. Gouliaev, *PCT Patent Appl.* WO 02002559, 2002.
[28] A. H. Gouliaev and D. Peters, *PCT Patent Appl.* WO 03014114, 2003.
[29] B. K. Madras, M. A. Fahey, G. M. Miller, R. De La Garza, M. Goulet, R. D. Spealman, P. C. Meltzer, S. R. George, B. F. O'Dowd, A. A. Bonab, E. Livni and A. J. Fischman, *Eur. J. Pharmacol.*, 2003, **479**, 41.
[30] S. V. Faraone, A. E. Doyle, E. Mick and J. Biederman, *Am. J. Psychiatry*, 2001, **158**, 1052.
[31] D. L. Grady, H. C. Chi, Y. C. Ding, M. Smith, E. Wang, S. Schuck, P. Flodman, M. A. Spence, J. M. Swanson and R. K. Moyzis, *Mol. Psychiatry*, 2003, **8**, 536.
[32] J. Swanson, C. Deutsch, D. Cantwell, M. Posner, J. L. Kennedy, C. L. Barr, R. Moyzis, S. Schuck, P. Flodman, M. A. Spence and M. Wasdell, *Clin. Neurosci. Res.*, 2001, **1**, 207.
[33] J. M. Swanson, P. Flodman, J. Kennedy, M. A. Spence, R. Moyzis, S. Schuck, M. Murias, J. Moriarity, C. Barr, M. Smith and M. Posner, *Neurosci. Behav. Rev.*, 2000, **24**, 21.
[34] J. S. Mill, A. Caspi, J. McClay, K. Sugden, S. Purcell, P. Asherson, I. Craig, P. McGuffin, A. Braithwaite, R. Poulton and T. E. Moffitt, *Mol. Psychiatry*, 2002, **7**, 383.
[35] K. Zhang, F. I. Tarazi and R. J. Baldessarini, *Neuropsychopharmacology*, 2001, **25**, 624.
[36] K. Zhang, F. I. Tarazi, E. Davids and R. J. Baldessarini, *Neuropsychopharmacology*, 2002, **26**, 625.
[37] K. Zhang, E. Davids, F. I. Tarazi and R. J. Baldessarini, *Psychopharmacology*, 2002, **161**, 100.
[38] K. Zhang, R. J. Baldessarini, F. I. Tarazi and J. L. Neumeyer, *Curr. Med. Chem. – Central Nerv. Syst. Agents*, 2002, **2**, 259.
[39] B. Bang-Andersen and J. Kehler, *PCT Patent Appl.* US 20030166665, 2003.
[40] B. Bang-Andersen, K. P. Hertel and P. G. Drewes, *PCT Patent Appl.* WO 04020437 (2004).

[41] J. Kehler and B. Bang-Andersen, *PCT Patent Appl.* US 20040044007, 2004.
[42] J. Felding, B. Bang-Andersen, G. P. Smith and K. Andersen, *PCT Patent Appl.* US 20030166664, 2003.
[43] B. Bang-Andersen, J. Felding and J. Kehler, *PCT Patent Appl.* US 20030232822, 2003.
[44] S. H. Zorn, M. A. Sanner, A. F. Fliri and P. A. Seymour, *US Patent* 6 548 502, 2003.
[45] L. Scahill, P. B. Chappell, Y. S. Kim, R. T. Schultz, L. Katsovich, E. Shepherd, A. F. T. Arnsten, D. J. Cohen and J. F. Leckman, *Am. J. Psychiatry*, 2001, **158**, 1067.
[46] F. B. Taylor and J. Russo, *J. Clin. Psychopharmacol.*, 2001, **21**, 223.
[47] S. F. Law and R. J. Schachar, *J. Am. Acad. Child Adolesc. Psychiatry*, 1999, **38**, 944.
[48] K. D. Gadow, J. Sverd, J. Sprafkin, E. E. Nolan and S. Grossman, *Arch. Gen. Psychiatry*, 1999, **56**, 330.
[49] J. S. Franowicz, L. E. Kessler, C. M. Dailey Borja, B. K. Kobilka, L. E. Limbird and A. F. T. Arnsten, *J. Neurosci.*, 2002, **22**, 8771.
[50] H. Tanila, K. Mustonen, J. Sallinen, M. Scheinin and P. Riekkinen, *Eur. J. Neurosci.*, 1999, **11**, 599.
[51] T. Roman, M. Schmitz, G. V. Polanczyk, M. Eizirik, L. A. Rohde and M. H. Hutz, *Am. J. Med. Genet. (Neuropsychiatr. Genet.)*, 2003, **120B**, 116.
[52] E. W. Baxter, R. E. Boyd, J. R. Carson, M. C. Jetter and A. B. Reitz, *PCT Patent Appl.* US 20020025974, 2002.
[53] E. W. Baxter and M. C. Jetter, *US Patent* 6 426 356, 2002.
[54] E. Schilicker, B. Malinowska, M. Kathmann and M. Gothert, *Fundam. Clin. Pharmacol.*, 1994, **8**, 128.
[55] J. A. Komater, K. E. Browman, P. Curzon, A. A. Hancock, M. W. Decker and G. B. Fox, *Psychopharmacology*, 2003, **167**, 363.
[56] H. Stark, *Expert Opin. Ther. Patents*, 2003, **13**, 851.
[57] R. G. Aslanian, N. Y. Shih, P. C. Ting, M. Y. Berlin, S. B. Rosenblum, K. D. McCormick, W. C. Tom, C. W. Boyce, P. Mangiaracina, M. W. Mutahi and J. J. Piwinski, *PCT Patent Appl.* US 20030045519, 2003.
[58] R. G. Aslanian, M. Y. Berlin, P. Mangiaracina, K. D. McCormick, M. W. Mutahi and S. B. Rosenblum, *PCT Patent Appl.* US 20040019099, 2004.
[59] P. C. Ting, R. G. Aslanian, M. Y. Berlin, C. W. Boyce, J. Cao, P. Mangiaracina, K. D. McCormick, M. W. Mutahi, S. B. Rosenblum, N. Y. Shih, D. M. Solomon, W. C. Tom and Q. Zeng, *PCT Patent Appl.* US 20040048843, 2004.
[60] Q. Zeng, R. G. Aslanian, M. Y. Berlin, C. W. Boyce, J. Cao, J. Kozlowski, P. Mangiaracina, K. D. McCormick, M. W. Mutahi, S. B. Rosenblum, N. Y. Shih, D. M. Solomon and W. C. Tom, *PCT Patent Appl.* WO 03088967, 2003.
[61] M. D. Cowert, Y. L. Bennani, R. Faghia and L. A. Black, *PCT Patent Appl.* US 20020183309, 2002.
[62] M. Bogenstaetter, N. I. Carruthers, J. A. Jablonowski, T. W. Lovenberg and K. S. Ly, *PCT Patent Appl.* US 20020198237, 2002.
[63] S. Wonnacott, *TINS*, 1997, **20**, 92.
[64] E. D. Levin, A. Bradley, N. Addy and N. Sigurani, *Neuroscience*, 2002, **109**, 757.
[65] E. D. Levin and A. H. Rezvani, *Eur. J. Pharmacol.*, 2000, **393**, 141.
[66] T. E. Wilens, J. Biederman, T. J. Spencer, J. Bostic, J. Prince, M. C. Monuteaux, J. Soriano, C. Fine, A. Abrams, M. Rater and D. Polisner, *Am. J. Psychiatry*, 1999, **156**, 1931.
[67] O. F. Pomerleau, K. K. Downey, F. W. Stelson and C. S. Pomerleau, *J. Subst. Abuse*, 1995, **7**, 373.
[68] E. Phillips, *US Patent* 6 569 865, 2003.
[69] H. F. Chang, Y. Li and E. Phillips, *PCT Patent Appl.* WO 03087102, 2003.
[70] H. F. Chang, Y. Li and E. Phillips, *PCT Patent Appl.* WO 03087103, 2003.
[71] H. F. Chang and E. Phillips, *PCT Patent Appl.* WO 03087104, 2003.
[72] D. Peters, G. M. Olsen, E. O. Nielsen, P. K. Ahring and T. D. Jorgensen, *PCT Patent Appl.* WO 04016608, 2004.
[73] D. Peters, G. M. Olsen, E. O. Nielsen, P. K. Ahring and T. D. Jorgensen, *PCT Patent Appl.* US 20040044026, 2004.

[74] D. Peters, G. M. Olsen, E. O. Nielsen, P. K. Ahring and T. D. Jorgensen, *PCT Patent Appl.* WO 03101987, 2003.
[75] D. G. Wishka, S. C. Reitz, D. W. Piotrowski and V. E. Groppi, *PCT Patent Appl.* US 20030045540, 2003.
[76] D. P. Walker, D. G. Wishka, J. W. Corbett, M. R. Rauckhorst, D. W. Piotrowski and V. E. Groppi, *PCT Patent Appl.* US 20030073707, 2003.
[77] J. K. Myers, V. E. Groppi and D. W. Piotrowski, *US Patent* 6 492 386, 2002.
[78] D. P. Walker, E. J. Jacobsen, D. W. Piotrowski, B. S. Acker, D. G. Wishka, J. W. Corbett, M. R. Rauckhorst and V. E. Groppi, *PCT Patent Appl.* US 20030176702, 2003.
[79] D. P. Walker, E. J. Jacobsen, D. W. Piotrowski, D. G. Wishka, J. W. Corbett, V. E. Groppi, B. S. Acker and M. R. Rauckhorst, *PCT Patent Appl.* US 2003037896, 2003.
[80] D. W. Piotrowski, J. K. Myers, B. N. Rogers, E. J. Jacobsen, A. L. Bodnar, V. E. Groppi, D. P. Walker and B. A. Acker, *PCT Patent Appl.* US 20030207913, 2003.
[81] E. J. Jacobsen, J. K. Myers, D. P. Walker, D. G. Wishka, S. C. Piotrowski, D. A. Acker and V. E. Groppi, *PCT Patent Appl.* US 20030236270, 2003.
[82] B. N. Rogers, D. W. Piotrowski, D. P. Walker, E. J. Jacobsen, B. A. Acker, D. G. Wishka and V. E. Groppi, *PCT Patent Appl.* US 20030236264, 2003.
[83] D. P. Walker, D. W. Piotrowski, E. J. Jacobsen, B. A. Acker and V. E. Groppi, *PCT Patent Appl.* US 20030232853, 2003.
[84] D. P. Walker, D. W. Piotrowski, E. J. Jacobsen, B. A. Acker and V. E. Groppi, *PCT Patent Appl.* US 20030130305, 2003.
[85] D. W. Piotrowski, E. J. Jacobsen, B. A. Acker, D. P. Walker, D. G. Wishka and V. E. Groppi, *PCT Patent Appl.* WO 04013137, 2004.
[86] D. P. Walker, D. W. Piotrowski, E. J. Jacobsen, B. A. Acker, J. K. Myers and V. E. Groppi, *PCT Patent Appl.* US 20030236279, 2003.
[87] D. G. Wishka, J. K. Myers, B. N. Rogers, E. J. Jacobsen, D. W. Piotrowski, J. W. Corbett, A. L. Bodnar and V. E. Groppi, *US Patent* 6 562 816, 2003.
[88] D. G. Wishka, D. P. Walker, J. W. Corbett, S. C. Reitz, M. R. Rauckhorst and V. E. Groppi, *PCT Patent Appl.* US 20030105089, 2003.
[89] D. G. Wishka, J. K. Myers and V. E. Groppi, *PCT Patent Appl.* US 20030069296, 2003.
[90] D. Peters, G. M. Olsen, E. O. Nielsen, P. K. Ahring and T. D. Jorgensen, *PCT Patent Appl.* WO 03004493, 2003.
[91] C. J. O'Donnell and B. T. O'Neill, *PCT Patent Appl.* US 20020177591, 2002.
[92] G. Ernst, E. Phillips and R. Schmiesing, *PCT Patent Appl.* WO 04016616, 2004.
[93] G. Ernst, E. Phillips and R. Schmiesing, *PCT Patent Appl.* WO 04016617, 2004.
[94] D. Peters, G. M. Olsen, E. O. Nielsen, P. K. Ahring and T. D. Jorgensen, *PCT Patent Appl.* WO 02096911, 2002.
[95] D. W. Piotrowski, B. N. Rogers, W. W. McWhorter, D. P. Walker, J. W. Corbett, V. E. Groppi and D. G. Rudmann, *PCT Patent Appl.* US 20030236287, 2003.

Ionotropic GABA Receptors as Therapeutic Targets for Memory and Sleep Disorders

Mary Chebib,[1] Jane R. Hanrahan,[1] Kenneth N. Mewett,[2]
Rujee K. Duke,[1,2] and Graham A. R. Johnston[2]

[1]*Faculty of Pharmacy, The Adrien Albert Laboratory of Medicinal Chemistry, The University of Sydney, Sydney, NSW 2006, Australia*
[2]*Department of Pharmacology, The Adrien Albert Laboratory of Medicinal Chemistry, The University of Sydney, Sydney, NSW 2006, Australia*

Contents

1. INTRODUCTION

The chemical diversity of agents acting on receptors for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, is substantial and increasing. This rich diversity offers both challenges and opportunities for medicinal chemists [1]. GABA produces neuronal inhibition by acting on two major types of receptors: ionotropic receptors that are ligand-gated ion channels ($GABA_A$ and $GABA_C$ receptors) [1], and metabotropic receptors that are G-protein coupled receptors ($GABA_B$ receptors) that act via second messengers [2]. The ionotropic $GABA_A$ and $GABA_C$ receptors belong to the nicotinicoid superfamily of ligand-gated ion channels that includes nicotinic acetylcholine, strychnine-sensitive glycine and $5HT_3$ receptors [3]. Although $GABA_C$ receptors are sometimes classified as subtypes of $GABA_A$ receptors, they differ in their ability to form endogenous heteromeric and homomeric receptors respectively, and in their physiological and pharmacological properties [4].

There is also a significant diversity of ionotropic GABA receptor subtypes composed of different protein subunits. The discovery of subtype specific agents is a major challenge in the continuing development of ionotropic GABA receptor pharmacology. Leads for the discovery of new chemical entities that selectively influence ionotropic GABA receptors come from using recombinant receptors of known subunit composition and the use of genetically modified mice [5]. This has been elegantly demonstrated in

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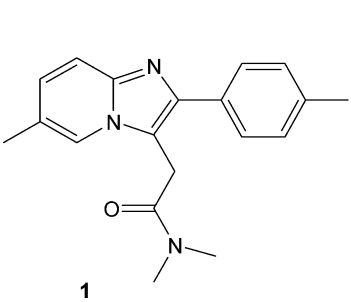
mice with mutant α1 subunits that show the normal anxiolytic responses to benzodiazepines but not the sedative effects. Mice with mutant α2 subunits show the sedative but not the anxiolytic effects of benzodiazepines. Positive allosteric modulators of $GABA_A$ receptor function that are selective for α1 subunits show non-anxiolytic sedative properties, e.g. **1** zolpidem [6]. While sufficiently selective α2 subunit agents are yet to be developed there are non-sedating anxiolytics, e.g. **2**, 7,8,9,10-tetrahydro-3-phenyl-6-(2-pyridinyl-methoxy)-7,10-ethano-1,2,4-triazolo[3,4-a]phthalazine that show selectivity for α2, α3 and α5 subunit containing receptors over α1 [7].



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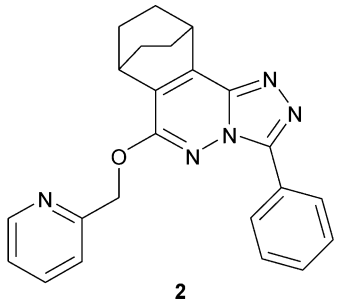

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GABA, as the major inhibitory neurotransmitter, is involved, directly or indirectly, in many disorders of brain function. The major disorders for which ionotropic GABA receptors represent important therapeutic targets include anxiety, depression, epilepsy, schizophrenia, sleep and memory disorders. Thus, therapeutic agents acting selectively on subtypes of ionotropic GABA receptors are much sought after. This review is concerned with ionotropic $GABA_A$ and $GABA_C$ receptors as therapeutic targets in particular for memory and sleep disorders. It is known from the actions of relatively non-selective agents that stimulating the function of ionotropic GABA receptors can enhance sleep while relatively non-selective agents that reduce the function of ionotropic GABA receptors can enhance aspects of memory. While classical benzodiazepines such as diazepam promote sleep by enhancing the function of $GABA_A$ receptors that contain a γ2 subunit, they do have some adverse effects on memory. The challenge is to discover more selective agents that influence only sleep and not memory, and vice versa.

There are many reviews on aspects of $GABA_A$ receptors including $GABA_A$ receptor subtypes [8–12], drug interactions [13], specific agonists and partial agonists [14], receptor recycling and regulation [15], novel modulators [16], medicinal chemistry [1,17], and analysis of $GABA_A$ receptors through mouse genetics [5]. $GABA_C$ receptors as therapeutic targets have been the subject of a recent review [18].

2. IONOTROPIC GABA RECEPTORS

Structurally, $GABA_A$ and $GABA_C$ receptors are similar to other members of the nicotinicoid superfamily of ligand-gated ion channels, consisting of five protein subunits arranged around a central pore that constitutes the actual ion channel [1]. Each subunit has a large extracellular N-terminal domain which incorporates part of the agonist/antagonist binding site, followed by three membrane spanning domains

(M1–3), an intracellular loop of variable length and a fourth membrane spanning domain (M4), with the C-terminal end being extracellular. Each subunit arranges itself such that the second membrane-spanning domain (M2) forms the wall of the channel pore and the overall charge of the domain determines whether the channel conducts anions or cations. Both $GABA_A$ and $GABA_C$ receptors are GABA-gated chloride ion channels causing inhibition of neuronal firing, with $GABA_A$ receptors being heteromeric, i.e., made up of different subunits (e.g. α1, β2 and γ2 subunits) and $GABA_C$ receptors being in general homomeric (e.g. made up exclusively of ρ1 subunits). The cytoplasmic loop, between the third and fourth transmembrane domains (M3 and M4), is believed to be the target for protein kinases, required for subcellular targeting and membrane clustering of the receptor.

2.1. Molecular composition of ionotropic receptors

There are 16 different subunits comprising the $GABA_A$ receptor family: α1–6, β1–3, γ1–3, δ, ε, and θ. In addition, there are splice variants of many of these subunits. If all of these subunits could co-assemble to form functional pentameric receptors the total number of $GABA_A$ receptors would be very large. Even if the combinations were restricted to those containing two α two β and one other subunit, then more than 2000 different $GABA_A$ receptors could exist. In fact, studies of native $GABA_A$ receptors suggest that there may be fewer than 20 widely occurring $GABA_A$ receptor subtype combinations, with the major combinations being α/β2/3γ2, α3β3γ2 and α2β3γ2 [8].

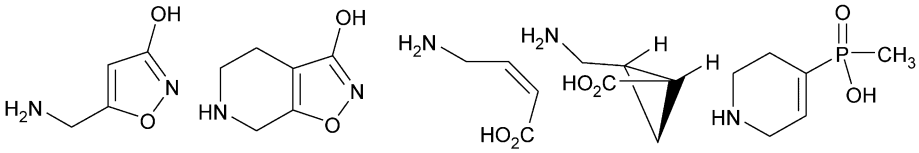
The molecular components of the $GABA_C$ receptors are the ρ-subunits. To date, two subunits (ρ1 and ρ2) have been cloned from human, while in rat three subunits (ρ1–3) have been cloned. There is a high degree of sequence homology (>92%) shared between human and rat ρ-subunits, while 60–74% sequence homology is exhibited between the various ρ-subunits [18]. The subunits form functional homomeric receptors (formed from ρ1, ρ2 or ρ3 subunits) [19] or pseudo-heteromeric receptors (formed from a combination of ρ1 and ρ2 subunits, or ρ2 and ρ3 subunits) [19,20]. Neither ρ1 or ρ2 subunits assemble with α or β subunits of the $GABA_A$ receptor and, thus, are generally not regarded as part of the $GABA_A$ receptor family [21], although coassembly of mutated $GABA_C$ ρ1 subunits with $GABA_A$ γ2S, glycine α1 and glycine α2 subunits was demonstrated *in vitro* [22] and ρ1 with γ2 subunits in white perch retina [23], suggesting that heteromeric assembly can exist.

While $GABA_A$ receptors are found throughout the central nervous system, $GABA_C$ receptors have a more restricted distribution, having been found in the retina, hippocampus, spinal cord, pituitary and gut [24–28]. Their role may include visual processing, regulation of sleep-waking rhythms, pain perception, memory, learning, regulation of hormones and neuroendocrine gastrointestinal secretion [18].

2.2. $GABA_A$ and $GABA_C$ receptor pharmacology

$GABA_A$ receptors are defined pharmacologically by their inhibition by the alkaloid bicuculline. In addition, muscimol **3** and THIP **4** (gaboxadol, 4,5,6,7-tetrahydroisoxazolo

(5,4-c)pyridin-3-ol) are widely used as selective $GABA_A$ receptor agonists [1]. However, they also have potent actions on $GABA_C$ receptors, which means that interpretation of studies with these agents should be treated with some caution [18]. No 'selective' $GABA_A$ receptor agonist is known that does not have significant action on either $GABA_B$ and/or $GABA_C$ receptors. For example, muscimol **3**, a conformationally restricted analog of GABA in which a hydroxyisoxazole moiety replaces the carboxyl group of GABA, is more potent at $GABA_C$ receptors than at $GABA_A$ receptors [29].



3 **4** **5** **6** **7**

$GABA_C$ receptors are not inhibited by bicuculline nor activated by the $GABA_B$ receptor agonist (−)-baclofen [1]. Instead, these receptors are selectively activated by CACA **5** (*cis*-4-amino-crotonic acid; (Z)-4-amino-2-butenoic acid) and (+)-CAMP **6** ((1S,2R)-2-(aminomethyl)cyclopropanecarboxylic acid) and inhibited by TPMPA **7** ((1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid) [1,30,31].

2.3. Modulators of ionotropic GABA receptors

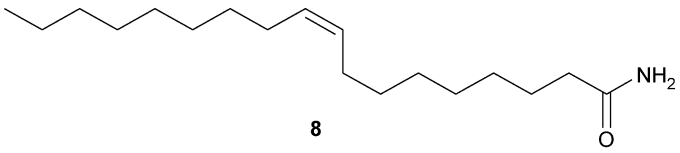
Allosteric modulators of GABA receptors are usually considered to be promising drug leads for two reasons. Firstly, the allosteric sites have greater diversity between receptor subtypes in amino acid sequence than on the GABA recognition sites (orthosteric sites). Secondly, they require the presence of the endogenous ligand to produce an effect and so are thought to be somewhat more 'gentle' drugs compared to ligands acting at the orthosteric site [32]. Benzodiazepines and barbiturates are examples of widely used therapeutic agents that act as positive allosteric modulators at $GABA_A$ receptors. Although there is a significant chemical diversity of positive allosteric modulators acting at $GABA_A$ receptors [1], allosteric modulators of $GABA_C$ receptors are relatively unknown. A number of early SAR studies of $GABA_C$ receptors reported that $GABA_A$ receptor modulators including benzodiazepine and steroids were inactive at these receptors [33,34]. However, such compounds were only tested at concentrations active at $GABA_A$ receptors, that is, at nM concentrations. More recent studies found that higher doses (μM concentrations) of certain steroids could indeed modulate this receptor [35].

3. IONOTROPIC GABA RECEPTORS AND SLEEP DISORDERS

The treatment of insomnia is regarded as a developing market for agents acting on $GABA_A$ and possibly $GABA_C$ receptors. GABA systems are known to play an important

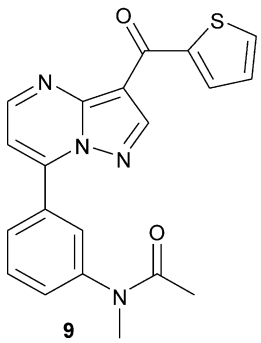
role in sleep and positive allosteric modulators of $GABA_A$ receptors are widely used to promote restful sleep [36].

The brain lipid oleamide (**8**, Z-9-octadecenamide) accumulates in the CSF of sleep-derived cats suggesting that it may be an endogenous sleep-inducing factor. Oleamide enhances the effect of GABA on rat cultured cortical neurons with an EC_{50} of 15 μM [37]. In studies on recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes, oleamide enhanced the effects of GABA only at those receptors containing a γ2 subunit that are susceptible to positive modulation by benzodiazepines. However, its enhancing action was not sensitive to the specific benzodiazepine antagonist flumazenil [37,38].



8

Oleamide is inactive in β3 knockout mice and a mutation in β3 $GABA_A$ receptor subunits has been described in a patient with chronic insomnia [39]. Oleamide and related compounds are being intensively investigated for use in sleep therapy [40]. Indiplon **9** acts in a similarly selective manner to oleamide and is in clinical trials for the treatment of insomnia [41,42].



9

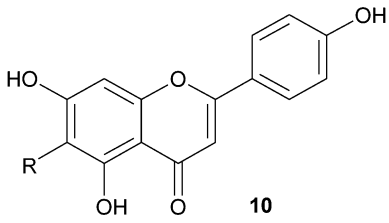
Also in clinical trials for the treatment of sleep disorders is THIP **4**, a directly acting $GABA_A$ receptor partial agonist that interacts with a $GABA_A$ receptor population that is insensitive to benzodiazepines, zolpidem, zaleplon and indiplon [43]. THIP is a moderately potent $GABA_C$ receptor antagonist [29]. Unlike GABA, THIP passes the blood–brain barrier on systemic administration and is a potent analgesic [44]. The side effects of THIP (including sedation, dizziness, and blurred vision) meant that it had too low a therapeutic index to be therapeutically useful as an analgesic [45,46] but it is being investigated for sleep therapy. Interestingly, THIP-induced analgesia is not sensitive to bicuculline suggesting that $GABA_A$ receptors are not involved [47]. Instead, the $GABA_C$ receptor antagonist action of THIP may contribute to its analgesic action [18] as the analgesic action of THIP in rats is blocked by subconvulsant doses of picrotoxinin [48], a potent $GABA_C$ receptor antagonist in addition to its well known action as a $GABA_A$ receptor antagonist [18].

THIP produces slow wave sleep and reduces spindling activity in non-rapid eye movement sleep in humans [49]. It does appear that receptors other than the classical benzodiazepine-sensitive, bicuculline-sensitive $GABA_A$ receptors are involved in the effects of THIP on both pain perception and sleep. Benzodiazepine-sensitive $GABA_A$ receptors do not appear to be involved in the effects of THIP on sleep patterns [49]. The binding of THIP to rat brain membranes, unlike that of GABA and muscimol, is not stimulated by diazepam [50]. THIP was devoid of the anticonvulsant and

antiepileptogenic effects shown by diazepam and alphaxalone in pentamethylenetetrazole-kindled mice [51].

Clinical studies with THIP have indicated that sleep quality improving effects are obtained at plasma concentrations of the order of 1 μM [49]. THIP shows considerable variation in potency on recombinant receptors: THIP acts on α1β3γ2S recombinant $GABA_A$ receptors expressed in oocytes as a partial agonist (EC_{50} 350 μM) and more potently and as a full agonist on α5β3γ3 (EC_{50} 40 μM) and α5β3γ3 (EC_{50} 29 μM) recombinant receptors [52]. On α4β3γ2 recombinant receptors THIP acts as a partial agonist (EC_{50} 102 μM) and on α4β3δ as a 'superagonist' (EC_{50} 6 μM) [53]. On recombinant $GABA_C$ receptors THIP acts as an antagonist (Kb 32 μM for ρ1 [29] and 10 μM for ρ3 receptors [54]). On this basis, α4β3δ $GABA_A$ and ρ3 $GABA_C$ receptors are the most likely GABA receptors to respond to clinically relevant 1 μM plasma concentrations of THIP. In addition, the $GABA_C$ receptor antagonist TPMPA **7** has been used to probe the involvement of $GABA_C$ receptors in sleep-waking behavior in rats on intraventricular administration [55]. TPMPA enhanced both active and quiet wakefulness and decreased total slow wave sleep and paradoxical sleep. $GABA_C$ receptors are also involved in sleep-waking regulation. It was concluded that since the sensitivity of $GABA_C$ receptors to GABA is much higher than that of $GABA_A$ and $GABA_B$ receptors, $GABA_C$ receptors modulators could be potential medications acting at low doses with fewer side effects [55].

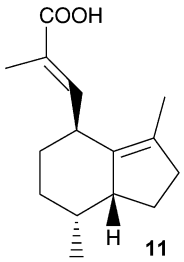
Many herbal preparations are used to promote sleep. Their active ingredients include flavonoids and terpenoids known to modulate $GABA_A$ receptor function. For example, chamomile tea contains the flavonoid apigenin, **10** R=H. Apigenin is known to have sedative effects in rats [56]. The effects of apigenin on $GABA_A$ receptors are complex and involve both flumazenil-sensitive and flumazenil-insensitive components. Apigenin has been shown to inhibit the activation of recombinant α1β1γ2S $GABA_A$ receptors in a flumazenil-insensitive manner and to have a similar effect on ρ1 $GABA_C$ receptors [57]. Other studies on recombinant α1β2γ2L $GABA_A$ receptors describe an inhibitory effect of apigenin on GABA responses and, in addition, describe an enhancement of the diazepam-induced positive allosteric modulation of GABA responses by apigenin [58]. Such a second order modulation by apigenin of benzodiazepine modulation of the activation by GABA of $GABA_A$ receptors may indicate that apigenin needs to work through an endogenous benzodiazepine system to produce sedation in a flumazenil-sensitive manner. 6-Methylapigenin, **10** R=CH_3, has been isolated from *Valeriana wallichii*, a known sedative herb, and may be a more potent positive modulator of $GABA_A$ receptors than apigenin [59].



10

Extracts of valerian (*Valeriana officinalis*) are widely used to reduce the latency of sleep onset, the depth of sleep and the perception of well-being. These extracts contain a variety of agents, including the sesquiterpenoid valerinic acid **11**, that act on $GABA_A$ receptors [60]. The monoterpenoid (+)-borneol **12**, found in high concentrations in extracts of valerian, is a flumazenil-insensitive positive allosteric modulator of recombinant $GABA_A$ receptors of low affinity but very high efficacy

producing a 12-fold enhancement of the action of 10 μM GABA at a concentration of 450 μM [58,61].



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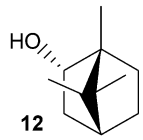
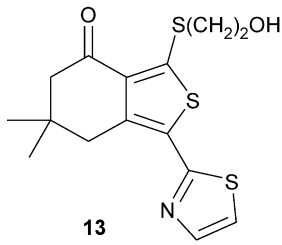


12

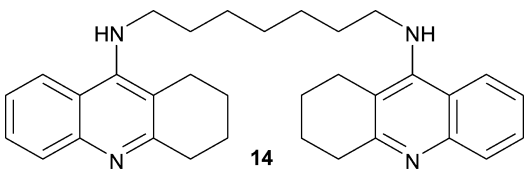
4. IONOTROPIC GABA RECEPTORS AND MEMORY DISORDERS

$GABA_A$ receptor α5-subunits are likely to be involved in aspects of memory. Less than 5% of $GABA_A$ receptors in the brain are thought to contain α5-subunits. They are localized mainly to the hippocampus where they may play a key role in cognitive processes by controlling a component of synaptic transmission in the CA1. Mice lacking the α5 gene show improved performance in the Morris water maze model of spatial learning, whereas the performance in non-hippocampal-dependent learning and in anxiety tasks were unaltered in comparison with wild-type controls [62]. Novel selective α5 negative allosteric modulators, e.g. 6,6-dimethyl-3-(2-hydroxyethyl)thio-1-(thiazol-2-yl)-6,7-dihydro-2-benzothiophen-4(5*H*)-one **13**, that enhance spatial learning but lack the convulsant or proconvulsant activity associated with non-selective $GABA_A$ receptor negative allosteric modulators have been developed [63].



13

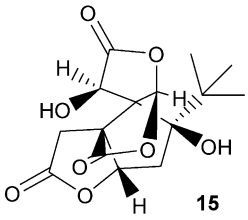
bis(7)-Tacrine (**14**, *N*,*N*′-bis(1,2,3,4-tetrahydro-9-acridinyl)-1,7-heptanediamine) is a potential Alzheimer's disease drug on the basis of its superior acetylcholinesterase inhibition and memory-enhancing potency relative to tacrine. It is a potent competitive $GABA_A$ receptor antagonist (IC_{50} 6 μM), some 18 times more potent than tacrine on these receptors, suggesting that its $GABA_A$ receptor antagonist activity may contribute to its memory-enhancing properties [64].



14

The sesquiterpene trilactone bilobalide **15** is one of the active constituents of the 50:1 *Ginkgo biloba* leaf extract widely used to enhance memory and learning. Bilobalide was

found to antagonize the direct action of GABA on recombinant α1β2γ2L $GABA_A$ receptors expressed in *Xenopus* oocytes [65,66]. Bilobalide is unusual in being a $GABA_A$ receptor antagonist that lacks overt convulsant properties; indeed it is an anticonvulsant [67,68]. As with **13**, the α5 subunit preferring negative allosteric modulator, the lack of convulsant action in an agent that reduces GABA action may be important for enhancement of memory and learning. The lack of convulsant action of bilobalide may result from subunit selectivity, but this has yet to be established. The structurally related ginkgolides act similarly to bilobalide as negative allosteric modulators of $GABA_A$ receptor function [69]. The biflavone amentoflavone **16** is negative allosteric modulator of $GABA_A$ receptor function [70]; it is found in *Ginkgo biloba* but is removed from the extracts used to enhance memory and learning.



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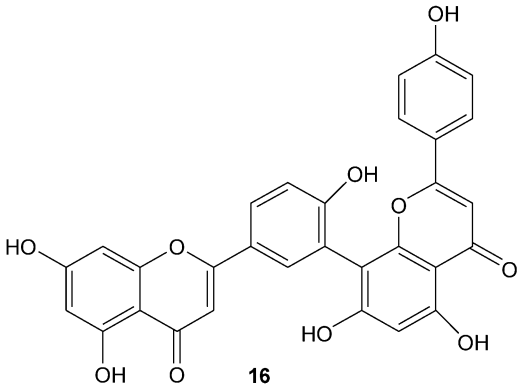
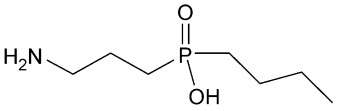


16

$GABA_C$ receptors have also been associated with learning and memory. (3-Aminopropyl)butylphosphinic acid (**17**, CGP36742), a moderately potent antagonist at the $GABA_B$ receptor ($IC_{50} = 38\ \mu M$), was found to also antagonize $GABA_C$ receptors. CGP36742 had memory-enhancing properties [71] and it was proposed that the memory enhancing effects of CGP36742 result from activity at $GABA_C$ receptors. The hypothesis that $GABA_C$ receptors enhanced memory was confirmed by the fact that TPMPA **7** was able to improve memory in rats and chicks [72]. (±)-*cis*-(3-Aminocyclopentyl)butylphosphonic acid **18**, a $GABA_C$ receptor antagonist that lacks $GABA_B$ receptor activity, has been patented as a memory enhancing agent [73].



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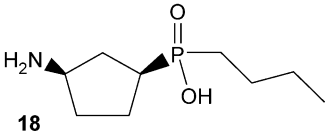
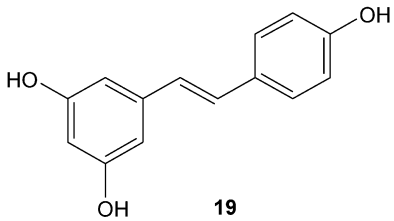


18

Resveratrol **19** is a moderately potent $GABA_C$ receptor non-competitive antagonist (IC_{50} 72 μM) [58]. It is an important constituent of red wine and has been patented for the treatment of mild cognitive disorders [74]. These studies provide some evidence that $GABA_C$ receptors play a role in memory.



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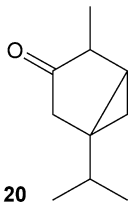

20

Preparations of sage have been used in herbal medicine to assist memory [75] and an extract of *Salvia lavandulaefolia* (Spanish sage) has been shown to enhance memory in healthy young volunteers [76]. Hispidulin, 6-methoxyapigenin **10** R=OCH_3, isolated from sage, has been shown to be a potent positive allosteric modulator of $GABA_A$ receptors [77]. Sage extracts are known to contain a variety of flavonoids (including apigenin, hispidulin and linarin) and terpenoids (including galdosol, miltirone, carnosic acid and carbosol) that are known to enhance the function of ionotropic GABA receptors. Possibly the most interesting terpenoid in extracts of sage is α-thujone **20** a known $GABA_A$ receptor antagonist [78]. The levels of α-thujone in individual sage plants are known to vary considerably [75]. The antagonistic effects of α-thujone on $GABA_A$ receptors may be an important component of the memory enhancing properties of sage extracts, while the constituents that enhance the functioning of $GABA_A$ receptors, such as hispidulin, are more likely to be involved in sedative actions [79].

5. CONCLUSION

Agents acting on $GABA_A$ or $GABA_C$ receptors have widespread therapeutic potential including therapy of sleep and memory disorders. The discovery of selective ionotropic GABA receptor agents as modulators acting on the allosteric sites or as agonists, partial agonists or antagonists acting on the orthosteric, site is a major challenge in the continuing development of therapeutic agents acting on specific subtypes of $GABA_A$ or $GABA_C$ receptors.

REFERENCES

[1] M. Chebib and G. A. R. Johnston, *J. Med. Chem.*, 2000, **43**, 1427.
[2] C. M. Vacher and B. Bettler, *Curr. Drug Targ. CNS Neurol. Disord.*, 2003, **2**, 248.
[3] N. Le Novere and J. P. Changeux, *Philos. Trans. R. Soc. London, Ser. B: Biol. Sci.*, 2001, **356**, 1121.
[4] J. Bormann, *Trends Pharmacol. Sci.*, 2000, **21**, 16.
[5] U. Rudolph and H. Möhler, *Ann. Rev. Pharmacol. Toxicol.*, 2004, **44**, 475.
[6] F. Crestani, J. R. Martin, H. Möhler and U. Rudolph, *Br. J. Pharmacol.*, 2000, **131**, 1251.
[7] R. W. Carling, K. W. Moore, L. J. Street, D. Wild, C. Isted, P. D. Leeson, S. Thomas, D. O'Connor, R. M. McKernan, K. Quirk, S. M. Cook, J. R. Atack, K. A. Wafford, S. A. Thompson, G. R. Dawson, P. Ferris and J. L. Castro, *J. Med. Chem.*, 2004, **47**, 1807.
[8] P. J. Whiting, *Drug Discovery Today*, 2003, **8**, 445.
[9] T. W. Rosahl, *Curr. Drug Targ. CNS Neurol. Disord.*, 2003, **2**, 207.
[10] H. Möhler, J. M. Fritschy and U. Rudolph, *J. Pharmacol. Exp. Ther.*, 2002, **300**, 2.

[11] U. Rudolph, F. Crestani and H. Möhler, *Trends Pharmacol. Sci.*, 2001, **22**, 188.
[12] W. Sieghart, *Trends Pharmacol. Sci.*, 2000, **21**, 411.
[13] E. R. Korpi, G. Grunder and H. Luddens, *Prog. Neurobiol.*, 2002, **67**, 113.
[14] P. Krogsgaard-Larsen, B. Frølund and T. Liljefors, *Chem. Record*, 2002, **2**, 419.
[15] M. Kneussel, *Brain Res. Brain Res. Rev.*, 2002, **39**, 74.
[16] E. Sigel and R. H. Dodd, *Drugs Future*, 2001, **26**, 1191.
[17] B. Frølund, B. Ebert, U. Kristiansen, T. Liljefors and P. Krogsgaard-Larsen, *Curr. Top. Med. Chem.*, 2002, **2**, 817.
[18] G. A. R. Johnston, M. Chebib, J. R. Hanrahan and K. N. Mewett, *Curr. Drug Targ. CNS Neurol. Disord.*, 2003, **2**, 260.
[19] R. Enz, *Biol. Chem.*, 2001, **382**, 1111.
[20] T. Ogurusu, K. Yanagi, M. Watanabe, M. Fukaya and R. Shingai, *Receptors Channels*, 1999, **6**, 463.
[21] A. S. Hackam, T. L. Wang, W. B. Guggino and G. R. Cutting, *J. Neurochem.*, 1998, **70**, 40.
[22] Z. H. Pan, D. X. Zhang, X. H. Zhang and S. A. Lipton, *Eur. J. Neurosci.*, 2000, **12**, 3137.
[23] H. H. Qian and Y. Pan, *Mol. Brain Res.*, 2002, **103**, 62.
[24] R. Enz, J. H. Brandstatter, E. Hartveit, H. Wassle and J. Bormann, *Eur. J. Neurosci.*, 1995, **7**, 1495.
[25] A. Rozzo, M. Armellin, J. Franzot, C. Chiaruttini, A. Nistri and E. Tongiorgi, *Eur. J. Neurosci.*, 2002, **15**, 1747.
[26] W. Zheng, W. Xie, J. Zhang, J. A. Strong, L. Wang, L. Yu, M. Xu and L. Lu, *J. Biol. Chem.*, 2003, **278**, 48321.
[27] E. Boue-Grabot, A. Taupignon, G. Tramu and M. Garret, *Endocrinology*, 2000, **141**, 1627.
[28] A. Jansen, M. Hoepfner, K. H. Herzig, E. O. Riecken and H. Scherubl, *Pflugers Archiv. Eur. J. Physiol.*, 2000, **441**, 294.
[29] R. M. Woodward, L. Polenzani and R. Miledi, *Mol. Pharmacol.*, 1993, **43**, 609.
[30] R. K. Duke, M. Chebib, V. J. Balcar, R. D. Allan, K. N. Mewett and G. A. R. Johnston, *J. Neurochem.*, 2000, **75**, 2602.
[31] D. Ragozzino, R. M. Woodward, Y. Murata, F. Eusebi, L. E. Overman and R. Miledi, *Mol. Pharmacol.*, 1996, **50**, 1024.
[32] A. Christopoulos, *Nature Rev. Drug Disc.*, 2002, **1**, 198.
[33] T. Kusama, C. E. Spivak, P. Whiting, V. L. Dawson, J. C. Schaeffer and G. R. Uhl, *Br. J. Pharmacol.*, 1993, **109**, 200.
[34] R. Shingai, K. Yanagi, T. Fukushima, K. Sakata and T. Ogurusu, *Neurosci. Res.*, 1996, **26**, 387.
[35] K. Morris, C. N. Moorefield and J. Amin, *Mol. Pharmacol.*, 1999, **56**, 752.
[36] C. Gottesmann, *Neuroscience*, 2002, **111**, 231.
[37] G. Lees, M. D. Edwards, A. A. Hassoni, C. R. Ganellin and D. Galanakis, *Br. J. Pharmacol.*, 1998, **124**, 873.
[38] C. S. Yost, A. J. Hampson, D. Leonoudakis, D. D. Koblin, L. M. Bornheim and A. T. Gray, *Anesth. Anal.*, 1998, **86**, 1294.
[39] A. Buhr, M. T. Bianchi, R. Baur, P. Courtet, V. Pignay, J. P. Boulenger, S. Gallati, D. J. Hinkle, R. L. Macdonald and E. Sigel, *Hum. Genet.*, 2002, **111**, 154.
[40] A. D. Laposky, G. E. Homanics, A. Basile and W. B. Mendelson, *NeuroReport*, 2001, **12**, 4143.
[41] A. J. Smith, L. Alder, J. Silk, C. Adkins, A. E. Fletcher, T. Scales, J. Kerby, G. Marshall, K. A. Wafford, R. M. McKernan and J. R. Atack, *Mol. Pharmacol.*, 2001, **59**, 1108.
[42] E. Sanna, F. Busonero, G. Talani, M. Carta, F. Massa, M. Peis, E. Maciocco and G. Biggio, *Eur. J. Pharmacol.*, 2002, **451**, 103.
[43] S. Storustovu and B. Ebert, *Eur. J. Pharmacol.*, 2003, **467**, 49.
[44] P. Krogsgaard-Larsen, B. Frølund, U. Kristiansen, K. Frydenvang and B. Ebert, *Eur. J. Pharm. Sci.*, 1997, **5**, 355.
[45] M. Kjaer and H. Neilson, *Br. J. Pharmacol.*, 1983, **16**, 477.
[46] J. L. Vaught, K. Pelley, L. G. Costa, P. Setler and S. J. A. Enna, *Neuropharmacology*, 1985, **24**, 211.
[47] S. H. Zorn and S. J. Enna, *Neuropharmacology*, 1987, **26**, 433.
[48] M. Tatsuo, C. M. Yokoro, J. V. Salgado, S. Pesquero, M. Santana and J. N. Francischi, *Braz. J. Med. Biol. Res.*, 1997, **30**, 251.

[49] J. Faulhaber, A. Steiger and M. Lancel, *Psychopharmacology*, 1997, **130**, 285.
[50] J. H. Skerritt and G. A. R. Johnston, *Neurosci. Lett.*, 1983, **38**, 315.
[51] S. L. Hansen, B. B. Sperling and C. Sanchez, *Prog. Neuropsychopharm. Biol Psych.*, 2004, **28**, 105.
[52] B. Ebert, P. J. Whiting, P. Krogsgaard-Larsen and J. A. Kemp, *Mol. Pharmacol.*, 1994, **46**, 957.
[53] N. Brown, J. Kerby, T. P. Bonnert, P. J. Whiting and K. A. Wafford, *Br. J. Pharmacol.*, 2002, **136**, 965.
[54] J. Vien, R. K. Duke, K. N. Mewett, G. A. R. Johnston, R. Shingai and M. Chebib, *Br. J. Pharmacol.*, 2002, **135**, 883.
[55] C. Arnaud, P. Gauthier and C. Gottesmann, *Psychopharmacology*, 2001, **154**, 415.
[56] P. Zanoli, R. Avallone and M. Baraldi, *Fitoterapia*, 2000, **71**, S117.
[57] J. D. Goutman, M. D. Waxemberg, F. Donate-Oliver, P. E. Pomata and D. J. Calvo, *Eur. J. Pharmacol.*, 2003, **461**, 79.
[58] G. A. R. Johnston, *J. Proc. R. Soc. NSW*, 2003, **135**, 57.
[59] C. Wasowski, M. Marder, H. Viola, J. H. Medina and A. C. Paladini, *Planta Med.*, 2002, **68**, 934.
[60] C. S. Yuan, S. Mehendale, Y. Xiao, H. H. Aung, J. T. Xie and M. K. Ang-Lee, *Anesth. Anal.*, 2004, **98**, 353.
[61] R. E. Granger, E. L. Campbell and G. A. R. Johnston, *J. Neurochem.*, 2004, **88** (Suppl. 1), 99.
[62] N. Collinson, F. M. Kuenzi, W. Jarolimek, K. A. Maubach, R. Cothliff, C. Sur, A. Smith, F. M. Otu, O. Howell, J. R. Atack, R. M. McKernan, G. R. Seabrook, G. R. Dawson, P. J. Whiting and T. W. Rosahl, *J. Neurosci.*, 2002, **22**, 5572.
[63] M. S. Chambers, J. R. Atack, H. B. Broughton, N. Collinson, S. Cook, G. R. Dawson, S. C. Hobbs, G. Marshall, K. A. Maubach, G. V. Pillai, A. J. Reeve and A. M. MacLeod, *J. Med. Chem.*, 2003, **46**, 2227.
[64] C. Y. Li, H. Wang, H. Xue, P. R. Carlier, K. M. Hui, Y. P. Pang, Z. W. Li and Y. F. Han, *NeuroReport*, 1999, **10**, 795.
[65] S. H. Huang, R. K. Duke, M. Chebib, K. Sasaki, K. Wada and G. A. R. Johnston, *Eur. J. Pharmacol.*, 2003, **464**, 1.
[66] L. Ivic, T. T. Sands, N. Fishkin, K. Nakanishi, A. R. Kriegstein and K. Stromgaard, *J. Biol. Chem.*, 2003, **278**, 49279.
[67] K. Sasaki, I. Oota, K. Wada, K. Inomata, H. Ohshika and M. Haga, *Comp. Biochem. Physiol.*, 1999, **124**, 315.
[68] K. Sasaki, S. Hatta, M. Haga and H. Ohshika, *Eur. J. Pharmacol.*, 1999, **367**, 165.
[69] S. H. Huang, R. K. Duke, M. Chebib, K. Sasaki, K. Wada and G. A. R. Johnston, *Eur. J. Pharmacol.*, 2004, in press.
[70] J. R. Hanrahan, M. Chebib, N. M. Davucheron, B. J. Hall and G. A. R. Johnston, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2281.
[71] W. Froestl, S. J. Mickel, G. Von Sprecher, P. J. Diel, B. J. Hall, L. Maier, D. Strub, V. Melillo, P. A. Baumann, R. Bernasconi, C. Gentsch, K. Hauser, J. Jaekel, G. Karlsson, K. Klebs, L. Maitre, C. Marescaux, M. F. Pozza, M. Schmutz, M. W. Steinmann, H. Van Riezen, A. Vassout, C. Monadori, H. R. Olpe, P. C. Waldmeier and H. Bittiger, *J. Med. Chem.*, 1995, **38**, 3313.
[72] G. A. R. Johnston, P.M. Burden, M. Chebib and K.N. Mewett, *WO Patent* 98/58939, 1998.
[73] M. Chebib, G.A.R. Johnston and J.R. Hanrahan, *WO Patent* 03/045897-A1, 2003.
[74] R. K. Wurtman and R.K.K. Lee, *WO Patent* 02/28141 A2, 2002.
[75] N. B. Perry, R. E. Anderson, N. J. Brennan, M. H. Douglas, A. J. Heaney, J. A. McGimpsey and B. M. Smallfield, *J. Agric. Food Chem.*, 1999, **47**, 2048.
[76] N. S. Perry, M.-J. Howes, P. Houghton and E. Perry, *Med. Arom. Plants Ind. Profiles*, 2000, **14**, 207.
[77] D. Kavvadias, P. Sand, K. A. Youdim, M. Z. Qaiser, C. Rice-Evans, R. Baur, E. Sigel, W.-D. Rausch, P. Riederer and P. Schreier, *Br. J. Pharmacol.*, 2004, **142**, 811.
[78] K. M. Hold, N. S. Sirisoma, T. Ikeda, T. Narahashi and J. E. Casida, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 3826.
[79] G. A. R. Johnston and P. M. Beart, *Br. J. Pharmacol.*, 2004, **142**, 809.

Melatonin Agonists for the Treatment of Sleep Disorders and Major Depression

Laura R. Fitzgerald and Jessica E. Reed

Pfizer Global Research and Development, Ann Arbor, MI 48105, USA

Contents

1. INTRODUCTION

Melatonin is widely used as an over-the-counter therapy for the treatment of sleep disorders. The discovery of novel potent melatonin agonists has led to the clinical development of a number of melatonin agonists for the treatment of sleep, major depression and anxiety disorders.

In this report, we will review recently published literature detailing some of the current understanding of the biology of melatonin receptors, the use of melatonin and detail some of potential new medicines being explored.

2. BIOLOGY OF MELATONIN

The hormone melatonin **1** (*N*-acetyl-5-methoxytryptamine) is produced and secreted from the pinealocytes of the pineal gland [1]. Melatonin is synthesized in a circadian mode as it is produced at high levels at night and low levels during the day. The suprachiasmatic nucleus (SCN) of the hypothalamus, which is considered the master clock, regulates the rhythmic secretion of melatonin. Melatonin, in turn, interacts at receptors in the SCN to suppress neuronal firing. The ability of melatonin to shift

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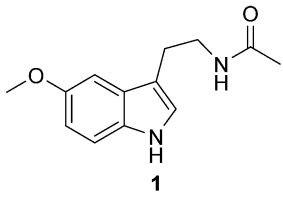
the circadian rhythm, modulate the sleep–wake cycle and circannual rhythms in many species, including man, is well documented as detailed in the recent literature [2–8]. Numerous recently published articles have also described the effect of melatonin on phase shifts, for example those associated with shift work or jet lag [9–13].



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Other effects of melatonin described in the literature include its neuroprotective, anti-inflammatory, pain modulatory, retinal, vascular and antioxidant properties [3,14–20]. This chapter focuses on the use of melatonin and synthetic melatonin agonists for the treatment of sleep, major depression and anxiety.

2.1. Melatonin receptors

Melatonin has high affinity for two G-protein coupled receptors (GPCRs), designated MT1 and MT2, and activates the inhibition of adenylyl cyclase via these receptors [21]. Melatonin receptors MT1 and MT2 have amino acid sequences which are 60% identical. *In situ* analysis has revealed that MT1 mRNA is present in human SCN. Autoradiographic localization studies have shown that ^{125}I-melatonin binding is present in the SCN of most mammalian species including human. MT2 mRNA is not as abundant as MT1 and has been found in SCN and retina by RT-PCR [22]. Most species, with the exception of humans, show specific ^{125}I-melatonin binding in the pars tuberalis of the pituitary. Pituitary melatonin receptors are believed to contribute to the regulation of seasonal changes in reproductive function [7].

The distinct function of MT1 and MT2 has yet to be clearly defined, however, their role in the regulation of sleep and in circadian rhythm is well established [22]. In mice lacking the MT1 receptor, melatonin does not inhibit suppression of neuronal firing in the SCN as in wild-type controls [23]. In contrast, melatonin inhibits the neuronal firing in the SCN of mice lacking the MT2 receptor [24]. These knockout mice studies reveal that the MT1 receptor is responsible for the regulation of neuronal activity in the SCN. MT1 and MT2 receptors both appear to regulate CREB-phosphorylation in the SCN showing that both receptors may be important in transcriptional regulation within the SCN and indicating that there may be some redundancy of melatonin receptors. As more MT1 or MT2 receptor specific ligands are discovered, the differing roles of these two subtypes may become more clearly defined.

A third melatonin receptor binding site, termed MT3, has lower affinity for ^{125}I-melatonin compared to MT1 and MT2 and does not fit the typical binding profile of a GPCR. Binding to MT3 displays enzyme-like kinetics of fast association and dissociation of ligand, is loosely associated with the membrane fraction and is not affected by GTPγS [25]. Recently, hamster MT3 binding site was identified as the hamster homologue of the human dihydronicotinamide riboside quinone oxidoreductase (NQO2)[26]. NQO2 is responsible for nitroreductase and detoxification of compounds. The activity of this enzyme is inhibited by melatonin and other compounds that interact at the ^{125}I-melatonin binding site. The physiological significance of melatonin's interaction with NQO2 has not been well explored. MT3 receptors are currently being investigated for their potential

role in regulating intraocular pressure [27]. Interestingly, a polymorphism in the NQO2/MT3 gene is associated with an increased risk of developing clozapine-induced agranulocytosis [28].

2.2. Regulation of melatonin receptors

Desensitization and down regulation of GPCRs by agonist ligands may effect the physiological responses mediated by the particular GPCR. The regulation of melatonin receptors by agonists has been studied in an effort to understand the mechanism regulating receptor expression. Desensitization of MT1 receptors by melatonin requires prolonged exposure and is not accompanied by significant observable receptor internalization [29]. Instead, a slight but significant desensitization of melatonin-induced GTPγS binding was revealed. Exposure of recombinant MT2 receptors to melatonin, however, leads to a rapid down-regulation of specific ^{125}I-melatonin binding and correlates with desensitization of MT2 receptor function [30]. The authors suggest that administration of high doses of MT2 agonists may desensitize the MT2 receptor and impair melatonin-induced phase shifts in circadian rhythms or reduce melatonin-induced vasodilation.

2.3. Melatonin and sleep

Sleep deprivation and poor sleep quality can reduce cognitive function, memory and negatively affect mood [31,32]. Understanding the relationship of melatonin levels to sleep disorders, depression and anxiety has become a topic of considerable recent research and the melatonin receptors MT1 and MT2 have emerged as therapeutic targets of interest [33]. Melatonin's effect on the duration and quality of sleep has been reported in the literature and in a recent clinical trial, the effects of exogenous melatonin on disturbed REM sleep in humans was studied [34,35]. The results showed that melatonin was significantly more effective than placebo in measures of sleep parameters with patients on melatonin experiencing significant increases in REM sleep percentage and improvements in subjective measures of daytime function [35]. Melatonin therapy could be a useful treatment for sleep disorders associated with shift work or patients with reduced melatonin levels.

Age-related reduction in serum melatonin levels in mice has been recently documented [15,36]. Also impaired production of melatonin, and sleep disorders are reported as common among elderly patients [37]. In a recent article, the excretion of the major melatonin metabolite, 6-sulfatoxymelatonin, was studied in patients aged 55 years or older [38]. It was concluded that low nocturnal melatonin production is associated with insomnia and these 'low excretors' had a significantly higher positive response to melatonin replacement therapy. However, previously published clinical trial data on the use of 5 mg of fast release melatonin taken at bedtime showed that it did not improve the quality of sleep in older people with reported age-related sleep maintenance problems [39].

In a recent review the beneficial use of melatonin in children with severe and intractable sleep disturbance associated with developmental disabilities was discussed [40]. It was concluded that melatonin use is a safe addition to psychological and social approaches for severe sleep disturbance. Similar clinical study reports indicate that melatonin facilitated sleep in individuals with mental retardation and insomnia [41]. Melatonin therapy has also been reported to dramatically improve sleep architecture in blind patients [42].

A disturbed sleep–wake rhythm is common in patients with Alzheimer Disease. This is reported to correlate with decreased melatonin levels and a disrupted circadian melatonin rhythm [43]. This recent article concludes that this disruption is caused by dysfunction of noradrenergic regulation and the depletion of serotonin by increased monoamine oxidase A, resulting in the loss of melatonin rhythm in preclinical Alzheimer Disease.

2.4. Melatonin and depression

Melatonin has been described in the literature as having an antidepressant effect in some animal models of depression. In the mouse tail suspension test (TST), the antidepressant activity of melatonin was proposed to be mediated through an interaction with NMDA receptors and the L-arginine-NO pathway [44]. In a rodent behavioral despair model, administration of melatonin decreased the immobility time that was observed on chronic exposure to forced swimming test [45,46]. Daily treatment of chronically stressed mice with melatonin was shown to reverse various perturbations induced by stress [47]. Recently, published results monitoring the levels of melatonin in trout, also seem to indicate it plays a role in the regulation of stress and anxiety [48].

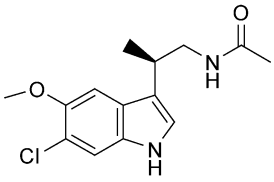
3. SYNTHETIC MELATONIN AGONISTS

Melatonin from the pineal gland was recently reported to be distributed to the brain via the cerebrospinal fluid [49]. Exogenously administered deuterated melatonin has low absolute bioavailability in man (1–37%) due to first pass metabolism and a short half life reported to be approximately 40 min [50]. The shortcomings of exogenously administered melatonin as a drug have led to the search for improved synthetic melatonin agonists. Some of the novel synthetic melatonin agonists previously reported in the literature include agomelatine (S 20098), ramelteon (TAK-375), LY-156735, GR196429, and BMS-214778 [51–55]. The efficacy of novel melatonin agonists in human sleep regulation and the treatment of depression has been well documented and is described in more detail for the specific ligands below [51–53]. The role of melatonin agonists in the treatment of anxiety is less clearly defined, however, some recently published results in anxiolytic animal models seem to indicate they play a significant role. For example melatonin MT1, MT2 dual receptor agonist, S23478 (structure not disclosed), was assessed in a rat model of anticipation of social defeat [56]. The behaviors induced in this animal model, immobility, ultrasonic vocalization and defensive postures

associated with anxiety, were reduced when the animal was pretreated with doses of S23478 at 40 mg/kg. This compound significantly reduced the duration of immobility and vocalization. The data indicates that a dual MT1/2 receptor agonist can exert anxiolytic-like effects.

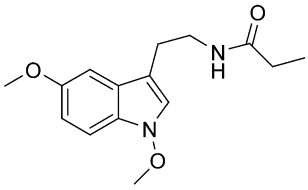
3.1. Indolic melatonin agonists

Melatonin has been studied extensively as described above and many close indole containing analogs have also been reported in the literature. The melatonin agonist LY-156735, **2** was recently reported to alleviate the symptoms of shift lag and to help resynchronize circadian rhythms at a dose of 5 mg/day in human clinical trials [53].



2

Indole derivative **3**, with 5-fold greater potency than melatonin was recently described [57]. The group also reported the synthesis and biological evaluation of tricyclic melatonin analogs with alkyl and cycloalkyl groups in the beta position of the ethylamido chain [58]. When two beta methyl groups were added to the side-chain of the methoxyl-substituted ligand, **4** an increase in agonist potency was observed. However, more bulky R group beta-substituents, reportedly led to antagonism.



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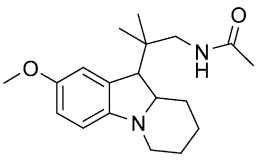
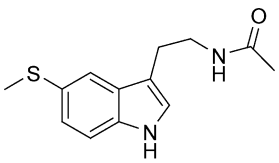


4

Recently sulfur analogs of melatonin, **5** and **6** were reported to have decreased binding affinity at human MT1 and MT2 receptors combined with reduced potency as melatonin agonists [59]. Introduction of the sulfur at the 5 position as seen in **5** resulted in a 35-fold reduction in binding to MT1 and 44-fold reduction in binding to MT2 compared to Melatonin. The thioamide **6** gave a 2.5- and 2-fold reduction in binding affinity for MT1 and MT2, respectively, indicating the greater importance of the 5-methoxy group to receptor affinity.



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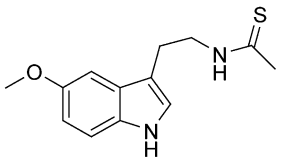


6

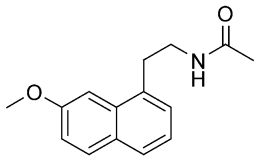
Computational methods have also been used to help determine the active conformation of melatonin and the structural requirements for both melatonin receptor subtype

specificity and intrinsic activity. In a recent report *ab initio* calculations were used to analyze the conformation of melatonin and the results were used to explain the different receptor affinities and subtype specificity of various melatonin analogs with restricted conformations [60,61]. Three dimensional quantitative structure–activity relationship comparative molecular field analysis (3D-QSAR CoMFA) was also recently reported with further exploration of the structural requirements for the melatonin receptor subtypes and intrinsic activity modulation [62].

3.2. Structurally diverse synthetic melatonin agonists

As well as literature describing melatonin and close indolic analogs, a number of more structurally diverse pharmacological tools and possible future drugs have been disclosed. The two most advanced drug development candidates are agomelatine (S 20098) and ramelteon (TAK-375) both are in Phase III studies for the treatment of depression and sleep, respectively.

3.3. Agomelatine and derivatives



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Agomelatine **7** is a potent agonist at melatoninergic receptors. It shows a high affinity for cloned human melatonin receptor MT1 and MT2 sub-types ($K_i = 61$ and 268 pM, respectively). The affinity of agomelatine at melatonin receptors is comparable to that of melatonin ($K_i = 85$ and 263 pM, respectively). Agomelatine also shows affinity ($IC_{50} = 270$ nM) for human cloned serotonin-2C (5-HT2C) receptors, and acts as an antagonist at this receptor subtype [63].

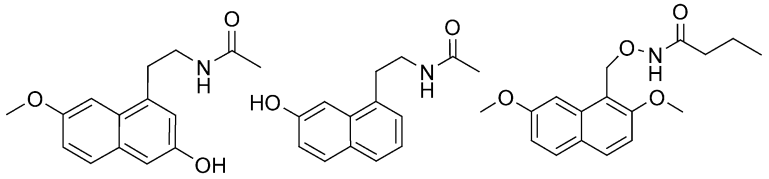
Agomelatine has shown antidepressant activity in animal models of depression, e.g. the rat forced swim test (FST) [63] and the rat chronic mild stress model of depression [64] Researchers have suggested that the antidepressant-like activity of agomelatine depends on some combination of its melatonin agonist and 5-HT2C antagonist properties. This novel dual pharmacological profile has been given the acronym: MASSA (Melatonin Agonist and Selective Serotonin Antagonist) [51].

Anti-depressant efficacy in human phase 2 clinical trials has been shown by agomelatine [65]. In a randomized placebo controlled trial, agomelatine (25 mg/day) after 2 and 4 weeks of treatment was significantly more effective than placebo in treating depression (based on HAM-D scores). Additionally, agomelatine was significantly more effective than placebo at treating a subpopulation of severely depressed patients whereas paroxetine was not significantly different from placebo. Agomelatine also decreased the severity of anxiety associated with depression and was well tolerated with adverse effects similar to placebo.

Agomelatine, is significantly more potent at melatonin receptors than at 5HT2c receptors, however, the role of 5HT2c in its efficacy for the treatment of depression is

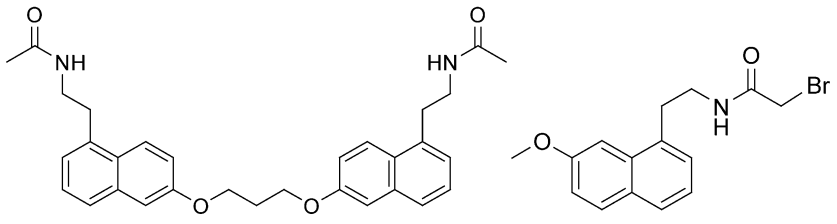
an interesting area of further investigation [66,67]. The potency of two major metabolites of agomelatine at 5HT2c has been reported in the literature. Metabolite S 21517, **8** has greater potency than agomelatine, 130 nM versus 210 nM at 5HT2c. However, potency, approximately 10-fold lower than agomelatine, was reported for the metabolite S 21540, **9** [68].

The synthesis of agomelatine-like arylalkoxy amido derivatives has been reported [69]. The introduction of an oxygen atom in the amido chain led to melatonin agonists that were less potent than the corresponding deoxy derivatives. The 2,7-dimethoxy-naphthalene derivative, **10** was reported to be 0.13 and 0.1 nM at hMT1 and hMT2 receptors, respectively.



8 **9** **10**

Agomelatine dimer derivatives have been described by Descamps-Francois *et al*. some of these dimers were reported to be MT1 selective, however, the most MT1 selective compound, S 26131, **11** showed an antagonist profile [70].



11 **12**

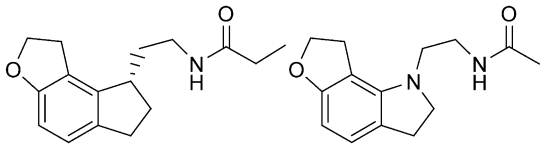
Recently inhibitors of serotonin *N*-acetyltransferase were found to be potent melatonin ligands [71]. As the ligands resemble serotonin and melatonin, the natural substrate and product of arylalkylamine *N*-acetyltransferase this activity was expected. These ligands were also structurally related to agomelatine. The most potent derivative, **12** was 90 and 80 pM at hMT1 and hMT2, respectively, and 2 μM at serotonin *N*-acetyltransferase.

3.4. Ramelteon

An exciting new drug candidate for the treatment of patients with insomnia and circadian rhythm dysfunction, ramelteon **13** has been reported [52]. It is a high-affinity agonist at MT1 and MT2 receptors with K_i values of 14 and 40 pM, respectively. In a randomized placebo-controlled phase 2 study, ramelteon (4, 8, 16 and 32 mg) significantly reduced the latency to persistent sleep, increased the mean total sleep

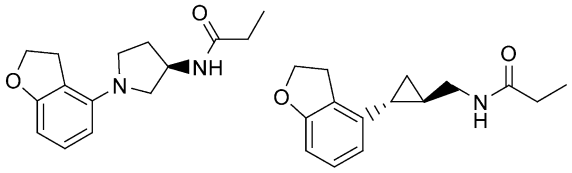
time, and improved sleep efficiency in patients with chronic insomnia [52]. Subjects experienced no residual pharmacological effects upon awakening. Its synthesis and SAR of related analogs has also been reported along with an enzymatic resolution route to the chiral indane [72,73].

Melatonin is a potent agonist and identifying novel structurally diverse ligands with equal or better potency is a considerable challenge. The tetrahydroindoline GR196429, **14** has been reported to have similar potency and efficacy to melatonin and was reported to readily penetrate the CNS and show modulation of circadian rhythm in rat [54]. Structurally it is very similar to ramelteon, but is reported to be less potent (hMT1, 126 pM; hMT2, 158 pM).



13 **14**

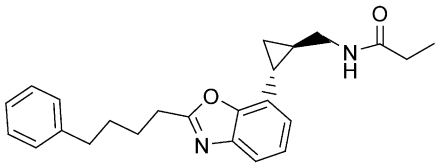
A series of chiral benzofuranyl pyrrolidinamine derivatives were also synthesized as novel melatoninergic ligands [74]. The potent dual agonist **15** was identified as an orally bioavailable agonist at MT1 and MT2 melatonin receptors and was described as a potential clinical trial candidate. The preclinical pharmacokinetics and metabolism of another related novel melatonin receptor agonist BMS-214778, **16** was also reported. The agonist showed high hepatic extraction, non-linear oral pharmacokinetics and its *in vitro* metabolism was reported to be by hydroxylation and dehydrogenation. The most likely human cytochrome P(450) (CYP) isoforms to be involved in its metabolism were reported to be CYP1A1, 1A2, 2D6, and 2C9 [55].



15 **16**

A novel series of benzoxazole melatoninergic ligands was recently described [75]. Compound **17**, (hMT1, 150 pM; hMT2, 70 pM) exhibited better MT1 and MT2 receptor affinities than melatonin (400 and 300 pM at hMT1 and hMT2, respectively). The benzoxazole nucleus was described as an effective isosteric replacement for the more traditional melatonin like alkoxyaryl cores. A series of *N*-acyl-4-indanyl-piperazines that bind to MT2 receptors was also recently reported [76]. The MT2 selective (R)-4-(2,3-dihydro-6-methoxy-1H-inden-1-yl)-*N*-ethyl-1-piperazine-carboxamide fumarate **18** produced advances in circadian phase in rats at doses of 1–56 mg/kg similar to those observed with melatonin at 1 mg/kg. The hMT2 receptor binding affinity of **18** was reported as 1.7 nM with melatonin reported as 0.3 nM in the same assays. The research group reported that unlike melatonin, compound **18**

produced only weak contractile effects in rat tail artery even at high doses reporting this was due to the enhanced hMT2 versus hMT1 selectivity.



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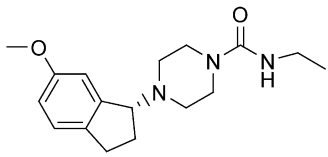
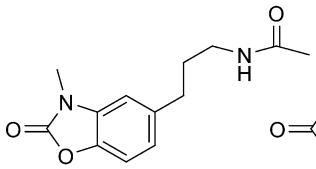
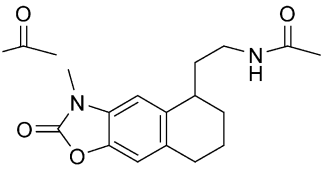


18

Mesangeau *et al*. recently described 2(3H)-benzoxazolone-based melatonin ligands, **19** and **20** in the literature [77]. They were described as exhibiting lower affinities for the hMT2 receptor than melatonin (**19**, 6.7 nM and **20**, 14.3 nM at hMT2). This was attributed to unfavorable steric bulk and hydrophilic interactions.

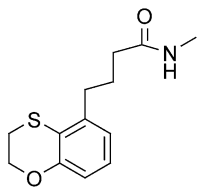


19



20

The novel heterocycle, 5-substituted-2,3-dihydro-1,4-benzoxathiin, **21** with a retroamide moiety was recently found to have similar affinity to melatonin on MT1 and MT2 receptors [78]. Structural modification of melatonin ligands has also been shown to alter melatonin receptor subtype specificity and ligand function resulting in the discovery of novel antagonists. Synthesis of tetrahydronaphthalenic melatonin analogs with a phenyl substituent in the 3-position of the tetraline ring led to the discovery of highly hMT2 selective ligands [79]. The (+)-(RR)-*cis* enantiomer, **22** is 880-fold more selective for hMT2 versus hMT1. It is also an antagonist.



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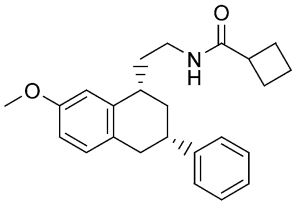
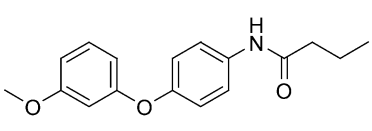


22

A series of 4-substituted anilide melatonin agonists was recently reported [80]. Compound **23** showed agonist function with subnanomolar hMT2 binding affinity of 750 pM and hMT2 selectivity of at least 70-fold over the hMT1 receptor. Melatonin was

reported to be 530 pM and 320 pM at hMT1 and hMT2, respectively, in their assays. A more potent non-selective compound, **24** was also described, with binding affinity at hMT1 and hMT2 reported as 50 and 230 pM, respectively.



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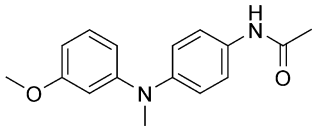
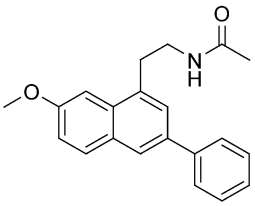


24

A variety of new selective and non-selective melatonin receptor agonists and antagonists have been reported [81]. The report highlighted the selective hMT2 antagonist S 24601, **25** as well as the non-selective antagonist, S 22153, **26**. The hMT1 selective agonist, S 24268, **27** was also described along with the potent melatonin non selective hMT1/2 receptor agonist, S 25150, **28**.



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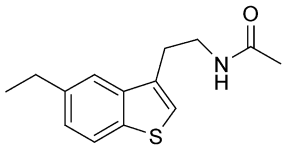

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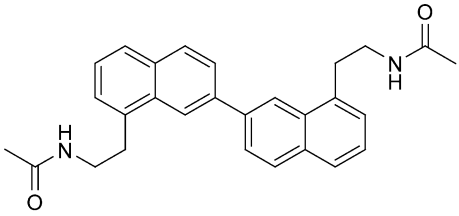

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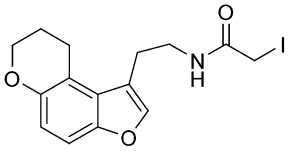

28

4. CONCLUSION

The last year has seen considerable progress in the understanding of the biology of melatonin, its receptors and the use of melatonin agonists. A rich array of chemical matter has also been described which provides great tools to further explore this exciting area of future research.

REFERENCES

[1] V. Csernus and B. Mess, *Neuroendocrinol. Lett.*, 2003, **24**, 404.
[2] D. Saito, Q. Shi, H. Ando and A. Urano, *Gen. Comp. Endocr.*, 2004, **137**, 62.

[3] C. B. Green and J. C. Besharse, *J. Biol. Rhythms*, 2004, **19**, 91.
[4] B. Pitrosky, P. Delagrange, M. C. Rettori and P. Pevet, *Behav. Brain Res.*, 2003, **138**, 145.
[5] C. Cajochen, K. Krauchi and A. Wirz-Justice, *J. Neuroendocrinol.*, 2003, **15**, 432.
[6] G. A. Lincoln, H. Andersson and A. Loudon, *J. Endocrinol.*, 2003, **179**, 1.
[7] C. Von Gall, *Ann. Anat.*, 2003, **185**, 301.
[8] J. H. Stehle and C. von Gall, *J. Neuroendocrinol.*, 2003, **15**, 383.
[9] U. Albrecht, *Neuroendocrinol. Lett.*, 2003, **24** (Suppl. 1), 188.
[10] C. Cajochen, M. E. Jewett and D. J. Dijk, *J. Pineal Res.*, 2003, **35**, 149.
[11] D. J. Skene, *J. Neuroendocrinol.*, 2003, **15**, 438.
[12] M. Beaumont, D. Batejat, C. Pierard, P. Van Beers, J. B. Denis, O. Coste, P. Doireau, F. Chauffard, J. French and D. Lagarde, *J. Appl. Physiol.*, 2004, **96**, 50.
[13] G. F. Oxenkrug and P. Requintina, *CNS Spectr.*, 2003, **8**, 139.
[14] V. S. Nedzvetsky, P. A. Nerush and S. V. Kirichenko, *Neurophysiology*, 2003, **35**, 422.
[15] E. H. Sharman, K. G. Sharman, Y.-W. Ge, D. K. Lahiri and S. C. Bondy, *J. Pineal Res.*, 2004, **36**, 165.
[16] P. K. Zahn, T. Lansmann, E. Berger, E.-J. Speckmann and U. Musshoff, *J. Pineal Res.*, 2003, **35**, 24.
[17] M. I. Masana, S. Doolen, C. Ersahin, W. M. Al-ghoul, S. P. Duckles, M. L. Dubocovich and D. N. Krause, *J. Pharmacol. Exp. Ther.*, 2002, **302**, 1295.
[18] C. Rodriguez, J. C. Mayo, R. M. Sainz, I. Antolin, F. Herrera, V. Martin and R. J. Reiter, *J. Pineal Res.*, 2004, **36**, 1.
[19] M. Mor, C. Silva, F. Vacondio, P. V. Plazzi, S. Bertoni, G. Spadoni, G. Diamantini, A. Bedini, G. Tarzia, M. Zusso, D. Franceschini and P. Giusti, *J. Pineal Res.*, 2004, **36**, 95.
[20] R. Hardeland, A. Coto-Montes and B. Poeggeler, *Chronobiol. Int.*, 2003, **20**, 921.
[21] P. Barrett, S. Conway and P. J. Morgan, *J. Pineal Res.*, 2003, **35**, 221.
[22] M. L. Dubocovich, M. A. Rivera-Bermude, M. J. Gerdin and M. I. Masana, *Front Biosci.*, 2003, **8d**, 1093.
[23] C. Liu, D. R. Weaver, X. Jin, L. P. Shearman, R. L. Pieschl, V. K. Gribkoff and S. M. Reppert, *Neuron*, 1997, **19**, 91.
[24] X. Jin, C. von Gall, R. L. Pieschl, V. K. Gribkoff, J. H. Stehle, S. M. Reppert and D. R. Weaver, *Mol. Cell. Biol.*, 2003, **23**, 1054.
[25] M. L. Dubocovich, *Trends Pharmacol.*, 1995, **16**, 50.
[26] O. Nosjean, M. Ferro, F. Coge, P. Beauverger, J.-M. Henlin, F. Lefoulon, J.-L. Fauchere, P. Delagrange, E. Canet and J. A. Boutin, *J. Biol. Chem.*, 2000, **274** (40), 31311.
[27] J. Pintor, T. Pelaez, C. H. V. Hoyle and A. Peral, *Br. J. Pharmacol.*, 2003, **138**, 831.
[28] O. Ostrousky, S. Meged, R. Loewenthal, A. Valevski, A. Weizman, H. Carp and E. Gazit, *Tissue Antigens*, 2003, **62**, 483.
[29] M. J. Gerdin, M. I. Masana and M. L. Dubocovich, *Biochem. Pharmacol.*, 2004, **67**, 2023.
[30] M. J. Gerdin, M. I. Masana, D. Ren, R. J. Miller and M. L. Dubocovich, *J. Pharmacol. Exp. Ther.*, 2003, **304**, 931.
[31] R. H. Silva, V. C. Abilio, A. L. Takatsu, S. R. Kameda, C. Grassl, A. B. Chehin, W. A. Medrano, M. B. Calzavara, S. Registro, M. L. Andersen, R. B. Machado, R. C. Carvalho, R. de A. Ribeiro, S. Tufik and R. Frussa-Filho, *Neuropharmacology*, 2004, **46**, 895.
[32] K. M. Koorengevel, D. G. M. Beersma, J. A. den Boer and R. H. van den Hoofdakker, *Psychiatr. Res.*, 2003, **117**, 57.
[33] I. Nir, *Receptor Channel*, 2003, **9**, 379.
[34] D. Aeschbach, L. Sher, T. T. Postolache, J. R. Matthews, M. A. Jackson and T. A. Wehr, *J. Clin. Endocr. Metab.*, 2003, **88**, 26.
[35] D. Kunz, R. Mahlberg, C. Mueller, A. Tilmann and F. Bes, *J. Clin. Endocr. Metab.*, 2004, **89**, 128.
[36] D. K. Lahiri, Y.-W. Ge, E. H. Sharman and S. C. Bondy, *J. Pineal Res.*, 2004, **36**, 217.
[37] Y. Touitou, *Chronotherapeutics*, 2003, 369.
[38] D. Leger, M. Laudon and N. Zisapel, *Am. J. Med.*, 2004, **116**, 91.
[39] J. J. Baskett, J. B. Broad, P. C. Wood, J. R. Duncan, M. J. Pledger, J. English and J. Arendt, *Age Ageing*, 2003, **32**, 164.

[40] J. Turk, *J. Med. Genet.*, 2003, **40**, 793.
[41] H. Niederhofer, W. Staffen, A. Mair and J. Autism, *Dev. Disord.*, 2003, **33**, 469.
[42] S. Fischer, R. Smolnik, M. Herms, J. Born and H. L. Fehm, *J. Clin. Endocr. Metab.*, 2003, **88**, 5315.
[43] Y.-H. Wu, M. G. P. Feenstra, J.-N. Zhou, R.-Y. Liu, J. S. Torano, H. J. M. Van Kan, D. F. Fischer, R. Ravid and D. F. Swaab, *J. Clin. Endocr. Metab.*, 2003, **88**, 5898.
[44] M. Mantovani, R. Pertile, J. B. Calixto, A. R. S. Santos and A. L. S. Rodrigues, *Neurosci. Lett.*, 2003, **343**, 1.
[45] M. N. Hill, L. A. Brotto, T. T.-Y. Lee and B. B. Gorzalka, *Prog. Neuropsychopharm. Biol. Psych.*, 2003, **27**, 905.
[46] V. Raghavendra, G. Kaur and S. K. Kulkarni, *Eur. Neuropsychopharmacol.*, 2000, **10**, 473.
[47] C. Kopp, E. Vogel, M.-C. Rettori, P. Delagrange and R. Misslin, *Behavior. Pharmacol.*, 1999, **10**, 73.
[48] E. T. Larson, S. Winberg, I. Mayer, O. Lepage, C. H. Summers and O. Overli, *Gen. Comp. Endocr.*, 2004, **136**, 322.
[49] H. Tricoire, A. Locatelli, P. Chemineau and B. Malpaux, *Endocrinology*, 2002, **143**, 84.
[50] J. B. Fourtillan, A. M. Brisson, P. Gobin, I. Ingrand, J. Ph. Decourt and J. Girault, *Biopharm. Drug Dispos.*, 2000, **21**, 15.
[51] K. Chilman-Blair, J. Castaner, M. Bayes and J. S. Silvestre, *Drug Future*, 2003, **28**, 7.
[52] K. Chilman-Blair, J. Castaner, J. S. Silvestre and M. Bayes, *Drug Future*, 2003, **28**, 950.
[53] T. Nickelsen, A. Samel, M. Vejvoda, J. Wenzel, B. Smith and R. Gerzer, *Chronobiol. Int.*, 2002, **19**, 915.
[54] I. J. Beresford, C. Browning, S. J. Starkey, J. Brown, S. M. Foord, J. Coughlan, P. C. North, M. L. Dubocovich and R. M. Hagan, *J. Pharmacol. Exp. Ther.*, 1998, **285**, 1239.
[55] N. N. Vachharajani, K. Yeleswaram and D. W. Boulton, *J. Pharm. Sci.*, 2003, **92**, 760.
[56] C. Becker, J. Andre, B. Zeau, M.-C. Rettori, B. Guardiola-Lemaitre, M. Hamon and J.-J. Benoliel, *Neuropharmacology*, 2004, **46**, 1158.
[57] A. Tsotinis, A. Eleutheriades, K. Hough and D. Sugden, *Chem. Commun.*, 2003, **3**, 382.
[58] A. Tsotinis, M. Panoussopoulou, K. Hough and D. Sugden, *Eur. J. Pharm. Sci.*, 2003, **18**, 297.
[59] D. J. Davies, R. Faust, P. J. Garratt, C. Marivingt-Mounir, K. Davidson, M.-T. Teh and D. Sugden, *Bioorg. Chem.*, 2004, **32**, 1.
[60] J. Csontos, M. Kalman and G. Tasi, *Theochem J. Mol. Struct.*, 2003, **640**, 69.
[61] J. Csontos, M. Kalman and G. Tasi, *Theochem J. Mol. Struct.*, 2003, **666–667**, 515.
[62] S. Rivara, M. Mor, C. Silva, V. Zuliani, F. Vacondio, G. Spadoni, A. Bedini, G. Tarzia, V. Lucini, M. Pannacci, F. Fraschini and P. V. Plazzi, *J. Med. Chem.*, 2003, **46**, 1429.
[63] M. Bourin, E. Mocaer and R. Porsolt, *J. Psychiatr. Neurosci.*, 2004, **29**, 126.
[64] M. Papp, P. Gruca, P.-A. Boyer and E. Mocaer, *Neuropsychopharmacology*, 2003, **28**, 694.
[65] H. Loo, A. Hale and H. D'haenen, *Int. Clin. Psychopharmacol.*, 2002, **17**, 239.
[66] A. Serretti, P. Artioli and D. De Ronchi, *Expert Opin. Ther. Targets*, 2004, **8**, 15.
[67] M. J. Millan, A. Gobert, F. Lejeune, A. Dekeyne, A. Newman-Tancredi, V. Pasteau, J.-M. Rivet and D. Cussac, *J. Pharmacol. Exp. Ther.*, 2003, **306**, 954.
[68] A. Chagraoui, P. Protais, T. Filloux and E. Mocaer, *Psychopharmacology*, 2003, **170**, 17.
[69] C. Pegurier, L. Morellato, E. Chahed, J. Andrieux, J. P. Nicolas, J. A. Boutin, C. Bennejean, P. Delagrange, M. Langlois and M. Mathe-Allainmat, *Bioorg. Med. Chem.*, 2003, **11**, 789.
[70] C. Descamps-Francois, S. Yous, P. Chavatte, V. Audinot, A. Bonnaud, J. A. Boutin, P. Delagrange, C. Bennejean, P. Renard and D. Lesieur, *J. Med. Chem.*, 2003, **46**, 1127.
[71] G. Ferry, C. Ubeaud, J. Mozo, C. Pean, P. Henning, M. Rodriguez, C. Scoul, A. Bonnaud, O. Nosjean, J.-P. Galizzi, P. Delagrange, P. Renard, J.-P. Volland, S. Yous, D. Lesieur and J. A. Boutin, *Eur. J. Biochem.*, 2004, **271**, 418.
[72] O. Uchikawa, K. Fukatsu, R. Tokunoh, M. Kawada, K. Matsumoto, Y. Imai, S. Hinuma, K. Kato, H. Nishikawa, K. Hirai, M. Miyamoto and S. Ohkawa, *J. Med. Chem.*, 2002, **45**, 4222.
[73] N. Tarui, Y. Nagano, T. Sakane, K. Matsumoto, M. Kawada, O. Uchikawa, S. Ohkawa and K. Nakahama, *J. Biosci. Bioeng.*, 2002, **93**, 44.

[74] L.-Q. Sun, J. Chen, R. J. Mattson, J. R. Epperson, J. A. Deskus, W.-S. Li, K. Takaki, D. B. Hodges, L. Iben, C. D. Mahle, A. Ortiz, D. Molstad, E. Ryan, K. Yeleswaram, C. Xu and G. Luo, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4381.
[75] L.-Q. Sun, J. Chen, K. Takaki, G. Johnson, L. Iben, C. D. Mahle, E. Ryan and C. Xu, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1197.
[76] R. J. Mattson, J. D. Catt, D. Keavy, C. P. Sloan, J. Epperson, Q. Gao, D. B. Hodges, L. Iben, C. D. Mahle, E. Ryan and F. D. Yocca, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1199.
[77] C. Mesangeau, J. H. Poupaert, P. Carato and S. Yous, *Heterocycles*, 2003, **60**, 2621.
[78] I. Charton, F. Suzenet, J. A. Boutin, V. Audinot, P. Delagrange, C. Bennejean, P. Renard and G. Guillaumet, *J. Enzyme Inhib. Med. Chem.*, 2003, **18**, 187.
[79] S. Yous, S. Durieux-Poissonnier, E. Lipka-Belloli, H. Guelzim, C. Bochu, V. Audinot, J. A. Boutin, P. Delagrange, C. Bennejean, P. Renard and D. Lesieur, *Bioorg. Med. Chem.*, 2003, **11**, 753.
[80] J. R. Epperson, J. A. Deskus, A. J. Gentile, L. G. Iben, E. Ryan and N. S. Sarbin, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1023.
[81] V. Audinot, F. Mailliet, C. Lahaye-Brasseur, A. Bonnaud, A. Le Gall, C. Amosse, S. Dromaint, M. Rodriguez, N. Nagel, J.-P. Galizzi, B. Malpaux, G. Guillaumet, D. Lesieur, F. Lefoulon, P. Renard, P. Delagrange and J. A. Boutin, *NS Arch. Pharmacol.*, 2003, **367**, 553.

Section 2
Cardiovascular and Metabolic Diseases

Editor: William J. Greenlee
Schering-Plough Research Institute
Kenilworth
New Jersey

Recent Advances in Therapeutic Approaches to Type 2 Diabetes

Ramakanth Sarabu, Jefferson Tilley,

Hoffmann-La Roche, Inc., Nutley, NJ 07110, USA

Contents

1. INTRODUCTION

Type 2 diabetes (T2D) affects an increasing proportion of populations of both the developed and developing parts of the world. According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) 17 million Americans – 6.2% of the U.S. population – have diabetes, and more than one third of these are undiagnosed. Another 16 million have insulin resistance or pre-diabetes. Worldwide figures are even more staggering: in 2000 the World Health Organization (WHO) reported a worldwide prevalence of 154.4 million diabetes patients. Hence, intense efforts in the discovery

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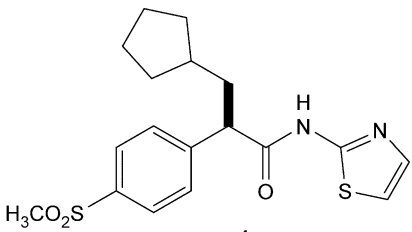
and development of more efficacious and safer diabetes therapies are underway in academic and industrial research organizations.

Since the appearance of the last review of diabetes in Annual Reports in Medicinal Chemistry in 2000, sales of troglitazone, the first peroxisome proliferator of activated receptor gamma (PPAR-γ) agonist on the market were halted due to hepatotoxicity in a small number of patients. Two more potent new PPAR-γ agonists, rosiglitazone and pioglitazone were introduced and appear to be free of the hepatic liability associated with troglitazone. One other new molecular entity (NME) is Starlix, an ATP-sensitive pancreatic potassium channel inhibitor. However, there have been many significant developments in the discovery and development of novel molecular entities that are in various phases of clinical and preclinical development. These recent developments (post 2000) discussed in this chapter, can be broadly classified into (1) enhancers of insulin release, (2) enhancers of insulin action, (3) inhibitors of hepatic glucose production, (4) inhibitors of glucose absorption from the gut.

2. ENHANCERS OF INSULIN RELEASE

2.1. Glucokinase activators

Glucokinase (GK) or hexokinase IV is one of the four hexokinases that phosphorylate glucose and plays a key role in whole-body glucose homeostasis through its action in β-cells and hepatocytes. The rationale for GK activators was derived from the study of GK mutations as manifested in maturity onset of diabetes of the young Type II (MODY II) in humans and persistent hyperinsulinemic hypoglycemia of infancy (PHHI) phenotypes associated with gene manipulation studies in mice. Recently, a potent GK activator RO0281675, **1**, which increased the enzymatic activity of recombinant human GK in a dose-dependent and stereospecific manner, was identified [1]. At a concentration of 3 μM, **1** increased the V_{max} of GK by a factor of about 1.5 and decreased the substrate concentration at $0.5V_{max}$ ($[S]_{0.5}$) for glucose from 8.6 to 2.0 mM. In numerous *in vivo* studies GK activators were shown to cause glucose-dependent insulin release in the pancreas, and also to increase glucose utilization in the liver.

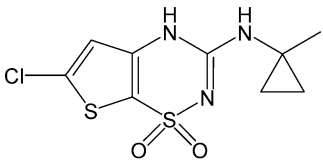


1

2.2. Potassium channel openers

Unlike the conventional sulfonylureas, which stimulate insulin secretion by blocking ATP sensitive potassium channels, NN414, **2**, is postulated to selectively open the pancreatic β-cell potassium channel, SUR1/Kir6.2 and consequently suppress overstimulation of insulin secretion resulting in an improvement in the insulin response to glucose

challenge. In ZDF (fa/fa) rats, **2** was shown to reduce fasting blood glucose levels and improve glucose tolerance in a 21 day study at a dose of 1.5 mg/kg, bid dosing [1]. NN414, **2** was advanced to Phase II clinical trials. However, further development was halted due to a finding of elevated liver enzymes in treated patients, according to a press release.



2

2.3. GLP-1 agonists and dipeptidyl peptidase IV (DPPIV) inhibitors

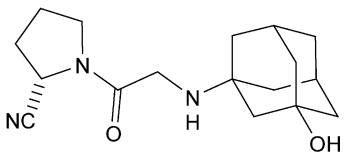
Glucagon-like peptide 1 (GLP-1) is a 36 amino acid peptide secreted by the gut in response to nutrients that exerts control over glucose levels by promoting insulin secretion, reducing glucagon levels, and slowing the rate of gastric emptying. However, GLP-1 is rapidly degraded by the endopeptidase dipeptidyl peptidase IV and has a short half-life. Approaches that are underway to potentiate GLP-1 activities include the preparation of stable GLP-1 analogs, and use of inhibitors of DPPIV, which slow degradation of the active form of GLP-1 and prevent the formation of the GLP-1 antagonist GLP1(9–36).

Exenatide **3**, a GLP-1 analog, exhibits several antidiabetic actions and is being developed as an injectable therapy. In Phase II clinical trials, exenatide **3** caused statistically significant reductions in post-prandial glucose and glucagon concentrations and reductions in the rate of gastric emptying [2]. These results, plus data from two ongoing pivotal studies of **3** in combination with sulfonyl ureas and metformin, will form the basis of an NDA submission to the FDA, anticipated for 2004. Three additional stable and potent GLP-1 analogs, liragutide, (NN-2211, Novo Nordisk) [3], BIM-51077, (Beaufour-Ipsen and Roche), and CJC-1131, (ConjuChem) are in phase II or III clinical testing.

H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2

3

Inhibitors of DPPIV are under investigation as orally active mediators of GLP-1 levels. NVP-LAF237, **4** a potent DPPIV inhibitor, was shown to increase active GLP-1 levels, and improve glucose tolerance in rodents. Chronic treatment with **4** had no effect on weight



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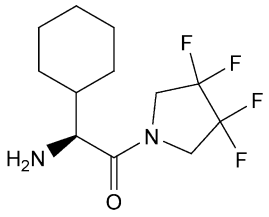


5

gain in mice and rats and delayed gastric emptying in cynomolgus monkeys [4]. In humans, **4** improved hyperglycemia in T2D patients at 100 mg TID, in a 4-week study.

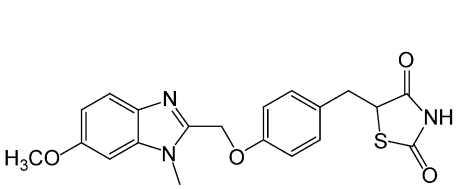
Another DPPIV inhibitor, CP-867534-01, **5** ($IC_{50} \sim 100$ nM), showed good efficacy in ob/ob and Balb/c mice at 10 mg/kg po and inhibited DPPIV activity in fed mice resulting in increased GLP-1 levels. Interestingly, CP-867534-01, **5** was associated with bloody stools in dogs after 5 mg/kg i.v. dosing. Although further investigation showed the effect is species-specific and not linked to DPPIV inhibition, **5** is no longer under development [19].

A number of other companies including Syrrx and Roche, are reported to have DPPIV inhibitors in development. The issues that remain to be addressed include breadth of action of DPPIV inhibitors, specificity of action, durability of effect and effects in combination with other drugs.

3. ENHANCERS OF INSULIN ACTION

3.1. Ligands for peroxisome-proliferator activated receptors (PPARs)

Investigation of the family of nuclear receptors PPAR-α, β, and γ remains a highly active area of research in the diabetes field and was recently reviewed in Annual Reports in Medicinal Chemistry [5]. Most advanced are the PPAR-γ agonists balaglitazone **7** (Dr Reddy's Laboratories/ Novo Nordisk, Phase II) rivoglitazone **6** (Sankyo, Phase II), FK614 (Fujisawa, Phase II), and R483 (Roche/Chugai, Phase II). PPAR-γ agonists promote adipocyte differentiation and consequently cause weight gain as a side effect. In attempts to minimize this side effect, there is interest in developing dual acting PPAR-α and γ co-activators which could be expected to simultaneously promote fatty acid oxidation and adipocyte differentiation. A leading entry was MK-0767 **8** (Merck, Phase III) until its suspension from development due to the formation of a rare hepatic tumor during long term safety studies. Tesaglitazar **9** (Astra Zeneca) is in Phase III clinical development, and three other candidates with dual PPAR-α and γ co-activators are in Phase II clinical development [6].



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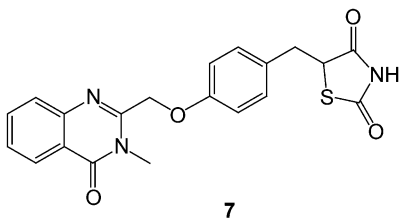

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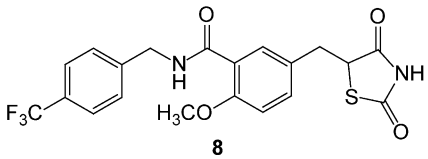

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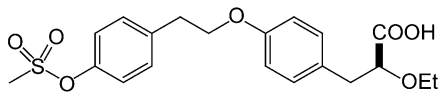

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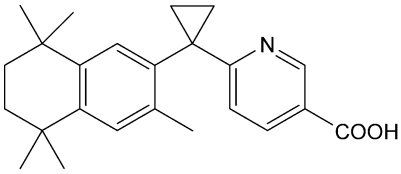
3.2. Retinoid X receptor (RXR) modulators



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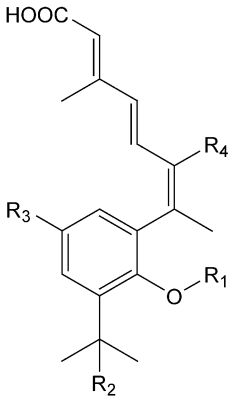
RXR is a nuclear receptor that plays a critical role in the activation of many genes by formation of functional heterodimers with other nuclear hormone receptors including the PPARs and LXR in the presence of small molecule ligands. The potent RXR modulator LG100268, **10**, activates the RXR:PPAR-γ heterodimer as efficiently as the PPAR-γ agonist (BRL-49653 – rosiglitazone) in *in vitro* assays. Compound **10**, was found to improve glucose tolerance in Zucker female fa/fa rats and also restrained weight gain in a 6 week study relative to the PPAR-γ agonist BRL-49653. However, **10** was found to raise triglyceride levels 2 h post-dose, and to lower TSH levels 24 h post-dose in Sprague Dawley rats following a single administration of 10 or 30 mg/kg, po. Because of this undesired activity, the team further optimized the molecule and identified, LG101506, **11**, a partial agonist that is selective for the RXR:PPAR-γ heterodimer. This compound did not increase TG levels and had no effect on TSH levels, while improving insulin sensitivity without weight gain [7]. However, compound **11**, had poor PK properties (low AUC, short T_{max}, and low C_{max}). Recently, compounds **12**, **13**, and **14** with better PK properties have been reported [8].



11 - $R_1 = CH_2CHF_2$, $R_2 = CH_3$, R_3 = t-butyl, $R_4 = H$

12 - $R_1 = CH_2CHF_2$, $R_2 = CH_3$, R_3 = t-butyl, $R_4 = F$

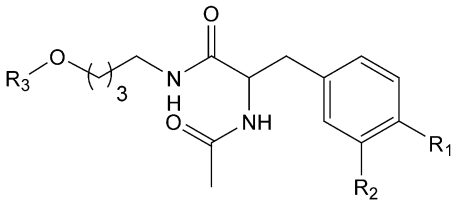
13 - $R_1 = CH_2CHF_2$, $R_2 = H$, R_3 = 2-fluorophenyl, $R_4 = H$

14 - $R_1 = CH_2CH_3$, $R_2 = H$, $R_3 = CF_2CF_3$, $R_4 = H$

3.3. Protein tyrosine phosphatase 1B (PTP1B) inhibitors

PTP1B negatively regulates insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) phosphorylation. Mice that lack the PTP1B gene have increased insulin sensitivity with resistance to weight gain on a high-fat diet and are otherwise normal. This unique combination of desired attributes has driven an intense search for PTP1B inhibitors for treatment of both T2D and obesity. The discovery of effective inhibitors of PTP1B has proven challenging, due to both the selectivity requirements over other protein tyrosine phosphatases, particularly T-cell protein tyrosine phosphatase (TC-PTP) with which it shares high sequence homology near the catalytic site, and the need for potent antagonists to incorporate polar phosphate mimics, thus limiting cell penetration. The Abbott team

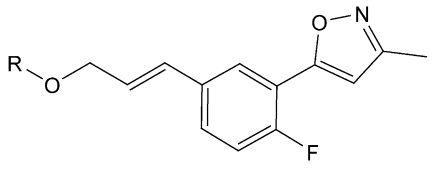
identified a peripheral site in the X-ray crystal structure of a PTP1B-inhibitor complex. It is located near the catalytic site where the substrate phosphotyrosine residues bind and by using an SAR by NMR approach, identified unique binders to each site. They then linked these fragments to obtain the potent inhibitor **15**, containing two free carboxylic acid groups.



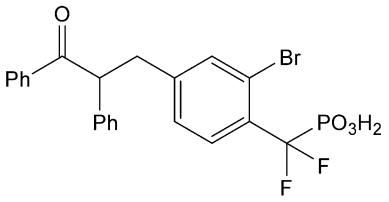
15 R_1 = N(COCOOH)(2-carboxy-phenyl), R_2 = CH_2CH_3
R_3 = (2-carbomethoxy-3-hydroxy)phenyl

16 R_1 = OCH_2COOH; R_2 = OH,
R_3 = (2-carbomethoxy-3-hydroxy)phenyl

This compound has a K_i of 18 nM against PTP1B and 65 nM against TC-PTP. Further optimization to improve transport properties led to the discovery of **16**, with one carboxylic acid group. Compound **16** showed modest potency against PTP1B (PTP1B K_i 9 μM), and good selectivity over TC-PTP (K_i 182 μM) and this inhibitor was active in a cell line in which PTP1B was over expressed [9,10]. Further work led to the identification of compound **17**, with improved cell permeability [11]. Other interesting antagonists include the deoxybenzoin bis-fluorophosphonate inhibitor, **18** (PTP1B IC_{50} 120 nM) which was found to have 13% oral bioavailability in rats. This PTP1B inhibitor **18**, in Zucker fa/fa rats, in an OGTT, caused a reduction of glucose AUC by 50% after a single dose of 30 mg/kg PO dose [12].



17 R = (2-carbomethoxy-3-hydroxy)phenyl



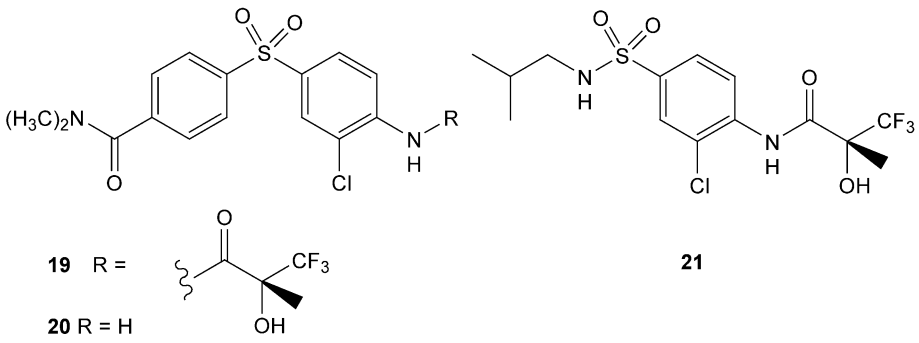
18

In a novel approach, researchers at ISIS Pharmaceuticals used a PTP1B anti-sense oligonucleotide (ASO) to target the mRNA to disrupt its transcription. *In vivo* studies with the PTP1B ASO showed that a 25 mg/kg ip dose either once or twice per week in ob/ob and db/db mice normalized plasma glucose levels, and postprandial glucose excursion and reduced HbA1c. PTP1B protein and mRNA were reduced in liver and fat with no effect in skeletal muscle, reasons for which are not fully understood [13]. This PTP1B ASO entered Phase II in September, 2003.

4. INHIBITORS OF HEPATIC GLUCOSE PRODUCTION (HPG) [14]

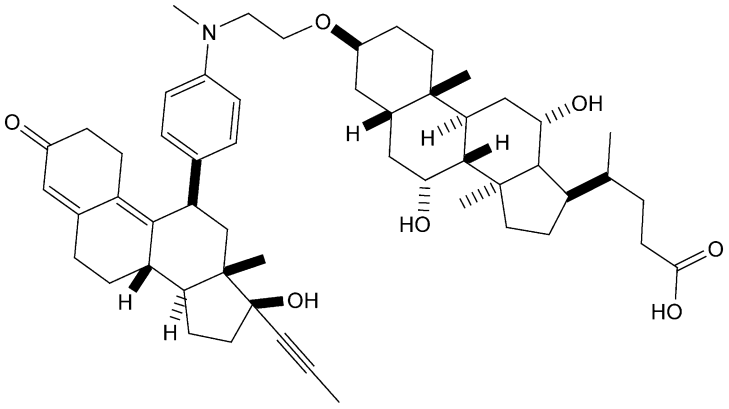
4.1. Inhibitors of pyruvate dehydrogenase kinase (PDHK)

Increasing the activity of pyruvate dehydrogenase (PDH) by inhibiting PDHK is expected to decrease blood glucose by increasing glucose oxidation in peripheral tissues and by decreasing the supply of the gluconeogenic precursors, lactate and alanine to the liver. Dichloroacetate (DCA), a known inhibitor of PDHK was shown to reduce plasma glucose levels both in animal models of diabetes and in patients. Administration of DCA for seven days to T2D patients decreased plasma glucose, and caused marked decreases in lactate and alanine levels. However, DCA was not suitable as therapeutic agent due to its low potency, lack of specificity, poor PK, and toxicity. AZD7545, **19** is a potent rat PDHK inhibitor (IC_{50} 0.021 μM) that increased PDH activity with an EC_{50} value of 0.105 μM in rat hepatocytes. Compound **19** was also found to markedly improve the 24 h glucose profile, by eliminating postprandial elevation in glucose levels, at 10 mg/kg, bid, in male obese Zucker fa/fa rats [15]. However, the development of **19** was stopped in view of the formation of corresponding aniline metabolite **20** which was considered to be undesirable. Further work to identify compounds without this liability is in progress. Interestingly, Novartis researchers did not observe lowered glucose levels with their PDHK inhibitors, for example **21**, in rodent models of type 2 diabetes [16,17], but observed significantly diminished blood lactate levels.



4.2. Liver-selective glucocorticoid receptor antagonist

The correlation between elevated hepatic glucose output and fasting hyperglycemia in type 2 diabetic patients is well established. Also, the link between elevated glucocorticoids (GCs) and their role in glucose control suggested the desirability of exploring glucocorticoid receptor antagonism as a potential therapy for T2D. However, the critical role played by GCs in the hypothalamic pituitary axis (HPA) and potential toxicities due to systemic GC antagonism, require liver-selective glucocorticoid receptor (GR) antagonism to safely treat T2D patients.



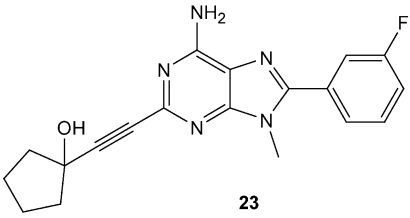
22

Another group used a novel strategy to design liver-selective GC receptor antagonists. They explored a bile-acid conjugation (BCA) approach by linking RU-486, a potent GC antagonist ($IC_{50} = 1.1$ nM) with cholic acid via a two carbon linker to give **22** (A-348441). Cholic acid is known to enter the liver and intestine via bile acid transporters and thus this approach could potentially minimize the systemic exposure to RU-486. The X-ray structure of GR ligand binding domain with RU-486 was determined. Modeling studies using a complex with the X-ray crystal structures helped in the design of **22**. This complex was found to retain potent GC antagonist activity ($IC_{50} = 9$ nM), and blocked GR mediated gene expression in primary hepatocytes (IC_{50}'s: **22** – 0.12 μM; RU486 – 0.21 μM). The conjugate **22** was also evaluated in various rodent models of type 2 diabetes and found to have desirable effects on glucose homeostasis and dyslypidemia [18,19]. Thus, it appears that a liver-targeted GC receptor antagonist has potential to have a beneficial effect in type 2 diabetics [20].

4.3. Adenosine A2B receptor antagonists

Adenosine is an autocoid produced in many tissues to mediate various functions through four receptor subtypes, A1, A2A, A2B and A3. Current literature reports suggest adenosine A2B receptor antagonism reduces hepatic glucose production and enhances glucose uptake in muscle. In human skeletal muscle cells, adenosine A2B and A2A but not A1 receptors were detected [21], while in rat skeletal muscle cells, A2A and A2B receptors but not A1 or A3 receptors were found [22]. Earlier reports ruled out a role for A2A receptors as modulators of muscle insulin sensitivity [23]. Using specific adenosine receptor agonists and antagonists, further evidence suggesting involvement of adenosine acting through A2B receptors in promoting hepatic glucose production, has been provided. The potent A2B receptor antagonist **23** (A2B CHO-cAMP 100 nM) was effective in lowering glucose levels in KK-Ay mice, at a 10 mg/kg po dose [24,25]. BWA1433, **24**, is a potent but non-selective A2B receptor antagonist that is efficacious in improving glucose clearance as measured through an ip glucose tolerance test (ipGTT) in Zucker fa/fa (obese phenotype) rats [28]. In hyperinsulinemic euglycemic clamp studies, **24** increased whole body glucose uptake in obese Zucker fa/fa rats [26]. Based on muscle

tissue A2B receptor distribution and the clamp study results, the authors conclude the effects of **24** are primarily mediated through adenosine A2B receptor antagonism in muscle [27]. Thus, adenosine A2B receptor antagonists may be useful in treating T2D through their action in both liver and muscle.



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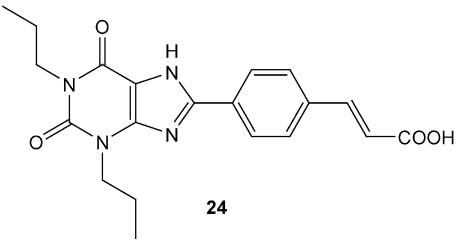

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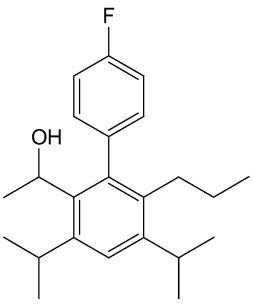
4.4. Glucagon receptor antagonists

Glucagon is a key hormone that acts as a counter regulator of the actions of insulin and, as a consequence, it contributes to insulin resistance in T2D. Glucagon is secreted by α-cells of the pancreas and it promotes hyperglycemia by increasing glycogenolysis and gluconeogenesis in liver. In T2D patients, circulating glucagon levels are normal or slightly elevated suggesting that elevated fasting glucagon levels that fail to appropriately decrease postprandially, contribute to hyperglycemia. Mice lacking glucagon receptors (GlucR $^{-/-}$ KO mice) were found to have normal glucose levels, and improved insulin sensitivity. Treatment of ob/ob mice or streptotozocin (STZ) induced diabetic rats with a glucagon monoclonal antibody (Glu-mAB) normalized or slightly lowered glucose levels. Recently, similar observations were made using a specific glucagon receptor antisense oligonucleotide (GR-ASO) [28]. Bay 27-9955, **25** a small molecule competitive glucagon receptor antagonist with moderate potency ($IC_{50} = 110$ nM) was advanced to phase I clinical trials. In healthy humans, **25** demonstrated efficacy in glucagon challenge experiments, wherein the elevated glucose levels were shown to be lower in the drug-treated group compared with the placebo group [29,30]. These results fueled further interest in this target and a selection of both competitive and non-competitive receptor antagonists such as **26**–**27** have been reported [31,32].



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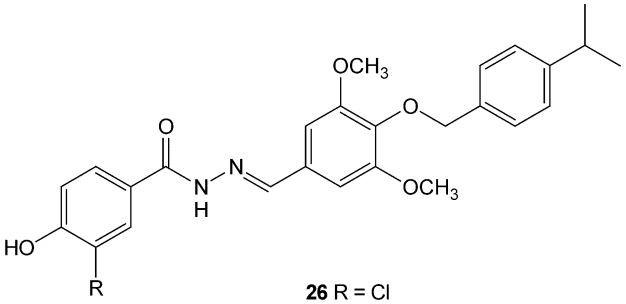
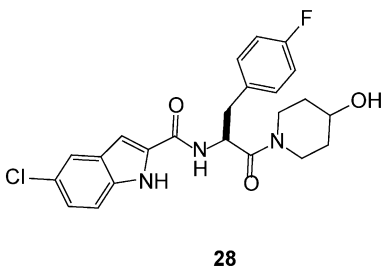


26 R = Cl

27 R = CN

4.5. Glycogen phosphorylase inhibitors

Glycogen phosphorylase is a dimeric enzyme which plays a key role in the breakdown of glycogen to glucose-1-phosphate, and its activity is modulated by signals that promote glycogen breakdown as well as its storage. The three isoforms of GP, brain, liver and muscle share about 80% homology. Inhibition of liver GP in T2D is considered to be desirable in view of its rate-limiting role in glycogenolysis and indirect inhibitory role in gluconeogenesis pathways. The activity of GP is known to be modulated by the affinity of ligands binding to its six different binding sites, thus offering multiple opportunities for its inhibition. The most interesting of these is the site which spans the GP dimer interface that was characterized by the identification of a potent inhibitor CP-320626, **28** [33]. Compound **28** was found to be efficacious at 10 mg/kg po dose in ob/ob mice [34]. Compounds from this class have been studied clinically, and preliminary results have confirmed their glucose lowering potential [35]. Other potent inhibitors of GP that bind at the allosteric effector site, **29** and the catalytic site, **30** are shown [37,38].



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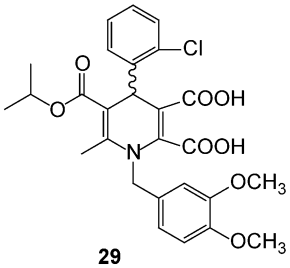

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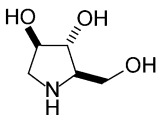

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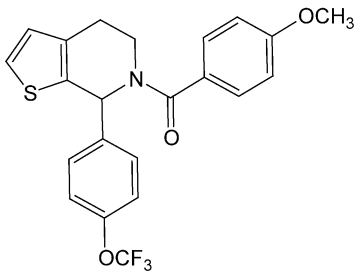
4.6. Glucose-6-phosphatase inhibitors

Glucose-6-phosphatase (G6Pase) catalyzes the terminal step in gluconeogenesis and glycogenolysis by converting glucose-6-phosphate to glucose and inorganic phosphate. G6Pase is a multicomponent enzyme, located in the endoplasmic reticulum, and has a wide tissue distribution. In T2D animal models, the G6Pase activity, GTPase protein content and mRNA levels are elevated. In humans, a rare autosomal recessive genetic disorder known as Type I Von Gierke's Disease, or type 1a glycogen storage disease is caused by the absence of G6Pase activity.



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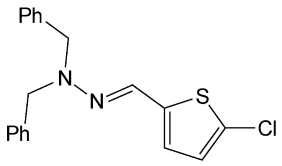
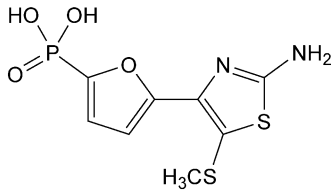


32

In these patients, hypoglycemia is observed as one of many clinical features. Two series of compounds, characterized by the tetrahydrothienopyridines, **31** (IC_{50} 350 nM) and the *N*,*N*-dibenzyl-*N*′-benzylidenehydrazines **32** (IC_{50} 170 nM), have moderate *in vitro* activity, but no *in vivo* activity has been reported [39,40].

4.7. Fructose-1,6-bisphosphatase inhibitors

Fructose-1,6-bisphosphatase (FBPase) catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate (F6P) and inorganic phosphate. FBPase is allosterically regulated by AMP and indirectly by glucagon and insulin. FBPase is a homotetramer and exists in active (R) and less active (T) states. Aminothiazole phosphinic acids (**33** IC_{50} 15 nM) and esters (**34**) represent the most potent inhibitors reported to date. The aminothiazole, **33** was shown reduce glucose levels relative to controls in db/db mice and Zucker diabetic fatty rats [41–43]. Thus, inhibitors of FBPase may provide therapeutic benefit for T2D patients, by lowering hepatic glucose production. A compound from the aminothiazole class has entered clinical trials.



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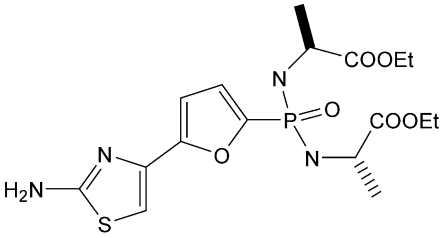
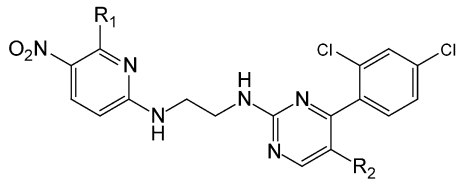
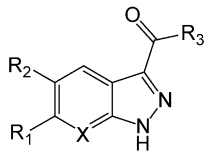


34

4.8. Glycogen synthase kinase-3 inhibitors

Glycogen synthase kinase (GSK-3) is a serine/threonine kinase that phosphorylates glycogen synthase and inhibits its activity. Conversely, inhibition of GSK3 is expected to activate glycogen synthase and promote glucose uptake into muscle. Human GSK-3 exists in two isoforms, α and β, encoded by two distinct genes, located on chromosomes 19 and 3, respectively. GSK-3 has wide tissue distribution and has multiple key biological functions. Although selective GSK-3 inhibitors with desired enzyme and tissue distribution may be beneficial in several indications, identification of sufficiently selective inhibitors has been challenging since most inhibitors are ATP site binders. Among the reported GSK-3 inhibitors, CHIR98014, **35** and CHIR98023, **36** increased glucose uptake in human skeletal muscle cell culture [36]. Other potent GSK-3 inhibitors include structures **37**–**42** [37,38]. There is a great deal of current interest in identifying GSK3 selective inhibitors with desired tissue distribution and further developments are eagerly awaited.



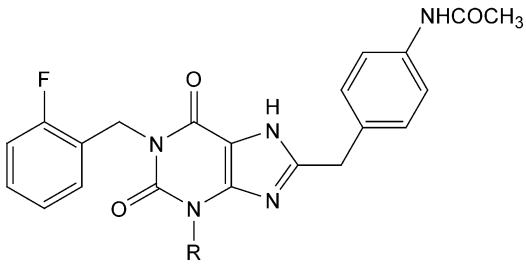


35 $R_1 = NH_2$, $R_2 = N_1$-imidazole
36 $R_1 = H$, R_2 = 2-(1H)-imidazole

37 X = N, R_1 = (3,4-di-OH)phenyl, R_2 = H, R_3 = cyclopropyl
38 X = N, R_1 = (4-OH)phenyl, R_2 = Cl, R_3 = cyclopropyl
39 X = N, R_1 = (4-OH)phenyl, R_2 = Br, R_3 = 4-piperidine-N-Me
40 X = N, R_1 = phenyl, R_2 = Br, R_3 = cyclopropyl
41 X = C, R_1 = 3-furanyl, R_2 = H, R_3 = cyclopropyl
42 X = N, R_1 = 2-thienyl, R_2 = Br, R_3 = cyclopentyl

4.9. PEPCK inhibitors

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the conversion of oxaloacetate (OAA) to phosphoenol pyruvate (PEP), the rate limiting step in gluconeogenesis. PEPCK is an intracellular enzyme and has two isoforms, PEPCK-C (cytosolic), and PEPCK-M (mitochondrial).

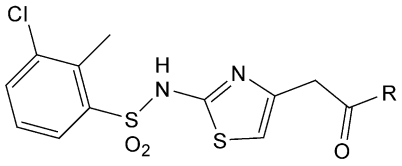


43 R = Propyl
44 R = Cyclopropylmethyl

In rodents, PEPCK-C accounts for 95% of total gluconeogenesis activity. However, in humans both isoforms are equally responsible and their functions are not interchangeable. This species difference in PEPCK activity complicates the discovery and evaluation of inhibitors. For obvious reasons, selective inhibition of PEPCK-C over the PEPCK-M form is desirable. Recently, the X-ray crystal structures of free and substrate-bound PEPCK were solved. 3-Mercaptopicolinic acid is a well known PEPCK inhibitor, and the xanthine based inhibitors, **43** and **44** were recently reported, although no in vivo data were provided [39,40]. A report on the viability of liver specific PEPCK KO mice suggests that at least in this extreme case alternative routes to glucose are utilized [41].

4.10. 11-β-Hydroxysteroid dehydrogenase-1 (11-β-HSD-1) inhibitors

In humans, the circulating levels and activity of cortisol and cortisone are tightly regulated. The enzyme 11-β-HSD-1 catalyzes the conversion of cortisone to cortisol, using NADPH as co-factor, while the reverse reaction is catalyzed by 11-β-HSD-2. Cortisol is the ligand for glucocorticoid receptors and modulates numerous biological functions, including the HPA axis. Studies using transgenic mice lacking either 11-β-HSD-1 or 11-β-HSD-2 indicated the desirability of selective inhibition of 11-β-HSD-1 to reduce hepatic glucose production, and improve glucose homeostasis. Numerous steroid based inhibitors have been discovered including glycyrrhetinic acid and carbenoxolone [42,43]. Recently, 2-aminothiazole based rat- and human-selective 11-β-HSD-1 inhibitors, **45** and **46** respectively were disclosed [44,45]. Compound **46**, was found to lower circulating glucose levels by 50–88% and insulin by 52–65% of control in ob/ob and KK-Ay mice after dosing at 200 mg/kg b.i.d for 4 days. BVT.3498, currently in Phase II clinical trials for NIDDM, is thought to belong to this class of 11-β-HSD-1 inhibitors.

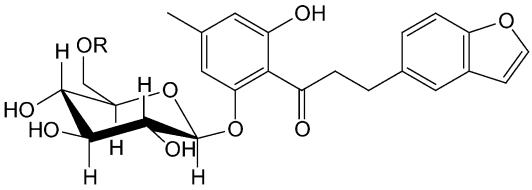


45 R = Diethylamino
46 R= 4-Methyl-1-piperazinyl

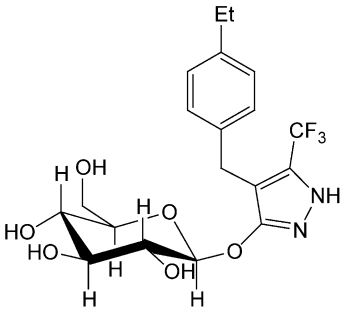
5. INHIBITORS OF GLUCOSE UPTAKE

5.1. Sodium-glucose transporter (SGLT) inhibitors

Both intestinal absorption and renal re-absorption of glucose are mediated by SGLTs. Three isoforms, SGLT-1, SGLT-2 and SGLT-3, have been reported to date. Phlorizin, a specific inhibitor of SGLTs is the earliest of the reported inhibitors to show efficacy in *in vivo* models of T2D. Based on these observations, stable analogs of phlorizin, T-1095A, **47** and the pro-drug T-1095, **48**, were evaluated in various T2D animal models. These studies suggested that **47** inhibited renal SGLTs. Thus, at 100 mg/kg po, **48** effectively suppressed renal reabsorption of glucose resulting in increased glucose excretion in urine in rats and mice. The compound was found to improve glucose homeostasis in yellow KK-mice and STZ-induced diabetic rats [46]. Recently, novel pyrazole-O-glucosides were also found to be potent inhibitors of SGLTs in vivo, as measured by development of glucosuria. For example, compound, **49**, induced 63 mg of urinary glucose excretion, at a 3 mg/kg iv dose, while at the same dose T-1095A induced 300 mg of urinary glucose excretion, in Wistar rats [47].



47 R = H
48 R = $COOCH_3$



49

6. SUMMARY AND OUTLOOK

T2D and associated morbidities are prevalent in an increasing proportion of populations of both the developed and the developing parts of the world. Major current therapies for T2D include sulfonylureas, metformin, and TZDs. Each of these therapies has limitations with regard to their efficacy or side-effect profile. Among the targets discussed in this chapter, the most advanced are those based on GLP-1 agonist activity, i.e., Exenatide, and DPPIV inhibitors. Both strategies are directed to potentiate the actions of GLP-1 on insulin secretion and have shown promise in Phase II/III clinical trials. These agents may avoid complications related to hypoglycemia and also may limit the potential for weight gain, thus complementing existing therapies. The discovery of potent PTP1B inhibitors remains a challenge; however, progress is being made and effective PTP1B inhibitors are expected to show beneficial effects in reducing insulin resistance and modulating weight gain, based on KO mice experiments. Glucokinase activation is the newest strategy disclosed. The lead molecule showed promising effects in glucose homeostasis in rodent models of T2D through a dual effect of improved glucose utilization in the liver and glucose-dependent insulin secretion by the pancreas. This strategy could offer an advantage over the current therapies where sulfonylureas and other insulin secretagogs are used. The failure of several promising new drugs in late stage clinical trials urges caution in predicting the impact of any of the new therapies, until they have been widely tested.

REFERENCES

[1] R. D. Carr, C. L. Brand, T. B. Bodvarsdottir, J. B. Hansen and J. Sturis, *Diabetes*, 2003, **52**, 2513.
[2] O. G. Kolterman, J. B. Buse, M. S. Fineman, E. Gaines, S. Heintz, T. A. Bicsak, K. Taylor, D. Kim, M. Aisporna, Y. Wang and A. D. Baron, *J. Clin. Endocrinol. Metab.*, 2003, **88**, 3082.
[3] L. B. Knudsen, P. F. Nielsen, P. O. Huusfeldt, N. L. Johansen, K. Madsen, F. Z. Pedersen, H. Thøgersen, M. Wilken and H. Agersø, *J. Med. Chem.*, 2000, **43**, 1664.
[4] E. B. Villhauer, J. A. Brinkman, G. B. Naderi, B. F. Burkey, B. E. Dunning, K. Prasad, B. L. Mangold, M. E. Russell and T. E. Hughes, *J. Med. Chem.*, 2003, **46**, 2774.

[5] D. D. Sternbach, *Ann. Rep. Med. Chem.*, 2003, **38**, 71.
[6] Annual Update 2003: endocrine and metabolic drugs, *Drugs Future*, 2003, **28**, 991.
[7] P. Y. Michellys, R. J. Ardecky, J. H. Chen, D. L. Crombie, G. J. Etgen, M. M. Faul, A. L. Faulkner, T. A. Grese, R. A. Heyman, D. S. Karanewsky, K. Klausing, M. D. Leibowitz, S. Liu, D. A. Mais, C. M. Mapes, K. B. Marschke, A. Reifel-Miller, K. M. Ogilvie, D. Rungta, A. W. Thompson, J. S. Tyhonas and M. F. Boehm, *J. Med. Chem.*, 2003, **46**, 2683.
[8] P. Y. Michellys, M. F. Boehm, J. H. Chen, T. A. Grese, D. S. Karanewsky, M. D. Leibowitz, S. Liu, D. A. Mais, C. M. Mapes, A. Reifel-Miller, K. M. Ogilvie, D. Rungta, A. W. Thompson, J. S. Tyhonas, N. Yumibe and R. J. Ardecky, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4071.
[9] G. Liu, Z. Xin, H. Liang, C. Abad-Zapatero, P. J. Hajduk, D. A. Janowick, B. G. Szczepankiewicz, Z. Pei, C. W. Hutchins, S. J. Ballaron, M. A. Stashko, T. H. Lubben, C. E. Berg, C. M. Rondinone, J. M. Trevillyan and M. R. Jirousek, *J. Med. Chem.*, 2003, **46**, 3437.
[10] Z. Xin, G. Liu, C. Abad-Zapatero, Z. Pei, B. G. Szczepankiewicz, X. Li, T. Zhang, C. W. Hutchins, P. J. Hajduk, S. J. Ballaron, M. A. Stashko, T. H. Lubben, J. M. Trevilliyan and M. R. Jirousek, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3947.
[11] G. Liu, Z. Xin, Z. Pei, P. J. Hajduk, C. Abad-Zapatero, C. W. Hutchins, H. Zhao, T. H. Lubben, S. J. Ballaron, D. L. Haasch, W. Kaszubska, C. M. Rondinone, J. M. Trevillyan and M. R. Jirousek, *J. Med. Chem.*, 2003, **46**, 4232.
[12] C. Dufresne, P. Roy, Z. Wang, E. Asante-Appiah, W. Cromlish, Y. Boie, F. Forghani, S. Desmarais, Q. Wang, K. Skorey, D. Waddleton, C. Ramachandran, B. P. Kennedy, L. Xu, R. Gordon, C. C. Chan and Y. Leblanc, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1039.
[13] B. A. Zinker, C. M. Rondinone, J. M. Trevillyan, R. J. Gum, J. E. Clampit, J. F. Waring, N. Xie, D. Wilcox, P. Jacobson, L. Frost, P. E. Kroeger, R. M. Reilly, S. Koterski, T. J. Opgenorth, R. G. Ulrich, S. Crosby, M. Butler, S. F. Murray, R. A. McKay, S. Bhanot, B. P. Monia and M. R. Jirousek, *Proc. Natl Acad. Sci. USA*, 2002, **99**, 11357.
[14] R. Kurukulasuriya, J. T. Link, D. J. Madar, Z. Pei, S. J. Richards, J. J. Rohde, A. J. Souers and B. G. Szczepankiewicz, *Curr. Med. Chem.*, 2003, **10**, 123.
[15] R. M. Mayers, R. J. Butlin, E. Kilgour, B. Leighton, D. Martin, J. Myatt, J. P. Orme and B. R. Holloway, *Biochem. Soc. Trans.*, 2003, **31**, 1165.
[16] G. R. Bebernitz, T. D. Aicher, J. L. Stanton, J. Gao, S. S. Shetty, D. C. Knorr, R. J. Strohschein, J. Tan, L. J. Brand, C. Liu, W. H. Wang, C. C. Vinluan, E. L. Kaplan, C. J. Dragland, D. DelGrande, A. Islam, R. J. Lozito, X. Liu, W. M. Maniara and W. R. Mann, *J. Med. Chem.*, 2000, **43**, 2248.
[17] W. R. Mann, C. J. Dragland, C. C. Vinluan, T. R. Vedananda, P. A. Bell and T. D. Aicher, *Biochim. Biophys. Acta*, 2000, **1480**, 283.
[18] B. K. Sorensen, J. T. Link, T. von Geldern, M. Emery, J. Wang, B. Hickman, M. Grynfarb, A. Goos-Nilsson and S. Carroll, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2307.
[19] R. Sarabu, *Expert Opin. Investig. Drugs*, 2003, **12**, 1721.
[20] T. W. von Geldern, J. T. Link, N. Tu, P. R. Kym, C. Lai, S. J. Richards, P. B. Jacobson, R. D. Bishop and D. B. Gates, *WO Patent* 2004000869, 2004.
[21] J. Lynge and Y. Hellsten, *Acta. Physiol. Scand.*, 2000, **169**, 283.
[22] A. K. Dixon, A. K. Gubitz, D. J. Srinathsinghui, D. J. Richardson and T. C. Freeman, *Br. J. Pharmacol.*, 1996, **118**, 1461.
[23] R. A. Challiss, S. J. Richards and L. Budohoski, *Eur. J. Pharmacol.*, 1992, **226**, 121.
[24] H. Harada, O. Asano, Y. Hoshino, S. Yoshikawa, M. Matsukura, Y. Kabasawa, J. Niijima, Y. Kotake, N. Watanabe, T. Kawata, T. Inoue, T. Hortzoe, N. Yasuda, H. Minami, K. Nagata, M. Murakami, J. Nagaoka, S. Kobayashi, I. Tanaka and S. Abe, *J. Med. Chem.*, 2001, **44**, 170.
[25] H. Harada, O. Asano, T. Kawata, T. Inoue, T. Horizoe, N. Yasuda, K. Nagata, M. Murakami, J. Nagaoka, S. Kobayashi, I. Tanaka and S. Abe, *Bioorg. Med. Chem. Lett.*, 2003, **9**, 2709.
[26] G. H. Crist, B. Xu, K. F. LaNoue and C. H. Lang, *FASEB J.*, 1998, **12**, 1301.
[27] K. F. LaNoue, G. H. Crist and J. M. Linden, *US Patent* 6 060 481, 2000.
[28] Y. Liang, M. C. Osborne, B. P. Monia, S. Bhanot, W. A. Gaarde, C. Reed, P. She, T. L. Jetton and K. T. Demarest, *Diabetes*, 2004, **53**, 410.
[29] K. F. Petersen and J. T. Sullivan, *Diabetologia*, 2001, **44**, 2018.

[30] W. R. Schoen, G. H. Ladouceur, J. H. Cook, T. G. Lease, D. J. Wolanin, R. H. Kramss, D. L. Hertzog and M. H. Osterhout, *US Patent* 6 218 431 B1, 2001.
[31] A. Ling, Y. Hong, J. Gonzalez, V. Gregor, A. Polinsky, A. Kuki, S. Shi, K. Teston, D. Murphy, J. Porter, D. Kiel, J. Lakis, K. Anderes, J. May, L. B. Knudsen and J. Lau, *J. Med. Chem.*, 2001, **44**, 3141.
[32] A. Ling, M. Plewe, J. Gonzalez, P. Madsen, C. K. Sams, J. Lau, V. Gregor, D. Murphy, K. Teston, A. Kuki, S. Shi, L. Truesdale, D. Kiel, J. May, J. Lakis, K. Anderes, E. Iatsimirskaia, U. G. Sidelmann, L. B. Knudsen, C. L. Brand and A. Polinsky, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 663.
[33] N. G. Oikonomakos, V. T. Skamnaki, K. E. Tsitsanou, N. G. Gavalas and L. N. Johnson, *Structure (London)*, 2000, **8**, 575.
[34] D. J. Hoover, S. Lefkowitz-Snow, J. L. Burgess-Henry, W. H. Martin, S. J. Armento, I. A. Stock, R. K. McPherson, P. E. Genereux, E. M. Gibbs and J. L. Treadway, *J. Med. Chem.*, 1998, **41**, 2934.
[35] J. L. Treadway, P. Mendys and D. J. Hoover, *Exp. Opin. Invest. Drugs*, 2001, **10**, 439.
[36] S. E. Nikoulina, T. P. Ciaraldi, S. Mudaliar, L. Carter, K. Johnson and R. R. Henry, *Diabetes*, 2002, **51**, 2190.
[37] J. Witherington, V. Bordas, A. Gaiba, N. S. Garton, A. Naylor, A. D. Rawlings, B. P. Slingsby, D. G. Smith, A. K. Takle and R. W. Ward, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3055.
[38] J. Witherington, V. Bordas, A. Gaiba, A. Naylor, A. D. Rawlings, B. P. Slingsby, D. G. Smith, A. K. Takle and R. W. Ward, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3059.
[39] L. H. Foley, P. Wang, P. Dunten, G. Ramsey, M. Gubler and S. J. Wertheimer, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3871.
[40] L. H. Foley, P. Wang, P. Dunten, G. Ramsey, M. Gubler and S. J. Wertheimer, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3607.
[41] P. She, S. C. Burgess, M. Shiota, P. Flakoll, E. P. Donahue, C. R. Malloy, A. D. Sherry and M. A. Magnuson, *Diabetes*, 2003, **52**, 1649.
[42] M. Hult, H. Jornvall and U. C. T. Oppermann, *FEBS Lett.*, 1998, **441**, 25.
[43] S. Diederich, C. Grossmann, B. Hanke, M. Quinkler, M. Herrmann, V. Bahr and W. Oelkers, *Eur. J. Endocrinol.*, 2000, **142**, 200.
[44] T. Barf, J. Vallgarda, R. Emond, C. Haggstrom, G. Kurz, A. Nygren, V. Larwood, E. Mosialou, K. Axelsson, R. Olsson, L. Engblom, N. Edling, Y. Ronquist-Nii, B. Ohman, P. Alberts and L. Abrahmsen, *J. Med. Chem.*, 2002, **45**, 3813.
[45] P. Alberts, C. Nilsson, G. Selen, L. O. M. Engblom, N. H. M. Edling, S. Norling, G. Klingstrom, C. Larsson, M. Forsgren, M. Ashkzari, C. E. Nilsson, M. Fiedler, E. Bergqvist, B. Ohman, E. Bjorkstrand and L. B. Abrahmsen, *Endocrinology*, 2003, **144**, 4755.
[46] K. Tsujihara, M. Hongu, K. Saito, H. Kawanishi, K. Kuriyama, M. Matsumoto, A. Oku, K. Ueta, M. Tsuda and A. Saito, *J. Med. Chem.*, 1999, **42**, 5311.
[47] K. Ohsumi, H. Matsueda, T. Hatanaka, R. Hirama, T. Umemura, A. Oonuki, N. Ishida, Y. Kageyama, K. Maezono and N. Kondo, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2269.

Recent Progress in Histamine H_3 Receptor Chemistry

Robert Aslanian and Neng-Yang Shih

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

Contents

1. INTRODUCTION

It has been over twenty years since the pioneering work of Arrang and co-workers demonstrated that histamine inhibited its own release from rat brain cortical slices via a novel receptor that is pharmacologically distinct from both the H_1 and H_2 receptors [1]. It is now well recognized that this third histamine receptor, the H_3 receptor, is a G-protein coupled receptor that functions both as an autoreceptor, controlling the release of histamine from histaminergic neurons, as well as a heteroreceptor, controlling the release of other neurotransmitters such as serotonin and noradrenalin. In the intervening years since the discovery of the H_3 receptor, considerable effort has gone into preparing novel H_3 receptor ligands and the field has evolved from one dominated by imidazole-derived analogs, with their attendant pharmacokinetic and toxicological issues, to one in which there are now several classes of non-imidazole compounds. Additionally, the potential therapeutic utility of these molecules in the treatment of disease, particularly CNS related disorders, is now starting to be tested in human clinical trials. The goal of this report is to update the current state of the field of H_3 receptor medicinal chemistry.

2. NON-IMIDAZOLE H_3 RECEPTOR ANTAGONISTS

The imidazole analog thioperamide, **1**, was the first selective H_3 antagonist identified [2], and most of the early literature in the field described compounds that were also 4-substituted imidazoles. However, 4-substituted imidazoles are not ideal drug

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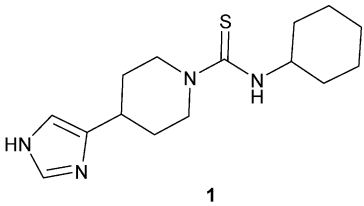
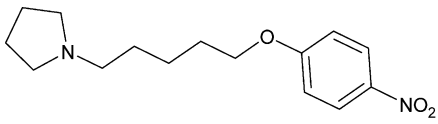


1

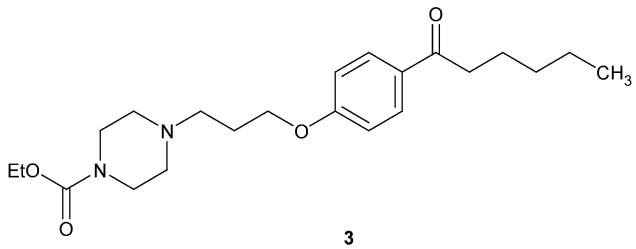
candidates for a number of reasons. For example, the imidazole moiety is an excellent ligand for the iron present in the heme group of the cytochrome P450 isozymes. Consequently, many 4-substituted imidazole analogs are potent inhibitors of these critical metabolic enzymes [3]. Additionally, the H_3 receptor is located primarily in the CNS and most of the proposed therapeutic applications for agonists and antagonists are in the treatment of CNS-related diseases. Therefore, good brain penetration is imperative for activity. However, many imidazole containing analogs do not display high brain levels due to the hydrogen bond donor and acceptor properties of the imidazole ring [4].

As a result of these issues, a significant amount of effort has been expended by a number of research groups in academia and industry on identifying ligands for the H_3 receptor that do not incorporate a 4-substituted imidazole ring. This work eventually led to the identification of the phenolic ether class of H_3 receptor antagonists, exemplified by **2**, as the first potent, non-imidazole H_3 receptor antagonist [4]. The general structure given by **2**, a tertiary amine, preferably cyclic, tethered to a phenolic ether via a hydrophobic chain is a common pharmacophore for the H_3 receptor, and this motif is repeated in a number of different series of compounds from several labs.



2

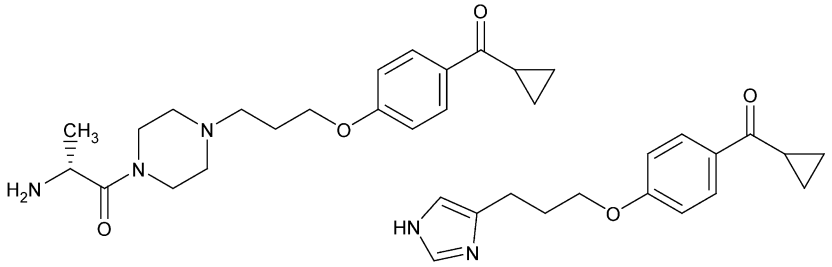
For example, high throughput screening of the Abbott compound collection led to the identification of the piperazine amide A-923 (**3**) as a high affinity ligand ($K_i = 2$ nM) for the rat H_3 receptor although it displayed poor oral bioavailability and selectivity [5].



3

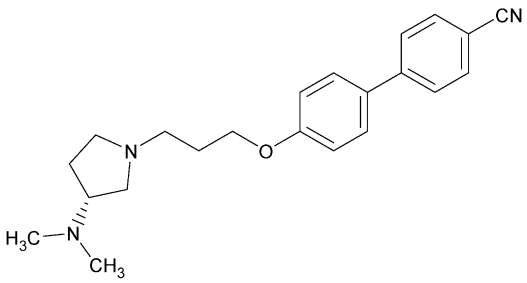
Systematic optimization of this lead led to the identification of a cyclopropyl ketone series exemplified by **4** [6]. Interestingly, this series of analogs incorporates the same tail group as ciproxifan (**5**) so it appears that the piperazine amide may be acting as a surrogate for the imidazole ring. Like ciproxifan, compound **4** was a more potent ligand for the rat full-length receptor than for the human full-length receptor and acts as an inverse agonist [7]. Compound **4** was also active in a variety of *in vivo* CNS models [8]. For example, rat pups treated with **4** (10 mg/kg, s.c.) showed improved cognitive

performance in an avoidance test while social memory was improved in adult rats treated with 3 and 10 mg/kg, i.p. [8].



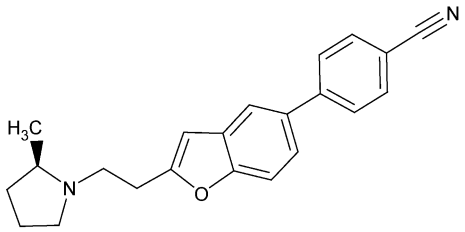
4 **5**

Further work in this series identified the biphenyl nitrile **6** [9]. Unlike the piperazine amide **4**, this compound is a potent ligand for both the rat and human H_3 receptor ($pK_{i\ rat} = 7.87$, $pK_{i\ human} = 8.56$) and is selective for the H_3 receptor over the H_1 and H_2 receptors. Furthermore, **6** was the first non-imidazole H_3 antagonist to show weight loss in a diet-induced obesity model [10]. In a 28-day study in mice fed a high fat diet, **6** (15 mg/kg) reduced weight to a level comparable to mice fed a low fat diet. Total body fat as measured in the fat pads was reduced and the animals displayed normal insulin tolerance. Consistent with a central mode of action, **6** attains extremely high concentrations in the brain (brain/plasma = 160 at 1 h following 5 mg/kg i.v. administration).



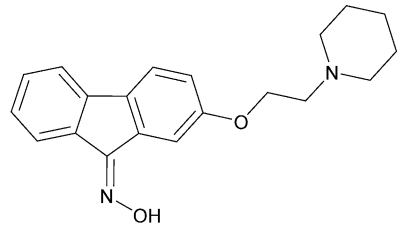
6

This lead structure was further optimized. In an attempt to prepare more rigid analogs and thus potentially improve the pharmacokinetic and selectivity profiles of the series, benzofuran analog **7** was obtained by cyclization of the alkyl ether chain back onto the central phenyl ring [11]. Compound **7** is a potent ligand for the human and rat H_3 receptors ($K_i = 0.45$ and 3.22 nM, respectively) and displays excellent brain concentrations after i.v. dosing (brain/plasma > 30). This compound is reported to be in Phase I clinical trials for the treatment of cognition related disorders.



7

This privileged H_3 pharmacophore has appeared in several other series of H_3 receptor ligands. Oxime **8** is structurally related to **6** in that the biphenyl moiety of **6** is now linked via a five-membered ring. In this case, the *Z*-oxime is essential for good H_3 activity ($K_i = 65$ nM). The *E*-oxime is approximately tenfold less active [12]. Imidazopyridine **9**, which was also derived from a lead identified via high throughput screening, is a potent ligand for the human H_3 receptor ($K_i = 2$ nM) with good selectivity over the H_1, H_2, and H_4 receptors as well as more than 50 other biogenic amine and neuropeptide receptors. Compound **9** also displayed good oral pharmacokinetics after dosing in the rat [13]. Sequential removal of each of the nitrogen atoms of **9** gives either an indolizine or indole analog, respectively [14]. The indolizine analogs were more potent than the corresponding indoles with the 3-methyl analog **10** being equipotent to **9** ($K_i = 2$ nM).



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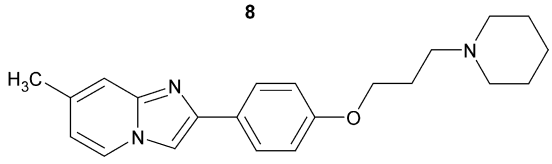

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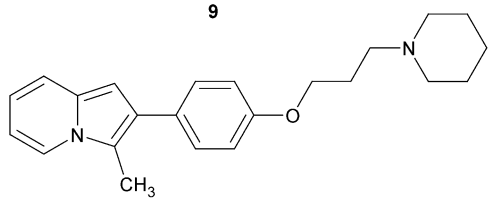
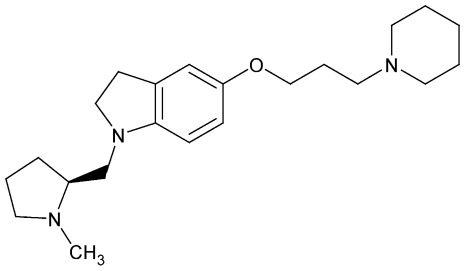


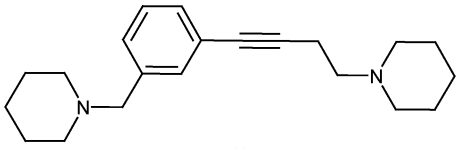
10

A number of other groups have also reported variations on this theme. For example, compound **11** is a potent ligand for the H_3 receptor ($K_i = 0.5$ nM) [15].



11

Not all non-imidazole H_3 antagonists fit this pharmacophore. A structurally related but novel series of piperidine alkynes exemplified by structure **12** has been prepared [16]. In this case an alkyne moiety has replaced the ether linker. Optimum binding activity seems to reside in those analogs with a basic tertiary amine on the 4-position of the butynyl chain. For example, compound **12** ($pK_a \approx 10.1$) binds to the H_3 receptor with subnanomolar affinity ($K_i = 0.8$ nM). The corresponding morpholine analog **13** ($pK_a \approx 7.4$) is a significantly weaker ligand ($K_i = 15$ nM). While the 4-position of the butynyl group seems to be sensitive to substitution, the 3-position of the central phenyl ring is more tolerant. For example, the left-hand piperidine of **12** can be replaced with the much bulkier indanyl amine as in **14** and still retain good binding affinity ($K_i = 1.3$ nM).



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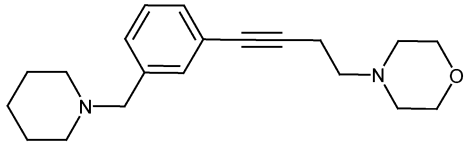

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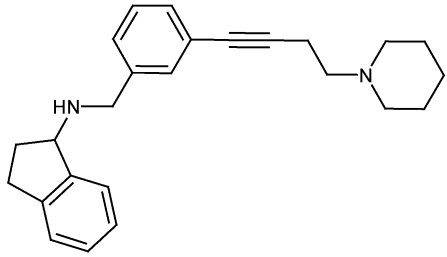
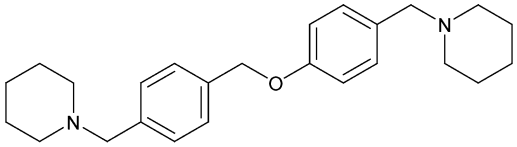


14

Pseudo-symmetric analog **15** represents another series of unique non-imidazole H_3 antagonists [17]. Compound **15** is a potent ligand for the H_3 receptor

($K_i = 2.8$ nM) although it was relatively weak *in vivo* in the mouse when dosed orally ($ED_{50} = 9.6$ mg/kg).

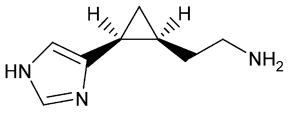


15

3. IMIDAZOLE DERIVED H_3 RECEPTOR AGONISTS AND ANTAGONISTS

3.1. Selective H_3 receptor agonists

Although there has been noteworthy progress in the discovery of non-imidazole H_3 receptor antagonists, significant interest still exists in imidazole based analogs as well. The discovery of the histamine H_4 receptor which shares an overall 43% identity homology to the histamine H_3 receptor has rekindled interest in discovery of highly selective H_3 receptor ligands [18]. Recently, a cyclopropane-based conformationally restricted analog of histamine, **16**, was found to be a potent H_3 receptor agonist which exhibits very good binding affinity ($K_i = 1.3$ nM) and functional activity ($EC_{50} = 10$ nM) but has virtually no effect on the H_4 receptor [19]. SAR investigations on immepip **17** ($pK_i = 9.32$; $pEC_{50} = 9.88$), a potent H_3 receptor agonist of moderate selectivity (46 fold favoring the H_3 receptor), resulted in the identification of olefin analogs **18** and **19** which exhibit decreased affinity and functional activity at the H_3 receptor ($pK_i = 8.23$ and 8.40; $pEC_{50} = 8.50$ and 8.63, respectively) but increased selectivity for the H_3 receptor over the H_4 receptor (300 and 700 fold, respectively) [20]. Alternatively, replacement of the piperidine ring of **17** with a 4-pyridine resulted in the discovery of a potent and highly selective H_3 receptor agonist, immethridine **20** ($pK_i = 9.07$, $pEC_{50} = 9.74$), which exhibits 300-fold H_3 receptor selectivity over the H_4 receptor [21]. In the search for novel H_3 receptor agonists, which lack a basic moiety in the side chain of the molecule in order to improve pharmacokinetic properties, a novel chiral carbamate **21** was prepared. Compared to BP294, a prodrug of (*R*)-α-methylhistamine, compound **21** was significantly more efficacious in all tissues investigated in the capsaicin-induced plasma extravasation models in rats ($ED_{50} = 0.07–0.1$ mg/kg p.o.) [21,22].



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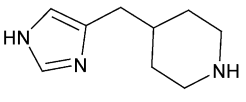
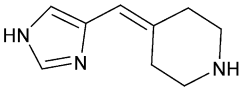


17 (Immepip)



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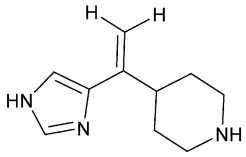

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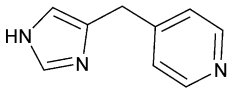

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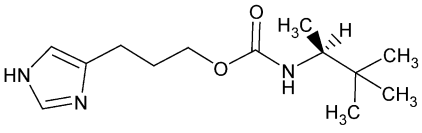
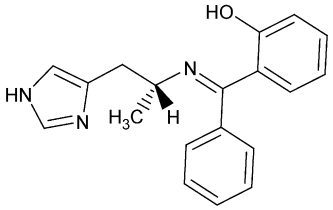


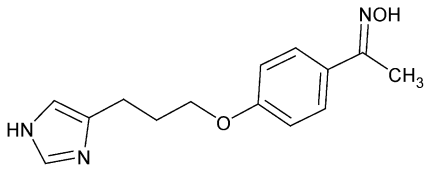
21 (S-)



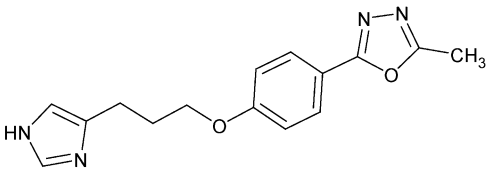
22 (BP 2-94) (R-)

3.2. Imidazole H_3 receptor antagonists

The proxifan class, compounds containing an imidazole heterocycle connected by a three-carbon alkyl chain to a *para*-substituted phenyl ether moiety, was previously reported to contain numerous potent and orally active H_3 antagonists, e.g., ciproxifan [5] ($K_i = 0.49$ nM; $ED_{50} = 0.14$ mg/kg p.o.) and imoproxifan (**23**) ($K_i = 0.26$ nM; $ED_{50} = 0.034$ mg/kg p.o. in mice) [23,24]. Recent work on the design of novel heterocyclic proxifan analogs has led to the identification of oxadiazole derivatives which displayed reduced potencies compared to those of ciproxifan and imoproxifan, but retain high *in vivo* efficacies, e.g., compound **24** ($K_i = 13$ nM; $ED_{50} = 0.57$ mg/kg p.o.) [25]. In an effort to discover a novel and selective H_3 antagonist that can be used in combination with an H_1 antihistamine for the treatment of nasal congestion, a novel series of H_3 receptor antagonists was prepared by incorporating urea and carbamate linkers to the 4-benzyl-(1*H*-imidazole-4-yl) template. The urea **25** is a potent H_3 antagonist ($K_i = 4$ nM) and it demonstrated excellent oral plasma levels in the rat (AUC = 18.1 μg h/ml at 10 mg/kg p.o.) and monkey (AUC = 12.6 μg h/ml at 3 mg/kg p.o.) [26].



23 (Imoproxifan)



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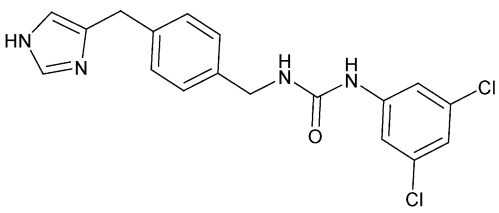

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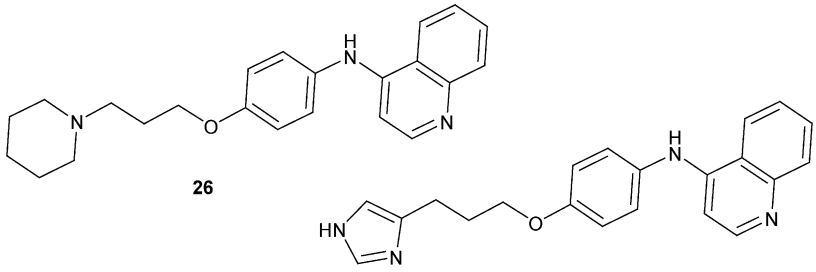
4. HISTAMINE H_3 ANTAGONISTS WITH A DUAL MODE OF ACTION

Three series of compounds which combine H_3 antagonism and another pharmacological profile in one molecule were recently reported. In contrast to the combined application of two molecules, these hybrid molecules have the advantage of a single pharmacokinetic and toxicological profile. In search of novel ways to enhance histaminergic neurotransmission in the central nervous system, a novel class of aminoquinoline histamine H_3 receptor ligands was developed that simultaneously possessed strong inhibitory activity on the main histamine metabolizing enzyme, histamine *N*-methyltransferase (HMT) as well as H_3 antagonist activity. Non-imidazole **26** (K_i (H_3) = 0.09 nM; IC_{50} (HMT) = 51 nM) and imidazole **27** (K_i (H_3) = 4.1 μM; IC_{50} (HMT) = 24 nM) exhibit dual H_3 antagonistic and HMT inhibitory activity [27,28]. Combining the first generation H_1 antihistamine chlorpheniramine with H_3 ligands of the alkylamine type has led to the discovery of dual ligands of the H_1 and H_3 receptors, e.g., **28** (K_i (H_1) = 7 nM; K_i (H_3) = 15 nM). Compounds such as **28** may be useful for the treatment of allergies and nasal congestion [29]. Nitric oxide (NO) is a recently discovered endogenous messenger. There is strong evidence that furoxan system (1,2,5-oxadiazole, 2-oxide) is able to release NO under the action of thiol cofactors [30]. Consequently, a series of H_3 antagonists endowed with NO-donor properties were designed by coupling the H_3 antagonist SKF91486 with the NO-donor furoxan moieties. Although behaving only as a weak partial H_2 agonist, compound **29** ($pA_2(H_3)$ = 7.02; pD_2 = 5.28) was able to trigger a dual NO-dependent muscle relaxation and H_3-antagonistic effect on guinea-pig ileum [31].



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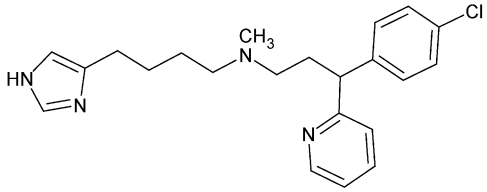

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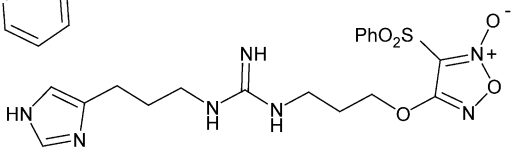

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REFERENCES

[1] J.-M. Arrang, M. Garbage and J.-C. Schwartz, *Nature*, 1983, **302**, 832.

[2] J.-M. Arrang, M. Garbage, J.-C. Lancelot, J.-M. Lecomte, H. Pollard, M. Robba, W. Schunack and J.-C. Schwartz, *Nature*, 1987, **327**, 117.

[3] P. R. Ortiz de Montellano and M. Almira Correia, in *Cytochrome P450, Structure, Mechanism and Biochemistry* (ed. P. R. Ortiz de Montellano), Plenum, New York, NY, 1995, p. 307.

[4] C. R. Ganellin, F. Leurquin, A. Piripitsi, J.-M. Arrang, M. Garbage, X. Ligneau, W. Schunack and J.-C. Schwartz, *Arch. Pharm. Pharm. Med. Chem.*, 1998, **331**, 395.

[5] R. Faghih, W. Dwight, R. Gentles, K. Phelan, T. A. Esbenshade, L. Ireland, T. R. Miller, C.-H. Kang, G. B. Fox, S. M. Gopalakrishnan, A. A. Hancock and Y. L. Bennani, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2031.

[6] R. Faghih, W. Dwight, L. Black, H. Liu, R. Gentles, K. Phelan, T. A. Esbenshade, L. Ireland, T. R. Miller, C.-H. Kang, K. M. Krueger, G. B. Fox, A. A. Hancock and Y. L. Bennani, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2035.

[7] T. A. Esbenshade, K. M. Krueger, T. R. Miller, C. H. Kang, L. I. Denny, D. G. White, B. B. Yao, G. B. Fox, R. Faghih, Y. L. Bennani, M. Williams and A. A. Hancock, *J. Pharmacol. Exp. Ther.*, 2003, **305**, 887.

[8] G. B. Fox, J. B. Pan, R. J. Radek, A. M. Lewis, R. S. Bitner, T. A. Esbenshade, R. Faghih, Y. L. Bennani, M. Williams, B. B. Yao, M. W. Decker and A. A. Hancock, *J. Pharmacol. Exp. Ther.*, 2003, **305**, 897.

[9] R. Faghih, W. Dwight, A. Vasudeven, J. Dinges, S. E. Conner, T. A. Esbenshade, Y. L. Bennani and A. A. Hancock, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3077.

[10] A. Hancock, Y. L. Bennani, E. N. Bush, T. A. Esbenshade, R. Faghih, G. B. Fox, P. Jacobson, V. K. Segel, K. M. Krueger, M. E. Nuss, J. B. Pan, R. Shapiro, D. G. Witte and B. B. Yao, *Eur. J. Pharmacol.*, 2004, **183**, 487.

[11] M. Cowart, J. K. Pratt, A. O. Stewart, Y. L. Bennani, T. A. Esbenshade and A. A. Hancock, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 689.

[12] P. C. Ting, J. F. Lee, M. M. Albanese, W. C. Tom, D. M. Solomon, R. Aslanian, N.-Y. Shih and R. West, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2643.

[13] C. Shah, L. McAtee, J. G. Breitenbucher, D. Rudolph, X. Li, T. W. Lovenberg, C. Mazur, S. J. Wilson and N. I. Carruthers, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3309.

[14] W. Chai, J. G. Breitenbucher, A. Kwok, X. Li, V. Wong, N. I. Carruthers, T. W. Lovenberg, C. Mazur, S. J. Wilson, F. U. Axe and T. K. Jones, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1767.

[15] L. S. Beavers, D. R. Finley, R. A. Gadski, P. A. Hipskind, C. D. Jesudason, R. T. Pickard and F. C. Stevens, *WO Patent* 026837-A2, 2004.

[16] R. Apodaca, W. Xiao and J. A. Jablonoski, *WO Patent* 050099-A1, 2003.

[17] T. Miko, X. Ligneau, H. H. Pertz, R. Ganellin, J.-M. Arrang, J.-C. Schwartz, W. Schunack and H. Stark, *J. Med. Chem.*, 2003, **46**, 1523.
[18] T. Nguyen, D. A. Shapiro, S. R. George, V. Setola, D. K. Lee, R. Cheng, L. Rauser, S. P. Lee, K. R. Lynch, B. L. Roth and B. F. O'Dowd, *Mol. Pharmcol.*, 2001, **59**, 427.
[19] Y. Kazuta, K. Hiano, K. Natsume, S. Yamada, R. Kimura, S. Matsumoto, K. Furuich, A. Matsuda and S. Shuto, *J. Med. Chem.*, 2003, **46**, 1980.
[20] R. Kitbunnadaj, O. P. Zuiderveld, I. J. P. DeEsch, R. C. Vollinga, R. Bakker, M. Lutz, A. L. Spek, E. Cavoy, M. Deltent, W. M. P. B. Menge, H. Timmerman and R. Leurs, *J. Med. Chem.*, 2003, **46**, 5445.
[21] R. Kitbunnadaj, O. P. Zuiderveld, B. Christophe, S. Hulscher, W. M. P. B. Menge, E. Gelens, E. Srip, R. A. Bakker, S. Celanire, M. Gillard, P. Talaga, H. Timmerman and R. Leurs, *J. Med. Chem.*, 2004, **47**, 2414.
[22] A. Sasse, X. Ligneau, A. Rouleau, S. Elz, C. R. Ganellin, J.-M. Arrange, J.-C. Schwartz, W. Schunack and H. Stark, *J. Med. Chem.*, 2002, **45**, 4000.
[23] H. Stark, B. Sadele, M. Krause, A. Huls, X. Ligneau, C. R. Ganellin, J.-M. Arrang, J.-C. Schwartz and W. Schunack, *J.Med. Chem.*, 2000, **43**, 3987.
[24] A. Sasse, B. Sadek, X. Ligneau, S. Elz, H. H. Pertz, P. Luger, C. R. Ganellin, J.-M. Arrang, J.-C. Schwartz, W. Schunack and H. Stark, *J. Med. Chem.*, 2000, **43**, 3335.
[25] S. Grabmann, B. Sadek, X. Ligneau, S. Elz, C. R. Ganellin, J.-M. Arrang, J.-C. Schwartz, H. Stark and W. Schunack, *Eur. J. Pharm. Sci.*, 2002, **15**, 367.
[26] R. Aslanian, M. W. Mutahi, N.-Y. Shih, K. D. McCormick, J. J. Piwinski, P. C. Ting, M. A. Albanese, M. Y. Berlin, X. Zhu, S.-C. Wong, S. B. Rosenblum, Y. Jiang, R. West, S. She, S. M. Williams, M. Bryant and J. Hey, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 937.
[27] J. Apelt, X. Ligneau, H. H. Pertz, J.-M. Arrang, C. R. Ganellin, J.-C. Schwartz, W. Schumack and H. Stark, *J. Med. Chem.*, 2002, **45**, 1128.
[28] S. Grabmann, J. Apelt, W. Sippl, X. Ligneau, H. H. Pertz, Y. H. Zhao, J.-M. Arrang, C. R. Ganellin, J.-C. Schwartz, W. Schunack and H. Stark, *Bioorg. Med. Chem. Lett.*, 2003, **11**, 2163.
[29] R. Aslanian, M. Mutahi, N.-Y. Shih, J. J. Piwinski, R. West, S. M. Williams, S. She, R.-L. Wu and J. Hey, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1959.
[30] K. Schonafinger, *Farmaco*, 1999, **54**, 316.
[31] M. Bertinaria, D. S. Antonella, T. Paolo, S. Giovanni, E. Poli, C. Pozzoli, G. Coruzzi, R. Frutters and A. Gasco, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1197.

Anti-Resorptive and Anabolic Bone Agents

George D. Hartman and Robert S. Meissner

Merck Research Laboratories, Department of Medicinal Chemistry,
West Point, PA 19486, USA

Contents

1. INTRODUCTION

Osteoporosis is a disease characterized by an increased risk of bone fractures that results from reduced bone mass and bone strength [1–3]. In humans, bone mass is maintained or increased through the balancing actions of continuous bone resorption and deposition. In healthy individuals, bone mass reaches a peak during early adulthood, and then begins to slowly decline with advancing age. Women, who generally have lower peak bone mass than men, experience a more rapid loss of bone following menopause. This accelerated loss of bone material is associated with an increasing risk of fracture over time. In the United States, 10 million persons are estimated to have osteoporosis, and a total of 34 million have low bone mass, indicating increased risk of disease. The estimated national direct expenditure for osteoporosis and associated fractures in 2001 was 17 billion USD.

Osteoporosis therapy seeks to maintain or increase bone mass and strength so that fracture risk is reduced. This can be done by decreasing the rate of bone resorption, increasing the rate of bone deposition, or by a combination of both actions. Several novel

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approaches for inhibition of bone resorption are detailed, including integrin $\alpha_v\beta_3$ antagonists, cathepsin K inhibitors, and inhibitors of the OPG/RANKL/RANK system. While parathyroid hormone-derived agents are the only approved therapy for the treatment of osteoporosis that work through a mechanism of increasing bone deposition, there are active research and development efforts to add to this paradigm. Those that will be reviewed include novel parathyroid hormone products, selective androgen receptor modulators (SARMs), growth hormone secretagogues, insulin-like growth factors, proteosome inhibitors, and statins.

2. BONE RESORPTION INHIBITORS

2.1. Hormone replacement therapy (HRT)

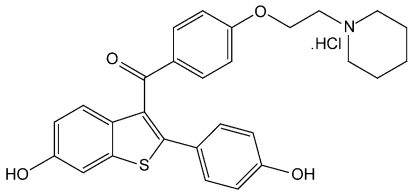
Because the decline in estrogen levels following menopause is a major cause of osteoporosis, estrogen therapy is a logical treatment. Estrogen is most often combined with progesterone in this regimen to reduce the risk of uterine cancer associated with unopposed estrogen. This combination therapy was shown to increase bone mineral density (BMD) but the effect on fracture rate was initially unclear [4,5]. The recently released results of the large, five year, multi-center Women's Health Initiative estrogen plus progesterone (E + P) study indicated that the most commonly prescribed HRT therapy reduces fractures in postmenopausal women by 24% overall, and 33% in the hip [6]. These positive results were tempered by the findings of increased incidences of dementia and ovarian cancer in the same study. Increased caution is thus now recommended when considering the use of HRT for the prevention and treatment of post-menopausal osteoporosis.

2.2. SERMs

The study and use of Selective Estrogen Receptor Modulators for the prevention and treatment of post-menopausal osteoporosis continues [7]. These compounds bind to the estrogen receptor and show agonist effects on bone, with antagonist effects on the endometrium and breast [8]. The only currently approved SERM is raloxifene **1**, which increased bone mass in the spine and hip while having no significant effect on endometrial thickness, breast pain, or hot flashes [9]. Raloxifene reduced the incidence of fracture in the spine, however, no effect on non-vertebral fractures was demonstrated [10]. Newer SERMs are currently under study in the clinic. Bazedoxifene **2** (TSE-424), lasofoxifene **3** (CP336156) and ospemifene **4** have entered late-stage clinical trials and show a beneficial effect on BMD and/or markers of bone turnover [11–14].

2.3. Bisphosphonates

The bisphosphonates are a class of drugs that bind to bone *via* a substituted $H_2O_3P-C(OH)-PO_3H_2$ backbone and inhibit resorption through inhibition of farnesyl pyrophosphate



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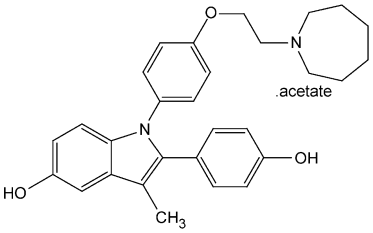

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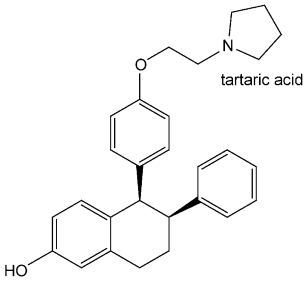

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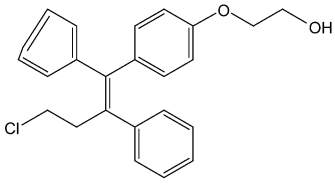

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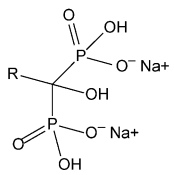
synthesis [15,16]. While bisphosphonates have low bioavailability and short plasma half-lives, the portion that is bound to bone has a long residence time. While etidronate **5a** was the first bisphosphonate to be used clinically, the aminoalkyl analogs pamidronate **5b** and alendronate **6a** soon followed, with **6a** becoming the most widely prescribed in the US. In clinical trials, **6a** increased BMD in the femoral neck, hip, and spine, and reduced the fracture rate at these locations [17]. The anti-osteoporotic effect of **6a** and risedronate **6c** was also shown to be additive to that of HRT and calcium [18,19]. Recently, however, **6a** treated patients gained no significant benefit from the inclusion of PTH therapy in their regimen [20]. Demonstration that **6a** and **6c** are effective following once-weekly dosing, has established these less-frequent dosing schedules as the standard [21,22]. The newer, more potent bisphosphonates ibandronate **6b**, minodronate **7**, and zoledronate **8** are currently in clinical trials [23,24]. Ibandronate is an oral agent that has been reported to increase spine and hip BMD in post-meonopausal women [25]. Oral **7** is under development in Japan and increases lumbar spine BMD. Intravenous **8** dosed once every three months, six months, or one year had beneficial effects on BMD in the spine and hip [26].

2.4. Integrin $\alpha_v\beta_3$ antagonists

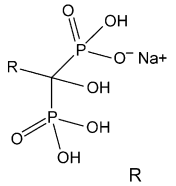
The vitronectin receptor $\alpha_v\beta_3$ is a member of the integrin superfamily of receptors that is highly expressed in osteoclast cells and has been shown to modulate osteoclast formation, adhesion and activity [27,28]. Anti-bodies to $\alpha_v\beta_3$, the peptide echistatin, and small-molecule $\alpha_v\beta_3$ antagonists inhibited bone resorption *in vitro* and prevented bone



R

5a CH_3

5b $(CH_2)_2NH_2$

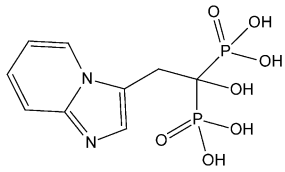


R

6a $(CH_2)_3NH_2$

6b $(CH_2)_3N(CH_3)(CH_2)_4CH_3$

6c CH_2(pyrid-3-yl)



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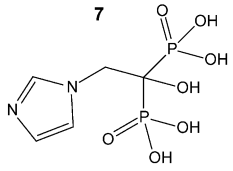

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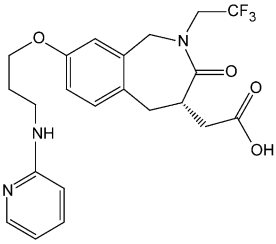
loss *in vivo* [29]. Recent reports describe significant progress toward the development of $\alpha_v\beta_3$ antagonists with drug-like attributes. The 2-benzazepine SB267268 **9** was a potent antagonist of $\alpha_v\beta_3$ in ligand displacement and osteoclast adhesion assays and had good efficacy in rats [30]. The urea MRK-4 **10** demonstrated potent antagonist activity *in vitro*, good oral bioavailability in rats, dogs and monkeys, and increased BMD in ovariectomized rats when dosed orally at 10 and 30 mp/kg. for 28 days [31]. In addition, **10** reduced urinary markers of bone resorption in rhesus monkeys by 39% over 15 days when dosed orally once daily at 15 mg/kg.



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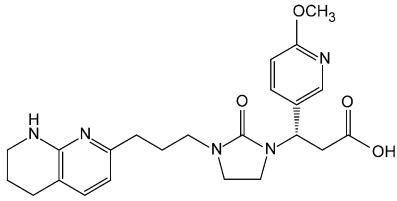

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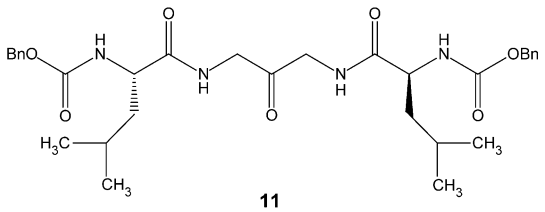
2.5. Cathepsin K inhibitors

Cathepsin K is a cysteine protease that is selectively expressed in osteoclast cells, where it plays a vital role in the degradation of bone [32]. Human deficiencies of cathepsin K are associated with abnormal stature, increased bone fracture rates, and osteosclerosis [33]. Inhibition of the enzyme with an anti-sense oligonucleotide led to decreased bone resorption, demonstrating a therapeutic proof-of concept [34]. The bis-aminoacyl ketone **11**, a selective, potent, and reversible covalent cathepsin K inhibitor, bound through a thiohemiketal formed between the ketone carbonyl and the enzyme active site Cys 25 [35]. The cyclic ketone **12** combined potency and selectivity with good oral bioavailability in rats. The central aminoazepinone of **12** was

identified as a key potency-enhancing and configurationally stable chiral constraint. The aminopyrrolidine CEL-1 **13** was a potent cathepsin K inhibitor providing a 20-fold selectivity over cathepsin L [36]. The cyanamide group of this class of compounds proved vital for the desired activity and was identified as the electrophilic 'warhead' for reaction with Cys 25. The non-peptidic biarylamide MRK-1 **14** utilizes a reactive (aminomethyl)nitrile group and reduced urinary markers of bone turnover by 80% in ovariectomized rhesus monkeys when dosed orally at 20 mpk daily [37]. Unique among the reported cathepsin K inhibitors is the arylaminoethyl amide class of agents exemplified by NVT-1 **15**, which shows good potency and selectivity despite the lack of an electrophilic warhead [38].



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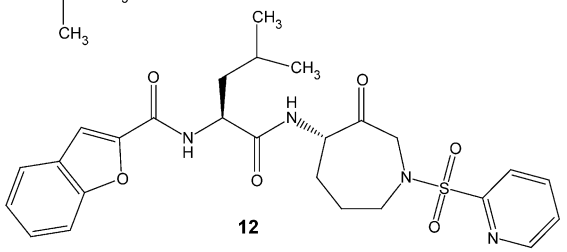

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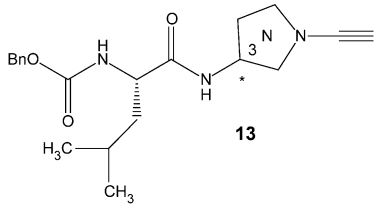

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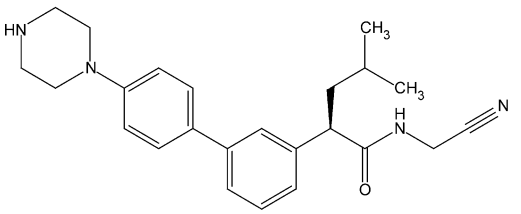

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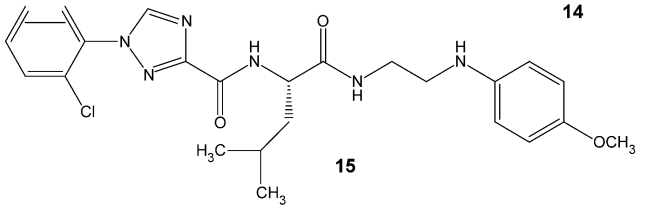

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2.6. OPG/RANKL/RANK inhibitors

Osteoprotegerin (OPG) is a naturally occurring protein that inhibits the activation of the receptor activator of NF-κB (RANK) pathway by its ligand (RANKL). The RANK pathway is important for the formation of osteoclast cells and their activation for bone

resorption [39]. OPG itself, dosed in a single SC injection to postmenopausal women, reduced urinary markers of bone resorption for 6 weeks [40]. An anti-body to RANKL, AMG 162 suppresses urinary markers of bone resorption in cynomolgus monkeys by 84–91% [41].

3. ANABOLIC BONE AGENTS (OSTEOANABOLICS)

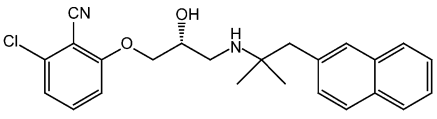
While anti-resorptive drugs have been the cornerstone of osteoporosis therapy, compounds that stimulate bone formation are already available. The potential of anabolic agents to restore and enhance bone density more substantially than anti-resorptives suggests that these agents may also reduce fracture risk to a greater degree. Comprehensive surveys on specific osteoanabolic agents have appeared [42–44].

3.1. Parathyroid hormone

Parathyroid hormone, hPTH(1-84), is the key endogenous hormone that controls calcium homeostasis. Intermittent, low doses of hPTH increase trabecular bone and cortical to a lesser extent, in animal models and humans via inhibition of osteoblast apoptosis and an increase in RANKL thus increasing both resorption and bone formation simultaneously. At a daily sc dose of 100 μg hPTH(1-84) lumbar spine BMD in postmenopausal women was increased by 7% [45–47].

The most promising PTH-derived osteoanabolic agent to date is the PTH fragment teriparatide, rhPTH(1-34) (Forteo), which was recently approved in the US for the treatment of osteoporosis in postmenopausal women with low bone density and osteoporosis. In a Phase III trial, rhPTH(1-34) at 40 μg sc increased BMD in lumbar spine and femoral neck by 9–13% and 3%, respectively [48]. Thus, the risk of new vertebral or non-vertebral fractures was reduced, compared to placebo, by 65% and 35%, respectively. Similar BMD effects were shown in men.

A related strategy for restoring bone involves enhancing secretion of endogenous PTH by inhibition of the calcium sensing receptor that is expressed in parathyroid and other cells. Administration of the calcilytic NPS-2143 **16** orally to osteopenic ovariectomized rats resulted in moderate, sustained increases in plasma PTH levels and marked increases in bone formation and resorption with no net gain or loss [49]. Combination of NPS-2143 with estrogen increased bone formation and density in rats to a greater extent than either agent alone [50]. The recent patent literature also contains reports of novel calcylitic **17** (WO 03099776).



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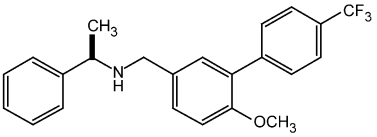

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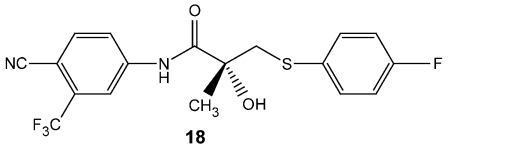
3.2. Selective androgen receptor modulators

It is well known that androgens, including dihydrotestosterone (DHT), increase periosteal bone formation in the cortical envelope and that the combination of testosterone with estrogen provides favorable effects in postmenopausal osteoporosis patients. However, the use of steroidal androgens has been limited by their potential for liver toxicity [51] and virilizing side effects including uterine/prostate hyperplasia, hirsutism, and acne. Given the proven osteoanabolic effect in man, there is tremendous potential therapeutic utility for a bone-specific, non-virilizing androgen receptor agonist [52].

Although the non-steroid anti-androgen bicalutamide **18** is well known in the treatment of hormone-dependent prostate cancer, only recently were non-steroidal androgen receptor agonists identified. Modification of bicalutamide, during a search for electrophilic ligands of the androgen receptor (AR), provided the chloroacetamido agonist **19**, which had $K_i = 1.65$ nM for AR binding and full transcriptional activity compared to DHT at 100 nM [53]. Agonist **19**, was 248-fold more potent in binding to the AR compared to its enantiomer and did not covalently (irreversibly) bind to the AR despite the potential for electrophilic reactivity [54]. Potency optimization for both AR binding and transcriptional activation was attained as shown in **20**–**22** [55].



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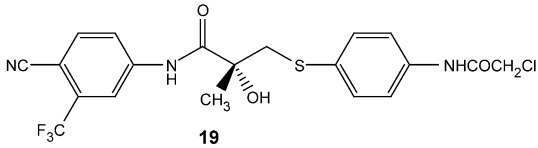

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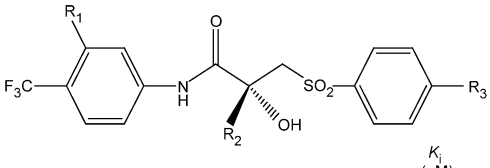
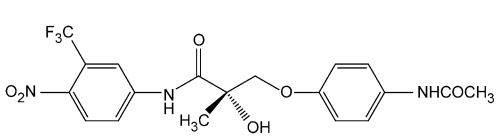


	K_i (nM)	Efficacy (% of DHT)
20 R_1 = CN, R_2 = CH_3, R_3 = F	11	100
21 R_1 = NO_2, R_2 = CH_3, R_3 = $NHCOCH_3$	9.3	500
22 R_1= CN, R_2 = CH_3, R_3 = $NHCOCH_3$	15.9	500

More recently, androxolutamide (GTx-007) **23** displayed anabolic effects on BMD when dosed at 1 mg/day sc in male and female gonadectomized rats [56]. In immature male rats, **23** showed partial agonist activity in prostate and seminal vesicles while acting as a full agonist in the levator ani muscle in castrated animals – a SARM-like profile

that suggests utility in hypogonal men. In dogs, **23** displayed dose-dependent PK and had 62% oral bioavailability at a dose of 1 mg/kg.



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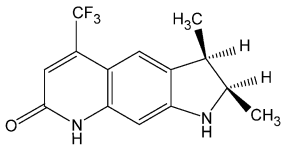

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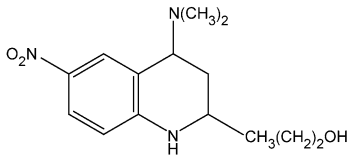
Optimization in the quinolinone series provided the novel and potent AR agonist **24** that mimicked the effects of DHT in transcription assays with similar potency and only slightly attenuated efficacy compared to DHT [57].

Using **18** as the template, tetrahydroquinoline **25** was discovered [58]. This compound bound to the AR with a similar potency to testosterone, had no measurable affinity for other nuclear receptors, and caused increases in BMD, but not prostate weight, in ORX rats after four weeks of administration. The SARM-like profile of **25** was confirmed in OVX rats where BMD and femoral cortical bone strength were increased during 2 months of dosing.



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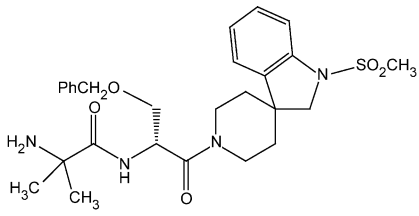

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3.3. Growth hormone secretagogues

Growth hormone levels decrease with age and restoration with the orally active GH-releasing secretagogue **26** (MK-677) is reported [59]. In postmenopausal, osteoporotic women, treatment with **26** for 16 months increased BMD in the femoral neck but not in the lumbar spine or total hip. GH-like side effects including weight gain, fluid retention, and abdominal distention were observed in addition to an increase in fasting glucose levels.

3.4. Insulin-like growth factor (IGF-1)

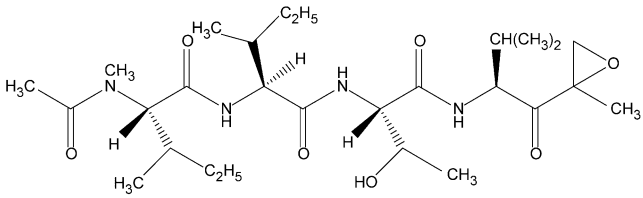
IGF-1 promotes chondrocyte and osteoblast differentiation and growth and is a key factor in bone turnover, but therapy has been limited due to side effects [60]. Beneficial effects of IGF-1 were seen in alleviating the osteopenia that is induced in OVX rats [61]. Subcutaneous infusions of rhIGF-1, in association with its binding protein IGFBP-3 at 1 mg/kg/day, for 2 months were well-tolerated and after six months post therapy BMD was significantly improved *vs* placebo [62].

3.5. Statins

Recent epidemiological data suggest that HMG coenzyme A reductase inhibitors cause a modest increase in BMD and a significant fracture risk reduction [63]. The mechanism appears to involve the stimulation of bone morphogenic protein-2 (BMP-2) *via* a reduction in Rho prenylation and a subsequent increase in eNOS [64]. Several clinical trials support an anti-resorptive bone effect for statins as shown by a decrease in bone resorption markers and no change in bone formation indices [65]. Since other large-scale, cross-sectional surveys failed to show fracture risk reduction in statin patients, further work is needed to firmly classify these agents as anabolic.

3.6. 20S proteasome inhibitors

A recent report indicates that epoxomicin **27** and other 20S proteasome inhibitors stimulated bone formation in bone organ cultures by enhancing BMP-2 expression in osteoblasts [66]. In addition, **27** increased trabecular bone volume and bone formation rates by more than 70% in mice treated for 5 days and then followed for 16 days.



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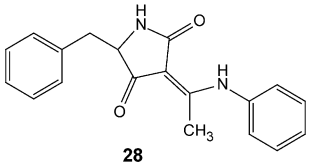
3.7. Miscellaneous

The sympathetic nervous system modulates bone metabolism *via* both impairment of osteoclast bone resorption and increased bone formation [67]. Adrenergic agonists activate β-receptors on osteoblasts and stimulate bone resorption in cultures of mouse calvariae [68]. A recent population-based study documented that β-blocker use was associated with higher BMD at the total hip and forearm and reduced fracture risk in older women [69].

A recent patent identifies compounds that are inhibitors of microtubule assembly as stimulators of bone growth [70]. Compound **28** administered sc increased BMD, bone volume, and bone formation rates in ICR Swiss mice at a dose of 8 mg/kg/day.



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4. CONCLUSION

Progress continues in the discovery and development of treatments for osteoporosis. The clinical use of bone resorption inhibitors and osteoanabolic agents is expanding and will allow for their use as single agents and in combination. These advances in the treatment of osteoporosis offer the promise of greater relief from this common and debilitating disease.

REFERENCES

[1] S. A. Doggrell, *Drugs Today*, 2003, **39**, 633.
[2] D. M. Biskobing, *Exp. Opin. Invest. Drugs*, 2003, **12**, 611–621.
[3] F. J. Lopez, *Curr. Opin. Chem. Biol.*, 2000, **4**, 383–393.
[4] T. L. Bush, H. B. Wells, M. K. James, E. Barrett-Connors, R. Marcus, S. Greendale, S. Hunsberger and J. McGowan, *J. Am. Med. Assoc.*, 1996, **276**, 1389.
[5] J. Cauley, D. Black, E. Barrett-Connor, F. Harris, K. Shields, W. Applegate and S. Cummings, *Am. J. Med.*, 2001, **110**, 442.
[6] J. Cauley, J. Robbins, Z. Che, C. Zhao, S. Cummings, R. Jackson, A. LaCroix, M. LeBoff, C. Lewis, J. McGowan, J. Neuner, M. Pettinger, M. Stefanick, J. Wactawski-Wende and N. Watts, *JAMA*, 2003, **290**, 1729.
[7] P. Albertazzi and D. Purdie, *Clin. Rheumatol.*, 2001, **15**, 451.
[8] S. Hendrixand and S. McNeeley, *Ann. NY Acad. Sci.*, 2001, **949**, 243.
[9] P. D. Delmas, N. H. Bjarnason, M. Mitlak, A.-C. Ravoux, A. S. Shah, W. J. Hustler, M. Draper and C. Christiansen, *N. Engl. J. Med.*, 1997, **337**, 1641.
[10] M. Maricic and O. Gluck, *Exp. Opin. Pharmacother.*, 2002, **3**, 767.
[11] S. Ronkin, L. Clarcke, P. Boudes and B. Constantine, *J. Bone Miner. Res.*, 2001, **16**, S413.
[12] L. A. Sorbera, P. A. Leeson and J. Castaner, *Drugs Future*, 1998, **23**, 1066.
[13] H. Z. Ke, V. M. Paralkar, W. A. Grasser, D. T. Crawford, H. Qi, H. A. Simmons, C. M. Pirie, K. L. Chidsey-Frink, T. A. Owen, S. L. Smock, H. K. Chen, W. S. S. Jee, K. O. Cameron, R. L. Rosati, T. A. Brown, P. Dasilva-Jardine and D. D. Thompson, *Endocrinology*, 1998, **139**, 2068.
[14] A. Moffitt, Symposium at International Osteporosis Foundation – World Congress on Osteoporosis, Lisbon, Portugal, 2002.
[15] C. Szabo, M. Martin and E. Oldfield, *J. Med. Chem.*, 2002, **45**, 2894.
[16] J. Dunford, K. Thompson and F. Coxon, *J. Pharm. Exp. Ther.*, 2001, **296**, 235.
[17] S. Cummings, D. Black and D. Thompson, *J. Am. Med. Assoc.*, 1998, **280**, 2077.
[18] R. Lindsay, F. Cosman and R. Lobo, *J. Clin. Endocrinol. Met.*, 1999, **84**, 3076.
[19] S. Harris, E. Eriksen and M. Davidson, *J. Clin. Endocrinol. Met.*, 2001, **86**, 1890.
[20] D. M. Black, S. L. Greenspan, L. Palermo, J. A. McGowan, T. F. Lang, P. Garnero, M. L. Bouxsein, J. P. Bilezikian and C. J. Rosen, *N. Engl. J. Med.*, 2003, **349**, 1207.
[21] M. Rossini, S. Gatti and V. Girardello, *Bone*, 2000, **27**, 119.
[22] T. Schnitzer, H. Bone and G. Crepaldi, *Aging*, 2000, **12**, 1.
[23] P. Ravn, B. Clemmensen, B. Riis and C. Christiansen, *Bone*, 1996, **19**, 527.
[24] H. Morii, Y. Nishizawa and Y. Taketani, *J. Bone Miner. Res.*, 2002, **17**, M324.
[25] M. Percherstorfer, N. Loho and S. Mirzai, *J. Bone Miner. Res.*, 2001, **16S1**, S406.
[26] I. R. Reid, J. P. Brown, P. Burckhardt, Z. Horowitz, P. Richardson, U. Trechsel, A. Widmer, J.-P. Devogelaer, J.-M. Kaufman, P. Jaeger, J.-J. Body and P. J. Meunier, *N. Engl. J. Med.*, 2002, **346**, 653.
[27] S. B. Rodan and G. A. Rodan, *J. Endocrinol.*, 1997, **154**, S47.
[28] J. Clover and R. Dodds, *J. Cell Sci.*, 1992, **103**, 267.
[29] G. D. Hartman and M. E. Duggan, *Exp. Opin. Invest. Drugs*, 2000, **9**, 1281.

[30] M. W. Lark, G. Stroup and R. D. Cousins, *J. Bone Miner.*, 1998, **23**, S219.
[31] J. J. Hutchinson, W. Halczenko, K. M. Brashear, M. J. Breslin, P. J. Coleman, L. T. Duong, M. A. Gentile, J. E. Fisher, G. D. Hartman, J. R. Huff, D. B. Kimmel, C.-T. Liu, R. S. Meissner, K. Merkle, R. Nagy, B. Pennypacker, J. J. Perkins, T. Prueksaritanont, G. Rodan, A. E. Zartman, S. Rodan and M. E. Duggan, *J. Med. Chem.*, 2003, **46**, 4790.
[32] F. H. Drake, R. A. Dodds, I. A. James, J. R. Connor, C. Debouck, S. Richardson, E. Lee-Rykaczewski, L. Coleman, D. Rieman, R. Barthlow, G. Hastings and M. Gowen, *J. Biol. Chem.*, 1996, **271**, 12511.
[33] B. D. Gelb, G.-P. Shi, H. A. Chapman and R. J. Desnick, *Science*, 1996, **273**, 1236.
[34] T. Inui, O. Ishibashi, Y. Origane, M. Kumigawa, T. Kokubo and T. Yamura, *J. Biol. Chem.*, 1997, **272**, 8109.
[35] D. S. Yamashita, W. W. Smith, B. Zhou, C. A. Janson, T. A. Tomaszek, M. J. Bossard, M. A. Levy, H.-J. Oh, T. J. Carr, S. A. Carr, M. McQueney, K. J. D'Alessio, B. U. Amegadzie, C. R. Hanning, S. Abdel-Meguid, R. L. DesJarlais, J. G. Gleason and D. F. Verber, *J. Am. Chem. Soc.*, 1997, **119**, 11351.
[36] R. Rydzewski, C. Bryant, R. Oballa, G. Wesolowski, S. Rodan, K. Bass and D. Wong, *Bioorg. Med. Chem. Lett.*, 2002, **10**, 3277.
[37] J. Robichaud, R. Oballa, P. Prasit, J.-P. Falgueyret, M. D. Percival, G. Wesolowski, S. B. Rodan, D. Kimmel, C. Johnson, C. Bryant, S. Venkatraman, E. Setti, R. Mendonca and J. T. Palmer, *J. Med. Chem.*, 2003, **46**, 3709.
[38] E. Altmann, J. Renaud, J. Green, D. Farley, B. Cutting and W. Jahnke, *J. Med. Chem.*, 2002, **45**, 2352.
[39] W. S. Simonet, D. J. Lacey, C. R. Dunstan, M. Kelley, M.-S. Chang, R. Luthy, H. Q. Nguyen, S. Wooden, L. Benett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.-L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T. M. Hughes, D. Hill, G. Van, J. Tarpley, P. Derby, R. Lee and W. J. Boyle, *Cell*, 1997, **89**, 309.
[40] P. J. Bekker, D. Holooway, A. Nakanishi, M. Arrighi, P. T. Leese and C. R. Dunstan, *J. Bone Miner. Res.*, 2001, **16**, 348.
[41] M. C. Peterson, B. J. Stouch, D. Chen, S. Baughman, A. M. DePaoli, C. R. Dunstan and S. W. Martin, International Bone and Mineral Society–Japan Bone and Mineral Society Meeting, Osaka, Japan, June 2003, abstract P275.
[42] M. R. Rubin and J. P. Bilezikian, *Curr. Opin. Rheumatol.*, 2002, **14**, 433.
[43] D. Biskobing, *Exp. Opin. Invest. Drugs*, 2003, **12**, 611.
[44] S. Doggrell, *Drugs Today*, 2003, **39**, 633.
[45] P. T. Cheng, C. Chen and K. Muller, *J. Bone Miner.*, 1995, **10**, 119.
[46] R. L. Jilka, R. S. Weinstein, T. Bellido, P. Roberson, A. M. Parfitt and S. C. Manolagas, *J. Clin. Invest.*, 1999, **104**, 439.
[47] R. S. Rittmaster, M. Bolognesse, M. P. Ettinger, D. A. Hanley, A. B. Hodsman, D. L. Kendler and C. J. Rosenet, *J. Clin. Endocrinol. Metab.*, 2000, **85**, 2129.
[48] R. M. Neer, C. D. Arnaud, J. R. Zarrchetta, R. Prince, G. A. Gaich, J. Y. Reginster, A. B. Hordsman, E. F. Ericksen, G. Ish-Shalom, H. K. Genant, O. Wang and B. H. Mitlak, *N. Engl. J. Med.*, 2001, **344**, 1434.
[49] E. F. Nemeth, E. G. Delmar, W. L. Heaton, M. A. Miller, L. D. Lambert, R. L. Conklin, M. Gowen, J. G. Gleason, P. K. Bhatnagar and J. Fox, *J. Pharmacol. Exp. Ther.*, 2001, **299**, 323.
[50] M. Gowen, G. B. Stroup, R. A. Dodds, I. E. James, B. J. Botta, B. R. Smith, P. K. Bhatnagar, A. M. Lago, J. F. Callahan, E. G. DelMar, M. A. Miller, E. F. Nemeth and J. Fox, *J. Clin. Invest.*, 2000, **105**, 1595.
[51] A. Negro-Vilar, *J. Clin. Endocrinol.*, 1999, **84**, 3459.
[52] J. Rosen and A. Negro-Vilar, *J. Musculoskel. Neuron. Interact.*, 2002, **2**, 222.
[53] J. T. Dadton, A. Mukherjee, Z. Zhu, L. Kirkovasky and D. D. Miller, *Biochem. Biophys. Res. Commun.*, 1998, **244**, 1.
[54] L. Kirkovsky, A. Mukherjee, D. Yin, J. T. Dalton and D. D. Miller, *J. Med. Chem.*, 2000, **43**, 581.
[55] Y. He, D. Yin, M. Perera, L. Kirkovsky, N. Stouran, W. Li, J. Dalton and D. D. Miller, *Eur. J. Med. Chem.*, 2002, **37**, 619.

[56] J. D. Kearbey, W. Gao, J. D. Landoll, V. Matkovic, K. Chung, D. D. Miller and J. T. Dalton, 84th Annual Meeting of the Endocrin Society, San Francisco, June 19–22, 2002, abstr P1-359.
[57] L. Zhi, C. M. Tegley, K. B. Marschke and T. K. Jones, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 1009.
[58] K. Hanada, K. Furuya, N. Yamamoto, H. Nejishima, K. Ichikawa, T. Nakamura, M. Miyakawa, S. Amano, Y. Sumita and N. Oguro, *Biol. Pharm. Bull.*, 2003, **26**, 1563.
[59] M. G. Murphy, S. Weiss, M. McClung, T. Schnitzer, K. Cerchio, J. Connor, D. Krupa and B. J. Gertz, *J. Clin. Endocrinol.*, 2001, **86**, 1116.
[60] L. Donohue and C. J. Rosen, *Proc. Soc. Exp. Biol. Med.*, 1998, **219**, 1.
[61] C. M. Bagi, E. DeLeon, R. Brommage, D. Rosen and A. Sommer, *Calcif. Tissue Int.*, 1995, **57**, 40.
[62] S. Boonen, C. Rosen, R. Bouillon, A. Sommer, M. McKay, D. Rosen, S. Adams, P. Broos, J. Lenaerts, J. Raus, D. Vanderschueren and P. Geusens, *J. Clin. Endocrinol.*, 2002, **87**, 1539.
[63] J. A. Pasco, M. A. Kotowicz, M. J. Henry, K. M. Sanders and G. C. Nicholson, *Arch. Med.*, 2002, **162**, 537.
[64] I. R. Garrett, G. Gutierrez and D. Chen, *J. Bone Miner.*, 2001, **16** (Suppl. 1), 1018.
[65] F. Cosman, J. Nieves, M. Zion, J. Cruz, S. Gordon and R. Lindsay, *J. Bone Miner.*, 2001, **16** (Suppl. 1), SA415.
[66] I. R. Garrett, D. Chen, G. Gutierrez, M. Zhao, A. Escobedo, G. Rossini, S. E. Harris, W. Gallwitz, K. B. Kim, S. Hu, C. M. Crews and G. R. Mundy, *J. Clin. Invest.*, 2003, **111**, 1771.
[67] S. Takeda, F. Elefteriou, R. Levasseur, X. Liu, L. Zhao, K. L. Parker, D. Armstrong, P. Ducy and G. Karsenty, *Cell*, 2002, **111**, 305.
[68] R. E. Moore, C. K. Smith, C. S. Bailey, E. F. Voelkel and A. H. Tashjian, *J. Bone Miner. Res.*, 1993, **23**, 301.
[69] J. A. Pasco, M. J. Henry, K. M. Sanders, M. A. Kotowicz, E. Seeman and G. C. Nicholson, *J. Bone Miner.*, 2004, **19**, 19.
[70] D. Chen, J. Rossini, Z. Gianni, M. Zhao, M. Qiao, G. R. Mundy, *WO Patent* 03059277 A2, 2003.

Inhibition of the Cysteine Protease Cathepsin K (EC 3.4.22.38)

Robert W. Marquis

GlaxoSmithKline, Collegeville PA, 19426, USA

Contents

1. INTRODUCTION

Bone is a tissue comprised of two principle constituents [1]. The first is an organic protein matrix made up of collagen fibers and other non-collagenase proteins. Approximately 90–95% of the protein matrix of bone is in the form of type 1 collagen. The second constituent is the mineral component of bone which is hydroxyapatite $[3Ca_3(PO_4)_2(OH)_2]$. This crystalline mineral component is found embedded throughout the protein matrix of the bone architecture. The equilibrium of remodeling bone tissue is a process maintained by the close interplay of two cell types. Osteoblasts are the cells that are responsible for the synthesis of new bone while osteoclasts are the principle cells mediating bone resorption. In order for effective bone resorption to take place, the osteoclast must serve the dual functions of being able to both dissolve the mineral portion of bone and enzymatically degrade the organic protein matrix. The mineral portion of bone is dissolved in the highly acidic environment maintained beneath the osteoclast in the resorption pit/lacuna. The organic matrix is degraded by proteolytic enzymes that are expressed within the osteoclast and then secreted into the resorption pit. When bone resorption by osteoclasts outpaces bone formation by osteoblasts, an overall fragility in the microarchitecture of bone results. This imbalance manifests itself clinically as osteoporosis, a disease characterized by an increased risk of bone fractures as a function

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of a reduction in bone mass. There are several treatments for osteoporosis that may be divided into two classes [2]. The first are a class of anabolic agents that act on the osteoblast to increase the rate of new bone formation. Teriparitide, the recombinant 1–34 amino acid fragment of human parathyroid hormone (h-PTH 1–84), as well as fluoride and androgen are examples of anabolic bone forming agents. The second class of agents used to treat osteoporosis is antiresorptives that serve to slow the rate of bone resorption. Here, examples include the use of supplemental calcium, vitamin D and its analogs, estrogen, selective estradiol receptor modulators (SERMs), calcitonin and bisphosphonates. Recently, the enzyme responsible for the degradation of the protein matrix of bone has been identified as the cysteine protease cathepsin K. Inhibition of this protease is thought to be an excellent antiresorptive target for the treatment of osteoporosis. This review will focus on the identification and characterization of cathepsin K as well as on the development of several classes of potent inhibitors of this protease as possible therapeutic agents to treat diseases of excess bone resorption such as osteoporosis.

2. CATHEPSIN K

Preceding the discovery of cathepsin K it had been thought that the proteolytic enzyme(s) utilized by the osteoclast to degrade the protein matrix of bone were the cysteine proteases cathepsins L, S and/or B. This thinking had arisen from several *in vitro* and *in vivo* studies that had utilized promiscuous, nonselective inhibitors of cysteine proteases to slow resorption. By measuring levels of hydroxyproline production following application of PTH to mouse calvaria, it was demonstrated that treatment with the known cysteine protease inhibitors leupeptin (**18**), Tos-Lys-CH_2Cl or Cbz-Phe-Ala-CHN_2 resulted in a 50% reduction in the loss of hydroxyproline, suggesting that the degradation of the protein matrix of bone is mediated by cysteine proteases [3]. In a similarly designed experiment, the epoxide inhibitor E-64 (**2**) was shown to inhibit bone resorption on mouse calvaria. Following i.p administration of a 2 mg dose of **2** in the mouse an 8% drop of serum calcium levels as well as a 50% drop of urinary hdroxyproline were observed, again suggesting that cysteine protease(s) may be involved in bone resorption [4]. It has been reported that the inhibitor of cathepsin L known as pig leucocyte cysteine protease inhibitor (PLCPI) stopped pit formation induced by parathyroid hormone on rat bone [5]. The reportedly selective peptide aldehyde inhibitor Cbz-Phe-Tyr-CHO (cathepsin L $IC_{50} = 0.74$ nM) was also shown to inhibit pit formation on rat bone [6]. In the ovariectomized mouse this aldehyde suppressed bone resorption as measured by levels of hydroxyproline. Recently, it was reported that the aldehyde *N*-1-naphthylenesulfonyl-i-Leu-Trp-CHO (cathepsin L $IC_{50} = 1.9$ nM) inhibited the release of $^{45}Ca^{+2}$ in the fetal rat long bone assay and prevented bone loss in the ovariectomized rat following p.o. administration of 50 mg/kg for 3 weeks [7]. As the majority of the studies described above were conducted before the identification of cathepsin K, none of the inhibitors discussed in these reports were assayed against this enzyme or any of the appropriate species orthologs.

2.1. Identification and characterization of cathepsin K

Prior to the official designation of this protein as cathepsin K it possessed several different names including cathepsin OC-2 (the rabbit ortholog), cathepsin O, cathepsin O2 as well as cathepsin X. A variety of molecular biology screening approaches have led to the identification of the gene expressing cathepsin K. Differential screening of a c-DNA library generated from isolated rabbit osteoclasts resulted in the identification of a gene initially termed rabbit OC-2 which was shown to encode a putative protein with high homology to cathepsins S and L [8,9]. *In situ* hybridization and northern blot analysis revealed that cathepsin OC-2 was highly and selectively expressed in osteoclasts, suggesting it may have a specific role in bone resorption by these cells. Screening of a c-DNA library derived from human osteoclastoma cells led to the identification of a human gene encoding a cysteine protease reported as cathepsin X [10,11]. This protein was shown to share 93.9% sequence homology at the amino acid level with rabbit cathepsin OC-2. The random screening of a library of human osteoclast-derived expressed sequence tags (EST) led to the identification of a gene encoding a cysteine protease highly expressed within these cells [12,13]. *In situ* hybridization experiments demonstrated that cathepsin K m-RNA was selectively and highly expressed in osteoclastoma and oesteophyte cells. Conversely, expression levels of the homologous cathepsins S, L and B were not detected in these cell lines suggesting that these proteases are not responsible for the resorption of bone matrix proteins. In a subsequent report, it was detailed that cathepsin K was expressed initially as pre-procathepsin K [14]. This protein included a 15-amino acid pre or signal sequence followed by a leader sequence of 99 amino acids. This pre-pro expression is typical of the expression of cysteine proteases of the papain family. Treatment of procathepsin K under low pH conditions at elevated temperatures resulted in the processing to mature, active cathepsin K. Mature cathepsin K was shown to proteolytically cleave both type 1 collagen and osteonectin. Detailed studies with collagen have determined the exact nature of the cleavage sites produced by cathepsin K [15–17]. Sequence alignments revealed that pre-procathepsin K possessed the highest degree of homology with cathepsin L (60% identity, 76% similarity) followed by cathepsin S (59% identity, 73% similarity). The high degree of identity and similarity between these proteases represents but one of the principle issues in the design of selective, potent inhibitors of cathepsin K. Screening of a monocyte-derived macrophage c-DNA library led to the identification of a 1670-bp gene encoding a 329 amino acid cysteine protease initially termed cathepsin O. This human ortholog was shown to share 94% structural identity to the rabbit ortholog cathepsin OC-2 [18]. A later report described the cloning of a 1.6 kb human c-DNA that encoded a 329 polypeptide predominantly expressed within both osteoclastomas and ovary which was termed cathepsin O2 [19]. A c-DNA library cloned from the bone of human osteoarthritic hips identified a gene encoding a 329-amino acid protein with 94% homology to rabbit cathepsin OC-2 [20]. Protein alignments revealed that cathepsin K identified from this study shared the highest sequence homology with cathepsin S. The expression of cathepsin K m-RNA in breast cancer bone metastases, synovium of rheumatoid arthritis patients and in giant cells of osteoarthritic synovial tissue derived from humans have also been detailed [21–23]. The cathepsin K gene derived from female *M. cynomolgus* monkey m-RNA produced a c-DNA sequence that encoded a polypeptide with 94.2% identity to human pre-procathepsin K [24].

Interestingly, sequence analysis of this protein revealed that the only differences in amino acid sequence occurred in the pre-pro region of this protein. The mature forms of both human and *M. cynomolgus* monkey cathepsins K have been shown to be identical. This finding has been crucial for the *in vivo* characterization of the bone resorptive effects of inhibitors of cathepsin K. Mature rat cathepsin K has been reported to be 88% identical to human cathepsin K [25]. The kinetic behavior of rat and human cathepsin K however varies considerably despite the high sequence homology. The k_{cat}/K_m for the fluorogenic substrate Cbz-Leu-Arg-aminomethylcoumarin is 12 to 35-fold lower for rat cathepsin K versus human cathepsin K. Additionally the K_m for this substrate is 6 μM for human cathepsin K while for rat cathepsin K it is 99 μM. These differences in specificities highlight the requirement of characterizing species orthologs to assure the accurate analysis of inhibitors in the appropriate animal models. The characterizations of mouse and rhesus monkey cathepsin K have also been reported [26–28].

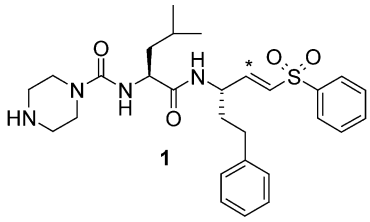
2.2. Target validation

The predominantly selective expression of cathepsin K in osteoclasts, as well as the ability of this enzyme to degrade type one collagen, suggests that this protease plays a specific role in the resorption phase of bone remodeling. Several additional lines of scientific evidence have provided further validation for the role of cathepsin K in this process. It was shown that a cathepsin K antisense oligonucleotide was able to decrease both the level of cathepsin K expression within osteoclasts as well as the resorptive activity of these cells [29]. Recently, mutations in the human gene expressing cathepsin K have been identified [30–32]. All of these mutations have been shown to result in the expression of a truncated, inactive version of cathepsin K which manifests itself as pycnodysostosis, a disease characterized by osteosclerosis, short stature and a high rate of bone fractures. Patients with pycnodysostosis have the normal number of osteoclasts but the function of these cells in resorbing bone is impaired. The structure and location of the human cathepsin K gene have been detailed [33,34]. Finally, additional validation of cathepsin K has been reported by two groups that have independently generated mice deficient in cathepsin K [35,36]. In both of these studies the cathepsin K deficient mice appeared normal, were viable and capable of breeding. More in depth histological examinations revealed that these mice displayed an osteopetrotic phenotype that became more distinct over time. Osteoclasts of the cathepsin K deficient mice were shown to be capable of demineralizing bone, but were not able to degrade the organic protein matrix, a finding similar to that seen in patients with pycnodysostosis [31]. In combination, these studies provide compelling validation for targeting the inhibition of cathepsin K as a potential therapy for osteoclast bone resorption-related diseases.

2.3. Protein structure

Independent X-ray co-crystal structures of human cathepsin K crystallized with either the potent, time dependent vinyl sulfone inhibitor 1 ($k_{obs}/[I] = 5.7 \times 10^{-6}\ M^{-1}\ s^{-1}$) or the cysteine protease pan-active epoxide inhibitor 2 bound covalently within the active site of the protein have been reported [37,38]. The overall protein folding of these structures revealed this protease to be a member of the papain family of cysteine proteases. Cathepsin

K shares the highest degree of sequence homology with the cysteine proteases cathepsins S and L with 56 and 42% identity, respectively. The X-ray co-crystal structure of cathepsin K with 1 (1.8 Å) bound in the active site showed that the nucleophilic thiol of Cys 25 had covalently attached to the inhibitor via Michael addition to the *si* face of the β-carbon atom of the vinyl sulfone (highlighted with an asterisk). The increased nucleophilicity of Cys 25 was ascribed to the influence of a helical dipole on this residue as well as hydrogen bonding interactions between residues of the catalytic triad (Cys 25, Asn 175 and His 159) within the active site. The peptide portion of the inhibitor was oriented on the unprimed side of the active site with the phenethyl moiety of the homophenylalanine bound within the S1 pocket. This shallow pocket was defined by Gly's 23, 64 and 65 and Cys 63. The iso-butyl group of the leucine residue was bound within the hydrophobic S2 binding pocket comprised of Tyr 67, Met 68, Ala 133, Leu 157 and Ala 160. It is the differences in the amino acid residues contained within the S2 binding pockets of the highly homologous members of the papain family cysteine proteases that is most relevant to the design of selective inhibitors for each of these respective enzymes. The piperizine moiety of **1** was bound within the S3 binding pocket defined by Tyr 67 and Asp 61. The phenyl sulfonyl group is oriented on the primed side of the active site with the phenyl moiety bound within the S1′ pocket comprised of Gln 142, Ser 137 and Ala 136. The X-ray co-crystal structure of cathepsin K complexed with the epoxide **2** (2.2 Å, 1ATK) showed this inhibitor also oriented on the unprimed side of the active site, covalently attached to the protein via attack of the thiol of Cys 25 to C2 of the inhibitor. As above, the iso-butyl moiety of **2** was bound within the S2 pocket. The basic guanidinium group was bound within the S3 binding pocket. The crystal structure of human pro-cathepsin K (2.60 Å, 1BY8) has also been described [39]. These structures have been instrumental in the structure-based design of potent and selective inhibitors of cathepsin K.



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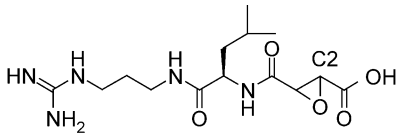


2

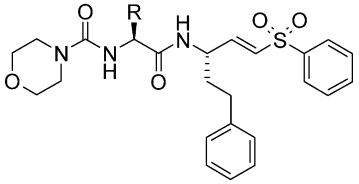
3. THE INHIBITION OF CATHEPSIN K

The inherent nucleophilic reactivity of the cysteine thiolate moiety, which in part defines cysteine proteases, has led to the design of a variety of inhibitor templates. These inhibitor classes may be divided broadly into four categories based upon their respective mechanistic modes of reactivity. Several reviews detailing inhibitor categorization have been published and the reader is referred to these accounts for a more critical description of inhibitor mechanism [40]. The first class of inhibitor template described is a series of irreversible alkylators of the active site cysteine thiol group. Representative members of this class include α-halo ketones, α-azido ketones, epoxides, α-aryloxy ketones, α,β unsaturated esters and most recently the vinyl sulfone inhibitor template. The second template category is comprised of a series of time dependent inhibitors that, by the nature

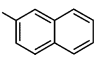
of their reactivity, form a stable thioacyl-enzyme intermediate that is slow to hydrolyze. The acyl hydrazide template originally described by Abeles [41], represents the prototype of this class of inhibitor. The third class of template are inhibitors which do not form a covalent interaction with the active site cysteine but rely almost entirely on the protein's substrate binding elements for effective inhibition. Here, the aminoethyl amides described below serve as a recent example of this type of inhibitor. Finally, the fourth class of template, which has seen recent widespread application, is a series of inhibitors that form a covalent, yet reversible, intermediate with the thiol group of the active site cysteine. Representative members of this class include peptide derived nitriles, aldehyde, α-ketoamides and α-heteroatom ketone-based inhibitors. Although not comprehensive in scope, the remainder of this review will detail recent, salient applications of these four mechanistic classes as inhibitors of cathepsin K.

3.1. Vinyl sulfone inhibitors

A series of peptidyl vinyl sulfone inhibitors, exemplified by **1**, have been reported as potent irreversible inactivators of cysteine proteases [42]. The vinyl sulfone moiety represents the second generation of irreversible inhibitors of cysteine proteases that incorporate a Michael acceptor motif [43]. Variation of the P2 residue of this inhibitor template permitted mapping of the S2 binding pockets of cathepsins K, S and L [44]. In this study it was shown that cathepsin K clearly prefers a branched hydrophobic moiety such as leucine in this position. This preference for leucine by cathepsin K does not impart selectivity over cathepsins L and S. Inhibitor **3**, which incorporates a P1 homophenylalanine and a P2 leucine, has been described as a potent inactivator of cathepsins K, L and S with a k_{inact}/K_I's of 772,700, 387,000 and 26,300,000 $M^{-1}\,s^{-1}$, respectively [45]. This compound is a modestly potent inactivator of cathepsin B with a k_{inact}/K_i of 4250 $M^{-1}\,s^{-1}$. Alternatively vinyl sulfone **4**, which incorporates a 2-naphthylalanine in the P2 binding position, is a weak inactivator of cathepsin K with a k_{inact}/K_i of less than 300 $M^{-1}\,s^{-1}$ and potently inactivates cathepsins S, L and B ($k_{inact}/K_i = 56,000,000$, 9,200,000 and 420,000 $M^{-1}\,s^{-1}$, respectively). The ability of these vinyl sulfones to inhibit bone resorption was assessed in the rat pit formation assay which measures the number of resorption pits per bone slice. In this assay the potent cathepsin S, L and B inhibitor **4** had no effect on resorption. Compound **3**, which is the potent K, S, L and B inhibitor, attenuated bone resorption with an IC_{50} of 0.05 μM. The antiresorptive effect of the cathepsin K, L, S and B inhibitor **3** was confirmed by its ability to inhibit the hydrolysis of the cathepsin K selective methylcoumarin substrate Cbz-Gly-Pro-Arg-MCA. Osteoclasts exposed to vehicle alone or to the potent cathepsin L, S and B inhibitor **4** hydrolyzed this substrate rapidly. However, no hydrolysis of this substrate was observed in osteoclasts treated with the cathepsin K, L, S and B inhibitor **3**. Although no data detailing the potencies of either **3** or **4** versus species orthologs of rat cathepsins K, L, S and B were reported, these data suggest that it is the inhibition of cathepsin K that is responsible for attenuating bone resorption *in vitro* in the rat.

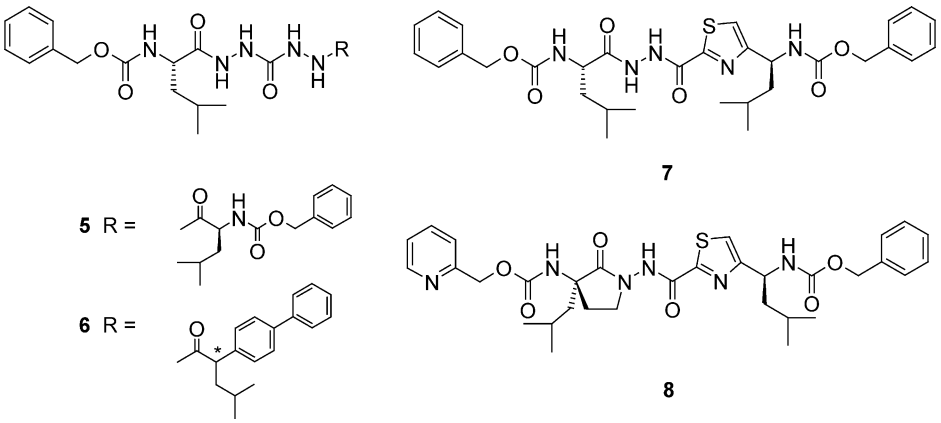


3 R = $CH_2CH(CH_3)_2$

4 R = CH_2 

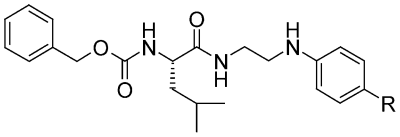
3.2. Hydrazide-based inhibitors

A series of acyl bis-hydrazide inhibitors exemplified by **5** have been reported as potent, time dependent inhibitors of cathepsin K [46]. The design of this inhibitor template was based upon substitution of the central diaminoketone inhibitor template contained within **23** (vide infra) with an acyl bis-hydrazide template. The prototypical acyl-bis-hydrazide **5** has been reported as a potent and selective inhibitor of cathepsin K with $k_{obs}/[I]'$s of 3.1×10^{-6} for cathepsin K, 1.3×10^{-3} for cathepsin B, 5.8×10^{-4} for cathepsin L and a $K_i = 11$ nM for cathepsin S. Compound **5** is also a potent inhibitor of cathepsin K activity in the osteoclast resorption assay with an $IC_{50} = 0.34$ μM. The X-ray co-crystal structure of **5** bound within the active site of cathepsin K (2.2 Å) revealed that this inhibitor, like the previously designed diaminoketone series, spanned the S3 to S3′ binding pockets of the active site. Additionally, this co-crystal structure suggested the inhibitor formed a covalent thio-acyl hydrazide intermediate with the active site cysteine thiol residue. Mechanistic studies have shown this inhibitor template to be time dependent, slow turn over inhibitors of cathepsin K [47]. Further molecular modeling analyses led to the identification of peptidomimetic groups to replace the Cbz-leucine moiety contained within **5**. Analogs **6**, as a 9:1 mixture of diastereomers at the carbon designated with an asterisk, are potent, time dependent inhibitors of cathepsin K with a $k_{obs}/[I]$ of 2,900,000 $M^{-1}\,s^{-1}$ [48]. The incorporation of other peptidomimetic moieties in to the acyl bis-hydrazide template has been detailed [49]. Thiazole **7** was identified as an effective replacement of the acyl hydrazide moiety resulting in potent inhibition of cathepsin K ($K_{i,app} = 10$ nM) with selectivity over cathepsins L ($K_{i,app} = 700$ nM), cathepsin S ($K_{i,app} > 1,000$ nM) and cathepsin B ($K_{i,app} = 5,200$ nM). Compound **7** has been characterized as an initially rapid and reversible inhibitor with a subsequent slow turnover step. Analog **8**, which incorporates the Freidinger lactam [50] was shown to be a 33 nM inhibitor of cathepsin K which has demonstrated greater stability to proteolitic processing by cathepsin K relative to **7**, the acylic version of this inhibitor template. Additional aza-peptide-based inhibitors of cathepsin K have been published recently [51,52].

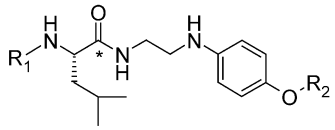


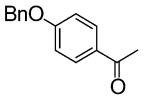
3.3. Aminoethyl amide-based inhibitors

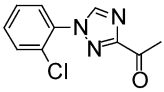
The identification of a novel series of aminoethyl amides based on the lead structure **9** have been detailed as potent, reversible and competitive inhibitors of both rabbit and human cathepsins K [53]. Unlike most of the inhibitors discussed within this review, the aminoethyl amide template does not contain an electrophilic moiety capable of forming a covalent bond with the thiol moiety of active-site cysteine residue. This inhibitor template attempts to take advantage of Van Der Waals interactions between itself and those contained within the respective binding pockets of active site of cathepsin K. Aminoethyl amide **9** inhibits rabbit cathepsin K with an $IC_{50} = 470$ nM. Incorporation of a 4-methoxy phenyl moiety to produce analog **10** resulted in a 7-fold increase in inhibitor potency with an $IC_{50} = 64$ nM versus rabbit cathepsin K. Further SAR studies to improve the potency of **10** by replacement of the P3 benzyloxycarbonyl moiety led to the identification of several potent and selective inhibitors. Analogs **12** and **13**, which incorporate either a P3 4-benzyloxyphenyl amide moiety or a 1-(2-chloro-phenyl)-1H-[1,2,4]triazole-3-carboxylic acid amide, are potent inhibitors of human cathepsin with K_I's of 15 and 11 nM, respectively. Compound **12** is selective for cathepsin K over cathepsin L and S with IC_{50}'s of >10 and 9.5 μM, respectively. Inhibitor **13** is somewhat less selective (cathepsin L $IC_{50} = 2.3$ μM; cathepsin S $IC_{50} = 1.9$ μM). Preincubation of **12** with excess cathepsin K followed by dialysis and redilution with substrate (Cbz-Phe-Arg-AMC) restored the proteolytic activity of the protein indicative of reversible inhibition. The Lineweaver-Burk plot of **12** displayed a constant 1/S axis intercept, characteristic of a competitive mode of inhibition. Evidence that this inhibitor template was not forming a covalent bond with the enzyme was established by ^{13}C NMR analysis of a ^{13}C radiolabeled analog of **13** (the position of the radiolabel is highlighted with an asterisk) in the presence of cathepsin K. In this experiment a small upfield shift and a broadening of the ^{13}C resonance in the radiolabeled amide carbonyl carbon of radiolabeled **12** were observed. These data are indicative of a non-covalent interaction between the inhibitor and the protein. Molecular modeling studies, in which inhibitor **10** was docked within the active site of cathepsin K, revealed the expected $\pi-\pi$ stacking interaction of the phenyl ring of the benzyloxycarbonyl moiety with Tyr 67 contained within the S3 binding pocket and occupation of the lipophilic S2 binding pocket by the iso-butyl group of the inhibitor [54]. Additionally, these studies suggested that an improvement in potency and/or selectivity could be realized by extension of **10** into the S1′ binding pocket of active site of cathepsin K. Towards this end, inhibitor **11**, which contains a benzyl ether moiety, is a potent inhibitor of cathepsin K ($IC_{50} = 54$ nM) with good selectivity over cathepsins L ($IC_{50} > 10$ μM) and S ($IC_{50} = 9.5$ μM). Analog **14**, which possesses a P3 1H-indo-2-yl amide and the lipophilic P1′ iso-butyloxy moiety, is approximately 3,300-fold selective for cathepsin K ($IC_{50} < 3$ nM) over cathepsins L and S (IC_{50}'s >10 μM). The potential for slow-turnover proteolytic processing of this inhibitor template by cathepsin K was not addressed in these accounts.

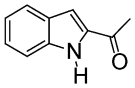


9 R = H
10 R = OCH_3
11 R = OBn

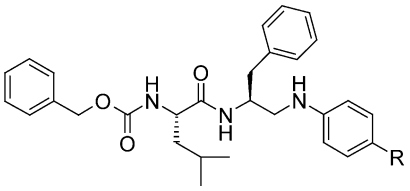


12 R_1 =  ; $R_2 = CH_3$

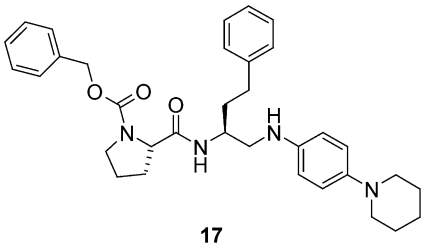
13 R_1 =  ; $R_2 = CH_3$

14 R_1 =  ; $R_2 = CH_2CH(CH_3)_2$

In a similar vein, a closely related extension of the aminoethyl amide inhibitor template described above has been reported [55]. Replacement of the aldehyde carbonyl moiety of the known, potent inhibitor of cathepsin L Cbz-Leu-Phe-CHO (cathepsin L $K_i = 13$ nM) with aniline provided the aminoethyl amide **15**. This compound inhibits cathepsin K with an $IC_{50} = 1.1$ μM and retains significant cathepsin L potency ($IC_{50} = 230$ nM). Incorporation of a 4-piperidinyl aniline moiety provided **16** which is a potent inhibitor of both cathepsins K and L ($IC_{50} = 10$ and 2 nM, respectively). Further SAR investigations of **16** led to **17** that incorporates a P1 homophenylalanine and a P2 proline residue. Compound **17** is a potent inhibitor of cathepsin K ($IC_{50} = 12$ nM) with selectivity over cathepsins L ($IC_{50} = 7.84$ μM) and B ($IC_{50} > 10$ μM). It has been characterized as a competitive and reversible inhibitor of cathepsin K that inhibited pit formation in murine and human bone resorption assays.



15 R = H
16 R = 

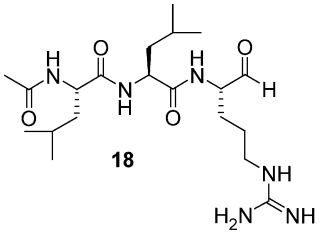


17

3.4. Carbonyl-based inhibitors

One of the first reports on the inhibition of cathepsin K described the utility of the classical cysteine protease tri-peptide aldehyde inhibitor leupeptin **(18)** which has been characterized as a potent, time-dependent inhibitor with an $IC_{50} = 70$ nM,

$k_{obs}/[I] = 273,00\ M^{-1}\ s^{-1}$ and a $k_{off} = 0.061\ min^{-1}$ [47]. Based on the potency of **18**, further evaluation of a series of structurally related peptide derived aldehydes identified **19** as a potent inhibitor of cathepsin K with a $K_{i,app} = 1.4$ nM [56]. The fluorometric assay utilized to assess the potency of these aldehyde inhibitors measured the rate of processing of the dipeptide aminomethylcoumarin substrate Cbz-Phe-Arg-AMC by cathepsin K. In this assay format, in which the aldehyde inhibitor and substrate (Cbz-Phe-Arg-AMC) were added simultaneously, the proteolytic activity of cathepsin K was observed to return over time with a concomitant loss of inhibitor potency. Preincubation of the aldehyde inhibitors with assay buffer, prior to the addition of cathepsin K and substrate, resulted in an approximate 10-fold loss of potency. This loss of inhibitor potency was attributed to the highly reactive nature of the electrophilic aldehyde moiety that had likely formed inactive intermediates with components of the assay buffer. Aldehyde **19** is a potent inhibitor of PTH-stimulated bone resorption in the fetal rat long bone assay ($IC_{50} = 20$ nM). In the human osteoclast resorption assay **19** inhibited bone resorption with an $IC_{50} = 100$ nM. *In vivo* in the adjuvent arthritis rat model a 61% inhibition of hindpaw inflammation was observed with **19** at a 30 mg/kg i.p. dose after 16 days. In the thyroparathyroidectomized (TPTX) rat model calcemic response to PTH [1–34] was inhibited significantly by compound **19** following i.p. administration at a 60 mg/kg dose. Due to the fact that aldehyde-based inhibitors such as **19** lack selectivity toward a variety of other enzyme systems, the attribution of an antiresorptive effect elicited by these compounds to the inhibition of a specific protease should be taken with some degree of caution.



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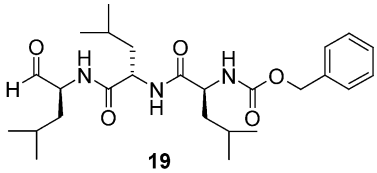
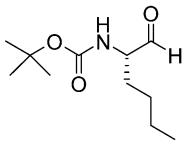


19

A screening effort led to the identification of aldehyde **20** as an inhibitor of cathepsin K with an $IC_{50} = 51$ nM [57]. Replacement of the aldehyde moiety of **20** with an α-ketoamide group provided a series of α-ketoamide inhibitors of cathepsin K [58]. The ketoamide **21** was substantially less potent than the parent aldehyde **20** with an $IC_{50} = 5.9$ μM. Optimization of the P2 and P1′ binding elements led to the identification of **22** which is a potent, reversible inhibitor of human and rat cathepsins K with IC_{50}'s of 0.77 and 13 nM, respectively [59]. Analog **22** is selective for cathepsin K over cathepsins L, V, H and B and modestly selective over cathepsin S. The 2.2 Å X-ray co-crystal structure of **22** bound within the active site of cathepsin K revealed that the pyrazole moiety interacted with Gln 18 through a bridging water molecule and with the S1′ Trp 184 aromatic moiety. One of the iso-propyl groups was bound within the S2 pocket while the second iso-propyl projects toward, but does not bind in, the S3 pocket. The *n*-butyl moiety of the norleucine is oriented toward the S1 site while the ketone of the inhibitor is engaged in a covalent interaction with the active site Cys 25. Oral administration in the rat showed **22** to

be 41% orally bioavailable with a high rate of clearance (Cl = 68 mL/min/kg) and a half-life of 700 min. In the neonatal rat calvaria assay 22 completely reversed PTH stimulated bone resorption at a dose of 10 μM. Evaluation in the TPTX rat model at an oral dose of 25 mg/kg demonstrated that **22** lowered serum calcium levels upon co-administration with PTH. No selectivity data versus serine proteases were reported in this account.



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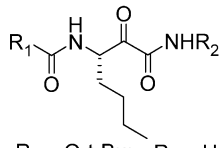
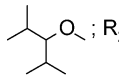
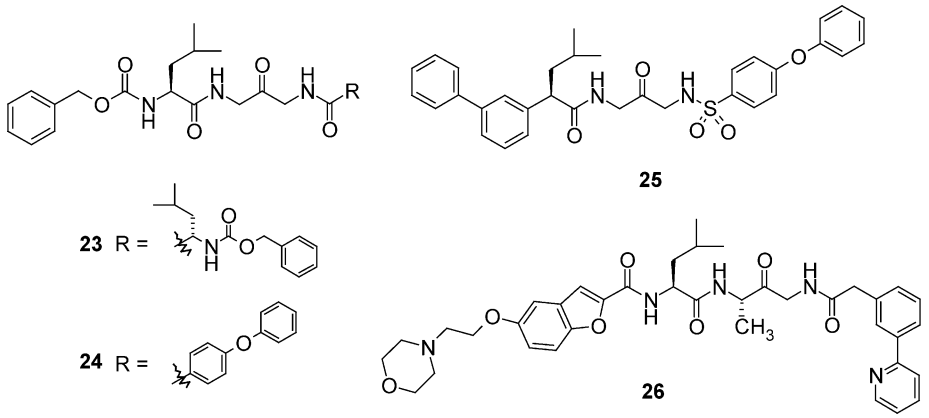


21 R_1 = O-t-Bu; R_2 = H

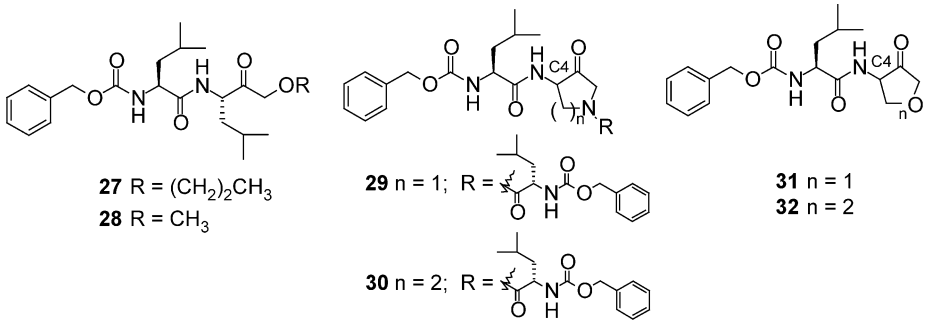
22 R_1 =  ; R_2 = 

The use of structure-based inhibitor design has led to the synthesis of a series of potent, reversible and competitive 1,3-bis(acylamino)-2-propanone inhibitors of cathepsin K exemplified by **23**. The intellectual underpinning of the design of this inhibitor template was based upon the anomalous binding orientations of two closely related peptide derived aldehyde inhibitors **18** and **19** within the active site of papain, the prototypical cysteine protease [60,61]. The X-ray co-crystal structure of aldehyde **18** bound within the active site of papain revealed that this inhibitor was oriented on the unprimed side of the active site of this protease [62]. Alternatively, the X-ray co-crystal structure of the closely related tri-peptide aldehyde **19**, again bound within the active site of papain, revealed this inhibitor to be bound on the primed side of the active site of papain. Molecular modeling of an overlay of these two structures within the active site of cathepsin K led to the design of the 1,3-bis(acylamino)-2-propanone **23** which has been characterized as a potent inhibitor of this enzyme with a $K_{i,app} = 22$ nM. Inhibitor **23** is selective for cathepsin K over cathepsins L, S and B with $K_{i,app}$'s of 340, 890 and 1300 nM, respectively. This compound is a weak inhibitor of papain ($K_{i,app} > 10$ μM) and inactive against the serine proteases trypsin and chymotrypsin. An X-ray co-crystal structure of **23** in the active site of cathepsin K showed the inhibitor bound from the S3 to the S3′ binding pockets confirming the elegant design of this inhibitor template. The synthesis of a variety of 1,3-bis(acylamino)-2-propanone inhibitors has been reduced to a solid-phase approach [63]. Further modeling studies led to the incorporation of peptidomimetics designed to replace one, and ultimately both, of the Cbz-leucine moieties contained within **23** [64]. Analog **24**, which incorporates the 4-phenoxyphenyl amide, is a 67 nM inhibitor of cathepsin K. Further utilization of iterative modeling/X-ray co-crystallography led to the design and synthesis of **25**, in which both of the Cbz-leucine peptide residues have been replaced by peptidomimetic binding elements, is a potent inhibitor of cathepsin K ($K_{i,app} = 1.4$ nM) which is selective over cathepsins L, S and B ($K_{i,app} > 1000$, 910, and > 10000 nM, respectively). Compound **26**, which contains the P3 morpholinobenzofuran moiety has been identified as a potent inhibitor of rat and

human cathepsins K with $K_{i,app}$'s of 69 and 0.082 nM, respectively [65]. This azepanone demonstrated potent inhibition of native cathepsin K in the osteoclast pit resorption assay with an $IC_{50} = 41$ nM.

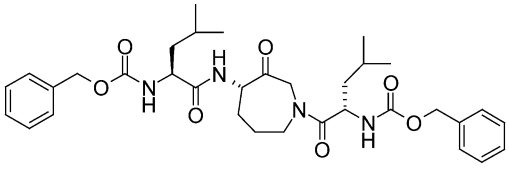


The α-alkoxy ketone template was originally reported in 1995 as a potent, reversible class of inhibitors of the serine protease thrombin [66]. This template has seen recent application for the inhibition of cathepsin K. The α-propyloxy ketone **27** was reported as a weakly time-dependent inhibitor of cathepsin K with a $k_{inact/I}$ of 4100 M^{-1} s^{-1} [67]. Incorporation of a P1 methoxymethyl ketone group provided **28** that is a 80 nM inhibitor of cathepsin K. The X-ray co-crystal structure of **27** bound within the active site of the enzyme revealed the dipeptide portion of the inhibitor oriented on the primed side of the active site with the P1′ and P2′ iso-butyl groups bound within the S1′ and S2′ binding pockets. The propoxymethyl ether group was seen oriented on the unprimed side of the active site barely penetrating the P1 binding pocket. The orientations of the P1 propyl and the P1′ iso-butyl moieties of **27** in this X-ray co-crystal structure suggested that cyclization of this template may be possible [68]. The 4-amidopyrrolidinone and 4-amidopiperidinone templates **29** and **30** were reported as potent, reversible inhibitors of cathepsin K with $K_{i,apps}$ of 2.3 and 2.6 nM, respectively, as mixtures of diastereomers [69,70]. The X-ray co-crystal structure of **29**, bound within the active site of cathepsin K, revealed that this inhibitor spanned the active site binding from the P3 to the P3′ pockets. Several peptidomimetic substitutions of the $N-1$ Cbz-leucine of both **29** and **30** were reported in these accounts. The rapid epimerization of the C-4 α-amido ketone chiral center limited the progression of this inhibitor template. A similar cyclization strategy led to the identification of the tetrahydrofuran and tetrahydropyran derivatives **31** and **32** both of which have been characterized as potent, reversible inhibitors of cathepsin K with K_i's of 140 and 150 nM, respectively, as mixtures of diastereomers at C4 [71]. Within this particular series of cyclic oxygen containing analogs, epimerization of the C-4 chiral center was demonstrated to be somewhat less problematic relative to **29** and **30**. A solid phase synthesis of a variety of analogs based on the furanone inhibitor template has been reported [72].



Incorporation of a 4-amidoazepanone was utilized in an effort to address the epimerization of the C-4 chiral center of the 4-amidopyrrolidinone/piperidinone inhibitor templates **29** and **30** [25]. The C4-S azepanone **33** has been characterized as a configurationally stable inhibitor of cathepsin K with a $K_{i,app} = 2.0$ nM. Inhibitor **33** is modestly selective versus cathepsins L and S with $K_{i,app}$'s of 47 and 26 nM, respectively. Incorporation of peptidomimetic elements into the azepanone template led to the identification of **34** which is a potent inhibitor of cathepsin K with a $K_i = 0.16$ nM. Compound **34** is modestly selective over cathepsins L and S with $K_{i,app}$'s of 2.2 and 4.0 nM, respectively. It is a potent inhibitor of native cathepsin activity as measured in the osteoclast pit resorption assay with an $IC_{50} = 70$ nM [73]. This assay measures the level of known collagen fragments that are released following active bone resorption. This compound was also shown to be a potent inhibitor in the *in situ* cytochemical assay, a measure of cathepsin activity in osteoclasts, with an $IC_{50} = 80$ nM [74]. The C4-R diastereomer (not shown) was significantly less potent against cathepsin K with a $K_{i,app} \geq 980$ nM. In the rat **34** was shown to be 42% orally bioavailable with clearance of 49.2 mL/min/kg and a $t_{1/2}$ of 29.8 min. It was the increase in oral bioavailability of cyclic azepanones such as **34** relative to their acyclic diaminoketone counterparts that, in part, led to a more general examination of the role of conformational constraint in determining this property and to the hypothesis relating systemic exposure to the importance of reducing the number of rotatable bonds [75,76]. The C4 R diastereomer was shown to have an oral bioavailability of 9.7% highlighting the role of stereochemistry as a determinant of systemic exposure. Although the oral bioavailability of **34** in the monkey was poor (4.8%), systemic levels of compound following s.c. administration were of significantly high levels to permit evaluation of the antiresorptive effect of this compound in the chemically ovariectomized monkey [77]. In this model, monkeys chemically ovariectomized following treatment with the gonadotropin releasing agonist lupron, were treated with **34** (12 mg/kg s.c dose) for 5 days. Following the first dose of azepanone **34** baseline levels of the bone biomarkers CTx and NTx were reported to be reduced 46 and 38%, respectively, after 24 h. After 4 days of dosing NTx and CTx levels were suppressed more than 50% relative to vehicle control animals. These studies suggest that a potent inhibitor of cathepsin K is an effective treatment of bone resorption diseases such as osteoporosis. *In vitro* and *in vivo* studies to determine the mechanisms limiting the systemic exposure of **34** in the monkey revealed that metabolism by cytochromes 3A were responsible for overall low systemic exposure. *In vitro* metabolism directed analog synthesis led to the identification of azepanone **35**, a potent inhibitor of cathepsin K with

a $K_i = 0.11$ nM [78]. Analog **35** is a potent inhibitor of *in vitro* osteoclast-mediated bone resorption with an $IC_{50} = 30$ nM. Compound **35** has been shown to be 66.3 and 23.4% orally bioavailable in the rat and the monkey, respectively. In the chemically ovariectomized monkey, a 32% reduction of urinary NTx levels were observed over the first 24 h period following oral administration (3 mg/kg) of **35**. A 32% drop in serum CTx levels were also observed 90 min after administration of **35**. These studies demonstrate that an orally administered inhibitor of cathepsin K may be an effective therapeutic for reducing bone loss due to excess resorption of the protein component of bone matrix collagen. Incorporation of peptidomimetic groups, originally identified in the acyclic 1,3-diaminoketone series (vida supra), to the 4-amido-azepanone template led to the identification of **36** a potent inhibitor of both human and rat cathepsins K with $K_{i,app}$'s of 0.0048 and 4.7 nM, respectively. This compound was one of the more potent inhibitors of rat cathepsin K identified within the 4-amidoazepanone series. No data versus rat cathepsins L, S or B was contained within these reports. Histologic evaluation of bones from ovariectomized rats treated with **36** for 4 weeks following intraperitoneal administration of 3, 10 and 30 mg/kg u.i.d for 4 weeks revealed that **36** inhibited OVX-induced bone loss *in vivo* at all of the dose levels examined [79].



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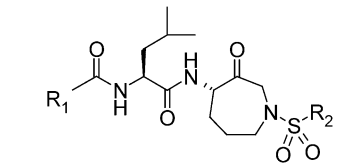
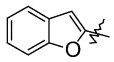
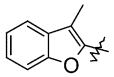
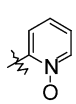
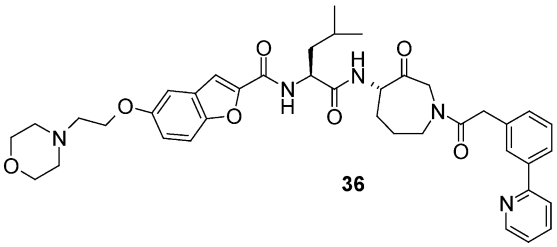


34 R_1 =  R_2 = 

35 R_1 =  R_2 = 

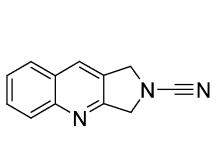


36

3.5. Nitrile-based inhibitors

Inhibitor templates utilizing a nitrile moiety as the electrophilic component were originally reported as inhibitors of papain in 1986 [80]. The nitrile motif has seen recent application in the inhibition of cathepsins B, S and K [81,82]. A screening effort led to the identification of cyanamide **37** as an inhibitor of cathepsins K, L and B with IC_{50}'s = 0.45, 0.37 and 2.3 μM, respectively [83]. Replacement of the quinoline moiety produced the benzenesulfonamide analog **38** that is a potent inhibitor of cathepsins K and L with IC_{50} values of 50 and 80 nM, respectively. The inhibitory potency of several related members of this class was assessed at pH 7 in the presence of excess glutathione. In these experiments no reduction in potency was observed suggesting that the nitrile moiety is stable to exogenous thiol nucleophiles. Pharmacokinetic analysis of **38** in the

rat, confirmed the stability of this inhibitor template, where it was shown to be 38% orally bioavailable with a half-life of 2 h. Nuclear magnetic resonance analysis of a ^{13}C labeled version of **38** (the position of the ^{13}C label is highlighted with an asterisk) with papain revealed the formation of two new resonance peaks indicative of isothiourea ester rotational isomers along with the disappearance of the nitrile resonance peak. Addition of E64 **(2)**, an irreversible epoxide-based inactivator of cysteine proteases, to this enzyme-inhibitor complex resulted in the reappearance of the nitrile resonance peak, indicative of a reversible mode of inhibition. Replacement of the benzenesulfonamide moiety with Cbz-leucine provided **39** that possesses the C3-R configuration. This compound is a potent inhibitor of cathepsin K ($K_{i,app} = 12$ nM) with excellent selectivities over cathepsins L ($K_{i,app} = 250$ nM), S ($K_{i,app} = 310$) and B ($K_{i,app} = 1500$ nM). In a functional assay of bone resorption, which utilized murine osteoclasts on bovine bone slices, **39** inhibited bone resorption with an $IC_{50} = 103$ nM [84].



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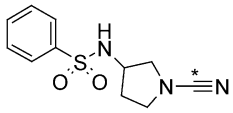

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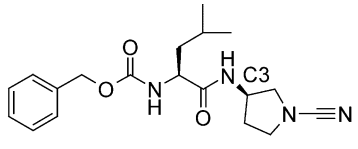
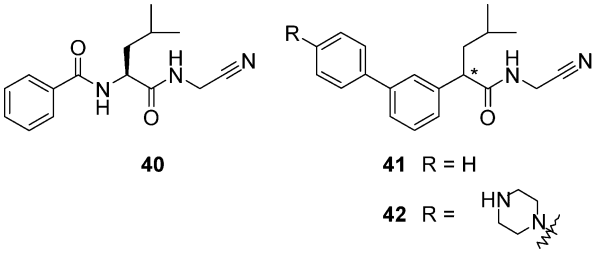


39

The peptidyl nitrile **40** was first described in a 1968 report on the synthesis of a series of inhibitors of carbon tetrachloride-induced necrosis of the liver in the rat [85]. It has been reported recently to be a potent inhibitor of cathepsins K, L and S (IC_{50}'s = 51, 42 and 41 nM, respectively) and a weakly potent inhibitor of cathepsin B ($IC_{50} = 3560$ nM) [86]. *In vitro* **40** inhibited bone resorption with an IC_{50} of 275 nM. A marked improvement in selectivity was realized upon replacement of the benzamide moiety of **40** with a biphenyl group. This led to racemic **41** which is a potent inhibitor of cathepsin K ($IC_{50} = 56$ nM) with good selectivities over cathepsins L ($IC_{50} = 498$ nM), S ($IC_{50} = 1578$ nM) and B ($IC_{50} = 3560$ nM). This compound was a weak inhibitor of bone resorption *in vitro* with an $IC_{50} = 3560$ nM. Further optimization of **41**, focusing on the SAR of the P3 binding element, led to piperazine **42** which now possesses the R stereochemistry at the indicated carbon. This analog is a potent and selective inhibitor of cathepsin K (cathepsin K $IC_{50} = 3$ nM, cathepsin L $IC_{50} = 3725$ nM, cathepsin S $IC_{50} = 2010$ nM and cathepsin B $IC_{50} = 3950$ nM) and is an active inhibitor of bone resorption *in vitro* ($IC_{50} = 95$ nM). Nitrile **42** has been characterized as a reversible inhibitor of cathepsin K with no detectable time dependent component. Incubation of **42** with 1 μM cathepsin K and 1 mM β-mercaptoethanol for 30 min demonstrated the inhibitor to be stable over this time period suggesting that the possibility of *in vivo* inhibitor inactivation by cysteine proteases or other thiol containing components was unlikely. Pharmacokinetic analysis in the rhesus monkey showed **42** to be 32% orally bioavailable with a half-life of 9.6 h. Oral administration of **42** for eight days at a dose of 20 mg/kg/day in the ovariectomized (OVX) rhesus monkey resulted in a 75–87% decrease of urinary *N*-teleopeptide/creatinine (uNTx/Cre), a biomarker of collagen breakdown observed during active resorption of bone. Three days after the dosing of **42**, uNTx/Cre levels were reduced 58%. Seventeen days after the last dose of **42** uNTX/Cre levels had returned to the same levels as those

observed in control group animals. The level of biomarker suppression observed in this study was comparable to that observed in human osteoporosis patients treated with bisphosphonates [87].



40 **41** R = H

42 R =

4. CONCLUSIONS

The identification of mutations of the human gene expressing cathepsin K as well as the phenotyping of the cathepsin K deficient mouse have provided exquisite validation of the role of this protease in osteoclast-mediated bone resorption. Potent and selective inhibitors of cathepsin K which demonstrate suitable pharmacokinetics have been identified and profiled *in vivo* to assess their ability to attenuate the production of biomarkers of bone resorption. These studies have suggested strongly that an inhibitor of cathepsin K may be an effective therapeutic agent for the prevention and treatment of osteoporosis.

REFERENCES

[1] R. E. Baron, in *Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism* (ed. M. J. Favus), 3rd edition, Lippincott-Raven, Philadelphia, PA, 1996, p. 3.
[2] R. Marcus, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (eds J. G. Hardman and L. E. Limbird), 10th edition, McGraw-Hill, New York, NY, 2001, p. 1715.
[3] J.-M. DeLaissé, Y. Eeckhout and G. Vaes, *Biochem. J.*, 1980, **192**, 365.
[4] J.-M. Delaissé, Y. Eeckhout and G. Vaes, *Biochem. Biophys. Res. Commun.*, 1984, **125**, 441.
[5] H. Kakegawa, T. Nikawa, K. Tagami, H. Kamioka, K. Sumitani, T. Kawata, M. Drobnič-Kosorok, B. Lenarčič, V. Turk and N. Katunuma, *FEBS Lett.*, 1993, **321**, 247.
[6] J.-T. Woo, K. Yamaguchi, T. Hayama, T. Kobori, S. Sigeizumi, K. Sugimoto, K. Kondo, T. Tsuji, Y. Ohba, K. Tagami and K. Sumatani, *Eur. J. Pharmacol.*, 1996, **300**, 131.
[7] T. Yasuma, S. Oi, N. Choh, Y. T. Nomura, N. Furuyama, A. Nisimura, Y. Fujisawa and T. Sohda, *J. Med. Chem.*, 1998, **41**, 4301.
[8] K. Tezuka, T. Sato, H. Kamioka, P. J. Nijweide, K. Tanaka, T. Matsuo, M. Ohta, N. Kurihara, Y. Hakeda and M. Kumegawa, *Biochem. Biophys. Res. Commun.*, 1992, **186**, 911.
[9] K. Tezuka, Y. Tezuka, A. Maejima, T. Sato, K. Nemoto, H. Kamioka, Y. Hakeda and M. Kumegawa, *J. Biol. Chem.*, 1993, **269**, 1106.
[10] Y.-P. Li, M. B. Alexander, A. L. Wucherpfennig, W. Chen, P. Yelick and T. Stashenko, *Mol. Biol. Cell*, 1994, **5**, 335a.
[11] Y.-P. Li, M. Alexander, A. L. Wucherpfennig, P. Yelick, W. Chen and P. Stashenko, *J. Bone Miner. Res.*, 1995, **10**, 1197.
[12] F. Drake, I. James, J. Conner, D. Rieman, R. Dodds, F. McCabe, D. Bertolini, R. Barthlow, G. Hastings and M. Gowen, *J. Miner. Res.*, 1994, **9**, 5177.
[13] F. H. Drake, R. A. Dodds, I. E. James, J. R. Connor, C. DeBouck, S. Richardson, E. Lee-Rykaczewski, L. Coleman, D. Rieman, R. Barthlow, G. Hastings and M. Gowen, *J. Biol. Chem.*, 1996, **271**, 12511.

[14] M. J. Bossard, T. A. Tomaszek, S. K. Thompson, B. Y. Amegadzie, C. R. Hanning, C. Jones, J. T. Kurdyla, D. E. McNulty, F. H. Drake, M. Gowen and M. A. Levy, *J. Biol. Chem.*, 1996, **21**, 12517.
[15] P. Garnero, O. Borel, I. Byrjalsen, M. Ferreras, F. Drake, M. S. McQueney, N. T. Foged, P. D. Delmas and J.-M. Delaissé, *J. Biol. Chem.*, 1998, **273**, 32347.
[16] A. Y. Nosaka, K. Kanaori, N. Teno, H. Togame, T. Inaoka, M. Takai and T. Kokubo, *Bioorg. Med. Chem.*, 1999, **7**, 375.
[17] W. Kafienah, D. Brömme, D. J. Buttle, L. J. Croucher and A. P. Hollander, *Biochem. J.*, 1998, **331**, 727.
[18] G.-P. Shi, H. A. Chapman, S. M. Bhairi, C. DeLeeuw, V. Y. Reddy and S. J. Weiss, *FEBS Lett.*, 1995, **357**, 129.
[19] D. Brömme and K. Okamoto, *Biol. Chem., Hoppe-Seyler*, 1995, **376**, 379.
[20] T. Inaoka, G. Bilbe, O. Ishibashi, K. Tezuka, M. Kumegawa and T. Kokubo, *Biochem. Biophys. Res. Commun.*, 1995, **206**, 89.
[21] A. J. Littlewood-Evans, G. Bilbe, W. B. Bowler, D. Farley, B. Wlodarski, T. Kokubo, T. Inaoka, J. Slone, D. B. Evans and J. A. Gallagher, *Cancer Res.*, 1997, **57**, 5386.
[22] K. M. Hummel, P. K. Petrow, J. K. Franz, U. Müller-Ladner, W. K. Aicher, R. E. Gay, D. Brömme and S. Gay, *J. Rheumatol.*, 1998, **25**, 1887.
[23] R. A. Dodds, J. R. Conner, F. H. Drake and M. Gowen, *Arthritis Rheum.*, 1999, **42**, 1588.
[24] M. S. McQueney, J. Field, C. R. Hanning, K. Brun, K. Ramachandran, J. Conner, F. Drake, C. S. Jones and B. Y. Amegadzie, *Prot. Express. Purif.*, 1998, **14**, 387.
[25] R. W. Marquis, Y. Ru, S. M. LoCastro, J. Zing, D. S. Yamashita, H.-J. Oh, K. F. Erhard, L. D. Davis, T. A. Tomaszek, D. Tew, K. Salyers, J. Proksch, K. Ward, B. Smith, M. Levy, M. D. Cummings, R. C. Haltiwanger, G. Trescher, B. Wang, M. E. Hemling, C. J. Quinn, H.-Y. Cheng, F. Lin, W. W. Smith, C. A. Janson, B. Zhao, M. S. McQueney, K. J. D'Alessio, C.-P. Lee, A. Marzulli, R. A. Dodds, S. Blake, S.-M. Hwang, I. E. James, C. J. Gress, B. R. Bradley, M. W. Lark, M. Gowen and D. F. Veber, *J. Med. Chem.*, 2001, **44**, 1380.
[26] B. D. Gelb, K. Moissoglu, J. Zhang, J. A. Martignetti, D. Brömme and J. Desnick, *Biochem. Mol. Med.*, 1996, **59**, 200.
[27] J. Rantakokko, H. T. Aro, M. Savontaus and E. Vuorio, *FEBS Lett.*, 1996, **393**, 307.
[28] J. Guay, D. Riendeau and J. A. Mancini, *Bone*, 1999, **25**, 205.
[29] T. Inui, O. Ishibashi, T. Inaoka, Y. Origane, M. Kumegawa, T. Kokubo and T. Yamamura, *J. Biol. Chem.*, 1997, **272**, 8109.
[30] M. R. Johnson, M. H. Polymeropoulos, H. L. Vos, R. I. Oritz da Luna and C. A. Francomano, *Genome Res.*, 1996, **6**, 1051.
[31] B. D. Gelb, G-P. Shi, H. A. Chapman and R. J. Desnick, *Science*, 1996, **273**, 1236.
[32] W.-S. Hou, D. Brömme, Y. Zhao, E. Mehler, C. Dushey, H. Weinstein, C. Sa Miranda, C. Fraga, F. Greig, J. Carey, D. L. Rimoin, R. J. Desnick and B. D. Gelb, *J. Clin. Invest.*, 1999, **103**, 731.
[33] B. C. Gelb, G.-P. Shi, M. Heller, S. Weremowicz, C. Morton, R. J. Desnick and H. A. Chapman, *Genomics*, 1997, **41**, 258.
[34] J. A. Rood, S. Van Horn, F. H. Drake, M. Gowen and C. Debouck, *Genomics*, 1997, **41**, 169.
[35] P. Saftig, E. Hunziker, O. Wehmeyer, S. Jones, A. Boyd, W. Rommerskirch, J. D. Moritz, P. Schu and K. von Figura, *Proc. Nat. Acad. Sci. USA*, 1998, **95**, 13453.
[36] M. Gowen, F. Lazner, R. Dodds, R. Kapadia, J. Field, M. Tavaria, I. Bertoncello, F. Drake, S. Zavarselk, I. Tellis, P. Hertzog, C. Debouck, I. Kola and J. Bone, *Miner. Res.*, 1999, **14**, 1654.
[37] M. E. McGrath, J. L. Klaus, M. G. Barnes and D. Brömme, *Nat. Struct. Biol.*, 1997, **4**, 105.
[38] B. Zhao, C. A. Janson, B. Y. Amegadzie, K. D'Alessio, C. Griffin, C. R. Hanning, C. Jones, J. Kurdyla, M. McQueney, X. Qiu, W. W. Smith and S. S. Abdel-Meguid, *Nat. Struct. Biol.*, 1997, **4**, 109.
[39] J. M. Lalonde, B. Zhao, W. W. Smith, C. A. Janson, R. L. DesJarlais, T. A. Tomaszek, T. J. Carr, S. K. Thompson, D. S. Yamashita, D. F. Veber and S. S. Abdel-Meguid, *Biochemistry*, 1999, **862**, 38.
[40] (a) D. S. Yamashita and R. A. Dodds, *Curr. Pharm. Des.*, 2000, **6**, 1; (b) D. F. Veber and S. K. Thompson, *Curr. Opin. Drug Disc. Des.*, 2000, **3**, 362; (c) A. A. Hernandez and

W. R. Roush, *Curr. Opin. Chem. Biol.*, 2002, **6**, 459; (d) R. W. Marquis, *Ann. Rep. Med. Chem.*, 2000, **35**, 309; (e) D. F. Veber and M. D. Cummings, in *Protein Crystallography in Drug Design* (eds R. Babine and S. S. Abdel-Meguid), Wiley-VCH Verlag, Weinheim, 2004, p. 127.
[41] J. Magrath and R. H. Abeles, *J. Med. Chem.*, 1992, **35**, 4297.
[42] J. T. Palmer, D. Rasnick, J. L. Klaus and D. Brömme, *J. Med. Chem.*, 1995, **38**, 3193.
[43] A. J. Barrett, A. A. Kembhavi, M. A. Brown, H. Kirschke, C. G. Knight, M. Tamai and K. Hanada, *Biochem. J.*, 1982, **201**, 189.
[44] D. Brömme, J. L. Klaus, K. Okamoto, D. Rasnick and J. T. Palmer, *Biochem. J.*, 1996, **315**, 85.
[45] L. Xia, J. Kilb, H. Wex, Z. Li, A. Lipyansky, V. Breuil, L. Stein, J. T. Palmer, D. W. Dempster and D. Brömme, *Biol. Chem.*, 1999, **380**, 679.
[46] S. K. Thompson, S. M. Halbert, M. J. Bossard, T. A. Tomaszek, M. A. Levy, B. Zhao, W. W. Smith, S. S. Abdel-Meguid, C. A. Janson, K. J. D'Alessio, M. S. McQueney, B. Y. Amegadzie, C. R. Hanning, R. L. DesJarlais, J. Briand, S. K. Sarkar, M. J. Huddleston, C. F. Ijames, S. A. Carr, K. T. Garnes, A. Shu, J. R. Heys, J. Bradbeer, D. Zembryki, L. Lee-Rykaczewski, I. E. James, M. W. Lark, F. H. Drake, M. Gowen, J. G. Gleason and D. F. Veber, *Proc. Nat. Acad. Sci. USA*, 1997, **94**, 14249.
[47] M. J. Bossard, T. A. Tomaszek, M. A. Levy, C. F. Ijames, M. J. Huddleston, J. Briand, S. Thompson, S. Halbert, D. F. Veber, S. A. Carr, T. D. Meek and D. G. Tew, *Biochemistry*, 1999, **38**, 15893.
[48] S. K. Thompson, S. M. Halbert, R. L. DesJarlais, T. A. Tomaszek, M. A. Levy, D. G. Tew, C. F. Ijames and D. F. Veber, *Bioorg. Med. Chem.*, 1999, **7**, 599.
[49] S. K. Thompson, W. W. Smith, B. Zhao, S. M. Halbert, T. A. Tomaszek, D. G. Tew, M. A. Levy, C. A. Janson, K. J. D'Alessio, M. S. McQueney, J. Kuryla, C. S. Jones, R. L. DesJarlais, S. S. Abdel-Meguid and D. F. Veber, *J. Med. Chem.*, 1998, **41**, 3923.
[50] R. M. Freidinger, D. S. Perlow and D. F. Veber, *J. Org. Chem.*, 1982, **47**, 104.
[51] K. J. Duffy, L. H. Ridgers, R. L. DesJarlais, T. A. Tomaszek, M. J. Bossard, S. K. Thompson, R. M. Keenan and D. F. Veber, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 1907.
[52] E. Wieczerzak, P. Drabik, L. Łankiewicz, S. Ołdziej, Z. Grzonka, M. Abrahamson, A. Grubb and D. Brömme, *J. Med. Chem.*, 2002, **45**, 4202.
[53] E. Altmann, J. Renaud, J. Green, D. Farley, B. Cutting and W. Jahnke, *J. Med. Chem.*, 2002, **45**, 2352.
[54] E. Altmann, J. Green and M. Tintelnot-Blomley, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1997.
[55] T.-S. Kim, A. B. Hague, T. I. Lee, B. Lian, C. M. Tegley, X. Wang, T. L. Burgess, Y.-X. Qian, S. Ross, P. Tagari, C.-H. Lin, C. Mayeda, J. Dao, S. Jordan, C. Mohr, J. Cheetham, V. Viswanadhan and A. Tasker, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 87.
[56] B. J. Votta, M. A. Levy, A. Badger, J. Bradbeer, R. A. Dodds, I. E. James, S. Thompson, M. J. Bossard, T. Carr, J. R. Connor, T. A. Tomaszek, L. Szewczuk, F. H. Drake, D. F. Veber and M. Gowen, *J. Bone Min. Res.*, 1997, **12**, 1396.
[57] J. Catalano, D. N. Deaton, E. S. Furfine, A. M. Hassell, R. B. McFadyen, A. B. Miller, L. R. Miller, L. M. Shewchuk, D. H. Willard, Jr. and Lois Wright, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 275.
[58] J. G. Catalano, D. N. Deaton, S. T. Long, R. B. McFadyen, L. R. Miller, A. J. Payne, K. J. Wells-Knecht and L. L. Wright, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 719.
[59] F. X. Tavares, V. Boncek, D. N. Deaton, A. M. Hassell, S. T. Long, A. B. Miller, A. A. Payne, L. R. Miller, L. M. Shewchuk, K. Wells-Knight, D. H. Willard, Jr., L. L. Wright and H.-Q. Zhou, *J. Med. Chem.*, 2004, **47**, 588.
[60] D. S. Yamashita, W. W. Smith, B. Zhao, C. A. Janson, T. A. Tomaszek, M. J. Bossard, M. A. Levy, H.-J. Oh, T. J. Carr, S. K. Thompson, C. F. Ijames, S. A. Carr, M. McQueney, K. J. D'Alessio, B. Y. Amegadzie, C. R. Hanning, S. Abdel-Meguid, R. L. DesJarlais, J. G. Gleason and D. F. Veber, *J. Am. Chem. Soc.*, 1997, **119**, 11351.
[61] J. M. LaLonde, B. Zhao, W. W. Smith, C. A. Janson, R. L. DesJarlais, T. A. Tomaszek, T. J. Carr, S. K. Thompson, H.-J. Oh, D. S. Yamashita, D. F. Veber and S. S. Abdel-Meguid, *J. Med. Chem.*, 1998, **41**, 4567.
[62] I. Schecter and A. Berger, *Biochem. Biophys. Res. Commun.*, 1967, **27**, 157.

[63] D. S. Yamashita, X. Dong, H.-J. Oh, C. S. Brook, T. A. Tomaszek, L. Szewczuk, D. G. Tew and D. F. Veber, *J. Comb. Chem.*, 1999, **1**, 207.
[64] R. L. DesJarlais, D. S. Yamashita, H.-J. Oh, I. N. Uzinskas, K. F. Erhard, A. C. Allen, R. C. Haltiwanger, B. Zhao, W. W. Smith, S. S. Abdel-Meguid, K. J. D'Alessio, C. A. Janson, M. S. McQueney, T. A. Tomaszek, M. A. Levy and D. F. Veber, *J. Am. Chem. Soc.*, 1998, **120**, 9114.
[65] D. F. Veber, D. S. Yamashita, H.-J. Oh, B. R. Smith, K. Salyers, M. Levy, C.-P. Lee, A. Marzulli, P. L. Smith, T. Tomaszek, D. Tew, M. McQueney, G. B. Stroup, M. W. Lark, I. E. James and M. Gowen, *Pept. New Millenium, Proc. Am. Pep. Symp.*, 2000, **16**, 4653.
[66] (a) D. M. Jones, B. Atrash, A.-C. Teger-Nilsson, E. Gyzander, J. Deinum and M. Szelke, *Lett. Pep. Sci.*, 1995, **2**, 147; (b) D. M. Jones, B. Atrash, H. Ryder, A.-C. Teger-Nilsson, E. Gyzander and M. Szelke, *J. Enzyme Inhibition*, 1995, **9**, 43.
[67] R. W. Marquis, Y. Ru, D. S. Yamashita, H.-J. Oh, J. Yen, S. K. Thompson, T. J. Carr, M. A. Levy, T. A. Tomaszek, C. F. Ijames, W. W. Smith, B. Zhao, C. A. Janson, S. S. Abdel-Meguid, K. J. D'Alessio, M. S. McQueney and D. F. Veber, *Bioorg. Med. Chem.*, 1999, **7**, 581.
[68] R.W. Marquis, Y. Ru, K.E. Erhard, A.D. Gribble, J. Witherington, A. Fenwick, B. Garnier, J. Bradbeer, M. Lark, M. Gowen, T.A. Tomaszek, D.G. Tew, B. Zhao, W.W. Smith, C.A. Janson, K.J. D'Alessio, M.S. McQueney, S.S. Abdel-Meguid, K.L. Salyers, B.R. Smith, M.A. Levy, D.F. Veber, 218th ACS National Meeting, New Orleans, Aug. 22–26 MEDI-019, 1999.
[69] R. W. Marquis, D. S. Yamashita, Y. Ru, S. L. LoCastro, H.-J. Oh, K. F. Erhard, R. L. DesJarlais, M. S. Head, W. W. Smith, B. Zhao, C. A. Janson, S. S. Abdel-Meguid, T. A. Tomaszek, M. A. Levy and D. F. Veber, *J. Med. Chem.*, 1998, **41**, 3563.
[70] R. W. Marquis, Y. Ru, J. Zing, R. E. L. Trout, S. M. LoCastro, A. D. Gribble, J. Witherington, A. E. Fenwick, B. Garnier, T. Tomaszek, D. Tew, M. E. Hemling, C. J. Quinn, W. W. Smith, B. Zhao, M. S. McQueney, C. A. Janson, K. D'Alessio and D. F. Veber, *J. Med. Chem.*, 2001, **44**, 725.
[71] A. E. Fenwick, A. D. Gribble, R. J. Ife, N. Stevens and J. Witherington, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 199.
[72] A. E. Fenwick, B. Garnier, A. D. Gribble, R. J. Ife, A. D. Rawlings and J. Witherington, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 195.
[73] I. E. James, M. W. Lark, D. Zembryki, E. Lee-Rykaczewski, S.-M. Hwang, T. A. Tomaszek, P. Belfiore and M. Gowen, *J. Bone Miner. Res.*, 1999, **14**, 1562.
[74] R. A. Dodds, I. E. James, D. Rieman, S.-M. Hwang, R. Ahern, J. R. Connor, S. Thomson, D. Veber, F. H. Drake, S. Holmes, M. W. Lark and M. Gowen, *J. Bone Miner. Res.*, 2001, **16**, 478.
[75] D. F. Veber, R. W. Marquis, D. S. Yamashita, H.-J. Oh, K. W. Ward and B. R. Smith, in *Peptides 2000, Proceedings of the European Peptide Symposium* (eds J. Martinez and J.-A. Fehrentz), Editions EDK, Paris, 2000, p. 113.
[76] D. F. Veber, S. R. Johnson, H.-Y. Cheng, B. R. Smith, K. W. Ward and K. D. Kopple, *J. Med. Chem.*, 2002, **45**, 2615.
[77] G. B. Stroup, M. W. Lark, D. F. Veber, A. Bhattacharyya, S. Blake, L. C. Dare, K. F. Erhard, S. J. Hoffman, I. E. James, R. W. Marquis, Y. Ru, J. A. Vasko-Moser, B. R. Smith, T. Tomaszek and M. Gowen, *J. Bone Miner. Res.*, 2001, **16**, 1739.
[78] R. W. Marquis, K. W. Ward, T. Roethke, B. R. Smith, Y. Ru, D. S. Yamashita, T. A. Tomaszek, P. D. Gorycki, H.-Y. Cheng, I. E. James, G. B. Stroup, M. W. Lark, M. Gowen and F. Veber, *Mol. Pharm.*, 2004, **1**, 98.
[79] M. W. Lark, G. B. Stroup, I. E. James, R. A. Dodds, S.-M. Hwang, S. M. Blake, B. A. Lechowska, S. J. Hoffman, B. R. Smith, R. Kapadia, X. Liang, K. Erhard, Y. Ru, X. Dong, R. W. Marquis, D. Veber and M. Gowen, *Bone*, 2002, **30**, 746.
[80] (a) S. A. Thompson, P. R. Andrews and R. P. Hanzlik, *J. Med. Chem.*, 1986, **29**, 104; (b) J. B. Moon, R. S. Coleman and R. P. Hanzlik, *J. Am. Chem. Soc.*, 1986, **108**, 1350.
[81] P. D. Greenspan, K. L. Clark, R. A. Tommasi, S. D. Cowen, L. W. McQuire, D. L. Farley, J. H. van Duzer, R. L. Goldberg, H. Zhou, Z. Du, J. J. Fitt, D. E. Coppa, Z. Feng, W. Macchia,

L. Zhu, M. P. Capparelli, R. Goldstein, A. M. Wigg, J. R. Doughty, R. S. Bohacek and A. K. Knap, *J. Med. Chem.*, 2001, **44**, 4524.
[82] Y. D. Ward, D. S. Thompson, L. L. Frye, C. L. Cywin, T. Morwick, M. J. Emmanuel, R. Zindell, D. McNeil, Y. Bekkali, M. Giradot, M. Harpchak, M. DeTuri, K. Crane, D. White, S. Pav, Y. Wang, M.-H. Hao, C. A. Grygon, M. E. Labadia, D. Freeman, W. Davidson, J. L. Hopkins, M. Brown and D. M. Spero, *J. Med. Chem.*, 2002, **45**, 5471.
[83] J.-P. Falgueyret, R. M. Oballa, O. Okamoto, G. Wesolowski, Y. Aubin, R. M. Rydzewski, P. Prasit, D. Riendeau, S. B. Rodan and M. D. Percival, *J. Med. Chem.*, 2001, **44**, 94.
[84] R. M. Rydzewski, C. Bryant, R. Oballa, G. Wesolowski, S. B. Rodan, K. E. Bass and D. H. Wong, *Bioorg. Med. Chem.*, 2002, **10**, 3277.
[85] S. Suzue and T. Irikura, *Chem. Pharm. Bull.*, 1968, **16**, 1417.
[86] J. Robichaud, R. Oballa, P. Prasit, J.-P. Falgueyret, M. D. Percival, G. Wesolowski, S. B. Rodan, D. Kimmel, C. Johnson, C. Bryant, S. Venkatraman, E. Setti, R. Mendonca and J. T. Palmer, *J. Med. Chem.*, 2003, **46**, 3709.
[87] S. Christgau, O. Bitsch-Jensen, N. Hanover-Bjarnason, E. Gamwell-Henriksen, P. Qvist, P. Alexandersen and D. Bang-Henriksen, *Bone*, 2000, **26**, 505.

Nonpeptide Gonadotropin Releasing Hormone Antagonists

Yun-Fei Zhu, Chen Chen, and R. Scott Struthers

Neurocrine Biosciences Inc., 10555 Science Center Dr., San Diego, CA 92121, USA

Contents

1. INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide amide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 that stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary [1,2]. The GnRH receptor has been cloned from a number of species and is a member of the G-protein coupled receptor super-family [3]. Chronic administration of peptide analogs of GnRH with increased potency and stability, such as leuprolide or goserelin, results in a down-regulation of gonadotropin secretion and subsequent gonadal suppression [4]. Depot formulations of these peptides are now routinely used for 'medical oophrectomy' where ablation of gonadal steroids has proven effective in treating a range of diseases including precocious puberty, endometriosis, prostate cancer, uterine fibroids, and breast cancer as well as for hormonal manipulation for assisted reproductive therapy [5,6]. More recently, peptide antagonists have been developed for clinical use in order to provide a more immediate gonadal suppression and avoid the initial rise in gonadal steroids which are a consequence of the initial stimulatory phase of agonist therapy [7].

Because of their peptidic nature, current GnRH based therapeutics require daily injection or implantation of long acting depots, although nasal formulations of some GnRH peptides are also available. This has prompted a number of groups to attempt to develop orally active, nonpeptide antagonists that conceptually may have several advantages over injectable GnRH peptide drugs. Obviously, injection site reactions

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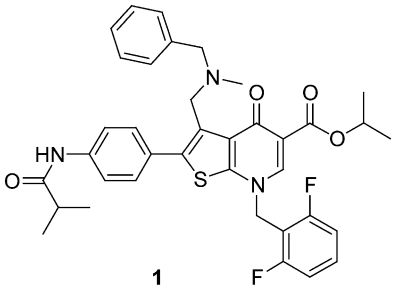
commonly observed in peptide depots are avoided and dosing can be rapidly discontinued if necessary – a clinical management option not available with long-acting injectable depots. Perhaps more importantly, it may be possible to vary the level of pituitary suppression by varying dosage and therefore allow titration of circulating estrogen levels to a therapeutic window which avoids symptoms of severe gonadal suppression, such as bone loss [8]. In this chapter, we review recent progress towards the development of nonpeptide GnRH antagonists which has resulted in a number of distinct chemical approaches to the problem.

2. THIENOPYRIDINONES AND THIENOPYRIMIDINONES

The first potent and orally active human GnRH receptor antagonist, T-98475 (**1**), was reported in 1998 [9]. It inhibits [^{125}I]leuprorelin binding to the cloned human GnRH receptor with an IC_{50} value of 0.2 nM. It also has potent binding affinity to membrane fractions of monkey pituitary with an IC_{50} of 4 nM, but somewhat lower activity on the rat pituitary (IC_{50} = 60 nM). It suppresses plasma LH concentration up to 25% level after oral administration in castrated male cynomolgus monkeys (60 mg/kg). A small set of SAR data suggested that the benzyl side-chain on the basic nitrogen contributes a 30-fold increase in binding affinity.



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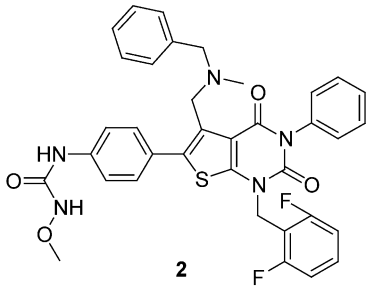

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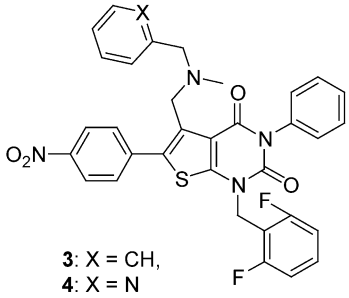
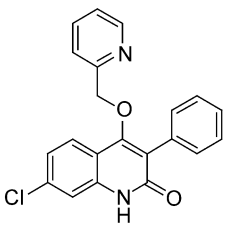


3: X = CH,
4: X = N

A slightly different series of compounds from T-98475, which removes the metabolically labile ester group, has been reported recently [10]. A representative compound from this series TAK-013 (**2**) binds with high affinity to human or monkey receptors expressed in CHO cells (IC_{50} values of 0.1 and 0.6 nM, respectively). Functionally, TAK-013 inhibits GnRH-stimulated arachidonic acid release with IC_{50}

values of 0.06 and 10 nM, for human and monkey receptors, respectively. In cynomolgus monkeys, after oral doses of 10 mg/kg, TAK-013 reaches maximal concentration of 140 ng/ml at 6 h with an AUC of 568 ng h/ml. This good oral exposure enables TAK-013 to almost completely suppress plasma LH levels (11% of pretreatment at 24 h) after oral administration of a 30 mg/kg dose in castrated male cynomolgus monkeys. Chronic administration (30 mg/kg, three times daily) over the course of four menstrual cycles in normal cycling female cynomolgus macaques resulted in a suppression of LH, estradiol and progesterone, but not FSH [11]. TAK-013 is highly lipophilic and poorly soluble in water with a log *D* value higher than 4. Structure–activity studies on the side-chain of the basic nitrogen reveals the benzyl group can be replaced by a more polar and slightly basic 2-pyridylmethyl group resulting in a 20-fold improvement in binding affinity (K_i values are 180 and 9 nM for **3** and **4**, respectively) [12].



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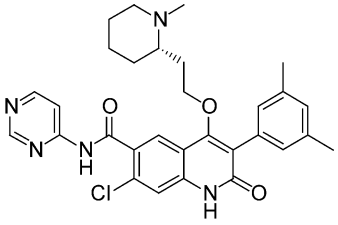

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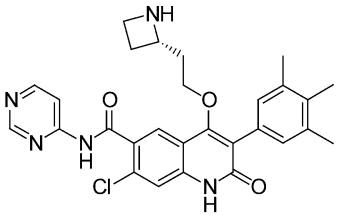


7

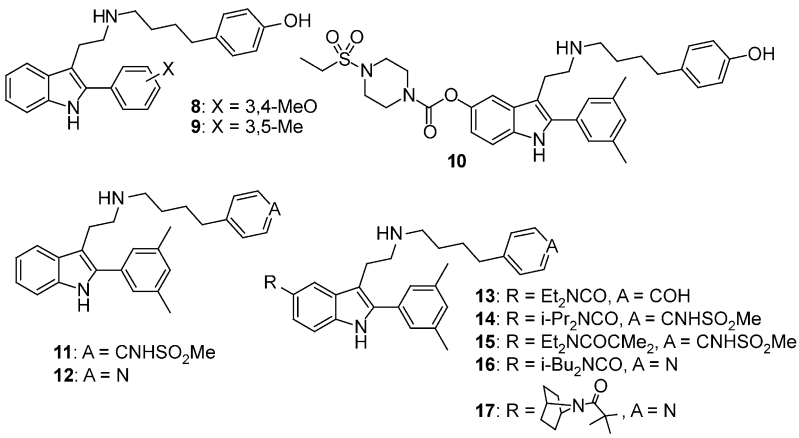
3. 3-ARYLQUINOLONES

An initial lead compound **5** was obtained from high throughput screening, and the IC_{50} of **5** at the rat GnRH receptor is reported to be 3 μM [13]. SAR studies of this series resulted in potent GnRH antagonists such as **6** with IC_{50} values of 60 nM on the rat GnRH receptor and 2.5 nM at the cloned human GnRH receptor [14–16]. Differences in affinities for GnRH receptors from different species have been reported for this and all other series of nonpeptide GnRH antagonists studied thus far. Functionally, **6** inhibits GnRH-stimulated LH release from rat pituitary cells with an IC_{50} of 85 nM, and GnRH-stimulated inositol phosphate hydrolysis in CHO cells expressing the human GnRH receptor ($IC_{50} = 16$ nM). A more potent human GnRH antagonist **7** (hGnRH $IC_{50} = 0.44$ nM, PI turnover $IC_{50} = 1.0$ nM) also binds very tightly to the rhesus monkey GnRH receptor ($IC_{50} = 0.5$ nM) and rat GnRH receptor ($IC_{50} = 4.0$ nM), but less at the dog GnRH receptor ($IC_{50} = 60$ nM). This compound suppresses circulating LH and testosterone levels in male rhesus macaques when given intravenously (0.5 mg/kg) [17].

4. 2-ARYLTRYPTAMINES

Initial SAR studies on a lead compound with a 2-aryltryptamine structure (**8**, $IC_{50} = 3$ μM) discovered from high throughput screening suggests that the optimal distance between the 4-phenol group and the basic amine is a four-carbon

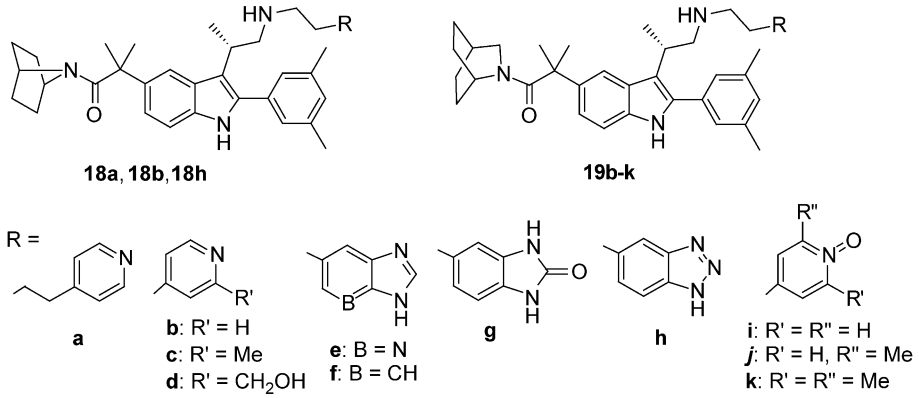
linkage [18]. When the 3,4-dimethoxyphenyl at the 2-position of tryptamine core is replaced with a 3,5-disubstituted phenyl, the binding affinity increases. For example, the 3,5-dimethoxy, 3,5-dichloro, and 3,5-dimethylphenyl analogs have IC_{50} values of 700, 170 and 50 nM, respectively. The dimethylphenyl compound (**9**) is over 25-fold better than the unsubstituted phenyl analog ($IC_{50} = 1.3\ \mu M$). However, the corresponding 4-fluorophenyl analog is about eightfold less active which suggests a lipophilic electron-rich phenyl group is favored at this position for interaction with the GnRH receptor, possibly at an aromatic residue. Further SAR study reveals a lipophilic group with hydrogen bonding capability at the 5-position of the tryptamine core improves binding affinity about 10-fold. Thus, **10** has an IC_{50} value of 4 nM [19]. When the OH group of the phenol was replaced with a methanesulfonamido group, the binding affinity further improved (**11**, $IC_{50} = 7$ nM). While the binding data above were obtained from crude membranes prepared from rat pituitary glands, the binding affinity of these compounds is less potent at the human GnRH receptor. For example, **11** exhibits an IC_{50} value of 170 nM at the cloned human receptor, an over 40-fold difference [20]. The phenol can also be replaced by a heteroaromatic ring with hydrogen bonding capability. Thus, the 4-pyridyl analog **12** has an IC_{50} value of 41 nM at the rat GnRH receptor [21].



Several very potent GnRH antagonists were synthesized by a combination of the previous SAR information. Thus, compounds with a 5-amide bulky group exhibit low nanomolar binding affinity at the rat receptor (IC_{50} values for **13**, **14** and **15** are 6, 3 and 2 nM, respectively). Functionally, they inhibit LH release from rat pituitary glands with IC_{50} values of 540, 72 and 42 nM. These data suggest the sulphonamides **14** and **15** are much more functionally active than the phenol such as **13**. Interestingly, the binding affinity of this series of compounds is greatly improved by the presence of 0.1% BSA in the binding assay, and the IC_{50} values obtained under this condition are 10-fold more potent. Thus, the IC_{50} values for **13**, **14** and **15** are now 0.5, 0.3 and 0.2 nM, respectively. The authors suggest that this shift is due to BSA preventing nonspecific loss of compound to glass assay tubes [22].

The function of the 5-amido group of the tryptamine seems not only to increase binding affinity of this series of compounds at the rat GnRH receptor, but also to narrow

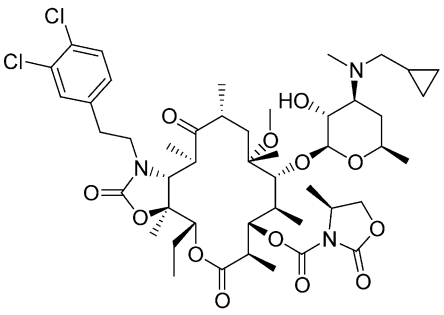
the gap in binding affinities between the rat and human GnRH receptors. For example, **16** has IC_{50} values of 0.6 and 7.1 nM at the rat and human GnRH receptors, respectively, which is only a 12-fold difference [23]. On the other hand, **11**, without a 5-substitution, has a 40-fold difference between these two species. Compound **17** is a very potent human GnRH receptor antagonist with an IC_{50} of 1.4 nM in receptor binding, and IC_{50} of 18 nM in inhibition of GnRH-stimulated inositol phosphate hydrolysis.



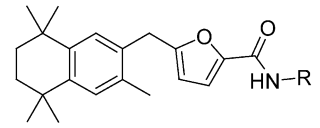
Because of the enhancement of both binding and function of this series of compounds from the substitution at the 5-position together with the introduction of (*S*)-methyl group at 2-aminoethyl side chain of the tryptamine core, the linker between the basic nitrogen and the aromatic side-chain can be reduced to 2-carbon without loss of activity (**18a**, $IC_{50} = 0.7$ nM and **18b**, $IC_{50} = 0.8$ nM). In contrast, the corresponding 2-carbon version of **12** is about 12-fold less active. This series of compounds have been further modified to improve pharmacokinetic profiles. In dogs, **19b** ($IC_{50} = 0.8$ nM) has an oral bioavailability of 25% and an elimination half-life of 3.9 h. It has a similar profile in monkeys ($F = 21\%$, $t_{1/2} = 3.3$ h), but is less bioavailable in rats ($F = 8\%$, $t_{1/2} = 1$ h) [24]. Introduction of a methyl group at the 2-position of the pyridine to reduce both 2- and *N*-oxidations of pyridine, improved oral bioavailability in dogs (**19c**, $F = 36\%$) but the 2-hydroxymethylpyridine analog **19d** with excellent *in vitro* profile did not improve bioavailability in dogs ($F = 15\%$) [25]. Despite limited oral bioavailability **19b** ($IC_{50} = 1.7$ nM, rat receptor) dose-dependently inhibits LH release for periods ranging from 5 to 7 h at 5 mg/kg po and >15 h at 20 mg/kg po in castrated male rats. Among compounds with different bicyclic side-chains, **19f** exhibits excellent oral bioavailability in dogs ($F = 63\%$), while the very close analog **19e** has much lower oral exposure ($F = 16\%$). Furthermore, low oral bioavailability of **19g** ($F = 5\%$) was observed [26]. The authors speculate that the urea structure of **19g** has very strong hydrogen-bonding capability that reduces cell permeability. Unlike most compounds in this series, **19h** has less risk of inhibition of cytochrome P450 3A4 ($IC_{50} = 7.5$ μM) while maintaining potent GnRH binding affinity ($IC_{50} = 0.3$ nM). This compound was also shown to be efficacious in the castrated male rats, where it reduced plasma LH levels for 14 h after a single oral dose at 10 mg/kg.

5. MACROLIDES

A-198401 (**20**) is a potent GnRH antagonist with a macrolide structure [27]. It inhibits ^{125}I-[Tyr^5]leuprolide binding to rat pituitary membranes with a pK_i value of 9.2. Functionally, A-198401 inhibits LH release with a pA_2 value of 8.8. It also tightly binds to the cloned human GnRH receptor, with a pK_i of 8.7. This compound has minimal antibacterial activity against *Staphylococcus* strains, and is roughly 100-fold less potent than erythromycin A. A-198401 causes no significant release of histamine from rat peritoneal mast cells, a concern which has hindered development of peptide GnRH antagonists. This compound suppresses plasma LH concentration in a dose-dependent manner after oral administration in castrated male rats. At a dose of 24 mg/kg po, A-198401 reduces the plasma LH to the 20% of pretreatment levels at approximately 3 h and this effect lasts up to the 24-h time point. Detailed SAR studies from an initial lead to yield the potent analogs **20**, and efforts on elimination of the antibacterial activities of this series of compounds have been published recently [28,29].

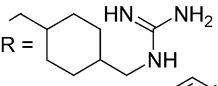


20 (A-198401)

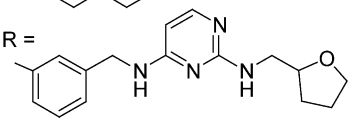


21 (AG-045572): R = 2,4,6-(MeO)3Ph

22: R =



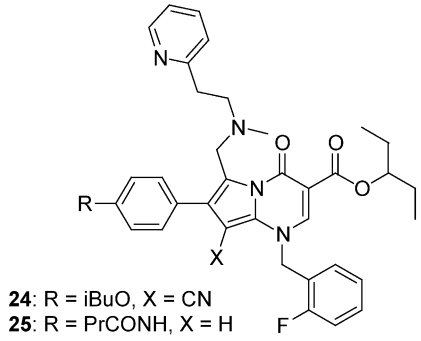
23: R =



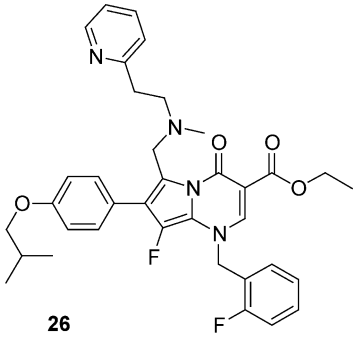
6. TETRALIN

A series of tetralins has been reported as GnRH antagonists. Among them, a potent analog AG-045572 (**21**) has been studied in detail. AG-045572 inhibits ^{125}I-[des-Gly^{10}, D-Ala^6]GnRH ethylamide binding to the membranes from rat pituitary glands with a K_i value of 3.8 nM. When tested with membranes prepared from HEK293 cells expressing mouse or human GnRH receptors, it has K_i values of 2.2 and 6.0 nM, respectively. In castrated male rats AG-045572 completely suppresses plasma LH concentration up to 8 h after oral administration of a single dose at 100 mg/kg. After a single intravenous administration at 10 mg/kg, this compound can suppress LH levels to almost baseline at the 0.5-h time point, but this effect lasts less than 2 h. AG-045572 also reduces testosterone concentrations in intact male rats after a single intravenous dose of 20 mg/kg. These data suggest AG-045572 is an efficacious GnRH antagonist [30]. Interestingly, this compound has varying oral bioavailability in rats depending on their endocrine status ($F = 8\%$ in intact male rats, and $F = 24\%$ in female and castrated male rats) [31]. Another potent analog from this series is **22** with a guanidine structure at right

side of the molecule [32]. Compound **22** is much more hydrophilic than the highly lipophilic AG-045572. This compound has K_i values of 40 nM at the human GnRH receptor and 520 nM at the rat receptor. The K_B value of this compound for inhibition of GnRH-stimulated inositol phosphate hydrolysis is 30 nM. Compound **23**, which is apparently designed to address the possible poor cell permeability of guanidine **22**, has an aminopyrimidine structure [33]. This compound has high binding affinity for both rat and human GnRH receptors (hGnRH $K_i = 9.3$ nM, rGnRH $K_i = 4.7$ nM). In cells expressing recombinant rat GnRH receptors, **23** is a competitive inhibitor of GnRH-stimulated extracellular acidification with a pA_2 value of 8.3, which is similar to its pK_i (8.3) at the rat pituitary receptor. In castrated male rats, **23** dose-dependently suppresses circulating LH levels when given intravenously (5–20 mg/kg). When administrated in intact male rats, **23** significantly lowers circulating testosterone levels for up to 24 h after a single intra-muscular dose of 20 mg/kg. At this dose, plasma levels of **23** is sustained at >1 μM for over 10 h, suggesting this compound is fairly stable in rats. **23** is also very selective among >40 receptor, enzyme and channel targets screened, with the exception of D_2 dopamine receptors (160 nM) and sodium channel site 2 (200 nM).



24: R = iBuO, X = CN
25: R = PrCONH, X = H

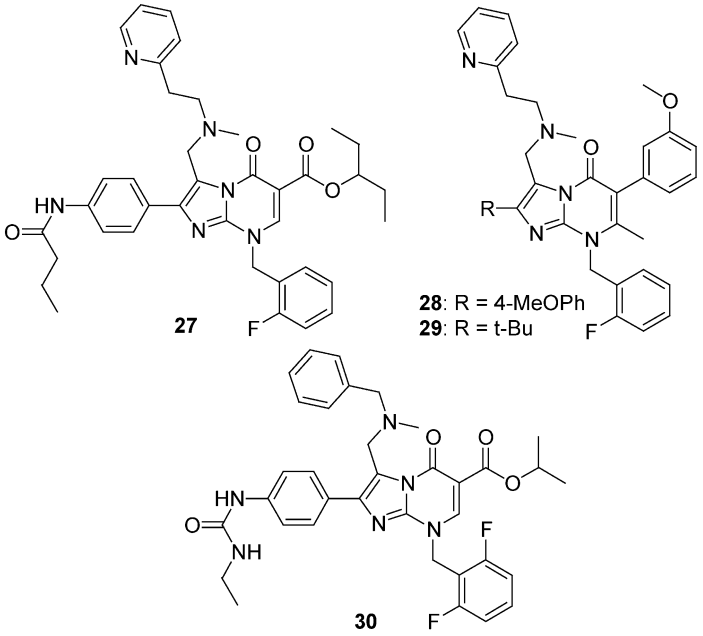


26

7. PYRROLOPYRIMIDINONE AND IMIDAZOPYRIMIDINONE

A series of pyrrolopyrimidinones as potent GnRH antagonists are described in recent publications. The initial set of compounds with 3-cyanopyrrolopyrimidinone structures have high affinity at the human GnRH receptor, but poor at the rat GnRH receptor [34]. Thus, compound **24** has K_i value of 25 nM in inhibition of des-Gly^{10}[^{125}I-Tyr^5, D-Leu^6, NMeLeu^7, Pro^9-Net]GnRH binding to the cloned human GnRH receptor stably expressed in HEK 293 cells, but only exhibits a K_i of 7.3 μM on the rat GnRH receptor. The binding affinities of this series of compounds are further enhanced by deleting the cyano group at the 3-position. Thus, **25** has K_i value of 2.7 nM on the human GnRH receptor, and completely inhibits GnRH stimulated calcium flux at 1 μM concentration [35]. While **25** exhibits much better *in vitro* activity than **24**, removal of the 3-cyano group results in analogs with less stability due to de-amination of the basic side-chain caused by the electron-rich bicyclic pyrrolopyrimidinone. This problem is resolved by introducing a much smaller but strongly electron-withdrawing fluorine group at this position.

Compound **26** has comparable binding affinity (K_i = 9 nM) to **25**, but is chemically much more stable than **25** [36].

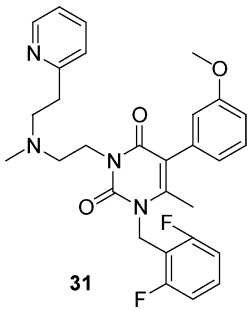


Another successful way to fix the stability of **25** is to replace the 3-carbon with a nitrogen to result in a less electron-rich imidazopyrimidinone to give such compounds as **27**. SAR studies reveal that the imidazopyrimidinone analogs are as potent as the pyrrolopyrimidinone compounds. For example, **27** has a K_i of 7.5 nM at the human GnRH receptor, and is stable under acidic conditions [37]. An independent report also demonstrates the imidazopyrimidinone such as **30** (IC_{50} = 0.3 nM) are potent GnRH antagonists [38]. Further SAR studies to address the potentially labile ester group reveals that a 3-methoxyphenyl group is a good substitution of the lipophilic isopropyl or 3-pentyl ester [39]. In combination with a methyl group at the 7-position of the bicyclic core, the binding affinity is enhanced about 50-fold. Thus, **28** with a smaller 4-methoxyphenyl group at the 2-position of imidazolopyrimidinone has a K_i value of 4.6 nM. Because of the binding enhancement from the right side of the molecule (**28**), the left side phenyl group with a hydrogen-bonding center can now be replaced by a smaller lipophilic group without losing binding affinity. Thus, **29** has K_i of 5.2 nM on the human GnRH receptor [40]. It also maintains potent functional activity in calcium flux assays (IC_{50} = 27 nM).

8. 5-ARYLURACILS

Since one of the main objectives for developing small molecule GnRH antagonists is clinical use as oral agents, a molecule with lower molecular weight might have improved pharmacokinetic properties. Towards this end, a series of monocyclic uracils have been

reported where the 5-numbered ring part of bicyclic compounds such as **28** and **29**, is essentially removed. The resulting uracils are moderately potent human GnRH receptor antagonists (**31**: $K_i = 34$ nM) [41]. Further SAR studies on the 3-side-chain reveal that introduction of a methyl group on the ethylene linkage improves binding affinity of this series, presumably by restriction of the free rotation of the ethylene group. Compounds **32** and **33** have K_i values of 5.5 and 20 nM, respectively, at the human GnRH receptor [42,43]. Compound **32** demonstrated a potent functional antagonism in inhibition of calcium flux with an IC_{50} of 2.5 nM. Continuous SAR at the 1-position of uracil also demonstrates the importance of a lipophilic, but electron-deficient benzyl group is favored for maximal binding [44]. More detailed SAR studies of this series of compounds have been published very recently [45].



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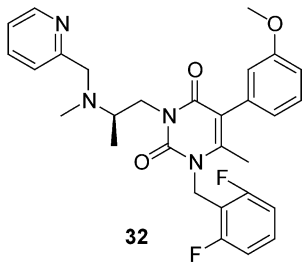

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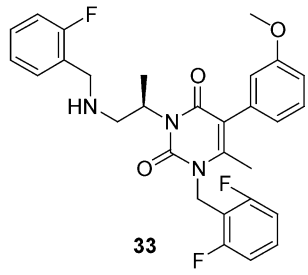

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9. COMPOUNDS IN HUMAN CLINICAL TRIALS

Early clinical results for two nonpeptide GnRH compounds, TAK-013 and NBI-42902 [46], have been reported at scientific meetings although reports have not appeared in a peer-reviewed journal as of this writing [47–50]. NBI-42902 showed good exposure following oral administration to post-menopausal women and dose-dependent suppression of LH [46]. TAK-013 was shown to suppress testosterone in healthy young men following a single oral dose [49]. In a 14-day study, premenopausal women receiving oral TAK-013 each day showed dose-dependent suppression of LH and estradiol, but no significant effect on FSH [48]. Comparison of day 1 and day 14 pharmacokinetics, as well as urinary 6-hydroxycortisol/cortisol ratios, suggested that the compound was inducing CYP3A4. A multiple dose study in post-menopausal women confirmed the potential for CYP3A4 induction, but in this population FSH, as well as LH, was suppressed [47].

10. CONCLUSIONS

The success of peptide GnRH agonists, and more recently, antagonists has prompted efforts to discover orally active GnRH antagonists. Since the publication of the first orally active GnRH antagonist in 1998, there has been tremendous progress in discovery of potent small molecule GnRH antagonists. As discussed above, at least eight distinct series of compounds have been reported in the literature. Several representative

molecules from these different series have demonstrated oral activity in suppressing LH in rats or monkeys. Recently, at least two small molecule GnRH antagonists have been evaluated in early human clinical trials. Because the well-described therapeutic mechanism of GnRH peptide based drugs is by suppression of the reproductive endocrine axis, the endocrine effects of the nonpeptide GnRH antagonists in these early clinical trials bodes well for their eventual therapeutic utility in a range of human reproductive related diseases.

REFERENCES

[1] R. Burgus, M. Butcher, M. Amoss, N. Ling, M. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale and R. Guillemin, *Proc. Natl Acad. Sci. USA*, 1972, **69**, 278.
[2] H. Matsuo, Y. Baba, R. M. Nair, A. Arimura and A. V. Schally, *Biochem. Biophys. Res. Commun.*, 1971, **43**, 1334.
[3] S. C. Sealfon, H. Weinstein and R. P. Millar, *Endocr. Rev.*, 1997, **18**, 180.
[4] P. E. Belchetz, T. M. Plant, Y. Nakai, E. J. Keogh and E. Knobil, *Science*, 1978, **202**, 631.
[5] P. M. Conn and W. F. Crowley, Jr., *N. Engl. J. Med.*, 1991, **324**, 93.
[6] D. H. Barlow, *J. Reprod. Med.*, 1998, **43**, 245.
[7] J. A. Huirne and C. B. Lambalk, *Lancet*, 2001, **358**, 1793.
[8] R. L. Barbieri, *J. Reprod. Med.*, 1998, **43**, 287.
[9] N. Cho, M. Harada, T. Imaeda, T. Imada, H. Matsumoto, Y. Hayase, S. Sasaki, S. Furuya, N. Suzuki, S. Okubo, K. Ogi, S. Endo, H. Onda and M. Fujino, *J. Med. Chem.*, 1998, **41**, 4190.
[10] S. Sasaki, N. Cho, Y. Nara, M. Harada, S. Endo, N. Suzuki, S. Furuya and M. Fujino, *J. Med. Chem.*, 2003, **46**, 113.
[11] T. Hara, H. Araki, M. Kusaka, M. Harada, N. Cho, N. Suzuki, S. Furuya and M. Fujino, *J. Clin. Endocrinol. Metab.*, 2003, **88**, 1697.
[12] Z. Guo, Y. Chen, D. Wu, Y. F. Zhu, R. S. Struthers, J. Saunders, Q. Xie and C. Chen, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3617.
[13] R. J. DeVita, D. D. Hollings, M. T. Goulet, M. J. Wyvratt, M. H. Fisher, J. L. Lo, Y. T. Yang, K. Cheng and R. G. Smith, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2615.
[14] R. J. DeVita, M. T. Goulet, M. J. Wyvratt, M. H. Fisher, J. L. Lo, Y. T. Yang, K. Cheng and R. G. Smith, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2621.
[15] T. F. Walsh, R. B. Toupence, J. R. Young, S. X. Huang, F. Ujjainwalla, R. J. DeVita, M. T. Goulet, M. J. Wyvratt, Jr., M. H. Fisher, J. L. Lo, N. Ren, J. B. Yudkovitz, Y. T. Yang, K. Cheng and R. G. Smith, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 443.
[16] J. R. Young, S. X. Huang, I. Chen, T. F. Walsh, R. J. DeVita, M. J. Wyvratt, Jr., M. T. Goulet, N. Ren, J. Lo, Y. T. Yang, J. B. Yudkovitz, K. Cheng and R. G. Smith, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1723.
[17] R. J. DeVita, T. F. Walsh, J. R. Young, J. Jiang, F. Ujjainwalla, R. B. Toupence, M. Parikh, S. X. Huang, J. A. Fair, M. T. Goulet, M. J. Wyvratt, J. L. Lo, N. Ren, J. B. Yudkovitz, Y. T. Yang, K. Cheng, J. Cui, G. Mount, S. P. Rohrer, J. M. Schaeffer, L. Rhodes, J. E. Drisko, E. McGowan, D. E. MacIntyre, S. Vincent, J. R. Carlin, J. Cameron and R. G. Smith, *J. Med. Chem.*, 2001, **44**, 917.
[18] L. Chu, J. E. Hutchins, A. E. Weber, J. L. Lo, Y. T. Yang, K. Cheng, R. G. Smith, M. H. Fisher, M. J. Wyvratt and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 509.
[19] L. Chu, J. L. Lo, Y. T. Yang, K. Cheng, R. G. Smith, M. H. Fisher, M. J. Wyvratt and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 515.
[20] P. Lin, D. Marino, J. L. Lo, Y. T. Yang, K. Cheng, R. G. Smith, M. H. Fisher, M. J. Wyvratt and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1073.
[21] P. Lin, M. Parikh, J. L. Lo, Y. T. Yang, K. Cheng, R. G. Smith, M. H. Fisher, M. J. Wyvratt and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1077.

[22] W. T. Ashton, R. M. Sisco, Y. T. Yang, J. L. Lo, J. B. Yudkovitz, K. Cheng and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1723.
[23] W. T. Ashton, R. M. Sisco, Y. T. Yang, J. L. Lo, J. B. Yudkovitz, P. H. Gibbons, G. R. Mount, R. N. Ren, B. S. Butler, K. Cheng and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1727.
[24] W. T. Ashton, R. M. Sisco, G. R. Kieczykowski, Y. T. Yang, J. B. Yudkovitz, J. Cui, G. R. Mount, R. N. Ren, T. J. Wu, X. Shen, K. A. Lyons, A. H. Mao, J. R. Carlin, B. V. Karanam, S. H. Vincent, K. Cheng and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 2597.
[25] J. P. Simeone, R. L. Bugianesi, M. M. Ponpipom, Y. T. Yang, J. L. Lo, J. B. Yudkovitz, J. Cui, G. R. Mount, R. N. Ren, M. Creighton, A. H. Mao, S. H. Vincent, K. Cheng and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3329.
[26] J. R. Young, S. X. Huang, T. F. Walsh, M. J. Wyvratt, Y. T. Yang, J. B. Yudkovitz, J. Cui, G. R. Mount, R. N. Ren, T. J. Wu, X. Shen, K. A. Lyons, A. H. Mao, J. R. Carlin, B. V. Karanam, S. H. Vincent, K. Cheng and M. T. Goulet, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 827.
[27] L. M. Besecke, G. J. Diaz, J. A. Segreti, K. M. Mohning, V. A. Cybulski, M. Rao, E. N. Bush, J. T. Randolph, P. L. Waid, F. Haviv, C. D. Wegner and J. Greer, *Drug Dev. Res.*, 2001, **52**, 485.
[28] J. T. Randolph, P. Waid, C. Nichols, D. Sauer, F. Haviv, G. Diaz, G. Bammert, L. M. Besecke, J. A. Segreti, K. M. Mohning, E. N. Bush, C. D. Wegner and J. Greer, *J. Med. Chem.*, 2004, **47**, 1085.
[29] J. T. Randolph, D. R. Sauer, F. Haviv, A. M. Nilius and J. Greer, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1599.
[30] K. L. Anderes, D. R. Luthin, R. Castillo, E. A. Kraynov, M. Castro, K. Nared-Hood, M. L. Gregory, V. P. Pathak, L. C. Christie, G. Paderes, H. Vazir, Q. Ye, M. B. Anderson and J. M. May, *J. Pharmacol. Exp. Ther.*, 2003, **305**, 688.
[31] E. A. Iatsimirskaia, M. L. Gregory, K. L. Anderes, R. Castillo, K. E. Milgram, D. R. Luthin, V. P. Pathak, L. C. Christie, H. Vazir, M. B. Anderson and J. M. May, *Pharm. Res.*, 2002, **19**, 202.
[32] D. R. Luthin, Y. Hong, V. P. Pathak, G. Paderes, K. D. Nared-Hood, M. A. Castro, H. Vazir, H. Li, E. Tompkins, L. Christie, J. M. May and M. B. Anderson, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3467.
[33] D. R. Luthin, Y. Hong, E. Tompkins, K. L. Anderes, G. Paderes, E. A. Kraynov, M. A. Castro, K. D. Nared-Hood, R. Castillo, M. Gregory, H. Vazir, J. M. May and M. B. Anderson, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3635.
[34] Y. F. Zhu, R. S. Struthers, P. J. Connors, Jr., Y. Gao, T. D. Gross, J. Saunders, K. Wilcoxen, G. J. Reinhart, N. Ling and C. Chen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 399.
[35] Y. F. Zhu, K. Wilcoxen, J. Saunders, Z. Guo, Y. Gao, P. J. Connors, Jr., T. D. Gross, F. C. Tucci, R. S. Struthers, G. J. Reinhart, Q. Xie and C. Chen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 403.
[36] F. C. Tucci, Y. F. Zhu, Z. Guo, T. D. Gross, P. J. Connors, Jr., R. S. Struthers, G. J. Reinhart, X. Wang, J. Saunders and C. Chen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3491.
[37] K. M. Wilcoxen, Y. F. Zhu, P. J. Connors, J. Saunders, T. D. Gross, Y. Gao, G. J. Reinhart, R. S. Struthers and C. Chen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2179.
[38] S. Sasaki, T. Imaeda, Y. Hayase, Y. Shimizu, S. Kasai, N. Cho, M. Harada, N. Suzuki, S. Furuya and M. Fujino, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2073.
[39] T. D. Gross, Y. F. Zhu, J. Saunders, K. M. Wilcoxen, Y. Gao, P. J. Connors, Jr., Z. Guo, R. S. Struthers, G. J. Reinhart and C. Chen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2185.
[40] Y. F. Zhu, Z. Guo, T. D. Gross, Y. Gao, P. J. Connors, Jr., R. S. Struthers, Q. Xie, F. C. Tucci, G. J. Reinhart, D. Wu, J. Saunders and C. Chen, *J. Med. Chem.*, 2003, **46**, 1769.
[41] Y. F. Zhu, T. D. Gross, Z. Guo, P. J. Connors, Jr., Y. Gao, F. C. Tucci, R. S. Struthers, G. J. Reinhart, J. Saunders, T. K. Chen, A. L. Killam Bonneville and C. Chen, *J. Med. Chem.*, 2003, **46**, 2023.
[42] Z. Guo, Y. F. Zhu, F. C. Tucci, Y. Gao, R. S. Struthers, J. Saunders, T. D. Gross, Q. Xie, G. J. Reinhart and C. Chen, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3311.
[43] F. C. Tucci, Y. F. Zhu, Z. Guo, T. D. Gross, P. J. Connors, Jr., R. S. Struthers, G. J. Reinhart, J. Saunders and C. Chen, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3317.
[44] M. W. Rowbottom, F. C. Tucci, Y.-F. Zhu, Z. Guo, T. D. Gross, G. J. Reinhart, Q. Xie, R. S. Struthers, J. Saunders and C. Chen, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 2269.

[45] Z. Guo, Y. F. Zhu, T. D. Gross, F. C. Tucci, Y. Gao, M. Moorjani, P. J. Connors, M. W. Rowbottom, Y. Chen, R. S. Struthers, Q. Xie, J. Saunders, G. Reinhart, T. K. Chen, A. L. Killam Bonneville and C. Chen, *J. Med. Chem.*, 2004, **47**, 1259.
[46] S. Struthers, H. Pan, S. Yen, T. Chen, B. Campbell, G. Reinhart, C. Chen, R. Jimenez and H. Bozigian, The Seventh International Symposium on GnRH Analogues in Cancer and Human Reproduction, Amsterdam, The Netherlands, February 6–9, 2003.
[47] M. Boyce, E. Clark, A. Johnston, M. George, J. Davies and M. Hibberd, Fifty-eighth Annual Meeting of the American Society for Reproductive Medicine, Seattle, WA, October 12–17, 2002.
[48] E. Clark, M. Boyce, A. Johnston, M. George, J. Davies and M. Hibberd, Fifty-eighth Annual Meeting of the American Society for Reproductive Medicine, Seattle, WA, October 12–17, 2002.
[49] E. Clark, M. Boyce, S. Warrington, A. Johnston, N. Suzuki, N. Cho, N. Dote, A. Nishizawa and S. Furuya, British Pharmacological Society Clinical Pharmacology Section, Proceedings published in Br. J. Clin. Pharmacol., January 7–10, 2003, p. 443.
[50] S. Furuya, N. Suzuki, N. Cho, S. Sasaki, T. Imada, M. Harada, T. Urushibara, S. Takekawa, A. Horinouchi, E. Clark, H. Onda, M. Boyce and M. Fujino, The Seventh International Symposium on GnRH Analogues in Cancer and Human Reproduction, Amsterdam, The Netherlands, February 6–9, 2003.

Section 3
Inflammatory, Pulmonary, and Gastrointestinal Diseases

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Merck Research Laboratories
West Point
Pennsylvania

Bradykinin B2 Receptor Antagonists for the Treatment of Pain

Edward K. Dziadulewicz

Novartis Institute for Medical Sciences, 5 Gower Place, London WC1E 6BS, UK

Contents

1. INTRODUCTION

Persistent pain is one of the most difficult pathologies to diagnose and treat effectively. Not only is pain perception very subjective in nature, but also the mechanistic redundancies inherent in pain neurobiology have led to a large number of emerging pain targets. The bradykinin B2 receptor is a mature G-protein coupled receptor target that has long offered the prospect of therapeutic intervention in pain and inflammation, but this has been notoriously difficult to achieve. This review charts the progress of bradykinin B2 antagonist medicinal chemistry and presents the clinical outlook for compounds in this area.

2. THE CHALLENGE OF PAIN

The alleviation of pain, especially persistent pain, is an urgent and unrelenting issue for physicians and the biopharmaceutical industry. Pain is initiated when noxious thermal, mechanical, or chemical stimuli excite the peripheral terminals of specialized primary afferent neurons (C and Aδ fibres) called 'nociceptors'. Under physiological conditions, acute painful stimuli serve as a protective mechanism, integral to survival or well-being. Such acute pain is generally short-lived and clinically controlled by removing the causative noxious stimulus or by treatment with conventional analgesics (non-steroidal anti-inflammatory drugs and opioids). However, under conditions of more prolonged tissue injury or peripheral sensory nerve damage (neuropathy), a number of complex adaptive changes occur within nociceptive pathways that together promote the clinical

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symptoms of chronic pain, including spontaneous pain, hyperalgesia (exaggerated sensitivity to pain) and allodynia (pain elicited by stimuli that are normally innocuous). Such pain, arising from metabolic disorders, infection or physical trauma, has outlived its biological usefulness, becoming a disease in itself and producing enormous deleterious effects on the physical and emotional states of the patient. However, at least 50% of patients given conventional therapy for their pain do not feel that the treatment is adequate. This unmet medical need, centred on the poor efficacy and poor side-effect profile of current therapies, has created a health problem of staggering proportions. The prevalence of these disorders has been forecast to rise, driven by the increasing age of the general population, the growth of obesity, improving diagnostic techniques and increasing physician (and patient) awareness. As a consequence, the annual economic burden of direct treatment (drugs, hospitalization, consultations) and the indirect societal costs (lost productivity, disability and retirement payments) are estimated to increase accordingly.

3. THE KALLIKREIN-KININ SYSTEM

Kinins are an endogenous family of potent vasoactive linear peptides acting as local (paracrine) hormones in the peripheral and central nervous systems. They are generated as short-lived components of the kallikrein-kinin system to regulate normal physiological processes and in response to pathophysiological stimuli such as inflammation or tissue damage [1,2]. The major human kinins are the nonapeptide bradykinin (BK; Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9}) and the physiologically related decapeptide kallidin (KD; [Lys^{0}]BK; Lys^{1}-Arg^{2}-Pro^{3}-Pro^{4}-Gly^{5}-Phe^{6}-Ser^{7}-Pro^{8}-Phe^{9}-Arg^{10}). The third human kinin, the undecapeptide Met-Lys-BK, is apparently only produced in inflammatory processes.

The kinin sequences are embedded in single-chain, multidomain glycoproteins called kininogens, which are synthesized in the liver and secreted into the mammalian circulation [3]. There are three molecular variants of these precursor proteins. High molecular weight kininogen (H-kininogen) is a 120-kDa α-globulin which is present in man, together with low molecular weight kininogen (L-kininogen), which is a 66-kDa β-globulin [4]. The third species, T-kininogen, is present only in rats (the precursor of Ile-Ser-BK or 'T-kinin'). The domain containing the kinin moiety has other specific functions, such as the ability to act as a cell binding site [5]. Thus, apart from extracellular fluid, kininogens have also been isolated on a number of cell types, such as human platelets [6], endothelial cells [7] and neutrophils [8]. The involvement of these cells in tissue injury makes this an efficient mechanism by which to deliver kinins to sites of inflammation.

Under pathophysiological conditions, elevated levels of kinins are rapidly produced from kininogen substrates by a variety of enzymatic activity [9,10]. However, the two most potent kininogen-metabolizing enzymes are the trypsin-like serine proteases, plasma kallikrein (PK) and tissue kallikrein (TK). The inactive precursors of these enzymes are normally present in all tissues and are ready to be activated by physiological or pathophysiological processes. BK is produced in plasma predominantly from H-kininogen. Although L-kininogen is a poor substrate for PK, it will form BK in

the presence of another serine protease, neutrophil elastase [11]. In fact, this enzyme combination may also generate Met-Lys-BK at inflammatory sites. KD, on the other hand, is preferentially released in tissues from L-kininogen, but TK can generate the peptide from both H- and L-kininogen [12].

Once formed, kinins exert their biological influences at two distinct cell surface receptors, designated B1 and B2. These membrane-bound receptors are members of the Type 1 GPCR superfamily and share the characteristic serpentine architecture and heptahelical topology. The receptors were initially distinguished on the basis of their differing pharmacological profiles [13,14], and this characterization was confirmed by molecular cloning techniques [15,16]. The cloned human B1 receptor is a 353 amino acid protein. B1 receptor density increases after exposure to inflammatory or noxious stimuli, i.e., B1 receptors are inducible, whereas they are poorly expressed under physiological conditions [17–19]. BK and KD are essentially inactive at the B1 receptor, which has higher affinity for their active metabolites. The cloned human B2 receptor is composed of 364 amino acids and is phylogenetically only 36% identical to the human B1 receptor. In contrast to the B1 subtype, B2 receptors are expressed constitutively in most cell and tissue types and mediate most of the known effects of BK and KD when these are produced in plasma and tissues, respectively [17,20].

A number of kininase enzymes rapidly degrade BK and KD to produce a variety of both active and inactive peptide metabolites [1]. Aminopeptidases remove amino acid residues from the N-terminus of the kinin sequences. In this fashion, KD can be converted to BK, but subsequent hydrolysis leads to kinin inactivation. At the C-terminus, kininase I-carboxypeptidases remove the terminal Arg^9 residue to produce des-Arg^9-BK and des-Arg^{10}-KD. These peptide fragments act as selective, full agonists for the B1 receptor and therefore prolong overall kinin activity. Carboxypeptidase N (CPN) is the soluble circulating form and gives BK a half-life of about 15 s in plasma. The kininase II enzyme, angiotensin converting enzyme (ACE), inactivates BK and KD by removing the C-terminal Phe-Arg dipeptide moiety. The enzyme inactivates kinins mainly during their passage through the lungs [1]. Finally, cleavage of BK and KD at Phe^5 and Phe^6, respectively, is facilitated by neutral endopeptidase 3.4.24.11 (NEP).

Recently, GPR100 was identified on human chromosome 1 from the human genome database as a new high-affinity BK receptor with binding features resembling those of the B2 receptor (BK $EC_{50} = 7.2$ nM; KD $EC_{50} = 6.6$ nM; Ca^{2+}-induced bioluminescence assay) [21]. Cloning of GPR100 revealed it to be a 374 amino acid protein with highest sequence identity (43%) to the human orphan somatostatin- and angiotensin-like peptide receptor (SALPR), although a very recent phylogenetic analysis placed SALPR between the B1 and B2 receptors [22]. There are suggestions that GPR100 and B2 may differ in their responses to BK given the involvement of different G-protein subunits in the signal transduction processes of the two receptors. If confirmed, this would lend credence to the frequently cited reports of B2 receptor heterogeneity [23], observations which are also very much dependent upon the cellular environments in which the B2 receptor is expressed [24]. Interestingly, GPR100 is highly expressed in a number of tumour-derived cell lines, hinting at a role in mediating the effects of BK on cell proliferation and tumourigenesis. Indeed, for quite some time, BK antagonists have been proposed as potential anti-cancer agents [25].

4. THE KININ B2 RECEPTOR AS A THERAPEUTIC TARGET

Although the role of B1 receptors in mediating various pain responses has been consolidated over the last ten years, and appears very compelling, the more physiologically prominent B2 subtype has attracted the most scrutiny to date as a drug target. This is also due to the fact that small molecule B1 antagonists have appeared only recently and have yet to achieve clinical candidacy [26].

One would expect B2 receptor antagonists to exhibit analgesic properties given the indisputable role of BK in evoking pain. By directly stimulating B2 receptors on nociceptors, BK exhibits an extremely potent pain-producing (algogenic) action. BK can also sensitizes these neurones to other noxious stimuli by lowering their firing thresholds [27,28]. Recent mechanistic studies in mice have demonstrated that BK produces its nociceptive effects, at least in part, through its ability to activate and sensitize the vanilloid receptor (TRPV1) [29]. It does this by stimulating the production of lipid-derived second messengers and this was proved to be a purely B2-mediated event. In contrast, B1 receptor agonists are not thought to directly excite or sensitize nociceptors, but contribute to nociception *via* other mechanisms [28].

As a pro-inflammatory mediator, BK elicits a wide variety of biological effects: oedema formation, arterial dilatation, venoconstriction and increased vascular permeability [30]. BK also facilitates the release of primary sensory neuropeptides such as substance P and calcitonin gene-related peptide, which can amplify the inflammatory response [31]. The action of BK on vascular smooth muscle promotes extravasation of plasma and inflammatory cells from the circulation into areas of infection, injury and inflammation. The cells may be activated to release other mediators such as prostaglandins, cytokines and nitric oxide [32]. These events are abetted by the enzyme elastase, released from neutrophils, which produces tissue degradation, allowing cellular infiltration of affected tissues, as well as helping to release Met-Lys-BK into the inflammatory milieu [33].

There is evidence that activation of B2 receptors engages multiple signal transduction pathways, leading to production of pro-inflammatory cytokines, such as interleukin-1β, and induction of the transcriptional nuclear factor-κB. These entities are subsequently capable of inducing the upregulation and expression of B1 receptors, potentiating the inflammatory response further [34,35]. In fact, interleukin-1β was also able to induce B2 receptor expression in human bronchial smooth muscle cells, establishing a positive feedback mechanism [36].

5. PEPTIDE BRADYKININ B2 RECEPTOR ANTAGONISTS

Peptide antagonists for B2 receptors were first discovered in 1984 following a systematic investigation of the BK sequence of amino acid residues [37]. The critical structural change which conferred antagonist action was the replacement of Pro^7 with D-Phe^7, ushering in the 'first generation' of antagonists. The affinity of these antagonists for B2 receptors could be significantly increased by replacing both Phe^5 and Phe^8 by thienylalanine (**1**, NPC-349, Table 1) [38]. In addition, the degrading action of aminopeptidase P could be blocked with the addition of an N-terminal D-Arg residue,

Table 1. Four generations of peptide antagonists (adapted from Ref. [48])

Compound	Sequence	Rat uterus[a]
BK	Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9]	7.9[b]
1	DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DPhe-Thi-Arg	6.9
2	DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg	9.5
3	DArg-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-Arg	10.0 (7.9[c])
4	DArg-Arg-Pro-Hyp-Gly-Igl-Ser-Df5f-Igl-Arg	10.5

Abbreviations: f5f, pentafluorophenylalanine; Hyp, 4-hydroxyproline; Igl, α-(2-indanyl)glycine; Oic, L-(3a*S*,7a*S*)-octahydroindole-2-carboxylic acid; Thi, β-(2-thienyl)alanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.
[a] pA_2 of antagonist activity.
[b] pD_2 of agonist activity.
[c] B1 receptor binding, pIC_{50}.

thereby prolonging the *in vivo* lifetime. Although action of ACE was blocked by D-Phe[7], the first generation antagonists were still substrates for CPN, which was able to remove the C-terminal Arg residue, and limited plasma duration of the intact structure to less than 20 min [39].

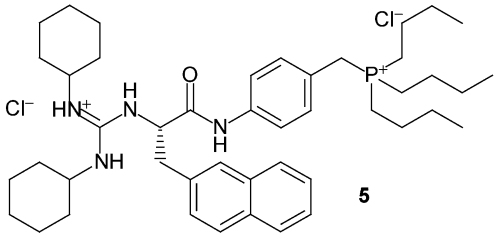
Successive generations of peptide antagonists incorporated unnatural amino acids in an effort to compensate for the particular weaknesses of their predecessors. The most well known of the 'second generation' antagonists is HOE-140 (**2**, icatibant), distinguished by the presence of the conformationally constrained dipeptide fragment D-Tic[7]-Oic[8] [40,41]. Compound **2** exhibited a much improved *in vivo* lifetime, mainly due to the Oic residue at position 8, which blocked the action of CPN. The difference in biological activity between peptides **2** and **1**, meanwhile, highlights the advantages of judicious conformational restriction, in that the 3D structure of the important C-terminus of **2** must be interacting favourably with complementary B2 receptor components.

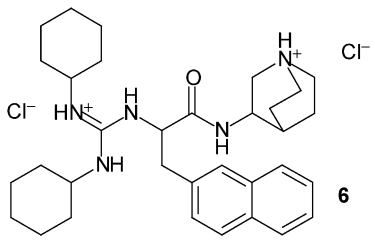
A subsequent modification to the structure of peptide **2** signalled the emergence of the 'third generation' antagonists. The non-proteinogenic amino acid, α-2-indanylglycine was introduced at positions 5 and 7 in L- and D-forms, respectively, to afford B-9430 (**3**) [42,43]. Not only is this peptide very potent in established kinin assay systems (Table 1), but also it is completely stable to enzymatic action (e.g., by lung or kidney homogenates) and orally bioavailable in rats [44]. The position 5 modification specifically stabilizes the sequence to hydrolysis by NEP. An interesting additional feature of peptide **3** is its antagonistic activity at both B2 and B1 receptors despite incorporating the C-terminal Arg residue [45]. This dual activity is an attribute shared by the 'fourth generation' antagonists (e.g., **4**, B-10056), which are the most potent peptide antagonists synthesized to date and characterized by the D-pentafluorophenylalanine residue at position 7 [46].

6. NON-PEPTIDE BRADYKININ B2 RECEPTOR ANTAGONISTS

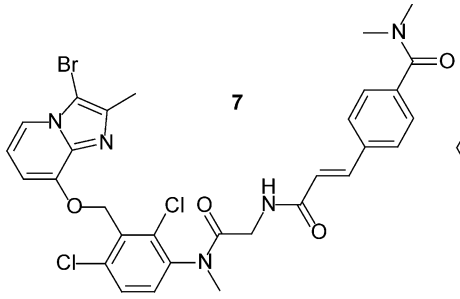
Non-peptide antagonists of the B2 receptor have been available since 1993, and have formed the subject of a number of previous reviews [9,47–51]. The first potent,

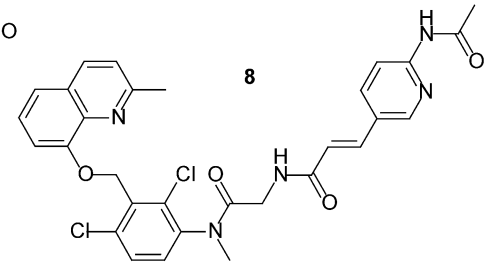
non-peptide antagonist of the human B2 receptor, **5** (WIN 64338), was based on a L-β-(2-naphthyl)alanine scaffold [52]. Compound **5** incorporates two terminal positively charged groups at a separation distance mimicking that between the terminal Arg residues in the proposed bioactive conformation of BK [53]. The affinity of compound **5** for B2 receptors in human IMR-90 fetal lung fibroblasts was 60 nM and the compound demonstrated a competitive antagonism in BK-mediated functional assays (pA_2 7.1 ± 0.5 in guinea pig ileum contractility assay). However, **5** exhibited an affinity for muscarinic receptors, a lack of oral bioavailability and variable B2 affinity across species [54]. Although modifications were made to **5** for suspected toxicological and bioavailability reasons (e.g., **6**: h-IMR-90 $K_i = 0.41$ μM), the design principle behind this compound class has only been extrapolated to a few other structural variants [49].





The imidazo[1,2-*a*]pyridine **7** (FR167344) and quinoline **8** (FR173657 or FK3657) were identified three years after the publication of compound **5** [55–57]. This series of antagonists arose from an appreciation of the relationship between the kinin system and the renin–angiotensin system [58,59]. The benzyloxy heteroaromatic substructure appears to be the common non-peptidic fragment recognized by the B2 and angiotensin II AT1 receptors. A key role was also attributed to the 2,6-dichloro-3-*N*-methylanilide moiety in stabilizing the active conformation suggested for these antagonists.

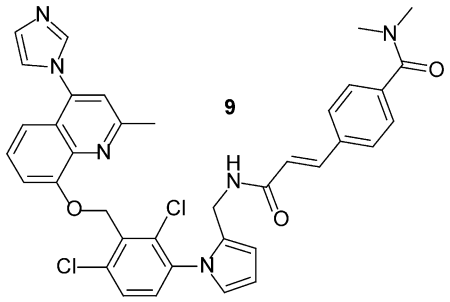


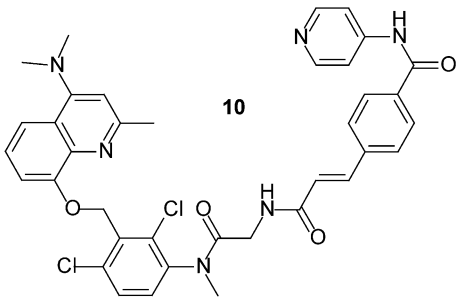


Compounds **7** and **8** inhibited the specific binding of [^{3}H]BK to human recombinant B2 receptors expressed in CHO cells with IC_{50} values of 2.2 and 1.4 nM, respectively. Intensive pharmacological evaluation of these compounds revealed pronounced beneficial efficacy in various animal models of inflammatory diseases. Thus, oral administration of **7** inhibited carrageenin-induced paw oedema in rats with an ID_{50} of 2.7 mg/kg at 2 h after carrageenin injection [60].

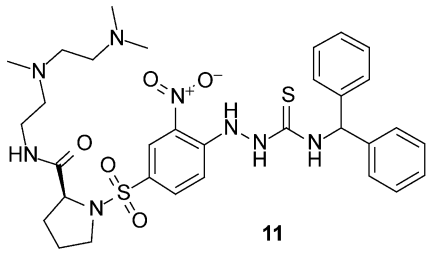
Compound **8** has recently been further elaborated with the introduction of nitrogen-containing heteroaromatic moieties and various aliphatic amino groups at

the quinoline 4-position [61,62]. By facilitating salt formation, it is hoped that an injectable form of the antagonist can be used in life-threatening inflammatory diseases such as acute pancreatitis, shock and brain oedema. The quinoline 4-position was also confirmed to be the key determinant of species selectivity, with structural changes enhancing compound affinities for the human B2 receptor compared to those at the guinea pig B2 receptor. Compound **9** had an IC_{50} value of 0.26 nM at the cloned human B2 receptor expressed in CHO cells, and was as potent as the peptide **2** in inhibiting BK-induced bronchoconstriction in guinea pigs at 1 μg/kg iv. The affinity of compound **10** for the cloned human B2 receptor ($IC_{50} = 0.7$ nM) was comparable to that of the peptide **2** ($IC_{50} = 0.49$ nM). In addition, **10** inhibited BK-induced bronchoconstriction *in vivo* at 10 μg/kg iv more efficaciously than the peptide **2** at 1 μg/kg iv, despite a 57-fold lower binding affinity for the guinea pig B2 receptor. These derivatives could be dissolved in 5% aqueous citric acid solution up to a concentration of 10 mg/mL.





The thiosemicarbazide **11** (bradyzide) represents a third, distinct non-peptide B2 antagonist class, and was optimized from a lead compound discovered by random screening [63,64]. Compound **11** had an affinity of 0.5 nM for the rat B2 receptor expressed in NG108-15 neuroblastoma–glioma hybrid cell membranes and was both competitive and selective. It displayed *in vivo* functional antagonist activity against Complete Freund's Adjuvant (CFA)-induced mechanical hyperalgesia in rats upon oral administration ($ED_{50} = 0.84$ μmol/kg, 0.57 mg/kg). However, the compound was much less potent at the human B2 receptor expressed in Cos-7 cells, with a K_i of 772 nM. Activity at the human B2 subtype could be restored by replacing the terminal diphenylmethyl moiety with dibenzosuberyl [65]. Compound **12** had an affinity of 2.8 nM at the human B2 receptor, and oral administration reversed CFA-induced and turpentine-induced mechanical hyperalgesia in rodents with ED_{50} values of 0.027 μmol/kg (0.02 mg/kg) and 0.32 μmol/kg (0.22 mg/kg), respectively. Subsequent binding and functional studies have also shown it to be unusually specific for the human form of the B2 receptor (e.g., rat B2R $K_i = 176$ nM, rabbit B2R $K_i = 44.4$ nM; pA_2 7.53 and <5 in human and rabbit venous contractility assays, respectively) [66].



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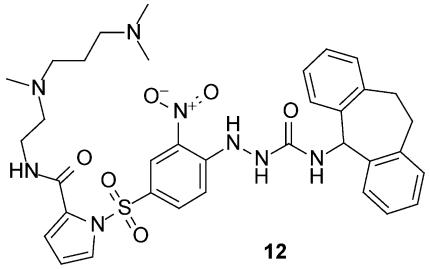
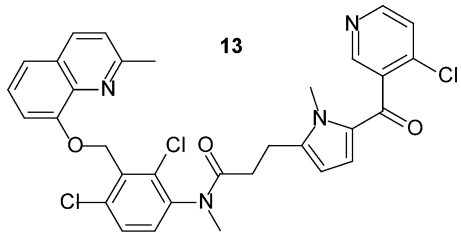


12

Compounds **7** and **8** have provided the basic framework for a number of related structures [67,68]. Most recently, a series of aroylpyrrole alkylamides (**13**) and pyrrolidine-containing B2 antagonists (**14**) has been disclosed [69,70]. Structural changes were introduced to replace the amide linkages found in **8** as well as substituting for the *N*-Me *cis* amide moiety, in an effort to modulate solubility, metabolic stability and *in vivo* properties. The compounds exhibited human B2 binding affinities (K_i) of 4–5500 nM and were functional antagonists of the B2 receptor in a GTP-γ-S assay (data not provided). In a kaolin-induced abdominal irritancy test, compound **13** (hB2R $K_i = 38$ nM) gave an ED_{50} of 23 μmol/kg po (14 mg/kg), whereas the (*S*)-enantiomer of compound **14** (hB2R $K_i = 33$ nM) showed a 59% inhibition of abdominal contractions at 160 μmol/kg po in the mouse, which was comparable to that elicited by compound **8** itself (69% inhibition at 100 mg/kg).



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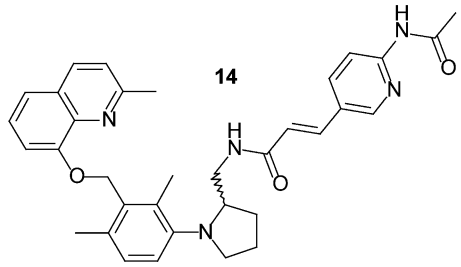
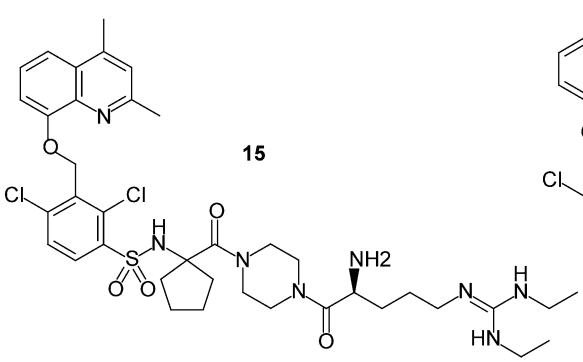
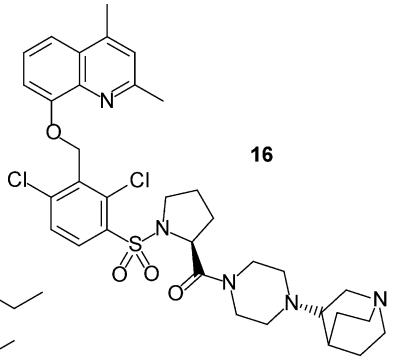


14

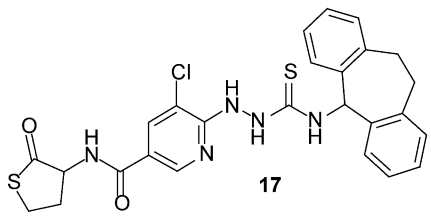
Replacement of the *N*-Me *cis* amide moiety of compound **8** has also appeared in the patent literature of the last year [71,72]. Compounds **15** and **16** incorporate *N*-benzenesulfonyl amino acids in this part of the molecule, a structural modification first introduced about five years ago [67]. Compound **15** is one of 90 examples characterized by the presence of an α,α-dialkyl amino acid residue and at least one additional amino group. Compound **15** exhibited a pK_i value of 10.1 at the human B2 receptor expressed in human WI38 fibroblasts. The α,α-dialkyl amino acid moiety is also claimed to confer a higher potency and longer duration of action in inhibiting BK-induced bronchospasm in guinea pigs, although no data are given. (*S*)-proline-containing compounds, such as **16**, were reported to give IC_{50} values of 0.1–4 nM in inhibiting the binding of [^{3}H]BK to CHO-K1 cell membranes, prepared from monkey ileum.

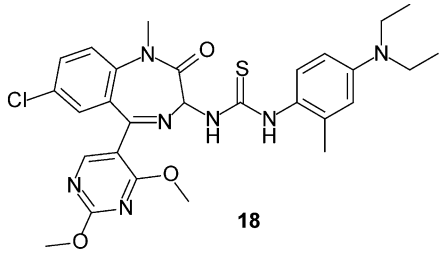




Compounds structurally related to the semicarbazide **12** were specifically exemplified in a recently published patent [73]. Most of the 165 analogues described therein were based on a pyridinecarboxamide core (e.g., **17**) and they exhibited K_i values in the range 5–5000 nM at the cloned human B2 receptor, expressed in HEK 293 S cells. The compounds are claimed to be useful for the treatment of pain of different aetiology, but no further data are presented.

The widely applicable 1,4-benzodiazepin-2-one core has been previously used as a scaffold for the design of B1 and B2 ligands [26,74,75]. Two recent patent disclosures extend this application, focussing on B2 antagonism as a treatment for pain [76,77]. A library of more than 1100 examples was assembled and K_i values of 43–3110 nM were obtained for the 33 compounds tested at the human B2 receptor (e.g., **18**), although no individual values are reported.





7. CONCLUSION

There is an urgent clinical need for new analgesic medicines with novel mechanisms of action. Within the last decade, potent, selective and orally active non-peptide B2 receptor antagonists have emerged as potential therapeutic agents and have demonstrated efficacy in various animal models of inflammatory pain. However, although most patent applications cite pain as a major indication, no B2 antagonist has been successfully shepherded through clinical trials to registration, despite a substantial industry-wide preclinical effort [23,78]. Indeed, kinin antagonists have been incisively described as 'therapeutic agents in search of an indication' [78]. Moreover, the ubiquitous nature of BK regulatory function in normal physiology has raised concerns about the possible

adverse consequences of B2 antagonism, especially within the cardiovascular system [23,50,51,78]. While these observations suggest caution in the clinical application of B2 antagonists, the tantalizing therapeutic opportunity afforded by animal studies should encourage the research community to continue to seek a resolution for these complications. It is hoped that the perennial question of potential clinical utility *versus* side-effect profile can be unequivocally addressed with the newer generation of antagonists or by incremental refinements of existing structures.

REFERENCES

[1] K. D. Bhoola, C. D. Figueroa and K. Worthy, *Pharmacol. Rev.*, 1992, **44**, 1.
[2] B. Cassim, G. Mody and K. D. Bhoola, *Pharmacol. Ther.*, 2002, **92**, 1.
[3] Y. Takagaki, N. Kitamura and S. Nakanishi, *J. Biol. Chem.*, 1985, **260**, 8601.
[4] A. H. Schmaier, in *The Kinin System* (ed. S. G. Farmer), Academic Press, London, 1997, p. 57.
[5] A. A. K. Hasan, D. B. Cines, J. Zhang and A. H. Schmaier, *J. Biol. Chem.*, 1994, **269**, 31822.
[6] A. H. Schmaier, P. M. Smith, A. D. Purdon, J. G. White and R. W. Colman, *Blood*, 1986, **67**, 119.
[7] F. Van Iwaarden, P. G. De Groot, J. J. Sixma, M. Berrettini and B. N. Bouma, *Blood*, 1988, **71**, 1268.
[8] C. D. Figueroa, L. M. Henderson, J. Kaufmann, R. A. Dela Cadena, R. W. Colman, W. Müller-Esterl and K. D. Bhoola, *Blood*, 1992, **79**, 754.
[9] J. M. Stewart, *Biopolymers*, 1995, **37**, 143.
[10] A. Kozik, R. B. Moore, J. Potempa, T. Imamura, M. Rapala-Kozik and J. Travis, *J. Biol. Chem.*, 1998, **273**, 33224.
[11] F. Sato and S. Nagasawa, *Biol. Chem. Hoppe Seyler*, 1988, **369**, 1009.
[12] O. A. Carretero and A. G. Scicli, *Am. J. Physiol.*, 1980, **238**, F247.
[13] J. M. Hall, *Pharmacol. Ther.*, 1992, **56**, 131.
[14] S. G. Farmer and R. M. Burch, *Ann. Rev. Pharmacol. Toxicol.*, 1992, **32**, 511.
[15] J. F. Hess, J. A. Borkowski, G. S. Young, C. D. Strader and R. W. Ransom, *Biochem. Biophys. Res. Commun.*, 1992, **184**, 260.
[16] J. G. Menke, J. A. Borkowski, K. K. Bierilo, T. MacNeil, A. W. Derrick, K. A. Schneck, R. W. Ransom, C. D. Strader, D. L. Linemeyer and J. F. Hess, *J. Biol. Chem.*, 1994, **269**, 21583.
[17] F. Marceau, *Immunopharmacology*, 1995, **30**, 1.
[18] C. E. Austin, A. Faussner, H. E. Robinson, S. Chakravarty, D. J. Kyle, J. M. Bathon and D. Proud, *J. Biol. Chem.*, 1997, **272**, 11420.
[19] F. Marceau, in *The Kinin System* (ed. S. G. Farmer), Academic Press, London, 1997, p. 143.
[20] D. Regoli and J. Barabé, *Pharmacol. Rev.*, 1980, **32**, 1.
[21] K. Boels and H. C. Schaller, *Br. J. Pharmacol.*, 2003, **140**, 932.
[22] R. Fredriksson, M. C. Lagerstrom, L. G. Lundin and H. B. Schioth, *Mol. Pharmacol.*, 2003, **63**, 1256.
[23] J. Howl and S. J. Payne, *Exp. Opin. Ther. Targets*, 2003, **7**, 277.
[24] D. Regoli, D. Jukic, F. Gobeil and N.-E. Rhaleb, *Can. J. Physiol. Pharmacol.*, 1993, **71**, 556.
[25] J. M. Stewart, *Curr. Pharm. Design*, 2003, **9**, 2036.
[26] M. G. Bock, J. F. Hess and D. J. Pettibone, *Ann. Reports Med. Chem.*, 2003, **38**, 111.
[27] L. R. Steranka, D. C. Manning, C. J. DeHaas, J. W. Ferkany, S. A. Borosky, J. R. Connor, R. J. Vavrek, J. M. Stewart and S. H. Snyder, *Proc. Natl Acad. Sci. USA*, 1988, **85**, 3245.
[28] A. Dray and M. N. Perkins, in *The Kinin System* (ed. S. G. Farmer), Academic Press, London, 1997, p. 157.
[29] J. Ferreira, G. L. da Silva and J. B. Calixto, *Br. J. Pharmacol.*, 2004, **141**, 787.
[30] J. M. Hall and I. K. M. Morton, in *The Kinin System* (ed. S. G. Farmer), Academic Press, London, 1997, p. 9.
[31] P. Geppetti, *Regul. Pept.*, 1993, **47**, 1.

[32] F. Marceau and D. R. Bachvarov, *Clin. Rev. Allergy Inflamm.*, 1998, **16**, 385.
[33] J. Travis, A. Dubin, J. Potempa, W. Watorek and A. Kurdowska, *Ann. NY Acad. Sci.*, 1991, **624**, 81.
[34] J. B. Calixto, D. A. Cabrini, J. Ferreira and M. M. Campos, *Pain*, 2000, **87**, 1.
[35] R. Couture, M. Harrisson, R. M. Vianna and F. Cloutier, *Eur. J. Pharmacol.*, 2001, **429**, 161, and references therein.
[36] F. Schmidlin, D. Scherrer, Y. Landry and J.-P. Gies, *Eur. J. Pharmacol.*, 1998, **354**, R7.
[37] R. J. Vavrek and J. M. Stewart, *Peptides*, 1985, **6**, 161.
[38] R. M. Burch, S. G. Farmer and L. R. Steranka, *Med. Res. Rev.*, 1990, **10**, 237.
[39] D. Regoli, G. Drapeau, P. Rovero, S. Dion, N.-E. Rhaleb, J. Barabé, P. D'Orleans-Juste and P. Ward, *Eur. J. Pharmacol.*, 1986, **127**, 219.
[40] F. J. Hock, K. Wirth, U. Albus, W. Linz, H. J. Gerhards, G. Wiemer, S. Henke, G. Breipohl, W. König, J. Knolle and B. A. Schölkens, *Br. J. Pharmacol.*, 1991, **102**, 769.
[41] K. Wirth, F. J. Hock, U. Albus, W. Linz, H. G. Alpermann, H. Anagnostopoulos, S. Henke, G. Breipohl, W. König, J. Knolle and B. A. Schölkens, *Br. J. Pharmacol.*, 1991, **102**, 774.
[42] J. M. Stewart and L. Gera, *Immunopharmacology*, 1996, **33**, 174.
[43] J. M. Stewart, L. Gera, D. C. Chan, E. T. Whalley, W. L. Hanson and J. S. Zuzack, *Can. J. Physiol. Pharmacol.*, 1997, **75**, 719.
[44] E. T. Whalley, W. L. Hanson, J. M. Stewart and L. Gera, *Can. J. Physiol. Pharmacol.*, 1997, **75**, 629.
[45] W. L. Hanson, R. G. McCullough, W. M. Selig, M. Wieczorek, S. Ross, E. T. Whalley, J. M. Stewart and L. Gera, *Immunopharmacology*, 1996, **33**, 191.
[46] L. Gera and J. M. Stewart, in *Peptides 1997. Proceedings of the 15th American Peptide Symposium* (eds J. P. Tam and P. T. P. Kaumaya), Kluwer, Dordrecht, The Netherlands, 1999, p. 730.
[47] J. E. Burke, J. C. Cheronis and E. T. Whalley, *Exp. Opin. Ther. Patents*, 1995, **5**, 331.
[48] J. M. Stewart, L. Gera, E. J. York, D. C. Chan and P. Bunn, *Immunopharmacology*, 1999, **43**, 155.
[49] M. Altamura, S. Meini, L. Quartara and C. A. Maggi, *Regul. Pept.*, 1999, **80**, 13.
[50] M. G. Bock and J. Longmore, *Curr. Opin. Chem. Biol.*, 2000, **4**, 401.
[51] H. Heitsch, *Curr. Med. Chem.*, 2002, **9**, 913.
[52] J. M. Salvino, P. R. Seoane, B. D. Douty, M. M. A. Awad, R. E. Dolle, W. T. Houck, D. M. Faunce and D. G. Sawutz, *J. Med. Chem.*, 1993, **36**, 2583.
[53] J. M. Salvino, P. R. Seoane and R. E. Dolle, *J. Comput. Chem.*, 1993, **14**, 438.
[54] D. G. Sawutz, J. M. Salvino, R. E. Dolle, P. R. Seoane and S. G. Farmer, *Can. J. Physiol. Pharmacol.*, 1995, **73**, 805.
[55] I. Aramori, J. Zenkoh, N. Morikawa, N. O'Donnell, M. Asano, K. Nakamura, M. Iwami, H. Kojo and Y. Notsu, *Mol. Pharmacol.*, 1997, **51**, 171.
[56] Y. Abe, H. Kayakiri, S. Satoh, T. Inoue, Y. Sawada, N. Inamura, M. Asano, C. Hatori, H. Sawai, T. Oku and H. Tanaka, *J. Med. Chem.*, 1998, **41**, 4053.
[57] Y. Abe, H. Kayakiri, S. Satoh, T. Inoue, Y. Sawada, N. Inamura, M. Asano, I. Aramori, C. Hatori, H. Sawai, T. Oku and H. Tanaka, *J. Med. Chem.*, 1998, **41**, 4062.
[58] Y. Abe, H. Kayakiri, S. Satoh, T. Inoue, Y. Sawada, K. Imai, N. Inamura, M. Asano, C. Hatori, A. Katayama, T. Oku and H. Tanaka, *J. Med. Chem.*, 1998, **41**, 564.
[59] D. Hoyer, M. M. A. Awad, J. M. Salvino, P. R. Seoane, R. E. Dolle, W. T. Houck and D. G. Sawutz, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 367.
[60] M. Asano, C. Hatori, N. Inamura, H. Sawai, J. Hirosumi, T. Fujiwara and K. Nakahara, *Br. J. Pharmacol.*, 1997, **122**, 1436.
[61] Y. Sawada, H. Kayakiri, Y. Abe, K. Imai, T. Mizutani, N. Inamura, M. Asano, I. Aramori, C. Hatori, A. Katayama, T. Oku and H. Tanaka, *J. Med. Chem.*, 2004, **47**, 1617.
[62] Y. Sawada, H. Kayakiri, Y. Abe, T. Mizutani, N. Inamura, M. Asano, I. Aramori, C. Hatori, T. Oku and H. Tanaka, *J. Med. Chem.*, 2004, **47**, 2667.
[63] E. K. Dziadulewicz, T. J. Ritchie, A. Hallett, C. R. Snell, S. Y. Ko, R. Wrigglesworth, G. A. Hughes, A. R. Dunstan, G. C. Bloomfield, G. S. Drake, M. C. Brown, W. Lee, G. M. Burgess, C. Davis, M. Yaqoob, M. N. Perkins, E. A. Campbell, A. J. Davis and H. P. Rang, *J. Med. Chem.*, 2000, **43**, 769.

[64] G. M. Burgess, M. N. Perkins, H. P. Rang, E. A. Campbell, M. C. Brown, P. McIntyre, L. Urban, E. K. Dziadulewicz, T. J. Ritchie, A. Hallett, C. R. Snell, R. Wrigglesworth, W. Lee, C. Davis, S. B. Phagoo, A. J. Davis, E. Phillips, G. S. Drake, G. A. Hughes, A. R. Dunstan and G. C. Bloomfield, *Br. J. Pharmacol.*, 2000, **129**, 77.
[65] E. K. Dziadulewicz, T. J. Ritchie, A. Hallett, C. R. Snell, J. W. Davies, R. Wrigglesworth, A. R. Dunstan, G. C. Bloomfield, G. S. Drake, P. McIntyre, M. C. Brown, G. M. Burgess, W. Lee, C. Davis, M. Yaqoob, S. B. Phagoo, E. Phillips, M. N. Perkins, E. A. Campbell, A. J. Davis and H. P. Rang, *J. Med. Chem.*, 2002, **45**, 2160.
[66] F. Marceau, J.-P. Fortin, G. Morissette and E. K. Dziadulewicz, *Int. Immunopharmacol.*, 2003, **3**, 1529.
[67] D. Pruneau, J. L. Paquet, J. M. Luccarini, E. Defrene, C. Fouchet, R.-M. Franck, B. Loillier, C. Robert, P. Belichard, H. Duclos, B. Cremers and P. Dodey, *Immunopharmacology*, 1999, **43**, 187.
[68] H. Heitsch, A. Wagner, B. A. Schölkens and K. Wirth, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 327.
[69] M. A. Youngman, J. R. Carson, J. S. Lee, S. L. Dax, S.-P. Zhang, R. W. Colburn, D. J. Stone, E. E. Codd and M. C. Jetter, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1341.
[70] J. Lee, C. Reynolds, M. C. Jetter, M. A. Youngman, D. J. Hlasta, S. L. Dax, D. J. Stone, S.-P. Zhang and E. E. Codd, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1879.
[71] F. Calvani, F. Catrambone, P. Felicetti, C. I. Fincham, A. Giolitti, C. A. Maggi, L. Quartara, C. Rossi and R. Terracciano, *PCT Patent Appl.* WO 03103671, 2003.
[72] S. Nukui, H. Koike, M. Kawai and Y. Katsu, *Jpn Patent JP* 03171377, 2003.
[73] Y.-X. Cheng, X. Luo, M. Tomaszewski and C. Walpole, *PCT Patent Appl.* WO 03051276, 2003.
[74] E. K. Dziadulewicz, M. C. Brown, A. R. Dunstan, W. Lee, N. B. Said and P. J. Garratt, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 463.
[75] D. R. Artis, C. Brotherton-Pleiss, J. H. B. Pease, C. J. Lin, S. W. Ferla, S. R. Newman, S. Bhakta, H. Ostrelich and K. Jarnagin, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2421.
[76] C. Leung, V. Santhakumar, M. Tomaszewski and S. Woo, *PCT Patent Appl.* WO 03051274, 2003.
[77] C. Leung, V. Santhakumar, M. Tomaszewski and S. Woo, *PCT Patent Appl.* WO 03051275, 2003.
[78] M. N. Perkins, in *Pain* (eds C. Bountra, R. Munglani and W. K. Schmidt), Marcel Dekker, New York, 2003, p. 539.

Selective Cyclooxygenase-2 Inhibitors

W. Cameron Black

Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Quebec, Canada H9R 4P8

Contents

1. INTRODUCTION

Cyclooxygenase (prostaglandin G/H synthase) catalyzes the conversion of arachidonic acid (AA) into prostaglandin H2 as the first committed step of prostaglandin biosynthesis. This enzyme has been known since the 1970s to be the target of acetyl salicylic acid (ASA) and non-steroidal anti-inflammatory drugs (NSAIDs). In 1991 it was recognized that this enzyme existed in two isoforms, designated COX-1, a constitutive enzyme, and COX-2, an enzyme induced in response to a variety of pro-inflammatory stimuli. This recognition led to the hypothesis that the anti-infla mmatory and analgesic effects of NSAIDs were due

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to the inhibition of COX-2, while many of the undesirable side effects of these drugs, particularly gastric irritation, were due to non-selective cyclooxygenase inhibition. The identification of selective inhibitors of COX-2 (coxibs) followed by clinical trials has largely validated this hypothesis. An extensive body of literature has accompanied the rapid development of this field and numerous reviews have been published [1–3]. The three-dimensional crystal structures of both COX-1 and COX-2 have been solved [4]. The two enzymes are highly homologous, with 63% identity and 77% similarity. The COX-1 active site contains an isoleucine residue (Ile523) which is replaced by a valine residue in COX-2 (Val523). This single amino acid difference allows access to a polar side pocket in COX-2, and binding in this pocket, along with a difference in binding kinetics, largely accounts for the selectivity observed with COX-2 inhibitors [5].

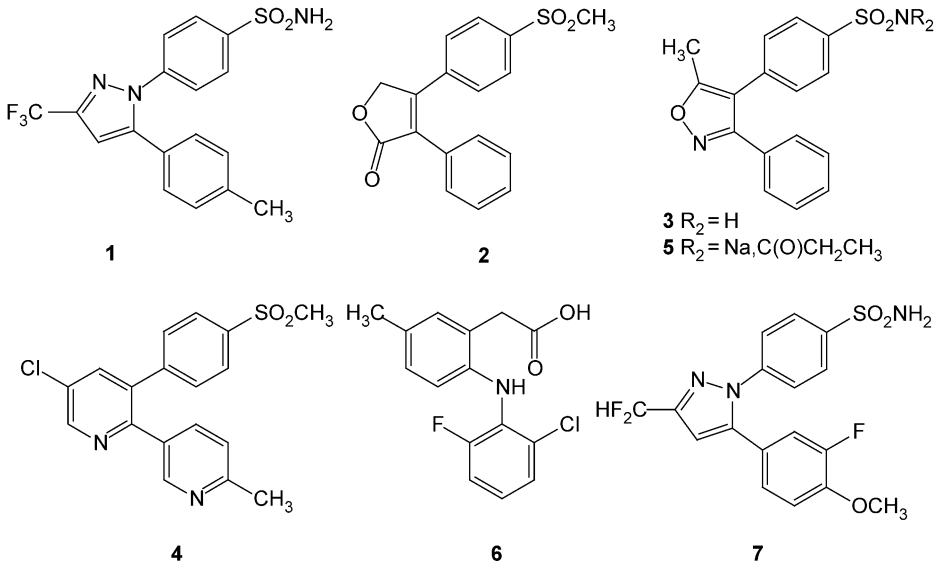
2. CLINICAL EXPERIENCE WITH COX-2 INHIBITORS

2.1. Marketed COX-2 inhibitors

Six COX-2 selective inhibitors (coxibs) have now reached the market, and a seventh is expected to be launched in the near future. Celecoxib (**1**) and rofecoxib (**2**) are the original entries from 1999 and are now widely prescribed for the treatment of the symptoms of osteoarthritis (OA) and rheumatoid arthritis. Valdecoxib (**3**) and etoricoxib (**4**) followed three years later, while parecoxib sodium (**5**), an injectable pro-drug of valdecoxib, was launched in 2003. Lumiracoxib (**6**) is expected to be the newest entry to the field and has already been approved for market in the United Kingdom. The relative potencies and selectivity of these compounds in human whole blood assays performed by two different groups are given in Table 1 along with the usual clinical dose for the treatment of pain due to osteoarthritis [6–9]. Additionally, deracoxib (**7**) is now available for the treatment of pain and inflammation associated with osteoarthritis in dogs. Multiple clinical trials have shown these drugs to have clinical efficacy comparable to that of NSAIDs [7].

Table 1. Coxibs: potency in whole blood assays, selectivity and OA clinical dose

	Patrignani *et al* [6,7] (IC_{50}, μM)			Brideau *et al* [8,9] (IC_{50}, μM)			OA clinical dose (mg)
	COX-2	COX-1	Ratio	COX-2	COX-1	Ratio	
Celecoxib (**1**)	0.54	16	30	0.87	6.7	7.6	200–400
Rofecoxib (**2**)	0.18	49	272	0.53	19	35	12.5–25
Valdecoxib (**3**)	0.65	40	61	0.87	26	30	10
Etoricoxib (**4**)	0.47	162	344	1.1	116	106	60
Lumiracoxib (**6**)	–	–	400	–	–	–	200–400



2.2. GI safety

The underlying hypothesis of selective COX-2 inhibitors is that there will be fewer serious gastric side-effects compared to NSAIDs. Endoscopy studies using therapeutic doses of celecoxib (**1**) or rofecoxib (**2**) have clearly shown significant reductions in upper GI ulceration relative to NSAID treatment, generally yielding ulceration rates equivalent to placebo [10,11]. However, since clinically important gastrointestinal events such as perforations and bleeds occur in only 1–4% of treated patients per year [12], large clinical trials are required to demonstrate an improved GI safety profile. Long-term outcome studies with 8000 patients each have been conducted with both **1** (CLASS) and **2** (VIGOR) in an effort to demonstrate their superior GI safety profile over NSAIDs. For **2** at the supraclinical chronic dose of 50 mg, the VIGOR study demonstrated a 50% reduction in confirmed gastrointestinal events over a 11-month study period relative to naproxen [13]. For **1** at the supraclinical dose of 400 mg bid, the CLASS study results were equivocal: a 40% reduction in upper GI complications and symptomatic ulcers was observed through 6 months relative to the combined group of ibuprofen and diclofenac, but no significant difference between these groups was achieved at the 9-month or 12-month points of the study [14–16].

2.3. Renal safety

An early hypothesis in the COX-2 program was that the well-established renal adverse effects of NSAIDs were also a result of COX-1 inhibition and that coxibs would be renal-sparing. This has not proven to be the case and studies with both **1** and **2** demonstrate dose-dependent adverse effects (hypertension and edema) as are observed with NSAIDs. This may be attributed to the constitutive expression of COX-2 within the kidney, and it

now appears that basal levels of COX-2 are required to maintain kidney function in renally impaired patients [17].

2.4. Cardiovascular safety

An unexpected finding that emerged from the VIGOR trial was a statistically significant increase in cardiovascular (CV) events among patients on rofecoxib relative to the naproxen group. It has been proposed that this may, in part, be due to a mechanism-based rebalancing of vasoactive prostacyclin and thromboxane A_2 in the face of COX-2 inhibition [18]. However, pooled analysis of data from VIGOR and other rofecoxib studies is consistent with the hypothesis that the observed difference in CV events is not due to a negative effect of rofecoxib, but rather a cardioprotective effect of naproxen associated with its sustained anti-platelet effects. Comparisons of patients on rofecoxib to patients on placebo or non-naproxen NSAIDs show similar rates of CV events between groups [19]. Differences in CV events between patients on celecoxib and those on ibuprofen or diclofenac were not observed in the CLASS study. Further studies are ongoing to clarify this issue.

2.5. Cancer therapy

A number of new indications have been explored clinically with celecoxib and/or rofecoxib. The role of selective COX-2 inhibitors in the prevention and treatment of a variety of cancers has been explored extensively. COX-2 is expressed at high levels in numerous cancer tissues and this expression has correlated with lower survival rates in patients [20]. COX-2 inhibitors may exert anti-tumor effects through a variety of mechanisms, including anti-angiogenesis and pro-apoptosis [21]. Several clinical trials are underway, and celecoxib has been approved for reducing polyp formation in patients with familial adenomatous polyposis.

2.6. Treatment of perioperative pain

The anti-platelet effects of NSAIDs and the corresponding increase in clotting times can be attributed to COX-1 inhibition. Thus, a selective COX-2 inhibitor can be considered for perioperative pain without the risk of increased bleeding times [22]. A study of rofecoxib administered prior to knee replacement surgery demonstrated a reduction in opioid consumption, less overall pain, and a more rapid recovery when compared to placebo [23].

2.7. Alzheimer's disease

Epidemiological evidence shows that a 80% reduction in the risk of Alzheimer's disease (AD) is observed in people who have used NSAIDs for more than 2 years [24]. Clinical trials have been conducted with COX-2 inhibitors to study the treatment of patients

with mild to moderate AD, but no effect on cognitive decline was observed [25,26]. To date, no trials studying the prevention of Alzheimer's disease have been reported with COX-2 inhibitors. *In vitro* studies have shown that only certain NSAIDs (ibuprofen, indomethacin, flurbiprofen and sulindac) are capable of reducing levels of $A\beta_{42}$ in neuronal culture, suggesting that this effect may be independent of COX activity [27,28].

3. NEW PHARMACOLOGY OF COX-2 INHIBITORS

3.1. Ischemia

The role of COX-2 inhibition in both cerebral and myocardial ischemia has also been explored. Much of the cellular damage caused by transient ischemic injury is not due to the initial ischemic event, but rather develops over hours and days following the injury in response to a variety of secondary mechanisms. Inflammation and the formation of reactive oxygen species through cyclooxygenase activity are thought to be two of these mechanisms. Increased levels of COX-2 expression in the brain have been reported to give an increase in infarct volume in a mouse model of transient focal cerebral ischemia [29]. The use of a COX-2 inhibitor in a gerbil model of cerebral ischemic injury has been successful in reducing infarct volume while a COX-1 inhibitor showed no effect [30]. In addition, the degree of hippocampal neuronal injury produced by global ischemia in COX-2 deficient mice was less than that in wild-type mice [31]. A rat model of acute myocardial infarction showed strong expression of COX-2 in the myocardium and treatment with a selective COX-2 inhibitor reduced infarct size and also gave an improvement in cardiac function [32].

3.2. PET tracers

PET ligands can be useful for localizing target proteins in whole animals or human patients. They can also allow the determination of fractional inhibition *in vivo* that is obtained with therapeutic doses of a pharmaceutical agent. Two COX-2 selective PET ligands have recently been characterized. ^{18}F-SC58125 has been used in biodistribution experiments in rat and baboon [33]. ^{18}F-desbromo Dup 697 has also been used for biodistribution studies in rat [34]. A significant amount of the ligand was found in the brain and ~40% of this could be competed with the COX-2 inhibitor NS398, potentially allowing a window for the study of COX-2 inhibition in neurological disorders.

4. RECENT ADVANCES IN INHIBITOR DESIGN

4.1. Evaluation of inhibitor potency and selectivity

Selective inhibitors of COX-2 generally exhibit slowly reversible, time-dependent inhibition of COX-2 and rapidly reversible, competitive inhibition of COX-1.

Thus, selectivity measurements are highly dependent on assay incubation time and substrate (arachidonic acid) concentration. A multitude of assays have been developed to measure COX-2 and COX-1 activity and coupled with these kinetic attributes, this has lead to great variation in the reported potencies and selectivity of standard inhibitors [2]. Both microsomal and intact cell assays are commonly used and are particularly subject to differences due to cell origin, stimulating agent, and culture conditions. Whole blood assays are also widely used and have the advantage of testing inhibitors in a biologically relevant milieu using endogenous substrate [35]. Even here, differences in selectivity are observed between assays, but the rank order is consistent (Table 1). A number of rat models of inflammation and pain have been used to assess *in vivo* efficacy of COX-2 selective inhibitors. In many cases, the results of these assays are a reflection of the pharmacokinetics of the test compound rather than of the compound's potency. All *in vivo* data reported here are from oral dosing.

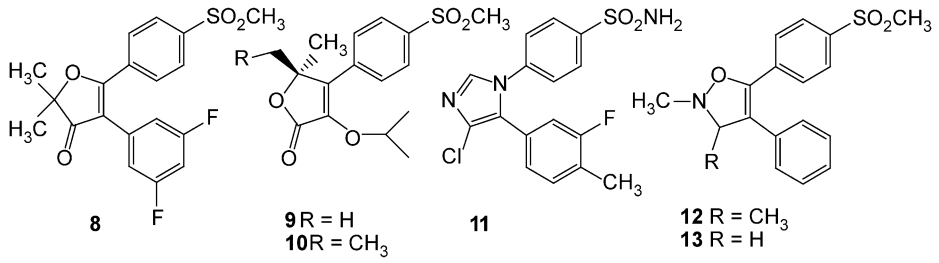
4.2. Tricyclic inhibitors

A large proportion of the research into selective COX-2 inhibitors over the past three years has been in the 'tricyclic sulfone/sulfonamide' class of compounds. All six of the currently marketed coxibs fall into this class, illustrating the importance of this structural motif. These compounds are in general lipophilic, neutral compounds with low aqueous solubility and moderate-to-high protein binding. The inherent selectivity of this class is critically dependent on the presence of a sulfone or sulfonamide group at the 4-position of one of the aromatic rings. This key pharmacophore has been shown by X-ray crystallography to bind to a polar pocket accessible only in COX-2 [5]. Within a given series, the sulfonamide derivatives generally have higher oral bioavailability than the corresponding sulfones, but also have greater activity against COX-1 and are often shifted in high protein environments such as whole blood assays.

4.3. 5-Membered central rings

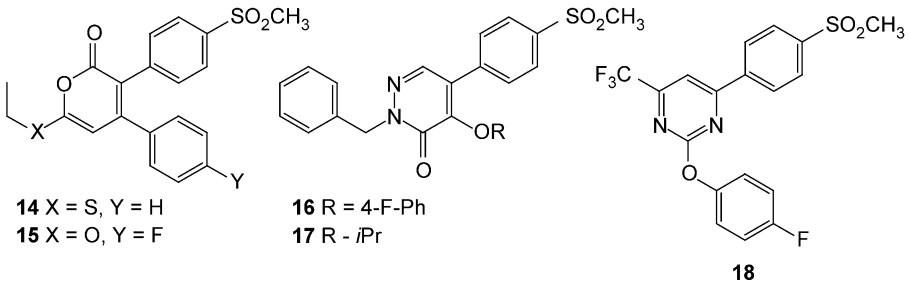
Variation of the central ring in the tricyclic series has been a traditional area of research and a great variety of heterocycles have been explored [35]. A number of new entries have been reported in recent years. A variation of the furanone ring in which the ring oxygen has been moved to the β-position has been described, giving potent and selective inhibitors of COX-2 [36]. Compound **8** is 667-fold selective for COX-2 in a mouse macrophage assay. It shows similar potency to celecoxib in the rat paw edema assay and is extremely potent in the rat adjuvant arthritis model ($ED_{50} = 0.03$ mg/kg). A chiral 5,5-disubstituted furanone was found to provide a metabolic advantage over previously described furanones [37]. The gem-dimethyl analogue DFP (**9**) had only 2% metabolism in human hepatocyte incubations, which translated into a 64 h half-life in clinic. The corresponding ethyl methyl analogue **10** showed increased *in vitro* metabolism (18% in human hepatocyte incubations) while maintaining the potency and selectivity of DFP in human whole blood assays (COX-2 $IC_{50} = 0.4$ μM, COX-1 $IC_{50} > 86$ μM). A study of 1,5-diarylimidazoles showed that

a halogen in the 4-position of the imidazole was crucial for activity, with chlorine being preferred [38]. Cimicoxib (UR-8880, **11**) is very potent in a human whole blood COX-2 assay (0.07 μM), shows 660-fold selectivity over COX-1 in whole cell assays and is active in three rat models of pain and inflammation. When dosed at 100 mg/kg for 5 days in a ^{51}Cr excretion model of ulcerogenicity in rats, minimal chromium leakage was observed indicating a low propensity for ulceration. An isoxazoline template has also been found to give potent, selective COX-2 inhibitors [39]. The 3-methyl isoxazoline **12** is 61,000-fold selective in ovine enzyme assays (COX-2 $IC_{50} = 0.004$ μM, COX-1 $IC_{50} = 258$ μM) but shows poor activity in the rat paw edema assay (50% inhibition at 50 mg/kg). Interestingly, the unsubstituted isoxazole **13** was inactive in both *in vitro* and *in vivo* assays. Molecular modeling suggests that the 3-methyl substituent allows a more favorable orientation of the inhibitor such that the sulfone group binds suitably in the polar side pocket of the COX-2 active site.



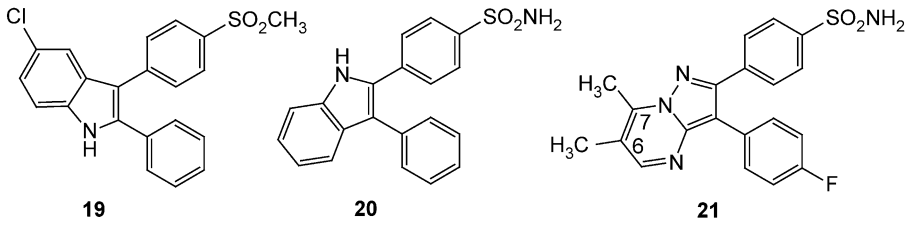
4.4. 6-Membered central rings

Several 6-membered central ring templates have been disclosed recently. The 6-thioethyl substituted pyranone **14** was potent and selective in an ovine enzyme assay (COX-2 $IC_{50} = 0.003$ μM, COX-1 $IC_{50} = 386$ μM) but showed little activity *in vivo*, possibly due to metabolic instability [40]. The corresponding ethoxy analogue **15**, while somewhat less potent *in vitro* (COX-2 $IC_{50} = 0.1$ μM, COX-1 $IC_{50} = 288$ μM), showed excellent activity in the rat paw edema assay (68% inhibition at 1 mg/kg). It is interesting to note that the fluorine substituent on the lower ring is not tolerated in the *S*-linked series but is essential to activity in the *O*-linked series. *N*-benzyl pyridazinone **16** is exceptionally potent in the human whole blood COX-2 assay ($IC_{50} = 0.09$ μM) but is inactive in the rat paw edema assay [41]. In contrast, the isopropoxy derivative **17** has only moderate potency in the human whole blood COX-2 assay ($IC_{50} = 1.7$ μM, >500-fold selective in whole cell assays) but has an ED_{50} of <0.3 mg/kg in the rat paw edema assay. The phenoxy-linked pyrimidine **18** is reported to be >81,000-fold selective in transfected COS cell assays (COX-2 $IC_{50} < 0.001$ μM, COX-1 $IC_{50} = 81$ μM) [42]. This 1,3-disubstitution pattern is unusual in the tricyclic class of compounds.



4.5. Bicyclic central rings

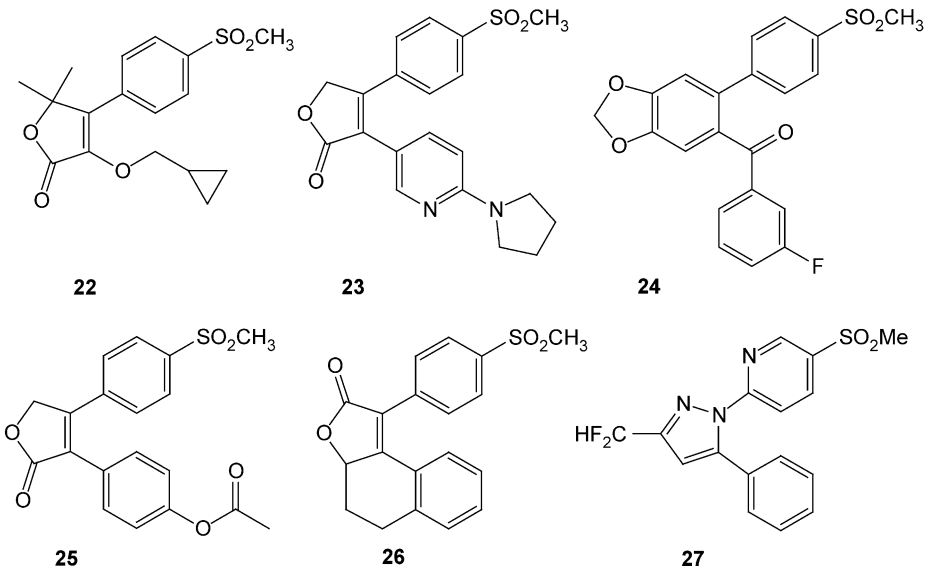
Several groups have explored bicyclic replacements of the central ring in recent years. One group has reported on two regioisomeric indoles. In compound **19** where the indole nitrogen is *anti* to the phenyl sulfone [43], selectivity comparable to that of celecoxib is observed in murine macrophage assays (COX-2 $IC_{50} = 0.0003$ μM, COX-1 $IC_{50} > 10$ μM) but no *in vivo* data is reported. In compound **20** where the indole nitrogen is *syn* to the phenyl sulfonamide, the COX-2 potency is threefold greater (COX-2 $IC_{50} = 0.00009$ μM, COX-1 $IC_{50} > 10$ μM) and this compound also shows activity in the rat paw edema assay ($ED_{50} = 5$ mg/kg) [44]. In these series, the preference for sulfone or sulfonamide was highly variable and depended strongly on the substituent on the second aryl ring. An extensive SAR study of the pyrazolopyrimidine ring system found that small substituents at the 6 and 7 positions were required for optimal potency and selectivity [45]. Compound **21** was more potent and selective than celecoxib in the human whole blood assay (COX-2 $IC_{50} = 0.08$ μM, COX-1 $IC_{50} > 10$ μM), but had poor oral bioavailability and had only a 1 h half-life in rats. As a result, little activity in the rat paw edema assay was observed.



4.6. Modifications to the lower ring

The non-sulfone bearing aryl ring of the tricyclic series has been an area in which a great variety of modification has been tolerated. Pyridine, alkoxy and cycloalkyl have been used successfully in the past [9,46]. Recently, the SAR that led to the cyclohexyl of JTE-522 has been published [47]. Also a series of alkyl amines and

alkyl thiols has been reported [48], but no biological data was disclosed. One noteworthy example of the alkoxy replacement for this aryl ring is the cyclopropylmethoxy group of firocoxib **22**, a compound currently being developed for use in dog [49]. **22** is potent and highly selective in a dog whole blood assay (COX-2 $IC_{50} = 0.3$ μM, COX-1 $IC_{50} = 105$ μM) and prevents lameness in a canine inflammatory synovitis model at 4 mg/kg. An analogue of **2** wherein the phenyl ring has been replaced with an aminopyridine to give **23** (UR-8962) has been described [50]. UR-8962 is highly selective in whole cell assays (COX-2 $IC_{50} = 0.022$ μM, COX-1 $IC_{50} = 100$ μM). An extensive SAR of methylene-linked carbocyclic and heterocylic replacements of the aryl ring has been published [51]. The authors noted that introducing an alkyl or carbonyl linker gave an increase in potency compared to the directly attached phenyl ring. The preferred compound **24** was comparable in potency and selectivity in human whole blood assays to that of celecoxib (COX-2 $IC_{50} = 1$ μM, COX-1 $IC_{50} = 20$ μM), but was inactive in the rat air pouch inflammation assay, apparently due to poor oral bioavailability. An acetoxy moiety has been attached to the lower phenyl ring of rofecoxib to generate compound **25** which could potentially acetylate COX-2 selectively in a manner analogous to the COX-2 selective ASA analogue APHS [52]. Although this has not been demonstrated mechanistically, **25** exhibits good potency and selectivity in ovine enzyme assays (COX-2 $IC_{50} = 0.001$ μM, COX-1 $IC_{50} > 100$ μM) while the corresponding phenol is inactive [53]. Finally, a report has been published in which the non-sulfone-bearing ring has been fused with a furanone central ring to form a novel tetracyclic inhibitor **26** [54]. In recombinant enzyme assays, this compound is >80-fold selective with potency comparable to that of rofecoxib (COX-2 $IC_{50} = 0.56$ μM, COX-1 $IC_{50} > 45$ μM).

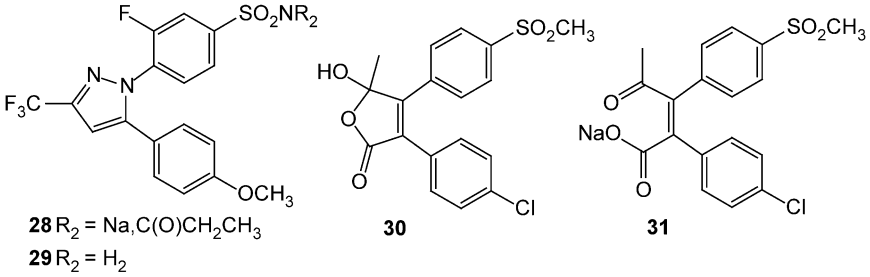


4.7. Modifications to the arylsulfonyl ring

Modification to the sulfone- or sulfonamide-bearing aryl group has met with limited success. The introduction of a fluorine *ortho* to the sulfonamide was found to increase COX-2 selectivity fourfold leading to JTE-522 while a *meta* fluorine substituent led to a twofold decrease in selectivity [55,56]. A pyridylsulfone was found to be an acceptable replacement for a phenylsulfone in a compound intended for use in dogs [57]. Compound **27** is 155-fold selective for COX-2 in a dog whole blood assay (COX-2 $IC_{50} = 0.3$ μM, COX-1 $IC_{50} = 48$ μM) and is active in a canine inflammatory synovitis model at 4 mg/kg.

4.8. Parenteral formulations

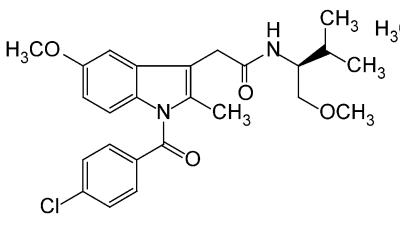
While the majority of COX-2 inhibitors are designed for oral use, there has been some effort towards identifying a compound suitable for parenteral administration. Due to the poor aqueous solubility associated with the tricyclic class of COX-2 inhibitors, this has led to an effort in pro-drugs. The best example is that of parecoxib **5** which features an acylsulfonamide capable of forming a sodium salt. Metabolic conversion to the active sulfonamide **3** is achieved *in vivo* following intravenous administration [58]. A similar approach has been taken in a pyrazole series of inhibitors giving compound **28** which is itself a weak inhibitor of COX-2, but in rats is rapidly converted to **29**, which has similar potency and selectivity to celecoxib in enzyme assays (COX-2 $IC_{50} = 0.15$ μM, COX-1 $IC_{50} > 30$ μM) [56]. Another solution to the solubility problem was the identification of hydroxyfuranone **30** which exists in a pH-dependent equilibrium with a water-soluble ketocarboxylate. At neutral pH the furanone predominates which is a moderately potent but highly selective inhibitor in a human whole blood assay (COX-2 $IC_{50} = 1.7$ μM, COX-1 $IC_{50} > 100$ μM). The sodium salt **31** is active *in vivo* with an ED_{50} of 1.8 mg/kg in the rat paw edema assay [59].



4.9. Modification of existing NSAIDs

A complementary strategy to the design of COX-2 inhibitors has been to modify conventional NSAIDs to take advantage of the larger COX-2 active site and thus impart selectivity. This effort has been aided greatly by the availability of structural information on both enzymes [60]. The most successful example of this approach has been

the identification of lumiracoxib **6**, a derivative of diclofenac. Several selective derivatives of indomethacin have been prepared. Two groups have introduced selectivity by modifying the carboxylic acid moiety to provide amides [61] or thiazoles [62]. Amide **32** is >500-fold selective in enzyme assays (COX-2 $IC_{50} = 0.11$ μM, COX-1 $IC_{50} > 63$ μM). Thiazole **33** is 30,000-fold selective in microsomal assays (COX-2 $IC_{50} = 0.0003$ μM, COX-1 $IC_{50} > 10$ μM). A similar approach preparing amide derivatives of meclofenamic acid has also been reported [63]. The resulting compound **34** is 440-fold selective in enzyme assays (COX-2 $IC_{50} = 0.15$ μM, COX-1 $IC_{50} = 66$ μM). No *in vivo* data was reported in these publications.



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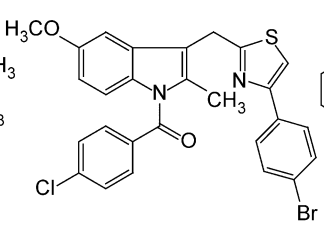

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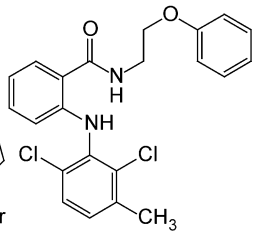

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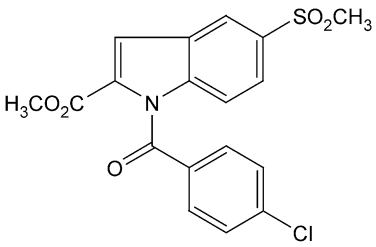
A second approach was to use a pharmacophore model to introduce beneficial features of the tricyclic series into the indomethacin skeleton [64]. The resulting compound **35** incorporates the sulfone group in the 5-position of the indole to provide >20-fold selectivity in whole blood assays (COX-2 $IC_{50} = 4.3$ μM, COX-1 $IC_{50} > 100$ μM). Applying the same approach to the ketoprofen skeleton led to the replacement of the profen group with a vinyl sulfonamide along with an alkyl substituent to improve selectivity [65]. Compound **36** (LM-1669) is eightfold selective in whole blood assays (COX-2 $IC_{50} = 4.3$ μM, COX-1 $IC_{50} > 100$ μM). Again, no *in vivo* data is reported.



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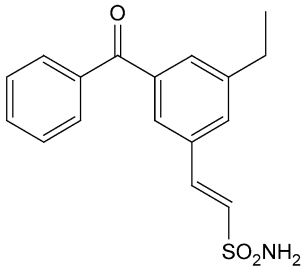

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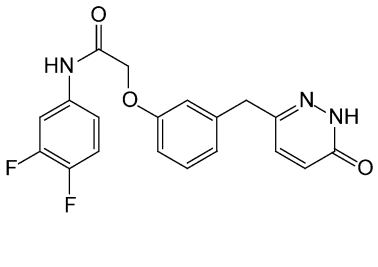
4.10. Miscellaneous structures

A series of benzyl pyrazinones has been described [66]. Compound **37** is 116-fold selective for COX-2 in microsomal assays (COX-2 $IC_{50} = 0.45$ μM, COX-1 $IC_{50} = 52$ μM) but had poor activity in the rat paw edema assay (32% inhibition at 30 mg/kg PO). The 2,5-diaryl pyrrole **38** is potent and 250-fold selective in a human whole blood assay (COX-2 $IC_{50} = 0.12$ μM, COX-1 $IC_{50} = 30$ μM) and is very active

in the rat adjuvant arthritis model ($ED_{50} = 0.15$ mg/kg) [67]. Introducing a methylsulfone group on the 4-position of one of the aryl rings led to 1000-fold loss in potency in the COX-2 assay, illustrating that the apparent structural similarity to the tricyclic class of inhibitors may not be reflected in their binding mode.

A 3D-QSAR approach based on known COX inhibitors led to the identification of sulfanilide **39** which was found to be 70-fold selective in mononuclear cell assays (COX-2 $IC_{50} = 0.37$ μM, COX-1 $IC_{50} = 26$ μM) [68].



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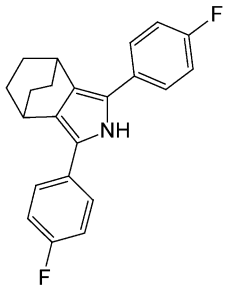

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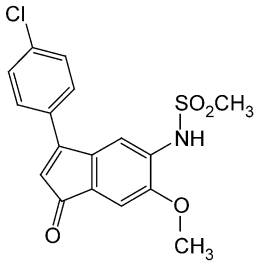

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5. CONCLUSION

The field of cyclooxygenase-2 inhibitors has progressed from infancy to maturity over the past decade. The clinical applications of the resulting drugs have already proceeded beyond that of NSAIDs and new indications and combinations continue to be explored. The pharmacophores resulting in selective inhibition of COX-2 are well established, although the subtleties of substituent effects and binding modes at times remain elusive. The future of the field may now lie in new structural classes and targeted inhibitors to address niche applications such as CNS indications.

REFERENCES

[1] J. R. Vane, Y. S. Bakhle and R. M. Blotting, *Ann. Rev. Pharmacol. Toxicol.*, 1998, **38**, 97.
[2] G. Dannhardt and W. Kiefer, *Eur. J. Med. Chem.*, 2001, **36**, 109.
[3] P. C. Isakson, *Prog. Exp. Tum. Res.*, 2003, **37**, 25.
[4] R. M. Garavito and A. M. Mulichak, *Annu. Rev. Biophys. Biomol. Struct.*, 2003, **32**, 183.
[5] R. G. Kurumbail, A. M. Stevens, J. K. Gierse, J. J. McDonald, R. A. Stegeman, J. Y. Pak, D. Gildehaus, J. M. Miyashiro, T. D. Penning, K. Seibert, P. C. Isakson and W. C. Stallings, *Nature*, 1996, **384**, 644.
[6] P. Patrignani, M. R. Panara, A. Greco, O. Fusco, C. Natoli, S. Iacobelli, F. Cipollone, A. Ganci, C. Creminon, J. Maclouf and C. Patrono, *J. Pharmacol. Exp. Ther.*, 1994, **271**, 1705.
[7] M. L. Capone, S. Tacconelli, M. G. Sciulli and P. Patrignani, *Int. J. Immunopathol. Pharmacol.*, 2003, **16**, 49.
[8] C. Brideau, S. Kargman, S. Liu, A. L. Dallob, E. W. Ehrich, I. W. Rodger and C.-C. Chan, *Inflamm. Res.*, 1996, **45**, 68.
[9] D. Riendeau, M. D. Percival, C. Brideau, S. Charleson, D. Dubé, D. Ethier, J.-P. Falgueyret, R. W. Friesen, R. Gordon, G. Grieg, J. Guay, J. Mancini, M. Ouellet, E. Wong, L. Xu,

S. Boyce, D. Visco, Y. Girard, P. Prasit, R. Zamboni, I. W. Rodger, M. Gresser, A. W. Ford-Hutchinson, R. N. Young and C.-C. Chan, *J. Pharmacol. Exp. Ther.*, 2001, **296**, 558.
[10] J. J. Deeks, L. A. Smith and M. D. Bradley, *Br. Med. J.*, 2002, **325**, 619.
[11] C. Hawkey, L. Laine, T. Simon, A. Beaulieu, J. Maldonado-Cocco, E. Acevedo, A. Shahane, H. Quan, J. Bolognese and E. Mortensen, *Arthritis Rheum.*, 2000, **43**, 370.
[12] G. Singh and D. R. Ramey, *J. Rheumatol.*, 1998, **25** (Suppl. 51), 8.
[13] C. Bombardier, L. Laine, A. Reicin, D. Shapiro, R. Burgos-Vargas, B. Davis, R. Day, M. B. Ferraz, C. J. Hawkey, M. C. Hochberg, T. K. Kvien and T. J. Schnitzer, *N. Engl. J. Med.*, 2000, **343**, 1520.
[14] F. E. Silverstein, G. Faich, J. L. Goldstein, L. S. Simon, T. Pincus, A. Whelton, R. Makuch, G. Eisen, N. M. Agrawal, W. F. Stenson, A. M. Burr, W. W. Zhao, J. D. Kent, J. B. Lefkowith, K. M. Verburg and G. S. Geis, *JAMA*, 2000, **284**, 1247.
[15] P. Juni, A. W. S. Rutjes and P. A. Dieppe, *BMJ*, 2002, **324**, 1287.
[16] G. S. Geis, *Br. Med. J. USA*, 2002, **2**, 522.
[17] G. Gambaro and M. A. Perazella, *J. Int. Med.*, 2003, **253**, 643.
[18] R. E. Fowles, *J. Pain Palliat. Care Pharmacother.*, 2003, **17**, 27.
[19] M. R. Wier, R. S. Sperling, A. Reicin and B. J. Gertz, *Am. Heart J.*, 2003, **146**, 591.
[20] H. B. Grossman, *Drugs Today*, 2003, **39**, 203.
[21] G. Gasparini, R. Longo, R. Sarmiento and A. Morabito, *Lancet Oncol.*, 2003, **4**, 605.
[22] L. Afflitto, *Plast. Surg. Nurs.*, 2000, **20**, 91.
[23] A. Buvanendran, J. S. Kroin, K. J. Tuman, T. R. Lubenow, D. Elmofty, M. Moric and A. G. Rosenberg, *JAMA*, 2003, **290**, 2411.
[24] B. A. in't Veld, A. Ruitenberg, A. Hofman, L. J. Launer, C. M. van Duijn, T. Stijnen, M. M. B. Breteler and B. H. C. Stricker, *N. Engl. J. Med.*, 2001, **345**, 1515.
[25] P. S. Aisen, *Curr. Neurol. Neurosci. Rep.*, 2002, **2**, 405.
[26] P. S. Aisen, K. A. Schafer, M. Grundman, E. Pfeiffer, M. Sano, K. L. Davis, M. R. Farlow, S. Jin, R. G. Thomas and L. J. Thal, *JAMA*, 2003, **289**, 2819.
[27] S. Weggen, J. L. Eriksen, P. Das, S. A. Sagi, R. Wang, C. U. Pietrzik, K. A. Findlay, T. E. Smith, M. P. Murphy, T. Bulter, D. E. Kang, N. Marquez-Sterling, T. E. Golde and E. H. Koo, *Nature*, 2001, **414**, 212.
[28] L. Gasparini, L. Rusconi, H. Xu, P. del Soldato and E. Ongini, *J. Neurochem.*, 2004, **88**, 337.
[29] S. Doré, T. Otsuka, T. Mito, N. Sugo, T. Hand, L. Wu, P. D. Hurn, R. J. Traystman and K. Andreasson, *Ann. Neurol.*, 2003, **54**, 155.
[30] E. Candelario-Jalil, A. González-Falcón, M. García-Cabrera, D. Alvarez, S. Al-Dalain, G. Martínez, O. S. León and J. E. Springer, *J. Neurochem.*, 2003, **86**, 545.
[31] T. Sasaki, K. Kitagawa, K. Yamagata, T. Takemiya, S. Tanaka, E. Omura-Matsuoka, S. Sugiura, M. Matsumoto and M. Hori, *J. Cereb. Blood Flow Metab.*, 2004, 107.
[32] T. Saito, I. W. Rodger, H. Shennib, F. Hu, L. Tayara and A. Giaid, *Can. J. Physiol. Pharmacol.*, 2003, **81**, 114.
[33] T. J. McCarthy, A. U. Sheriff, M. J. Graneto, J. J. Talley and M. J. Welch, *J. Nucl. Med.*, 2002, **43**, 117.
[34] E. F. J. de Vries, A. van Waarde, A. R. Buursma and W. Vaalburg, *J. Nucl. Med.*, 2003, **44**, 1700.
[35] P. Prasit and D. Riendeau, *Ann. Rep. Med. Chem.*, 1997, **32**, 211.
[36] S. S. Shin, M.-S. Noh, Y. J. Byun, J. K. Choi, J. Y. Kim, K. M. Lim, J.-Y. Ha, J. K. Kim, C. H. Lee and S. Chung, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 165.
[37] Y. Leblanc, P. Roy, Z. Wang, C. S. Li, N. Chauret, D. A. Nicoll-Griffith, J. M. Silva, Y. Aubin, J. A. Yergey, C. C. Chan, D. Riendeau, C. Brideau, R. Gordon, L. Xu, J. Webb, D. M. Visco and P. Prasit, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3317.
[38] C. Almansa, J. Alfón, A. F. de Arriba, F. L. Cavalcanti, I. Escamilla, L. A. Gómez, A. Miralles, R. Soliva, J. Bartrolí, E. Carceller, M. Merlos and J. García-Rafanell, *J. Med. Chem.*, 2003, **46**, 3463.
[39] A. Habeeb, P. N. P. Rao and E. E. Knaus, *J. Med. Chem.*, 2001, **44**, 2921.
[40] P. N. P. Rao, M. Amini, H. Li, A. Habeeb and E. E. Knaus, *J. Med. Chem.*, 2003, **46**, 4872.
[41] C. S. Li, C. Brideau, C. C. Chan, C. Savoie, D. Claveau, S. Charleson, R. Gordon, G. Greig, J. Y. Gauthier, C. K. Lau, D. Riendeau, M. Thérien, E. Wong and P. Prasit, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 597.

[42] A. Naylor, J. J. Payne and N. A. Pegg, *WO Patent Appl.* 02096885-A, 2002.
[43] W. Hu, Z. Guo, X. Yi, C. Guo, F. Chu and G. Cheng, *Bioorg. Med. Chem.*, 2003, **11**, 5539.
[44] W. Hu, Z. Guo, F. Chu, A. Bai, X. Yi, G. Cheng and J. Li, *Bioorg. Med. Chem.*, 2003, **11**, 1153.
[45] C. Almansa, A. F. de Arriba, F. L. Cavalcanti, L. A. Gómez, A. Miralles, M. Merlos, J. García-Rafanell and J. Forn, *J. Med. Chem.*, 2001, **44**, 350.
[46] Y. Leblanc, P. Roy, S. Boyce, C. Brideau, C. C. Chan, S. Charleson, R. Gordon, E. Grimm, J. Guay, S. Léger, C. S. Li, D. Riendeau, D. Visco, Z. Wang, J. Webb, L. J. Xu and P. Prasit, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2207.
[47] H. Hashimoto, K. Maeda, K. Ozawa, J. Haruta and K. Wakitani, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 65.
[48] S. M. Sakya and B. Rast, *Tetrahedron Lett.*, 2003, 7629.
[49] W. C. Black, D. R. Andersen, C. Brideau, N. Chauret, R. T. Chern, J. Chen, M. Drag, R. Fortin, P. Hanson, M. Haven, G. J. Hickey, M. E. McCann, D. Patrick, Z. Wang and R. N. Young, *Drugs Fut.*, 2002, **27** (Suppl. A), 136.
[50] C. Almansa, J. Alfón, F. L. Cavalcanti, L. Gómez and A. Miralles, *Drugs Fut.*, 2002, **27** (Suppl. A), 106.
[51] S. P. Khanapure, D. S. Garvey, D. V. Young, M. Ezawa, R. A. Earl, R. D. Gaston, X. Fang, M. Murty, A. Martino, M. Shumway, M. Trocha, P. Marek, S. W. Tam, D. R. Janero and L. G. Letts, *J. Med. Chem.*, 2003, **46**, 5484.
[52] A. S. Kalgutkar, B. C. Crews, S. W. Rowlinson, C. Garner, K. Seibert and L. J. Marnett, *Science*, 1998, **280**, 1268.
[53] M. A. Rahim, P. N. P. Rao and E. E. Knaus, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2753.
[54] M. Pal, V. R. Veeramaneni, M. Nagabelli, S. R. Kalleda, P. Misra, S. R. Casturi and K. R. Yeleswarapu, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1639.
[55] H. Hashimoto, K. Imamura, J. Haruta and K. Wakitani, *J. Med. Chem.*, 2002, **45**, 1511.
[56] M. Pal, M. Madan, S. Padakanti, V. R. Pattabiraman, S. Kalleda, A. Vanguri, R. Mullangi, N. V. S. R. Mamidi, S. R. Casturi, A. Malde, B. Gopalakrishnan and K. R. Yeleswarapu, *J. Med. Chem.*, 2003, **46**, 3975.
[57] J. Li, K. M. L. DeMello, H. Cheng, S. M. Sakya, B. S. Bronk, R. J. Rafka, B. H. Jaynes, C. B. Ziegler, C. Kilroy, D. W. Mann, E. L. Nimz, M. P. Lynch, M. L. Haven, N. L. Kolosko, M. L. Minich, C. Li, J. K. Dutra, B. Rast, R. M. Crosson, B. J. Morton, G. W. Kirk, K. M. Callaghan, D. A. Koss, A. Shavnya, L. A. Lund, S. B. Seibel, C. F. Petras and A. Silvia, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 95.
[58] J. J. Talley, S. R. Bertenshaw, D. L. Brown, J. S. Carter, M. J. Graneto, M. S. Kellogg, C. M. Koboldt, J. Yuan, Y. Y. Zhang and K. Seibert, *J. Med. Chem.*, 2000, **43**, 1661.
[59] W. C. Black, C. Brideau, C.-C. Chan, S. Charleson, W. Cromlish, R. Gordon, E. L. Grimm, G. Hughes, S. Leger, C.-S. Li, D. Riendeau, M. Thérien, Z. Wang, L.-J. Xu and P. Prasit, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1195.
[60] G. Trummlitz and J. van Ryn, *Curr. Opin. Drug Disc. Dev.*, 2002, **5**, 550.
[61] K. R. Kozak, J. J. Prusakiewicz, S. W. Rowlinson and L. J. Marnett, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 1315.
[62] K. W. Woods, R. W. McCroskey, M. R. Michaelides, C. K. Wada, K. I. Hulkower and R. L. Bell, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1325.
[63] A. S. Kalgutkar, S. W. Rowlinson, B. C. Crews and L. J. Marnett, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 521.
[64] A. Palomar, F. Cabré, J. Pascual, J. Campos, M. A. Trujillo, A. Entrena, M. A. Gallo, L. García, D. Mauleón and A. Espinosa, *J. Med. Chem.*, 2002, **45**, 1402.
[65] A. Palomar, J. Pascual, M. Cabré, L. Borràs, G. González, M. Aparici, A. Carabaza, F. Cabré, M. L. García and D. Mauleón, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 533.
[66] V. K. Chintakunta, V. Akella, M. S. Vedula, P. K. Mamnoor, P. Mishra, S. R. Casturi, A. Vangoori and R. Rajagopalan, *Eur. J. Med. Chem.*, 2002, **37**, 339.
[67] B. Portevin, C. Tordjman, P. Pastoureau, J. Bonnet and G. De Nanteuil, *J. Med. Chem.*, 2000, **43**, 4582.
[68] C. H. Park, X. Siomboing, S. Yous, B. Gressier, M. Luyckx and P. Chavatte, *Eur. J. Med. Chem.*, 2002, **37**, 461.

The Discovery of Small Molecule C5a Antagonists

Alan J. Hutchison and James E. Krause

Neurogen Corporation, Branford, CT 06405, USA

Contents

1. INTRODUCTION

In host defense, the complement system of blood-borne proteins initiates a series of cellular and inflammatory responses to divergent stimuli. These can vary from infectious agents (e.g., bacteria, viruses and parasites) to biochemical, chemical and physical injury [1]. Excessive activation of the complement system in man results in elevated C5a, a glycosylated 74-amino acid protein that is associated with several acute and chronic clinical situations or disease states. Due to the pro-inflammatory nature of C5a receptor-mediated responses, this receptor has been investigated as a potential drug target by the pharmaceutical industry for some time [2]. Solid evidence, based on animal models, has only recently been generated to demonstrate the crucial involvement of the C5a receptor in disease pathogenesis or progression. For example, it has been shown in the K/B×N T cell receptor transgenic mouse model of inflammatory arthritis, that the arthritic state is critically dependent on the complement system and in particular the C5a receptor [3]. Further, several disease models in rat including an antigen-induced monarticular arthritis model, an acute limb ischemia-reperfusion model and a model of inflammatory bowel disease have been explored with peptide-based C5a receptor antagonists, and the results indicate promising activity of C5a receptor antagonists [4–6]. C5a receptor antagonists have been proposed to be useful in rheumatoid arthritis, respiratory distress syndrome, ischemia-reperfusion injury, sepsis, psoriasis, and inflammatory bowel disease [1–3]. In this review, we provide an overview of the complement pathway in man and the role of the C5a effector arm in anaphylatoxin biology. Recent work on the identification of peptide and small molecule C5a receptor antagonists is summarized.

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2. THE COMPLEMENT SYSTEM, C5a AND THE C5a RECEPTOR

The complement system consists of more than 30 serum and cellular proteins and encompasses three biochemical cascades (classic, mannose binding lectin and alternative pathways), that culminate in the activation C5 convertase, the enzyme that converts C5 to two products, C5a and C5b. The latter pathway and its multiple arms of activation are illustrated in Fig. 1. The specific molecular activators of the classical complement pathway are immune complexes; however, β-amyloid peptide has also been shown to interact with C1q under apparent physiological conditions to activate the cascade [7]. Complex polysaccharides activate the mannose binding lectin pathway, while microbial surfaces, IgA antibodies, certain Ig light chains, as well as

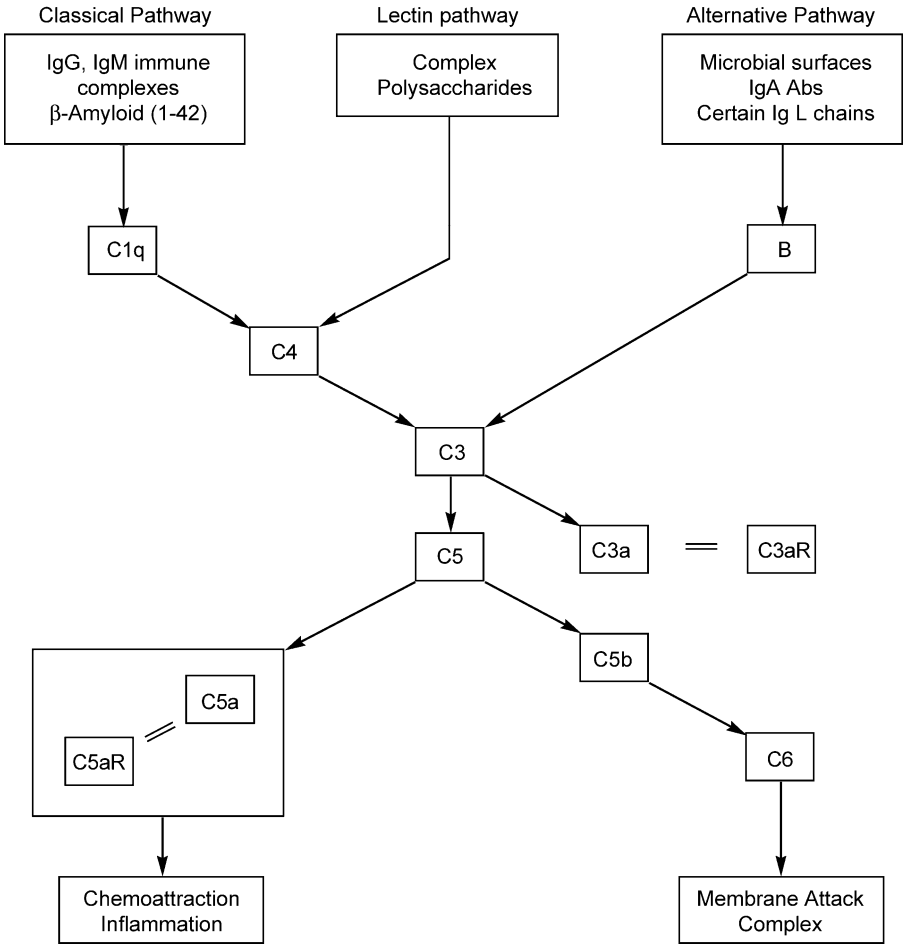
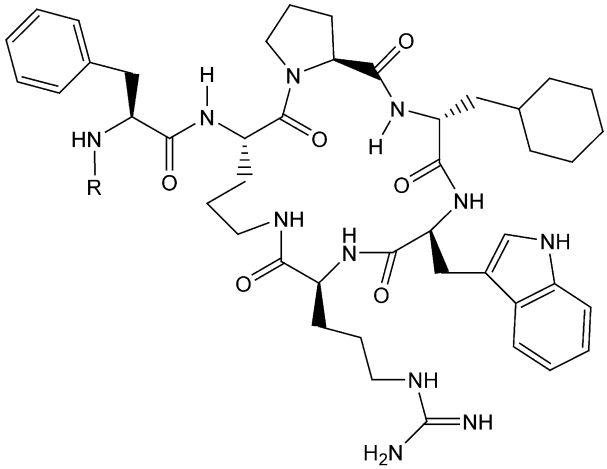


Fig. 1. Schematic illustration of the complement system. For the purpose of clarity, not all complement components are depicted. This figure is adapted from Ref. [3].

foreign surfaces such as the tubing of the cardiopulmonary bypass-oxygenator activate the alternative pathway. In most animal species, there are in essence three effector arms of the complement pathway, the C5a receptor, the C3a receptor and the membrane attack complex. The two anaphylatoxins, C3a and C5a, activate G-protein coupled receptors and as such are the specific cellular effectors of the complement system. C3a and its receptor are also potential drug targets [8]. C5a is a glycosylated 74-amino acid containing protein that activates the G-protein coupled receptor (GPCR) known as CD88 or C5a receptor. C5b combines with C6, C7, C8 and C9 serum complement proteins to form C5b-9. The latter is also known as the membrane attack complex, and mediates lysis of invading microorganism membranes as well as clearing tissue debris from sites of tissue destruction or necrosis. The C5a receptor is expressed on neutrophils, mast cells, other cells of myeloid lineage, smooth muscle cells and endothelial cells. It initiates several biochemical cascades, including decreases in intracellular cAMP levels and increases of intracellular calcium levels, PI3 kinase activity and MAP kinase activity [2]. These biochemical effects of C5a receptor activation result in the classic neutrophil effects including chemotaxis, degranulation and cellular adhesion. More recently, receptor activation has been shown to influence cytokine and chemokine gene expression and even apoptosis, depending on the specific cellular site of C5a receptor expression [9,10].

3. PEPTIDE ANTAGONISTS

The search for potent small C5a peptide antagonists has proven to be a difficult challenge. Initial efforts in protein SAR focused on determining the minimum peptide fragment possessing potent binding affinity. The C-terminal octapeptide was identified as the shortest linear sequence possessing reasonable C5a binding affinity [11]. A major complication associated with C5a antagonists arises from the fact that historically, most C5a receptor active ligands display some partial agonist activity. In spite of this precedent, there has recently been significant progress in the development of pure antagonist C5a peptide ligands. The discovery that the relatively weak linear peptide antagonist MePhe-Lys-Pro-dCha-Trp-dArg, **1**, is a pure antagonist has served as the springboard for the exploration of a series of small cyclic peptide analogs of C5a with potent C5a blocking activity [12–15]. Compounds **2** and **3** have been reported to competitively displace ^{125}I-rhC5a from rat, dog and human polymorphonuclear leukocytes (PMN's) with IC_{50} values in the 40–200 nM range [16]. However, in a functional assay in human PMN's the peptide **2** displayed insurmountable antagonism against C5a [17]. In addition, **2** was reported to block C5a- or endotoxin-induced neutropenia in the rat after iv administration [18]. Compound **3** was reported to be orally bioavailable with a 3 mg/kg po dose generating peak plasma levels of 300 ug/mL and a terminal half-life of about 70 min [19]. The latter analog was reported to have efficacy in a variety of disease models following oral and topical administration [4–6,19–21]. It was also disclosed that **3**, designated as PMX53, is currently being evaluated in Phase II clinical trials for rheumatoid arthritis and psoriasis [22]. In this connection, an improved, scalable synthesis of **3**, suitable for large-scale production was reported [23].

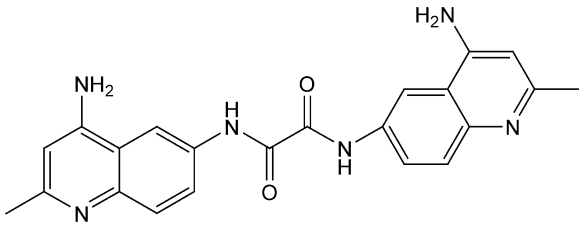


2, (R = H): F-[OP-dCha-WR]
3, (R = Ac): AcF-[OP-dCha-WR]

dCha = cyclohexyl-D-alanine

3.1. Non-peptide antagonists

The first reports of non-peptide compounds with C5a blocking activity appeared in the early 1990s. A series of 4,6-diaminoquinolines exemplified by **4** and **5** possessed weak (IC_{50} values, 4–40 μM) activity in inhibiting ^{125}I-rhC5a binding as well as in antagonizing C5a-induced neutrophil activation [24]. Subsequent reports cast doubt on the specificity of these antagonists in view of the fact that the diaminoquinolines also inhibit the binding of the chemotactic agent IL-8 to its receptors [25].



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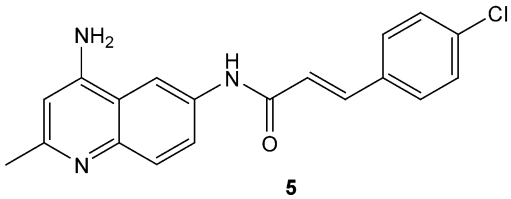

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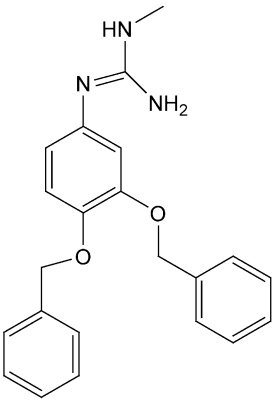
The optimization of screening hit **6**, which displayed weak activity ($IC_{50} = 30\ \mu M$) in inhibiting ^{125}I-rhC5a binding to its receptor was also documented [26]. These efforts resulted in the identification of RPR121154, **7**. This biaryl guanidine showed a modest boost in binding affinity ($IC_{50} = 800$ nM) and demonstrated functional antagonism of C5a in an oxidative burst assay at 30 μM.



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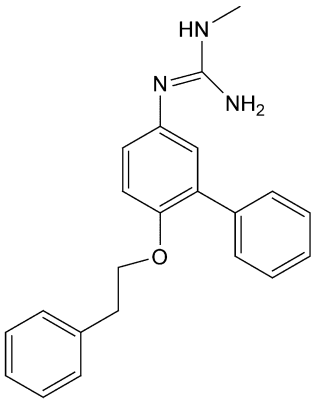

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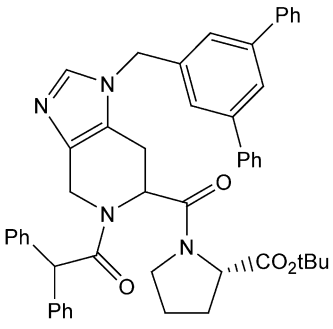
Tetrahydro imidazopyridines **8**, L-164,710 and benzodiazepines **9**, L-747,981 were evaluated as C5a antagonist ligands [27,28]. Similar to the experience with early peptide ligands, **9** and related analogs were found to be partial to full agonists [28]. A further report from the same group described a hydantoin series of C5a ligands, the most potent of which is the analog **10**. This compound is potent in a binding assay ($IC_{50} = 20$ nM), but displays agonist properties in functional assays [29]. Extensive efforts to convert each of these lead series to potent and selective pure antagonists were ultimately unsuccessful. Thus, despite these promising early reports, none of the first generation non-peptide lead compounds proved to be a viable starting point for the generation of potent and selective low molecular weight C5a antagonists devoid of agonist activity and having suitable pharmacokinetic parameters.



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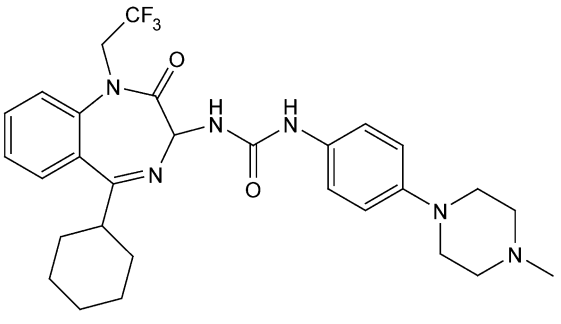

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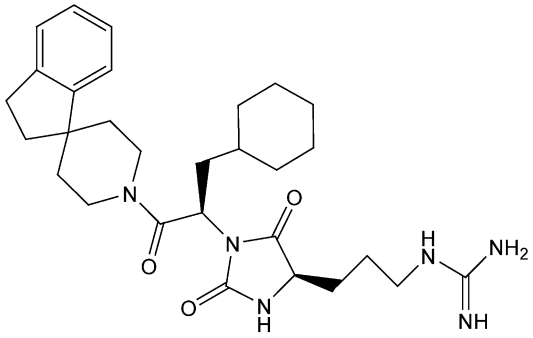

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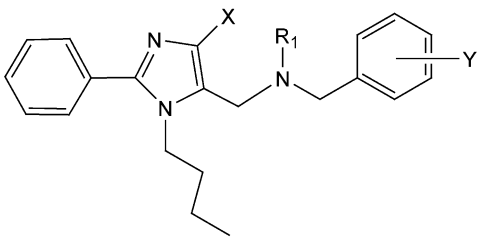
The small molecules described thus far were all identified utilizing displacement binding assays. This strategy generally yielded weak ligands for the C5a receptor with partial to full agonist profiles. More recently, library screening with functional assays was successfully employed to identify pure C5a receptor antagonists. In the first account of this approach, structures were not disclosed [30]. However, the lead compounds were characterized as antagonists/inverse agonists and reported to inhibit Ca^{2+} flux and chemotaxis in a monocytic cell line (differentiated to a neutrophil-like state) with IC_{50}s in the 0.3–5 nM range. In addition, one of these newer generation antagonists blocked C5a-induced neutropenia in a primate model at oral doses as low as 1 mg/kg. These compounds were reported to show potent C5a blocking effects only at the human and primate C5a receptors. Following the initial report, several patent applications from the same research group claimed potent C5a antagonists [31–33]. Some structural classes that are disclosed with specific examples include aminomethyl arylimidazoles, pyrazoles and pyridines such as **11**, acylaminomethyl azabenzimidazoles such as **12**, simple biarylcarboxamides such as **13**, and tetrahydroisoquinoline derivatives such as **14**. One compound, presumably within this patent estate, NGD 2000-1, is in Phase II clinical trials for asthma and rheumatoid arthritis [34].



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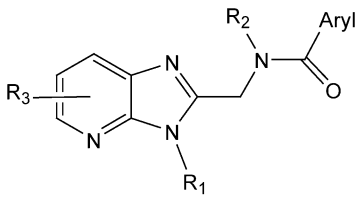

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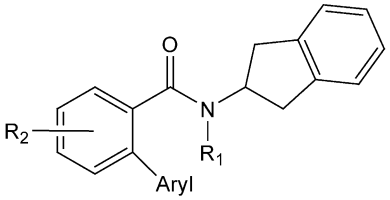

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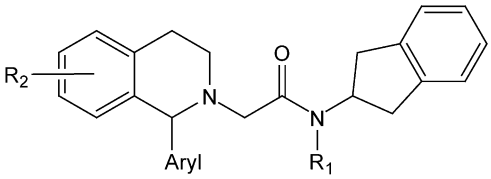

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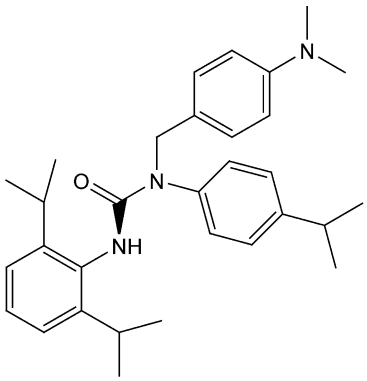
The most recent examples of small molecule C5a antagonists consist of diaryl ureas such as compound **15** and their isosteric amide analogs, exemplified by W-54011, **16** [35–37]. The potent C5a antagonist **16** displaces ^{125}I-hC5a from human PMN's with a K_i value of 2.2 nM. It has similar potency in inhibiting C5a-induced Ca^{2+} mobilization, chemotaxis and generation of reactive superoxide species in human neutrophils. Compound **16** also displays species specificity. It is a potent antagonist at human, primate and gerbil C5a receptors, whereas it is essentially inactive at mouse, rat, guinea pig, rabbit and dog C5a receptors [37]. In addition, **16** is active in a C5a-induced neutropenia model in the gerbil (3–30 mg/kg, po).



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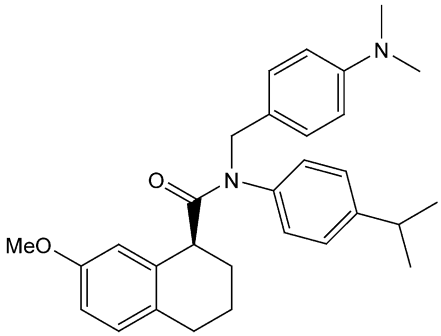

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4. CONCLUSIONS

The generation of small molecule C5a antagonists has been a recalcitrant problem for medicinal chemists. However, recent reports suggest that several blueprints now exist for the potential solution of this elusive problem. At least two orally bioavailable small molecule C5a antagonists are reported to be in Phase II clinical trials, NGD 2000-1 and PMX53 [3]. Preliminary reports of the efficacy of PMX53 in psoriasis have been reported [23]. Thus, the long awaited proof of the clinical utility for this promising class of potential therapeutic agents may soon be available.

REFERENCES

[1] S. C. Makrides, *Pharmacol. Rev.*, 1998, **50**, 59.
[2] C. Gerard and N. P. Gerard, *Ann. Rev. Immunol.*, 1994, **12**, 775.
[3] H. Ji, K. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Boackle, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, A. Ezekowitz, M. C. Carroll, M. Brenner, R. Weissleder, J. S. Verbeek, V. Duchatelle, C. Degott, C. Benoist and D. Mathis, *Immunity*, 2002, **16**, 157.
[4] T. M. Woodruff, A. J. Strachan, N. Dryburgh, I. A. Shiels, R. C. Reid, D. P. Fairlie and S. M. Taylor, *Arthritis Rheumat.*, 2002, **46**, 2476.
[5] T. M. Woodruff, T. V. Arumugam, I. A. Shiels, R. C. Reid, D. P. Fairlie and S. M. Taylor, *J. Surg. Res.*, 2004, **116**, 81.
[6] T. M. Woodruff, T. V. Arumugam, I. A. Shiels, R. C. Reid, D. P. Fairlie and S. M. Taylor, *J. Immunol.*, 2003, **171**, 5514.
[7] P. Tacnet-Delorme, S. Chevallier and G. J. Arlaud, *J. Immunol.*, 2001, **167**, 6374.
[8] A. A. Humbles, B. Lu, C. A. Nilsson, C. Lilly, E. Israel, Y. Fujiwara, N. P. Gerard and C. Gerard, *Nature*, 2000, **406**, 998.
[9] E. P. Grant, D. Picarella, T. Burwell, T. Delaney, A. Croci, N. Avitahl, A. A. Humbles, J. C. Gutierrez-Ramos, M. Briskin, C. Gerard and A. J. Coyle, *J. Exp. Med.*, 2002, **196**, 1461.
[10] R. F. Guo, M. Huber-Lang, X. Wang, X. Sarma, V. A. Padgaonkar, R. A. Craig, N. C. Riedemann, S. D. McClintock, T. Hlaing, M. M. Shi and P. A. Ward, *J. Clin. Invest.*, 2000, **10**, 1271.
[11] M. Kawai, D. A. Quincy, B. Lane, K. W. Mollison, J. R. Luly and G. W. Carter, *J. Med. Chem.*, 1991, **34**, 2068.
[12] J. A. DeMartino, Z. D. Konteatis, S. J. Siciliano, G. Van Riper, D. J. Underwood, P. A. Fischer and M. A. S. Springer, *J. Biol. Chem.*, 1995, **270**, 15966.
[13] A. K. Wong, A. M. Finch, G. K. Pierens, D. J. Craik, S. M. Taylor and D. P. Fairlie, *J. Med. Chem.*, 1998, **41**, 3417.
[14] A. M. Finch, A. K. Wong, N. J. Paczkowski, S. K. Wadi, D. J. Craik, D. P. Fairlie and S. M. Taylor, *J. Med. Chem.*, 1999, **42**, 1965.
[15] S. Taylor and I. A. Shiels, *WO Patent* 033528-A1, 2003.
[16] T. M. Woodruff, A. J. Strachan, S. D. Sanderson, P. N. Monk, A. K. Wong, D. P. Fairlie and S. D. Taylor, *Inflammation*, 2001, **25**, 171.
[17] N. J. Paczkowski, A. M. Finch, J. B. Whitmore, A. J. Short, A. K. Wong, P. N. Monk, S. A. Cain, D. P. Fairlie and S. D. Taylor, *Br. J. Pharmacol.*, 1999, **128**, 1461.
[18] A. J. Short, A. K. Wong, A. M. Finch, G. Haaima, I. A. Shiels, D. P. Fairlie and S. D. Taylor, *Br. J. Pharmacol.*, 1999, **125**, 551.
[19] A. J. Strachan, I. A. Shiels, R. C. Reid, D. P. Fairlie and S. M. Taylor, *Br. J. Pharmacol.*, 2001, **134**, 1778.
[20] T. V. Arumugam, I. A. Shiels, T. M. Woodruff, R. C. Reid, D. P. Fairlie and S. M. Taylor, *J. Surg. Res.*, 2002, **103**, 260.
[21] T. V. Arumugam, I. A. Shiels, A. J. Strachan, G. Abbenante, D. P. Fairlie and S. M. Taylor, *Kidney Intl*, 2003, **63**, 134.
[22] See www.promics.com and press releases therein.
[23] R. C. Reid, G. Abbenante, S. M. Taylor and D. P. Fairlie, *J. Org. Chem.*, 2003, **68**, 4464.
[24] T. J. Lanza, P. L. Durette, T. Rollins, S. Siciliano, D. N. Cianciarulo, S. V. Kobayashi, C. G. Caldwell, M. S. Springer and W. K. Hagmann, *J. Med. Chem.*, 1992, **35**, 252.
[25] S. J. Siciliano, T. E. Rollins, J. Demartino, Z. Konteatis, L. Malkowitz, G. Van Riper, S. Bondy, H. Rosen and M. S. Springer, *Proc. Natl Acad. Sci. USA*, 1994, **91**, 1214.
[26] P. C. Astles, T. J. Brown, P. Cox, F. Halley, P. M. Lockey, C. McCarthy, I. M. McLay, T. N. Majid, A. D. Morley, B. Porter, A. J. Ratcliffe and R. J. A. Walsh, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 907.
[27] D. Kim, R. A. Rivero, S. E. deLaszlo, J. Tran, J. Fair, W. T. Ashton, S. M. Hutchins, L. Malkowitz, C. Molineaux, S. Siciliano, M. S. Springer, W. J. Greenlee and N. B. Mantlo, *210th ACS National Meeting*, Chicago, IL, MEDI-086, 1995.

[28] K. L. Flanagan, L. L. Chang, W. T. Ashton, S. M. Hutchins, L. Malkowitz, C. Siciliano, M. S. Springer, W. J. Greenlee and N. B. Mantlo, 210th ACS National Meeting, Chicago, IL, MEDI-085, 1995.
[29] S. E. de Laslo, E. E. Allen, B. Li, D. Ondeyka, R. Rivero, L. Malkowitz, C. Molineaux, S. J. Siciliano, M. A. Springer, W. J. Greenlee and N. B. Mantlo, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 213.
[30] A. J. Hutchison and K. R. Shaw, 220th ACS National Meeting, Washington, DC, MEDI-321, 2000.
[31] A. Thurkauf, X. Zhang, X. He, H. Zhao, J. Peterson, G. Maynard and R. Ohliger, *WO Patent* 049992-A2, 2002.
[32] G. P. Luke, G. Maynard, S. Mitchell, A. Thurkauf, L. Xie, L. Zhang, S. Zhang, H. Zhao, B. L. Chenard, Y. Gao, B. Han and X. He, *WO Patent* 082829-A1, 2003.
[33] A. Thurkauf, H. Zhao, S. Zhang and Y. Gao, *WO Patent* 018460-A1, 2004.
[34] See www.nrgn.com and press releases therein.
[35] S. Ishibuchi, H. Sumichika, K. Itoh and Y. Naka, *WO Patent* 014265-A1, 2002.
[36] M. Nakamura, T. Kamahori, S. Ishibuchi, Y. Naka, H. Sumichika and K. Itoh, *WO Patent* 022556-A1, 2002.
[37] H. Sumichika, K. Sakata, N. Sato, S. Takeshita, S. Ishibushi, M. Nakamura, T. Kamahori, S. Ehara, K. Itoh, T. Ohtsuka, T. Mishina, H. Komatsu and Y. Naka, *J. Biol. Chem.*, 2002, **277**, 49403.

CCR1 Antagonists

Kenneth G. Carson, Bruce D. Jaffee, and Geraldine C. B. Harriman

Millennium Pharmaceuticals, 40 Landsdowne St, Cambridge, MA 02139, USA

Contents

1. INTRODUCTION

Chemokines are members of a superfamily of proteins whose primary function is to control leukocyte activity and trafficking through tissues. The biological cascade leading to leukocyte trafficking has been reviewed [1,2]. The current understanding of leukocyte recruitment to sites of inflammation infers that the initial contacts of leukocytes with the blood vessel wall are weak interactions with selectins which lead to the rolling of leukocytes along the vessel wall. This is followed by a tighter binding event, "sticking", that is mediated by integrins and various Cell Adhesion Molecules (CAMs). The final event is the chemokine-induced leukocyte extravasation through the vessel wall, followed by their migration toward the site of injury/inflammation. This is mediated through secretion of chemokines at the inflammatory site and the directional migration of leukocytes towards this chemotactic gradient. Figure 1 illustrates the recruitment of monocytes to Mip-1α (macrophage inflammatory protein-1α through the CC Chemokine Receptor-1 (CCR1) receptor.

The increased expression of chemokines and their respective receptors is associated with inflammatory diseases, autoimmune and allergic disorders, infectious diseases, and cancer. CCR1 is one of 27 known CC chemokine receptors. CCR1 is a seven-transmembrane (7-TM) cell surface G-protein coupled receptor (GPCR) from the C-C family of chemokine receptors that bind chemokines containing adjacent cysteine residues. In humans, CCR1 is expressed at high levels on monocytes, basophils, and a subset of activated memory T cells [1]. Chronic inflammatory diseases are characterized by target tissue CCR1 positive mononuclear cell infiltrates. CCL3 (MIP-1α, macrophage inflammatory protein-1α) and CCL5 (RANTES, regulated on activation normal T cell expressed and secreted) chemokines are specific ligands for the CCR1 receptor. Together, both CCR1 chemokines and their receptor are expressed

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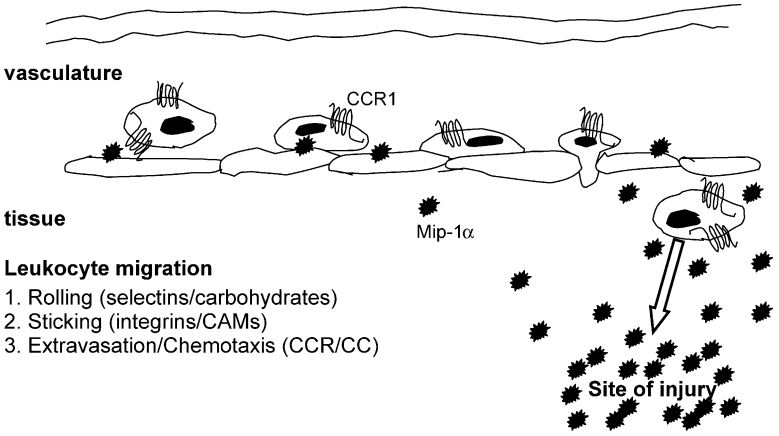


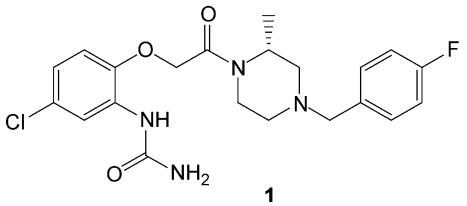
Fig. 1. The three-step model of leukocyte recruitment.

at high levels in chronic inflammatory diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS) and psoriasis [3–7]. CCR1 antagonists should reduce monocyte/macrophage and memory T cell infiltration into inflammatory sites, as well as the subsequent pathological sequelae. CCR1 has recently been shown to co-localize with amyloid beta plaques in Alzheimer disease (AD) patients leading to its possible application as a diagnostic tool for AD [8]. Therefore, antagonists of CCR1 may provide a novel and specific mechanism for therapeutic intervention in RA, MS and other autoimmune or inflammatory diseases. In this chapter, we provide a brief summary of the chemical structures and allied data of recently published, small-molecule CCR1 antagonists.

2. PIPERAZINE DERIVATIVES

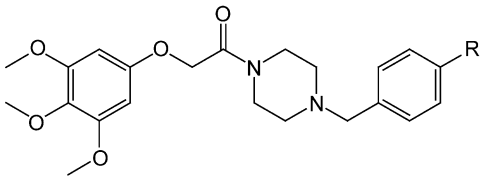
The structure and primary pharmacology of BX-471 (**1**) was disclosed in 1998–2000, and has been reviewed [9,10]. One recurring theme evident in much of the CCR1 antagonist literature is the disconnect between affinity for the human receptor and activity on rodent orthologs. Compound **1** is a prime example, as it shows a marked difference in potency between the human receptor and those of other species [11]. While very potent against the human CCR1 (1 nM), and the rabbit CCR1 (60 nM), compound **1** only inhibits the rat CCR1 with a K_i value of 121 nM; no results have been published for the murine CCR1. Compound **1** was reported to have efficacy in various inflammatory disease models such as rat EAE (experimental autoimmune encephalitis), rabbit allograft rejection, rat heart allograft rejection [13], and mouse renal fibrosis [9,12–14]. Although compound **1** is reported to have acceptable pharmacokinetics in dog (60% bioavailability, $T_{1/2} = 3$ h), studies with most rodent models were performed using sub-cutaneous (s.c.) dosing.

The SAR that led to compound **1** was recently presented [15]. The initial hit from a high-throughput screen was the 3,4,5-trimethoxy derivative **2**, which had a receptor



1

binding K_i value of 328 nM. Subsequent optimization of the right-hand benzyl group led to compound **3**, which showed an increase in potency to 18 nM. Exploration of the left-hand phenoxy ring led from the 3,4,5-trimethoxy substituted analog to the equipotent 4-chloro substituted derivative, **4**. The receptor binding affinity was enhanced further with the addition of a 2-carboxamide substituent leading to the 5 nM inhibitor, **5**. Subsequent elaboration of the piperazine ring was then performed, where it was shown that the 2-(*R*)-methyl derivative **7** was more potent (6 nM) than 2-(*S*)-methyl enantiomer **6** (307 nM). Disubstitution of the piperazine gave analog **8** which retained good potency (3 nM). Further refinement of the 2-carboxamide substituent in **8** yielded the fully optimized urea, compound **1**.



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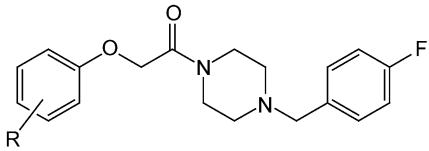
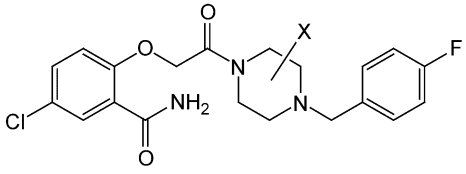
3, R = F

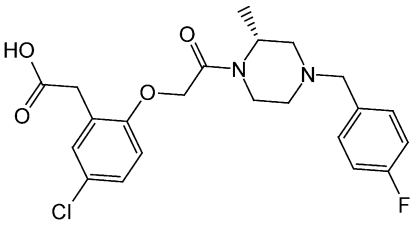


4, R = 4-Cl
5, R = 4-Cl-2-$CONH_2$



6, X = 2-(*S*)-methyl
7, X = 2-(*R*)-methyl
8, X = 2-(*R*)-methyl 5-(*S*)-methyl

Several series of substituted piperazines similar to compound **1** have been reported in the patent literature. The compounds are exemplified by carboxylic acids such as **9**, as well as by a series of sulfonamides, acylsulfonamides and sulfonic acids such as **10** and **11** [16,17]. In addition, related bicyclic structures such as **12** have been reported to have CCR1 activity (CCR1 chemotaxis $IC_{50} < 25$ μM [18]).



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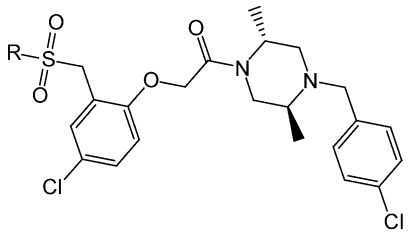
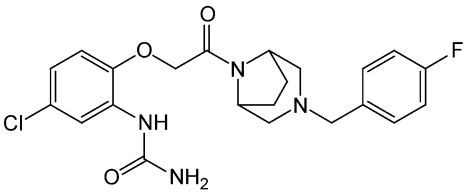
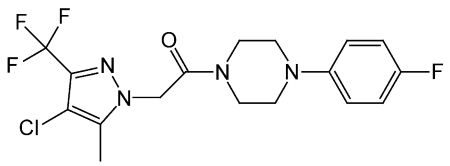


10 R = NHAc
11 R = OH



12

Still other piperazine-containing CCR1 antagonists have been published and presented recently [19]. A series of piperazine-pyrazole antagonists was discovered by utilizing the initial screening hit CCX-105 (**13**), for which a CCR1 binding potency of less than 500 nM was reported. Other active compounds among the family include compound **14**, which was listed as having a binding IC_{50} value of less than 500 nM. Compound **14** was reported to show *in vivo* activity in a rabbit model of lipopolysaccharide (LPS)-induced destructive joint inflammation when administered via direct intra-articular injection in the knee joint. Compound **14** was also active in a rat collagen-induced arthritis (CIA) model. Using a prophylactic dose of 25 mg/kg (s.c. once/day), the compound showed a reduction in joint diameter upon collagen-induced ankle joint inflammation. No compound-specific ortholog activity data has been reported for this series of compounds.



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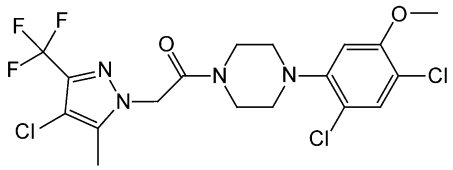
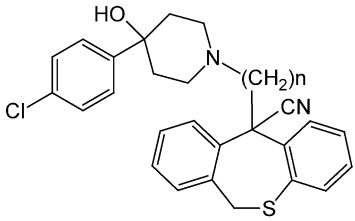


14

3. PIPERIDINE DERIVATIVES

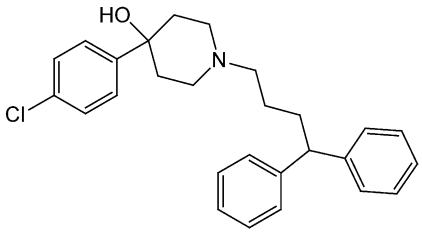
As in the case of piperazines, such as compound **1**, rodent ortholog cross-reactivity differences have been observed in other structural series of CCR1 antagonists. Although



15, n = 3
16, n = 4
17, n = 5

4-hydroxy-4-chlorophenyl piperidine CCR1 antagonists have been reported previously, new data concerning their murine CCR1 cross-reactivity was recently published [20,21]. In the 6-cyano-dibenzothiepine series, linker length showed a correlation with murine CCR1 affinity. While compound **15** was reasonably potent against the human CCR1 (21 nM), it had diminished potency (9150 nM) against the murine ortholog. Conversely, compound **16** had higher affinity for the mCCR1 (332 nM) than for the hCCR1 (417 nM). Inclusion of a 5-carbon-linker in compound **17** resulted in affinities of 96 nm for the hCCR1 and 635 nM for the mCCR1.

Another interesting SAR trend was revealed in rodents in the related non-bridged series exemplified by structures **18** and **19** [21]. The incorporation of a quaternary ammonium group increased the binding affinity to the murine CCR1. Accordingly, compound **18** has a binding affinity of 840 nM for the mCCR1, whereas the quaternary analog **19** displays affinity of 50 nM for the mCCR1. A similar potency shift was seen on the human receptor, with K_i values of 116 and 6 nM, respectively.



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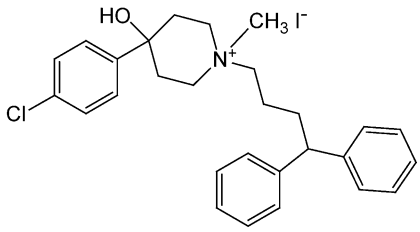

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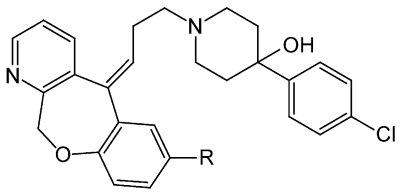
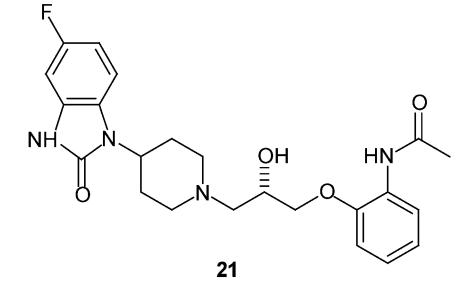
Tricyclic heterocycles have also been reported in the patent literature as CCR1 antagonists [22]. These molecules display the typical GPCR pharmacophore. For example, **20** is a pyridinebenzoxapine tethered to an aryl piperidine. This series of CCR1 antagonists utilizes an exocyclic olefin as the spacer to the piperidine functionality.



20

The functionalized propoxy piperidines **21**–**24** were reported in the patent literature as a structurally divergent class of antagonists that include chemokine receptors whose natural ligands are MIP-1α and RANTES, implying activity against CCR1 [23–26]. This series of molecules contains common structural motifs, among which is the well-known GPCR antagonist pharmacophore, comprising the aromatic—basic amine—aromatic motif and separated by an appropriate spacer. The left hand aromatic ring is halogen substituted, the central basic amine is commonly a piperidine ring, and the right hand aromatic ring

contains polar substituents such as an amide or urea. In the patent application reporting compounds which include piperidine **24**, quaternizing the carbinol carbon was important in successfully decreasing the cross reactivity to the hERG channel; the latter activity could potentially lead to cardiac liabilities.



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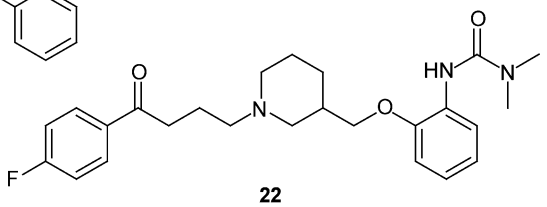

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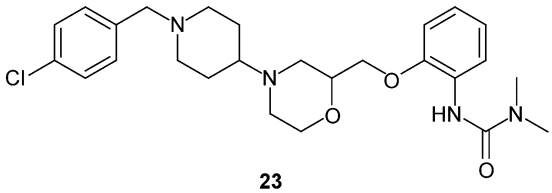

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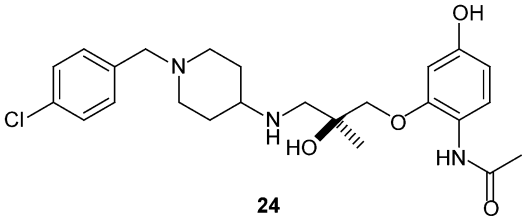
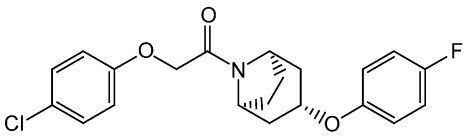


24

A series of acylated piperidine derivatives has recently been disclosed [27–28]. Compounds such as **26** and **27** are reported to inhibit CCR1-mediated chemotaxis at concentrations of less than 10 μM.



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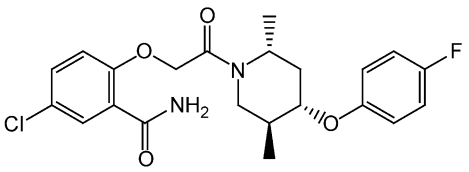
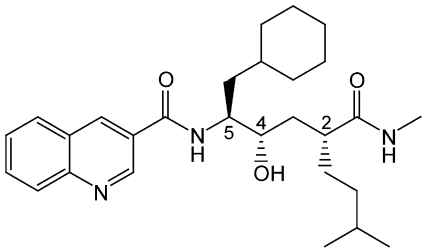


26

4. QUINOXALINE AMIDE DERIVATIVES

A considerable amount of activity has centered on the quinoxaline-amide class of CCR1 antagonists, exemplified by CP-481,715 (**28**). This series of compounds was first disclosed in the patent literature in 1998 [29–31] and the scope of the medicinal chemistry effort has recently been reported [33–35]. High-throughput screening revealed CCR1 activity in a class of hydroxyethylene peptide isosteres which were designed as renin inhibitors [33]. The initial screening hit **28** was selected as a lead compound due to its CCR1 potency ($IC_{50} = 2300$ nM) and selectivity vs rennin ($IC_{50} > 10$ μM). The stereochemistry of compound **28** was investigated, and the 2R, 4S, 5S, stereochemistry was determined to be optimal. The N-5 quinoline amide linkage was judged critical, since its replacement with a sulfonamide group led to an inactive compound. Substitution of the cyclohexyl group showed a strong effect, with acyclic aliphatics leading to inactive compounds. Alternatively, the phenylalanine derivative **29** showed improvement in potency ($IC_{50} = 650$ nM). Other modifications demonstrated that the hydrophobic C-2 substituent and the C-1 amide NH are critical for activity. Substitution of the quinoline amide with various acyl groups [34] led to a significant potency gain in quinoxaline derivative **30** ($IC_{50} = 64$ nM). Further SAR refinements confirmed the importance of the hydrophobic group at C-2 and showed that a C-terminal primary amide can also lead to active analogs as in **31** ($IC_{50} = 28$ nM). Finally, efforts to improve PK properties led to derivative **32** ($IC_{50} = 46$ nM) [35]. While oral absorption was identified as a major



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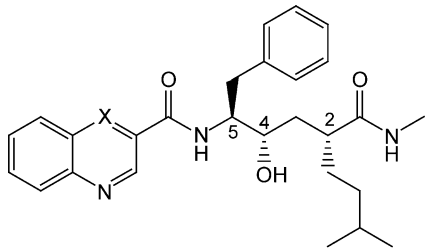
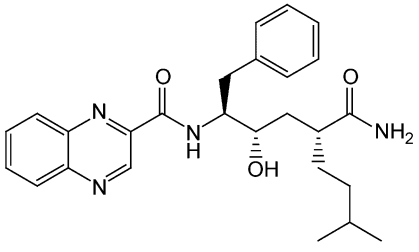


28 X = CH
29 X = N



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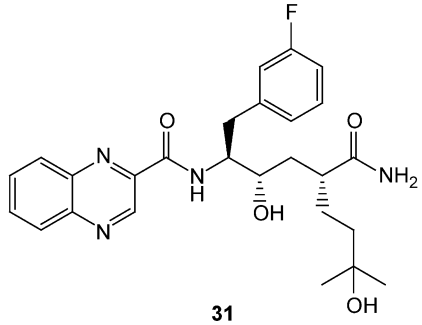

31

liability, compound **32** was reported to have favorable PK properties in dogs where it had moderate clearance (13 mL/min/kg), a low volume of distribution (Vdss = 1.0 L/kg), and 49% bioavailability.

Analog **32** was reported to have an IC_{50} value of 74 nM in a CCR1/Mip-1α radioligand binding assay [32]. In a separate study with radiolabeled compound, **32** was shown to have a binding Kd of 9 nM to hCCR1. Compound **32** was shown to be an antagonist using a GTPγS binding assay using ^{35}S-GTP. Compound **32** was also shown to inhibit calcium mobilization induced by Mip-1α. In support of receptor antagonism, **32** did not directly induce calcium flux. Compound **32** was also shown to inhibit chemotaxis in a dose-dependent manner in human peripheral blood monocytes using both Mip-1α and RANTES. In addition, the compound was shown to be inactive in a panel of GPCR's and other chemokine receptors such as CCR2, CCR3, and CCR5. Activity in whole blood was demonstrated via inhibition of CD11b upregulation in human whole blood, with an average IC_{50} of 160 nM. Further validation of the utility of using a CCR1 antagonist for the treatment of RA was demonstrated experimentally when compound **32** was shown to inhibit monocyte chemotactic activity induced by RA synovial fluid *in vitro* [36]. A clinical proof of concept study was conducted with a panel of 12 active RA patients who were treated with the CCR1 antagonist **32** for 14 days [36]. The results indicated that on day 15 a significant reduction in the number of macrophages and CCR1 + cells was observed in synovial biopsy samples. Moreover, no severe side effects were reported [36]. These preliminary data support the potential clinical utility of a CCR1 antagonist for the treatment of chronic inflammatory diseases.

5. CONCLUSION

Within the past few years there has been a dramatic increase in patent and publication activity on CCR1 antagonists. Several different structural classes of CCR1 antagonists have been advanced to clinical trials. It is a critical time-point for the biology of CCR1 to be manifested in clinical effect in humans. Although different classes of molecules have shown efficacy in various animal models of inflammation (CIA, EAE, transplantation, etc.), this is only indicative of their potential clinical utility. The availability of different and effective molecular probes will undoubtedly help in unraveling the therapeutic potential for CCR1 antagonists in human disease.

REFERENCES

[1] A. E. I. Proudfoot, *Nat. Rev. Immunol.*, 2002, **2**, 106.
[2] A. D. Luster, *New. Engl. J. Med.*, 1998, **338**, 436.
[3] L. Boiardi, P. Macchioni, R. Meliconi, L. Pulsatelli, A. Facchini and C. Salvarani, *Clin. Exp. Rheumatol.*, 1999, **17**, 419.
[4] A. E. Koch, S. L. Kunkel, L. A. Harlow, D. D. Mazarakis, G. K. Haines, M. D. Burdick, R. M. Pope and R. M. Strieter, *J. Clin. Invest.*, 1994, **93**, 921.

[5] T. Ellingsen, A. Buus, B. K. Moller and K. Stengaard-Pedersen, *Scand. J. Rheumatol.*, 2000, **29**, 216.
[6] C. Trebst, T. L. Sorensen, P. Kivisakk, M. K. Cathcart, J. Hesselgesser, R. Horuk, F. Sellebjerg, H. Lassmann and R. M. Ransohoff, *Am. J. Pathol.*, 2001, **159**, 1701.
[7] S. P. Raychaudhuri, W. Y. Jiang, E. M. Farber, T. J. Schall, M. R. Ruff and C. B. Pert, *Acta Derm Venereol.*, 1999, **79**, 9.
[8] M. Halks-Miller, M. L. Schroeder, V. Haroutunian, U. Moenning, M. Rossi, C. Achim, D. Purohit, M. Mahmoudi and R. Horuk, *Ann. Neurol.*, 2003, **54**, 638.
[9] M. Liang, C. Mallari, M. Rosser, H. P. Ng, K. May, S. Monahan, J. G. Bauman, I. Islam, A. Ghannam, B. Buckman, K. Shaw, G.-P. Wei, W. Xu, Z. Zhao, E. Ho, J. Shen, H. Oanh, B. Subramanyam, R. Vergona, D. Taub, L. Dunning, S. Harvey, R. M. Snider, J. Hesselgesser, M. M. Morrissey, H. D. Perez and R. Horuk, *J. Biol. Chem.*, 2000, **275**, 19000.
[10] M. J. Elices, *Curr. Opin. Invest. Drugs*, 2002, **865**.
[11] M. Liang, M. Rosser, H. P. Ng, K. May, J. G. Bauman, I. Islam, A. Ghannam, P. J. Krestchmer, H. Pu, L. Dunning, R. M. Snider, M. M. Morrissey, J. Hesselgesser, H. D. Perez and R. Horuk, *Eur. J. Pharmacol.*, 2000, **389**, 41.
[12] R. Horuk, S. Shurey, H. P. Ng, K. May, J. G. Baumann, I. Islam, A. Ghannam, B. Buckman, G. P. Wei, W. Xu, M. Liang, M. Rosser, L. Dunning, J. Hesselgesser, R. M. Snider, M. Morrissey, H. D. Perez and C. Green, *Immunol. Lett.*, 2001, **76**, 193.
[13] R. Horuk, C. Clayburger, A. M. Krensky, Z. Wang, H.-J. Grone, C. Weber, K. S. C. Weber, P. J. Nelson, K. May, M. Rosser, L. Dunning, M. Liang, B. Buckman, A. Ghannam, H. Ng, I. Islam, J. G. Bauman, G.-P. Wei, S. Monahan, W. Xu, R. M. Snider, M. M. Morrissey, J. Hesselgesser and H. D. Perez, *J. Biol. Chem.*, 2001, **276**, 4199.
[14] H.-J. Anders, V. Vielhauer, M. Frink, Y. Linde, C. D. Cohen, S. M. Blattner, M. Kretzler, F. Strutz, M. Mack, H.-J. Grone, J. Onuffer, R. Horuk, P. J. Nelson and D. Schlondorff, *J. Clin. Invest.*, 2002, **109**, 251.
[15] I. Imadul, K. May, J. Bauman, A. Ghannam, H.P. Ng, S. Monahan, G.-P. Wei, W. Xu, M. Liang, L. Dunning, B. Subramanyam, J. Shen, J. Walters, E. Ho and R. Horuk, Abstracts of Papers, 224th National ACS Meeting, Boston, MA, August 18–22, 2002, MEDI-334.
[16] L.C. Blumberg, M.F. Brown, M.M. Hayward, C.S. Poss and G.D. Lundquist, *WO Patent* 035627, 2003.
[17] M.M. Hayward, *WO Patent* 102787, 2002.
[18] L.C. Blumberg, M.F. Brown, R.P. Gladue and C.S. Poss, *WO Patent* 32901, 2002.
[19] A.M.K. Pennell, J.B. Aggen, J.J.K. Wright, S. Sen, B.E. McMaster and D.J. Dairaghi, *WO Patent* 105853, 2003.
[20] H. P. Ng, K. May, J. G. Bauman, A. Ghannam, I. Islam, M. Liang, R. Horuk, J. Hesselgesser, R. M. Snider, H. D. Perez and M. M. Morrissey, *J. Med. Chem.*, 1999, **42**, 4680.
[21] J. Onuffer, M. A. McCarrick, L. Dunning, M. Liang, M. Rosser, G.-P. Wei, H. Ng and R. Horuk, *J. Immunol.*, 2003, **170**, 1910.
[22] J.R. Luly, Y. Nakasato, E. Ohshima, G.C.B. Harriman, K.G. Carson, S. Ghosh, E.M. Elder and K.M. Mattia, *WO Patent* 045942, 2003.
[23] T. Eriksson, S. Ivanova and H. Lonn, *WO Patent* 074763-A1, 2002.
[24] J. Gustafsson, N. Hossian and S. Nilsson, *WO Patent* 018566-A1, 2003.
[25] J. Gustafsson, N. Hossian and S. Nilsson, *WO Patent* 018576-A1, 2003.
[26] T. Eriksson and G. Noori, *WO Patent* 051839-A1, 2003.
[27] L.C. Blumberg, M.F. Brown, M.M. Hayward, C.S. Poss and G.D. Lundquist, *WO Patent* 009588, 2004.
[28] L.C. Blumberg, M.F. Brown, M.M. Hayward and C.S. Poss, WO 009550, 2004.
[29] M. F. Brown, J.C. Kath and C.S. Poss, *WO Patent* 38167, 1998.
[30] J. C. Kath, M.F. Brown and C.S. Poss, *WO Patent* 40061, 1999.
[31] M.F. Brown and C.S. Poss, *WO Patent* 057023-A1, 2001.
[32] R. P. Gladue, L. A. Tylaska, W. H. Brisette, P. D. Lira, J. C. Kath, C. S. Poss, M. F. Brown, T. J. Paradis, M. J. Conklyn, K. T. Ogborne, M. A. McGlynn, B. M. Lillie, A. P. DiRico, E. N. Mairs, E. B. McElroy, W. H. Martin, I. A. Stock, R. M. Shepard, H. J. Showell and K. Neote, *J. Biol. Chem.*, 2003, **278**, 40473.

[33] J. C. Kath, A. P. DiRico, R. P. Gladue, W. H. Martin, E. B. McElroy, I. A. Stock, L. A. Tylaska and D. Zheng, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 2163.
[34] J. C. Kath, W. H. Brissette, M. F. Brown, M. Conklyn, A. P. DiRico, P. Dorff, R. P. Gladue, B. M. Lillie, P. D. Lira, E. N. Mairs, W. H. Martin, E. B. McElroy, M. A. McGlynn, T. J. Paradis, C. S. Poss, I. A. Stock, L. A. Tylaska and D. Zheng, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 2169.
[35] M. F. Brown, M. Avery, W. H. Brisette, J. H. Chang, K. Colizza, M. Conklyn, A. P. DiRico, R. P. Gladue, J. C. Kath, S. S. Krueger, P. D. Lira, B. M. Lillie, G. D. Lundquist, E. N. Mairs, E. B. McElroy, M. A. McGlynn, T. J. Paradis, C. S. Poss, M. I. Rossulek, R. M. Shepard, J. Sims, T. J. Strelevitz, S. Truesdell, L. A. Tylaska, K. Yoon and D. Zheng, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 2175.
[36] J. J. Haringman, M. C. Kraan, T. J. M. Smeets, K. H. Zwindermann and P. P. Tak, *Ann. Rheum. Dis.*, 2003, **62**, 715.

Section 4
Cancer and Infectious Diseases

Editor: Jacob J. Plattner
Anacor Pharmaceuticals
Palo Alto
California

Recent Advances in Antimetabolite Cancer Chemotherapies

James R. Henry and Mary M. Mader

Lilly Research Laboratories, Indianapolis, IN 46285, USA

Contents

1. INTRODUCTION

An antimetabolite interferes with the formation or utilization of a normal cellular metabolite. Most antimetabolites interfere with the enzymes involved in the synthesis of new DNA, are incorporated into the newly formed DNA, or in some cases both processes are important to an agent's efficacy. As a result, many antimetabolites are derivatives of the building blocks of DNA itself, such as the nucleoside based inhibitors, or analogs of critical cofactors such as the antifolates. A variety of key cellular pathways have been disrupted with antimetabolite therapy, including inhibition of the thymidine and purine nucleotide biosynthesis pathway, and the inhibition of ribonucleoside reductase. Given their mechanism of action, it is not surprising that the observed benefits of antimetabolites are often accompanied by significant toxicity, due to the fact that the affected cellular metabolites are critical to both normal and cancer cells. Single antimetabolite agents can act on a single pathway, or on multiple pathways at once, but in either instance, they are often used in combination with other therapies in the clinic.

2. MECHANISMS OF ANTIMETABOLITE CANCER CHEMOTHERAPY

2.1. Thymidine biosynthesis

Critical to the cell's process of replication is its ability to synthesize thymidine. This process involves several key enzymes including thymidylate synthase (TS),

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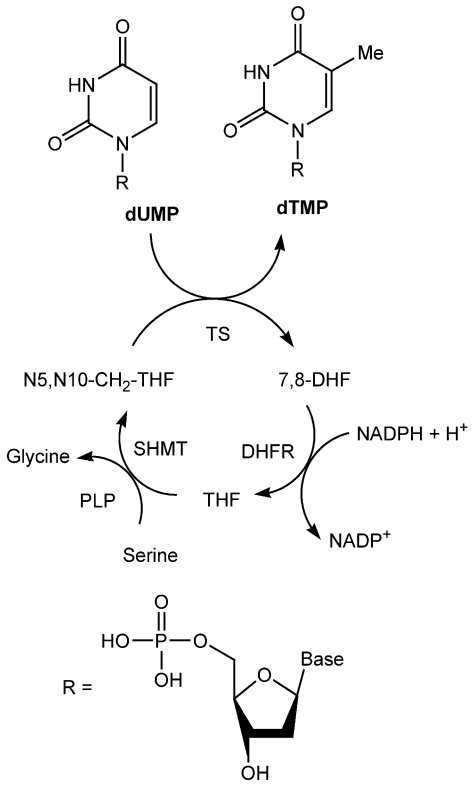


Fig. 1. Key steps in thymidine biosynthesis.

dihydrofolate reductase (DHFR), and serine hydroxymethyl transferase (SHMT) (Fig. 1).

The methylation of deoxyuridine 5′ monophosphate (dUMP) to produce deoxythymidine 5′ monophosphate (dTMP) is mediated by TS [1]. The methyl group for dTMP is provided by N5,N10-methylene tetrahydrofolate (N5,N10-CH_2-THF) through its conversion to 7,8-dihydrofolate (7,8-DHF). The 7,8-DHF must then be converted to tetrahydrofolate (THF) by DHFR [2], followed by further transformation back to N5,N10-CH_2-THF through the action of SHMT [3]. Therefore, inhibition of TS, DHFR, or SHMT with an appropriate agent would interrupt the process of thymidine biosynthesis. Low thymidine levels cause defects in DNA which in turn activates stress response elements, such as the Fas ligand/Fas death pathway leading to apoptosis [4]. It has also been proposed that defects in this Fas-dependent apoptotic signaling pathway are one cause of cellular resistance to drugs.

2.2. Purine nucleotide synthesis

The cell's ability to provide the needed purine nucleotides for DNA and RNA synthesis is also critical to its survival. The *de novo* biosynthesis of purine nucleotides involves 10

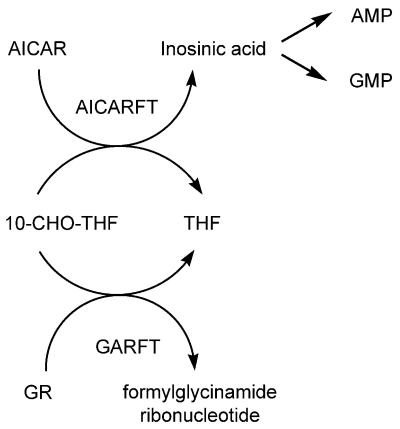


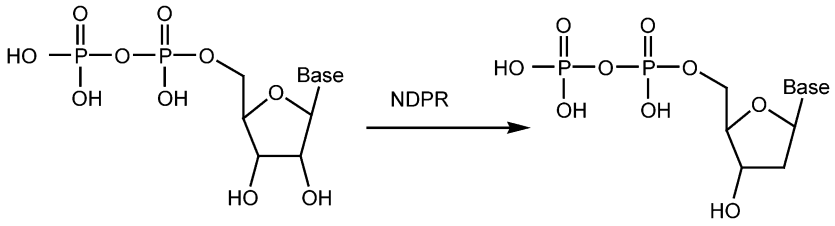
Fig. 2. Key step in purine biosynthesis.

separate enzyme catalyzed reactions starting with 5-phosphoribosyl-1-pyrophosphate and leading to inosinic acid [5]. Both adenosine monophosphate (AMP) and guanine monophosphate (GMP) are then derived from inosinic acid (Fig. 2). The third step in this process is the biosynthesis of formylglycinamide ribonucleotide from glycinamide ribonucleotide (GR) via glycinamide ribonucleotide formyltransferase (GARFT). The last two steps in the synthesis of inosinic acid occur via a bifunctional enzyme having both aminoimidazolecarboxamide ribonucleotide formyltransferase (AICARFT) and inosine monophosphate cyclohydrolase (IMPCH) activity. This enzyme has been shown to be made up of a 39 kDa carboxy-terminal AICARFT active fragment along with a 25 kDa amino-terminal IMPCH active fragment [6]. Both GARFT and AICARFT catalyze the transfer of a formyl group from 10-CHO-tetrahydrofolate (10-CHO-THF) to GR or aminoimidazolecarboxamide ribonucleotide (AICAR) respectively, returning THF as the second product of the reaction.

2.3. Ribonucleoside reductase

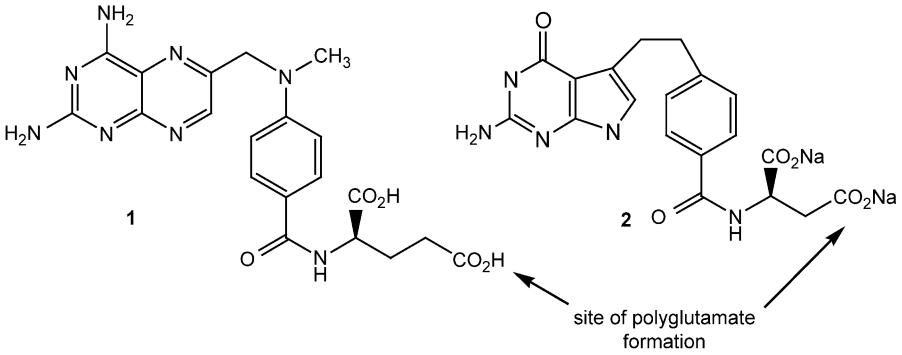
The synthesis of new DNA within a cell requires the production of deoxynucleotides. The four required deoxynucleotides (adenosine, guanosine, cytidine, and thymidine) are produced as by reduction of the appropriate ribonucleotide substrate with ribonucleoside reductase, also referred to a nucleoside diphosphate reductase (NDPR) [7]. The resulting oxidized form of NDPR can then be reduced back to NDPR by the action of glutaredoxin, which is in turn oxidized to thioredoxin [8]. NDPR is a dimer, with each monomer made up of two subunits: a larger (M1) and a smaller (M2) subunit. The M1 subunit contains two allosteric sites involved in regulation of the overall activity of the enzyme and the enzyme's substrate specificity. The deoxynucleoside triphosphates bind to this allosteric site, and regulate their own synthesis. The M2 subunit is responsible for the key reduction reaction, carrying a tightly bound iron atom that stabilizes the tyrosyl free radical critical to reduction. Deoxynucleotide pools in proliferating cells are

sufficient for only a few minutes of DNA synthesis without regeneration, thus making NDPR inhibition an attractive candidate for cancer chemotherapy [9].



2.4. Intracellular transformations of antimetabolites

Most antimetabolites must undergo modification within the cell before they are active agents, and so in essence are prodrugs. Methotrexate (MTX, **1**), an antifolate agent targeting DHFR, and pemetrexed (Alimta, **2**), a multi-targeted antifolate, exert much of their pharmacological effects as a polyglutamate, as do most classical antifolates [10]. This transformation is carried out by the enzyme folylpolyglutamate synthetase (FPGS). Formation of the polyglutamate of antifolate drugs can cause a dramatic increase in the activity of the agent toward its intended target. Further, polyglutamates (above diglutamate) are less susceptible to cellular efflux, thus providing a long-lived pool of drug within the cell [11]. Most natural folate cofactors exist as polyglutamates, and so the beneficial action of FPGS on antifolate drugs is not surprising. It also follows that any cellular change leading to decreased FPGS activity could lead to antifolate resistance.



Many nucleoside derived antimetabolite analogs also undergo intracellular transformations to become active agents (Fig. 3). The earliest of these agents, 5-fluorouracil (5-FU, **3**) is converted into three major metabolites that are responsible for its activity. 5-FU is changed into 5-fluoro-2′-deoxyuridine monophosphate (FdUMP, **4**) that acts as a mimic for the natural substrate of TS, dUMP, thus inhibiting TS activity. 5-FU can also be tranformed to 5-fluoro-2′-deoxyuridine triphosphate, which is eventually incorporated into DNA causing DNA damage, and finally to 5-fluorouridine triphosphate (**5**), which is incorporated into RNA leading to impaired RNA function [12]. Deoxyribonucleoside

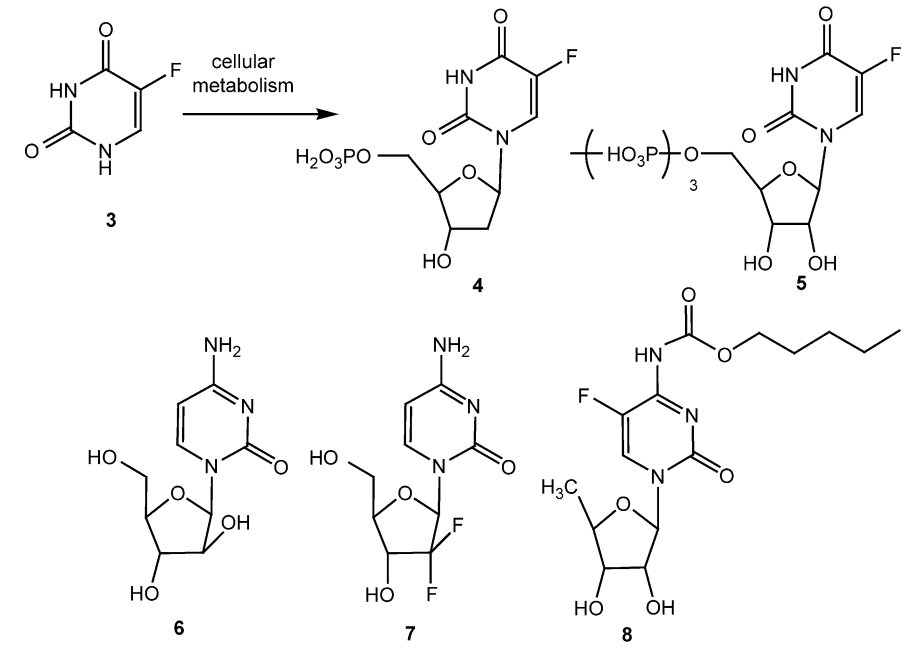


Fig. 3. Metabolism of 5-FU **3** and other nucleoside antimetabolites.

analogs such as cytarabine (**6**) and gemcitabine (**7**) are converted to their triphosphate derivatives *in vivo* before showing their effects [13,14], while capecitabine (**8**) is metabolized *in vivo* to **3** (Fig. 4). **8** is readily absorbed in the gastrointestinal tract, then passes intact through the intestinal mucosa. It is subsequently converted to 5′-deoxycytidine **9** by carboxylesterase in the liver, 5′-deoxy-5-fluorouridine **10** by cytidine deaminase, and finally to 5-FU **3** by thymidine phosphorylase.

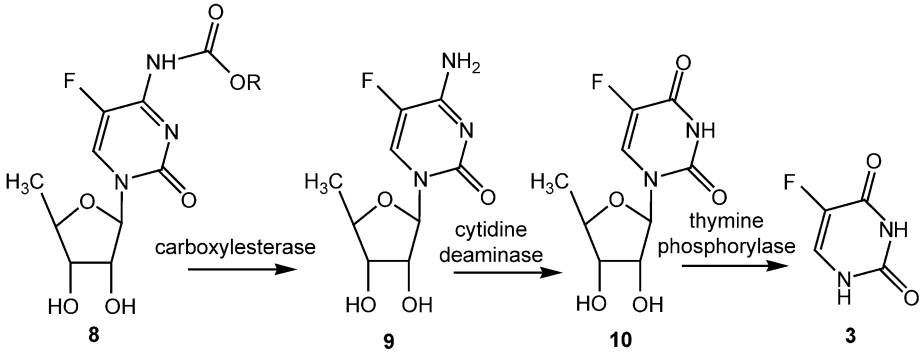


Fig. 4. Metabolism of capecitabine to 5-FU.

3. ANTIMETABOLITES

Historically, antimetabolites have been useful agents in hematopoietic therapy. However, in recent years, they have been shown to be effective in the treatment of solid tumors (Table 1). Three antimetabolites have been approved for clinical use since 1996: gemcitabine, capecitabine and pemetrexed. A new method for the delivery of cytarabine, DepoCyt, was given accelerated approval in 1999. Additional oncolytic indications for some of the approved antimetabolites are in clinical trials, and other antimetabolites, including nolatrexed and decitabine, are currently undergoing Phase III study. An antifolate, raltitrexed, has been approved for use outside of the US, but its development as a single agent, as well as that of the nucleoside analog eniluracil has been discontinued in the USA [15,16].

Although 5-FU is widely used, its pharmacokinetic profile is not ideal. Its optimal method of delivery is by continuous intravenous infusion, as its bioavailability after oral administration is variable. 5-FU is rapidly metabolized, with a mean half-life of elimination of approximately 16 min. Within 3 h, no intact drug can be detected in plasma. 5-FU is more effective when co-administered with leucovorin, a prodrug of 5,10-CH_2-THF. Inhibition of TS by FdUMP is dependent on the cofactor 5,10-CH_2-THF, which combines with TS and FdUMP to form a covalent ternary complex. Excess cofactor decreases the dissociation rate of this complex, and consequently addition of

Table 1. Selected oncolytic antimetabolites in clinical use

Clinical agent	Trade name	Year approved (USA)	Indication	Ref.
Folate Antagonists				
Methotrexate (MTX)		1953, 1959, 1971	Leukemia	[18]
Pemetrexed	Alimta®	2004	Mesothelioma	[19]
Nolatrexed	Thymitaq®	(Phase III)	Liver cancer	[20]
Nucleoside analogs				
5-Fluorouracil	Adrucil®	1962	Colorectal cancer	[21]
Cytarabine	Cytosar-U®	1969	Leukemia	[22]
Fludarabine	Fludara®	1991	Leukemia	[23]
Pentostatin	Nipent®	1991	Leukemia	[24]
Cladribine	Leustatin®	1993	Leukemia	[25]
Gemcitabine	Gemzar®	1996	Pancreatic cancer	[26]
		1998	Non-small cell lung cancer	[27]
Cytarabine lyposomal	DepoCyt®	1999	Lymphomatous meningitis	[28]
Capecitabine	Xeloda®	1998	Breast cancer	[29]
		2001	Colorectal cancer	[30]
Decitabine	Dacogen®	(Phase III)	Leukemia	[31]

leucovorin increases the cytotoxicity of 5-FU. The major toxicities of 5-FU are to bone marrow and mucous membranes [17].

Capecitabine (**8**) is an orally administered fluoropyrimidine carbamate that is metabolized *in vivo* to 5-FU (**3**) [32]. Pharmacokinetic studies in patients showed rapid gastrointestinal absorption of capecitabine, followed by extensive conversion to **10**, with only low systemic levels of 5-FU [33]. Significantly, capecitabine appears to deliver drug selectively to tumors. Analysis of the tumor:plasma AUC ratios of capecitabine vs. 5-FU in four human tumor xenografts in mice (HCT116, CXF280, COLO205, and WiDr) at the maximum tolerated dose (p.o.) showed that although the half-life of 5-FU was similar in all four tumors, the tumor:plasma AUC ratio of 5-FU was significantly higher for animals dosed with capecitabine. For example, in HCT116, 5-FU exposure was 127-fold higher in tumor than plasma in animals treated with capecitabine, and 209-fold higher in CXF280 [34]. In the clinic, the efficacy of capecitabine equals or exceeds 5-FU, although its pharmacology in the murine models might have predicted consistently superior efficacy. Capecitabine is approved for the treatment of breast and colorectal cancer, and its ease of dosing by mouth is attractive to patients.

Gemcitabine (**7**) is a nucleoside analog that exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cells through the G1/S-phase boundary. The cytotoxic effect of gemcitabine is attributed to a combination of two actions of the diphosphate and the triphosphate nucleosides, which leads to inhibition of DNA synthesis. First, gemcitabine diphosphate inhibits NDPR, which causes a reduction in the concentrations of deoxynucleotides, including deoxycytidine triphosphate (dCTP) [35]. Second, gemcitabine triphosphate competes with dCTP for incorporation into DNA. The reduction in the intracellular concentration of dCTP (by the action of the diphosphate) enhances the incorporation of gemcitabine triphosphate into DNA (self-potentiation). After the gemcitabine nucleotide is incorporated into DNA, only one additional nucleotide is added to the growing DNA strands. After this addition, there is inhibition of further DNA synthesis. Because of the addition of this final nucleotide, DNA polymerase epsilon is unable to remove the gemcitabine nucleotide and repair the growing DNA strands (masked chain termination). First approved by the FDA in 1996, **7** was demonstrated to have a significant clinical benefit response in advanced pancreatic cancer patients compared to 5-FU, with a survival advantage of 5.6 months vs. 4.4 months in the 5-FU-treated patients [26]. The clinical benefit was measured as improvement in three symptoms present in most pancreatic cancer patients: pain, functional impairment and weight loss. Gemcitabine has become accepted as the standard of care for the treatment of advanced pancreatic cancer, and in 1998 the FDA approved its combination with cisplatin for the treatment of non-small cell lung cancer (NSCLC).

DepoCyt is an injectable, sustained release form of cytarabine (ara-C; **6**), for the treatment of antineoplastic meningitis (NM) arising from lymphoma (lymphomatous meningitis) [28]. Ara-C acts by inhibiting DNA polymerase as well as through incorporation of its triphosphate into DNA. A phase III trial of DepoCyt in lymphomatous NM showed it to be more convenient and associated with a higher positive response rate than ara-C. The DepoCyt formulation of ara-C is encapsulated in the aqueous chambers of a spherical 20 μM matrix comprised of lipids biochemically similar to normal human cell membranes (phospholipids, triglycerides, and cholesterol).

When injected into the cerebral spinal fluid (CSF) at room temperature, the particles spread throughout the neuroaxis and slowly release ara-C. A single injection of free unencapsulated ara-C maintains cytotoxic concentrations in the CSF for <24 h [36], whereas a single injection of 50 mg of DepoCyt maintains cytotoxic concentrations of ara-C in most patients in the CSF for >14 days [37]. As cytotoxicity is a function of both drug concentration and duration of exposure, this formulation maintains high concentrations of ara-C in the cancer cell for prolonged periods of time and increases the efficacy of the agent.

Pemetrexed is an antifolate that inhibits multiple folate-requiring enzymes including TS, DHFR, GARFT and to a lesser extent, AICARFT [38,39]. Having multiple sites of inhibition results in an activity profile that differs from the TS inhibitor, 5-FU, or the DHFR inhibitor, MTX. Folic acid and vitamin B-12 supplementation modulate pemetrexed's overall toxicity while enhancing its cytotoxic effects, and pre-treatment with folic acid is a component of the clinical regimen [40]. In a Phase II study of pemetrexed as a single agent in patients with malignant pleural mesothelioma (MPM), a 17% response rate was observed (9 of 64 patients). In combination with cisplatin, however, a Phase III trial found a response rate of 41.3% and a median survival of 12.3 months [19]. The control arm of the study received cisplatin monotherapy, and the response rate in these patients was 16.7% with median survival of 9.3 months [41]. Based on these findings, the pemetrexed/cisplatin combination was approved by the FDA in February 2004 as a treatment for MPM. Clinical trials of pemetrexed are underway as a therapy for solid tumors including non-small cell lung, pancreatic, metastatic breast, colorectal, and gastric cancers [42].

4. CLINICAL ADVANCES WITH COMBINATIONS OF ANTIMETABOLITES

Anti-cancer agents are rarely given singly, as combinations of drugs have proven to be far superior to single agent therapy for a variety of cancers. Antimetabolites are no exception; they have been combined with other antimetabolites and with other chemotherapeutic agents. The goal of combination therapy is to find agents whose activities are synergistic, i.e., a regime where the combined effect is greater than what would be expected from the sum of the two individual agent's activities, and have non-overlapping toxicities. Since most antimetabolites interfere with the process of DNA synthesis or growth, many combinations with drugs that react with DNA have been used [43]. An illustration of this is the combination of pemetrexed with cisplatin. Other potential combinations would be with compounds targeted towards inducing apoptosis, preventing angiogenesis, or with antimetabolites targeting different enzymes in the same pathway. An example is the combination of cyclophosphamide (a DNA alkylating agent), MTX, and 5-FU. This regimen, referred to as CMF, was an early standard of care in the treatment of metastatic breast cancer [44]. 5-FU targets the thymine biosynthesis pathway by inhibiting TS, while MTX targets the same general pathway by inhibiting DHFR. Combinations are first studied preclinically in cellular and animal models, but application of the models to patients in the clinical setting is complicated by ADMET phenomena that are not well predicted by the models [45]. Clinical success for therapy

(combination or single agent) is assessed by a variety of measures relative to the standard of care for the indication, including toxicities, survival benefit, response rate, median survival, and survival to a determined point in time (e.g. 12 months). Our focus will be on recent clinical outcomes with combinations of antimetabolites and the biochemical rationale behind them.

Thymidylate synthase inhibitors including 5-FU, capecitabine, and raltitrexed have been tested in the clinic in combination with gemcitabine. Gemcitabine and the TS inhibitors inhibit DNA and RNA synthesis by different mechanisms and possess almost no overlapping toxicity profiles. Gemcitabine inhibits NDPR, depleting cellular dUMP pools, thereby decreasing the dUMP competition with 5-FdUMP at TS. Raltitrexed prevents binding of the folate cofactor on TS, and also has little overlapping toxicity with gemcitabine. At least additive, and possibly synergistic cytotoxic effects for gemcitabine/5-FU were anticipated based on preclinical results with the combination in HT29 colon cancer cells [46]. To date, several Phase III trials in pancreatic cancer have found gemcitabine/5-FU regimens to be tolerable in terms of toxicities, but an optimal dosing schedule has yet to be found which improves the median survival of patients with advanced pancreatic carcinoma compared with single-agent gemcitabine [47]. A Phase II trial of bi-weekly high dose gemcitabine plus capecitabine in pancreatic cancer similarly found a good therapeutic index for the combination, but no advantage was observed in terms of efficacy parameters compared to monotherapy with gemcitabine [48,49]. The combination of gemcitabine with raltitrexed has been investigated in a Phase II trial involving pancreatic cancer patients, and was found to be convenient with little symptomatic toxicity. However, like the combinations with the nucleoside TS inhibitors, the dual therapy of gemcitabine with the antifolate TS inhibitor was no more efficacious in terms of survival benefit than single gemcitabine monotherapy [50].

Gemcitabine has also been investigated with pemetrexed in pancreatic cancer and NSCLC. Pemetrexed depletes the intracellular supply of both purine and thymidine deoxynucleotides, while gemcitabine is incorporated into nascent DNA strands ultimately resulting in strand termination. Thus, the two agents together would interfere with DNA replication at both the nucleotide and strand synthesis level. Early *in vitro* cell assays and tumor xenograft models indicated that gemcitabine/pemetrexed would show synergism *in vivo*, but the degree of activity was dependent on cell type and dosing schedule [40,51,52]. Each drug is active as a single agent in pancreatic cancer (Table 2) [26,53], and a Phase I trial of the gemcitabine/pemetrexed combination in advanced pancreatic cancer was initiated. In patients, the recommended schedule and doses were found to be gemcitabine on days 1 and 8 @ 1250 mg/m^2 with pemetrexed on day 8 only @ 500 mg/m^2 [51]. Phase II results for the gemcitabine/pemetrexed combination have been reported, with patients showing a 15% partial response rate, with 29% of the evaluable patients surviving for 12 months. As the three measures (partial response, median survival, and 1-yr survival) for the combination showed improvement relative to therapy with gemcitabine or pemetrexed alone, a Phase III trial was initiated. Enrollment for the Phase III trial has concluded, and a final data analysis is anticipated in the coming year. The combination has demonstrated efficacy in an ongoing Phase II trial for NSCLC, employing the same schedule, with evidence of improved median survival and 1-yr survival in comparison to the gemcitabine/cisplatin combination or single agent pemetrexed.

Table 2. Comparisons of selected anti-metabolite single agent and combination clinical trials

Ref.	Patients total/eval.	Treatment	Agent	PR[a] *n* (%)	Median survival (mo)	1-yr survival (%)
Advanced pancreatic cancer						
[26]	63/56	Phase III	Gemcitabine	3 (5.4)	5.7	18
[53]	42/35	Phase II	Pemetrexed	2 (5.7)	6.5	28
[54]	42/40	Phase II	Gemcitabine/ pemetrexed	6 (15)	6.5	29
Non-small cell lung cancer						
[55]	301/288	Phase III	Gemcitabine/ cisplatin	60 (21)	8.1	36
[56]	33/30	Phase II	Pemetrexed	7 (23)	9.2	25
[57]	60/54	Phase II	Gemcitabine/ pemetrexed	9 (17)[b]	11.3	46

[a] PR = partial response.
[b] Objective response.

5. CONCLUSIONS

Much progress has been made in the last decade in the development and clinical use of antimetabolites as chemotherapeutics for the treatment of solid tumors. Both mono- and combination therapies have been found to be efficacious, and clinical trials are underway to determine efficacies against a greater variety of tumor types, and of regimens involving two, three and four-drug combinations.

REFERENCES

[1] D. V. Santi and P. V. Danenberg, Folates in pyrimidine nucleotide biosynthesis. in *Folates and Pterins* (eds R. L. Blakely and S. J. Benkovic), Wiley, New York, 1984, p. 345.
[2] R. L. Blakely, Dihydrofolate reductase. in *Folates and Pterins* (eds R. L. Blakely and S. J. Benkovic), Wiley, New York, 1984, p. 191.
[3] V. Schirch, Folates in serine and glycine metabolism. in *Folates and Pterins* (eds R. L. Blakely and S. J. Benkovic), Wiley, New York, 1984, p. 399.
[4] J. A. Houghton, F. G. Harwood and D. M. Tillman, *Proc. Natl. Acad. Sci.*, 1997, **94**, 8144.
[5] P. B. Rowe, Folates in the biosynthesis and degradation of purines. in *Folates and Pterins* (eds R. L. Blakely and S. J. Benkovic), Wiley, New York, 1984.
[6] J. L. Kan and R. G. Moran, *Nucl. Acids. Res.*, 1997, **25**, 3118.
[7] J. G. Cory and A. H Cory (eds), *Inhibitors of Ribonucleoside Diphosphate Reductase Activity*, Pergamon, New York, 1989.
[8] A. Holmgren, *Annu. Rev. Biochem.*, 1985, **54**, 237.
[9] J. G. Cory and P. Chiba, *Pharmacol. Ther.*, 1985, **29**, 111.
[10] L. H. Matherly, R. L. Seither and I. D. Goldman, *Pharmacol. Ther.*, 1987, **35**, 27.
[11] J. J. McGuire, Antifolate polyglutamation in preclinical and clinical antifolate resistance. in *Antifolate Drugs in Cancer Therapy* (ed. A. L. Jackman), Humana Press, Totowa, 1999, p. 339.

[12] J. A. Montgomery, Antimetabolites. in *Cancer Chemotherapeutic Agents* (ed. W. O. Foye), American Chemical Society, Washington, DC, 1995, p. 49.
[13] P. P. Major, E. M. Egan, G. P. Beardsley, M. D. Minden and D. W. Kufe, *Proc. Natl. Acad. Sci.*, 1981, **78**, 3235.
[14] P. Huang, S. Chubb, L. W. Hertel, G. B. Grindey and W. Plunkett, *Cancer Res.*, 1991, **51**, 6110.
[15] D. Cunningham, J. Zalcberg, J. Maroun, R. James, S. Clarke, T. S. Maughan, M. Vincent, J. Schulz, M. Gonzalez Baron and T. Facchini, *Eur. J. Cancer*, 2002, **38**, 478.
[16] H. L. Kindler and R. L. Schilsky, Clinical trials of the eniluracil/5-fluorouracil combination. in *Fluoropyrimidines in Cancer Therapy* (ed. Y. M. Rustum), Humana Press, Totowa, NJ, 2003, p. 235.
[17] M. J. Moore and C. Erlichman, Pharmacology of anticancer drugs. in *The Basic Science of Oncology* (eds I. F. Tannock and R. P. Hill), 3rd edition, McGraw-Hill, New York, 1998, p. 370.
[18] R. Gorlick and J. R. Bertino, Clinical pharmacology and resistance to dihydrofolate reductase inhibitors. in *Antifolate Drugs in Cancer Therapy* (ed. A. L. Jackman), Humana Press, Totowa, NJ, 1999, p. 37.
[19] N. J. Vogelzang, J. J. Rusthoven, J. Symanowski, C. Denham, E. Kaukel, P. Ruffie, U. Gatzemeier, M. Boyer, S. Emri, C. Manegold, C. Niyikiza and P. Paoletti, *J. Clin. Oncol.*, 2003, **21**, 2636.
[20] K. Stuart, J. Tessitore, J. Rudy, N. Clendennin and A. Johnston, *Cancer*, 1999, **86**, 410.
[21] S. Ananthan, Fluoropyrimidines. in *Cancer Chemotherapeutic Agents* (ed. W. O. Foye), American Chemical Society, Washington, DC, 1995, p. 49.
[22] J. A. Secrist, III, 2′-Deoxyribonucleoside analogs. in *Cancer Chemotherapeutic Agents* (ed. W. O. Foye), American Chemical Society, Washington, DC, 1995, p. 71.
[23] J. C. Adkins, D. H. Peters and A. Markham, *Drugs*, 1997, **53**, 1005.
[24] C. E. Dearden, E. Matutes and D. Catovsky, *Semin. Oncol.*, 2000, **27**, 22.
[25] M. S. Tallman and D. Hakimian, *Sem. Hematol.*, 1996, **33**, 23.
[26] H. A. Burris, III, M. J. Moore, J. Andersen, M. R. Green, M. L. Rothenberg, M. R. Modiano, M. C. Cripps, R. K. Portenoy, A. M. Storniolo, P. Tarassoff, R. Nelson, F. A. Dorr, C. D. Stephens and D. D. Von Hoff, *J. Clin. Oncol.*, 1997, **15**, 2403.
[27] A. Sandler and D. S. Ettinger, *Oncologist*, 1999, **4**, 241.
[28] K. A. Jaeckle, S. Phuphanich, M. J. van den Bent, R. Aiken, T. Batchelor, T. Campbell, D. Fulton, M. Gilbert, D. Heros, S. J. O'Day, W. Akerley, J. Allen, S. Baidas, S. Z. Gertler, H. S. Greenberg, S. LaFollette, G. M. Lesser, W. L. Recht, E. Wong, M. C. Chamberlain, A. Cohn, M. J. Glantz, J. C. Gutheil, B. Maria, P. Moots, P. New, C. Russell, W. Shapiro, L. Swinnen and S. B. Howell, *Br. J. Cancer*, 2001, **84**, 157.
[29] P. M. Hoff, R. Ansari, G. Batist, J. Cox, W. Kocha, M. Kuperminc, J. Maroun, D. Walde, C. Weaver, E. Harrison, H. U. Burger, B. Osterwalder, A. O. Wong and R. Wong, *J. Clin. Oncol.*, 2001, **19**, 2282.
[30] C. Twelves, *Eur. J. Cancer*, 2002, **38** (Suppl. 2), 15.
[31] J.-P. Issa, *Curr. Opin. Oncol.*, 2003, **15**, 446.
[32] N. Shimma, I. Umeda, M. Arasaki, C. Murasaki, K. Masubuchi, Y. Kohchi, M. Miwa, M. Ura, N. Sawada and H. Tahara, *Bioorg. Med. Chem.*, 2000, **8**, 1697.
[33] M. Mackean, A. Planting, C. Twelves, J. Schellens, D. Allman, B. Osterwalder, B. Reigner, T. Griffin, S. Kaye and J. Verweij, *J. Clin. Oncol.*, 1998, **16**, 2977.
[34] T. Ishikawa, M. Utoh, N. Sawada, M. Nishida, Y. Fukase, F. Sekiguchi and H. Ishitsuka, *Biochem. Pharmacol.*, 1998, **55**, 1091.
[35] C. H. Baker, J. Banzon, J. M. Bollinger, J. Stubbe, V. Samano, M. J. Robins, B. Lippert, E. Jarvi and R. Resvick, *J. Med. Chem.*, 1991, **34**, 1879.
[36] S. Zimm, J. M. Collins, J. Miser, D. Chatterji and D. G. Poplack, *Clin. Pharmacol. Ther.*, 1984, **35**, 826.
[37] S. Kim, E. Chatelut, J. C. Kim, S. B. Howell, C. Cates, P. A. Kormanik and M. C. Chamberlain, *J. Clin. Oncol.*, 1993, **11**, 2186.
[38] E. C. Taylor, D. Kuhnt, C. Shih, S. M. Rinzel, G. B. Grindey, J. Barredo, M. Jannatipour and R. G. Moran, *J. Med. Chem.*, 1992, **35**, 4450.

[39] C. Shih, V. J. Chen, L. S. Gossett, S. B. Gates, W. C. Mackellar, L. L. Habeck, K. A. Shackelford, L. G. Mendelsohn, D. J. Soose, V. F. Patel, S. L. Andis, J. R. Bewley, E. A. Rayl, B. A. Moroson, G. P. Beardsley, W. Kohler, M. Ratnam and R. M. Schultz, *Cancer Res.*, 1997, **57**, 1116.
[40] J. F. Worzalla, C. Shih and R. M. Schultz, *Anticancer Res.*, 1998, **18**, 3235.
[41] G. V. Scagliotti, D.-M. Shin, H. L. Kindler, M. J. Vasconcelles, U. Keppler, C. Manegold, H. Burris, U. Gatzemeier, J. Blatter, J. T. Symanowski and J. J. Rusthoven, *J. Clin. Oncol.*, 2003, **21**, 1556.
[42] L. Paz-Ares, S. Bezares, J. M. Tabernero, D. Castellanos and H. Cortes-Funes, *Cancer (Supplement)*, 2003, **97**, 2056.
[43] S. P. Ackland and R. Kimbell, Antifolates in combination therapy. in *Antifolate Drugs in Cancer Therapy* (ed. A. L. Jackman), Humana Press, Totowa, NJ, 1999, p. 365.
[44] G. Bonadonna, P. Valagussa, A. Rossi, G. Tancini, C. Brambilla, M. Zambetti and U. Veronesi, *Breast Cancer Res. Treat.*, 1985, **5**, 95.
[45] A. L. Jackman, R. Kimbell and H. E. R. Ford, *Eur. J. Cancer*, 1999, **35**, S3.
[46] L. Schulz, A. Schalhorn, W. Wilmanns and V. Heinemann, *Proc. Am. Soc. Clin. Oncol.*, 1998, *abstract* 965.
[47] V. Heinemann, *Semin. Oncol.*, 2002, **29**, 25.
[48] V. Hess, M. Salzberg, M. Borner, R. Morant, A. D. Roth, C. Ludwig and R. Herrmann, *J. Clin. Oncol.*, 2003, **21**, 66.
[49] W. Scheithauer, B. Schull, H. Ulrich-Pur, K. Schmid, M. Raderer, K. Haider, W. Kwasny, D. Depisch, B. Schneeweiss, F. Lang and G. Kornek, *Ann. Oncol.*, 2003, **14**, 97.
[50] E. Kralidis, S. Aebi, H. Friess, M. W. Buchler and M. M. Borner, *Ann. Oncol.*, 2003, **14**, 574.
[51] A. A. Adjei, C. Erlichman, J. A. Sloan, J. M. Reid, H. C. Pitot, R. M. Goldberg, P. Peethambaram, P. Atherton, L. J. Hanson, S. R. Alberts and J. Jett, *J. Clin. Oncol.*, 2002, **18**, 1748.
[52] B. A. Teicher, V. Chen, C. Shih, K. Menon, P. A. Forler, V. G. Phares and T. Amsrud, *Clin. Cancer Res.*, 2000, **6**, 1016.
[53] K. D. Miller, J. Picus, C. Blanke, W. John, J. Clark, L. N. Shulman, D. Thornton, E. Rowinsky and P. J. S. Loehrer, *Ann. Oncol.*, 2000, **11**, 101.
[54] H. L. Kindler, II, W. Dugan, H. Hochster, D. Strickland, A. Jacobs and W. J. John, *Am. Soc. Clin. Onc.* Orlando, FL, 2002, abstract 499.
[55] J. H. Schiller, D. Harrington, C. P. Belani, C. Langer, A. Sandler, J. Krook, J. Zhu and D. H. Johnson, *N. Engl. J. Med.*, 2002, **346**, 92.
[56] J. J. Rusthoven, E. Eisenhauer, C. Butts, R. Gregg, J. Dancey, B. Fisher and J. Iglesias, *J. Clin. Oncol.*, 1999, **17**, 1194.
[57] D. S. Ettinger, C. Monnerat, K. Kelly, S. Novello, J. R. Brahmer, D. Readett, J. J. Rusthoven, P. A. Bunn and T. Le Chevalier, *Am. Soc. Clin. Onc.*, Orlando, FL, 2002, abstract 1243.

Mitotic Kinesin Inhibitors as Novel Anti-cancer Agents

Kenneth W. Wood and Gustave Bergnes

Cytokinetics, Inc., South San Francisco, CA 94080, USA

Contents

1. INTRODUCTION

Kinesins are microtubule motor proteins that translate energy released by hydrolysis of ATP into mechanical force for directional movement of a wide variety of cellular cargoes along microtubules [1,2]. The hallmark of kinesin family members is the catalytic kinesin motor domain, a relatively compact domain of approximately 350–450 amino acids, depending upon the specific kinesin in question. The motor domains of Kin-N family kinesins mediate motility toward microtubule minus ends. Kin-C motors mediate movement toward microtubule plus ends, and Kin-I family members destabilize microtubule ends [1].

Kinesin-mediated movement along microtubules involves a complex enzymatic cycle that requires coordination of nucleotide hydrolysis and turnover with microtubule interaction, and conformational change resulting in force generation [3]. Small molecule perturbation of any one of these events would be expected to impair kinesin function, highlighting kinesin motor domains as a focus for drug discovery efforts.

Curation of the human genome indicates the existence 45 kinesin family members that may be grouped into approximately 15 phylogenetic subclasses based on primary amino acid sequence [4,5]. Although these proteins are members of a common superfamily, their motor domains share only ~45% identity at the amino acid level (R. Freedman, personal communication). Considering that a substantial portion of this identity resides within conserved core structural elements of the motor domains the solvent-exposed

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surfaces of kinesin family members may be quite different, offering the potential for discovery of inhibitors specific to various kinesins.

Perhaps the largest single functional class of kinesins are those involved in cell division [6]. These kinesins, termed mitotic kinesins, play important roles in assembly and function of the mitotic spindle. The mitotic spindle has proven a fruitful target for the discovery of anti-neoplastic agents. All approved anti-mitotic cancer therapies, including the taxanes and vinca alkaloids, target β-tubulin, thus disrupting the function of microtubules, the major structural element of the mitotic spindle. Microtubule dysfunction results in a failure to form a functional mitotic spindle resulting in cell cycle arrest and subsequent cell death [6]. The effectiveness of anti-microtubule agents in the treatment of various cancers is thought to be a direct consequence of their anti-mitotic actions. However, the action of these drugs on microtubules of non-proliferating cells such as neurons results in substantial toxicities which can be dose limiting [7]. As a target class, kinesins functioning exclusively in mitosis offer the potential for discovery of novel anti-mitotic cancer therapies with novel mechanisms of action and enhanced specificity for proliferating cells.

One mitotic kinesin, known as KSP or *Hs* Eg5, has seen significant attention as a drug target, yielding several classes of inhibitors. At least one of these KSP inhibitors has advanced to clinical trial. The biological roles and characteristics of these mitotic kinesins are summarized below, as are recent progress in identification and advancement of KSP inhibitors.

2. MITOTIC KINESIN BIOLOGY

Mitosis is divided into several phases, each characterized by specific mechanical events (Fig. 1). The integrity of mitosis is monitored and enforced by the mitotic checkpoint. This checkpoint prevents sister chromatid separation and anaphase onset until all chromosomes are attached to spindle microtubules and aligned at the spindle midzone. Mitotic spindle dysfunction in prometaphase results in failure to satisfy this checkpoint, resulting in prolonged cell cycle arrest and eventual cell death (reviewed in Ref. [6]). The consequences of mitotic spindle dysfunction later in mitosis, for example during anaphase, telophase or cytokinesis are not as well characterized as responses to early mitotic dysfunction, but are expected to result in chromosome missegregation and multinucleation. Mitotic kinesins have been shown to play important roles in nearly all aspects of mitotic spindle function.

A table of known and putative mitotic kinesins is presented below (Table 1). Functional characterization in multiple organisms strongly supports mitotic roles for nine of these fourteen kinesins: CENP-E, HSET, Kid, Kif4, KSP, MCAK, MKLP1, MPP1 and RabK6. Functional characterization in human cells is summarized in Table 1. These findings will not be reviewed in detail here, but are covered in the references cited and in several recent reviews [8–12]. Systematic profiling of kinesin family mRNA expression levels in human tissues, and in proliferating and quiescent human fibroblasts revealed a positive correlation of expression levels of these well-characterized mitotic kinesins with proliferative index [13]. These kinesins were also found to be more highly expressed in tumor tissue relative to normal adjacent tissue and

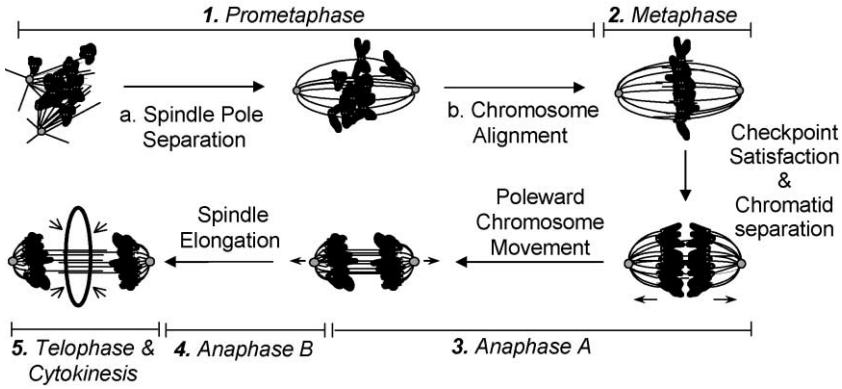


Fig. 1. During prometaphase (1) chromosomes attach to microtubules, spindle poles separate (1a) and chromosomes align at the spindle midzone (1b). Following satisfaction of the mitotic cell cycle checkpoint at metaphase (2), sister chromatids separate and migrate toward their respective spindle poles (3), which then move apart resulting in spindle elongation (4). Contraction of the cytokinetic furrow in the final stage of mitosis divides the parent cell into two daughter cells each retaining one copy of the parental genome (5).

were absent from post-mitotic neurons. Several kinesins of uncharacterized function, including Kif15/HKIp2, KinI3, Kip3a and Kip3d exhibited similar expression profiles and were identified as probable mitotic kinesins.

A recent systematic survey of genes periodically expressed in the cell cycle identified the mitotic kinesins CENP-E, HSET, Kid, KSP, MCAK and MKLP1 as exhibiting increased mRNA levels during mitosis [14]. Two other kinesins, CmKrp (KIAA0042) and MPP1, were found to display similar patterns of expression. Independent efforts have demonstrated that CENP-E and RabK6 exhibit similar profiles [15–17].

2.1. Identification of mitotic kinesin inhibitors

In addition to the catalytically active motor domain, mitotic kinesins also include substantial additional regions responsible for protein–protein interaction, including regions responsible for dimerization, for localization to intracellular cargo and for interaction with upstream regulators. The variety of inhibitory mechanisms that may be identified in small molecule discovery screening efforts may vary greatly depending on the specific protein fragment used and the functional or binding event monitored. Discovery efforts reported to date have been grounded in both biochemical [41,42] and cell-based approaches [43,44]. Biochemical screens have focused on motor domain function. Such screens may identify inhibitors of kinesin motor interaction with nucleotide or microtubule substrates, as well as inhibitors of conformational changes associated with coupling of substrate interactions and motility. Cell-based approaches may identify inhibitors both of motor domain function, and of other critical functions mediated by non-motor regions

Table 1. Mitotic kinesin target validation

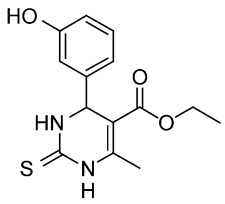
Kinesin name	Alias	Phenotype upon dysfunction or modulation (*mammalian cells*)		References
		Mitotic arrest?	Morphology	
CENP-E	–	Yes	Bipolar prometaphase spindle with misaligned XC[a]	[18–21]
CmKrp	KIAA0042, KIF14	ND	ND	[22]
HKLP2	HsKif15	ND	ND	[23,24]
HSET	KNSL2; HsCHO2, HsKIFC1	No	Unfocussed spindle poles?	[25–27]
Kid	KNSL4, KIF22	No	More diffuse metaphase XC alignment	[26,28]
Kif4	HSA271784; chromokinesin	ND	ND	[29,30]
KinI3	KIF24, AL353662	ND	ND	[31]
Kip3a	HsKif18B	ND	ND	[32]
Kip3d	HsKif18A	ND	ND	[33]
KSP	KNSL1; HsEg5; KIF11	Yes	Monopolar spindle	[27,34] see below
MCAK	KNSL6, KIF2C	Yes	Lagging anaphase chromosomes	[35,36]
MKLP1	KNSL5; HsCHO1; KIF23	Yes	Cytokinesis failure	[37,38]
MPP1	MPHOSPH1, KRMP1; DKFZP434B0435	No	Cytokinesis failure	[39]
RabK6	MKLP2, Kif20A, Rab6KIFL	Yes	Cytokinesis failure	[16,17,40]

[a] XC = Chromosomes, ND = not determined.

of a particular mitotic kinesin. Clearly, compounds identified by either approach must be carefully characterized with respect to specificity of action since, for example, microtubule-active compounds may also alter kinesin biochemical activity but cause cell cycle arrest of living cells as a consequence of their microtubule activity.

Described below are chemical structures reported to specifically inhibit KSP, together with available biological data.

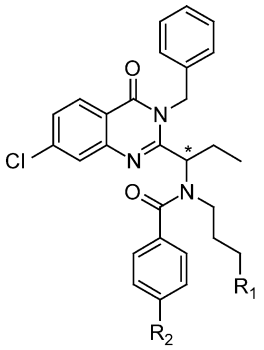
KSP INHIBITORS



1

2.2. Dihydropyrimidines

Using a cell-based screen for compounds producing mitotic arrest, the dihydropyrimidinethione **1**, termed monastrol ($IC_{50} = 14-20\ \mu M$), was identified [43]. Characterization of monastrol revealed it to be an allosteric inhibitor of KSP ATPase function, with the *S*-enantiomer being bound preferentially by the motor [43,45]. Mechanistic analysis revealed that this compound prevents ADP release, the rate-limiting step for KSP, by forming a ternary complex with KSP and ADP. Recently, a 1.9 Å resolution X-ray crystal structure of the ADP/monastrol/KSP motor domain complex was solved revealing the precise binding locus of the monastrol/KSP interaction [46]. The site to which monastrol binds is in a region 12 Å away from the nucleotide binding pocket between helices α1 and α2, making contact with flexible loop L5. As many as 20 residues (Met115, Glu116, Gly117, Glu118, Arg119, Trp127, Asp130, Leu132, Ala133, Gly134, Ile136, Pro137, Leu160, Tyr211, Leu214, Glu215, Gly217, Ala218, Arg221, and Phe239) from various parts of the protein's secondary structure are involved in the binding. Comparison of this ternary complex structure with the previously solved inhibitor-free ADP/KSP binary complex structure [47] shows that monastrol lies in an induced fit pocket not normally present in unbound KSP. Overall, loop L5 moves 7 Å, with its Trp127 side-chain shifting 10 Å to effectively cap the entrance of the pocket. The consequence of this binding mode appears to be a complex set of structural changes that, while not affecting the structure of the nucleotide-binding pocket itself, could prevent the conformational changes required for nucleotide exchange and the generation of mechanical force. Some of the largest conformational changes are observed in the putative microtubule binding region known as switch-2 which is located ~30 Å away. Findings demonstrating monastrol-resistance of KSP motor domain in which loop L5 was substituted with the corresponding region of naturally



	R_1	R_2	Stereo
2:	NMe_2	Br	rac
3:	NH_2	Me	*S*

monastrol-insensitive conventional kinesin heavy chain loop5 support the essential role of L5 in monastrol binding [48].

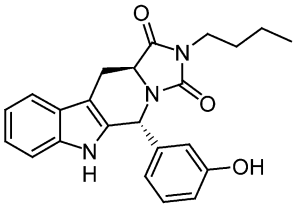
In addition to monastrol, other dihydropyrimidines have also been reported as inhibitors of KSP, supporting the notion that this is a tractable scaffold [49,50].

2.3. Quinazolinones and related compounds

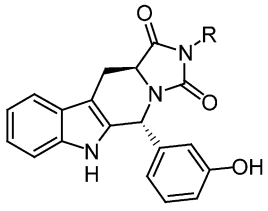
Another series of inhibitors of KSP catalytic function are quinazolinone-containing compounds such as CK0106023, **2**, which has a K_i of 12 nM and produces 50% growth inhibition (GI_{50}) of a panel of 11 human tumor cell lines of at concentrations ranging from 126 to 582 nM (mean = 362 nM) [42] Like monastrol, these compounds are reported to function via an allosteric mechanism, also stabilizing the ADP-bound form of the enzyme. Analog **3** has been reported to have a biochemical IC_{50} below 1 nM and a cellular IC_{50} below 10 nM [51]. Synthetic methods for accessing these structures in stereo-controlled fashion have been reported [52,53].

One member of this series, SB-715992 (CK0238273) has been reported as the subject of two phase I clinical trials in patients with refractory solid tumors. Preclinical studies suggested that intermittent dosing was most effective [54,55]. The two reported ongoing clinical trials explored pharmacokinetics and safety of escalating doses on two dosing schedules: once weekly × 3 every 28 days (days 1, 8 and 15) [56], and once every 21 days [57]. Drug related toxicities were described as primarily hematologic and gastrointestinal, consistent with unpublished preclinical safety studies. Three patients had experienced stable disease for six or more cycles.

Compounds related to this series have been reported in which the quinazolinone core has been replaced with various carbocycle- and heterocycle-fused pyrimidinones, substituted monocyclic pyrimidinone, substituted chromenone, substituted pyrrolotriazinone, substituted isoquinolin-1-one, and pyridopyrimidinone, suggesting a degree of tolerance for core modification within this structural class [58–65]. In addition, replacement of the benzamide portion with heterocycles such as piperazines, diazepines, imidazoles and imidazolines has also been reported [66,67].

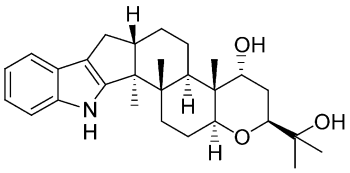


HR22C16 (4)



	IC_{50} (nM)
5: R = $(CH_2)_2NH_2$	90
6: R = $CHCH_2CO_2H$	300
7: R = $CH_2CH_2CH_3$	400
4: R = $CH_2CH_2CH_2CH_3$	800

2.4. Tetrahydro-β-carbolines

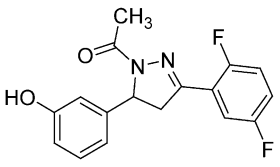


Terpendole E (**8**)

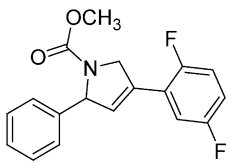
Using a cell-based screen for anti-mitotic compounds, HR22C16 (**4**), was identified as a potent ($IC_{50} = 800$ nM) inhibitor of KSP from among a collection of 16,000 synthetic samples. Like the structures above, it caused monopolar spindles in dividing cells without affecting microtubules [44,68]. Also termed monastroline, HR22C16 served as the progenitor for an optimization effort that has led to more potent analogs such as **5**, which inhibits KSP movement on microtubules with an IC_{50} of 90 nM. In order to assert a degree of temporal control over the effects of HR22C16 in a cellular assay, the pendant phenol was 'caged' using an *o*-nitrobenzyl protecting group that when irradiated with a He–Cd laser for 21 s, converted inactive, caged HR22C16 into its active component in a tissue culture environment, causing monoastral figures only after photolysis. Using this strategy, it is predicted that both temporal and spatial control over inhibition of cell division in a living organism can be accomplished. Ongoing investigations involve application of 'caged' HR22C16 toward a better understanding of time dependence of inhibitor release during cell division.

2.5. Terpendole E

In a screen for cell cycle inhibitors, over 4000 fungal extracts were tested against mouse temperature-sensitive cell line tsFT210 leading to the identification of a complex mixture consisting of terpendoles C, E, H and I that blocked M phase progression [69]. Among them, only terpendole E (TerE), **8**, was found to cause M phase accumulation at a concentration of 50 μM, generating monopolar spindles without affecting interphase microtubules or tubulin polymerization *in vitro*. It is interesting to note that terpendoles have previously been reported to be inhibitors of acyl-CoA:cholesterol *O*-acyltransferase (ACAT); however, **8** was found to be a selective inhibitor of KSP with an IC_{50} of 14.6 μM in a microtubule sliding assay and an IC_{50} of 23.0 μM in an ATP hydrolysis assay. Though its precise biochemical mechanism of action has not been elucidated, **8** inhibits both the microtubule-catalyzed and basal ATPase rates, supporting a direct interaction with KSP. TerE also represents the only known example of a selective, natural product inhibitor of KSP.



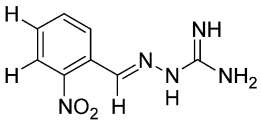
9



10

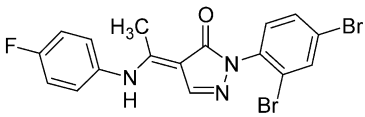
2.6. Dyhydropyrazoles and Dihydropyrroles

Recently, diaryl azaheterocycles such as dihydropyrazoles and dihydropyrroles have been reported to inhibit KSP [70–72]. Both are suggested to bind at the same allosteric binding site to which monastrol binds and may find use as competitive ligands for screens looking to identify compounds that also bind at this site [73]. Examples **9** and **10** are both reported to inhibit KSP at concentrations below 50 μM.



Monastrol Suppressor

11



Monastrol Enhancer

12

2.7. Suppressors and enhancers of KSP inhibitor action

In addition to inhibitors of KSP, a cell-based screen of 20,000 synthetic samples led to the identification of compounds that either enhance or suppress monastrol-induced mitotic arrest while having no such effect when dosed alone [74]. Treatment of synchronized BSC1 cells pre-treated with monastrol led to the identification of compounds such as amidinohydrazide **11**, which was found to promote escape from monastrol-induced mitotic block at concentrations below 50 μM. In all, 32 suppressors were found of which 15 had activity below 50 μM. Conversely, pyrazolone **12** was notable for its ability to extend monastrol's effect on these cells at 25 μM without affecting KSP ATPase activity at functional concentrations. A total of 12 enhancers were identified with potencies below 50 μM with **12** being the most potent. The targets of these compounds were not discerned.

3. CONCLUSIONS

Efforts toward the discovery of novel anti-tumor therapeutic agents over the last decade have increasingly focused on cell cycle targets, with anti-mitotic strategies providing the greatest impact. The appearance of phase I and II clinical candidates against the mitotic kinesin KSP has only served to spark interest in this novel family of proteins, evidenced by increased numbers of publications within the chemistry and biology literature within the last year. As discussed in this review, a variety of structures have now been reported as inhibitors of one mitotic kinesin, KSP. Multiple other mitotic kinesins remain to be explored. As the biology surrounding these remarkable proteins is discerned it will become feasible to test the hypothesis that inhibition of specific mitotic kinesins will be an effective mode for the treatment of human cancer.

REFERENCES

[1] L. S. Goldstein and A. V. Philp, *Ann. Rev. Cell Dev. Biol.*, 1999, **15**, 141.
[2] R. D. Vale, *Cell*, 2003, **112**, 467.
[3] R. A. Cross, I. Crevel, N. J. Carter, M. C. Alonso, K. Hirose and L. A. Amos, *Phil. Trans. R. Soc. Lond. B Biol. Sci.*, 2000, **355**, 459.
[4] H. Miki, M. Setou, K. Kaneshiro and N. Hirokawa, *Proc. Natl Acad. Sci. USA*, 2001, **98**, 7004.
[5] E. M. Dagenbach and S. A. Endow, *J. Cell Sci.*, 2004, **117**, 3.
[6] K. W. Wood, W. D. Cornwell and J. R. Jackson, *Curr. Opin. Pharmacol.*, 2001, **1**, 370.
[7] E. K. Rowinsky, E. A. Eisenhauer, V. Chaudhry, S. G. Arbuck and R. C. Donehower, *Semin. Oncol.*, 1993, **20**, 1.
[8] D. T. Miyamoto, Z. E. Perlman, T. J. Mitchison and M. Shirasu-Hiza, *Prog. Cell Cycle Res.*, 2003, **5**, 349.
[9] T. M. Kapoor and D. A. Compton, *J. Cell Biol.*, 2002, **157**, 551.
[10] R. Heald, *Cell*, 2000, **102**, 399.
[11] D. J. Sharp, G. C. Rogers and J. M. Scholey, *Nature*, 2000, **407**, 41.
[12] M. Glotzer, *Ann. Rev. Cell Dev. Biol.*, 2001, **17**, 351.
[13] J. Mak, R. Freedman and C. Beraud, *Proc. Am. Assoc. Cancer Res.*, 2002, **43**, Abstr. 5375.
[14] M. L. Whitfield, G. Sherlock, A. J. Saldanha, J. I. Murray, C. A. Ball, K. E. Alexander, J. C. Matese, C. M. Perou, M. M. Hurt, P. O. Brown and D. Botstein, *Mol. Biol. Cell*, 2002, **13**, 1977.
[15] K. D. Brown, R. M. Coulson, T. J. Yen and D. W. Cleveland, *J. Cell Biol.*, 1994, **125**, 1303.
[16] E. Hill, M. Clarke and F. A. Barr, *EMBO J.*, 2000, **19**, 5711.
[17] R. D. Fontijn, B. Goud, A. Echard, F. Jollivet, J. van Marle, H. Pannekoek and A. J. Horrevoets, *Mol. Cell. Biol.*, 2001, **21**, 2944.
[18] X. Yao, K. L. Anderson and D. W. Cleveland, *J. Cell Biol.*, 1997, **139**, 435.
[19] B. T. Schaar, G. K. Chan, P. Maddox, E. D. Salmon and T. J. Yen, *J. Cell Biol.*, 1997, **139**, 1373.
[20] F. R. Putkey, T. Cramer, M. K. Morphew, A. D. Silk, R. S. Johnson, J. R. McIntosh and D. W. Cleveland, *Dev. Cell*, 2002, **3**, 351.
[21] B. A. Weaver, Z. Q. Bonday, F. R. Putkey, G. J. Kops, A. D. Silk and D. W. Cleveland, *J. Cell Biol.*, 2003, **162**, 551.
[22] N. Nomura, T. Nagase, N. Miyajima, T. Sazuka, A. Tanaka, S. Sato, N. Seki, Y. Kawarabayasi, K. Ishikawa and S. Tabata, *DNA Res.*, 1994, **1**, 223.
[23] M. Sueishi, M. Takagi and Y. Yoneda, *J. Biol. Chem.*, 2000, **275**, 28888.
[24] H. J. Heidebrecht, S. Adam-Klages, M. Szczepanowski, M. Pollmann, F. Buck, E. Endl, M. L. Kruse, P. Rudolph and R. Parwaresch, *Mol. Cancer Res.*, 2003, **1**, 271.
[25] M. B. Gordon, L. Howard and D. A. Compton, *J. Cell Biol.*, 2001, **152**, 425.
[26] A. A. Levesque, L. Howard, M. B. Gordon and D. A. Compton, *Mol. Biol. Cell*, 2003, **14**, 3541.
[27] V. Mountain, C. Simerly, L. Howard, A. Ando, G. Schatten and D. A. Compton, *J. Cell Biol.*, 1999, **147**, 351.
[28] A. A. Levesque and D. A. Compton, *J. Cell Biol.*, 2001, **154**, 1135.
[29] Y. M. Lee, S. Lee, E. Lee, H. Shin, H. Hahn, W. Choi and W. Kim, *Biochem. J.*, 2001, **360**, 549.
[30] Y. M. Lee and W. Kim, *Biochem. J.*, 2003, **374**, 497.
[31] C. Beraud, J. Guo, R. Freedman, U. Patel and K. Davies, *US Patent* 6,514,738, 2003.
[32] C. Beraud, A. Craven, M. Yu, R. Sakowicz, U. A. Patel and K. A. Davies, *US Patent* 6,680, 369, 2001.
[33] C. Beraud and R. Freedman, *PCT Int. Appl.* WO 02/12268, 2002.
[34] A. Blangy, H. A. Lane, P. d'Herin, M. Harper, M. Kress and E. A. Nigg, *Cell*, 1995, **83**, 1159.
[35] S. L. Kline-Smith, A. Khodjakov, P. Hergert and C. E. Walczak, *Mol. Biol. Cell*, 2004.
[36] T. Maney, A. W. Hunter, M. Wagenbach and L. Wordeman, *J. Cell Biol.*, 1998, **142**, 787.
[37] R. Saint and W. G. Somers, *J. Cell Sci.*, 2003, **116**, 4277.
[38] J. Matuliene and R. Kuriyama, *Mol. Biol. Cell*, 2002, **13**, 1832.

[39] A. Abaza, J. M. Soleilhac, J. Westendorf, M. Piel, I. Crevel, A. Roux and F. Pirollet, *J. Biol. Chem.*, 2003, **278**, 27844.
[40] R. Neef, C. Preisinger, J. Sutcliffe, R. Kopajtich, E. A. Nigg, T. U. Mayer and F. A. Barr, *J. Cell Biol.*, 2003, **162**, 863.
[41] G. Bergnes, E. Ha, B. Feng, B. Yao, W. W. Smith, T. Tochimoto, E. R. Lewis, Y. Y. Lee, R. Moody, R. A. Turincio, J. T. Finer, K. W. Wood, R. Sakowics, A. M. Crompton, J. C. Chabala, D. J. Morgans, N. H. Sigal and J. Sabry, *Proc. Am. Assoc. Cancer Res.,* San Francisco, CA, 2002, **43**, Abstr. 3648.
[42] R. Sakowicz, J. T. Finer, C. Beraud, A. Crompton, E. Lewis, A. Fritsch, Y. Lee, J. Mak, R. Moody, R. Turincio, J. C. Chabala, P. Gonzales, S. Roth, S. Weitman and K. W. Wood, *Cancer Res.*, 2004, 3276.
[43] T. U. Mayer, T. M. Kapoor, S. J. Haggarty, R. W. King, S. L. Schreiber and T. J. Mitchison, *Science*, 1999, **286**, 971.
[44] S. Hotha, J. C. Yarrow, J. G. Yang, S. Garrett, K. V. Renduchintala, T. U. Mayer and T. M. Kapoor, *Angew. Chem. Int. Ed. Engl.*, 2003, **42**, 2379.
[45] Z. Maliga, T. M. Kapoor and T. J. Mitchison, *Chem. Biol.*, 2002, **9**, 989.
[46] Y. Yan, V. Sardana, B. Xu, C. Homnick, W. Halczenko, C. A. Buser, M. Schaber, G. D. Hartman, H. E. Huber and L. C. Kuo, *J. Mol. Biol.*, 2004, **335**, 547.
[47] J. Turner, R. Anderson, J. Guo, C. Beraud, R. Fletterick and R. Sakowicz, *J. Biol. Chem.*, 2001, **276**, 25496.
[48] Z. Maliga and T. J. Mitchison, 43rd Annu. Meet. Am. Soc. Cell Biol., 2003, Abstr. L103.
[49] L. J. Lombardo and L. I. Wu, *PCT Int. Appl.* WO 02/079169-A1, 2002.
[50] S. D. Kimball, L. J. Lombardo, D. B. Rawlins, H.-Y. Xiao, J. R. Schmidt and D. K. Williams, *PCT Int. Appl.* WO 02/079149-A2, 2002.
[51] G. Bergnes, E. Ha, B. Feng, W. W. Smith, B. Yao, T. Tochimoto, E. R. Lewis, Y. Lee, R. S. Moody, J. T. Finer, R. A. Turincio, K. W. Wood, R. Sakowicz, A. M. Crompton, J. C. Chabala, D. J. Morgans, N. H. Sigal and J. H. Sabry, 223rd American Chemical Society National Meeting, Orlando, FL, 2002.
[52] G. Bergnes, E. Ha, G. Yiannikourous, P. Kalaritis, B. E. Yonce and K. A. Welday, *PCT Int. Appl.* WO 03/070701-A2, 2003.
[53] P. Coleman, G. D. Hartman and L. A. Neilson, *PCT Int. Appl.* WO 03/099211-A2, 2003.
[54] P. Gonzales, A. Bienek, M. Boehme, C. Rivali, S. Roth, H. Wynne, G. Piazza, S. Weitman, K. Wood, A. Crompton, R. S. Moody, S. Schauer, B. Feng, G. Bergnes, N. H. Sigal and D. Morgans, *Proc. Am. Assoc. Cancer Res.*, 2002, **43**, Abstr. 1337.
[55] R. K. Johnson, F. L. McCabe, E. Cauder, L. Innlow, M. Whitacre, J. D. Winkler, G. Bergnes, B. Feng, W. Smith, D. Morgans, K. Wood and J. R. Jackson, *Proc. Am. Assoc. Cancer Res.,* San Francisco, CA, 2002, 43, Abstr. 1335.
[56] H. Burris, P. LoRusso, S. Jones, J. McCormick, N. Willcutt, J. Hodge, P. Bush, L. Pandite, J. Sabry and P. Ho, *Eur. J. Cancer Suppl.*, 2003, **1**, S172.
[57] Q. Chu, K. D. Holen, E. K. Rowinsky, D. B. Alberti, P. Montroe, J. P. Volkman, J. P. Hodge, J. Sabry, P. T. C. Ho and G. Wilding, *Proc. Am. Assoc. Clin. Oncol.*, 2003, **22**, Abstr. 525.
[58] M. E. Fraley and G. D. Hartman, *PCT Int. Appl.* WO 03/050122-A2, 2003.
[59] M. E. Fraley, G. D. Hartman and W. F. Hoffman, *PCT Int. Appl.* WO 03/050064-A2, 2003.
[60] M. E. Fraley and R. M. Garbaccio, *PCT Int. Appl.* WO 03/049678-A2, 2003.
[61] D. J. Morgans, S. D. Knight, K. A. Newlander, D. Dhanak, H.-J. Zhou and N. D. Adams, *PCT Int. Appl.* WO 03/094839-A2, 2003.
[62] A. McDonald, G. Bergnes, B. Feng, D. J. C. Morgans, S. D. Knight, K. A. Newlander, D. Dhanak and C. S. Brook, *PCT Int. Appl.* WO 03/088903-A2, 2003.
[63] L. J. Lombardo, R. S. Bhide, K. S. Kim and S. Lu, *PCT Int. Appl.* WO 03/0999286-A1, 2003.
[64] A. McDonald, P.-P. Lu, G. Bergnes, D. Morgans, D. Dhanak and S. D. Knight, *PCT Int. Appl.* WO 03/106426-A1, 2003.
[65] D. Dhanak, S. D. Knight, P.-P. Lu, D. J. Morgans and B. Yao, *PCT Int. Appl.* WO 03/103575-A2, 2003.
[66] M. E. Fraley and W. F. Hoffman, *PCT Int. Appl.* WO 03/039460-A2, 2003.

[67] B. Feng, G. Bergnes, D. J. C. Morgans, D. Dhanak, S. D. Knight and M. G. Darcy, *PCT Int. Appl.* WO 03/097053-A1, 2003.
[68] S. Hotha, J. G. Yang, S. Garrett, K. V. Renduchintala and T. M. Kapoor, 42nd Annu. Meet. Am. Soc. Cell Biol., San Francisco, CA, 2002, Abstr. L73.
[69] J. Nakazawa, J. Yajima, T. Usui, M. Ueki, A. Takatsuki, M. Imoto, Y. Y. Toyoshima and H. Osada, *Chem. Biol.*, 2003, **10**, 131.
[70] K. L. Arrington and M. E. Fraley, *PCT Int. Appl.* WO 03/106417-A1, 2003.
[71] K. L. Arrington, P. J. Coleman, C. D. Cox, M. E. Fraley, R. M. Garbaccio, G. D. Hartman, W. F. Hoffman and E. S. Tasber, *PCT Int. Appl.* WO 03/105855-A1, 2003.
[72] M. J. Breslin, P. J. Coleman, C. D. Cox, J. C. Culberson, G. D. Hartman, B. J. Mariano and M. Torrent, *PCT Int. Appl.* WO 03/079973-A2, 2003.
[73] C. A. Buser-Doepner, P. J. Coleman, C. D. Cox, M. E. Fraley, R. M. Bargaccio, G. D. Hartman, D. C. Heimbrook, L. C. Kuo, H. E. Huber, V. V. Sardana, M. Torrent and Y. Yan, *PCT Int. Appl.* WO 04/004652-A2, 2004.
[74] Z. E. Perlman, T. J. Mitchison and T. U. Mayer, *43rd Annu. Meet. Am. Soc. Cell Biol.*, 2002, Abstr. L114.

Recent Advances in Medicinal Chemistry of Histone Deacetylase Inhibitors

Hilmar Weinmann and Eckhard Ottow

Schering AG, Medicinal Chemistry, D-13342 Berlin, Germany

Contents

1. INTRODUCTION

Over the last decade, histone deacetylase inhibitors have become the subject of considerable attention due to a remarkable increase in understanding of the role that acetylation of histone and non-histone proteins plays in the regulation of normal and cancer cell behavior. Histone deacetylases (HDACs) represent a family of enzymes that compete with histone acetyltransferases (HATs) to modulate chromatin structure and to regulate gene transcriptional activity by changing the acetylation status of lysines of nucleosomal histones. Several forms of cancer are characterized by an altered expression or mutation of genes that encode HATs or HDACs. Similarly, aberrant repression of genes mediated by HDACs, is associated with the pathogenesis of various types of solid tumors and haematological malignancies. HDAC inhibitors induce histone hyperacetylation, reactivate suppressed genes and consequently, inhibit the cell cycle, activate differentiation programmes or induce apoptosis and several HDAC inhibitors of various structural families have now advanced into phase I and II clinical trials. Comprehensive reviews on histone deacetylase as a new target for cancer chemotherapy and the current status of HDAC inhibitors in clinical development have appeared [1–4].

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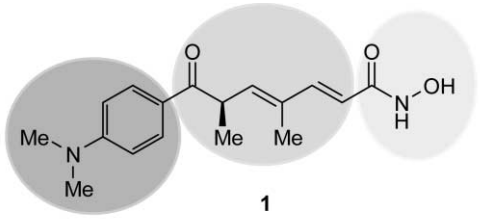
1.1. Histone deacetylases and structural studies

Mammalian HDACs can be divided into three distinct classes [5]. Class I deacetylases, HDACs 1, 2, 3 and 8, share homology in their catalytic sites with molecular weights of 42–55 kDa. Class II deacetylases includes HDACs 4, 5, 6, 7, 9 and 10. They have molecular weights between 120–130 kDa. HDACs 4, 5, 7 and 9 share homology in two regions, the C-terminal catalytic domain and a N-terminal regulatory domain [6]. Recently, HDAC 11 was cloned and characterized [7]. It contains conserved residues in the catalytic core regions shared by both Class I and Class II mammalian enzymes. HDAC 6 and 10 have two regions of homology with the Class II catalytic site. The third class of deacetylases is the conserved Sir2 family of proteins which are dependent on NAD^+ for activity whereas Class I and II HDACs operate by zinc-dependent mechanisms.

A structural rationale for HDAC inhibition is suggested from the X-ray crystal structure of Trichostatin A – bound HDAC-like protein (HDLP), a homologue of Class I/II HDAC with ca. 35% sequence identity [8]. In this structure, the hydroxamic acid of Trichostatin A (TSA), **1**, penetrates a narrow, hydrophobic channel and chelates a buried zinc ion. Despite the variety of their structural characteristics most HDAC inhibitors can be rationalized in the light of the HDLP structure. Inhibitors typically possess a metal-binding moiety and a surface recognition part which interacts with amino acids at the entrance of the *N*-acetyl lysine binding channel. These two parts of the molecule are connected by a linker which is often a 5–6 hydrocarbon chain.



1

Surface recognition **Linker** **Metal binding**

2. HDAC INHIBITORS AS ANTICANCER AGENTS

HDAC inhibitors bear great potential as new drugs because of their ability to modulate transcription, and are endowed with cytodifferentiating, antiproliferative and apoptogenic properties [9]. Furthermore the anticancer activity of HDAC inhibitors may be mediated in part by the inhibition of angiogenesis, since it was shown recently that TSA, **1**, specifically inhibited hypoxia-induced angiogenesis by reducing the expression of genes required for angiogenesis [10]. Several structural classes of compounds have been described as HDAC inhibitors [11–14]. The most important of these compound classes are short-chain fatty acids, hydroxamic acids, benzamides, and cyclic tetrapeptides.

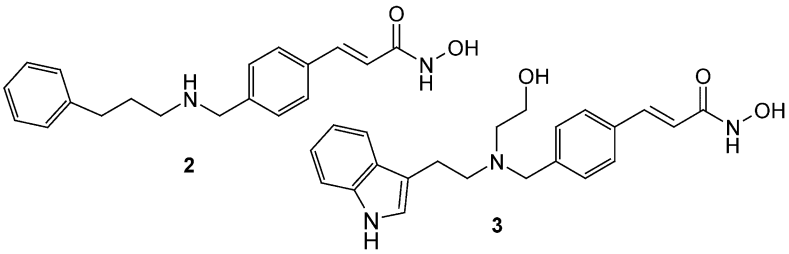
2.1. Short-chain fatty acids

This compound class with butyrate, phenyl butyrate, or valproic acid as most important examples exhibits the least potency, with IC_{50} in the millimolar range [15–17]. In a recent structural optimization study valproate, butyrate, phenylacetate, and phenylbutyrate

were coupled with Zn^{2+} chelating motifs (hydroxamic acid and *o*-phenylenediamine) through aromatic amino acid linkers [18]. This strategy has led to a novel class of short-chain fatty acid derivatives that exhibited varying degrees of HDAC inhibitory potency with the best compounds in the nanomolar range.

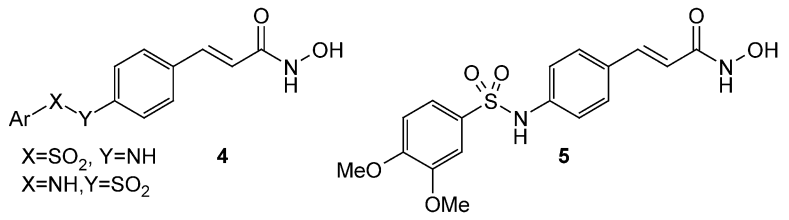
2.2. Hydroxamic acids

Hydroxamic acids constitute the largest class of HDAC inhibitors with novel compounds being added rapidly. Solution- and solid-phase synthesis methods as well as potential therapeutic applications of hydroxamic acids [19,20] have been reviewed recently. A fully automated multi-step solution-phase synthesis using polymer supported reagents for the preparation of hydroxamic acid HDAC inhibitors has been described which might have the potential to accelerate the output of medicinal and combinatorial chemistry groups in this field even further [21]. The established hydroxamic acid HDAC inhibitors TSA (**1**), Suberoyl anilide hydroxamic acid (SAHA), CBHA, Pyroxamide, Oxamflatin and Scriptaid which are partially undergoing clinical trials were extensively reviewed elsewhere [11–14] and therefore will not be discussed here.

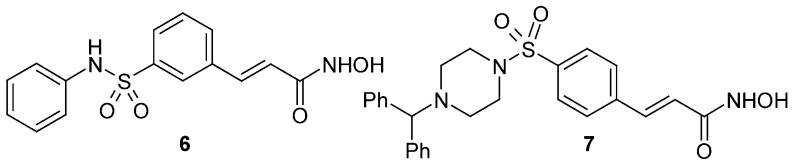


The cinnamyl hydroxamic acid NVP-LAK974 (**2**) was found as a high-throughput screening hit. This compound had good enzyme and cellular potency, but poor efficacy *in vivo*. A systematic structural exploration of cinnamyl hydroxamates based on NVP-LAK974 was undertaken with the goal of finding a novel, well-tolerated and efficacious HDAC inhibitor [22,23]. NVP-LAQ824 (**3**) showed activity both *in vitro* (HDAC $IC_{50} = 32$ nM, HCT116 $GI_{50} = 10$ nM) and *in vivo* and is currently undergoing phase I clinical trials against both solid tumors and leukemia [24].

A new set of sulfonamide hydroxamic acids (**4**) and anilides have been synthesized [25,26]. One of the best compounds was **5** which had an IC_{50} in the HDAC1 *in vitro* assay of 90 nM and showed 57% tumor growth inhibition against A549 tumors. The reversal of the sulfonamide functionality resulted in similar activity.

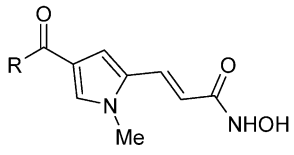


PXD101 (**6**) is a new hydroxamate-type HDAC inhibitor that inhibits histone deacetylase activity with an IC_{50} of 27 nM [27]. It was found that replacing the cinnamic acid moiety with an alkyl chain leads to a reduction in activity and substitution of the sulfonamide nitrogen also reduces activity. In a further elaboration of this series piperazine sulfonamide derivatives (e.g. **7**) have been prepared [28]. Most of these compounds inhibit HDAC with $IC_{50} = 20-200$ nM.



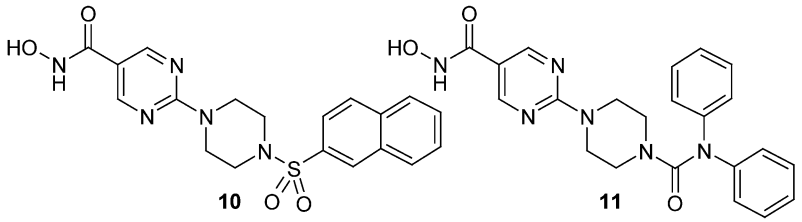
Novel 3-(4-substituted-phenyl)-*N*-hydroxy-2-propenamides have been prepared recently [29,30]. Incorporation of a 1,4-phenylene carboxamide linker and a 4-(dimethylamino)phenyl or 4-(1-pyrrolidinyl)phenyl group as a cap substructure generated highly potent hydroxamic acid-based HDAC inhibitors.

Aroyl-pyrrole-hydroxy-amides (APHA's) are another important class of α,β-unsaturated hydroxamic acid inhibitors in which the benzene ring was replaced by a pyrrole ring [31]. Pyrrole N-substitution with groups larger than methyl gave a reduction in HDAC inhibiting activity, and replacement of hydroxamate function with various non-hydroxamate, metal ion-complexing groups yielded poorly active or totally inactive compounds. On the contrary, proper substitution at the pyrrole C(4)-position favorably affected enzyme inhibiting potency, leading to **9** which was 38-fold more potent than **8** in *in vitro* anti-HD2 assay. Such enhancement of inhibitory activity can be explained by the higher flexibility of the pyrrole C(4)-substituent of **9** which accounts for a considerably better fit into the HDAC1 pocket compared to **8**. The enhanced fit allows a closer positioning of the hydroxamate moiety to the zinc ion. These findings were supported by extensive docking studies performed on both APHAs and reference drugs (TSA and SAHA) [32].



8: R = -Ph
9: R = - CH_2-Ph

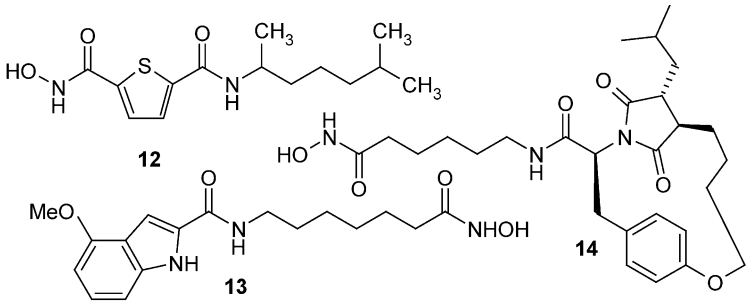
A new class of pyrimidine derived hydroxamic acid HDAC inhibitors was disclosed recently [33–36]. JNJ-16241199 (**10**) was found to be a potent ($IC_{50} = 6$ nM) and orally bioavailable HDAC inhibitor. Orally administered JNJ-16241199 was associated with strong inhibition of the growth of ovarian A2780, human non-small cell lung carcinoma NCI-H460 and human colon carcinoma HCT 116 xenografts in immunodeficient mice. Urea derivatives (e.g. **11**) of these pyrimidine hydroxamic acids also showed potency in the HDAC inhibitor assay as well as compounds in which the sulfonamide group in **10** was replaced by an amide or an alkyl or aryl moiety [37–39].



10 **11**

A series of novel aromatic dicarboxylic acid derivatives was prepared and tested as HDAC inhibitors [40]. The *in vitro* cytotoxic activity of these compounds against HT29 human colon carcinoma cells was determined (**12**, $IC_{50} = 0.03$ μM).

The synthesis of a series of heterocyclic-amide hydroxamic acids demonstrated the highly potent HDAC inhibitory activity of indole-amides [41]. Compounds with a 2-indole amide moiety were found to be the most active inhibitors among the different regioisomers. Introduction of substituents on the indole ring further improved the potency and generated a series of low nanomolar HDAC inhibitors (e.g. **13**, $IC_{50} = 3.1$ nM) with significant antiproliferative activity (HT1080 $IC_{50} = 120$ nM).



12 **13** **14**

A series of succinimide hydroxamic acids which contain a macrocyclic surface recognition domain was prepared [42]. The best compound **14** had an HDAC IC_{50} of 38 nM and in the antiproliferation assay using HT1080 fibrosarcoma cells an IC_{50} of 250 nM was measured. The number, identity and disposition of macrocycle substituents appears to be critical for activity. The removal of the succinimide substituents or the phenylalanine side chain depresses activity. Replacement of the succinimide with a lactam or phthalimide also led to reduced activity. The length of the linker domain alkyl group was also found to be critical, with the five-methylene analogue showing maximal activity. The pharmacokinetic and *in vivo* tumor study data for **14** were rather disappointing. In mouse or monkey, **14** had a very short half-life (ca. 20 min) and little or no measurable exposure after oral or intraperitoneal dosing due to rapid hydrolysis to the inactive carboxylic acid [43]. In the HT1080 mouse model **14** had only marginal anti-tumor activity.

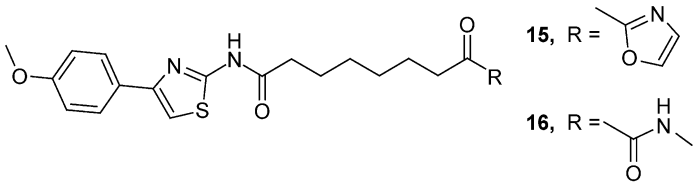
2.3. Non-hydroxamate HDAC inhibitors

Although hydroxamic acids are frequently employed as zinc-binding groups, they often present metabolic and pharmacokinetic problems such as glucuronidation and sulfation that result in a short *in vivo* half-life. Many hydroxamates are unstable *in vivo*, and are

prone to hydrolysis giving hydroxylamine which has potential mutagenic properties. Because of such concerns with the metabolic stability and the toxicity associated with hydroxamic acids, it has become increasingly desirable to find replacement groups with strong HDAC inhibitory potency.

Trifluoromethyl ketones were found to be active as HDAC inhibitors [44]. It is likely that for these and other electrophilic ketones the hydrated form of the ketone acts in a similar way as a transition state analogue and coordinates the zinc-ion in the active site. Optimization of this series led to the identification of submicromolar inhibitors that demonstrated antiproliferative effects.

In another series various heterocyclic ketones were tested for their HDAC inhibitory potency [45]. α-Keto oxazoles were found to be submicromolar inhibitors. One of the most potent compounds was **15** with HDAC $IC_{50} = 30$ nM and anti-proliferative activity in MDA 435 cells ($IC_{50} = 2.3$ μmol). However, both types of electrophilic ketones exhibited short half-lives *in vivo* and *in vitro* due to rapid reduction to the corresponding inactive alcohols. Recently α-keto acids, α-keto esters and α-keto amides were studied [46,47].Compound **16** is an HDAC inhibitor ($IC_{50} = 9$ nM) that shows antiproliferative activity *in vitro*, as well as significant efficacy in an HT1080 mouse tumor model. However, the α-keto amide **16** was also rapidly metabolized to the inactive α-hydroxy amide. Overall exposure was low with only transient concentrations of inhibitor above the cellular proliferation IC_{50} value. Despite the poor pharmacokinetics of the α-keto amides, they exhibit significant anti-tumor effects in xenograft models, suggesting that transient exposure to HDAC inhibitors may be sufficient for anti-tumor effects.

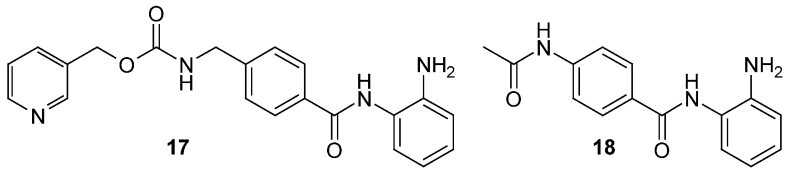


In another attempt to substitute the hydroxamic acid moiety three analogs of suberoyl anilide hydroxamic acid (SAHA) with phosphorus metal-chelating functionalities were synthesized as inhibitors of histone deacetylases [48]. The compounds showed only weak HDAC inhibitory potency in the millimolar range, suggesting that the transition state of HDAC is not analogous to zinc proteases. Also derived from SAHA HDAC inhibitors with a *N*-formyl hydroxylamine head group have been prepared [49]. Replacing the hydroxamic acid group by this moiety led to a fifty-fold drop in potency. This loss in activity could be offset by increasing the size of the hydrophobic region to afford HDAC inhibitors with low micromolar activity in cellular histone hyperacetylation assay. Further studies will be necessary to determine whether these compounds offer enhanced pharmacological properties relative to SAHA.

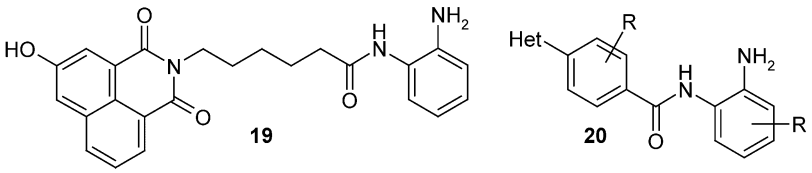
2.4. Benzamides

The benzamide class of HDAC inhibitors, which is generally less potent than the corresponding hydroxamic acid and tetrapeptide classes, includes MS-275, **17**, [50] and

CI-994, **18**, [11]. The SAR of MS-275 revealed that a 2′-amino or 2′-hydroxyl moiety is critical for inhibitory activity.

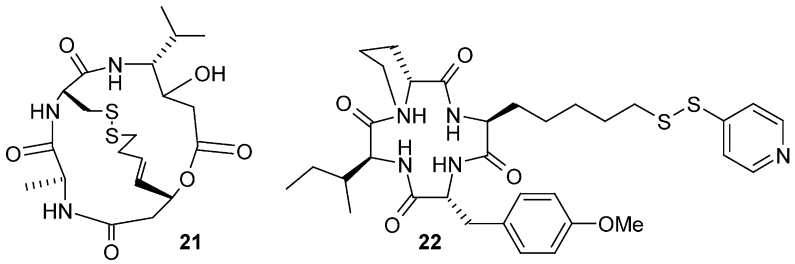


Both compounds are currently under clinical evaluation. A variety of ω-substituted alkanoic acid (2-amino-phenyl)-amides were shown to inhibit recombinant human HDACs with IC_{50} values in the low micromolar range [51]. Compounds in this class (e.g. **19**) showed efficacy in human tumor xenograft models. However the activity was lower than that of MS-275. A heterocyclic series of benzamides (**20**) derived from *o*-phenylene diamine has been reported recently and found to be active in the *in vitro* HDAC assay in the micromolar range [52].



2.5. Cyclic peptides

Compounds containing cyclic peptide structures constitute the most structurally complex class of HDAC inhibitors. Well-known examples are the natural product FK-288 which is currently undergoing clinical evaluation, CHAP31 and Trapoxin A and B and derivatives [11–14].

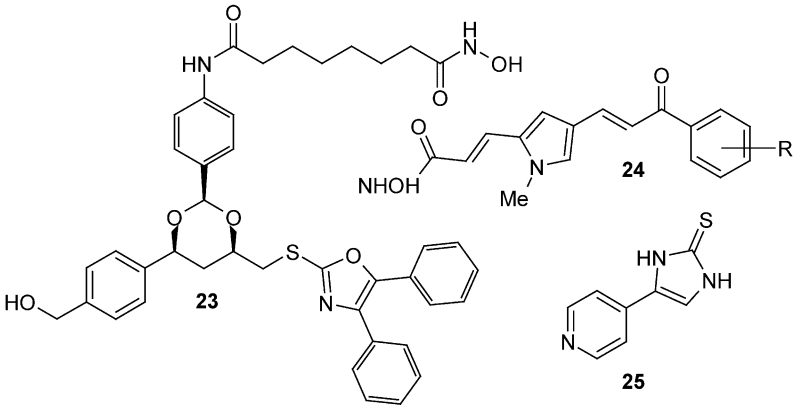


Recently the total synthesis of Spiruchostatin A (**21**) was accomplished, unambiguously confirming its structure [53]. Spiruchostatin A is shown to have biological activity similar to that of FK228.The Spiruchostatin A analog, epimeric at the β-hydroxy acid, is inactive, highlighting the importance of stereochemistry at this position for interactions with HDACs. New inhibitors of histone deacetylase containing a sulfhydryl group (e.g. **22**) were designed based on the CHAP31 skeleton and the HDAC binding functional group of FK228 [54]. HDAC inhibitor potency was in the nanomolar range.

3. INHIBITORS SELECTIVE FOR SPECIAL HDAC ISOFORMS

Despite the progress made in the last few years the role of the various individual HDAC isoforms has only started to unravel. Therefore new isoform selective HDAC inhibitors could be useful as tools for probing the biology of these enzymes and eventually as new anticancer agents with low toxicity. Class II HDACs differ from class I HDACs depending on their tissue expression, subcellular localization, and biological roles. Class I HDACs are ubiquitously expressed, whereas class II enzymes display tissue-specific expression in humans and mice [55].

There is evidence for differences in sensitivity of different members of class I and class II HDACs to different inhibitors. Few HDAC inhibitors (e.g., Trapoxin A, Trapoxin B, CHAP1 and FK-228) are known to be selective for class I HDACs. Recently Tubacin, **23**, which has been discovered through a multidimensional, chemical genetic screen of 7392 molecules, has been described as specific α-tubulin deacetylation inhibitor in mammalian cells [56–58]. Between the two HDAC6 catalytic domains, Tubacin selectively binds only that with tubulin deacetylase activity, without affecting histone acetylation, gene expression, or cell-cycle progression [59]. These results suggest that small molecules that selectively inhibit HDAC6-mediated α-tubulin deacetylation might have therapeutic applications as antimetastatic and antiangiogenic agents. Interestingly, Histacin, a closely related *o*-aminoanilide derivative of **23**, is inactive toward HDAC6 while apparently inhibiting deacetylases that act upon histone substrates [60].



A series of (aryloxopropenyl)pyrrolyl hydroxyamides **24** (R = H, 2-Cl, 3-Cl, 4-Cl, 2-Me, 3-Me, 4-Me) were designed as the first representatives of selective inhibitors of class IIa histone deacetylase (HDAC4, HDAC5, HDAC 7, and HDAC9) [61]. **24** showed better inhibitory activity against maize HD1-A than HD1-B (two homologues of mammalian class IIa and I HDACs) in the submicromolar range with 7–78-fold selectivity. The unsubstituted compound showed good inhibitory activity against both HD1-B and HD1-A, lacking in class selectivity. Insertion of a chlorine atom or a methyl group at any position of the benzene ring caused a dramatic change in the inhibiting effect on the two deacetylases. 2-substituted compounds were the most potent against both enzymes whereas 3-substitution gave rise to the highest selectivities.

In a HDAC8 enzyme-based high-throughput screen several class I HDAC selective hits were found [62]. SB-42920 preferentially inhibited HDAC1 (IC_{50} ~1.5 μM) whereas SB-379872, **25**, only inhibited HDAC8 (IC_{50} ~0.5 μM). Recently a method for selectively inhibiting HDAC7 and HDAC8 was described by either inhibiting expression at the nucleic acid level using antisense oligonucleotides or by inhibiting enzymatic activity at the protein level with small molecule inhibitors derived from the phenylenediamine benzamide series [63].

4. FURTHER CLINICAL OPPORTUNITIES FOR HDAC INHIBITORS

In addition to their application in cancer, HDAC inhibitors also show promise in neurological and immunological disorders [64]. HDAC inhibitors alone or in combination with other drugs were claimed to be useful for the treatment of multiple sclerosis, amyotrophic lateral sclerosis and Alzheimer's disease [65]. HDAC inhibitors were found to be beneficial in treating neurodegenerative diseases caused by polyglutamine toxicity, such as Huntington's disease [66,67]. Recently, this was confirmed in a mouse model of Huntington's disease in which the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) was found to improve motor deficits [68].

In addition to polyglutamine-induced neurodegeneration, HDAC inhibitors may find application in immunological disorders. Cytokine production is regulated through selective patterns of histone acetylation [69]. The HDAC inhibitor SAHA exhibits anti-inflammatory properties both *in vitro* and *in vivo* [70,71]. In a model of lipopolysaccharide induced inflammatory response in mice, SAHA decreases the levels of circulating pro-inflammatory cytokines, such as TNF-α, IL-1-β, IL-6 and IFN-γ. Recently, TSA and SAHA have also been shown to antagonize systemic lupus erythematosus in a mouse model [72,73].

Another therapeutic strategy uses HDAC inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis [74]. The current treatment options, which suppress immune responses or ameliorate inflammation, do not halt the destructive process. It was found that the HDAC inhibitors phenylbutyrate and Trichostatin A causing histone hyperacetylation to modulate multiple gene expression and inhibited the expression of TNF-α in an animal model of rheumatoid arthritis.

5. CONCLUSION

HDAC inhibitors represent a prototype of molecularly targeted agents that perturb signal transduction, cell cycle-regulatory and survival-related pathways. Newer generation HDAC inhibitors have been introduced into the clinics that are considerably more potent than their predecessors and are beginning to show early evidence of activity, particularly in hematopoietic malignancies. It is now becoming evident that each HDAC enzyme has a particular role in controlling transcription, the cell cycle, cell motility, DNA damage response, and senescence by deacetylating histone and non-histone proteins. Therefore enzyme sub-type specific inhibitors will pave a new way to therapeutic drugs that control the specific function and the downstream pathway of HDACs.

REFERENCES

[1] R. Rosato and S. Grant, *Expert Opin. Invest. Drugs*, 2004, **13**, 21.
[2] P. A. Marks, V. M. Richon, R. Breslow and R. A. Rifkind, *Curr. Opin. Oncol.*, 2001, **13**, 477.
[3] W. K. Kelly, O. A. O'Connor and P. A. Marks, *Expert Opin. Invest. Drugs*, 2002, **11**, 1695.
[4] M. Yoshida, R. Furumai, M. Nishiyama, Y. Komatsu, N. Nishino and S. Horinouchi, *Cancer Chemother. Pharmacol.*, 2001, **48** (Suppl. 1), S20.
[5] A. J. M. De Ruijter, A. H. van Gennip, H. N. Caron, S. Kemp and A. B. P. van Kuilenburg, *Biochem. J.*, 2003, **370**, 737.
[6] G. G. Gray and T. J. Ekstrom, *Exp. Cell. Res.*, 2001, **262**, 75.
[7] L. Gao, M. A. Cueto, F. Asselbergs and P. Ataoja, *J. Biol. Chem.*, 2002, **277**, 25748.
[8] M. S. Finnin, J. R. Donigian, A. Cohen, V. M. Richon, R. A. Rifkind, P. Marks, R. Breslow and N. P. Pavletich, *Nature*, 1999, **401**, 188.
[9] M. Yoshida, A. Matsuyama, Y. Komatsu and N. Nishino, *Curr. Med. Chem.*, 2003, **10**, 2351.
[10] M. S. Kim, H. J. Kwon, Y. M. Lee, J. H. Baek, J. E. Jang, S. W. Lee, E. J. Moon, H. S. Kim, S. K. Lee, H. Y. Chung, C. W. Kim and K. W. Kim, *Nature Med.*, 2001, **7**, 437.
[11] T. A. Miller, D. J. Witter and S. Belvedere, *J. Med. Chem.*, 2003, **46**, 5097.
[12] J. A. Gomez-Vidal, J. Campos, J. A. Marchal, H. Boulaiz, M. A. Gallo, E. Carrillo, A. Espinosa and A. Aranega, *Curr. Topics Med. Chem.*, 2004, **4**, 175.
[13] S. W. Remiszewski, *Curr. Opin. Drug Disc. Dev.*, 2002, **5**, 487.
[14] P. A. Marks, T. Miller and V. M. Richon, *Curr. Opin. Pharmacol.*, 2003, **3**, 344.
[15] H. L. Newmark, J. R. Lupton and C. W. Young, *Cancer Lett.*, 2001, **78**, 1.
[16] J. A. DiGiuseppe, L. J. Wenig, K. H. Yu, S. Fu, M. B. Kastan, D. Samid and S. D. Gore, *Leukemia*, 1999, **13**, 1243.
[17] M. Göttlicher, S. Minucci, P. Zhu, O. H. Krämer, A. Schimpf, S. Giavara, J. P. Sleeman, F. Lo Coco, C. Nervi, P. G. Pelicci and T. Heinzel, *EMBO J.*, 2001, **20**, 6969.
[18] Q. Lu, Y.-T. Yang, C.-S. Chen, M. Davis, J. C. Byrd, M. R. Etherton, A. Umar and C.-S. Chen, *J. Med. Chem.*, 2004, **47**, 467.
[19] K. Yang and B. Lou, *Mini Rev. Med. Chem.*, 2003, **3**, 349.
[20] B. Lou and K. Yang, *Mini Rev. Med. Chem.*, 2003, **3**, 609.
[21] E. Vickerstaffe, B. H. Warrington, M. Ladlow and S. V. Ley, *Org. Biomol. Chem.*, 2003, **1**, 2419.
[22] S. W. Remiszewski, *Curr. Med. Chem.*, 2003, **10**, 2393.
[23] S. Remiszewski, L. Sambucetti, K. Bair, J. Bontempo, D. Cesarz, N. Chandramouli, R. Chen, M. Cheung, S. Cornell-Kennon, K. Dean, G. Diamantidis, D. France, M. A. Green, K. L. Howell, R. Kashi, P. Kwon, P. Lassota, M. S. Martin, Y. Mou, L. B. Perez, S. Sharma, T. Smith, E. Sorensen, F. Taplin, N. Trogani, R. Versace, H. Walker, S. Weltchek-Engler, A. Wood, A. Wu and P. Atadja, *J. Med. Chem.*, 2003, **46**, 4609.
[24] P. Atadja, L. Gao, P. Kwon, N. Trogani, H. Walker, M. Hsu, L. Yeleswarapu, N. Chandramouli, L. Perez, R. Versace, A. Wu, L. Sambucetti, P. Lassota, D. Cohen, K. Bair, A. Wood and S. Remiszewski, *Cancer Res.*, 2004, **64**, 689.
[25] G. Bouchain, S. Leit, S. Frechette, E. A. Khalil, R. Lavoie, Rico, O. Moradei, S. H. Woo, M. Fournel, P. T. Yan, A. Kalita, M.-C. Trachy-Bourget, C. Beaulieu, Z. Li, M.-F. Robert, A. R. MacLeod, J. M. Besterman and D. Delorme, *J. Med. Chem.*, 2003, **46**, 820.
[26] G. Bouchain and D. Delorme, *Curr. Med. Chem.*, 2003, **10**, 2359.
[27] J. A. Plumb, P. W. Finn, R. J. Williams, M. J. Bandara, M. R. Romero, C. J. Watkins, N. B. La Thangue and R. Brown, *Mol. Canc. Ther.*, 2003, **2**, 721.
[28] C. J. Watkins, M.-R. Romero-Martin, J. Ritchie, P. W. Finn, I. Kalvinsh, E. Loza, K. Dikovska, I. Starchenkov, D. Lolya and V. Gailite, *PCT Int. Patent Appl.*, WO 03082288-A1, 2003.
[29] D.-K. Kim, J. Y. Lee, J.-S. Kim, J.-H. Ryu, J.-Y. Choi, J. W. Lee, G.-J. Im, T.-K. Kim, J. W. Seo, H.-J. Park, J. Yoo, J.-H. Park, T.-Y. Kim and Y.-J. Bang, *J. Med. Chem.*, 2003, **46**, 5745.
[30] D.-K. Kim, J. Y. Lee, N. K. Lee, J.- S. Kim, J.-H. Ryu, J. W. Lee, S. H. Lee, J. Y. Choi, N. H. Kim, G.- J. Im, T.- K. Kim, J. W. Seo and Y.-J. Bang, *PCT Int. Patent Appl.*, WO 03087066-A1, 2003.

[31] A. Mai, S. Massa, R. Ragno, I. Cerbara, F. Jesacher, P. Loidl and G. Brosch, *J. Med. Chem.*, 2003, **46**, 512.
[32] A. Mai, S. Massa, I. Cerbara, S. Valente, R. Ragno, P. Bottoni, R. Scatena, P. Loidl and G. Brosch, *J. Med. Chem.*, 2004, **47**, 1098.
[33] J. Arts et al., AACR/NCI/EORTC Intl. Conf. Mol. Targets Cancer Ther., Boston, Abstr. A153, 2003.
[34] K. Van Emelen, J. Arts, L. J. J. Backx, H. L. J. De Winter, S. F. A. Van Brandt, M. G. C. Verdonck, L. Meerpoel, I. N. C. Pilatte, V. S. Poncelet and A. B. Dyatkin, *PCT Int. Patent Appl.*, WO 03076422-A1, 2003.
[35] K. Van Emelen, *PCT Int. Patent Appl.*, WO 03076438-A1, 2003.
[36] K. Van Emelen, L. J. J. Backx, S. F. A. Van Brandt, P. R. Angibaud, I. N. C. Pilatte, M. G. C. Verdonck and H. L. J. De Winter, *PCT Int. Patent Appl.*, WO 03076401-A1, 2003.
[37] K. Van Emelen, H. L. J. De Winter, A. B. Dyatkin, M. G. C. Verdonck and L. Meerpoel, PCT Int. Patent Appl., WO 03076421-A1, 2003.
[38] K. Van Emelen, M. G. C. Verdonck, S. F. A. Van Brandt, P. R. Angibaud, L. Meerpoel and A. B. Dyatkin, PCT Int. Patent Appl., WO 03075929-A1, 2003.
[39] P. R. Angibaud, I. N. C. Pilatte, S. F. A. Van Brandt, B. Roux, P. Ten Holte, M. G. C. Verdonck, L. Meerpoel and A. B. Dyatkin, *PCT Int. Patent Appl.*, WO 03076400-A1, 2003.
[40] U. Leser-Reiff, T. Sattelkau and G. Zimmermann, *Patent*, WO 03011851-A2, 2003.
[41] Y. Dai, Y. Guo, J. Guo, L. J. Pease, J. Li, P. A. Marcotte, K. B. Glaser, P. Tapang, D. H. Albert, P. L. Richardson, S. K. Davidsen and M. R. Michaelides, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1897.
[42] M. L. Curtin, R. B. Garland, H. R. Heyman, R. R. Frey, M. R. Michaelides, J. Li, L. J. Pease, K. B. Glaser, P. A. Marcotte and S. K. Davidsen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 2919.
[43] M. Curtin and K. Glaser, *Curr. Med. Chem.*, 2003, **10**, 2373.
[44] R. R. Frey, C. K. Wada, R. B. Garland, M. Curtin, M. R. Michaelides, J. Li, L. J. Pease, K. B. Glaser, P. A. Marcotte, J. J. Bouska, S. S. Murphy and S. K. Davidsen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 3443.
[45] A. Vasudevan, Z. Ji, R. R. Frey, C. K. Wada, D. Steinman, H. R. Heyman, Y. Guo, M. L. Curtin, J. Guo, J. Li, L. Pease, K. B. Glaser, P. A. Marcotte, J. J. Bouska, S. K. Davidsen and M. R. Michaelides, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3909.
[46] C. K. Wada, R. R. Frey, Z. Ji, M. L. Curtin, R. B. Garland, J. H. Holms, J. Li, L. J. Pease, J. Guo, K. B. Glaser, P. A. Marcotte, P. L. Richardson, S. S. Murphy, J. J. Bouska, P. Tapang, T. J. Magoc, D. H. Albert, S. K. Davidsen and M. R. Michaelides, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3331.
[47] Y. Dai, Y. Guo, M. L. Curtin, J. Li, L. J. Pease, J. Guo, P. A. Marcotte, K. B. Glaser, S. K. Davidsen and M. R. Michaelides, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3817.
[48] G. V. Kapustin, G. Fejer, J. L. Gronlund, D. G. McCafferty, E. Seto and F. A. Etzkorn, *Org. Lett.*, 2003, **5**, 3053.
[49] T. Y. H. Wu, C. Hassig, Y. Wu, S. Ding and P. G. Schultz, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 449.
[50] T. Suzuki, T. Ando, K. Tsuchiya, N. Fukazawa and A. Saito, *J. Med. Chem.*, 1999, **42**, 3001.
[51] J. Wang, S. H. Woo, M. Fournel, P. Yan, M. C. Trachy-Bourget, A. Kalita, C. Beaulieu, Z. Li, A. R. MacLeod, J. M. Besterman and D. Delorme, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 283.
[52] E. S. E. Stokes, C. A. Roberts and M. J. Waring, *PCT Patent*, WO 03087057-A1, 2003.
[53] A. Yurek-George, F. Habens, M. Brimmell, G. Packham and A. Ganesan, *J. Am. Chem. Soc.*, 2004, **26**, 1030.
[54] N. Nishino, B. Jose, S. Okamura, S. Ebisusaki, T. Kato, Y. Sumida and M. Yoshida, *Org. Lett.*, 2003, **5**, 5079.
[55] N. R. Bertos, A. H. Wang and X. J. Yang, *Biochem. Cell Biol.*, 2001, **79**, 243.
[56] S. M. Sternson, J. C. Wong, C. M. Grozinger and S. L. Schreiber, *Org. Lett.*, 2001, **3**, 4239.
[57] K. M. Koeller, S. J. Haggarty, B. D. Perkins, I. Leykin, J. C. Wong, M. J. Kao and S. L. Schreiber, *Chem. Biol.*, 2003, **10**, 397.
[58] J. C. Wong, R. Hong and S. L. Schreiber, *J. Am. Chem. Soc.*, 2003, **125**, 5586.

[59] S. J. Haggarty, K. M. Koeller, J. C. Wong, C. M. Grozinger and S. L. Schreiber, *Proc. Natl Acad. Sci. USA*, 2003, **100**, 4389.
[60] S. J. Haggarty, K. M. Koeller, J. C. Wong, R. A. Butcher and S. L. Schreiber, *Chem. Biol.*, 2003, **10**, 383.
[61] A. Mai, S. Massa, R. Pezzi, D. Rotili, P. Loidl and G. Brosch, *J. Med. Chem.*, 2003, **46**, 4826.
[62] E. Hu, E. Dul, C. Sung, Z. Chen, R. Kirkpatrick, G.-F. Zhang, K. Johanson, R. Liu, A. Lago, G. Hofmann, R. MacArron, M. de los Frailes, P. Perez, J. Krawiec, J. Winkler and M. Jaye, *J. Pharmacol. Exp. Ther.*, 2003, **307**, 720.
[63] J. M. Besterman, Z. Li, D. Delorme and C. Bonfils, *PCT Patent Appl.* WO 005513-A2, 2004.
[64] J. Arts, S. de Scheper and K. van Emelen, *Curr. Med. Chem.*, 2003, **10**, 2343.
[65] F. Dangond, PCT Int. Patent Appl., WO 03083067-A2, 2003.
[66] A. McCampbell, A. A. Taye, L. Whitty, E. Penney, J. S. Steffan and K. H. Fischbeck, *Proc. Natl Acad. Sci. USA*, 2001, **98**, 15179.
[67] J. S. Steffan, L. Bodai, J. Pallos, M. Poelmann, A. McCampbell, B. L. Apostol, A. Kazantsev, E. Schmidt, Y.-Z. Zhu, M. Greenwald, R. Kurokawa, D. E. Housman, G. R. Jackson, J. L. Marsh and L. M. Thompson, *Nature*, 2001, **13**, 739.
[68] E. Hockly, V. M. Richon, B. Woodman, D. L. Smith, X. Zou, E. Rosa, K. Sathasivam, S. Ghazi-Noori, A. Mahal, P. A. S. Lowden, J. S. Steffan, J. L. Marsh, L. M. Thompson, C. M. Lewis, P. A. Marks and G. P. Bates, *Proc. Natl Acad. Sci. USA*, 2003, **100**, 2041.
[69] O. Avni, D. Lee, F. Macian, S. J. Szabo, L. H. Glimcher and A. Rao, *Nature Immunol.*, 2002, **3**, 643.
[70] F. Leoni, A. Zaliani, G. Bertolini, G. Porro, P. Pagani, P. Pozzi, G. Donà, G. Fossati, S. Sozzani, T. Azam, P. Bufler, G. Fantuzzi, I. Goncharov, S.-H. Kim, B. J. Pomerantz, L. L. Reznikov, B. Siegmund, C. A. Dinarello and P. Mascagni, *Proc. Natl Acad. Sci. USA*, 2002, **99**, 2995.
[71] P. Mascagni, F. Leoni, G. Porro, P. Pagani, G. Dona, P. Pozzi, C. Dinarello, G. Fantuzzi, B. Siegmund, L. Reznikov, P. Bufler and S.-H. Kim, *PCT Int. Patent Appl.*, WO 03013493-A1, 2003.
[72] G. M. Kammer and N. Mishra, *PCT Int. Patent Appl.*, WO 0255017-A2, 2002.
[73] N. Mishra, C. M. Reilly, D. R. Brown, P. Ruiz and G. S. Gilkeson, *J. Clin. Invest.*, 2003, **111**, 539.
[74] Y.-L. Chung, M.-Y. Lee, A.-J. Wang and L.-F. Yao, *Mol. Ther.*, 2003, **8**, 707.

Therapeutic Opportunities for the Treatment of Biofilm-Associated Infections

Zhenkun Ma, Timothy W. Morris, and Keith D. Combrink

Cumbre Inc., 1502 Viceroy Drive, Dallas, TX 75235-2304, USA

Contents

1. INTRODUCTION

Bacterial biofilms are cellular communities adherent to an inert or living surface that can tolerate both antibiotic and immune attacks [1–3]. The most commonly recognized biofilm infections are prosthetic or medical device infections where the device surface serves as safe harbor for bacterial adhesion and biofilm formation [3]. Device associated infections are difficult to eradicate by the available antibiotic therapies. Standard treatment requires surgical removal of infected devices plus prolonged antibiotic therapy [4–6]. Biofilms have also been linked to many forms of non-device, chronic infections such as cystic fibrosis, endocarditis, osteomylitis and otitis media [2,3]. Based on some estimates, biofilms are associated with about half of the two million cases of nosocomial infections that occur each year in the United States and responsible for the increased morbidity, mortality and medical cost [6,7]. In addition, biofilm organisms live in an ideal niche for the transfer of drug resistant plasmids and are more likely to be exposed to a prolonged suboptimal drug concentration, which lead to the rapid development of antibiotic resistance [8–10]. There is a clear unmet need for effective therapeutic agents that can prevent or eradicate biofilm infections.

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2. MECHANISMS OF BIOFILM FORMATION AND PERSISTENCE

2.1. Quorum sensing and biofilm formation

The processes by which bacteria colonize artificial and natural surfaces have been extensively studied [11–13]. Key stages identified include primary attachment, microcolony formation, growth/maturation into multilayer biofilm, and ongoing detachment of pathogenic cells back into the fluid phase. Despite general agreement that these stages characterize most biofilms, the molecular details of key attachment and maturation processes are extremely variable and clearly depend on the species, the choice of biofilm model system, the source of a particular isolate, and often poorly understood genetic differences between strains. For example, although a variety of mutants in multiple species have been characterized with substantial defects in specific steps of *in vitro* and *in vivo* biofilm formation, the clinical and therapeutic relevance of such biofilm specific genes or targets even within a single bacterial species remains uncertain [14–20].

One area that has received particular recent attention is the role that quorum sensing (QS) based cell-to-cell signaling systems play in biofilm formation and maturation. To date three different families of bacterial QS signal transduction pathways have been characterized, each of which produces a chemically distinct set of small molecule pheromones that are excreted for the purpose of signaling and regulating the behavior of nearby cells in dense bacterial communities. Because genetic or chemical interference with QS signaling has been found to influence biofilm behavior in a broad variety of bacteria, disruption of these bacterial specific signal pathways has recently emerged as an alternative strategy for discovery of new chemotherapeutics [16,19,21,22].

In staphylococci, a family of closely related oligopeptides is excreted as key signal molecules that coordinate a complex, overlapping network of signal transduction components and transcription factors. Although the downstream signal effectors and overlapping pathways are still being elucidated, the key signals themselves seem to be auto inducer peptides (AIP), and RNA III inhibiting peptides (RIP), which are all excreted and function by specific activation or inhibition of membrane bound histidine kinase receptors of adjacent cells. Downstream phosphorylation cascades initiated by these sensor kinases then modulate transcription of a large number of staphylococcal genes involved in virulence, metabolism, and biofilm formation [20,23–25]. In *Pseudomonas aeruginosa* and other Gram-negative bacteria, the major QS signal molecules are small molecule acyl homoserine lactones (AHLs), which regulate cell density dependent behavior of other cells. AHLs diffuse into nearby cells and bind to specific transcription factors that in turn regulate a broad variety of cellular processes, again including environmental responses, metabolism, virulence, and biofilm functions [19,22]. The Gram-negative AHL family is also known as auto inducer-1 (AI-1) group. A third type of QS signal molecule has also been found in both Gram-positive and negative bacteria [26]. This molecule incorporates an unusual borate functionality and is part of the auto inducer-2 (AI-2) system, which has been primarily characterized in the marine symbiont *Vibrio harveyi* [27,28]. AI-2 signals can regulate nearby cells by external binding

to a membrane-bound sensor kinase or by directly binding to cytoplasmic transcription factors [26].

2.2. Biofilm drug tolerance

In general, *in vitro* surface associated and *in vivo* device associated bacterial biofilms can be remarkably tolerant to conventional antibiotics, although some conventional agents still retain substantial efficacy against bacteria embedded in biofilms [3,10,11,29–34]. Table 1 demonstrates that a variety of otherwise effective antibacterial drugs lose substantial activity when tested against *in vitro* models of surface associated *Staphylococcus aureus* or *P. aeruginosa* biofilms. The distinguishing feature of such biofilms is that when they are disrupted and resuspended into planktonic culture, they typically revert to their original drug susceptible state, indicating that biofilm organisms are not usually drug-resistant in the classical sense. Instead, these results make plain that one or more aspects of the biofilm growth state result in temporary, environmentally dependent non-susceptibility to otherwise highly effective antibacterials. Biofilm drug tolerance occurs for multiple antibacterial drug classes against both Gram-positive and Gram-negative pathogen (Table 1). Biofilm efficacy can also vary substantially depending on the antibiotic tested, the age of the biofilm and the duration of drug exposure [35].

The unusual drug tolerance of bacterial biofilms has stimulated extensive research regarding molecular mechanisms of biofilm formation and their resulting non-susceptibility to antimicrobials. Despite considerable effort, the precise molecular and physiological basis for biofilm mediated drug tolerance remains to be fully understood. To date the major hypotheses for biofilm mediated drug tolerance include: reduced penetration rates; inactivation of antibacterials during transit through the biofilm; altered growth, physiology, and stress responses of bacterial cells in biofilm communities; and the existence of cryptic persister cells that reemerge when drug treatment is stopped [3,10–12,37].

Table 1. Representative planktonic *vs* biofilm potency of selected antibiotics

Organism	Antibiotic	Class	Planktonic MBC[a] (μg/mL)	Biofilm/adherent MBC[b] (μg/mL)	Ref.
S. aureus	Vancomycin	Glycopeptide	2	>20	[35]
	Tetracycline	Tetracycline	0.25	>2.5	[35]
	Rifampin	Rifamycin	0.03	0.03	[35]
P. aeruginosa	Tobramycin	Aminoglycoside	8	400	[29]
	Ciprofloxacin	Fluoroquinolone	4	50	[29]
	Meropenem	Carbapenem	8	128	[36]

[a] Minimal bactericidal concentration to kill 99.9% of planktonic bacteria.
[b] Minimal bactericidal concentration to kill 99.9% of biofilm/adherent bacteria.

2.3. Biofilm drug penetration

Although reduced penetration of antibiotics into biofilms provides a simple, intuitive explanation for drug tolerance, multiple studies show that most drugs still penetrate a broad variety of bacterial biofilms quite effectively, and that the observed modest reductions in penetration rates cannot account for large reductions in drug potency [3,10,11,33,38]. In two recent examples, rifampin quantitatively diffused into and across *S. epidermidis* biofilms [31] while tetracycline was directly observed to rapidly penetrate throughout *Escherichia coli* biofilms [30]. There is some evidence, however, for what are termed reaction-diffusion effects on drug permeation that suggest enhanced trapping of aminoglycosides in extracellular polysaccharides of *P. aeruginosa* biofilms [29,32], and enhanced inactivation of some β-lactam antibiotics in *Klebsiella pneumoniae* biofilms [39]. Nevertheless, the results of most biofilm penetration studies indicate that simply optimizing antibiotic penetration into biofilms may not be a generally effective strategy for discovery of improved broad spectrum biofilm agents.

2.4. Biofilm growth states

For simplicity, many of the competing hypotheses for reduced drug susceptibility in biofilms can be grouped together and easily understood as resulting from the architecture, various growth states and heterogeneous subpopulations of bacterial cells that characterize biofilm communities. The concept of multicellular bacterial biofilm communities has become an increasingly accepted paradigm for understanding growth patterns and the dynamics of drug effects on surface associated bacteria [13,40,41], particularly with regard to describing how diffusion of aqueous solutes (primarily nutrients, waste products, and drugs in and out of the biofilm) may account for biofilm properties [33,42]. Biofilms are now seen as heterogeneous populations, with layers of metabolically dormant and dead cells embedded in the biofilm whereas more metabolically active cells are typically found on the periphery. While the specific details vary considerably, multiple studies consistently conclude that it is the dormant cells that seem to account for much of the recalcitrance to drug treatments, whereas the more active cells are consistently more drug sensitive, showing faster and more complete killing upon exposure to therapeutic drugs [3,10,11,13,30,37]. From a drug discovery perspective, the current literature clearly suggests that decreased growth rate is the most significant factor in biofilm mediated drug tolerance, as well as in the reemergence of drug sensitive persister cells and overtly drug resistant clones following drug treatments.

The growing realization that many human infections likely include previously unappreciated biofilm properties [11,43–45] has stimulated two new general strategies detailed below for discovery of new therapies targeting such infections. The first approach seeks to exploit recent progress in understanding the role of bacterial cell-to-cell communication, particularly QS systems in the attachment, development/differentiation, and persistence of bacterial biofilms in a variety of settings. The second approach seeks to refocus traditional medicinal chemistry methods on systematically

optimizing the biofilm specific efficacy of compounds that are already active against planktonic bacteria.

3. PROPHYLACTIC STRATEGIES FOR BIOFILM INFECTIONS

Various prophylactic strategies have been utilized to minimize initial colonization of devices by biofilm-forming organisms. As recently reviewed, the primary strategies include co-administration of prophylactic antibiotics and use of antibiotic device coatings [46]. Disruption of QS based cell-to-cell signaling in biofilms has also been investigated as an alternative therapeutic strategy in a number of recent studies [47].

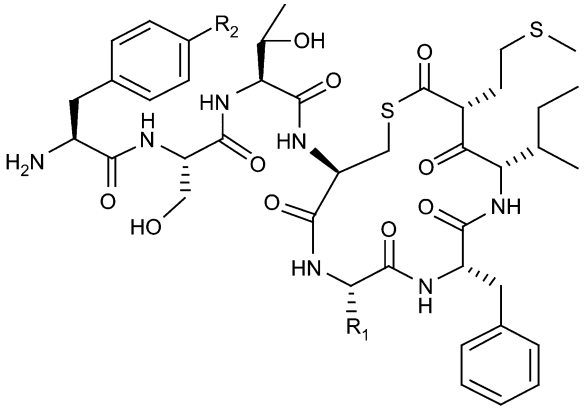
3.1. Staphylococcal QS inhibitors

Synthetic heptapeptide analogs of the QS signal molecule RIP enhanced activity of antibiotics against drug resistant *S. aureus* and *S. epidermidis* by blocking phosphorylation of a critical membrane-bound sensor kinase known as TRAP [23, 48]. In these studies, a synthetic form of RIP, YSPWTNF-NH_2, was tested in a rat model. Dacron grafts soaked with RIP were implanted under the skin, infected with *S. aureus*, and rats were co-administered various antibiotics. In all cases the peptide treated Dacron grafts had a 1–2 log additional reduction in viable bacteria compared to drug alone [48]. Similar efficacy was reported for synthetic RIP against *S. epidermidis* [23]. Others have described analogs of the cyclized thiolactone peptide **1a**, which is also known as the key QS signal molecule autoinducer peptide (AIP) from *S. aureus* [49]. While native AIP is an agonist of the QS sensor kinase AgrC, the synthetic analogs **1b**–**1e** shown in Table 2 exhibited substantial inhibition of both isoforms of the membrane-bound AgrC histidine kinase [49]. AIP analogs designed to act as AgrC antagonists were also investigated by another group, who suggested that synthetic AIP analogs show promise as general inhibitors of *agr* dependent virulence gene expression in *S. aureus* [24]. However, an earlier study of the QS system in *S. aureus* found that *agr* deficient strains were actually better biofilm forming strains than the *agr* positive strains [50]. The authors of this study suggest that treating

Table 2. AIP derivatives as quorum sensing inhibitors

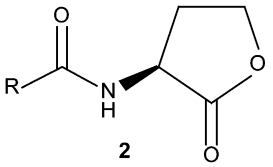
			Inhibitory IC_{50} (nM)	
Compd	R_1	R_2	AgrC-1	AgrC-2
1a	CH_2CO_2H	OH	Agonist	Agonist
1b	CH_3	OH	21	4
1c	CH_2CH_3	OH	137	2.8
1d	CH_3	CH_2Ph	295	19
1e	CH_3	C(O)Ph	303	18

infections with QS inhibitors of the *agr* system would likely enhance the adhesion properties of the pathogens and could be counterproductive [50].



3.2. Gram-negative QS inhibitors

N-Acyl-homoserine lactone (AHL, **2**) is a family of signaling molecules produced by a variety of biofilm forming bacteria. Table 3 illustrates that each species has unique AHLs that govern signaling specificity based on the length and substitution of the fatty acyl side chain [51]. Moreover, subtle structural changes can greatly diminish the ability of AHL analogs to stimulate production of biofilm and virulence factors in nearby bacterial cells [51]. In principle, QS inhibitors could therefore be used as therapeutics to directly disperse biofilms and enhance the efficacy of conventional antibiotics.



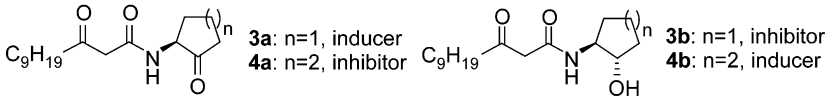
2

Recent reports have described synthesis and evaluation of AHL analogs as antagonists of QS signaling. Bioisosteric replacement of the lactone oxygen of the *P. aeruginosa* AI molecule **2** led to an interesting series of compounds in which slight structural modifications produced compounds that inhibited QS mediated expression of virulence factors [51]. The carbon analog **3a** was a weak autoinducer. Reduction of the ketone to an

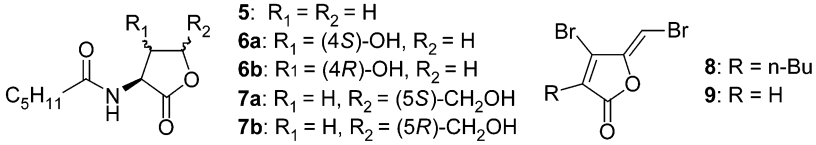
Table 3. AHL autoinducers of various biofilm forming organisms

Organism	R group structure
V. fisheri/*E. chrysanthemi*/*Y. enterocolitica*	$-CH_2C(O)(CH_2)_2CH_3$
A. tumefaciens	$-CH_2C(O)(CH_2)_4CH_3$
P. aeruginosa	$-CH_2C(O)(CH_2)_8CH_3$
P. aeruginosa/*S. liquefaciens*	$-(CH_2)_2CH_3$
B. cepacia	$-(CH_2)_5CH_3$
R. leguminosarum	$-CH_2CH(OH)(CH_2)_3CH{=}CH(CH_2)_5CH_3$

alcohol gave **3b** that bound to the AI receptor but did not induce the QS system. Expansion of the five-membered ring as with **4a** produced a QS inhibitor and reduction of the 6-membered ketone to **4b** gave an inducer.



A series of 4-hydroxy derivatives of the AHL (**5**) were also prepared and had QS signaling potency similar to their parent structure, with the *S*-isomer **6a** being more potent than the *R*-isomer **6b**. In contrast, the 5-hydroxymethyl derivative inhibited QS signaling, with the *S*-isomer **7a** showing slightly more inhibition than the *R*-isomer **7b**. However, neither isomer was as potent as compound **8**, a naturally occurring QS inhibitor isolated from *Delisea pulchra* [52]. At 40 μg/ml, compound **8** completely inhibited growth of *Bacillus subtilis*on LB agar plates, while at 10 μg/ml the growth was reduced by 28% [53]. Compound **9** is a des-butyl analog of **8** that demonstrated QS inhibitory activity. *In vitro*, **9** partially or completely suppressed production of virulence factors in *P. aeruginosa* cultures at 1 to 10 μM without causing any direct growth effects on planktonic cells. Further, cells treated with **9** were 2–3 orders of magnitude more sensitive to tobramycin [47]. In an *in vivo* model, *P. aeruginosa* infected mice were treated with 0.7 mg/kg subcutaneous doses of **9**, resulting a 3 log reduction of viable bacteria compared to placebo treated animals. A dose of 0.4 mg/kg gave a 1 log reduction and 0.2 mg/kg had no effect. No estimate of the dose that would produce a toxic effect in mice was discussed for this compound, nor were separate antibiotic controls reported [47].



A recent patent reported the co-crystal structure of the *Vibrio harveyi* AI-2 signal molecule **10** with its kinase receptor LuxP [27], and a series of inhibitory analogs based on an oxyanion binding motif where X represented B, S or P (**11**–**13**), were claimed in a related patent [28]. The proposed binding mode of the borate adduct is shown in Fig. 1. No biological data were reported for compounds **11**–**13**.

Macrolides have been reported to benefit patients with chronic *P. aeruginosa* infections despite the absence of bactericidal or bacteriostatic activity [54,55]. Azithromycin at 2 μg/ml inhibited QS in *P. aeruginosa*, possibly by preventing expression of the AHL synthetase for *P. aeruginosa* [55]. Prolonged exposure to azithromycin was necessary to observe this effect, and the authors noted that the resulting reduction of virulence factors may reduce the inflammatory response in chronic *P. aeruginosa* lung infections [55]. In a later report, μg/ml azithromycin caused a 45% reduction in biofilm formation of wild-type *P. aeruginosa* [56].

In summary, targeted disruption of QS systems could prevent the establishment of infection, which could be an adjuvant to the existing therapies for biofilm infections, or even an effective monotherapy if further research indicates that *in vivo* biofilms

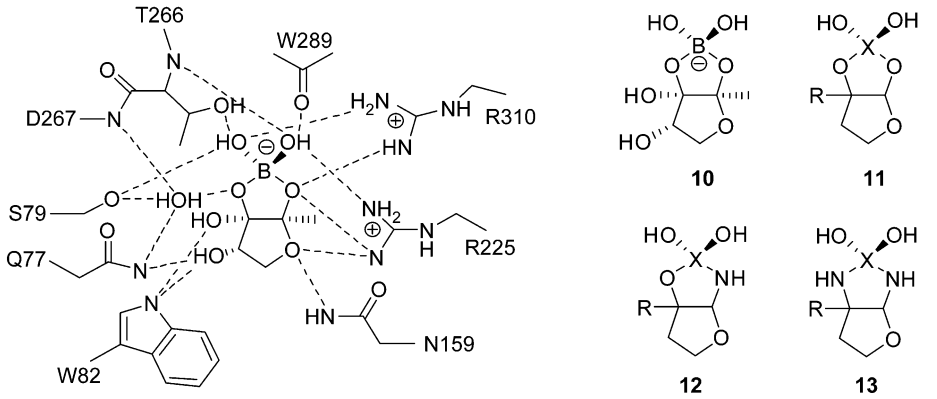


Fig. 1. Proposed binding of *V. harveyi* AI-2 signal with LuxP kinase receptor.

disperse when their signals are jammed. To date however, there are conflicting reports regarding the clinical significance of QS signaling in both Staphylococci and *P. aeruginosa*. Indeed, recent studies have found that interference with QS based signaling in *S. aureus* [15,16,18] and *S. epidermidis* [21] often yields undesirable and unpredictable effects, including enhanced virulence *in vivo* and stimulation of biofilm production, depending on the genetic background of the bacterial strain. These results are especially troubling because staphylococci are the most frequent cause of serious device-related infections [48,57]. With *P. aeruginosa* infections, the QS inhibition strategy presents two additional new challenges to drug discovery scientists. First, existing QS inhibitors such as furanones or natural products often show direct antibacterial effects against planktonic cells which can be rather nonselective, cytotoxic, and mechanistically quite difficult to distinguish from the desired signal transduction effects. Finally, QS inhibitors with the appropriate mechanistic selectivity to become drugs may also be expected to be narrow spectrum agents with limited clinical application [19,22,58].

4. THERAPEUTIC STRATEGIES FOR BIOFILM INFECTIONS

Some advancement has been made in understanding the mechanisms of biofilm formation and persistence, based on which new therapeutic strategies can be derived [11,12,58]. However, progress towards identifying a new, effective agent against biofilms has been limited. Efforts in this area have been mainly focused on evaluation of the known antibiotics or compounds that have already been in clinical development. Despite having potent activity against planktonic cells, the existing agents are much less effective against organisms in a biofilm [3]. The biofilm potency of these agents varies significantly according to their structures and shows little correlation with the planktonic activity [59].

The poor predictability of conventional planktonic assays for biofilm activity supports the notion that a new approach is needed for advancing lead compounds against biofilms

[3]. Ideally, a new approach would instead be driven by a series of predictive *in vitro* and *in vivo* biofilm models that can accurately reproduce the clinical outcomes of antibacterial agents. Such models need to have enough throughput to allow the rapid development of structure-activity relationships against biofilms. However, standard models suitable for biofilm lead optimization have not been developed to date. Researchers in this area have independently developed a variety of *in vitro* and *in vivo* models to address specific questions [3]. However, the available models utilize different organisms, experimental conditions and end points for measuring biofilm efficacy. For example, cell viability is often determined by different methods such as plate counts (CFU), light emission and microscopy [3].

As summarized in Table 4, many commercial antibiotics and compounds in clinical development have been evaluated by using the available *in vitro* models. A relative potency score is used here so that the results from different models can be compared. The results obtained from various *in vitro* models are not always consistent and sometimes conflicting, although each model may reflect certain true aspects of a real biofilm infection. It is clear that a drug's biofilm killing effect is not only strain but also model dependent. Therefore, caution must be made to carefully select the right models before launching a biofilm lead optimization program. Despite the discrepancies between different models, certain commonalities do exist. *S. aureus* and *S. epidermidis* are responsible for the majority of device-related biofilm infections in hospital. Rifampin, a RNA polymerase inhibitor, exhibited good activity almost uniformly against these pathogens in various biofilm models. In the few instances that rifampin performed poorly, resistance to this drug was observed and believed to be responsible for the poor activity [66]. On the opposite side, antibiotics in the glycopeptide and lipopeptide families showed inferior potency against biofilm organisms. They were either inactive or weakly active in various biofilm models. One exception was ramoplanin which demonstrated good killing against *S. aureus* and *S. epidermidis* biofilms but only when the drug concentration reached and exceeded 16 μg/ml [62]. Members of the μ-lactam family were also ineffective against Gram-positive biofilms although aztreonam, a monobactam, appeared to be active against biofilms formed by *E. coli* [66]. Members from the quinolone, macrolide, oxazolidinone, aminoglycoside and tetracycline families showed a wide range of biofilm killing activities in different models. Quinolones and aminoglycosides appeared to be more active against biofilms formed by Gram-negative organisms than Gram-positive organisms. Rufloxacin exhibited the best biofilm killing effect among seven quinolones tested against a biofilm model formed by *Stenotrophomonas maltophilia*, which is an important nosocomial pathogen with increasing prevalence [69].

Many antibacterial agents have also been tested in a number of *in vivo* biofilm models. It is worth noting that the reported efficacy data are not always consistent and vary significantly depending on the model and condition used. Table 5 shows the efficacy of various antibacterial agents in three different biofilm infection models of *S. aureus*. Again, a relative efficacy score is used in order to compare the results from different models. The *in vivo* studies indicated that biofilm associated infections were extremely hard to eradicate. Very few agents demonstrated meaningful efficacy in these models. One of the main observations from these studies was that rifampin or rifampin-containing regiments were more efficacious than other drugs or drug combinations against

Table 4. Potency of various antibiotics in selected *in vitro* biofilm models

Mode	Organism	Relative potency score of antibiotics[a]				Ref.
		3 Most active	2 Moderately active	1 Weakly active	0 Not active	
Adherence model	*S. aureus S. epi.*	Rif, Nov, Pho	Tet	Cefu, Cefa	Van, Tei, Cep, Cip, Gen, Ery	[60,61]
Adherence model	*S. aureus S. epi.*		Rif, Ram	Tei, Cip, Ofl	Van, Lin	[62]
Calgary model	*S. aureus*	Gen		Cip, Clin, Pen	Van, Oxa, Cefa	[36,59]
Sobarod model	*S. aureus S. epi.*		Cip, Max	TD-6424, Lin, Q/D	Van, Tei	[63,64]
Glass bead model	*S. aureus S. epi.*	Rif		Cip, Van, Tei, Dap	Fle, Net	[65,66]
MRD model	*S. epi.*	Lin	Epe	Van	Gen	[67]
Silicone disk model	*S. aureus*	Rif	Min	Q/D	Van, Lin	[68]
Adherence model	*S. maltophilia*	Ruf	TMP/SMX, Ofl	Grep, Nor	Cip, Lev, Mox, Cefta	[69]

[a] Ami: amikacin, Amp: ampicillin, Azt: aztreonam, Cefa: cefazolin, Cefu: cefuroxime, Cefta: ceftazidime, Cep: cephalothin, Cip: ciprofloxacin, Clin: clindamycin, Dap: daptomycin, Epe: eperezolid, Ery: erythromycin, Fle: fleroxacin, Gen: gentamicin, Gre: grepafloxacin, Lev: levofloxacin, Lin: linezolid, Min: minocyclin, Mox: moxifloxacin, Net: netilmicin, Nor: norfloxacin, Nov: novobiocin, Ofl: ofloxacin, Ram: ramoplanin, Pen: penicillin, Pip: piperacillin, Pho: phosphomycin, Rif: rifampin, Ruf: rufloxacin, Q/D: quinupristin/dalfopristin, Tei: teicoplanin, Tet: tetracyclin, TMP/SMX: trimethoprim/sulfamethoxazole, Van: vancomycin.

Table 5. Efficacy of selected agents against *S. aureus* biofilms in animal models

		Relative potency score of antibiotics				
		3	2	1	0	
Model	Animal	Most efficacious	Moderately efficacious	Weakly efficacious	Not efficacious	Ref.
Tissue cage model	Guinea pig	Rif		Dap, Cip, Fle	Van, Tei, Net	[66,70]
Catheter implant model	Mouse	Rif			Cip, Tob	[71,72]
Osteomyelitis model	Rat	Rif	Cefu		Van, Dap Cip, Tob	[73,74]

staphylococci biofilm infections. This finding is consistent with the previous conclusions based on various *in vitro* biofilm models. The clinical efficacy of the rifampin-containing regiments against device associated infections was also illustrated by randomized placebo-controlled clinical trials [4,75–77]. However, drug resistance developed rapidly if rifampin was used alone [75].

Various *in vitro* and *in vivo* studies reveal that the biofilm killing effect of a given compound is highly dependent on the drug class as well as the chemical structure. Certain structure-activity relationships can be developed for biofilm potency. Unfortunately, biofilm potency cannot be predicted by conventional planktonic assays. To identify a better biofilm agent, a new drug discovery platform utilizing predictive biofilm models must be developed. The inconsistency of the *in vitro* results from different biofilm models indicates that developing a predictive *in vitro* model will continue to be a challenge. The *in vitro* models will require extensive validation by using relevant animal models in conjunction with pharmacodynamic considerations. Ultimately, both *in vitro* and *in vivo* biofilm models must be validated by the clinical outcomes of the reference agents.

5. CONCLUSION

The current antibiotic therapies are insufficient against infections associated with bacterial biofilms, which have become a serious and growing problem in the infectious disease area. Considerable advancement has been made in understanding the mechanism of biofilm formation and persistence, based on which two specific strategies, targeting quorum sensing and biofilm persistence have emerged. However, progress on both fronts has been slow. One of the major factors for this slow progress is that the current antibiotic discovery platform based on planktonic assays is unreliable in predicting potency against biofilms. A new platform that utilizes predictive *in vitro* and *in vivo* biofilm models must be developed. Appropriate use of these models should allow for rapid development of structure–activity relationships for biofilm potency and accelerate the discovery of effective therapeutic agents against biofilm infections.

An effective biofilm agent would improve the quality of care for patients with device and non-device associated biofilm infections and significantly reduce the cost for the treatment of such infections. Because most forms of bacterial infections, both biofilm and non-biofilm related, involve organisms in various metabolic states, the conventional approach focusing on the fast growing cell populations will likely lead to agents with suboptimal efficacy [78]. In contrast, bacterial biofilms involve cells in various metabolic states. A new approach based instead on biofilm activity will likely lead to therapeutic agents with improved efficacy against all forms of bacterial infections. Such a biofilm effective agent will have potential to shorten the duration of antibiotic therapy and minimize the chance for bacteria to develop resistance.

REFERENCES

[1] J. W. Costerton, P. S. Stewart and E. P. Greenberg, *Science*, 1999, **284**, 1318.
[2] W. Costerton, R. Veeh, M. Shirtliff, M. Pasmore, C. Post and G. Ehrlich, *J. Clin. Invest.*, 2003, **112**, 1466.
[3] R. M. Donlan and J. W. Costerton, *Clin. Microbiol. Rev.*, 2002, **15**, 167.
[4] L. Bernard, P. Hoffmeyer, M. Assal, P. Vaudaux, J. Schrenzel and D. Lew, *J. Antimicrob. Chemother.*, 2004, **53**, 127.
[5] B. Gottenbos, H. J. Busscher, H. C. Van Der Mei and P. Nieuwenhuis, *J. Mater. Sci. Mater. Med.*, 2002, **13**, 717.
[6] R. O. Darouiche, *N. Engl. J. Med.*, 2004, **350**, 1422.
[7] (a) M. Chicurel, *Nature*, 2000, **408**, 284; (b) C. Potera, *Science*, 1999, **283**, 1837.
[8] R. M. Donlan, *Emerg. Infect. Dis.*, 2002, **8**, 881.
[9] T.-F. Mah and G. A. O'Toole, *Trends Microbiol.*, 2001, **9**, 34.
[10] P. S. Stewart, *Int. J. Med. Microbiol.*, 2002, **292**, 107.
[11] D. Davies, *Nat. Rev. Drug Discovery*, 2003, **2**, 114.
[12] W. M. Dunne, *Clin. Microbiol. Rev.*, 2002, **15**, 155.
[13] B. Schacter, *Nat. Biotechnol.*, 2003, **361**, 21.
[14] K. E. Beenken, J. S. Blevins and M. S. Smeltzer, *Infect. Immun.*, 2003, **7**, 4206.
[15] M. Otto, *Peptides*, 2001, **22**, 1603.
[16] J. M. Yarwood, D. J. Bartels, E. M. Volper and E. P. Greenberg, *J. Bacteriol.*, 2004, **186**, 1838.
[17] J. Valle, A. Toledo-Arana, C. Berasain, J.-M. Ghigo, B. Amorena, J. R. Penades and I. Lasa, *Mol. Microbiol.*, 2003, **48**, 1075.
[18] N. C. Caiazza and G. A. O'Toole, *J. Bacteriol.*, 2003, **184**, 3214.
[19] M. Hentzer, H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby and M. Givskov, *EMBO J.*, 2003, **22**, 3803.
[20] P. Dufour, S. Jarraud, F. Vandenesch, T. Greenland, R. P. Novick, M. Bes, J. Etienne and G. Lina, *J. Bacteriol.*, 2002, **184**, 1180.
[21] C. Vuong, C. Gerke, G. A. Somerville, E. R. Fischer and M. Otto, *J. Infect. Dis.*, 2003, **188**, 706.
[22] K. M. Smith, Y. Bu and H. Suga, *Chem. Biol.*, 2003, **10**, 81.
[23] N. Balaban, A. Giacometti, O. Cirioni, Y. Gov, R. Ghiselli, F. Mocchegiani, C. Viticchi, M. S. Del Prete, V. Saba, G. Scalise and G. Dell'Acqua, *J. Infect. Dis.*, 2003, **187**, 625–630.
[24] G. L. Lyon, P. Mayville, T. W. Muir and R. P. Novick, *Proc. Natl Acad. Sci. USA*, 2000, **97**, 13330.
[25] N. Balaban, T. Goldkorn, Y. Gov, M. Hirshberg, N. Koyfman, H. R. Matthews, R. T. Nhan, B. Singh and O. Uziel, *J. Biol. Chem.*, 2001, **276**, 2658.
[26] M. J. Federle and B. Bassler, *J. Clin. Invest.*, 2003, **112**, 1291.

[27] B. Bassler, S. Schauder, X. Chen, F. Hughson, S. Cooper and K. Yager, *WO Patent* 0318046, 2003.
[28] S. Cooper and K. Yager, *WO Patent* 0318029, 2003.
[29] T.-F. Mah, B. Pitts, B. Pollock, G. C. Walker, P. S. Stewart and G. A. O'Toole, *Nature*, 2003, **426**, 306.
[30] G. Stone, P. Wood, L. Dixon, M. Keyhan and A. Matin, *Antimicrob. Agents Chemother.*, 2002, **46**, 2458.
[31] Z. Zheng and P. S. Stewart, *Antimicrob. Agents Chemother.*, 2002, **46**, 900.
[32] M. C. Walters, III, F. Roe, A. Bugnicourt, M. J. Franklin and P. S. Stewart, *Antimicrob. Agents Chemother.*, 2003, **47**, 317.
[33] P. S. Stewart, *J. Bacteriol.*, 2003, **185**, 1485.
[34] P. Labthavikul, P. J. Petersen and P. A. Bradford, *Antimicrob. Agents Chemother.*, 2003, **47**, 3967.
[35] I. Williams, W. A. Venables, D. Loyd, F. Paul and I. Critchley, *Microbiology*, 1997, **143**, 2407.
[36] S. D. Aaron, W. Ferris, K. Ramotar, K. Vandemheem, F. Chan and R. Saginur, *J. Clin. Microbiol.*, 2002, **40**, 4172.
[37] N. Sufya, D. G. Allison and P. Gilbert, *J. Appl. Microbiol.*, 2003, **95**, 1261.
[38] P. Gilbert, T. Mairia-Litran, A. J. McBain, A. H. Rickard and F. W. White, *Adv. Microbiol. Physiol.*, 2002, **46**, 202.
[39] J. N. Anderl, M. J. Franklin and P. S. Stewart, *Antimicrob. Agents Chemother.*, 2000, **44**, 1818.
[40] G. D. Ehrlich, F. Z. Hu, Q. Lin, J. W. Costerton and J. C. Post, *Am. Soc. Microbiol. News*, 2004, **70**, 127.
[41] J. S. Webb, M. Givskov and S. Kjelleberg, *Curr. Opin. Microbiol.*, 2003, **6**, 578.
[42] M. E. Roberts and P. Stewart, *Antimicrob. Agents Chemother.*, 2004, **48**, 48.
[43] I. I. Raad and H. A. Hanna, *Arch. Inter. Med.*, 2002, **162**, 871.
[44] G. D. Ehrlich, R. Veeh, X. Wang, J. W. Costerton, J. D. Hayes, F. Z. Hu, B. J. Daigle, M. D. Ehrlich and J. C. Post, *JAMA*, 2002, **287**, 1710.
[45] N. Fergie, R. Bayston, J. P. Pearson and J. P. Birchall, *Clin. Otolaryngol.*, 2004, **29**, 38.
[46] P. N. Danese, *Chem. Biol.*, 2002, **9**, 873.
[47] M. Hentzer and M. Givskov, *J. Clin. Invest.*, 2003, **112**, 1300.
[48] A. Giacometti, O. Cirioni, Y. Gov, R. Ghiselli, M. S. Del Prete, F. Mocchegiani, V. Saba, F. Orlando, G. Scalise, N. Balaban and G. Dell'Acqua, *Antimicrob. Agents Chemother.*, 2003, **47**, 1979.
[49] R. J. Scott, L.-Y. Lian, S. H. Muharram, A. Cockayne, S. J. Wood, B. W. Bycroft, P. Williams and W. C. Chan, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2449.
[50] C. Voung, H. L. Saenz, F. Gotz and M. Otto, *J. Infect. Dis.*, 2000, **182**, 1688.
[51] H. Suga and K. M. Smith, *Curr. Opin. Chem. Biol.*, 2003, **7**, 586.
[52] J. A. Olsen, R. Severinsen, T. B. Rasmussen, M. Hentzer, M. Givskov and J. Nielsen, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 325.
[53] D. Ren, J. J. Sims and T. K. Wood, *Lett. Appl. Microbiol.*, 2002, **34**, 293.
[54] J.- C. Pechere, C. Van Delden and O. Menekse, *WO Patent* 0320290, 2003.
[55] K. Tateda, R. Comte, J.-C. Pechere, T. Kohler, K. Yamaguchi and C. Van Delden, *Antimicrob. Agents Chemother.*, 2001, **45**, 1930.
[56] S. Favre-Bonte, T. Kohler and C. Van Delden, *J. Antimicob. Chemother.*, 2003, **52**, 598.
[57] W. Zimmerli and P. E. Ochsner, *Infections*, 2003, **31**, 99.
[58] K. Lewis, *Antimicrob. Agent Chemother.*, 2001, **45**, 999.
[59] H. Ceri, M. E. Olson, C. Stremick, R. R. Read, D. Morck and A. Buret, *J. Clin. Microbiol.*, 1999, **37**, 1771.
[60] B. Amorena, E. Gracia, M. Monzon, J. Leiva, C. Oteiza, M. Perez, J.-L. Alabart and J. Hernandez-Yago, *J. Antimicrob. Chemother.*, 1999, **44**, 43.
[61] M. Monzon, C. Oteiza, J. Leiva, M. Lamata and B. Amorena, *Diagnostic Microbiol. Inf. Dis.*, 2002, **44**, 319.
[62] T. J. Opperman, 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2003, Abstract C1-1119.

[63] S. Gander, K. Hayward and R. Finch, *J. Antimicrob. Chemother.*, 2002, **49**, 301.
[64] S. Gander, A. Kinnaird, L. Baklavadaki and R. Finch, 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2003, Abstract F2110.
[65] A. F. Widmer, R. Frei, Z. Rajacic and W. Zimmerli, *J. Infect. Dis.*, 1990, **162**, 96.
[66] J. Blaser, P. Vergeres, A. F. Widmer and W. Zimmerli, *Antimicrob. Agents Chemother.*, 1995, **39**, 1134.
[67] J. Curtin, M. Cormican, G. Fleming, J. Keelehan and E. Colleran, *Antimicrob. Agents Chemother.*, 2003, **47**, 3145.
[68] I. I. Raad, G. Chaiban, H. A. Hanna, T. Dvorak, R. Lewis, R. Darouiche and R. Y. Hachem, 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 2003, Abstract C1-118.
[69] G. D. Bonaventura, I. Spedicato, D. D'Antonio, I. Robuffo and R. Piccolomini, *Antimicrob. Agents Chemother.*, 2004, **48**, 151.
[70] W. Zimmerli, R. Frei, A. F. Widmer and Z. Rajacic, *J. Antimicrob. Chemother.*, 1994, **33**, 959.
[71] J. L. Kadurugamuwa, L. V. Sin, J. Yu, K. P. Francis, R. Kimura, T. Purchio and P. R. Contag, *Antimicrob. Agents Chemother.*, 2003, **47**, 3130.
[72] N. A. Kuklin, G. D. Pancari, T. W. Tobery, L. Cope, J. Jackson, C. Gill, K. Overbye, K. P. Francis, J. Yu, D. Montgomery, A. S. Anderson, W. McClements and K. U. Jansen, *Antimicrob. Agents Chemother.*, 2003, **47**, 2740.
[73] R. Dworkin, G. Modin, S. Kunz, R. Rich, O. Zak and M. Sande, *Antimicrob. Agents Chemother.*, 1990, **34**, 1014.
[74] M. Monzon, F. Garcia-Alvarez, A. Lacleriga, E. Gracia, J. Leiva, C. Oteiza and B. Amorena, *J. Orthop. Res.*, 2001, **19**, 820.
[75] W. J. Zimmerli, *J. Antimicrob. Chemother.*, 1993, **31**, S97.
[76] A. F. Widmer, A. Gaechter, P. E. Ochsner and W. Zimmerli, *Clin. Infect. Dis.*, 1992, **14**, 1251.
[77] W. Zimmerli, A. F. Widmer, M. Blatter, R. Frei and P. E. Ochsner, *JAMA*, 1998, **279**, 1537.
[78] A. Coates, Y. Hu, R. Bax and C. Page, *Nat. Rev. Drug Discovery*, 2002, **1**, 895.

Biowarfare Pathogens. Is the Research Flavor Different Than That of Clinically Relevant Pathogens?

Ving J. Lee

Anacor Pharmaceuticals, Inc., Palo Alto, CA 94303, USA

Contents

1. INTRODUCTION

Significant progress has been achieved in the 20th century in the implementation of antiinfective therapies that have improved the quality of life of people throughout the world. These advances have also given both immuno-competent and immunosuppressed patients significant enhancements in life span. Numerous scientific and lay publications have focused with high emphasis on the problems of antibiotic-resistant clinical infections. However, there has been little balanced discussion on the etiological impact of the subgroups of pathogens called biowarfare agents [1–3].

In contrast to most clinically-relevant pathogens, biowarfare agents have intrinsic features which make their etiology and clinical treatment unique. These features range from production of exotoxins which promote rapid organ failure (anthrax) to simply a lack of small molecule therapeutics (smallpox and hemorrhagic fever viruses).

The events of October 2001, where dispersement of anthrax spores in key US government facilities crippled their function for weeks, highlighted the specter of societal traumas due to these agents. Aside from the medical challenges, effect on the public psyche is immeasurable [4]. Some of these agents have left their impact on the history of civilization through natural distribution. Notable examples are (i) smallpox (*Variola major*) since the early times and (ii) the Black Death (*Yersinia pestis*) in the Middle

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Ages, which caused significant political shifts in Europe. In some cases, especially the hemorrhagic viruses, the originating loci fortuitously have been a Third World region where the epidemic self-extinguishes. However, transoceanic travel can facilitate the disbursement of some etiological agents as recently shown with Lassa Fever and Severe Acute Respiratory Syndrome (SARS)[1].

Biological weapons have been a societal problem since their first use in the sixth century BC. In the Middle Ages, during some military conflicts, these agents were intentionally dispersed by throwing diseased animal carcasses into an opposition's encampments. In the 20th century, several notable incidences with severe consequences were: (i) Japanese dispersal of *Y. pestis* on Chinese population in Manchuria in late 1930s [5,6] and (ii) the accidental release of anthrax in Sverdlovsk, Russia in 1979 [7,8].

Weaponization further enhances the aerosoling of these agents, which Biopreparat (Soviet Union's biological warfare program) undertook on industrial-scale. The Soviets weaponized anthrax, tularemia, brucellosis, plague, typhus, Q fever, smallpox, botulinum toxin, Ebola and Marburg virus, and Venezuelan equine encephalitis [9,10].

Intentional construction of multiply-resistant mutants (chimeric constructs) by genetic recombination of dissimilar agents with individual resistance mechanisms was also a cornerstone of the Biopreparat technology. Agents that were more virulent or multiply resistant to various classes of therapeutics were attained. Veepox, which is a combination of Venezuelan equine encephalitis and smallpox, is a frightening example of a chimeric construct [11].

Table 1. NIAID priority pathogens

Class A	Class B
Bacillus anthracis (anthrax)	*Burkholderia pseudomallei*
Clostridium botulinium	*Burkholderia mallei* (glanders)
Yersinia pestis (plague)	*Brucella* species (brucellosis)
Francisella tularensis (tularemia)	*Coxiella burnetti* (Q fever)
Variola major (smallpox), other pox viruses	Ricin toxin
Viral hemorrhagic fevers	Epsilon toxin of Clostridium perfringens
Arena viruses	Staphylococcus enterotoxin B
LCM, Junin Virus, Machupo Virus	*Rickettsia prowazekii* (typhus)
Lassa Fever	**Food and Waterborne Pathogens**
Bunyaviruses	*Clostridium perfringens*
Hantaviruses	*Vibrio cholerae*
Rift Valley Fever	*Cryptosporidium parvum*
Flaviviruses	Viral encephalitides
Dengue	
Filoviruses	
Ebola	
Marburg	

Starting in 1998, as part of coordinated effort by US government agencies for preparedness in the event of a biowarfare incident, a pathogen classification system was adopted. The more pertinent categories have been reproduced in Table 1, with the most dangerous listed as Class A agents because of rapid death. The Class B agents cause debilitating diseases, slow death, or panic driven events [12].

The Class A pathogens have characteristics which are medically and operationally challenging: (i) high morbidity or mortality, (ii) inter-personal transmissibility (except anthrax and tularemia), (iii) lack of effective or safe vaccines, and (iv) lack of effective or available treatments. In the absence of pathogen speciation data, the initial clinical symptoms can be confused with those of more benign organisms. While early speciation is crucial, the lack of small molecule therapeutics is abundantly clear.

A recent survey of the Investigational Drugs Database (IDDB) for various research initiatives against Class A agents showed a dearth of small molecule leads (highlighted in grey in Table 2). The majority of the experimental therapeutics are either vaccines or monoclonal antibodies.

While the Class A agents (except for the hemorrhagic viruses) are the focus of this chapter, research and clinical knowledge obtained from the Class A and B agents could provide new treatment modalities for clinically relevant pathogens. Some examples are (i) the interplay between virulence factors and host immune systems which could lead to better understanding of the physiology of sepsis; and (ii) knowledge from *Burkholderia pseudomallei* and *Burkholderia mallei* could provide insights into treatments for *Burkholderia cepacia*, a pulmonary pathogen found in patients with cystic fibrosis.

Drug discovery efforts against the biowarfare agents listed above have historically been limited, either because the consequences of these pathogens were not high priority for policy-makers agenda, or because of technical difficulties. The technical difficulties in working with biowarfare agents include (i) inadequate knowledge of genomic sequences of some of these agents, (ii) the need for use of specialized bio-containment facilities, (iii) inadequate or lack of in-life models that approximate human circumstances, and (iv) also a lack of scientists with relevant research expertise.

Clearly, there is a need for new agents that are potent and minimally toxic against the biowarfare agents. This synopsis describes recent progress towards identifying small molecule inhibitors.

2. BACILLUS ANTHRACIS (ANTHRAX)

2.1. Background

Anthrax is a dimorphic bacterium that normally exists as spores. The clinical presentation can be as cutaneous, inhalational or gastrointestinal forms that are fortuitously not transmissible from person to person. As the October 2001 anthrax cases showed, the insidious nature of anthrax has both a vegetative and spore morphology. The vegetative state, being the growth phase, is typically responsive to most classes of antibiotics, while the spore phase is not. Thus, long treatment modalities with systemic antibiotics for

Table 2. R and D initiatives for modulators of biowarfare agents

Therapeutic	Company or organization	Status	Ref.
Anthrax			
Antitoxin antibodies	Alexion Pharmaceuticals	Discovery	NA
Anthrax vaccines	DynPort/AVANT	Phase I	[13]
Anthrax-plague vaccine	DynPort/AVANT	Discovery	[14]
Abthrax monoclonal antibody	Cambridge Antibody Technology	Phase I	[15,16]
Lethal factor inhibitor	Cengent Pharmaceuticals	Discovery	[17]
Anthrax vaccines	DOR Biopharmaceuticals	Discovery	NA
ETI-205 monoclonal antibody	EluSys Therapeutics	Discovery	[18]
Human monoclonal antibodies	IQ Corporation	Discovery	[19]
Anthrax vaccines	Medarex	Discovery	NA
Anthrax vaccines	Microscience	Discovery	NA
Anthrax vaccines	NIAID/USAMRIID/VaxGen	Phase I	NA
Anthrax vaccines	Vaxin Pharmaceuticals	Discovery	NA
Polyclonal anti-RcPA IgC	NEXT Therapeutics	Discovery	NA
NAD inhibitors, VDDI	U. Alabama	Discovery	[20]
DNA vaccine	Vical	Discovery	[21]
AVP-21D9 monoclonal antibody	Xenerex Biosciences	Discovery	NA
Plague			
Anthrax-plague vaccine	DynPort/AVANT	Discovery	[22]
Plague vaccine	DERA/Provalis	Discovery	[23–26]
Proteosome-formulated vaccine	ID Biomedical/USAMRMC	Discovery	NA
Smallpox			
CMX-001	Chimerix	Discovery	[27,28]
ACAM-2000 vaccine	Acambis	Phase III	[29]
MVA vaccine	Acambis	Discovery	NA
ACY-111 vaccine	Acceptys	Discovery	NA
Elstrein-BN vaccine	Bavarian Nordic	Phase I	NA
Smallpox vaccine	Kaketsuken/VaxGen	Launched	NA
Smallpox vaccine	DynPort	Phase I	[30]
Smallpox vaccine	NIH	Phase I	[31]
Smallpox vaccine	SIGA Technologies	Discovery	NA

NA, Primary technical reference(s) not available yet, but product theme has been discussed in government forums (cited in Investigational Drugs Database).

the deadly inhalation form are necessary in order to inhibit all of the organisms that convert from the spore state to the vegetative state [4]. This requires anti-infectives with safety profiles adequate for several months of administration. A major advance would be the discovery of antibiotics that are effective against the spore phase or stationary phase of these pathogens.

A second unique aspect of anthrax, which alters the clinical treatment, is the presence of anthrax toxin which is responsible for rapid on-set of organ and cardiac failure.

Cessation of anthrax growth through antibiotic action does not stop the downstream biological effects of the toxin components, especially when pathogen overload results in large titers of anthrax toxin release into the body.

After inhalation, *Bacillus anthracis* spores germinate in alveolar macrophages and then migrate to lymph nodes where they propagate. The vegetative bacteria secrete a tripartite toxin, which consists of three proteins: lethal factor (LF, 90 kDa), edema factor (EF, 89 kDa), and protective antigen (PA, 83 kDa), all of which work in concert to kill host cells. While the mode-of-action of anthrax toxin itself is not yet well understood, small molecule inhibitors or monoclonal antibodies to inhibit toxin assembly and/or function represent potentially useful approaches.

Lethal Factor (LF) is a zinc dependent protease which targets the Mitogen Activated Protein Kinase Kinases (MAPKK), that are involved in intracellular signaling pathways. The protease inactivates the MAPKK which cannot then signal the p38 MAPKs. Lack of P-38 activity causes lysis of macrophages, which then facilitates the propagation of the anthrax [32,33].

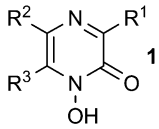
Edema factor (EF) is a calmodulin-mediated adenylate cyclase that impairs the host defenses through a variety of mechanisms inhibiting phagocytosis. These include interference with the host's immune response which facilitates the bacteria propagation, and induction of massive tissue necrosis, including pulmonary fluid retention. The EF-calmodulin complex is an exquisitely potent and hyperactive adenylyl cyclase. Cells activated by the adenyl cyclase lose the ability to regulate their environment, release water, and die [34–36].

Protective antigen (PA) binds to the cellular receptor, Tumor Endothelium Marker-8 (TEM8). Upon binding to TEM8, PA is cleaved into 20 and 63 kDa fragments (PA20 and PA63) by furin or furin-like proteases. The PA63 fragment re-associates and binds either LF or EF. The resulting complexes of PA63-EF or PA63-LF are internalized into endosomes followed by translocation of LF and OF into cytosol of the cells. PA receptor TEM8 (also known as Anthrax Toxin receptor, ATR1) is a glycoprotein with extracellular (1-321aa), cytosolic (343-564aa) and TM (322-342) domains [37,38]

Nearly half (5/11) of the pulmonary anthrax patients from the Oct 2001 incidents died, while the survivors have ongoing symptoms such as fatigue, shortness of breath and memory loss [4]. This highlights the need for more effective therapies [39].

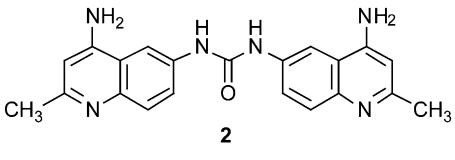
While various initiatives are ongoing to examine the efficacy of existing antibiotics against anthrax, this may not address the fears of a chimeric construct of anthrax which is multiply-resistant to various classes of existing antibiotics. Thus, parallel small molecule approaches for clinical use are needed [40]. There is a need for new antibiotics which function against new anthrax targets, and also agents to inhibit various toxin components.

While the multiplicative biological effects of the various toxin components is daunting, even inhibition of a single component would be beneficial. Several approaches have been reported. As a potential treatment for pulmonary indications, substituted 3-hydroxyhydropyrazine-2-ones **1** are being investigated as inhibitors of LF [17]. A small-molecule LF inhibitor demon-strated *in vivo* protection against anthrax toxin-induced disease processes, and also partial restoration



R^1 = benzyl, *i*-butyl, 4-phenylbenzyl
R^2 = alkyl, substituted phenyl, 2-thienyl, 2-naphtyl, biphenyl
R^3 = H, alkyl, phenyl

of anthrax lethal toxin-suppressed immunological function in mice. In addition, treating lethal toxin-exposed rats with the inhibitors increased the life span of the rats significantly.



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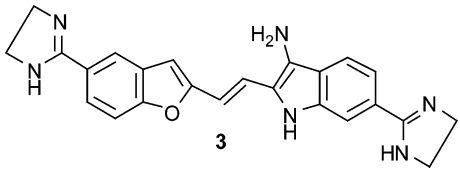

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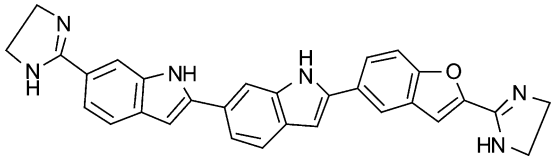

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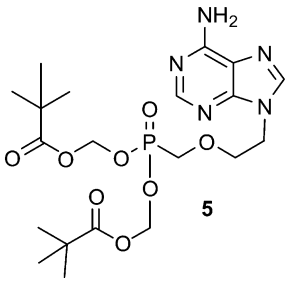
In examining a subset of the NCI compound library, researchers have identified inhibitors **2**–**4** of LF with a common pharmacophore, which may be amenable to further structural modifications to provide more potent analogs [41,42]. Other earlier screening leads of peptidic origin have yet to evolve into more drug-like molecules [43–45].



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The search for new drugs can also be serendipitous. Using *de novo* design strategies, researchers at the University of Chicago have identified adefovir dipivoxil (**5**, Hepsera®), a hepatitis B antiviral, as an *in vitro* inhibitor of EF [46]. Adefovir fits 10,000 times better then the natural substrate into a pocket on the surface of EF. While *in vitro* success does not necessarily translate to *in vivo* success, a new use for a recently approved drug represents an attractive strategy.

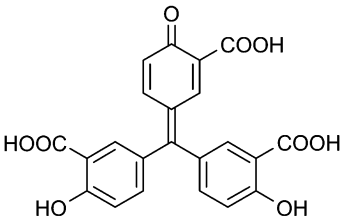
3. YERSINIA PESTIS (PLAGUE)

3.1. Background

Plague is caused by a bacterium carried by a rodent flea [47–49]. While current antibiotics are effective against plague, the worry is the possibility of a bioengineered chimeric construct that would be resistant to all classes of antibiotics.

Wild rodents in certain areas around the world are infected with plague. Human plague in the United States occurs mostly in rural areas with an average of 10–15 cases/annum *vs*. 1000–3000 cases worldwide. Most human cases in the United States occur in two regions: (i) northern New Mexico, northern Arizona, and southern Colorado, and (ii) California, southern Oregon, and far western Nevada. Plague also exists in Africa, Asia, and South America [50].

Aside from citations on the use of older antibiotics for the treatment of plague, there is one key report on the molecular and cellular basis of plague's virulence [51]. An attempt to identify inhibitors of *Yersinia* protein tyrosine phosphatase (YopH) [52], one of the virulence factors, has been reported the activity of aurintricarboxylic acid **6** [53].



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4. FRANCESELLA TULARENSIS (TULAREMIA)

4.1. Background

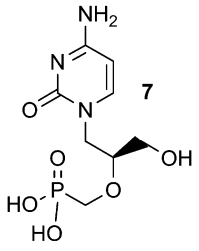
Tularemia is a zoonosis that occurs naturally in the United States, with animal (especially rodents, rabbits, and hares) transmission to man. Sometimes an insect vector may also be the primary route of infection. It is highly pathogenic and the inhalation of 10 organisms would be adequate for infection [54–55]. It is resilient to various environmental factors such as low temperatures and resides in the natural environment (moist hay, grass, water) when distributed. It exists in two subspecies: (i) *F. tularensis* biovar tularensis (type A) and (ii) *F. tularensis* biovar palaearctica (type B). Type A, the more virulent form, exists predominantly in North America whereas type B, a less virulent form, is found in Europe and Asia. Aerosol release of a virulent form of tularemia would be expected to lead to substantial morbidity and mortality. There are no reported approaches to a small molecule therapy.

5. VARIOLA MAJOR (SMALLPOX)

5.1. Background

Smallpox, which originated in northern Africa, has been known for *ca.* 10,000 years, and the last known case occurred in Somalia in mid-1970s. It was declared eradicated in 1977 by the WHO. Historically it has caused more deaths then any other cause of mortality. Smallpox is the most feared of all biowarfare pathogens, primarily due to its high transmissibility versus other pathogens whose etiologic affects are episodic (e.g., *Y. pestis* or *M. tuberculosis*) [1,56].

This area has prompted substantial interest in the last several years, especially in the use of vaccinia viruses as surrogates for smallpox. A number of nucleoside analogs were identified that inhibited various stages of viral reproduction by various mechanisms, including (i) inosine monophosphate (IMP) dehydrogenase inhibitors (EICAR), (ii) *S*-adenosylhomocysteine (SAH) hydrolase inhibitors (5′-noraristeromycin, 3-deazaneplanocin A and neplanocin A analogs), (iii) orotidine



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monophosphate (OMP) decarboxylase inhibitors (pyrazofurin), (iv) cytidine triphosphate (CTP) synthetase inhibitors (cyclopentenyl cytosine), (v) thymidylate synthetase (TS) inhibitors (2′-deoxyuridines), (vi) DNA synthetase inhibitors (Ara-A), (vii) acyclic nucleoside (cidofovir), and (viii) polyacrylic acid.

Cidofovir **7**, an approved drug for the treatment of cytomegalovirus (CMV) infections, is a potent inhibitor of the smallpox virus [57–59]. However, cidofovir must be administered by intravenous infusion making it impractical for broad and rapid distribution in the event of a smallpox outbreak. Further, in some patients, the parenteral formulation shows nephrotoxic events. Lipophosphate prodrugs of cidofovir have been reported to be orally bioavailable [27,28]. CMX-001 (structure undisclosed) is being studied for the potential treatment of smallpox infections and complications resulting from smallpox vaccination. It is more potent and less toxic than cidofovir. CMX-001 is 100 times more potent than cidofovir, as shown by antiviral assays, and it is active against a variety of pox viruses including smallpox and monkeypox. When given orally, it was fully effective in mouse models of pox virus infection. The development of CMX-001 for smallpox therapy is compelling since earlier clinical data, from the regulatory approval for CMV indications, would facilitate its use for smallpox.

6. CONCLUSION

While few small molecular entities were discussed in this review, the increased research activity along with enhanced government support of biowarfare agents is encouraging. We can hope that within a few years there will be significant developments in small molecule discovery to fill this void.

The events from 9/11, in conjunction with expanded government funding for biowarfare research, has led to shifts in anti-infectives research operations. These include:

- Interest in microbiology, molecular microbiology, and adjunctive technologies has increased in academic institutions. Especially evident is the use of comparative genomic and post-genomic strategies to identify pathogen targets.
- There is an increase in collaborations between private industry, academics, and government labs. As more 3D crystallographic data is obtained on target proteins from biowarfare pathogens, massive parallel virtual screening efforts are being encouraged through government-sponsored programs [60]. This will foster a better utilization of government-sponsored BL3 facilities.
- There is the opportunity for researchers to work creatively by using clinically-relevant pathogens as surrogates for biowarfare pathogens: (i) *B. cereus* or *B. subtilis* for *B. anthracis* activity or (ii) *Yersinia pseudotuberculosis* for *Y. pestis*.
- In contrast to the more traditional drug development sequence that requires Phase I–III studies, the FDA has created a new paradigm for approval of drugs for use against biowarfare agents [61].
- A new paradigm for funding of biotech start-ups has occurred. Several companies have been started with minimal levels of venture support, but with substantial non-dilutive government funding.

Ironically, one of the anti-infective successes in 20th century included the final eradication of smallpox in 1977 in Somalia. Within one generation, we now fear the possible use of smallpox as a biowarfare agent.

REFERENCES

[1] L. Garrett, *The Coming Plague*, Farrar, Straus and Giroux, New York, NY, 1994.
[2] P Farmer, *Infections and Inequalities: The Modern Plagues, Updated Edition with New Preface*, University of California Press, Berkeley, CA, 1999.
[3] R. Preston, *The Demon in the Freezer: A True Story*, Random House, New York, NY, 2002.
[4] T. V. Inglesby and T O'Toole (eds), *Bioterrorism – Guidelines for Medical and Public Health Management*, American Medical Association Press, Chicago, 2002.
[5] http://www.cbwinfo.com/biological/pathogens.
[6] H. Gold, in *Unit 731 Testimony* (ed. E. Charles), Tuttle Press, Boston, MA, 1996.
[7] (a) M. Meselson, J. Guillemin, M. Hugh-Jones, A. Langmuir, I. Popova, A. Shelokov and O. Yampolskaya, *Science*, 1994, **266**, 1202; (b) L. M. Grinberg, F. A. Abramova, O. V. Yampolskaya, D. H. Walker and J. H. Smith, *Mod. Pathol.*, 2001, **14**, 482.
[8] A. J. Venter, *Sverdlovsk Outbreak: A Portent of Disaster*, Jane's Intelligence Review, 1998, p. 36.
[9] http://judiciary.senate.gov/oldsite/preston.htm.
[10] C. J. Davis, *Emerging Infect. Dis.*, 1999, **5**, 509.
[11] http://www.zkea.com/archive10003.html.
[12] http://www.niaid.nih.gov/biodefense/bandc_priority.htm.
[13] R. Bhatnagar (poster), 5th International Conference on Anthrax and 3rd International Workshop on Molecular Biology of Bacillus cereus, Bacillus anthracis and Bacillus thuringiensis, Nice, FR, 2003.
[14] K. P. Killeen, 3rd International Conference on Vaccines for Enteric Diseases (Part II), Montego Bay, Jamaica, 2004.
[15] G. Choi, X. Zhang, J. Askins, R. Fleming, B. Strum, C. Poortman, P. Virlassov, B. Peterson, M. Flynn, Y. Miao, D. Zakauskas and R. Smith, 43th ICAAC Abstracts, Chicago, IL, 2003, pp. 277, Abstract G-280.
[16] L. E. Beebe, J. Zhong, M. Clagett, M. Babin, Y. Ou, V. Roschke, A. Yu, M. Subramanian and G. Choi, 43th ICAAC Abstracts, Chicago, IL, 2003, pp. 47, Abstract B-331.
[17] D. Rideout, V. V. Yalamoori, K. Ramnarayan, M. Shenderovich, J. H. Zheng, J. Sun and C. Niemeyer, US Patent 6 436 933, 2002.
[18] L. S. Casey, N. Mohamed, J. Li, C. Ferreira, G. D'Alia, K. Curtis, J. Porter, S. E. Pincus, S. Jones, L. Nardone, M. Clagett, R. Barnewall, M. Babin, C. Lyons and G. L. Spitalny, 2nd ASM Biodefense Meeting Abstracts, Baltimore, MD, 2004, pp. 61, Abstract 60.
[19] H. Groen, 5th International Conference on Anthrax and 3rd International Workshop on Molecular Biology of Bacillus cereus, bacillus anthracis and Bacillus thuringiensis Abstracts, Nice, FR., Abstract 0610, 2003.
[20] W. J. Brouillette, S. E. Velu, C. G. Brouillette, C.-H. Luan and L. J. DeLucas, 224th ACS Meeting Abstracts, Boston, MA., 2002. Abstract MEDI 51.
[21] T. Evans, G. Hermanson, V. Whitlow, P. Lalor, M. Ferrari, K. Brenneman, M. Bell, D. Kaslow, D. Galloway and P. Hobart, 43th ICAAC Abstracts, Chicago, IL, 2003, pp. 298, Abstract G-1666.
[22] M. Enserink and E. Marshall, *Science*, 2002, **296**, 639.
[23] E. D. Williamson, S. M. Eley, K. F. Griffin, M. Green, P. Russell, S. E. Leary, P. C. Oyston, T. Easterbrook, K. M. Reddin, A. Robinson, *et al.*, *FEMS Immunol. Med. Microbiol.*, 1995, **12**, 223.
[24] G. W. Anderson, Jr., S. E. Leary, E. D. Williamson, R. W. Titball, S. L. Welkos, P. L. Worsham and A. M. Friedlander, *Infect. Immun.*, 1996, **64**, 4580.

[25] R. W. Titball and S. E. Leary, *Br. Med. Bull.*, 1998, **54**, 625.
[26] R. W. Titball and E. D. Williamson, *Vaccine*, 2001, **19**, 4175.
[27] K. Y. Hostetler, R. Kumar and L. M. Stuhmiller, *US Patent* 6 448 392, 2002.
[28] K. Y. Hostetler, R. Kumar and L. M. Stuhmiller, *US Patent* 6 599 887, 2003.
[29] R. Weltzin, J. Liu, K. V. Pugachev, G. A. Myers, B. Coughlin, P. S. Blum, R. Nichols, C. Johnson, J. Cruz, J. S. Kennedy, F. A. Ennis and T. P. Monath, *Nature Med.*, 2003, **9**, 1125.
[30] R. J. Hopkins, R. N. Greenberg, E. A. Plummer, L. Hague, D. Clanton, V. Johnson, D. Fine and M. Langford, 43rd ICAAC Abstracts, Chicago, IL, 2003, pp. 286, Abstract G-1086.
[31] L. H. McCurdy, M. Chen, J. Rutigliano, T. Johnson and B. S. Graham, *43rd ICAAC Abstracts,* Chicago, IL, 2003, pp. 297, Abstract G-1662.
[32] A. D. Pannifer, T. V. Wong, R. Schwarzenbacher, M. Renatus, C. Petosa, J. Bienkowska, D. B. Lacy, R. J. Collier, S. Park, S. H. Leppla, P. Hanna and R. C. Liddington, *Nature*, 2001, **414**, 229.
[33] L.-C. Hsu, J. M. Park, K. Zhang, J.-L. Luo, S. Maeda, R. J. Kaufman, L. Eckmann, D. G. Guiney and M. Karin, *Nature*, 2004, **428**, 341.
[34] D. B. Lacy, M. Mourez, A. Fouassier and R. J. Collieret, *J. Biol. Chem.*, 2002, **227**, 3006.
[35] J. Mogridge, K. Cunningham, D. B. Lacy, M. Mourez and R. J. Collier, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 7045.
[36] K. Cunningham, D. B. Lacy, J. Mogridge and R. J. Collier, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 7049.
[37] C. L. Drum, S.-Z. Yan, J. Bard, Y.-Q. Shen, D. Lu, S. Soelaiman, Z. Grabarek, A. Bohm and W.-J. Tang, *Nature*, 2002, **415**, 396.
[38] L. B. Price, M. Hugh-Jones, P. J. Jackson and P. Keim, *J. Bacteriol.*, 1999, **181**, 2358.
[39] K. A. Bradley, J. Mogridge, M. Mourez, R. J. Collier and A. T. Young, *Nature*, 2001, **414**, 225.
[40] A. M. Friedlander, *Nature*, 2001, **414**, 160.
[41] B. E. Turk, T. Y. Wong, R. Schwarzenbacher, E. T. Jarrell, S. H. Leppla, R. J. Collier, R. C. Liddington and L. C. Cantley, *Nat. Struct. Biol.*, 2004, **11**, 60.
[42] R. G. Panchal, A. R. Hermone, T. L. Nguyen, T. Y. Wong, R. Schwarzenbacher, J. Schmidt, D. Lane, C. McGrath, B. E. Turk, J. Burnett, M. J. Aman, S. Little, E. A. Sausville, D. W. Zaharevitz, L. C. Cantley, R. C. Liddington, R. Gussio and S. Bavari, *Nat. Struct. Biol.*, 2004, **11**, 67.
[43] M. Mourez, R. S. Kane, J. Mogridge, S. Metallo, P. Deschatelets, B. R. Sellman, G. M. Whitesides and R. J. Collier, *Nat. Biotechnol.*, 2001, **19**, 958–961.
[44] F. Tonello, M. Seveso, O. Marin, M. Mock and C. Montecucco, *Nature*, 2002, **418**, 386.
[45] M. Glick, G. H. Grant and G. Richards, *Nat. Biotechnol.*, 2002, **20**, 118.
[46] Y. Shen, N. L. Zhukovskaya, M. I. Zimmer, S. Soelaiman, P. Bergson, C.-R. Wang, C. S. Gibbs and W.-J. Tang, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 3242.
[47] K. L. Gage, in *Topley and Wilson's Microbiology and Microbiological Infections* (eds L. Colliers, A. Balows, M. Sussman and W. J. Hausles), Edward Arnold Press, London, England, 1998, Vol. 3, p. 885.
[48] G. L. Campbell and D. T. Dennis, in *Harrison's Principles of Internal Medicine* (eds E. Braunwald, A. Fauci, D. Kasper, S. Hauser, D. Longo and J. Jameson), 14th edition, McGraw Hill, New York, NY, 1988, p. 975.
[49] R. D. Perry and J. D. Fetherston, *Clin. Microbiol. Rev.*, 1997, **10**, 35.
[50] M. Bahmanyar and D. C. Cavanaugh, *Plague Manual*, World Health Organization, Geneva, 1976.
[51] G. R. Cornelius, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 8778.
[52] J. Phan, K. Lee, S. Cherry, J. E. Tropea, T. R. Burke, Jr. and D. S. Waugh, *Biochemistry*, 2003, **42**, 13113.
[53] F. Liang, A. Huang, S.-Y. Lee, J. Liang, M. I. Ivanov, A. Alonso, J. B. Bliska, D. S. Lawrence, T. Mustelin and Z.-Y. Zhang, *J. Biol. Chem.*, 2003, **278**, 41734.
[54] J. Chin (ed.), *Control of Communicable Diseases Manual*, American Public Health Association, Washington, DC, 2002, p. 532.
[55] J. M. Boyce, *J. Infect. Dis.*, 1975, **131**, 197.
[56] J. G. Breman and D. A. Henderson, *N. J. Med. Engl.*, 2002, **346**, 1300.

[57] E. de Clercq, *Clin. Microbiol. Rev.*, 2001, **14**, 382.
[58] E. de Clercq, *Trends Pharmacol. Sci.*, 2002, **23**, 456.
[59] E. de Clercq, *Antiviral Res.*, 2002, **55**, 1.
[60] A. Yarnell, *Chem. Eng. News*, 2003, **81**, 9.
[61] The Food and Drug Administration's Strategic Action Plan Protecting and Advancing America's Health – Responding to new challenges and opportunities, http://www.fda.gov/oc/mcclellan/strategic.html, 2003.

HCV Anti-viral Agents

Ronald C. Griffith, Lillian Lou, Christopher D. Roberts, and Uli Schmitz

Genelabs Technologies Inc., 505 Penobscot Drive, Redwood City, CA 94063, USA

Contents

1. INTRODUCTION

Chronic infection with HCV has become a major health problem associated with liver cirrhosis, hepatocellular carcinoma and liver failure. An estimated 170 million chronic carriers worldwide are at risk of developing liver disease [1]. In the US alone 2.7 million are chronically infected with HCV and the death-toll in 2000 was estimated between 8000 and 10,000, a number that is expected to increase significantly over the next years [2]. Considering the slow course of the infection and the nature of the current, insufficient therapy, treatment of HCV-related illness is a heavy burden on any public health system. Current therapy involving pegylated interferon-α alone (Pegasys, PEG-Intron™) or in combination with ribavirin (**1**) (Rebetrol™, Copegus™) [3], is expensive, associated with side-effects and exhibits only modest success rates, which are strongly dependent on the particular genotype of the virus. For the most prevalent HCV subtypes in the US, **1a** and **1b** (72%), the above co-therapy yields response rates between 40 and 60%. Note that the above therapy is not aimed at inhibiting viral targets directly, in contrast to the successful approaches in HIV therapy. Future therapies need to include the more elusive HCV subtypes and should benefit from targeting viral replication directly. HCV is a small, enveloped virus with a single strand RNA genome coding for a 3000 a.a. polyprotein which is processed into at least 10 individual viral proteins of which the six C-terminal ones are non-structural proteins termed NS2, NS3, NS4a, NS4b, NS5a and NS5b [4]. Assembly of the final

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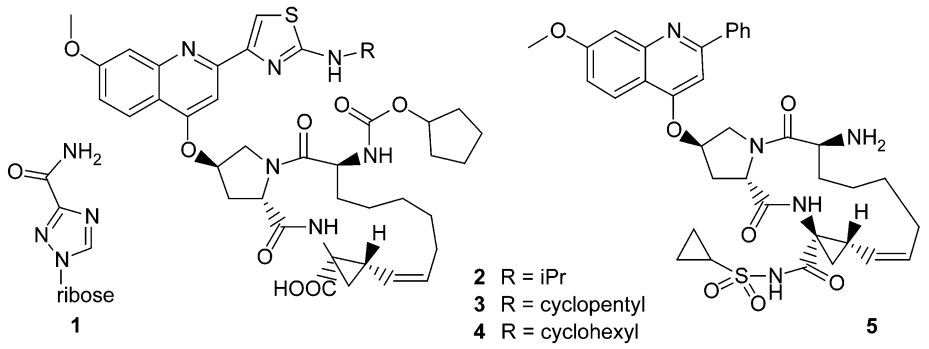
replication complex around the NS5b RNA polymerase requires five proteolytic steps of which the first is carried out by the metalloprotease NS2. Subsequently, the protease module of NS3 delivers the remaining cuts which most require NS4a as a cofactor for NS3. Fueled by the success of targeting the HIV protease and reverse transcriptase, the largest anti-HCV drug discovery efforts have been directed towards NS3 protease and NS5b RNA polymerase.

2. INHIBITORS OF VIRAL PROTEIN TARGETS

2.1. NS3 protease

NS3 is a relatively small serine protease of the chymotrypsin family and peptide sequence requirements for both efficient cleavage and competitive inhibition are well known [5]. Since low micromolar inhibition could be achieved with hexapeptides derived from N-terminal P-site cleavage products [6], the vast majority of work has not strayed from competitive peptidic inhibitors with either a covalent ('serine traps') or non-covalent mode of interaction. Most promising is the group of constrained tripeptides, **2**–**5**, which arose from linking the P1 and P3 a.a. side chains and optimization of the P2 proline substituents [7]. Trailblazer BILN-2061, **2**, yielded clinical proof-of-concept data as its outstanding *in vitro* activity in enzyme inhibition assays ($K_i = 0.3$ and 0.7 nM for subtype **1a** and **1b**, respectively) and the surrogate cell-based replicon assay ($EC_{50} = 3$ and 4 nM for subtype **1a** and **1b**, respectively) was mirrored by a dramatic 2–3 $\log_{10}$ reduction of viral loads within 24 h in the majority of patients that had received **2** (200 mg in an oral solution of a PEG 400/ethanol 80:20 mixture, twice-daily for 2 days) [8]. While **2** was well tolerated up to 2000 mg and no serious clinical or laboratory findings were obtained in a human safety study, further clinical trials are currently on hold because cardiac lesions were observed in routine chronic safety testing in monkeys at supra-therapeutic doses [9,10]. The primary interaction site of BILN 2061 with NS3, as revealed by a crystal structure, involves largely the residues conserved across HCV subtypes [7]. This explains why K_i values for subtypes **2a**, **2b** and **3a** are still below 100 nM. Oral bioavailability for **2** was below 20% in rats and rhesus monkeys; for dogs, values were as high as 38% [8]. Some of the clinical activity of **2** might in fact be the restoration of the IFN related cellular anti-viral response. NS3 has been shown to mediate

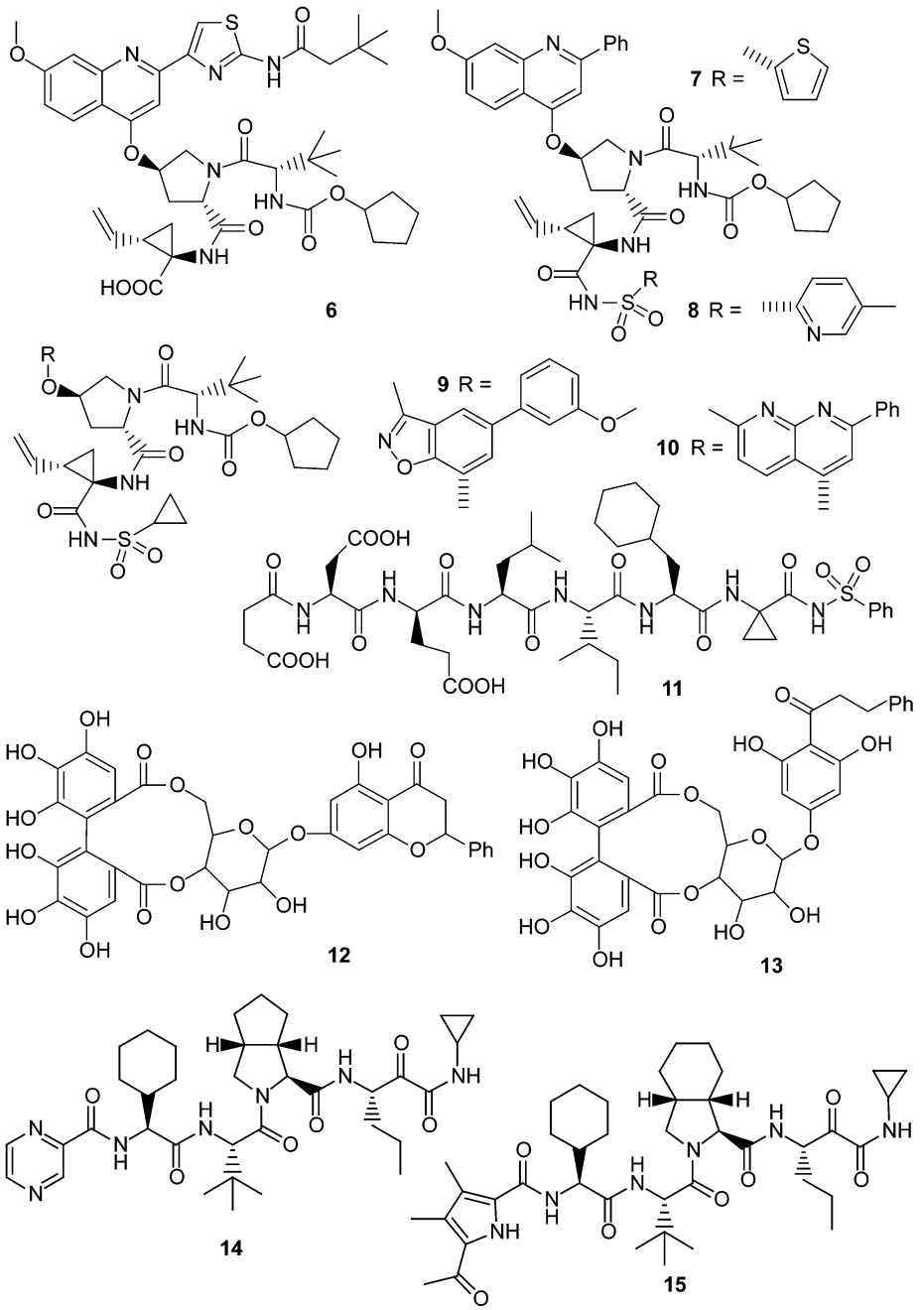


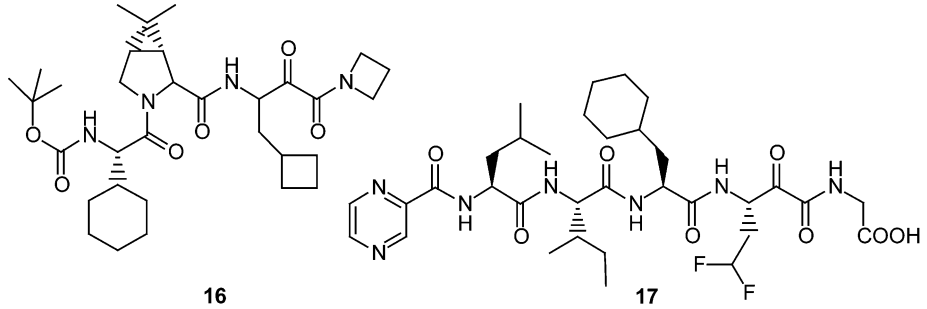
inhibition of the phosphorylation, hence activation of IFN regulatory factor-3 (IRF-3), a key anti-viral signaling molecule [11].

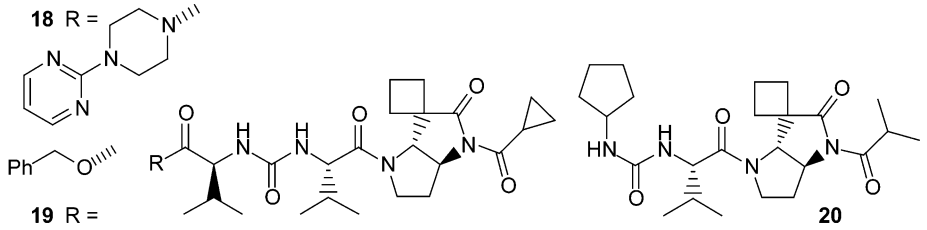
The scaffold of **2** tolerates small changes in several places. IC_{50} and replicon EC_{50} values of **3** and **4** are both below 10 nM [12]. When the carboxylate group in **3** is replaced by a cyclopropyl-acylsulfonamide moiety no loss in activity is observed. This latter class of macrocycles is further exemplified by **5**, which exhibits IC_{50} and EC_{50} values of 21 nM and 124 nM, respectively [13]. Note that an unprotected amino group and a shortened aliphatic linker are compatible with good potency. Also unconstrained proline-substituted tripeptides, **6**–**10**, retain activity. **6** represents a group of compounds wherein various bulky aliphatic substituents (e.g., *t*-butyl, cyclobutyl, cyclopentyl, and cyclohexyl) can be interchanged [13,14]. In this scaffold, the carboxylate group can be replaced by methyl- and phenyl-acylsulfonamide moieties. A wide range of six-membered and especially five-membered heterocycles, exemplified by **7** and **8**, can be used in conjunction with the acylsulfonamide grouping [13]. **9** and **10** are examples of a large group of compounds with both IC_{50} and EC_{50} below 100 nM in which the heterocyclic proline-substituent assumes a range of substituted condensed ring systems [15]. Studies with acylsulfonamide bearing hexapeptides demonstrated that a cluster of carboxylate groups at the N-terminus contributes greatly to inhibitory activity; **11** exhibited an IC_{50} value of 3.8 nM [16]. Among the non-covalent NS3 inhibitors, two non-peptidic natural products, **12** and **13**, showed submicromolar inhibition [17].

The class of covalent inhibitors is largely comprised of peptides bearing a reactive center at the cleavage site trapping the hydroxyl of Ser139, i.e., α-keto, aldehyde, boronic acid or lactam moieties. Most advanced is VX-950, **14**, which binds slowly and covalently ($K_i^* = 3$ nM, $T_{1/2} = 58$ min), yet reversibly to NS3 with a multistep mechanism. The replicon EC_{50} is 400 nM and a cell-based viral clearance assay showed 3 $\log_{10}$ reductions of viral RNA levels at concentrations above 3.5 μM [18]. With good oral bioavailability and high liver exposure, **14** is slated for clinical studies [18]. The co-crystal structure of **14** shows that it only partially overlaps with the region accessed by BILN-2061, which explains why **14** retains full activity against NS3 mutants (e.g., D168V) that are more than 1000-fold less sensitive to BILN 2061 [19]. Modification of the bicycloproline moiety and the N-terminal capping group as seen in **15**, yields potent derivatives ($IC_{50} < 500$ nM) [20]. Even the replacement of the cyclopentane moiety in **14** with the bulkier bicyclo[2.2.1]hept-2-ene is compatible with good potency [21]. Replacement of norvaline in **14** with difluoro-Abu and methylbenzyl instead of cyclopropyl yields potent anti-viral activity (replicon $IC_{50} = 630$ nM) [22]. Other examples of new α-keto-peptide inhibitors are **16** and **17** [23]. Compounds bearing only an aldehyde group replacing the entire α-keto-amide moiety also show *in vitro* activity in the low micromolar range [24]. Another class of covalent inhibitors is that of the pyrrolidine-*trans*-lactams, exemplified by **18** and **19**. The crystal structure of a smaller example showed that the serine hydroxyl opens the lactam ring, forming a hemiketal [25]. As older compounds in this series exhibited carbamate moieties at the N-terminus, newer designs favor the urea linker. There is crystallographic evidence that both amide hydrogens engage in hydrogen bonding to A157 [26]. **18** and **19** exhibit replicon IC_{50} values of 300 and 100 nM, respectively, although **18** was found to be fivefold more active in the enzyme assay [26]. Due to the chemical instability of the lactam moiety, compounds like **18** and **19** exhibit

undesirable, fast intravenous (IV) clearance rates. GW0014, **20**, however, although less potent, showed significantly improved *in vivo* pharmacokinetic properties and demonstrated anti-viral activity in a surrogate animal model with GBV-B infected marmosets following subcutaneous administration [27].







2.2. NS5b RNA dependent RNA polymerase

As an RNA virus, HCV must encode its own RNA dependent RNA polymerase, NS5b [28]. NS5b is essential for viral replication and uses single-stranded HCV RNA as a template to initiate *de novo* synthesis [29]. The enzyme contains a hydrophobic C-terminus which anchors the polymerase to the ER membrane in a replicase complex composed of viral and host proteins [30]. Under enzymatic screening conditions the hydrophobic C-terminus of NS5b causes several important assay complications. The solubility of expressed protein is decreased by the presence of this tail as well as shifts in K_m for both RNA template and nucleotide substrates (Table 1). The usual solution is to truncate this tail as either the Δ21 or Δ57 construct. N- or C-terminal His_6 tags as purification aids can further change the affinities. This has the effect, particularly in the case of some non-nucleoside inhibitors, of causing IC_{50} values to vary dramatically depending on the exact construct used (Table 1) [31].

Furthermore, the choice of homopolymeric vs. heteropolymeric templates with or without primers can further shift the substrate K_ms and consequently the inhibitor's

Table 1. Effecrt of NS5b enzyme constructs on kinetics and inhibition

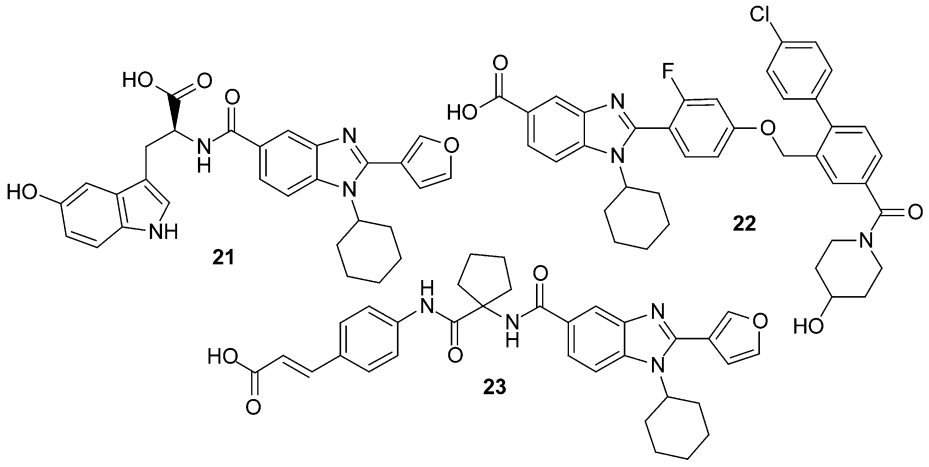
	HT[a]-NS5b	HT-NS5bΔ21	NS5bΔ57-HT	NS5bΔ21-HT	NS5b
K_m (P-T)[b] (nM)	210	58	34	25	25
K_m (UTP) (nM)	6200	12,000	1800	5200	3300
IC_{50} (nM)[c]	54	440	2200	3000	5700

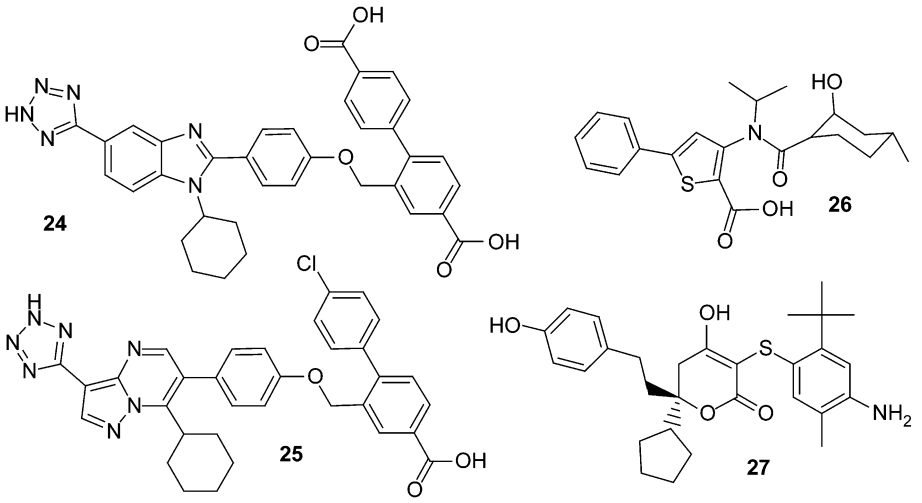
[a] His_6-tagged.
[b] Primer-template.
[c] IC_{50} of compound **21**.

IC_{50}. Therefore one must clearly understand the enzymatic systems of reported results to properly analyze and interpret SAR. Crystal structures of the enzyme reveal similarities to the classic polymerase right-handed model with fingers, thumb and the active site palm subdomains [32,33]. Thus the unique structure, *de novo* mechanism and the absence of a homologous mammalian enzyme should allow potent and selective anti-virals to be discovered. Anti-viral therapy strategies targeting this polymerase have been approached with parallel efforts in both nucleoside and non-nucleoside inhibitor discovery.

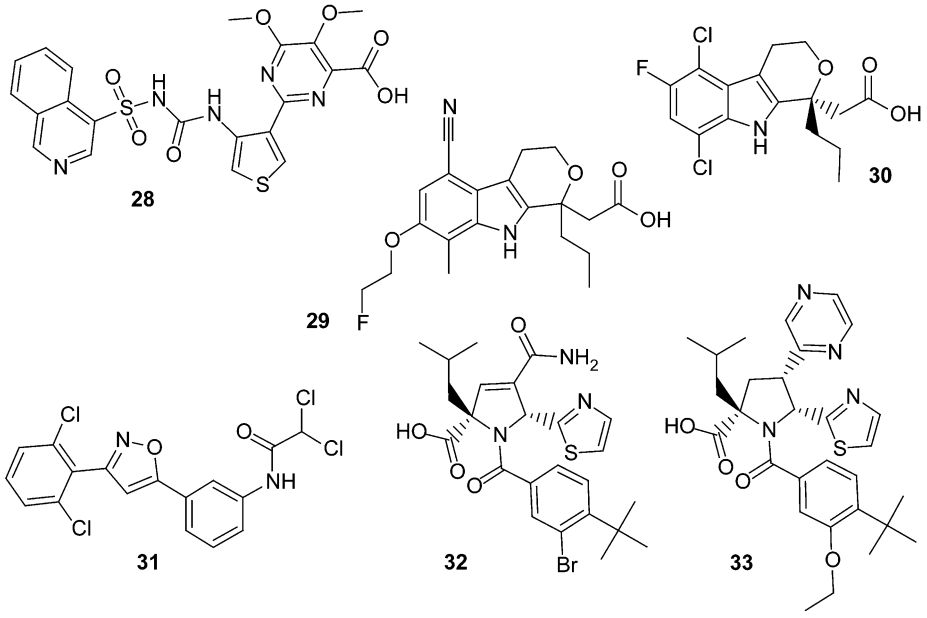
2.3. Non-nucleoside NS5b inhibitors

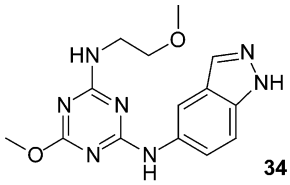
Benzimidazole containing compounds were some of the first non-nucleoside inhibitors discovered to be active against NS5b and to date represent the most potent. **22** was reported to be active at <0.01 μM [34] but under different enzyme assay conditions was reported at 0.28 μM [35] demonstrating the importance of understanding the enzyme and polymer constructs. Similarly, the initially reported IC_{50} of **21** using one enzyme construct was 0.054 μM but using a different construct resulted in a 0.25 μM IC_{50} [31,35]. Kinetic studies show that they are non-competitive with NTP and competitive with the RNA template [31]. Further kinetic analysis demonstrated that **21** and **22** were exclusive inhibitors [36]. Finally, **22** was active in the HCV replicon ($EC_{50} = 0.35$ μM) and multiple resistant replicon mutants generated to **22** displayed a common P495L/A mutation in NS5b [35]. This proline residue is located in a recently reported allosteric GTP binding site [32] and may represent the binding site for this benzimidazole class of compounds. Further optimization of this class of allosteric inhibitors has yielded the optimized analog **23** ($IC_{50} < 0.5$ μM) [37–39] and the tetrazole **24** ($IC_{50} = 0.01$ μM) [40]. Interestingly, replacement of the benzimidazole with a pyrazolo-pyrimidine ring and reversal of its relative orientation (6:5 to 5:6 ring system) resulted in active compounds such as **25** ($IC_{50} < 1$ μM) [41].





A second allosteric binding site has been reported with inhibitors bound to crystalized NS5b [42,43]. Further optimization of one of these two compounds led to the discovery of **26** (NS5b $IC_{50} = 0.7$ μM, replicon $EC_{50} = 0.2$ μM). The crystal structure reveals the cyclohexyl ring binding tightly in a deep hydrophobic pocket [44]. Similarly, **27** binds to the same site with the cyclopentyl group binding to the same pocket. Recently, analogs of **27** were reported with activities less than 1 μM [45,46].





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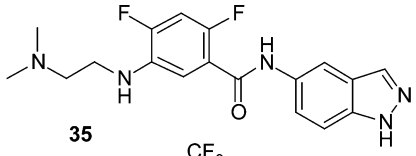

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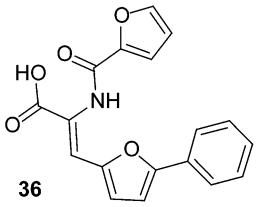

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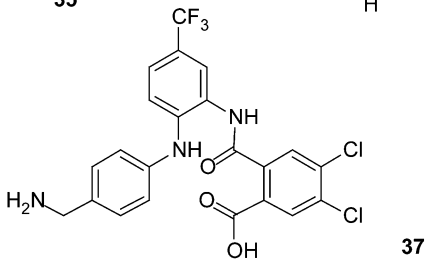

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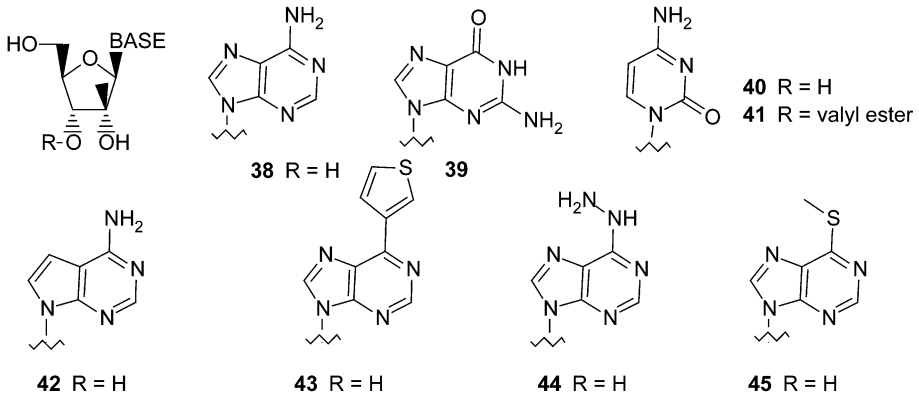
Other non-nucleoside inhibitors have been reported and characterized. A previously described benzothiadiazine (NS5b $IC_{50} = 0.08$ μM, replicon $EC_{50} = 0.5$ μM) was used to generate resistant replicons which mapped the key mutation to M414T, implicating a new inhibitor site on NS5b [47,48]. Similarly, a dimethoxypyrimidine analog **28** previously described [49] was used to generate resistant replicons containing either P156L or G152E mutations [47]. Interestingly, these mutations map the binding site of this compound to the active site, the first reported active-site non-nucleoside inhibitor. A non-nucleoside compound, HCV-371, was reported to have failed a phase I/II clinical trial [28,50]. While the compound was safe, it failed to reduce HCV RNA levels. However, follow-up compounds such as racemic **29** and (*R*)-pyranoindole **30** (NS5b $IC_{50} = 0.004$ and 0.06 μM, respectively) have recently been reported [51,52]. An analog of dichloroacetamide **31**, R803, is active in the replicon ($EC_{50} < 10$ μM) and is claimed to be an NS5b inhibitor [53]. Phase I results for this compound were reported and a phase I/II study scheduled to assess anti-viral efficacy [54]. Dihydropyrroles such as **32** (NS5b $IC_{50} < 5$ μM for best compounds) and related pyrrolidine analogs such as **33** (NS5b $IC_{50} < 0.3$ μM for best compounds) have been reported [55–57]. Triazine **34** (%inhibition at 0.1 μg/mL: HCV NS5b = 46% and HBV RT = 52%) and difluorobenzamide **35** (%inhibition at 0.1 μg/mL: HCV NS5b = 46% and HBV RT = 48%) are two reported promiscuous inhibitors with activity against both HCV NS5b and HBV RT [58,59]. Finally, compounds such as **36** (NS5b $IC_{50} < 10$ μM for best compounds) [60] and 1,2-diaminophenyl compound **37** (NS5b $IC_{50} = 1.42$ μM) have recently been disclosed [61].

2.4. Nucleoside NS5b inhibitors

Nucleoside anti-virals are pro-drugs in that they are actively transported into cells and then activated by cellular kinases to the nucleotide triphosphate. This NTP is now able to competitively inhibit the enzyme or, more commonly, act as a substrate and be incorporated into the nascent RNA chain. Chemical or structural features of

the incorporated nucleoside subsequently prevents (chain-terminates) the further replication of the viral genome. While nucleoside inhibitors can vary in either (or both) the ribose or base portion of the molecule, the initially reported anti-HCV inhibitors were modified ribose analogs such as 2′-fluoro [62], 4′-azido [63] and dioxolane cytidine analogs [64].

To date, the most significant amount of work has been reported on 2′-methylribose nucleosides. Adenosine (**38**, $IC_{50} = 1.9\ \mu M$, $EC_{50} = 0.17\ \mu M$) and guanosine (**39**, $IC_{50} = 0.13\ \mu M$, $EC_{50} = 1.4\ \mu M$) analogs inhibit both NS5b (as the NTP) and the HCV replicon [65]. Mechanism of action work clearly demonstrates that these nucleosides act as chain-terminators. Modeling of **38** into the RNA primer portion of the NS5b crystal structure suggests how these 3′-hydroxyl containing nucleosides are able to act as chain-terminators. The 2′-methylribose nucleoside is incorporated as a substrate but the 2′-methyl group prevents subsequent incorporation *via* steric clashes with either the O4′ or H8 of purines of the incoming nucleotide. Resistant replicons were generated against **38** and showed a S282T mutation in the NS5b sequence. When this mutation is modeled with **38** into the crystal structure, the addition of a methyl group to the wild-type serine (i.e., threonine) clearly prevents the incorporation of this nucleotide into the resistant mutant [65]. The cytidine analog (**40**) is claimed to be active *in vitro* as the active metabolite of its 3′-valyl ester NM-283 (**41**) [66,67] which is currently undergoing a phase Ib/phase IIa clinical trial. Initial data from a 50 patient study (doses from 25 to 800 mg) demonstrated an approximately 0.5 $\log_{10}$ reduction in HCV RNA levels after 100 mg once-daily dosing for 15 days. Clinical pharmacokinetics at both day 1 and 15 showed a half-life slightly longer than 4 h with no apparent accumulation [66,68].



Recently, base modified analogs containing 2′-methylribose have been reported, including the tubercidin (**42**) and 6-thiophene-purine (**43**) [69,70]. Multiple modified-base nucleosides including a hydrazine analog series of **44** and thiomethylpurine analog **45** were reported to have HCV replicon EC_{50}s less than 10 μM [71–74]. Mouse PK data for **45** demonstrated half-lives of 1.2 h and 0.43 h for oral and IV dosing, respectively, with no toxicity observed up to 160 mg/kg.

2.5. Other viral targets

HCV IRES inhibitors are mostly nucleic acid based molecules and no small molecule with promising activity has been reported. The only inhibitor that has demonstrated clinical effectiveness is ISIS 14803 (anti-sense oligonucleotide). In a phase II trial involving 43 patients with chronic hepatitis C, ISIS 14803 reduced viral loads in 7 patients from ≥ 1 to 3.8 $\log_{10}$ copies/ml during a 12-week treatment period [75]. Gene silencing by RNA interference is being explored as a new approach to inhibit HCV replication. Duplex small interfering RNAs directed at the HCV genome has been shown to be effective in dramatically inhibiting HCV replication and protein expression in human hepatoma cells [76]. *In vivo* application is likely to require gene delivery.

Despite certain efforts in targeting the HCV NS3 helicase, no compound that selectively and directly inhibits the NS3 ATP-hydrolyzing or nucleic acid-unwinding activities has been reported. Several nucleoside compounds including ring-expanded nucleosides **46**–**48** were synthesized and evaluated for their abilities to affect helicases of *Flaviviridae* viruses. Mixed results were obtained. The compounds can cause either enzyme inhibition or activation depending on the assay condition, source of the enzyme and nucleic acid template [77]. While these compounds may be interesting tools to elucidate putative allosteric sites on the helicase, their ability to inhibit HCV replication remains to be shown.

The HCV P7 protein forms an ion channel in lipid bilayers. It is not essential for viral replication, but it has been speculated that cation permeation across membranes may be important for the maturation and release of infectious virions. There is interest in evaluating P7 as an anti-viral target. Amantidine, **49**, an anti-influenza agent that inhibits the ion channel of influenza virus, inhibits the HCV P7 ion channel [78]. Amantidine has been tested clinically against HCV and as a single agent has not shown anti-viral activity; in combination with interferon or interferon and ribavirin has yielded mixed results [79,80]. Long-alkyl-chain iminosugar derivatives inhibit bovine viral diarrhea virus (BVDV). **50**–**52** were recently shown to inhibit the HCV P7 ion channel in black lipid membrane [81].

3. INHIBITORS FOR NON-VIRAL TARGETS

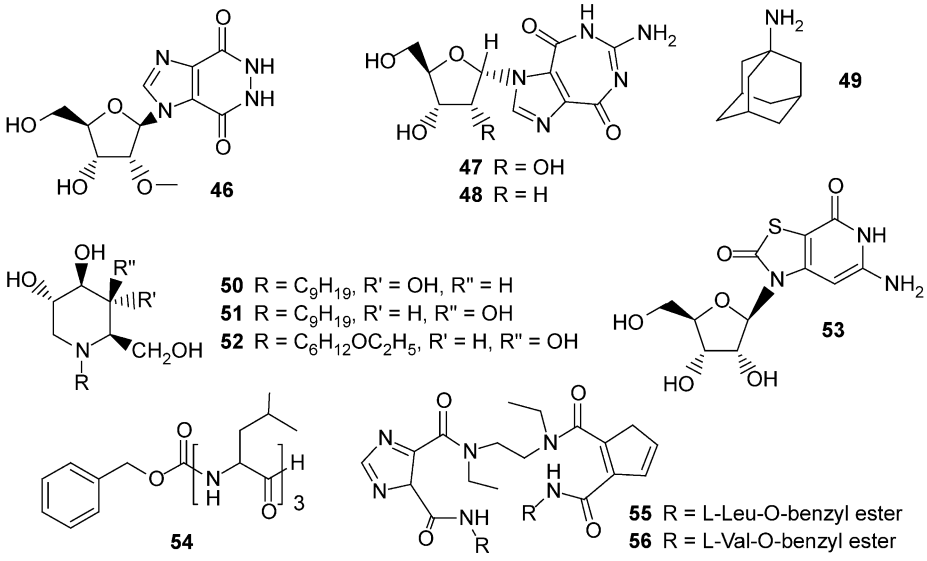
3.1. Caspase

Liver disease caused by conditions such as HCV infection is characterized by excessive apoptosis. Oxamyl dipeptide IDN-6556 is an anti-apoptotic irreversible caspase inhibitor with potencies against several caspases in the low to subnanomolar range [82]. In a phase IIa trial with chronic hepatits C patients, oral dosing of IDN-6556 for 14 days significantly lowered liver enzyme levels in plasma at all doses [83]. While IDN-6556 is not an anti-viral agent, it may help infected patients to preserve the function and health of their livers.

3.2. Toll-like receptor 7

Certain C8-substituted and N7, C8-substituted guanosine analogs stimulate immune responses. Some of these compounds activate toll-like receptor 7 (TLR7), a member

of a group of receptors that take part in the host defense against viruses. Isotoribine (**53**, ANA245) is a TLR7 agonist that is currently under evaluation in the clinic and so is its oral pro-drug ANA971 [84]. While it has no direct anti-viral activity, it induces hepatic 2′,5′-oligoadenylate synthetase (OAS) leading to activation of the innate immune response. In a phase Ib trial with a 7-day treatment by IV administration, the compound reduced viral load in patients with chronic HCV infection. Viral load reduction was maximal in the 800 mg arm at approximately $1-3\ \log_{10}$ copies/ml and was associated with induction of the OAS system [85].



3.3. Proteasome inhibitors

A fortuitous discovery recently implicated the human 20S proteasome subunit α-PSMA7 in HCV IRES mediated translation. In fact, a proteasome inhibitor MG132 **54** showed dose-dependent inhibition of HCV IRES activity in human hepatoma cells [86].

3.4. CD81

CD81 is a member of the tetraspanin family of integral membrane protein and is thought to be a binding partner for the HCV envelope E2 protein. Although binding alone is not enough for infection, prevention of binding does coincide with lack of infection in human hepatocytes. An extracellular region (D helix) on CD81 is important for binding to E2. Small molecule mimics **55**, **56** of the D helix inhibit binding of E2 to CD81 on Molt-4 cells [87]. Cellular activities of these compounds are unknown and may be difficult to determine since they require 6% DMSO for solubilization.

4. CONCLUSION

There has been a dramatic increase in patents and publications describing new HCV inhibitors last year as many research organizations have turned their attention to this important viral disease. Compounds which directly target viral specific proteins are beginning to enter clinical trials and the recent encouraging proof-of-principle results with BILN 2061 will hopefully mark the beginning of an era of new high efficacy treatments for HCV.

REFERENCES

[1] E. Szabo, G. Lotz, C. Paska, A. Kiss and Z. Schaff, *Pathol. Oncol. Res.*, 2003, **9**, 215.
[2] W. R. Kim, J. B. Gross, Jr., J. J. Poterucha, G. R. Locke, III and E. R. Dickson, *Hepatology*, 2001, **33**, 201.
[3] G. Lake-Bakaar, *Curr. Drug Targets. Infect. Disord.*, 2003, **3**, 247.
[4] S. Rosenberg, *J. Mol. Biol.*, 2001, **313**, 451.
[5] L. Pacini, A. Vitelli, G. Filocamo, L. Bartholomew, M. Brunetti, A. Tramontano, C. Steinkuhler and G. Migliaccio, *J. Virol.*, 2000, **74**, 10563.
[6] A. Lahm, A. Yagnik, A. Tramontano and U. Koch, *Curr. Drug Targets*, 2002, **3**, 281.
[7] Y. S. Tsantrizos, G. Bolger, P. Bonneau, D. R. Cameron, N. Goudreau, G. Kukolj, S. R. LaPlante, M. Llinas-Brunet, H. Nar and D. Lamarre, *Angew. Chem. Int. Ed.*, 2003, **42**, 1356.
[8] D. Lamarre, P. C. Anderson, M. Bailey, P. Beaulieu, G. Bolger, P. Bonneau, M. Boes, D. R. Cameron, M. Cartier, M. G. Cordingley, A. M. Faucher, N. Goudreau, S. H. Kawai, G. Kukolj, L. Lagace, S. R. LaPlante, H. Narjes, M. A. Poupart, J. Rancourt, R. E. Sentjens, R. St. George, B. Simoneau, G. Steinmann, D. Thibeault, Y. S. Tsantrizos, S. M. Weldon, C. L. Yong and M. Llinas-Brunet, *Nature*, 2003, **426**, 186.
[9] Y. Benhamou, HEP DART 2003, Kauai, HI, December 14–18, Abstract 025, 2003.
[10] Boehringer Ingelheim Pharmaceuticals, http://us.boehringer-ingelheim.com/about/pressreleases/102803_hepatitis.html (last accessed 02/10/04).
[11] E. Foy, K. Li, C. Wang, R. Sumpter, Jr., M. Ikeda, S. M. Lemon and M. Gale, Jr., *Science*, 2003, **300**, 1145.
[12] M. Llinas-Brunet and V. J. Gorys, *PCT Patent Appl.* WO 2003064455 A2, 2003.
[13] J. A. Campbell and A. C. Good, *PCT Patent Appl.* WO 2003053349 A2, 2003.
[14] M. Llinas-Brunet and V. J. Gorys, *PCT Patent Appl.* WO 2003064456 A1, 2003.
[15] X. A. Wang, L.-Q. Sun, S.-Y. Sit, N. Sin, P. M. Scola, P. Hewawasam, A. C. Good, Y. Chen and J. A. Campbell, *PCT Patent Appl.* WO 2003099274 A1, 2003.
[16] A. Johansson, A. Poliakov, E. Akerblom, K. Wiklund, G. Lindeberg, S. Winiwarter, U. H. Danielson, B. Samuelsson and A. Hallberg, *Bioorg. Med. Chem.*, 2003, **11**, 2551.
[17] V. R. Hegde, H. Pu, M. Patel, P. R. Das, N. Butkiewicz, G. Arreaza, V. P. Gullo and T. M. Chan, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2925.
[18] R. B. Perni, G. Chandorkar, P. R. Chaturvedi, L. F. Courtney, C. J. Decker, C. A. Gates, S. L. Harbeson, A. D. Kwong, C. Lin, Y. P. Luong, W. Markland, G. Rao, R. D. Tung and J. A. Thomson, 54th Annu. Meet. Am. Assoc. Study Liver Dis., October 24–28, Boston, MA, Abstract 972, 2003.
[19] C. Lin, K. Lin, C. A. Gates, D. Ma, D. Brennan, J. Fulghum, H.-M. Hsiao, G. Rao, Y. Wei, J. Alford, R. B. Perni and A. D. Kwong, 54th Annu. Meet. Am. Assoc. Study Liver Dis., October 24–28, Boston, MA, Abstract 1000, 2003.
[20] J. Pitlik, K. Cotrell, L. Farmer, R. Perni, J. Van Drie and M. Murcko, *PCT Patent* Appl. WO 2003087092 A2, 2003.
[21] R. B. Pernie, J. Pitlik, J. H. Van Drie and L. J. Farmer, *PCT Patent Appl.* WO 2003035060 A1, 2003.
[22] F. Victor, J. Lamar, N. Snyder, Y. Yip, D. Guo, N. Yumibe, R. B. Johnson, Q. M. Wang, J. I. Glass and S. H. Chen, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 257.

[23] W. Han, Z. Hu, X. Jiang, Z. R. Wasserman and C. P. Decicco, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1111.
[24] R. B. Perni, S. D. Britt, J. C. Court, L. F. Courtney, D. D. Deininger, L. J. Farmer, C. A. Gates, S. L. Harbeson, J. L. Kim, J. A. Landro, R. B. Levin, Y. P. Luong, E. T. O'Malley, J. Pitlik, B. G. Rao, W. C. Schairer, J. A. Thomson, R. D. Tung, J. H. Van Drie and Y. Wei, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4059.
[25] D. M. Andrews, S. J. Carey, H. Chaignot, B. A. Coomber, N. M. Gray, S. L. Hind, P. S. Jones, G. Mills, J. E. Robinson and M. J. Slater, *Org. Lett.*, 2002, **4**, 4475.
[26] M. J. Slater, E. M. Amphlett, D. M. Andrews, P. Bamborough, S. J. Carey, M. R. Johnson, P. S. Jones, G. Mills, N. R. Parry, D. O. Somers, A. J. Stewart and T. Skarzynski, *Org. Lett.*, 2003, **5**, 4627.
[27] D. M. Andrews, H. M. Chaignot, B. A. Coomber, M. D. Dowle, S. Lucy Hind, M. R. Johnson, P. S. Jones, G. Mills, A. Patikis, T. J. Pateman, J. E. Robinson, M. J. Slater and N. Trivedi, *Eur. J. Med. Chem.*, 2003, **38**, 339.
[28] J. Z. Wu and Z. Hong, *Curr. Drug Targets. Infect. Disord.*, 2003, **3**, 207.
[29] D. O'Farrell, R. Trowbridge, D. Rowlands and J. Jager, *J. Mol. Biol.*, 2003, **326**, 1025.
[30] J. Schmidt-Mende, E. Bieck, T. Hugle, F. Penin, C. M. Rice, H. E. Blum and D. Moradpour, *J. Biol. Chem.*, 2001, **276**, 44052.
[31] G. McKercher, P. L. Beaulieu, D. Lamarre, S. LaPlante, S. Lefebvre, C. Pellerin, L. Thauvette and G. Kukolj, *Nucl. Acids Res.*, 2004, **32**, 422.
[32] S. Bressanelli, L. Tomei, F. A. Rey and R. De Francesco, *J. Virol.*, 2002, **76**, 3492.
[33] C. A. Lesburg, R. Radfar and P. C. Weber, *Curr. Opin. Invest. Drugs*, 2000, **1**, 289.
[34] H. Hashimoto, K. Mizutani and A. Yoshida, *Eur. Patent Appl.* EP1162196 A1, 2001.
[35] L. Tomei, S. Altamura, L. Bartholomew, A. Biroccio, A. Ceccacci, L. Pacini, F. Narjes, N. Gennari, M. Bisbocci, I. Incitti, L. Orsatti, S. Harper, I. Stansfield, M. Rowley, R. De Francesco and G. Migliaccio, *J. Virol.*, 2003, **77**, 13225.
[36] D. Latour, C. Hancock, E. Michelotti, L. Lou, D.-F. Shi, C. D. Roberts, R. Griffith and J. J. Pouliot, HEP DART 2003, Kauai, HI, December 14–18, Abstract 032, 2003.
[37] P. L. Beaulieu, G. Fazal, G. Kukolj, E. Jolicoeur, J. Gillard, M. A. Poupart and J. Rancourt, *PCT Patent Appl.* WO 03010140 A2, 2003.
[38] P. L. Beaulieu, G. Fazal, S. Goulet, G. Kukolj, M. Poirier, Y. S. Tsantrizos, E. Jolicoeur, J. Gillard, M. A. Poupart and J. Rancourt, *PCT Patent Appl.* WO 03010141 A2, 2003.
[39] P. L. Beaulieu, G. Fazal, S. Goulet, G. Kukolj, M. Poirier and Y. S. Tsantrizos, *PCT Patent Appl.* WO 03007945 A1, 2003.
[40] E. S. Priestly, C. P. Decicco, T. W. Hudyma and X. Zheng, *PCT Patent Appl.* WO 03026587 A2, 2003.
[41] G. W. Shipps Jr., K. E. Rosner, J. Popovici-Muller, Y. Deng, T. Wang and P. J. Curran, *PCT Patent Appl.* WO 03101993 A1, 2003.
[42] R. A. Love, H. E. Parge, X. Yu, M. J. Hickey, W. Diehl, J. Gao, H. Wriggers, A. Ekker, L. Wang, J. A. Thomson, P. S. Dragovich and S. A. Fuhrman, *J. Virol.*, 2003, **77**, 7575.
[43] M. Wang, K. K. Ng, M. M. Cherney, L. Chan, C. G. Yannopoulos, J. Bedard, N. Morin, N. Nguyen-Ba, M. H. Alaoui-Ismaili, R. C. Bethell and M. N. James, *J. Biol. Chem.*, 2003, **278**, 9489.
[44] L. Chan, T. J. Reddy, M. Proulx, S. K. Das, O. Pereira, C. Yannopoulos, C. Poisson, N. Nguyen-Ba, L. Halab, M. Courchesne, C. Roy, R. Bethell, A. Siddiqui, W. Wang, M. Q. Zhang, M. David, M. Hamel, L. L'Heureux, J. Bedard, O. Nicolas, M. N. James, M. Wang, M. M. Cherney and K. K. Ng, 16th ICAR, Savannah, GA, April 27–May 1, Abstract 124, 2003.
[45] A. J. Borchardt and M. P. Goble, *PCT Patent Appl.* WO 03082848 A1, 2003.
[46] J. Gonzalez, A. J. Borchardt, P. S. Dragovich, T. M. Jewell, M. A. Linton, R. Zhou, H. Li, J. H. Tatlock, M. A. Abreo and T. J. Prins, *PCT Patent Appl.* WO 03095441 A1, 2003.
[47] G. Migliaccio, 16th ICAR, Savannah, GA, April 27–May 1, LB-12, 2004.
[48] T. T. Nguyen, A. T. Gates, L. L. Gutshall, V. K. Johnston, B. Gu, K. J. Duffy and R. T. Sarisky, *Antimicrob. Agents Chemother.*, 2003, **47**, 3525.
[49] C. Gardelli, C. Giuliano, S. Harper, U. Koch, F. Narjes, J. M. Ontoria Ontoria, M. Poma, S. Ponzi, I. Stansfield and V. Summa, *PCT Patent Appl.* WO 0206246 A1, 2002.

[50] Viropharma, http://www.irconnect.com/vphm/pages/news_releases.html?d = 42687 (last accessed 03/26/04).
[51] A. Gopalsamy, J. W. Ellingboe and T. S. Mansour, *PCT Patent Appl.* WO 03099824 A1, 2003.
[52] A. Gopalsamy, M. S. Collette, J. W. Ellingboe, M. G. Laporte, T. S. Mansour, C. J. Burns, K. Park and S. M. Condon, *PCT Patent Appl.* WO 03099275 A1, 2003.
[53] D. Goff, H. Lu, R. Singh, T. Sun and S. D. Issankani, *PCT Patent Appl.* WO 03040112, 2003.
[54] Rigel Pharmaceuticals, http://www.rigel.com/genomics/pr_1074900710 (last accessed 03/26/04).
[55] G. Bravi, H. S. Goodland, D. Haigh, C. D. Hartley, V. L. H. Lovegrove, P. Shah and M. J. Slater, *PCT Patent Appl.* WO 03037894 A1, 2003.
[56] G. Bravi, R. Guidetti, D. Haigh, C. D. Hartley, P. D. Howes, D. L. Jackson, V. L. H. Lovegrove, P. Shah, M. J. Slater and K. J. Wareing, *PCT Patent Appl.* WO 03037895 A1, 2003.
[57] G. Bravi, P. D. Howes, V. L. H. Lovegrove, P. Shah and M. J. Slater, *PCT Patent Appl.* WO 03037893 A1, 2003.
[58] Y. H. Chung, C. W. Lee, W. Y. Yang, H. D. Lee, W. J. Park, Y. Y. Chang and J. S. Lee, *PCT Patent Appl.* WO 02079187 A1, 2002.
[59] Y. H. Chung, C. W. Lee, W. Y. Yang, H. D. Lee, W. J. Park, Y. Y. Chang and J. S. Lee, *PCT Patent Appl.* WO 03002518 A1, 2003.
[60] H. Gao, M. L. Greene, R. J. Gross, R. A. Nugent and J. Pfefferkorn, *PCT Patent Appl.* WO 2004002977 A1, 2004.
[61] B. C. Finzel, L. A. Funk, R. C. Kelly, M. T. Reding and N. A. Wicnienski, *PCT Patent Appl.* WO 2004002944 A1, 2004.
[62] R. R. Devos, *PCT Patent Appl.* 6,660,721 B2, 2003.
[63] R. R. Devos, *PCT Patent Appl.* WO 2002100415 A2, 2002.
[64] R. Storer, *PCT Patent Appl.* WO 200132153 A2, 2001.
[65] G. Migliaccio, J. E. Tomassini, S. S. Carroll, L. Tomei, S. Altamura, B. Bhat, L. Bartholomew, M. R. Bosserman, A. Ceccacci, L. F. Colwell, R. Cortese, R. De Francesco, A. B. Eldrup, K. L. Getty, X. S. Hou, R. L. LaFemina, S. W. Ludmerer, M. MacCoss, D. R. McMasters, M. W. Stahlhut, D. B. Olsen, D. J. Hazuda and O. A. Flores, *J. Biol. Chem.*, 2003, **278**, 49164.
[66] J.-P. Sommadossi, *PCT Patent Appl.* WO 2004002422 A2, 2004.
[67] J.-P. Sommadossi, *PCT Patent Appl.* WO 2004003000 A2, 2004.
[68] Idenix Pharmaceuticals, http://www.sec.gov/Archives/edgar/data/1093649/000095013503006012/0000950135-03-006012.txt.
[69] S. S. Carroll, J. E. Tomassini, M. Bosserman, K. Getty, M. W. Stahlhut, A. B. Eldrup, B. Bhat, D. Hall, A. L. Simcoe, R. LaFemina, C. A. Rutkowski, B. Wolanski, Z. Yang, G. Migliaccio, R. De Francesco, L. C. Kuo, M. MacCoss and D. B. Olsen, *J. Biol. Chem.*, 2003, **278**, 11979.
[70] S. S. Carroll, R. L. Lafemina, D. L. Hall, A. L. Himmelberger, L. C. Kuo, M. Maccoss, D. B. Olsen, C. A. Rutkowski, J. E. Tomassini, H. An, B. Bhat, N. Bhat, P. D. Cook, A. B. Eldrup, C. J. Guinosso, M. Prhavc and T. P. Prakash, *PCT Patent Appl.* 02057425 WO, 2002.
[71] H. An and Y. Ding, *PCT Patent Appl.* WO 2003061576 A2, 2003.
[72] H. An, Y. Ding, S. Shaw and Z. Hong, *PCT Patent Appl.* WO 03062256 A1, 2003.
[73] Z. Hong, H. An, Y. Ding, J. L. Girardet and W. Zhong, *PCT Patent Appl.* WO 03062255 A2, 2003.
[74] H. An, Z. Hong, K. Smith, Y. Ding and J. L. Girardet, *PCT Patent Appl.* WO 03061385 A1, 2003.
[75] S. C. Gordon, B. R. Bacon, I. M. Jacobson, L. Shiffman, N. H. Afdhal, R. Z. Yu, J. G. McHutchison and J. K. Kwoh, 54th Annu. Meet. Am. Assoc. Study Liver Dis., October 24–28, Boston, MA, Abstract 312, 2003.
[76] T. Yokota, N. Sakamoto, N. Enomoto, Y. Tanabe, M. Miyagashi, S. Maekawa, M. Kurosaki, L. Yi, K. Taira, M. Watanabe and H. Mizusawa, *EMBO Rep.*, 2003, **4**, 602.

[77] N. Zhang, H. M. Chen, V. Koch, H. Schmitz, C. L. Liao, M. Bretner, V. S. Bhadti, A. I. Fattom, R. B. Naso, R. S. Hosmane and P. Borowski, *J. Med. Chem.*, 2003, **46**, 4149.
[78] S. D. Griffin, D. J. Rowlands, D. S. Clarke, J. Jaeger, L. P. Beales, M. P. Harris, O. Worsfold and S. D. Evans, *FEBS Lett.*, 2003, **535**, 34.
[79] G. Ideo, G. Antonucci, A. F. Atilli, S. Babudieri, A. Chirianni, A. Craxi, G. DiPerri, A. Francavilla, A. Mangia, A. Piccotto, M. Rizzetto, G. Ruggiero, F. Suter, S. Tripi and M. Sarracino, 54th Annu. Meet. Am. Assoc. Study Liver Dis., October 24–28, Boston, MA, Abstract 1186, 2003.
[80] E. J. Lawitz, N. S. Cantu, M. Davis, N. Afdahl, M. Curry, M. Maillard, F. Adams, N. Tsai, A. Gasic, T. Kothari, J. Cox, D. P. Ganeshappa, B. Silverman and S. C. Kadakia, 54th Annu. Meet. Am. Assoc. Study Liver Dis., October 24–28, Boston, MA, Abstract 997, 2003.
[81] D. Pavlovic, D. C. A. Neville, O. Argaud, B. Blumberg, R. A. Dwek, W. B. Fischer and N. Zitmann, *PNAS*, 2003, **100**, 6104.
[82] D. S. Karanewsky and R. J. Teranosky, *PCT Patent Appl.* US 6,199,750, 2001.
[83] D. Shapiro, E. Schiff, P. Pockros, M. Shiffman, J. McHutchison, A. Muir, R. Gish, N. Afdahl, M. Huyghe, D. Hecht and T. Oltersdorf, HEP DART, December 14–18, Kaudi, HI, Abstract 087, 2003.
[84] J. Lee, T. H. Chuang, V. Redecke, P. M. Pitha, D. A. Carson, E. Raz and H. B. Cottam, *PNAS*, 2003, **100**, 6646.
[85] B. Kerr, L. Bauman, W. Liaw, S. Webber, L. Kirkovsky, K. Steffy, S. Fletcher, V. Banh, J. Theiss, Y. Horsmans, J. P. Desager, T. Berg, T. Mueller, S. Worland and D. Averett, HEP DART, December 14–18, Kauki, HI, Abstract 063, 2003.
[86] M. Kruger, C. Berger, P. J. Welch, J. R. Barber, M. P. Manns and F. Wong-Staal, *Mol. Cell. Biol.*, 2001, **24**, 8657.
[87] S. E. VanCompernolle, A. V. Wiznycia, J. R. Rush, M. Dhanasekaran, P. W. Baures and S. C. Todd, *Virology*, 2003, **314**, 371.

Section 5
Topics in Biology

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London
United Kingdom

Pleiotropic Effects of Statins

Andres Floto,[1] John Overington,[2] Janet Allen,[2]

[1]*Cambridge Institute for Medical Research, Department of Medicine, University of Cambridge, School of Medicine, Cambridge CB2 2XY, UK*
[2]*Inpharmatica, 60 Charlotte Street, London W1T 2NU, UK*

Contents

1. INTRODUCTION

Elevation of serum LDL cholesterol is a major risk factor for the development of atherosclerosis and subsequent development of myocardial infarction. Cardiovascular disease is the leading cause of morbidity and mortality in the industrialised world and as such has a major impact on health economics. A variety of therapeutic approaches have been developed to lower serum cholesterol including bile acid sequesterants, cholesterol absorption inhibitors, nicotinic acid derivatives, fibrates and statins. Statins are drugs that inhibit the enzyme, 3-hydroxy-3 methylglutaryl-coenzyme A reductase (HMG-CoA reductase), which is a key step in the *de novo* synthesis of cholesterol. Reduced hepatocyte cholesterol concentrations trigger increased expression of hepatic LDL receptors which clear LDL and LDL precursors from the circulation. Given that statins still lower LDL in patients with homozygous familial hypercholesterolemia lacking

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functional LDL receptors, this indicates that statins can lower cholesterol by their action on the hepatic cholesterol biosynthetic pathway alone [1]. Large scale clinical trials have repeatedly demonstrated the efficacy of statins in lowering serum cholesterol in man and long term trials consistently demonstrate significant reductions in coronary heart disease and total mortality [2–4].

The widespread use of statins and the multitude of large clinical trials have suggested anecdotally that these drugs may have additional benefits to patients over and above those expected from only lowering serum LDL cholesterol. *In vitro* studies, initiated by these clinical observations, have frequently identified unanticipated activity of statins on cell types distinct from the liver and consistent with the additional benefit observed in trials. This article will review these non-hepatic, non-LDL cholesterol lowering effects of statins and discuss why the sometimes dramatic effects of statins in model systems do not necessarily translate back into man.

2. MECHANISM OF ACTION OF STATINS

Statins are competitive inhibitors of HMG-CoA reductase which converts HMG-CoA to mevalonate. This enzyme is recognised to be the rate limiting enzyme in the cholesterol biosynthetic pathway (Fig. 1). Analysis of the human genome has shown that there is only one gene for HMGCoA reductase which is found in all cell types. The enzyme is anchored in the endoplasmic reticulum through the amino terminal part of the protein which comprises 8 transmembrane spanning domains (Fig. 2).

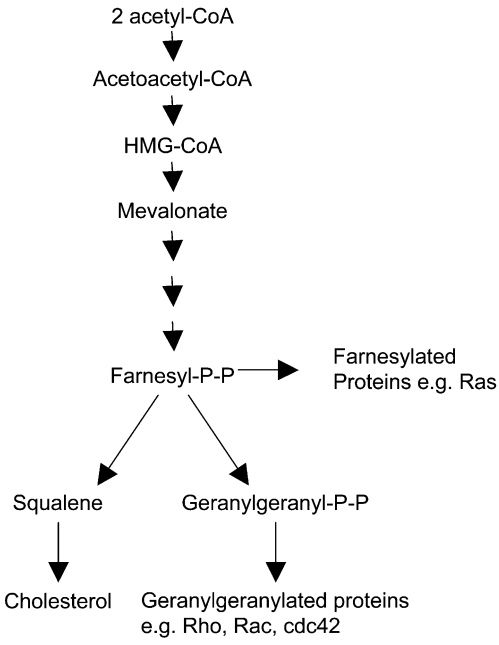

Fig. 1. Scheme for cholesterol biosynthetic pathway.

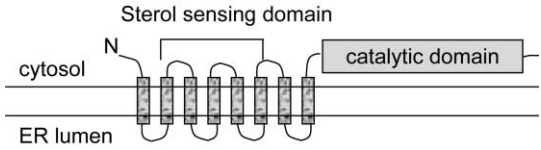

Fig. 2. Schematic depiction of HMG-CoA reductase enzyme.

The catalytic domain comprises the C terminal half of the protein and resides in the cytosol. Cellular levels of HMG-CoA reductase are highly regulated: high intracellular levels of sterols and nonsterol endproducts of mevalonate metabolism promote the ubiquitination and degradation of HMG-CoA reductase in the proteasome [5]. Of the 8 transmembrane domains of HMG-CoA reductase, 5 from the sterol sensing domain of the protein required to mediate the regulated degradation of the enzyme [6].

3. MECHANISTIC MULTIPLICITY OF STATIN EFFECTS

This mevalonate pathway is important not only for cholesterol biosynthesis but also for the formation of the isoprenoids and their precursors, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), both of which are involved in regulatory post-translational isoprenylation of proteins involved in many signal transduction pathways. For example, Ras is farnesylated at the C terminal CAAX box, whereas the Rho family members of small molecular weight GTPases, including Rho, Rac and cdc42, are modified by geranylgeranyl transferase. These proteins are key intracellular signalling molecules that play a pivotal role in regulating cellular activities such as cell polarity, intracellular vesicle trafficking, progression through cell cycle, differentiation, gene transcription, cell migration (chemotaxis) [7–9]. Isoprenylation of proteins alters subcellular localization and facilitates their association with membranes and downstream effectors such as the actin cytoskeleton and p160 Rho kinase [7–10]. Failure to isoprenylate these proteins impairs intracellular signalling functions. There is an increasing body of evidence that the effects of statins, not explained through their action on serum LDL cholesterol levels, may be due to altered isoprenylation.

Furthermore, there are additional mechanisms by which statins can alter signal transduction pathways by altering cholesterol levels within inflammatory cells. The majority of cholesterol and cholesterol esters are derived following uptake through specific transporters or receptors from the extracellular space. However, HMG-CoA reductase is required by every cell for the *de novo* synthesis of plasma membrane cholesterol which is an essential component of sphingolipid rafts [11,12]. These detergent-insoluble microdomains concentrate molecules involved in signal transduction enriched in these raft structures include GPCRs, heterotrimeric G proteins, ion channels, Src-family kinases and PI-3 kinases [13]. Disruption of these rafts by physical depletion of cholesterol uncouples receptors such as the T cell receptor (TCR), B cell receptor (BCR), IgG and IgE receptors from intracellular signalling cascades [14–18]. Inhibition of *de novo* synthesis of cholesterol would be expected to similarly disrupt raft function [11,19]. This may account for some of the direct effects of statins on membrane proteins

such as TCR, Fc receptors, GPCRs and ion channels [20–24]. Thus, simvastatin and lovastatin have been found to relax rat basilar artery precontracted with either 5HT or high potassium. Electrophysiology of the basilar artery demonstrated that lovastatin acts to directly inhibit L-type calcium current [23]. In a separate study, cerivastatin was found to activate human endothelial calcium-activated potassium channels (BK(Ca)). Cerivastatin caused significant membrane hyperpolarization at very low concentrations (1–50 nM) [24].

An additional mode of action of statins is the direct interaction with cell surface molecules. Lovastatin was identified from a screening programme aimed at finding molecules that specifically interfered with LFA-1 [25]. Subsequent crystallography has shown that lovastatin binds to LFA-1 at a distance from its adhesion site but that the drug inhibits LFA-1:ICAM-1 interactions and leukocyte adherence. Other statins are active in the same assays but the SAR differs to that observed for HMG-CoA reductase [26].

4. NON-LDL CHOLESTEROL LOWERING ACTIONS OF STATINS

The efficacy of statins in lowering serum LDL cholesterol is well-recognised [2–4]. The widespread clinical use of statins has led to observations that statins have additional effects not explicable through their primary role of lowering serum LDL cholesterol. Initial clinical trial data in patients with hypercholesterolemia indicated beneficial effects of statins on cardiovascular parameters beyond that expected for their ability to directly lower LDL cholesterol [3,27–31]. There is now clear-cut evidence that statins act locally on the vascular wall to directly reduce inflammation. Additional anecdotal clinical data has emerged from trials to suggest that statins benefit other disease processes. Epidemiological data hinted that statins may influence the incidence and severity of transplant rejection episodes, progression of Alzheimers dementia (AD), the incidence of hip fracture and, possibly neoplasia (see below). Although the clinical trial data remains equivocal, studies to explore the underlying action of statins in these conditions have unearthed novel mechanisms consistent with and supportive of the initial clinical observations.

5. INFLAMMATION

5.1. Atherosclerosis

The improvement in cardiovascular endpoints in many of these trials was superior to estimations calculated from the effect on LDL cholesterol loading [3,27–31]. The clinical improvement was observed faster than was explicable on the basis of lowering serum LDL cholesterol levels compared to other cholesterol lowering therapies. Statins were found to reduce the incidence of stroke despite the fact that serum LDL is not an important risk factor for this condition [27–31]. In addition, there is increasing evidence for benefit in patients with atherosclerotic thrombo-embolic conditions in terms of plaque stabilization [32]. These effects are so striking that the criteria for statin use are constantly being re-evaluated to include patients with vascular risk such as carotid stenosis,

intermittent claudication and vascular complications linked to type II diabetes [33–36]. For particular patients at risk, such as those with type II diabetes mellitus, the recommendation is now that treatment with statins be based on their vascular score rather than their serum cholesterol levels [37].

How were statins having these effects in man? This has been the subject of intense investigation and has led to the concept for a pleiotropic role of statins in cardiovascular tissues where statins act to stop the inflammation process [1,38–43]. Evidence that statins influence the inflammatory component of cardiovascular disease has been provided by trials by measuring markers such as CRP, interleukin 6 (IL-6), soluble ICAM-1, soluble phospholipase-2 (sPLA2) [44–47]. The beneficial effect on these markers was frequently observed shortly after starting statin treatment mirroring the clinical improvements in vascular status which occurred more rapidly than expected from lowering cholesterol alone. These studies are on-going and will have important implications for selection of patients for statin treatment [48–50].

The relationship of cholesterol and inflammation to the development of atherosclerotic lesions is well-recognised and is not within the scope of this review. Briefly, macrophages laden with cholesterol become foam cells which are a hallmark of the atherosclerosis. Many different cell types of the vascular wall and immune systems including Th1 lymphocytes and myeloid cells have been demonstrated to play a role in atherosclerosis. Focus has been on the relationship of monocytes to the vascular endothelium as a principal determinant in the development of atherosclerosis. Thus, chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) have been implicated in promoting migration of monocytes to arterial intima. In the arterial intima, monocytes differentiate into macrophages and these accumulate cholesterol esters to form foam cells. Macrophages secrete pro-inflammatory cytokines resulting in the increased surface expression of adhesion molecules on the endothelial cells. This in turn recruits more monocytes to the lesion. These cells also secrete growth factors, which stimulate vascular smooth muscle proliferation, and matrix metalloproteinases (MMP-2 and MMP-9) leading to matrix degradation and unstable plaques. Accepted clinical markers of atherosclerosis now include the classic markers of inflammation such as C reactive protein (CRP) and cytokines [44–50]. Laser dissected macrophage-rich shoulder regions of the atherosclerotic plaques have been analysed for gene expression and compared to normal human intima. A number of inflammatory genes were found to be upregulated [51]. This study also demonstrated that HMG-CoA reductase enzyme expression levels were increased in these shoulder regions of the atherosclerotic plaques [51].

Experimental work has demonstrated that statins intervene at many different levels in the inflammatory process. The overall cumulative effect is to reduce the development of atherosclerotic lesions, to increase local blood flow by increased local production of nitric oxide and to stabilise plaques by reducing their liability for rupture. In addition, they may have local actions on the thrombotic process. Histopathology of plaques from patients with carotid stenosis has shown that statin treatment confers benefit even after a relatively short treatment period. In one study, patients with carotid stenosis were treated with pravastatin (40 mg/day) for 3 months and, in another study, with simvastatin (40 mg/day) for 4 months prior to endarterectomy [52,53]. Surgical samples were analysed for markers of atherosclerosis and compared to samples from non-statin treated patients.

In both studies, there were significant improvements in the histological and biochemical markers of atherosclerosis. Thus, in the pravastatin study, the plaques had less lipid as judged by oil red O staining, less oxidised LDL, fewer macrophages, fewer T cells, less MMP-2 and increased collagen content. In the second study, plaques from the statin treated patients demonstrated reduced lipid and oxidised LDL content, increased collagen content, fewer macrophages, T lymphocytes and HLA-DR+ cells and reduced immunoreactivity for inducible cyclooxygenase (COX-2) and PGE synthase. In both studies, the amount of collagen was reported to be increased. As plaque rupture is believed to result from thinning of the collagen, fibrous cap of plaques, this data provides histological evidence for plaque stabilisation by statins.

The effect of statins on the individual cellular components that comprise the atherosclerotic lesion has also been studied in some detail. The major cellular players mediating this inflammatory process are the endothelium, vascular smooth muscle cells (VSMC) and immune cells such as monocytes/macrophages, T cells and dendritic cells.

5.1.1. Endothelial cells

Local production of cytokines increases the surface expression of various adhesion molecules on endothelial cells which promotes the adherence of circulating cells such as monocytes. Thus, statins have been demonstrated to inhibit the cell surface expression of ICAM-1, E-selectin, MHC class II and CD40 on human endothelial cells [54–57]. Intravital microscopy of rat mesenteric microvasculature superfused with thrombin has shown that statin treatment (rosuvastatin 1.25 mg/kg) attenuated leukocyte rolling, adherence and transmigration [58]. Rosuvastatin treatment was associated with a 70% reduction in E-selectin expression on the endothelial cells [58]. Using genome-wide microarrays to analyse gene expression patterns in human endothelial cells (HUVEC), statins (either 6 μM atorvastatin or 1.1 μM pitavastatin) were found to suppress the expression of both IL-8 and MCP-1 [59]. Statins also suppress expression of endothelin-1 (ET-1) and elevate endothelial type III nitric oxide synthase (eNOS) which synthesizes the vasodilator, nitric oxide, NO [59,60]. The combined effect of suppressing endothelin and increasing eNOS activity will increase local blood flow and explains some of the beneficial effects of statins observed in both man and experimental animals. Thus, in man, simvastatin has been demonstrated to increase endothelium-dependent vasodilation [61]. This response was observed within one month of starting treatment. In an experimental model of stroke, statins were found to be neuroprotective [62,63]. This effect of statins was attributed to its activity on eNOS as the neuroprotection conferred by statins was lost in eNOS deficient mice [62]. Using cultured bovine aortic endothelial cells, NO release was increased 4-fold following treatment with statins [64]. This effect of statins on local NO production by the endothelium explains observations that these drugs protect vascular beds against ischemia (coronary, cerebral, renal and lung) independent of LDL cholesterol as protection is still observed in normocholesterolemic animals [63].

Oxidised LDL and hypoxia are both factors that reduce eNOS expression and reduction is reversed by statins [60,65,66]. The ability of statins to reverse the inhibition of eNOS by oxidised LDL is further clear evidence for a role for these drugs independent of their action on the liver to lower LDL cholesterol. Endothelial NOS activity is

regulated by phosphorylation by the kinase, Akt, and by the small GTPase, Rho which negatively regulates expression and activity of eNOS in endothelial cells. Thus, constitutively active Rho down-regulates expression of eNOS [67,68]. A number of studies indicate that statins act to disinhibit eNOS regulation by Rho by limiting the formation of GGPP (Fig. 1) and, thereby, prevent geranylgeranylation of Rho. Thus, treatment of human endothelial cells with mevastatin *in vitro* increased eNOS protein and mRNA levels [68]. Co-treatment with either mevalonate or GGPP reversed the action of mevastatin whereas FPP or LDL had no effect. Consistent with the concept that the isoprenylation drives the subcellular localization of Rho and its interaction with effector proteins, mevastatin inhibited both Rho translocation and GTP binding activity [68].

5.1.2. Vascular smooth muscle cells (VSMC)

Proliferation and migration of VSMC are key events in atherogenesis. VSMC proliferation has been shown to be inhibited by statins *in vitro* and in animal models [69]. In addition, statins have been shown to promote apoptosis of VSMC [70]. In man, statins decrease restenosis after successful angioplasty and, in animal models of vascular injury, statins reduce arterial neointimal thickening [71–73]. Mevalonate completely reversed the effect of statins both *in vivo* and *in vitro*. The mechanism of action of statins is again thought to be via the inhibition of formation of GGPP and the failure to geranylgeranylate Rho [74]. The Rho family of proteins play an important role in regulating cell cycle progression through activity of the cyclin dependent kinase (CDK) inhibitors, p27 and p21 [7–9]. Rho has been shown to mediate PDGF-induced cell cycle progression in human VSMC *in vitro* by down-regulating the Cdk inhibitor, $p27^{Kip1}$ and stimulating the activity of Cdk-2 [75]. In addition, for successful cell division, an actin myosin contractile band constricts to separate the two daughter cells. Active Rho and cdc42 are required for this process of cytokinesis [7,8]. Statins by inhibiting the production of GGPP prevent these actions of Rho and thereby inhibit both cell cycle progression and cell division. Lovastatin has been shown to arrest cells in G1 through the induction of cyclin-dependent kinase (Cdk) inhibitors, p27 and p21, thereby disinhibiting the effects of Rho on cell cycle control. Mevalonate releases arrested cells from lovastatin induced G1 block.

5.1.3. Immune cells

Migration of T cells and monocytes into vascular tissue is a key feature of the progressive inflammatory process that underlies atherosclerosis. As detailed above, endarterectomy samples from patients treated with either simvastatin or pravastatin exhibited fewer macrophages and T cells compared to the non-statin treated control samples [52,53]. Adhesion and chemotaxis of monocytes into the vessel wall is mediated through cell surface adhesion molecules. There are numerous studies *in vitro* and *in vivo* showing consistently that statins inhibit expression of cell surface adhesion molecules on circulating immune cells or endothelial cells and the production of chemokines and cytokines [76,77]. Statins have been shown to inhibit expression of ICAM-1, P-selectin, CD40, CD11 and LFA-1. In hypercholesterolemic patients, simvastatin (20 mg/day) treatment for 8 weeks reduced monocyte expression of TNFα and IL-1β by 49 and 35%,

respectively [67]. Many studies have documented the *in vitro* effect of statins in causing a reduction in pro-inflammatory cytokines such as IL-6, IL-1β, IL-8, TNFα and chemokines such as MCP-1 [78]. Statins have been found to inhibit T cell function *in vitro* in man [20]. In addition, statins have been found to inhibit the maturation of dendritic cells and interfere with their ability to present antigen to T cells and cause T cell activation [79,80].

5.1.4. Plaque rupture

Plaque rupture leads to adverse cardiovascular events such as stroke and myocardial infarction. The main component that prevents rupture is the collagen-rich fibrous cap. Activated macrophages in these lesions secrete a variety of inflammatory agents including proteases, such as MMP-2 and MMP-9, which degrade collagen. Statins have been shown to reduce production of MMP-9 and MMP-2 by macrophages *in vitro* and as detailed above, *in vivo* [52,53,81].

5.2. Transplant rejection

Early clinical studies suggested that statin treatment conferred benefit to transplant patients by altering the incidence and severity of transplant rejection episodes particularly for patients receiving cardiac transplants. These reports co-incided with the experimental data as outlined above, that statins were modulating the inflammatory response. As Th1 lymphocytes play a key role in transplant rejection, the beneficial effect on transplant rejection was taken as further evidence for an immunomodulatory role for statins [85]. Randomized trials have demonstrated that use of statins in heart transplant patients significantly lowers mortality. However, it seems increasingly that the benefit of statins after cardiac transplantation reflects the effect of these drugs on the development of coronary vasculopathy although some studies report benefit of stains in severe rejection episodes [82–84]. This vasculopathy is associated with high levels of plasma CRP and levels are lower in patients treated with statins [86,87]. In a mouse model of transplant rejection, the effect of cerivastatin was tested on the rejection parameters in the mouse cardiac heterotopic transplant model. Cerivastatin was placed in the drinking water but plasma levels in the mouse ranged from 10–22 nM. Cerivastatin was found to reduce graft arterial disease and this was associated with a significant decrease in graft-infiltrating cells and levels of expression of the chemokines, RANTES and MCP-1, in the graft [88].

The effect of statins on transplant rejection episodes has been studied for other allografts. Thus, in one study, the incidence of rejection was evaluated in patients who received lung transplants and were treated with statins for hypercholesterolemia and compared to the patients who did not receive statins. In this study, the nature of the statin treatment was not standardised. This study demonstrated a markedly improved 6-year survival rate amongst the patients treated with statins compared to the control group [89]. Histological parameters of inflammation showed benefit in the statin treated group in terms of immune cell lung infiltration in broncho-alveolar lavage, in particular the appearance of inflammatory neutrophils, and in the development of obilterative

bronchiolitis. Measurements of lung function were similarly improved in the statin treated group compared to the control group. The data for renal transplants is variable. Pravastatin has been shown to reduce the incidence of acute rejection, being 25% in the treated group compared to 58% in the control group. In this same study, *in vitro* studies demonstrated that statin treatment was assoicated with a decrease in T cell mediated cytotoxicity [90]. In another study, patient survival was improved in statin treated patients compared to controls [91]. However, in a large randomised trial of 364 renal transplant patients, fluvastatin (40 mg/day) was found to have no effect on the incidence or severity of acute rejection [92].

5.3. Arthritis

Collagen-induced arthritis (CIA) in rodents is an accepted model for rheumatoid arthritis (RA) in man. Two injections of type II collagen separated by 21 days results in the onset of a classic Th1 lymphocyte driven arthritis: inflammation becomes apparent 5–10 days after the second dose of collagen. The ability of simvastatin to modulate the disease process was tested in both a prophylactic protocol, where the drug treatment started before the second dose of collagen, and in a therapeutic protocol, where the drug was administered after the development of the arthritis [93]. In both cases, simvastatin suppressed the incidence and severity of the arthritis. In the therapeutic group, a significant reduction in arthritis progression was observed with 3 days of starting treatment. In both groups, plasma levels of IL-6 were significantly reduced. The studies demonstrated that simvastatin specifically suppressed Th1 cytokine (TNFα, IL-12 and IL-6) responses to collagen and the serum levels of anti-type II collagen antibodies indicating that B cell responses were also suppressed in these animals. The effective dose of simvastatin in this study was 40 mg/kg being considerably higher than that used in man. Plasma levels in the mice were not measured. In the same study, the effect of simvastatin was tested *in vitro* on PBMC and synovial fluid from patients with RA. Simvastatin (10 μM) reduced T cell proliferation in response to TCR cross-linking, release of IFN-γ and the ability of T cells to activate macrophages.

Published phase IV clinical trial data for patients with hypercholesterolemia has not highlighted any unexpected beneficial effects on the severity of arthritis. However, in one very limited open trial, benefit was reported in RA patients treated with simvastatin (40 mg/day) [94].

5.4. Multiple sclerosis

This is an auto-immune inflammatory disease mediated through Th1 lymphocytes resulting in demyelination within the central nervous system. The effect of atorvastatin on a mouse model, experimental autoimmune encephalitis (EAE), was studied [95]. Atorvastatin treatment (1–10 mg/kg) was found to prevent both the onset of the disease and also to modify established disease. In this model, secretion of pro-inflammatory cytokines, IL-2, IL-12, IFN-γ and TNFα, was suppressed. Treatment of microglia with atorvastatin suppressed up-regulation of Class II MHC complexes by IFN-γ.

The suggestion from this study is that atorvastatin has pleiotropic effects on the immune process to modify the auto-immune response to antigen in the development of EAE by intervening at many different steps of the process. In a similar study, lovastatin was found to reduce the severity and duration of EAE by acting on both T cells and on antigen presenting cells (APC) [96]. In an additional study, statins were shown to inhibit proliferation of PBMCs derived from patients with multiple sclerosis and to down-regulate MMP-9, chemokine receptors and adhesion molecules [97]. Curiously, in two separate studies in mice, statins promoted differentiation of Th0 lymphocytes towards a Th2 phenotype [95,98].

Plasma concentrations of statins were not measured in these studies. However, the findings were striking enough to stimulate a number of formal clinical trials for statin treatment in multiple sclerosis [99,100]. In one very recent report, simvastatin (80 mg) was given to 30 patients with relapsing-remitting multiple sclerosis in an open study to assess the effect of statins on gadolinium-enhancing lesions by MRI at 4, 5 and 6 months of treatment [100]. In this study, improvement in was observed in both volume and number of lesions over the 6 month treatment course suggesting benefit, however, there was no placebo control group in this study.

5.5. Asthma

Th2 lymphocytes are thought to play a key role in the initiation and maintenance of the inflammatory response in asthma. The standard mouse model of allergic asthma is the ovalbumin (OVA) challenge model where acute eosinophilic airway inflammation is measured. Simvastatin (40 mg/day) was found to suppress eosinophil recruitment to the lung in response to OVA in animals pre-sensitised to the protein [101]. Both eosinophils and macrophage lung infiltrates were reduced and levels of the Th2 cytokines, IL-3 and IL-5, in the bronchoalveolar fluid were reduced. *In vitro*, simvastatin inhibited OVA specific secretion of IL-4 and IL-5. As a general marker of inflammation, IL-6 levels were also decreased in the mice. In this study, in contrast to the CIA and EAE models, statin treatment did not significantly reduce antigen induced cell proliferation.

5.6. Glomerulonephritis

Auto-immune inflammation of the renal glomeruli results in renal failure. A number of studies have shown that statins inhibit the inflammatory process and the progression of renal disease [102]. This is in part through the action of statins on Th1 lymphocytes and in part due to the local action of statins on mesangial cells in the glomerulus [98,103].

6. OSTEOPOROSIS

A role for statins in regulating bone metabolism has been suggested from some clinical trial data. The mixed results coming from clinical trials may, in part, be a result of the fact that the data is derived from a secondary analysis of existing trials where patient entry

selection was based on hypercholesterolemia. The patient population is very varied as patient recruitment and monitoring was not specifically aimed at measuring bone parameters or outcomes. The incidence of fractures is influenced dramatically by factors such as age and sex. Smaller and shorter trials have been completed in post-menopausal women but often the trial size or duration has been insufficient to come to a firm conclusion.

Initial studies implied that there was a reduced risk of fracture in patients treated with statins compared to their controls [104]. In this study, the history of fractures in women over the age of 60 years was related to the statin dispensing history. Statins appeared to be protective, particularly amongst women treated for 2 years or more. No clear-cur protection was observed in patients with shorter history of statin use. Other studies have been published that associate statin treatment with a reduced rate of fracture [105]. A smaller study of 36 women measured mean bone density at the femoral neck and found that this was increased significantly during 15 months statin treatment [106]. At the same time, other clinical studies have failed to demonstrate this association; in particular, secondary analysis of the LIPID study showed no difference in fractures occurring in the pravastatin group compared to the controls [107]. Thus, observational studies have suggested that the risk of hip fracture is lower among older women taking statins but secondary analysis of the large cardiovascular clinical trials do not necessarily support this. On a smaller scale, studies have been reported examining specifically bone parameters in man; again with conflicting results [108–110].

Statins have been shown to influence bone formation in animal models and *in vitro*. Osteoblasts promote bone formation and osteoclasts cause bone resorption and both aspects of bone metabolism may be influenced by statins. Of particular interest is the action of statins on osteoblasts, as therapeutic agents targeted at this cell type, promote bone formation and are likely to have a profound influence on osteoporosis. Bone Morphogenic Proteins (BMPs) are a family of differentiation factors that have bone-forming activity and account for the osteo-inductive activity found in bone extracts. An increase in BMP activity would therefore promote bone formation. In a screening programme to identify small molecules that enhance BMP-2 transcription, a collection of 30,000 natural products or drugs were screened. Lovastatin was identified as a potent and specific stimulator of the BMP-2 transcription [111]. Lovastatin and simvastatin were subsequently shown to induce BMP-2 expression in osteoblasts and stimulate bone formation *in vitro* and *in vivo* [112]. In MC3T3-E1 cells, a mouse clonal osteoblastic primary cell line, simvastatin was found to promote osteoblast differentiation and mineralization as measured by the induction of BMP-2 and bone alkaline phosphatase and by the accumulation of bone matrix proteins, such as type I collagen [112]. In this *in vitro* study, simvastatin was found to be active at concentrations in the range of 10–100 nM. Using primary human osteoblasts, pitavastatin was found to increase expression of BMP-2 and osteocalcin. This stimulation by pitavastatin was abolished by addition of mevalonate or GGPP. Furthermore, pitavastatin treatment prevented Rho translocation following receptor activation. Results using these cells implied that Rho kinase inhibits bone formation and that statins, by preventing Rho activity, act as dis-inhibitors [113]. In animal studies, simvastatin (20 mg/kg) was found to induce an increase in cortical bone formation and reduce the loss of cancellous bone induced by ovariectomy, consistent with activity of statins in both osteoblasts and osteoclasts [114].

7. DEMENTIA

Epidemiological studies have suggested that statin treatment in man may be beneficial in the treatment of dementia [115–119]. This has been extensively reviewed in previous years [120]. As with all epidemiological data which relies on historical data, the answer is not clear-cut and a number of prospective studies are now underway. An added complexity for analysing trial data for a role of statins in treating dementia is their variable penetration across the blood brain barrier. The etiology of Alzheimers-type dementia is complex but recognised to be associated with an accumulation of the amyloid β peptide (Aβ) which is neurotoxic. Aβ is formed through the cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. An issue relating to the retrospective studies for efficacy of statins in modifying disease progression for dementia is that the pathophysiology leading to dementia can vary enormously and statins can act through different mechanisms dependent on the nature of the underlying cause. Thus, trial design and patient selection criteria for measuring the influence of statins on cardiovascular outcome may not be suitable for subsequently analysing effects specifically on AD. For example, vascular disease is a common cause of dementia in the elderly population. By improving vascular status and, thereby, blood flow, statins may independently improve dementia scores through the mechanisms relating to their impact on the vascular wall described above. In addition, an inflammatory component for AD has been described and NSAIDs have been reported to be beneficial. Statins may have benefit through their anti-inflammatory activity, such as has been observed for NSAIDs [120].

In effort to examine Alzheimer dementia directly, the effect of statin treatment on plasma levels of Aβ have been measured although it is debatable as to whether plasma levels *per se* correlate with dementia. The results are not clear cut. In one study, lovastatin was found to reduce Aβ levels significantly by up to 40% [121]. In another study, 39 patients were treated with increasing doses of either simvastatin or atorvastatin reaching a final dose of 80 mg/day and then maintained at this level for 24 weeks. Plasma levels of both Aβ (1–40) and Aβ (1–42) were unchanged [122].

There is a complex relationship between serum cholesterol, serum lipoproteins and the development of dementia in man and animal studies [123,124]. Thus, transgenic mice overexpressing a mutant form of APP develop neuritic plaques. This process is accelerated when the mice are fed a high cholesterol diet and treatment of mice with atorvastatin reduces appearance of plaques by about 50% [125]. However, *in vitro* data, points to a role for intraneuronal cellular cholesterol pools in regulating APP processing. Depletion of cholesterol from primary rat hippocampal neurones expressing APP resulted in a reduction of Aβ formation [126]. The mechanism underlying this was proposed to relate to the partitioning of APP and β-secretase into the cholesterol-sphingolipid rich membrane microdomains known as lipid rafts.

Further evidence for a role of statins in modulating disease processes has come from studies of transmissible spongiform encephalopathies (TSE) characterised by Creutzfeld-Jacob disease, scrapie and bovine spongiform encephalopathy (BSE). Neurodegeneration follows the accumulation of protease-resistant prion protein (PrP) aggregates. Screening of a cell model of scrapie for molecules that influenced the production of aggregated protease-resistant PrP resulted in the identification of lovastatin, out of a collection of 2,000 drugs, as active in this model. Subsequent dose responses demonstrated that

lovastatin inhibited the model with an IC_{50} of 500 nM [127]. In a separate study, lovastatin (300 nM) was found to inhibit the PrP processing in the same cells. The effect was attributed to the depletion of cellular cholesterol by lovastatin [128].

8. CANCER

Previous epidemiological studies have pointed to an association between low serum cholesterol levels and the incidence of gastro-intestinal cancers, in particular colorectal cancer [129]. This topic is still controversial [130,131]. However, this data taken together with the increasing evidence that statins can act as immunomodulators, in both animal models and *in vitro*, and the role of statins in reversing hypoxic inhibition of eNOS, thereby promoting increased local blood flow through eNOS activity, have raised concerns over the long term use of statins in terms of immune surveillance for tumours. However, large scale prolonged clinical trials have given no evidence for an association of statin use with cancer [132].

As discussed above, statins have been shown to cause cell cycle arrest and induce apoptosis of VSMC through the inhibition of geranylgeranylation of Rho, thereby disinhibiting the effect of Rho on Cdk inhibitors, p21 and p27 [7–9,74,75]. The role of Rho-family proteins in mediating intracellular signalling pathways involved in cell cycle progression, cytokinesis and cell migration/motility makes this family an attractive target for oncology [7–9,133,134]. Thus, the potential for statins to interfere with the isoprenylation of Rho-family proteins in cells has been studied and a number of observations reported that indicate that statins *in vitro* cause cell cycle arrest and change the behaviour of cells in invasion models such as Boyden chambers [135,136].

9. TARGETING NON-HEPATIC HMG-COA REDUCTASE

Statins are now widely used in clinical practice and have proven to be well-tolerated and safe. As detailed above, the widespread use of statins has led to observations of anecdotal benefit unrelated to their inhibition of hepatic HMG-CoA reductase. The principal adverse effects are recognised to be liver and muscle toxicity [137]. Elevated hepatic transaminase has been observed in 0.5–2% of cases and is dose-dependent being reversible with a reduction in dose. Myopathy is recognised to be the major significant adverse effect for statins. This is frequently a non-specific, mild myalgia comprising muscle aches and cramps without elevation of creatine kinase. Rarely, statin treatment can lead to a myositis with muscle soreness and weakness associated with an elevated creatine kinase. This can lead to rhadomyolysis with myoglobinuria and acute renal necrosis. This side effect, although very rare, resulted in the voluntary withdrawal of cerivastatin in 2001. Understanding the mechanism of myopathy has proven difficult because it is rare. However, the effect does appear to relate to higher dose levels of the drugs.

In efforts to reduce the risk of myopathy, statins are increasingly targeted specifically to the liver via uptake systems to maximise effects on hepatic HMG-CoA reductase and minimise systemic exposure [138–142]. It seems likely that with increasing success of this approach, the opportunity to identify non-hepatic, non-LDL cholesterol effects will

diminish, both through empirical observation and directed trials. This factor may already account for the variability in the results from some clinical trials. Taken together, these previous data suggest that there may be further opportunity for chemistry optimisation of statin class drugs achieving higher systemic exposure, to exploit these potential novel mechanisms, assuming that the mechanism behind muscle toxicity can be understood and avoided. Alternatively, there exists the possibility of delivering statins at high relative concentrations to local sites and optimising the structure of the statin to ensure it is rapidly metabolised to an inactive form, either on exposure to the systemic circulation, or through efficient clearance on first pass metabolism, i.e., a 'soft statin' approach. Clinical conditions amenable to this strategy include asthma, allergic rhinitis or dermatitis, psoriasis, or even arthritis.

REFERENCES

[1] D. J. Maron, S. Fazio and F. L. Linton, *Circulation*, 2000, **101**, 207.
[2] B. M. Cheung, I. J. Lauder, C. P. Lau and C. R. Kumana, *Br. J. Pharmacol.*, 2004, **57**, 640.
[3] F. M. Sacks, M. A. Pfeffer, L. A. Moye, J. L. Rouleau, J. D. Rutherford, T. G. Cole, L. Brown, J. W. Warnica, J. M. Arnold, C. C. Wun, B. R. Davis and E. Braunwald, *N. Engl. J. Med.*, 1996, **335**, 1001.
[4] J. C. LaRosa, J. He and S. Vupputuri, *JAMA*, 1999, **282**, 2340.
[5] R. Y. Hampton and H. Bhakta, *Proc. Natl Acad. Sci. USA*, 1997, **94**, 12944.
[6] N. Sever, B. L. Song, D. Yabe, J. L. Goldstein, M. S. Brown and R. A. DeBose-Boyd, *J. Biol. Chem.*, 2003, **278**, 52479.
[7] S. Etienne-Manneville and A. Hall, *Nature*, 2002, **420**, 629.
[8] K. Burridge and K. Wennerberg, *Cell*, 2004, **116**, 167.
[9] A. J. Ridley, *Trends Cell Biol.*, 2001, **11**, 471.
[10] G. Molnar, M. C. Dagher, M. Geiszt, J. Settleman and E. Ligeti, *Biochemistry*, 2001, **40**, 10542.
[11] F. Schroeder, A. M. Gallegos, B. P. Atshaves, S. M. Storey, A. L. McIntsh, A. D. Petrescu, H. Huang, O. Starodub, H. Chao, H. Yang, A. Frelov and A. B. Kier, *Exp. Biol. Med.*, 2001, **226**, 873.
[12] M. Dykstra, A. Cherukuri, H. W. Sohn, S. J. Tzeng and S. K. Pierce, *Annu. Rev. Immunol.*, 2003, **21**, 457.
[13] L. J. Foster, C. L. De Hoog and M. Mann, *Proc. Natl Acad. Sci. USA*, 2003, **100**, 5813.
[14] T. Magee, N. Pirinen, J. Adler, S. N. Pagakis and I. Parmryd, *Biol. Res.*, 2002, **35**, 127.
[15] A. E. Schade and A. D. Levine, *J. Immunol.*, 2002, **168**, 2233.
[16] M. A. Alonso and J. Millan, *J. Cell Sci.*, 2001, **114**, 3957.
[17] H. Kono, T. Suzuki, K. Yamamoto, M. Okada, T. Yamamoto and Z. Honda, *J. Immunol.*, 2002, **169**, 193.
[18] T. Harder, *Curr. Opin. Immunol.*, 2004, **16**, 353.
[19] C. Kirsch, G. P. Eckert and W. E. Mueller, *Biochem. Pharmacol.*, 2003, **65**, 843.
[20] D. Z. Hillyard, A. J. Cameron, A. H. McIntyre, M. H. Hadden, H. E. Marshall, N. Johnston and A. G. Jardine, *Clin. Exp. Pharmacol. Physiol.*, 2002, **29**, 673.
[21] D. H. Nguyen and D. Taub, *Blood*, 2002, **99**, 4298.
[22] K. M. O'Connell, J. R. Martens and M. M. Tamkun, *Trends Cardiovasc. Med.*, 2004, **124**, 37.
[23] A. Bergdahl, E. Persson, P. Hellstrand and K. Sward, *Pharmacol. Toxical.*, 2003, **93**, 128.
[24] C. R. Kuhlmann, C. Gast, F. Li, M. Schafer, H. Tillmanns, B. Waldecker and J. Wiecha, *J. Am. Soc. Nephrol.*, 2004, **15**, 868.
[25] J. Kallen, K. Welzenbach, P. Ramage, D. Geyl, R. Kriwacki, G. Kegge, S. Cottens, G. Weitz-Schmidt and U. Hommel, *J. Mol. Biol.*, 1999, **292**, 1.
[26] G. Weitz-Schmidt, K. Welzenbach, V. Brinkmann, T. Kamata, J. Kallen, C. Bruns, S. Cottens, Y. Takada and U. Hommel, *Nat. Med.*, 2001, **7**, 687.

[27] J. Shepherd, S. M. Cobbe, I. Ford, C. G. Isles, A. R. Lorimer, P. W. MacFarlane, J. H. McKillop and C. J. Packard, *N. Engl. J. Med.*, 1995, **333**, 1301.
[28] Prospective Studies Collaboration, *Lancet*, 1995, **346**, 1647.
[29] J. F. Plehn, B. R. Davis, F. M. Sacks, J. L. Rouleau, M. A. Pfeffer, V. Bernstein, T. E. Cuddy, L. A. Moye, L. B. Piller, J. Rutherford, L. M. Simpson and E. Braunwald, *Circulation*, 1999, **99**, 216.
[30] P. M. Ridker, N. Rifai, M. A. Pfeffer, F. M. Sacks, L. A. Moye, S. Goldman, G. C. Flaker and E. Braunwald, *Circulation*, 1998, **98**, 839.
[31] J. W. Jukema, A. V. Bruschke, A. J. van Boven, J. H. Reiber, E. T. Bal, A. H. Zwinderman, H. Jansen, G. J. Boerma, F. M. van Rappard and K. I. Lie, *Circulation*, 1995, **91**, 2528.
[32] P. Libby and M. Aikawa, *Am. J. Cardiol.*, 2003, **20**, 4B–8B.
[33] C. D. Furberg, H. P. Adams, W. B. Applegate, B. P. Byington, M. A. Espeland, T. Harwell, D. B. Hunninghake, D. S. Lefkowitz, J. Prostfield and W. A. Riley, *Circulation*, 1994, **90**, 1679.
[34] H. Iso, D. R. Jacobs, D. Wentworth, J. D. Neation and J. D. Cohen, *N. Eng. J. Med.*, 1989, **320**, 904.
[35] S. Warshafsky, D. Packard, S. J. Marks, N. Sachdeva, D. M. Terashita, G. Kaufman, K. Sang, A. J. Deluca, S. J. Peterson and W. H. Frishman, *J. Gen. Intern. Med.*, 1999, **14**, 763.
[36] R. Collins, J. Armitage, S. Parish, P. Sleigh, R. Peto and Heart Protection Study Collaborative Group, *Lancet*, 2003, **361**, 2005.
[37] V. Snow, M. D. Aronson, E. R. Hornblake, C. Mottur-Pilson and K. B. Weiss, Clinical Efficacy Assessment Subcommittee of American College of Physicians, *Ann. Intern. Med.*, 2004, **140**, 644.
[38] A. S. Wierzbicki, R. Poston and A. Ferro, *Pharmacol. Ther.*, 2003, **99**, 95.
[39] G. J. Blake and P. M. Ridker, *Curr. Control. Trials Cardiovasc. Med.*, 2000, **1**, 161.
[40] C. Novela and C. H. Hennekens, *J. Cardiovasc. Pharmacol. Ther.*, 2004, **9**, 61.
[41] B. R. Kwak, F. Mulhaupt and F. Mach, *Autoimmun. Rev.*, 2003, **2**, 332.
[42] J. C. Mason, *Clin. Sci.*, 2003, **105**, 251.
[43] W. Marz and W. Koenig, *J. Cardiovasc. Risk*, 2003, **10**, 169.
[44] S. Kinlay, G. G. Schwartz, A. G. Olsson, N. Rifai, S. J. Leslie, W. J. Sasiela, M. Szarek, P. Libby and P. Ganz, Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering Study Investigators, *Circulation*, 2003, **108**, 1560.
[45] W. Marz, K. Winkler, M. Nauck, B. O. Bohm and B. R. Winkelmann, *Am. J. Cardiol.*, 2003, **92**, 305.
[46] M. A. van de Ree, M. V. Huisman, H. M. Princen, A. E. Meinders and C. Kluft, *Atherosclerosis*, 2003, **166**, 129.
[47] C. C. Case and C. M. Ballantyne, *Curr. Atheroscler. Rep.*, 2002, **4**, 42.
[48] M. A. Albert, E. Danielson, N. Rifai and P. M. Ridker, PRINCE Investigators, *JAMA*, 2001, **286**, 64.
[49] M. A. Albert, J. Staggers, P. Chew and P. M. Ridker, PRINCE Investigators, *Am. Heart J.*, 2001, **141**, 893.
[50] P. Libby and P. M. Ridker, *Am. J. Med.*, 2004, **116** (Suppl. 6A), 9S.
[51] T. T. Tuomisto, A. Korkeela, J. Rutanen, H. Viita, J. H. Brasen, M. S. Riekkinen, T. T. Rissanen, K. Karkola, Z. Kiraly, K. Kolble and S. Yla-Herttuala, *Arterioscler. Thromb. Vasc. Biol.*, 2003, **23**, 2235.
[52] F. Cipollone, M. Fezia, A. Iezzi, M. Zucchelli, B. Pini, D. De Cesare, S. Ucchino, F. Spigonardo, G. Bajocchi, R. Bei, R. Muraro, L. Artese, A. Piattelli, F. Chiarelli, F. Cuccurullo and A. Mezzetti, *Circulation*, 2003, **107**, 1479.
[53] M. Crisby, G. Nordin-Fredriksson, P. K. Shah, J. Yano, J. Zhu and J. Nilsson, *Circulation*, 2001, **103**, 926.
[54] T. Nubel, W. Dippold, H. Kleinert, B. Kaina and G. Fritz, *FASEB J.*, 2004, **18**, 140.
[55] Y. Dimitrova, S. Dunoyer-Geindre, G. Reber, F. Mach, E. K. Kruithof and P. De Moerloose, *J. Thromb. Haemost.*, 2003, **1**, 2290.
[56] U. Schonbeck, N. Gerdes, N. Varo, R. S. Reynolds, D. B. Horton, U. Bavendicek, L. Robbie, P. Ganz, S. Kinlay and P. Libby, *Circulation*, 2002, **106**, 2888.

[57] B. Kwak, F. Mulhaupt, N. Veillard, G. Pelli and F. Mach, *Swiss Med. Wkly*, 2001, **131**, 41.
[58] T. J. Stalker, A. M. Lefer and R. Scalia, *Br. J. Pharmacol.*, 2001, **133**, 406.
[59] S. Morikawa, W. Takabe, C. Mataki, T. Kanke, T. Itoh, Y. Wada, A. Izumi, Y. Saito, T. Hamakubo and T. Kodama, *J. Atheroscler. Thromb.*, 2002, **9**, 178.
[60] O. Hernandez-Perera, D. Perez-Sala, J. Navarro-Antolin, R. Sanchez-Pascuala, G. Hernandez, C. Diaz and S. Lamas, *J. Clin. Invest.*, 1998, **101**, 2711.
[61] G. O'Driscoll, D. Green and R. R. Taylor, *Circulation*, 1997, **95**, 1126.
[62] M. Endres, U. Laufs, Z. Huang, T. Nakamura, P. Huang, M. A. Moskowitz and J. K. Liao, *Proc. Natl Acad. Sci. USA*, 1998, **95**, 8880.
[63] U. Laufs, K. Gertz, P. Huang, G. Nickenig, M. Bohm, U. Dirnagl and M. Endres, *Stroke*, 2000, **31**, 2442.
[64] M. B. Harris, M. A. Blackstone, S. G. Sood, C. Li, J. M. Goolsby, V. J. Venema, B. E. Kemp and R. C. Venema, *Am. J. Physiol. Heart Circ. Physiol.*, 2004, **286**.
[65] J. K. Liao, W. S. Shin, W. Y. Lee and S. L. Clark, *J. Biol. Chem.*, 1995, **270**, 319.
[66] U. Laufs, V. La Fata and J. K. Liao, *J. Biol. Chem.*, 1997, **272**, 31725.
[67] U. Laufs, *Eur. J. Clin. Pharmacol.*, 2003, **58**, 719.
[68] U. Laufs and J. K. Liao, *J. Biol. Chem.*, 1998, **273**, 24266.
[69] R. Baetta, E. Donetti, C. Comparato, M. Calore, A. Rossi, C. Teruzzi, R. Paoletti, R. Fumagalli and M. R. Soma, *Pharmcol. Res.*, 1997, **36**, 115.
[70] C. Guijarro, L. M. Blanco-Colio, M. Ortego, C. Alonso, A. Ortiz, J. J. Plaza, C. Diaz, G. Hernandez and J. Edigo, *Circ. Res.*, 1998, **83**, 490.
[71] R. Sahni, A. R. Maniet, G. Voci and V. S. Banka, *Am. Heart J.*, 1991, **121**, 1600.
[72] D. H. Walter, V. Schachinger, M. Elsner, S. Mach, W. Auch-Schwelk and A. M. Zeiher, *Am. J. Cardiol.*, 2000, **85**, 962.
[73] M. R. Soma, E. Donetti, C. Parolini, G. Mazzini, C. Ferrari, R. Fumagalli and R. Paoletti, *Arterioscler. Thromb.*, 1993, **13**, 571.
[74] M. R. Soma, C. Parolini, E. Donetti, R. Fumagalli and R. Paoletti, *J. Cardiovasc. Pharmacol.*, 1995, **25** (suppl. 4), S20.
[75] U. Laufs, D. Marra, K. Node and J. K. Liao, *J. Biol. Chem.*, 1999, **274**, 21926.
[76] S. Niwa, T. Totsuka and S. Hayashi, *Int. J. Immunopharmacol.*, 1996, **18**, 669.
[77] D. Ferro, S. Parrotto, S. Basili, C. Alessandri and F. Violi, *J. Am. Coll. Cardiol.*, 2000, **36**, 427.
[78] M. Romano, L. Diomede, M. Sironi, L. Massimiliano, M. Sottocorno, N. Polentarutti, A. Guglielmotti, D. Albani, A. Bruno, P. Fruscella, M. Salmona, A. Vecchi, M. Pinza and A. Mantovani, *Lab. Invest.*, 2000, **80**, 1095.
[79] D. Sun and G. Fernandes, *Cell Immunol.*, 2003, **223**, 52.
[80] A. Yilmaz, C. Reiss, O. Tantawi, A. Weng, C. Stupf, D. Raaz, J. Ludwig, T. Berger, A. Steinkasserer, W. G. Daniel and C. D. Garlichs, *Atherosclerosis*, 2004, **172**, 85.
[81] Z. Luan, A. J. Chase and A. C. Newby, *Arteriscler. Thromb. Vasc. Biol.*, 2003, **23**, 769.
[82] F. Mach, *Transpl. Immunol.*, 2002, **9**, 197.
[83] K. Wenke, B. Meiser, J. Thiery, D. Nagel, W. von Scheidt, K. Krobot, G. Steinbeck, D. Seidel and B. Reichart, *Circulation*, 2003, **197**, 93.
[84] M. Weis, S. Pehlivanli, B. M. Meiser and W. Von Scheidt, *J. Am. Coll. Cardiol.*, 2001, **38**, 814.
[85] J. A. Kobashigawa, S. Katznelson, H. Laks, J. A. Johnson, L. Yeatman, X. M. Wang, D. Chia, P. I. Teraski, A. Sabad and G. A. Cogert, *N. Engl. J. Med.*, 1995, **333**, 621.
[86] A. Hognestad, K. Endressen, R. Wergeland, O. Stokke, O. Geiran, T. Holm, S. Simonsen, J. K. Kjekshus and A. K. Andreassen, *J. Am. Coll. Cardiol.*, 2003, **42**, 477.
[87] M. S. Eisenberg, H. J. Chen, M. K. Warshofsky, R. R. Sciacca, H. S. Wasserman, A. Schwartz and L. E. Rabbani, *Circulation*, 2000, **102**, 2100.
[88] K. Shimizu, M. Aikawa, K. Takayama, P. Libby and R. N. Mitchell, *Circulation*, 2003, **108**, 2113.
[89] B. A. Johnson, A. T. Iacono, A. Zeevi, K. R. McCurry and S. R. Duncan, *Am. J. Resp. Care Med.*, 2003, **167**, 1271.
[90] S. Katznelson and J. A. Kobashigawa, *Kidney Int.*, 1995, **48**, S112.

[91] F. G. Cosio, T. E. Pesavento, R. P. Pelletier, M. Henry, R. M. Fergusin, S. Kim and S. Lemeshow, *Am. J. Kidney Dis.*, 2002, **40**, 638.
[92] H. Holdaas, A. G. Jardine, D. C. Wheeler, I. B. Brekke, P. J. Conlon, B. Fellstrom, A. Hammad, I. Holme, H. Isoniemi, R. Moore, P. A. Rowe, P. Sweny, D. A. Talbot, J. Wadstrom and O. Ostraat, *Kidney Int.*, 2001, **60**, 1990.
[93] B. P. Leung, N. Sattar, A. Crilly, M. Prach, D. W. McCarey, H. Payne, R. Madhok, C. Campbell, J. A. Gracie, F. Y. Liew and I. B. McInnes, *J. Immunol.*, 2003, **170**, 1524.
[94] C. Abud-Mendoza, H. de la Fuente, E. Cuevas-Orta, L. Baranda, J. Cruz-Rizo and R. Gonzalez-Amaro, *Lupus*, 2003, **12**, 607.
[95] S. Youssef, O. Stuve, J. C. Patarroyo, P. J. Ruiz, J. L. Radosevich, E. M. Hur, M. Bravo, D. J. Mitchell, R. A. Sobel, L. Steinman and S. S. Zamvil, *Nature*, 2002, **420**, 78.
[96] N. Nath, S. Giri, R. Prasad, A. K. Singh and I. Singh, *J. Immunol.*, 2004, **172**, 1273.
[97] O. Nerhaus, S. Strasser-Fuchs, F. Fazekas, B. C. Kleseier, G. Niederwieser, H. P. Hartung and J. J. Archelos, *Neurology*, 2002, **59**, 990.
[98] R. Hakamada-Taguchi, Y. Uehara, K. Kuribayashi, A. Numabe, K. Saito, H. Negoro, T. Fujita, T. Toyo-oka and T. Kato, *Circ. Res.*, 2003, **93**, 948.
[99] C. H. Polman and B. M. Uitdehaag, *Lancet Neurol.*, 2003, **2**, 563.
[100] T. Vollmer, L. Key, V. Duralski, W. Tyor, J. Corboy, S. Markovic-Plese, J. Preuningerova, M. Rizzo and I. Singh, *Lancet*, 2004, **363**, 1607.
[101] A. McKay, B. P. Leung, I. B. McInnes, N. C. Thomson and F. Y. Liew, *J. Immunol.*, 2004, **172**, 2903.
[102] R. Coppo and A. Amore, *Pediatr. Nephrol.*, 2004, **19**, 256.
[103] C. Blume, D. Sabuda-Widemann, J. Pfeilschifter, J. Plum, K. Schror, B. Grabensee and K. F. Beck, *Eur. J. Pharmacol.*, 2004, **485**, 1.
[104] K. A. Chan, S. E. Andrade, M. Boles, D. S. M. Buist, G. A. Chase, J. G. Donaghue, M. J. Goodman, J. H. Gurwitz, A. Z. LaCroix and R. Platt, *Lancet*, 2000, **355**, 2185.
[105] D. C. Bauer, G. R. Mundy, S. A. Jamal, D. M. Black, J. A. Cauley, K. E. Ensrud, M. van der Klift and H. A. Pols, *Arch. Intern. Med.*, 2004, **164**, 146.
[106] Y.-S. Cheung, M.-D. Lee, S.-K. Lee, H.-M. Kim and L. A. Fitzpatrick, *J. Clin. Endocrinol. Metab.*, 2000, **85**, 1137.
[107] I. R. Reid, W. Hague, J. Emberson, J. Baker, A. Tonkin, D. Hunt, S. MacMahon and N. Sharpe, *Lancet*, 2001, **357**, 509.
[108] L. Rejnmark, N. H. Buus, P. Vestergaard, F. Andreasan, M. L. Larsen and L. Mosekilde, *Eur. J. Clin. Invest.*, 2002, **32**, 581.
[109] J. A. Pasco, M. A. Kotowicz, M. J. Henry, K. M. Saunders and G. C. Nicholson, *Arch. Intern. Med.*, 2002, **162**, 537.
[110] A. Z. LaCroix, J. A. Cauley, M. Pettinger, J. Hsia, D. C. Bauer, J. McGowan, Z. Chen, C. E. Lewis, S. G. McNeeley, M. D. Passaro and R. D. Jackson, *Ann. Intern. Med.*, 2003, **139**, 97.
[111] I. R. Garrett and G. R. Mundy, *Arthritis Res.*, 2002, **4**, 237.
[112] T. Maeda, A. Matsunuma, T. Kawane and N. Horiuchi, *Biochem. Biophys. Res. Commun.*, 2001, **280**, 874.
[113] K. Ohnaka, S. Shimoda, H. Nawata, H. Shimokawa, K. Kaibuchi, Y. Iwamaoto and R. Takayangi, *Biochem. Biophys. Res. Commun.*, 2001, **287**, 337.
[114] H. Oxlund and T. T. Andreassen, *Bone*, 2004, **34**, 609.
[115] B. Wolozin, W. Kellman, P. Rousseau, G. G. Celesia and G. Siegel, *Arch. Neurol.*, 2000, **57**, 1439.
[116] H. Jick, G. L. Zomberg, S. S. Jick, S. Seshadri and D. A. Drachman, *Lancet*, 2000, **356**, 1627.
[117] K. Yaffe, E. Barrett-Connor, F. Lin and D. Grady, *Arch. Neurol.*, 2002, **59**, 378.
[118] M. Etminan, S. Gill and A. Samii, *Pharmacotherapy*, 2003, **23**, 726.
[119] E. Zamrini, G. McGwin and J. M. Roseman, *Neuroepidemiology*, 2004, **23**, 94.
[120] R. B. Nelson, in *Ann. Rep. Med. Chem.* (ed. A. M. Doherty), Elsevier, 2002, Vol. 37, p. 197.
[121] B. Wolozin, *Biochem. Soc. Trans.*, 2002, **30**, 525.
[122] K. Hoglund, O. Wiklund, H. Vanderstichele, O. Eikenberg, E. Vanmechelen and K. Blennow, *Arch. Neurol.*, 2004, **61**, 333.
[123] J. Poirier, *Trends Mol. Med.*, 2003, **9**, 94.

[124] B. Wolozin, *Neuron*, 2004, **41**, 7.
[125] L. M. Refolo, M. A. Pappolla, B. Malester, J. La Francois, T. Bryant-Thomas, R. Wang, G. S. Tint, K. Sambamurti and K. Duff, *Neurobiol. Dis.*, 2000, **7**, 321.
[126] M. Simons, P. Keller, B. De Strooper, K. Beyreuther, C. G. Dotti and K. Simons, *Proc. Natl Acad. Sci. USA*, 1998, **95**, 6460.
[127] D. A. Kocisko, G. S. Baron, R. Rubenstein, J. Chen, A. S. Kuizon and B. Caughey, *J. Virol.*, 2003, **77**, 10288.
[128] A. Taraboulos, M. Scott, A. Semenov, D. Avrahami, L. Laszlo and S. B. Prusiner, *J Cell Biol.*, 1995, **129**, 121.
[129] B. E. Kreger, K. M. Anderson, A. Schatzkin and G. L. Splansky, *Cancer*, 1992, **70**, 1038.
[130] G. A. Mannes, A. Maier, C. Thieme, B. Wiebecke and G. Paumgartner, *N. Engl. J. Med.*, 1986, **315**, 1634.
[131] M. Gaard, S. Tretli and P. Urdal, *Scan. J. Gastroenterol.*, 1997, **32**, 162.
[132] L. M. Bjerre and J. LeLorier, *Am. J. Med.*, 2001, **110**, 716.
[133] E. Sahai and C. J. Marshall, *Nat. Rev. Cancer*, 2002, **2**, 133.
[134] A. Besson, M. Gurian-West, A. Schmidt, A. Hall and J. M. Roberts, *Genes Dev.*, 2004, **18**, 862.
[135] E. A. Collisson, C. Kleer, M. Wu, A. De, S. S. Gambhir, S. D. Merajver and M. S. Kolodney, *Mol. Cancer Ther.*, 2003, **2**, 941.
[136] T. Kusama, M. Mukai, T. Iwasaki, M. Tatsuta, Y. Matsumoto, H. Akedo and H. Nakamura, *Cancer Res.*, 2001, **61**, 4885.
[137] R. C. Pasternak, S. C. Smith, C. N. Bairey-Merz, S. M. Grundy, J. I. Cleeman and C. Lenfant, ACA/AHA/NHlB, *Circulation*, 2002, **106**, 1024.
[138] J. M. McKenney, *Clin. Cardiol.*, 2003, **26** (4 Suppl. 3), III32.
[139] S. Vickers, C. A. Duncan, I. W. Chen, A. Rosegay and D. E. Duggan, *Drug Metab. Dispos.*, 1990, **18**, 138.
[140] M. J. Garcia, R. F. Reinoso, A. Sanchez Navrro and J. R. Prous, *Methods Find. Exp. Clin. Pharmacol.*, 2003, **25**, 457.
[141] K. Nezasa, K. Higaki, T. Matsumura, K. Inazawa, H. Hasegawa, N. Nakano and M. Koike, *Drug Metab. Dispos.*, 2002, **30**, 1158.
[142] T. Hatanaka, *Clin. Pharmacokinet.*, 2000, **39**, 397.

Comparative Protein Structure Modeling and its Applications to Drug Discovery

Matthew Jacobson[1] and Andrej Sali[1,2]

[1]*Department of Pharmaceutical Chemistry, California Institute for Quantitative Biomedical Research, Mission Bay Genentech Hall, 600 16th Street, University of California, San Francisco, CA 94143-2240, USA*
[2]*Department of Biopharmaceutial Sciences, California Institute for Quantitative Biomedical Research, Mission Bay Genentech Hall, 600 16th Street, University of California, San Francisco, CA 94143-2240, USA*

Contents

1. INTRODUCTION

Homology or comparative protein structure modeling constructs a three-dimensional model of a given protein sequence based on its similarity to one or more known structures. In this perspective, we begin by describing the comparative modeling technique and the accuracy of the models. We then discuss the significant role that comparative prediction plays in drug discovery. We focus on virtual ligand screening against comparative models and illustrate the state-of-the-art by a number of specific examples.

The genome sequencing efforts are providing us with complete genetic blueprints for hundreds of organisms, including humans. We are now faced with describing,

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controlling, and modifying the functions of proteins encoded by these genomes. This task is generally facilitated by protein three-dimensional structures [1], which are best determined by experimental methods such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Despite significant advances in these techniques, many protein sequences are not easily accessible to structure determination by experiment. Over the last two years, the number of sequences in the comprehensive public sequence databases, such as SwissProt/TrEMBL [2] and GenPept [3], increased by a factor of 2.3 from 522,959 to 1,215,803 on 26 April 2004. In contrast, despite structural genomics, the number of experimentally determined structures deposited in the Protein Data Bank (PDB) increased by only a factor of 1.4 over the same period, from 16,612 to 23,793 [4]. Thus, the gap between the numbers of known sequences and structures continues to grow.

Protein structure prediction methods attempt to bridge this gap [5]. The first class of protein structure prediction methods, including threading and comparative modeling, rely on detectable similarity spanning most of the modeled sequence and at least one known structure [6]. The second class of methods, *de novo* or *ab initio* methods, predict the structure from sequence alone, without relying on similarity at the fold level between the modeled sequence and any of the known structures [7]. Despite progress in *ab initio* protein structure prediction [7,8], comparative modeling remains the most reliable method to predict the 3D structure of a protein with an accuracy that can be comparable to a low-resolution, experimentally determined structure [6].

Comparative modeling is carried out in four sequential steps: finding known structures (templates) related to the sequence to be modeled (target), aligning the target sequence with the templates, building the model, and assessing the model. Therefore, comparative modeling is only applicable when the target sequence is detectably related to a known protein structure. Using automated comparative modeling, the fraction of sequences with comparative models for at least one domain remained at ~57% over the last two years [9].

A number of servers for automated comparative modeling are available (http://salilab.org/bioinformatics_resources.shtml). Automation makes comparative modeling accessible to both experts and the non-specialists alike. Many of the servers are tested at the bi-annual CAFASP meetings and continually by the LiveBench and EVA [10–14] web servers for assessment of automated structure prediction methods. However, in spite of automation, manual intervention is generally still needed to maximize the accuracy of the models.

Comparative modeling will benefit from structural genomics [15]. Structural genomics aims to structurally characterize most protein sequences by an efficient combination of experiment and prediction [16,17]. This aim will be achieved by careful selection of target proteins and their structure determination by X-ray crystallography or NMR spectroscopy. There are a variety of target selection schemes, ranging from focusing on only novel folds to selecting all proteins in a model genome [1]. A model-centric view requires that targets be selected such that most of the remaining sequences can be modeled with useful accuracy by comparative modeling. Even with structural genomics, the structure of most proteins will be modeled, not determined by experiment. As discussed below, the accuracy of comparative models, and correspondingly the variety of their applications, decreases sharply below the 30% sequence identity cutoff, mainly as a result of a rapid increase in alignment errors. Thus, structural genomics aims

to determine protein structures such that most of the remaining sequences are related to at least one known structure at higher than 30% sequence identity [1,15,17]. It was recently estimated that this cutoff requires a minimum of 16,000 structures to cover 90% of all protein domain families, including those of membrane proteins [17]. These 16,000 structures will allow the modeling of a very much larger number of proteins. For example, the New York Structural Genomics Research Consortium measured the impact of its structures by documenting the number and quality of the corresponding models for detectably related proteins in the non-redundant sequence database. For each new structure, on average ~100 protein sequences without any prior structural characterization could be modeled at least at the fold level [9]. This large leverage of structure determination by protein structure modeling illustrates and justifies the premise of structural genomics.

We begin by reviewing the methods needed for each of the four steps of comparative modeling. While we only briefly touch on fold assignment, sequence-structure alignment, and model assessment, we elaborate on model building; we emphasize the modeling of loops and sidechains, because of their importance in ligand docking and rational drug discovery. We continue by describing MODBASE, a comprehensive database of comparative models. Next, we describe the role of comparative modeling in drug discovery, focusing on ligand docking against comparative models. We compare successes of docking against models and x-ray structures, and illustrate the computational docking against models with a number of examples, including kinases and G-protein coupled receptors.

2. FOLD ASSIGNMENT AND SEQUENCE-STRUCTURE ALIGNMENT

The templates for modeling may be found by pairwise sequence alignment methods, such as BLAST and FASTA, profile-sequence alignment methods, such as PSI-BLAST, profile–profile alignment methods, such as SALIGN, Hidden Markov Models, such as SAM-T02, and sequence-structure threading methods that can sometimes reveal more distant relationships than purely sequence-based methods [18–23]. Threading methods assign the fold by threading the sequence through each of the structures in a library of all known folds; each sequence-structure alignment is assessed by the energy of a corresponding coarse model, not by sequence similarity as in sequence comparison methods. Recently, the accuracy of aligning a sequence to a remotely related protein structure has been improved by a genetic algorithm protocol that iterates through alignment, model building, and model assessment [24].

3. COMPARATIVE MODEL BUILDING

Comparative protein structure prediction produces an all-atom model of a sequence, based on its alignment to one or more related protein structures. Comparative model building includes either sequential or simultaneous modeling of the core of the protein, loops, and side-chains. In the original comparative approach, a model is constructed from

a few template core regions, and from loops and side-chains obtained from either aligned or unrelated structures [25–27]. Another family of comparative methods relies on approximate positions of conserved atoms from the templates to calculate the coordinates of other atoms [28]. A third group of methods uses either distance geometry or optimization techniques to satisfy spatial restraints obtained from the sequence-template alignment [29–31]. Next, we review a large variety of specialized methods that focus on the modeling of loops in a fixed environment of the rest of the protein and the modeling of sidechains on a fixed backbone.

4. LOOP MODELING

In comparative modeling, target sequences often have residues inserted relative to the template structures or have regions that are structurally different from the corresponding regions in the templates. Thus, no structural information about these segments can be extracted from the template structures. These regions frequently correspond to surface loops. Loops often play an important role in defining the functional specificity of a given protein, forming the active and binding sites. The accuracy of loop modeling can be a major factor determining the usefulness of comparative models in applications such as ligand docking. Loop modeling can be seen as a mini protein folding problem because the correct conformation of a given segment of a polypeptide chain has to be calculated mainly from the sequence of the segment itself. However, loops are generally too short to provide sufficient information about their local fold. Even identical decapeptides in different proteins do not always have the same conformation [32,33]. Some additional restraints are provided by the core anchor regions that span the loop and by the structure of the rest of a protein that cradles the loop. Although many loop modeling methods have been described, it is still challenging to model correctly and confidently loops longer than approximately 8–10 residues [34,35].

There are two main classes of loop modeling methods: (i) database search approaches that scan a database of all known protein structures to find segments fitting the anchor core regions [36,37]; (ii) conformational search approaches that rely on optimizing a scoring function [38–40]. There are also methods that combine these two approaches [41,42].

The database search approach to loop modeling is accurate and efficient when a database of specific loops is created to address the modeling of the same class of loops, such as β-hairpins [43], or loops on a specific fold, such as the hypervariable regions in the immunoglobulin fold [37,44]. There are attempts to classify loop conformations into more general categories, thus extending the applicability of the database search approach [45–47]. However, the database methods are limited because the number of possible conformations increases exponentially with the length of a loop. As a result, only loops up to 4–7 residues long have most of their conceivable conformations present in the database of known protein structures [48,49]. This limitation is made even worse by the requirement for an overlap of at least one residue between the database fragment and the anchor core regions, which means that modeling a five residue insertion requires at least a seven residue fragment from the database [50]. Despite the rapid growth of the database of known structures, it does not seem possible to cover most

of the conformations of a 9-residue segment in the foreseeable future. On the other hand, most of the insertions in a family of homologous proteins are shorter than 10–12 residues [34].

To overcome the limitations of the database search methods, conformational search methods were developed [38,39]. There are many such methods, exploiting different protein representations, objective functions, and optimization or enumeration algorithms. The search algorithms include the minimum perturbation method, molecular dynamics simulations, genetic algorithms, Monte Carlo and simulated annealing, multiple copy simultaneous search, self-consistent field optimization, and enumeration based on graph theory [41,51–59]. The accuracy of loop predictions can be further improved by clustering the sampled loop conformations and partially accounting for the entropic contribution to the free energy [60]. Another way to improve the accuracy of loop predictions is to consider the solvent effects. Improvements in implicit solvation models, such as the Generalized Born solvation model, motivated their use in loop modeling. The solvent contribution to the free energy can be added to the scoring function for optimization, or it can be used to rank the sampled loop conformations after they are generated with a scoring function that does not include the solvent terms [34,61–64].

5. SIDECHAIN MODELING

Two simplifications are frequently applied in the modeling of sidechain conformations. First, amino acid residue replacements often leave the backbone structure almost unchanged [65], allowing us to fix the backbone during the search for the best sidechain conformations. Second, most sidechains in high-resolution crystallographic structures can be represented by a limited number of conformers that comply with stereochemical and energetic constraints [66]. This observation motivated Ponder and Richards to develop the first library of sidechain rotamers for the 17 types of residues with dihedral angle degrees of freedom in their sidechains, based on 10 high-resolution protein structures determined by X-ray crystallography [67]. Subsequently, a number of additional libraries have been derived [68–72].

Rotamers on a fixed backbone are often used when all the sidechains need to be modeled on a given backbone. This approach reduces the combinatorial explosion associated with a full conformational search of all the sidechains, and is applied by some comparative modeling [27] and protein design approaches [73]. However, ~15% of the sidechains can not be represented well by these libraries [74]. In addition, it has been shown that the accuracy of sidechain modeling on a fixed backbone decreases rapidly when the backbone errors are larger than 0.5 Å [75].

Earlier methods for sidechain modeling often put less emphasis on the energy or scoring function. The function was usually greatly simplified, and consisted of the empirical rotamer preferences and simple repulsion terms for non-bonded contacts [69]. Nevertheless, these approaches have been justified by their performance. For example, a method based on a rotamer library compared favorably with that based on a molecular mechanics force field, and new methods continue to be based on the rotamer library approach [72,76,77]. The various optimization approaches include a Monte Carlo simulation, simulated annealing, a combination of Monte Carlo and simulated annealing,

the dead-end elimination theorem, genetic algorithms, neural network with simulated annealing, mean field optimization, and combinatorial searches [69,78–86]. Several recent papers focused on the testing of more sophisticated potential functions for conformational search [86,87] and development of new scoring functions for side chain modeling [77,88], reporting higher accuracy than earlier studies.

6. COMPARATIVE MODELING BY MODELLER

MODELLER is a computer program for comparative protein structure modeling [30,34]. In the simplest case, the input is an alignment of a sequence to be modeled with the template structures, the atomic coordinates of the templates, and a short script file. MODELLER then automatically calculates a model containing all non-hydrogen atoms, without any user intervention and within minutes on a Pentium processor.

MODELLER implements comparative protein structure modeling by satisfaction of spatial restraints [30]. The spatial restraints include (i) homology-derived restraints on the distances and dihedral angles in the target sequence, extracted from its alignment with the template structures [30], (ii) stereochemical restraints such as bond length and bond angle preferences, obtained from the CHARMM-22 molecular mechanics force-field [89], (iii) statistical preferences for dihedral angles and non-bonded inter-atomic distances, obtained from a representative set of known protein structures [90], and (iv) optional manually curated restraints, such as those from NMR spectroscopy, rules of secondary structure packing, cross-linking experiments, fluorescence spectroscopy, image reconstruction from electron microscopy, site-directed mutagenesis, and intuition. The spatial restraints, expressed as probability density functions, are combined into an objective function that is optimized by a combination of conjugate gradients and molecular dynamics with simulated annealing. This model building procedure is similar to structure determination by NMR spectroscopy.

Apart from model building, MODELLER can perform additional auxiliary tasks, including alignment of two protein sequences or their profiles, multiple alignment of protein sequences and/or structures, calculation of phylogenetic trees, and *de novo* modeling of loops in protein structures [34].

7. PHYSICS-BASED APPROACHES TO COMPARATIVE MODEL CONSTRUCTION AND REFINEMENT

In principle, an accurate and efficient method for estimating the free energy of a given protein conformation could substantially improve the accuracy of comparative models. That is, an accurate energy function combined with efficient sampling could be used to refine initially constructed comparative models and thus decrease their RMS error, assuming that the native state represents the lowest free energy state. We suggest that accurate energy-based scoring may be particularly important for accurately reproducing the fine details (e.g., specific hydrogen bonding interactions) of protein active sites, which in turn will be critical for success of virtual screening using comparative models. Two key challenges confronting this approach are (i) efficient but accurate methods for

treating solvent (many methods have entirely ignored solvent or used low-accuracy but efficient distance-dependent dielectric representations); and (ii) the estimation of entropic contributions to free energy differences among states, which generally requires extensive sampling.

As discussed above, loop and sidechain prediction algorithms rely on scoring functions to guide the sampling, aiming to identify favorable conformations and reject unfavorable ones. These scoring functions typically do not explicitly attempt to estimate the free energy of a given conformation. Rather, most scoring functions have been based on statistical analyses of native protein structures encoded in the so-called potentials-of-mean-force, heuristic functional forms, or highly simplified energetic models (e.g., only van der Waals energy terms). Electrostatic interactions and, especially, the effect of solvent are infrequently used in such algorithms, largely due to their computational expense; a large number of conformations of a protein model must typically be scored during comparative model construction, and thus the scoring function must be rapid to compute.

Nonetheless, a number of groups have made efforts to use more sophisticated energetic scoring functions for comparative model construction and refinement. Recently, the convergence of increased computing power as well as accurate and efficient implicit solvent models (i.e., Poisson–Boltzmann and Generalized Born models) has bolstered these efforts. In the realm of loop modeling, there have been several groups reporting recent studies [62,91–93]; all four of these studies employed all-atom force fields and Generalized Born solvent models for scoring. An alternative recent approach is that of Hornak and Simmerling, which uses molecular dynamics methods [94]. Xiang and Honig developed a function that attempts to mimic the entropic contribution to free energy without rigorous sampling [72]. These studies have focused on reproducing loop conformations in native protein structures.

The problem of refining comparative models to improve accuracy is more challenging, and early attempts to use molecular dynamics or energy minimization in this context had mixed success, frequently increasing the RMS error of the models [95,96]. Nonetheless, physics-based energy functions, although certainly not perfect, have shown impressive abilities to distinguish native from non-native protein structures [61,97], suggesting that the model accuracy may be currently limited by incomplete sampling rather than the accuracy of the scoring function. A few recent reports about successful refinement of comparative models by restrained molecular dynamics [98,99] support this viewpoint.

A complete software package for physics-based comparative model construction and refinement has been developed [146]. The energy function employed is based on the OPLS all-atom force field and Surface Generalized Born solvent model with a non-polar estimator [100–103]. The sampling algorithms, which all use this energy function, include side chain and loop and helix prediction; the latter capability is novel and addresses the fact that corresponding helices in homologous proteins frequently adopt differing conformations, especially at relatively low sequence identity [64,87,104]. The loop prediction algorithm is, to our knowledge, the most accurate yet reported in the literature when tested by reconstructing hundreds of loops in native structures. Of particular relevance to virtual ligand screening against comparative models (as discussed below), the comparative model construction algorithm permits inclusion of co-factors and ligands as the model is built, which can help to improve accuracy of binding site conformations.

8. ACCURACY OF COMPARATIVE MODELS

The accuracy of the predicted model determines the information that can be extracted from it. Thus, estimating the accuracy of 3D protein models in the absence of the known structures is essential for interpreting them. The model can be evaluated as a whole as well as in the individual regions. There are many model evaluation programs and servers [147].

The accuracy of comparative modeling is related to the percentage sequence identity on which the model is based, correlating with the relationship between the structural and sequence similarities of two proteins [6,96,105]. High accuracy comparative models are generally based on more than 50% sequence identity to their templates. They tend to have approximately 1 Å RMS error for the main-chain atoms, which is comparable to the accuracy of a medium resolution NMR structure or a low-resolution X-ray structure. The errors are mostly mistakes in side-chain packing, small shifts or distortions of the core main-chain regions, and occasionally larger errors in loops. Medium accuracy comparative models are based on 30–50% sequence identity. They tend to have approximately 90% of the main-chain modeled with 1.5 Å RMS error. Errors in side-chain packing, core distortion, and loop modeling errors are more frequent, and there are occasional alignment mistakes [105]. Finally, low accuracy comparative models are based on less than 30% sequence identity. Alignment errors increase rapidly below 30% sequence identity and become the most significant source of errors in comparative models. In addition, when a model is based on an almost insignificant alignment to a known structure, it may also have an entirely incorrect fold. Accuracies of the best model building methods are relatively similar when used optimally [96,106]. Other factors such as template selection and alignment accuracy usually have a larger impact on the model accuracy, especially for models based on less than 40% sequence identity to the templates.

9. MODELING ON A GENOMIC SCALE

Threading and comparative modeling methods have been applied on a genomic scale [105, 107,108]. Domains in approximately one half of all 1,300,000 known protein sequences were modeled with MODPIPE [105,107] and MODELLER [30], and deposited into a comprehensive database of comparative models, MODBASE [9]. The web interface to the database allows flexible querying for fold assignments, sequence-structure alignments, models, and model assessments. An integrated sequence/structure viewer, Chimera [110], allows inspection and analysis of the query results. MODBASE is inter-linked with other applications and databases such that structures and other types of information can be easily used for functional annotation. For example, MODBASE contains binding site predictions for small ligands and a set of predicted interactions between pairs of modeled sequences from the same genome. Other resources associated with MODBASE include a comprehensive database of multiple protein structure alignments (DBALI) as well as web servers for automated comparative modeling with MODPIPE (MODWEB), modeling of loops in protein structures (MODLOOP) [112], and predicting functional consequences of single nucleotide polymorphisms (SNPWEB) [109,111,113].

While the current number of modeled proteins may look impressive given the early stage of structural genomics, usually only one domain per protein is modeled

(on the average, proteins have slightly more than two domains) and two thirds of the models are based on less than 30% sequence identity to the closest template.

10. APPLICATIONS OF COMPARATIVE MODELING TO DRUG DISCOVERY

There is a wide range of applications of protein structure models [5]. For example, high and medium accuracy comparative models frequently are helpful in refining functional predictions that have been based on a sequence match alone because ligand binding is more directly determined by the structure of the binding site than by its sequence. It is often possible to correctly predict features of the target protein that do not occur in the template structure. The size of a ligand may be predicted from the volume of the binding site cleft, and the location of a binding site for a charged ligand can be predicted from a cluster of charged residues on the protein. Fortunately, errors in the functionally important regions in comparative models are many times relatively low because the functional regions, such as active sites, tend to be more conserved in evolution than the rest of the fold. Even low accuracy comparative models may be useful, for example, for assigning the fold of a protein. Fold assignment can be very helpful in drug discovery, because it can shortcut the search for leads by pointing to compounds that have been previously developed for other members of the same family [114,115].

10.1. Comparative models vs experimental structures in virtual screening

The remainder of this review focuses on the use of comparative models for ligand docking [116–118]. It is widely accepted that docking to comparative models is more challenging and less successful than docking to crystallographic structures. However, surprisingly little work has been done to quantify the accuracy of docking to comparative models, to determine in detail why the results are inferior to those obtained with crystal structures, and to improve methods for docking to comparative models. Efforts along these lines, directed at the important kinase and GPCR drug targets, are described in separate sections below.

We begin our discussion with a study that compared the success of docking against ten enzymes, each in three different conformations: holo (ligand bound), apo, and homology modeled [119]. Each enzyme had multiple known inhibitors in the MDDR database, a library of drug-like molecules where each molecule has been annotated by the receptor to which it binds. Success of the docking, carried out with the this group's version of DOCK, was assessed by enrichment: the ability to distinguish known inhibitors from a large set of 'decoys' ($\sim$100,000 of them in this case), relative to random selection. As might be expected, the holo structures were the best at selecting the known ligands from among the MDDR decoys based on docking score. Unexpectedly, the comparative models, all taken from the MODBASE website without any special preparation, often ranked known ligands among the top-scoring database molecules. In four targets, this enrichment was better than 20-fold over random for top-scoring molecules [119]. In one

case, purine nucleoside phosphorylase, the modeled structure actually performed better than the holo structure. For the comparative model, 25% of the known ligands were found in the top 1.2% of the ranked database, whereas for the holo conformation 2.8% of the ranked list had to be searched before 25% of the ligands were found. Thus, whereas X-ray crystallographic structures remain the first choice in docking, in many cases comparative models seem good enough to highly rank known ligands from among a very large list of possible alternatives.

10.2. Use of comparative models to obtain novel drug leads

Comparative models have been used in conjunction with virtual screening to successfully identify novel inhibitors over the past few years. We briefly review a few of these 'success stories' to highlight the potential of this approach. The role of comparative modeling in the design of new kinase inhibitors is discussed separately, below.

A series of papers demonstrate the successful use comparative protein structure models to aid rational drug design against parasites. Comparative models were used for computational docking studies that identified low micromolar nonpeptidic inhibitors of proteases in malaria and the schistosome [53]. Subsequently, further work applied similar methods to develop nanomolar inhibitors of falcipain that are active against chloroquine-resistant strains of malaria [120]. Additionally, comparative models were used to predict new nonpeptide inhibitors of cathepsin L-like cysteine proteases in *L. major*[121]. Sixty-nine compounds were selected by DOCK 3.5 as strong binders to a comparative model of protein cpB, and of these, 21 had experimental IC_{50} values below 100 μM. Finally, comparative models were used to rationalize ligand binding affinities of cysteine proteases in *E. histolytica* [122]. Specifically, this work provided an explanation for why proteins ACP1 and ACP2 had substrate specificity similar to that of cathepsin B, although their overall structure is more similar to that of cathepsin D.

Fifteen new inhibitors of matriptase have been discovered by docking against its comparative model [123]. The comparative model employed thrombin as the template, sharing only 34% sequence identity with the target sequence. Moreover, some residues in the binding site are significantly different, including a trio of Asp residues in thrombin that are modified to Tyr and Trp in matriptase. Thrombin was chosen as the template, in part, because it prefers substrates with positively charged residues at the P1 position, as does matriptase. The comparative model was constructed using MODELLER and refined with MD simulations in CHARMM. The NCI database was used for virtual screening that targeted the S1 site with the DOCK program. The 2000 best-scoring compounds were manually inspected to identify positively charged ligands (the S1 site is negatively charged), and 69 compounds were experimentally screened for inhibition, identifying 15 inhibitors. One of them, hexamidine, was used as a lead to identify additional compounds selective for matriptase relative to thrombin. Another group has also used similar methods to discover seven new, low-micromolar inhibitors of Bcl-2, using a comparative model based on the NMR solution structure of Bcl-X_L [124].

A novel inhibitor of a retinoic acid receptor by virtual screening using a comparative model [125]. In this case, the target (RAR-α) and template (RAR-γ) are very closely

related; only three residues in the binding site are not conserved. The ICM program was used for virtual screening of ligands from the Available Chemicals Directory, using a hierarchical approach. As with other studies, 300 good-scoring compounds from the virtual screening were manually inspected to choose the final 30 for testing. Two novel agonists were identified, with 50 nanomolar activity.

Novel inhibitors of DHFR in *T. cruzi* (the parasite that causes Chagas' disease) were discovered by docking into a comparative model based on ~50% sequence identity to DHFR in *L. major*, a related parasite [126]. The virtual screening procedure used DOCK for rigid docking of over 50,000 selected compounds from the Cambridge Structural Database. Visual inspection of the top 100 hits was used to select 36 compounds for experimental testing. This work identified several novel scaffolds with micromolar IC_{50} values. The authors report attempting to use virtual screening results to identify compounds with greater affinity for *T. cruzi* DHFR than human DHFR, but it is not clear how successful they were.

10.3. Comparative models of kinases in virtual screening

Protein kinases have been intensely investigated as drug targets. The large number of kinases in the human genome (~500) is both an asset and a liability for drug development efforts. The catalytic domain of kinases (i.e., the domain that binds ATP and effects phosphorylation of substrates) is well conserved structurally, making it possible to construct comparative models for this domain for many of the kinases in the human genome. On the other hand, the high level of sequence conservation around the ATP binding site-the site most frequently targeted for inhibitor development-raises concerns about selectivity. Drugs that inadvertently inhibit kinases unrelated to the disease state one intends to treat may lead to side effects (although activity against several kinases may be desirable for treating some diseases). Proteins other than kinases can also contain ATP binding sites, of course, although it is not clear whether selectivity across protein families is a problem in practice. Reliable comparative models of kinases can in principle help to address issues of selectivity and aid drug development against the many kinases lacking experimental structures. Work along these lines remains at an early stage. Here we highlight five studies in which kinase comparative models were used in virtual screening applications; two of these studies developed new kinase inhibitors, while the remaining three focused on methodological issues.

A selective inhibitor of human CK2 was discovered by virtual screening [144]. Because no experimental structure of the human protein was available at the time, a comparative model of this kinase based on the structure of CK2 from *Zea mays*, using the WhatIf program [127]. The level of sequence identity between the two proteins is very high: 72% overall, and 82% in the ATP binding site. The crystal structure of human CK2 was subsequently solved, and the Cα RMS error of the model was determined to be only 0.92 Å overall, and 0.64 Å in the binding site. DOCK was used to screen a corporate database of 400,000 compounds against the comparative model. As with many other studies, the hit list was subjected to extensive filtering based on a variety of factors including visual inspection. A dozen compounds were tested, yielding several inhibitors with activity at 10 μM, and one with an IC_{50} of 80 nM – the most potent inhibitor of CK2

yet reported. Fortuitously, this inhibitor has excellent selectivity, showing much weaker inhibition of other kinases.

In another successful use of kinase comparative models for inhibitor development, was in the virtual screening of a model of CDK4, a member of the important cyclic-dependent kinase class of drug targets (CDKs play a central role in regulation of the cell cycle) [145]. The comparative model was built from the crystal structure of CDK2 in its activated (phosphorylated) form (45% sequence identity). The *de novo* design program LEGEND was then used to predict inhibitors, which were subsequently filtered based on commercial availability. Of 382 tested compounds, 18 had IC_{50} values better than 500 μM. After several rounds of optimization, one inhibitor had an IC_{50} of 42 nM. The predicted binding mode of this inhibitor was subsequently supported by a crystal structure of the inhibitor bound to CDK2.

Two recent papers have evaluated the enrichment of known kinase inhibitors (relative to randomly selected drug-like molecules) obtained by virtual screening against kinase comparative models. Construction of a total of 17 comparative models of six kinases, using templates with sequence identity ranging from 30 to 77% was used in the first study [128]. Four of these kinases had crystal structures available for comparison. Known inhibitors for the kinases (ranging from 46 for VEGFR1 to 387 for EGFr) were combined with 32,000 random compounds for the virtual screening, which used the LibDock program [129]. In five of six cases, the known inhibitors were found to be enriched by factors of 4–5 in the top 5% of the docking hits. These results are worse than typical results with kinase crystal structures (although for SRC, the comparative model performed much better than the crystal structure, for reasons that are not entirely clear). This study also compared poses of the docked ligands with crystallographically determined positions, when available, and suggested structural reasons for the difference in enrichment between crystal structures and comparative models.

A further study compared enrichment for models of CDK2 built from several different templates with results for the crystal structure [130]. Comparative models were constructed with MOE, and docking was performed with DOCK. This study concluded that comparative models built from templates with $>50\%$ sequence identity provide useful proxies of crystal structures, generating enrichment factors of ~ 5, similar to the results with the crystal structure. Results for virtual screening against comparative models of factor VIIa were also presented and are similar to those for CDK2.

In a study investigating the differential binding of the drugs Gleevec, purvalanol A, and hymenialdisine to their intended kinase targets and many other kinases, most receptor structures were constructed by comparative modeling [131]. This study represents an early effort toward using comparative models to help assess possible side effects of drugs caused by unintended binding to homologs of the intended target protein.

10.4. GPCR comparative models for drug development

Comparative modeling takes on special importance for the G-protein coupled receptors (GPCRs). This class of membrane proteins has played an enormously important role in drug development; approximately 40% of all drugs target a member of this class. However, they are very difficult to crystallize, and most of the comparative modeling

effort has been based on the atomic-resolution structure of bovine rhodopsin. Comparative modeling methods for GPCRs capable of accuracy sufficient for structure-based drug design would have an enormous impact on drug discovery. Several serious challenges confront this goal. Many targets of interest for drug development share rather low sequence identity (<30%) with rhodopsin. Although the transmembrane helices can frequently be aligned with reasonable certainty (aided by certain highly conserved residues), the extracellular loops are much more divergent in sequence. Finally, model building algorithms typically do not take the membrane environment into account explicitly, although it is not clear whether this approximation is a significant factor for reconstructing the ligand binding sites. Despite these challenges, encouraging early results have been reported for docking into comparative models of GPCRs. In fact, enrichment of known ligands in virtual screening has become an important means of validating GPCR comparative models.

A rather extensive test of docking methods with rhodopsin-based comparative models, using three different docking programs and seven different scoring functions has recently been reported [132]. The comparative modeling protocol is intricate, and includes manual adjustment of sequence alignments, specialized loop search methods for the second extracellular loop, and energy minimization of the model using AMBER. Models were separately prepared for agonists and antagonists; that is, known ligands were manually 'docked' into the binding site in reasonable poses, and the protein structure was again minimized. Several different docking protocols were attempted, and the authors report that the use of a 'consensus' scoring procedure involving three scoring functions provides the best results. Under optimal conditions, the rather elaborate procedures produced encouraging results in cross-docking experiments involving known ligands of the β2 and D3 receptors, and in enrichment tests using 990 randomly chosen compounds from the ACD.

The ICM flexible docking algorithm could successfully reproduce the conformation of the retinal ligand in rhodopsin and rank this ligand in the top 2% relative to a database of decoy ligands [133]. Encouragingly, the method appears to be robust with respect to entirely deleting the extracellular loop regions (which are difficult to model) and errors in the binding site side chain conformations. GPCR models generated by the MembStruk [134] and PREDICT [135,136] methods, which do not explicitly rely on the rhodopsin structure, also appear to be useful for virtual screening purposes.

10.5. Other uses of comparative models in drug development

Clearly, as the previous discussion shows, comparative models are finding increasing application in virtual ligand screening. However, comparative models have been put to use in many other ways in the context of drug development, generally to obtain qualitative insights to guide inhibitor development. In contrast to the literature on docking to comparative models, the number of papers in this category is quite large, and we aim to provide only a flavor of the many ways in which comparative models have been used.

One common use of comparative models is to rationalize trends in binding affinities among known inhibitors or structure-activity relationships, with an aim of guiding future

lead discovery and optimization efforts. In these studies, docking algorithms are used primarily to generate possible binding modes of known inhibitors in comparative models, providing a structural context for understanding existing data on these inhibitors. Examples include recent studies of the cysteine protease falcipain-3 in malaria, thymidine phosphorylase, thymidine kinase, and androgen receptor [137–140]. Similar studies have also combined comparative modeling and docking to help guide the improvement of the pharmacokinetic properties of inhibitors, rationalize the role of mutations in the development of drug resistance, and understand selectivity of drugs against homologous proteins [141–143].

10.6. Future directions

Although recent reports of successful virtual screening against comparative models are encouraging, such efforts are not yet a routine part of rational drug design efforts. Even the successful efforts appear to rely strongly on visual inspection of the docking results, and it is clear that much work remains to improve the robustness and accuracy of docking against comparative models. Although a number of studies have compared docking against comparative models with docking against crystal structures of the same proteins, little work has been done to compare the accuracy achievable by different approaches to comparative modeling, or to identify the specific structural reasons why comparative models produce less accurate virtual screening results. Among the many issues that deserve consideration are the following:

- The inclusion of co-factors and bound waters in protein receptors is often critical for success of virtual screening; however, co-factors are not routinely included in comparative models.
- The accuracy of comparative models is frequently judged by the Cα RMS error, or similar measures of backbone accuracy. For virtual screening, however, the precise positioning of side chains in the binding site is likely to be critical; measures of accuracy for binding sites are needed to help evaluate the suitability of comparative modeling algorithms for constructing models for docking.
- Knowledge of known inhibitors, either for the target protein or the template, can be used to evaluate and improve virtual screening against comparative models. For example, comparative models constructed from ‘holo’ template structures implicitly preserve some information about the ligand-bound receptor conformation.
- Improvement in the accuracy of models produced by comparative modeling will require methods that finely sample protein conformational space using a free energy or scoring function that has sufficient accuracy to distinguish the native structure from the non-native conformations. Despite many years of development of molecular simulation methods, attempts to refine models that are already relatively close to the native structure have met with relatively little success. This failure is likely to be due to inaccuracies in the potential functions used in the simulations, particularly in the treatment of electrostatics and solvation effects. Improvements in sampling strategies may also be necessary. Combination of physical chemistry with the vast amount of

information in known protein structures may provide a route to development of improved potential functions.

11. CONCLUSIONS

High-resolution crystallographic structures becoming available for all human and pathogen proteins and complexes is a very unlikely scenario. The only practical manner of exploring ligand–protein interactions for most systems is to use comparative protein structure models. It is now clear that comparative models, based on as little as 30% sequence identity to known template structures, can be useful. New methods that improve and combine existing modeling and docking techniques to further advance the utility of comparative models will no doubt be developed in the future.

REFERENCES

[1] S. E. Brenner, *Nat. Struct. Biol.*, 2000, **7**(Suppl.), 967.
[2] B. Boeckmann, A. Bairoch, R. Apweiler, M. C. Blatter, A. Estreicher, E. Gasteiger, M. J. Martin, K. Michoud, C. O'Donovan, I. Phan, *et al.*, *Nuclic Acids Res.*, 2003, **31**, 365.
[3] D. A. Benson, I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp and L. Wheeler, *Nucleic Acids Res.*, 2002, **30**, 17.
[4] H. M. Berman, T. Battistuz, T. N. Bhat, W. F. Bluhm, P. E. Bourne, K. Burkhardt, Z. Feng, G. L. Gilliland, L. Iype, S. Jain, *et al.*, *Acta Crystallogr. D.*, 2002, **58**, 899.
[5] D. Baker and A. Sali, *Science*, 2001, **294**, 93.
[6] M. A. Marti-Renom, A. Stuart, A. Fiser, R. Sanchez, F. Melo and A. Sali, *Annu. Rev. Biophys. Biomol. Struct.*, 2000, **29**, 291.
[7] R. Bonneau and D. Baker, *Annu. Rev. Biophys. Biomol. Struct.*, 2001, **30**, 173.
[8] D. Baker, *Nature*, 2000, **405**, 39.
[9] U. Pieper, N. Eswar, H. Braberg, M. S. Madhusudhan, F. P. Davis, A. C. Stuart, N. Mirkovic, A. Rossi, M. A. Marti-Renom, A. Fiser, *et al.*, *Nucleic Acids Res.*, 2004, **32**, D217, Database issue, http://www.salilab.org/modbase/ and http://www.nysqxrc.org/.
[10] D. Fischer, A. Elofsson, L. Rychlewski, F. Pazos, A. Valencia, B. Rost, A. R. Ortiz and R. L. Dunbrack, Jr, *Proteins*, 2001, **45** (Suppl. 5), 171.
[11] P. E. Bourne, *Methods Biochem. Anal.*, 2003, **44**, 501.
[12] J. M. Bujnicki, A. Elofsson, D. Fischer and L. Rychlewski, *Prot. Sci.*, 2001, **10**, 352.
[13] V. A. Eyrich, M. A. Marti-Renom, D. Przybylski, M. S. Madhusudhan, A. Fiser, F. Pazos, A. Valencia, A. Sali and B. Rost, *Bioinformatics*, 2001, **17**, 1242.
[14] I.-Y. Y. Koh, V. A. Eyrich, M. A. Marti-Renom, D. Przybylski, M. S. Madhusudhan, E. Narayanan, O. Graca, F. Pazos, A. Valencia, A. Sali, *et al.*, *Nucleic Acids Res.*, 2003.
[15] A. Sali, *Nat. Struct. Biol.*, 1998, **5**, 1029.
[16] S. K. Burley, S. C. Almo, J. B. Bonanno, M. Capel, M. R. Chance, T. Gaasterland, D. Lin, A. Sali, F. W. Studier and S. Swaminathan, *Nat. Genet.*, 1999, **23**, 151.
[17] D. Vitkup, E. Melamud, J. Moult and C. Sander, *Nat. Struct. Biol.*, 2001, **8**, 559.
[18] S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman, *J. Mol. Biol.*, 1990, **215**, 403.
[19] W. R. Pearson, *Methods Enzymol.*, 1990, **183**, 63.
[20] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman, *Nucleic Acids Res.*, 1997, **25**, 3389.
[21] M. A. Marti-Renom, M. S. Madhusudhan and A. Sali, *Prot. Sci.*, 2004, **13**, 1071.
[22] R. Karchin, M. Cline, Y. Mandel-Gutfreund and M. Karplus, *Proteins*, 2003, **51**, 504.

[23] A. E. Torda, *Curr. Opin. Struct. Biol.*, 1997, **7**, 200.
[24] B. John and A. Sali, *Nucleic Acids Res.*, 2003, **31**, 3982–3992.
[25] W. J. Browne, A. C. T. North, D. C. Phillips, K. Brew, T. C. Vanaman and R. C. Hill, *J. Mol. Biol.*, 1969, **42**, 65.
[26] J. Greer, *J. Mol. Biol.*, 1981, **153**, 1027.
[27] T. L. Blundell, B. L. Sibanda, M. J. Sternberg and J. M. Thornton, *Nature*, 1987, **326**, 347.
[28] M. Levitt, *J. Mol. Biol.*, 1992, **226**, 507.
[29] T. F. Havel and M. E. Snow, *J. Mol. Biol.*, 1991, **217**, 1.
[30] A. Sali and T. L. Blundell, *J. Mol. Biol.*, 1993, **234**, 779.
[31] A. Kolinski, M. R. Betancourt, D. Kihara, P. Rotkiewicz and J. Skolnick, *Proteins*, 2001, **44**, 133.
[32] W. Kabsch and C. Sander, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 1075.
[33] M. Mezei, *Prot. Eng.*, 1998, **11**, 411.
[34] A. Fiser, R. K. Do and A. Sali, *Prot. Sci.*, 2000, **9**, 1753, http://www.salilab.org/modeller.
[35] M. P. Jacobson, D. L. Pincus, C. S. Rapp, T. J. Day, B. Honig, D. E. Shaw and R. A. Friesner, *Proteins*, 2004, **55**, 351.
[36] T. A. Jones and S. Thirup, *EMBO J.*, 1986, **5**, 819.
[37] C. Chothia and M. Lesk, *J. Mol. Biol.*, 1987, **196**, 901.
[38] J. Moult and M. N. James, *Proteins*, 1986, **1**, 146.
[39] R. E. Bruccoleri and M. Karplus, *Biopolymers*, 1987, **26**, 137.
[40] P. S. Shenkin, D. L. Yarmush, R. M. Fine, H. J. Wang and C. Levinthal, *Biopolymers*, 1987, **26**, 2053.
[41] H. W. van Vlijmen and M. Karplus, *J. Mol. Biol.*, 1997, **267**, 975.
[42] C. M. Deane and T. L. Blundell, *Prot. Sci.*, 2001, **10**, 599.
[43] B. L. Sibanda, T. L. Blundell and J. M. Thornton, *J. Mol. Biol.*, 1989, **206**, 759.
[44] C. Chothia, A. M. Lesk, A. Tramontano, M. Levitt, S. J. Smith-Gill, G. Air, S. Sheriff, E. A. Padlan, D. Davies, W. R. Tulip, *et al.*, *Nature*, 1989, **342**, 877.
[45] C. S. Ring, D. G. Kneller, R. Langridge and F. E. Cohen, *J. Mol. Biol.*, 1992, **224**, 685.
[46] B. Oliva, P. A. Bates, E. Querol, F. X. Aviles and M. J. Sternberg, *J. Mol. Biol.*, 1997, **266**, 814.
[47] S. D. Rufino, L. E. Donate, L. H. Canard and T. L. Blundell, *J. Mol. Biol.*, 1997, **267**, 352.
[48] K. Fidelis, P. S. Stern, D. Bacon and J. Moult, *Prot. Eng.*, 1994, **7**, 953.
[49] U. Lessel and D. Schomburg, *Prot. Eng.*, 1994, **7**, 1175–1187.
[50] M. Claessens, E. Van Cutsem, I. Lasters and S. Wodak, *Prot. Eng.*, 1989, **2**, 335.
[51] R. M. Fine, H. Wang, P. S. Shenkin, D. L. Yarmush and C. Levinthal, *Proteins*, 1986, **1**, 342.
[52] R. E. Bruccoleri and M. Karplus, *Biopolymers*, 1990, **29**, 1847.
[53] C. S. Ring, E. Sun, J. H. McKerrow, G. K. Lee, P. J. Rosenthal, I. D. Kuntz and F. E. Cohen, *Proc. Natl Acad. Sci. USA*, 1993, **90**, 3583.
[54] J. Higo, V. Collura and J. Garnier, *Biopolymers*, 1992, **32**, 33.
[55] V. Collura, J. Higo and J. Garnier, *Prot. Sci.*, 1993, **2**, 1502.
[56] R. Abagyan and M. Totrov, *J. Mol. Biol.*, 1994, **235**, 983.
[57] Q. Zheng, R. Rosenfeld, S. Vajda and C. DeLisi, *Prot. Sci.*, 1993, **2**, 1242.
[58] P. Koehl and M. Delarue, *Nat. Struct. Biol.*, 1995, **2**, 163.
[59] R. Samudrala and J. Moult, *J. Mol. Biol.*, 1998, **279**, 287.
[60] Z. Xiang, C. S. Soto and B. Honig, *Proc. Natl Acad. Sci. USA*, 2002, **99**, 7432–7437.
[61] A. K. Felts, E. Gallicchio, A. Wallqvist and R. M. Levy, *Proteins*, 2002, **48**, 404.
[62] P. I. de Bakker, M. A. DePristo, D. F. Burke and T. L. Blundell, *Proteins*, 2003, **51**, 21.
[63] M. A. DePristo, P. I. de Bakker, S. C. Lovell and T. L. Blundell, *Proteins*, 2003, **51**, 41.
[64] M. P. Jacobson, D. L. Pincus, C. S. Rapp, T. J. F. Day, B. Honig, D. E. Shaw and R. A. Friesner, *Proteins*, 2004, **55**, 351.
[65] C. Chothia, A. M. Lesk, M. Levitt, A. G. Amit, R. A. Mariuzza, S. E. Phillips and R. J. Poljak, *Science*, 1986, **233**, 755.
[66] J. Janin and C. Chothia, *Biochemistry*, 1978, **17**, 2943.
[67] J. W. Ponder and F. M. Richards, *J. Mol. Biol.*, 1987, **193**, 775.
[68] P. Tuffery, C. Etchebest, S. Hazout and R. Lavery, *J. Biomol. Struct. Dyn.*, 1991, **8**, 1267.

[69] R. L. Dunbrack and M. Karplus, *J. Mol. Biol.*, 1993, **230**, 543.
[70] R. L. Dunbrack, Jr and F. E. Cohen, *Prot. Sci.*, 1997, **6**, 1661.
[71] J. Mendes, A. M. Baptista, M. A. Carrondo and C. M. Soares, *Proteins*, 1999, **37**, 530.
[72] Z. Xiang and B. Honig, *J. Mol. Biol.*, 2001, **311**, 421–430.
[73] J. R. Desjarlais and T. M. Handel, *J. Mol. Biol.*, 1999, **290**, 305.
[74] V. De Filippis, C. Sander and G. Vriend, *Prot. Eng.*, 1994, **7**, 1203.
[75] S. Y. Chung and S. Subbiah, *Pac. Symp. Biocomput.*, 1996, 126.
[76] D. Cregut, J. P. Liautard and L. Chiche, *Prot. Eng.*, 1994, **7**, 1333.
[77] A. A. Canutescu, A. A. Shelenkov and R. L. Dunbrack, Jr, *Prot. Sci.*, 2003, **12**, 2001.
[78] F. Eisenmenger, P. Argos and R. Abagyan, *J. Mol. Biol.*, 1993, **231**, 849.
[79] G. M. Lee, A. Varma and B. O. Palsson, *Biotechnol. Prog.*, 1991, **7**, 72.
[80] L. Holm and C. Sander, *Proteins*, 1992, **14**, 213.
[81] I. Lasters and J. Desmet, *Prot. Eng.*, 1993, **6**, 717.
[82] L. L. Looger and H. W. Hellinga, *J. Mol. Biol.*, 2001, **307**, 429.
[83] J. K. Hwang and W. F. Liao, *Prot. Eng.*, 1995, **8**, 363.
[84] P. Koehl and M. Delarue, *J. Mol. Biol.*, 1994, **239**, 249.
[85] M. J. Bower, F. E. Cohen and R. L. Dunbrack, Jr, *J. Mol. Biol.*, 1997, **267**, 1268.
[86] R. J. Petrella, T. Lazaridis and M. Karplus, *Fold. Des.*, 1998, **3**, 353.
[87] M. P. Jacobson, G. A. Kaminski, R. A. Friesner and C. S. Rapp, *J. Phys. Chem. B*, 2002, **106**, 11673.
[88] S. Liang and N. V. Grishin, *Prot. Sci.*, 2002, **11**, 322.
[89] A. D. MacKerell, Jr, D. Bashford, M. Bellott, R. L. Dunbrack, Jr, J. D. Evanseck, M. J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, *et al.*, *J. Phys. Chem. B*, 1998, **102**, 3586.
[90] A. Sali and J. P. Overington, *Prot. Sci.*, 1994, **3**, 1582–1596.
[91] C. S. Rapp and R. A. Friesner, *Proteins*, 1999, **35**, 173.
[92] A. Fiser, M. Feig, C. L. Brooks, III and A. Sali, *Acc. Chem. Res.*, 2002, **35**, 413.
[93] M. P. Jacobson, R. A. Friesner, Z. X. Xiang and B. Honig, *J. Mol. Biol.*, 2002, **320**, 597.
[94] V. Hornak and C. Simmerling, *Proteins*, 2003, **51**, 577.
[95] J. Moult, *Curr. Opin. Biotechnol.*, 1996, **7**, 422.
[96] P. Koehl and M. Levitt, *Nat. Struct. Biol.*, 1999, **6**, 108.
[97] B. N. Dominy and C. L. Brooks, *J. Comp. Chem.*, 2002, **23**, 147.
[98] H. Li, R. Tejero, D. Monleon, D. BassolinoKlimas, C. AbateShen, R. E. Bruccoleri and G. T. Montelione, *Prot. Sci.*, 1997, **6**, 956.
[99] J. A. Flohil, G. Vriend and H. J. C. Berendsen, *Proteins*, 2002, **48**, 593.
[100] W. L. Jorgensen and J. Tiradorives, *J. Am. Chem. Soc.*, 1988, **110**, 1666.
[101] G. A. Kaminski, R. A. Friesner, J. Tirado-Rives and W. L. Jorgensen, *J. Phys. Chem. B*, 2001, **105**, 6474.
[102] A. Ghosh, C. S. Rapp and R. A. Friesner, *J. Phys. Chem. B*, 1998, **102**, 10983.
[103] E. Gallicchio, L. Y. Zhang and R. M. Levy, *J. Comp. Chem.*, 2002, **23**, 517.
[104] X. Li, M. P. Jacobson and R. A. Friesner, *Proteins*, 2004.
[105] R. Sanchez and A. Sali, *Proc. Natl Acad. Sci. USA*, 1998, **95**, 13597.
[106] M. A. Marti-Renom, M. S. Madhusudhan, A. Fiser, B. Rost and A. Sali, *Structure*, 2002, **10**, 435.
[107] D. Fischer and D. Eisenberg, *Proc. Natl Acad. Sci. USA*, 1997, **94**, 11929.
[108] N. Guex, A. Diemand and M. C. Peitsch, *Trends Biochem. Sci.*, 1999, **24**, 364.
[109] N. Eswar, B. John, N. Mirkovic, A. Fiser, V. A. Ilyin, U. Pieper, A. C. Stuart, M. A. Marti-Renom, M. S. Madhusudhan, B. Yerkovich, *et al.*, *Nucleic Acids Res.*, 2003, **31**, 3375, http://www.salilab.org/modweb.
[110] C. C. Huang, W. R. Novak, P. C. Babbitt, A. I. Jewett, T. E. Ferrin and T. E. Klein, *Pac. Symp. Biocomput.*, 2000, 230.
[111] M. A. Marti-Renom, V. A. Ilyin and A. Sali, *Bioinformatics*, 2001, **17**, 746, http://www.salilab.org/dbali.
[112] A. Fiser and A. Sali, *Bioinformatics*, 2003, **19**, 2500.
[113] N. Mirkovic, M. A. Marti-Renom, A. Sali and A. N. A. Monteiro, *Cancer Res.*, 2004, **64**, 3790.
[114] R. K. Gordon, K. Ginalski, W. R. Rudnicki, L. Rychlewski, M. C. Pankaskie, J. M. Bujnicki and P. K. Chiang, *Eur. J. Biochem.*, 2003, **270**, 3507.

[115] M. von Grotthuss, L. S. Wyrwicz and L. Rychlewski, *Cell*, 2003, **113**, 701.
[116] A. Schafferhans and G. Klebe, *J. Mol. Biol.*, 2001, **307**, 407–427.
[117] A. Evers, H. Gohlke and G. Klebe, *J. Mol. Biol.*, 2003, **334**, 327–345.
[118] A. Evers and G. Klebe, *Angew Chem. Int. Ed. Engl.*, 2004, **43**, 248–251.
[119] S. L. McGovern and B. K. Shoichet, *J. Med. Chem.*, 2003, **46**, 2895.
[120] R. Li, X. Chen, B. Gong, P. M. Selzer, Z. Li, E. Davidson, G. Kurzban, R. E. Miller, E. O. Nuzum, J. H. McKerrow, *et al.*, *Bioorg. Med. Chem. Lett.*, 1996, **4**, 1421.
[121] P. M. Selzer, X. Chen, V. J. Chan, M. Cheng, G. L. Kenyon, I. D. Kuntz, J. A. Sakanari, F. E. Cohen and J. H. McKerrow, *Exp. Parasitol.*, 1997, **87**, 212.
[122] X. Que, L. S. Brinen, P. Perkins, S. Herdman, K. Hirata, B. E. Torian, H. Rubin, J. H. McKerrow and S. L. Reed, *Mol. Biochem. Parasitol.*, 2002, **119**, 23.
[123] I. J. Enyedy, S. L. Lee, A. H. Kuo, R. B. Dickson, C. Y. Lin and S. Wang, *J. Med. Chem.*, 2001, **44**, 1349.
[124] I. J. Enyedy, Y. Ling, K. Nacro, Y. Tomita, X. Wu, Y. Cao, R. Guo, B. Li, X. Zhu, Y. Huang, *et al.*, *J. Med. Chem.*, 2001, **44**, 4313.
[125] M. Schapira, B. M. Raaka, H. H. Samuels and R. Abagyan, *BMC Struct. Biol.*, 2001, **1**, 1.
[126] F. Zuccotto, M. Zvelebil, R. Brun, S. F. Chowdhury, R. Di Lucrezia, I. Leal, L. Maes, L. M. Ruiz-Perez, D. Gonzalez Pacanowska and I. H. Gilbert, *Eur. J. Med. Chem.*, 2001, **36**.
[127] G. Vriend, *J. Mol. Graph.*, 1990, **8**, 52.
[128] D. J. Diller and R. Li, *J. Med. Chem.*, 2003, **46**, 4638.
[129] D. J. Diller and K. M. Merz, *Proteins*, 2001, **43**, 113.
[130] C. Oshiro, E. K. Bradley, J. Eksterowicz, E. Evensen, M. L. Lamb, J. K. Lanctot, S. Putta, R. Stanton and P. D. Grootenhuis, *J. Med. Chem.*, 2004, **47**, 764.
[131] W. M. Rockey and A. H. Elcock, *Proteins*, 2002, **48**, 664.
[132] C. Bissantz, P. Bernard, M. Hibert and D. Rognan, *Proteins*, 2003, **50**, 5.
[133] C. N. Cavasotto, A. J. Orry and R. A. Abagyan, *Proteins*, 2003, **51**, 423.
[134] N. Vaidehi, W. B. Floriano, R. Trabanino, S. E. Hall, P. Freddolino, E. J. Choi, G. Zamanakos and W. A. Goddard, III, *Proc. Natl Acad. Sci. USA*, 2002, **99**, 12622.
[135] S. Shacham, M. Topf, N. Avisar, F. Glaser, Y. Marantz, S. Bar-Haim, S. Noiman, Z. Naor and O. M. Becker, *Med. Res. Rev.*, 2001, **21**, 472.
[136] O. M. Becker, S. Shacham, Y. Marantz and S. Noiman, *Curr. Opin. Drug. Discov. Devel.*, 2003, **6**, 353.
[137] Y. A. Sabnis, P. V. Desai, P. J. Rosenthal and M. A. Avery, *Prot. Sci.*, 2003, **12**, 501.
[138] M. L. Price, W. C. Guida, T. E. Jackson, J. A. Nydick, P. L. Gladstone, J. C. Juarez, F. Donate and R. J. Ternansky, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 107.
[139] L. Spadola, E. Novellino, G. Folkers and L. Scapozza, *Eur. J. Med. Chem.*, 2003, **38**, 413.
[140] C. A. Marhefka, B. M. Moore, T. C. Bishop, II, L. Kirkovsky, A. Mukherjee, J. T. Dalton and D. D. Miller, *J. Med. Chem.*, 2001, **44**, 1729.
[141] M. H. Rabinowitz, R. C. Andrews, J. D. Becherer, D. M. Bickett, D. G. Bubacz, J. G. Conway, D. J. Cowan, M. Gaul, K. Glennon, M. H. Lambert, *et al.*, *J. Med. Chem.*, 2001, **44**, 4252.
[142] R. T. Delfino, O. A. Santos-Filho and J. D. Figueroa-Villar, *Biophys. Chem.*, 2002, **98**, 287.
[143] E. A. Salter, A. Wierzbicki, G. Sperl and W. J. Thompson, *Int. J. Quantum Chem.*, 2004, **96**, 402.
[144] E. Vangrevelinghe, K. Zimmermann, J. Schoepfer, R. Portmann, D. Fabbro and P. Furet, *J. Med. Chem.*, 2003, **46**, 2656.
[145] T. Honma, K. Hayashi, T. Aoyama, N. Hashimoto, T. Machida, K. Fukasawa, T. Iwama, C. Ikeura, M. Ikuta, I. Suzuki-Takahashi, Y. Iwasawa, T. Hayama, S. Nishimura and H. Morishima, *J. Med. Chem.*, 2001, **44**, 4628.
[146] http://www.francisco.compbio.ucsf.edu/~jacobson/plop_overview.htm.
[147] http://www.salilab.org/bioinformatics_resources.shtml.

Section 6
Topics in Drug Design and Discovery

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California

Drug Transporters and Their Role in Tissue Distribution

Anne H. Dantzig,[1] Kathleen M. Hillgren,[1] Dinesh P. de Alwis,[2]

[1]*Lilly Research Laboratories, Indianapolis, IN 46285, USA*
[2]*Eli Lilly and Co. Limited, Surrey GU20 6PH, UK*

Contents

1. INTRODUCTION

For efficacy, a drug must be absorbed and move from the site of administration across several tissues to reach the site of action. The cellular membrane forms a formidable barrier for the movement of compounds into and out of the cell. By the chemical nature of hydrophilic compounds, these molecules prefer residing in water and are less soluble in hydrophobic environments such as the phospholipid bilayer of cellular membranes and therefore cannot easily cross the membrane. By contrast, hydrophobic compounds easily diffuse into lipid membranes. This poses a dichotomy since hydrophobic compounds prefer a lipid environment then why do they diffuse through the cellular membrane into the hydrophilic cytosol of cells? In the past few decades, transport proteins have been recognized to play a major role in determining whether hydrophilic and hydrophobic compounds are allowed passage across the membrane barrier. Drugs and/or their metabolites are now known to be substrates of membrane transporters. This knowledge has enhanced our understanding of absorption, tissue distribution, and excretion from the body. This review describes the properties of transporters and their importance in drug transport and disposition, highlighting specific clinical examples.

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2. BIOLOGY OF TRANSPORTERS

2.1. General characteristics

The human genome initiative has estimated that humans possess 2000 or more transporters and ion channels [1,2]. As illustrated in Fig. 1, transporters are integral membrane proteins typically containing 12 transmembrane spanning regions and are important in the movement of both solutes and ions from one side of a membrane to other without chemical modification.

Functionally, three classes of transporters exist: *influx* transporters facilitate the movement of compounds into the cell, *efflux* transporters remove cytosolic substances from the cell, and *exchangers* permit the simultaneous movement of solutes across the membrane in opposite directions. Like enzymes, transporters display substrate specificity normally recognizing structurally related compounds and saturate with increasing concentrations as typically described by Michaelis-Menten kinetics with addition of a simple diffusion component (Fig. 2).

$$v = \frac{V_{\max}[S]}{K_m + [S]} + k_d[S]$$

The uptake rate (v) is related to the affinity of the transporter for its substrate (K_m) at the concentration of half maximal transport $(V_{\max})$. Simple diffusion is directly proportional to the substrate concentration by a kinetic constant (k_d) and does not saturate with increasing concentrations. Transporters may have overlapping specificities but may differ in substrate affinity, regulation of expression, and tissue or cellular distribution.

2.2. Energetics

Transporters receive energy from a variety of sources (Fig. 3A). *Active* transporters concentrate a substrate to higher concentrations within a cell than what is present extracellularly; *passive* transporters facilitate the movement of a solute to achieve equilibrium across the membrane. In mammalian cells, primary active transporters are directly coupled to ATPase activity and hydrolyze ATP to derive energy for pumping substrates across the membrane.

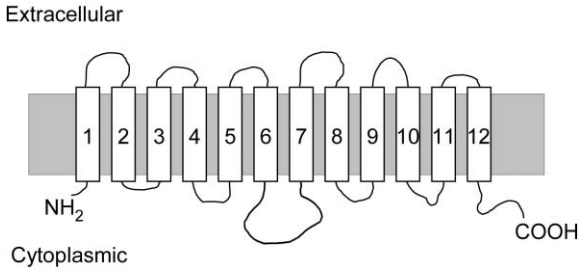

Fig. 1. Transporter membrane topology.

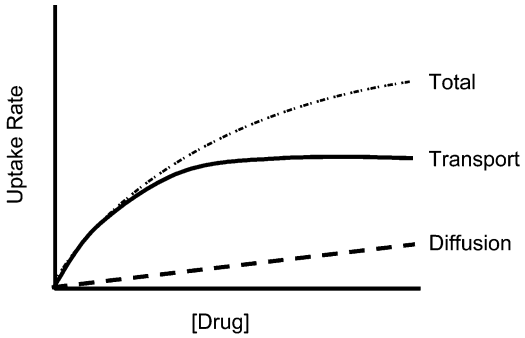

Fig. 2. Concentration dependence.

All other active transporters use an energy source that is indirectly coupled to Na^+/K^+-ATPase. As shown in Fig. 3B, Na^+/K^+-ATPase creates an inwardly directed sodium chemical gradient by simultaneously effluxing 3 Na^+ out of the cell and influxing 2 K^+ into the cell, thereby, creating an intracellular negative membrane potential due to the imbalance in charge movement. Since Na^+/K^+-ATPase is present in all mammalian cells, a number of transporters use this electrochemical gradient either directly or indirectly to drive the concentrative accumulation of solutes within the cell (Fig. 3A). Secondary active transporters are Na^+-dependent, cotransporting Na^+ with a substrate (S). Similarly, transporters can be coupled to a proton gradient created by the Na^+/H^+ exchanger that utilizes the Na^+ gradient created by the Na^+/K^+-ATPase to export H^+ from the cell thereby creating an acid microenvironment at the cell surface. Examples of tertiary active transporters are the proton-dependent peptide transporters (PEPT1, PEPT2) and the amino acid transporter, L-system (LAT2) (Table 1) [3–5]. Alternatively, active transporters can also be exchangers permitting the movement of one solute for another such as the Na^+/H^+ antiporter. By contrast, facilitative transporters are not coupled to any energy source and are 'passive', facilitating the diffusion of molecules

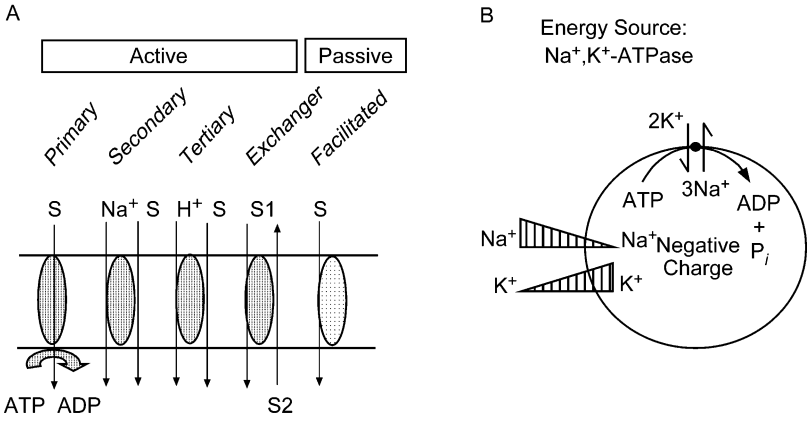

Fig. 3. Energetics of transporters.

Table 1. Examples of solute carriers that mediate drug transport [8]

Transporter	Location	Natural substrates	Drug substrates	References
CNT1[a] (SLC28A1)	Liver, kidney, intestine, brain	Pyrimidine nucleosides	2′-Deoxycitidine, 5-fluoro-5′-deoxyuridine, gemcitabine	[9]
ENT1[p] (SLC29A1)	Ubiquitous	Nucleosides, free nucleobases	Gemcitabine, fludarabine, cytarabine, ribavirin	[9–11]
LAT1[p] (SLC7A5)	Brain, testis, ovaries, placenta	Large neutral amino acids with bulky or branched side chains	L-Dopa, melphalan, gabapentin, triiodothyronine	[4,12]
LAT2[a] (SLC7A8)	Ubiquitous	Large and small neutral amino acids	L-Dopa	[4,13]
PEPT1[a] (SLC15A1)	Intestine, kidney proximal tubule	Di/tripeptides	Cephalosporins, ACE inhibitors, renin inhibitors, bestatin, valacyclovir,	[14,15]
PEPT2[a] (SLC15A2)	Kidney distal tubule, brain	Di/tripeptides	Cephalosporins, ACE inhibitors, valacyclovir	[16,17]
HPT-1[a] (CDH17)	Intestine	Di/tripeptides?	Cephalexin, bestatin, valacyclovir	[6,7]
MCT1[a] (SLC16A1)	Ubiquitous	Anions, Pyruvate, lactic acid, ketone bodies, biotin	Ceftibuten, carindacillin, atorvastatin acid	[18–23]
RFC-1[a] (SLC19A1)	Intestine, kidney, retina	Folic acid, thiamine	Methotrexate, premetrexed	[24–29]
OATP-C[a], OATP2, LST-1 (SLC21A6)	Liver	Organic anions, bile acids, bilirubin, estrone sulfate or estradiol-17-β-D-glucuronide	Pravastatin, troglitazone sulfate conjugate, Atorvastatin acid	[22,30–32]

[a] Active transport; [p] passive transport.

Abbreviations: ACE, angiotensin converting enzymes; CNT, concentrative nucleoside transporter; DOPA, dopamine; ENT, equilibrative nucleoside transporter; HPT, human peptide transporter; LAT, L-system-amino acid transporter; MCT, monocarboxylic acid transporter; OATP, organic anion transport protein; PEPT, peptide transporter; RFC, reduced folate transporter.

across the membrane down their concentration gradients permitting solutes to rapidly equilibrate across the membrane.

2.3. Solute carriers

In mammalian cells, the largest group of influx transporters is classified as the solute carriers (designated SLC) [2]. These membrane proteins are important for the transport of nutrients such as amino acids, peptides, sugars, nucleosides, vitamins, neurotransmitters, bile acids and organic and inorganic anions and cations. There are presently 298 genes assigned to the SLC gene classification. They are divided into 43 families (designated SLC1-43). Table 1 lists a number of different drug influx transporters and some of their known substrates. An exception noted in Table 1 is CDH17, commonly known as HPT-1. HPT-1 transports the β-lactam antibiotic, cephalexin, bestatin and valacyclovir [6,7]; however, it is in the cadherin (CDH) gene classification.

2.4. ATP-binding cassette (ABC) transporter superfamily

A second major group of drug transporters are members of the ABC transporter superfamily that are responsible for the efflux of many drugs and drug conjugates. These ABC membrane proteins are ATP-dependent transporters that contain a highly conserved ATP signature domain and multiple transmembrane spanning domains. Humans possess ~50 members of this superfamily which are divided into seven families (designated ABCA–ABCG) and to date four families contain drug efflux transporters (Table 2) [33–37]. These ABC drug transporters are listed in Table 2 with their tissue distribution and substrates. MDR1 (ABCB1, P-glycoprotein, Pgp) is the most extensively studied and effluxes drugs for a wide array of therapeutic indications. Genetic polymorphisms exist in several members that influence their substrate specificity and/or transport activity [38–42]. The role of several of these transporters in tissue distribution will be discussed below.

2.5. Tissue distribution

Transporters may work in concert to allow movement through polarized cells such as epithelial cells of the intestine, kidney, liver, and lung. Transporters in these cell types are asymmetrically distributed to cell surfaces (Fig. 4). One example of this is the movement of nucleosides across the intestinal mucosa. Uptake at the apical (luminal) side is mediated by the Na^+-dependent concentrative nucleoside transporters (CNTs) while efflux is mediated by the Na^+-independent equilibrative nucleoside transporters, ENTs. These transporters work in concert to permit the vectorial transport across the mucosa of nucleosides into the blood stream. Expression of CNTs, PEPT1 and HPT-1 in the intestine is regulated by diet or disease [67–69].

Also, ABC transporters are often key in maintaining the barrier function of various tissues and excretion of substances from the body. For example, Pgp is highly expressed in the luminal membrane of the intestine, the canalicular membrane of the liver and the blood–brain barrier (BBB). At the intestinal luminal membrane, Pgp

Table 2. ABC drug efflux transporters [43]

Transporter	Location	Natural substrates	Drug substrates	References
ABCA8	Heart, skeletal tissue, liver, others	LTC_4, 17-β-glucuronide-estradiol, taurocholate	*p*-Aminohippuric acid (ochratoxin-A)	[44]
MDR1 (ABCB1) Pgp	Intestine, kidney, lung, liver, adrenal gland, BBB	Amphiphilic cationic and neutral compounds	Natural product anticancer agents; STI-571; immunosuppressive agents, HIV protease inhibitors; anti-CVS drugs; CNS, morphine, fexofenadine, loperamide	[45–47]
BSEP (ABCB11) Sister Pgp	Liver	Bile acids	Vinblastine	[48,49]
MRP1 (ABCC1)	Ubiquitous (<< liver)	Organic anionic conjugates, X + GSH LTC_4 GSSG peptides	Anticancer agents, Anthracyclines, Epipodophyllo-toxins, Melphalan, SN-38	[50]
MRP2 (ABCC2) cMoat, cMRP	Liver, intestine, kidney	Organic anions and anionic conjugates Glucuronsul-bilirubin LTC_4	Anticancer drugs, SN-38, premetremed; cephalosporins, HIV protease inhibitors	[51–53]
MRP3 (ABCC3) Moat-D	Liver, intestine, kidney, placenta (pancreas)	Organic anionic conjugates Bile Salts, LTC_4, 17-β-glucuronide-estradiol	Anticancer drugs, etoposide MTX, vincristine	[54,55]
MRP4 (ABCC4) Moat-B	Prostate, lung, testis, ovary, bladder, pancreas	Nucleoside Phosphonates cAMP, cGMP	Antiviral agents, PMEA, PMEG AZT, 6-MP, 6-TG	[56–59]
MRP5 (ABCC5) Moat-C	Ubiquitous	Base and Nucleotide Analogs, camp, cGMP	PMEA, 6-MP, 6-TG, premetrexed	[53,56,60]
MRP8 (ABCC11) SUR1	Low levels except in kidney, spleen, colon	Purine and pyrimidine nucleotide analogs, cAMP, cGMP	Antiviral, PMEA; anticancer fluoropyrimidine	[61,62]
BCRP (ABCG2) MXR, ABCP	Placenta, breast, liver, intestine, brain	Organic anions, porphyrins and steroids	Anticancer, mitoxantrone, doxorubicin, MTX, topotecan, SN-38, AZT	[63–66]

Abbreviations: 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; AZT, zidovudine; LTC_4, leukotriene C4; MTX, methyltrexate, PMEA 9-(2-phosphonylmethoxyethyl)adenine; PMEG, 9-(2-phosphonylmethoxyethyl)guanosine; SN-38, 7-ethyl-10-hydroxycamptothecin.

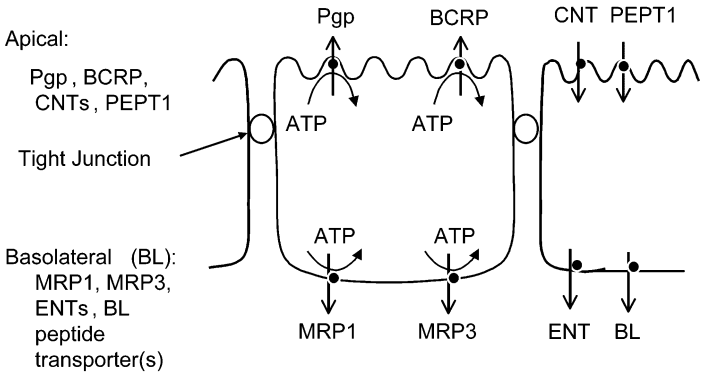

Fig. 4. Asymmetric distribution of transporters in epithelial cells.

as well as Breast Cancer Resistance Protein (BCRP) is known for limiting the bioavailability of certain drugs (Table 2). At the BBB, Pgp is a major player in excluding substances from the brain, thereby protecting the brain and maintaining the BBB. In addition, Pgp is responsible for the excretion of some drugs from the liver into the bile.

3. ROLES IN CLEARANCE AND TISSUE DISTRIBUTION

Transporters are important in determining the pharmacokinetics (e.g. absorption, distribution, metabolism and excretion) of their substrates [70]. Their roles become apparent when polymorphisms exist in transporters that alter their transport activity or when two drugs are co-administered that compete for the same transporter. Under these circumstances altered pharmacokinetics of the transported compound(s) can be seen by changes in pharmacokinetic parameters such as bioavailability, plasma clearance and apparent volume of distribution. Bioavailability can be defined as the fraction of the dose reaching the systemic circulation as the intact unmetabolized drug. Clearance describes the efficiency of irreversible elimination of the drug from the body and can be defined as the volume of blood cleared of drug per unit time. The apparent volume of distribution relates the concentration of drug in the plasma to the total amount of the drug in the body. The major determinant is the relative strength of drug binding to tissue components compared to binding to plasma proteins.

3.1. Impact on oral absorption

Both influx and efflux transporters may have a dramatic effect on oral absorption. Early in the development of oral cephalosporins, an intestinal peptide transporter was recognized to be important for enhancing the oral absorption of these hydrophilic antibiotics [3]. For some β-lactams, cephalexin, cepharadrine, and cefadroxil, nearly 90% is orally absorbed in humans [71,72]. Because excellent bioavailability generally is

afforded PEPT1 substrates, peptidyl mimetic prodrug strategies have been used to enhance absorption relative to the parent molecule. An example is the development of valacyclovir a prodrug of acyclovir that enhanced oral bioavailability of acyclovir from 22 to 70% and reduced the interindividual variability from 49 to 21% in patients.

By contrast, the ABC drug transporters can dramatically limit the oral absorption of their substrates by enhancing their clearance via the intestine, kidney and/or liver. For example, the anticancer agent, paclitaxel, a Pgp substrate has poor oral bioavailability in humans, only 5%, but when dosed with the Pgp substrate, cyclosporin A, the oral bioavailability of paclitaxel is increased to 50% [73]. When a Pgp substrate, talinolol, that is not appreciably metabolized, was co-administered with a Pgp inhibitor, verapamil, oral absorption of talinolol was enhanced by blocking intestinal secretion and decreasing plasma clearance (between 30 and 60%) as well as reducing the volume of distribution of talinolol [74]. Absorption can also be altered by agents that enhance the expression of transporters such as rifampicin, St. John's wort or phenobarbital for Pgp and MRP2 [75–77]. Clinical studies have examined the effect of rifampicin on talinolol [78]. After 9 days of rifampicin treatment, duodenal Pgp expression was enhanced by ~4-fold and talinolol plasma exposure was reduced after either oral or intravenous administration, 35 or 21%, respectively. Following 1 mg talinolol with 1 mg digoxin by IV administration, the volume of distribution was enhanced modestly by 13%. A similar result was seen with digoxin administered orally after dosing rifampicin for 10 days [79]. The oral bioavailability was decreased by 30% and was shown to correlate to an increase in Pgp expression in the duodenum of the subjects. Taken together, these studies demonstrate the important role that Pgp has in oral absorption, clearance as well as distribution.

Another ABC drug transporter important in limiting oral absorption is BCRP. The anticancer agent topotecan is a substrate of BCRP and not Pgp. In clinical studies, cancer patients were dosed topotecan orally or intravenously with and without a single oral dose of a potent, mixed inhibitor of both Pgp and BCRP, GF120918 [80]. Co-administration of GF120918 significantly increased the apparent bioavailability of topotecan from 40 to 97%.

3.2. Impact on the kidney

The kidney plays an important role in the elimination of certain compounds. The intestine and kidney share many of the same transporters that are located to the same epithelial cell surfaces [81]. These include PEPT1, OATP, MDR and MRPs, organic anion transporters (OAT), organic cation transporters (OCT). PEPT1 and PEPT2 function for renal tubular reabsorption across the kidney brush-border membranes for drugs such as the β-lactam antibiotics [81]. Actively secreted renal drugs may be affected by co-administered drugs that share a common transporter. For example probenecid, an OAT substrate inhibits the OAT-mediated secretion of anionic drugs (penicillins, ACE inhibitors, antiviral drugs) at the basolateral membrane of renal proximal tubules [82–84]. OAT transporters are thought be important for mediating cephaloridine nephrotoxicity which is reversible with the co-administration of probenecid [85].

3.3. Impact on blood–brain distribution

An important tissue barrier for agents acting on the CNS is the BBB. Although much is still to be learned about the transporters in the BBB, a number of amino acid, organic anion, organic cation and ABC transporters, Pgp, MRPs, and BCRP are expressed there [86–88]. Only lipophilic drugs cross the BBB by way of simple diffusion and a strong correlation between lipophilicity and brain penetration of drugs exists although factors such as number of hydrogen bonds and molecular size can also influence the transport of drugs across the BBB [89–92]. Pgp is highly expressed in the endothelia that comprise the BBB and plays a critical role in restricting the passage of lipophilic compounds into the brain [93,94]. *In vitro* studies examining the permeability of 93 marketed drugs through MDCK cells expressing human Pgp demonstrated that both simple diffusion of drugs and their transport by Pgp could distinguish between drugs marketed for CNS and non-CNS indications [95]. In recent studies, several Pgp inhibitors (cyclosporin A, PSC-833 and GF120918) were co-administered with paclitaxel to increase the BBB penetration of wild type mice and *mdr*1a(−/−) knockout mice which lack Pgp in the BBB [96,97]. Brain levels of paclitaxel were 11-fold greater in knockout mice than wild type mice. Brain levels in wild type mice were enhanced by co-administration of cyclosporin A (3-fold), PSC-833 (6.5-fold) or GF120918 (5-fold). This indicates that drug combinations may alter the CNS pharmacodynamics. This has been demonstrated with loperamide, a Pgp substrate that is a potent opiate. Loperamide does not normally elicit CNS effects due to Pgp limiting its access to the brain, however, when co-administered with the Pgp inhibitor, quinidine, respiratory depression, was observed which is an effect common to centrally acting opiates [98]. The role of Pgp in this interaction was confirmed by studies in *mdr*1a(−/−) knockout mice. The brain concentration of loperamide after oral administration was 10-times greater in the knockout mice than in wild type mice [99]. Together, these clinical data support Pgp limiting the brain penetration of loperamide and paclitaxel.

3.4. Impact on liver

The liver is responsible for the majority of first pass metabolism that detoxifies orally absorbed xenobiotics. As such, the liver has a large number of transporters on the sinusoidal and canalicular membranes to not only extract xenobiotics from the blood into the liver, but to release the drug and/or its metabolites into the bile (Fig. 5) [51,100,101]. Liver-specific forms of some transporters exist such as the Bile salt export pump (BSEP) and the OATPs (C and 8) [32,77,102,103]. OATP-C is an influx transporter present on the sinusoidal membrane of hepatocytes that transports pravastatin. Genetic polymorphisms of OATP-C result in decreased transport capacity for pravastatin. A recent clinical study in the Japanese population showed that the transporter allele (Asp130/Ala174) resulted in a reduced clearance of pravastatin leading to greater plasma exposure [31,104]. This study confirms the role of OATP-C in the uptake of pravastatin into the liver in man.

MDR1 is located on the bile canalicular membrane of hepatocytes and is responsible for the biliary secretion of some drugs from the liver. In clinical studies, patients were given

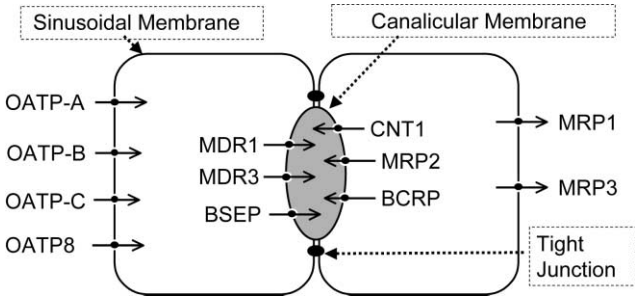

Fig. 5. Distribution of transporters in hepatocytes.

digoxin, a Pgp substrate, with and without concomitant administration of the Pgp inhibitor, verapamil [105]. Verapamil increased the steady-state plasma concentrations of digoxin by 44% by decreasing the biliary clearance by 43% without altering the renal clearance. Thus, Pgp can have an important role in the biliary clearance of certain drugs.

An important function of the liver is the metabolism of xenobiotics. Both Pgp and cytochrome P450 3A enzyme (CYP3A) are expressed in hepatocytes and their substrate specificities overlap considerably unlike CYP2C another isoform with more limited substrate specificity for these drugs. Expression of CYP3A and Pgp in the liver and the intestine are co-ordinately regulated by the pregnane (steroid) xenobiotic receptor to eliminate xenobiotics, i.e., Pgp effluxes the drug out of the cell while CYP3A further removes the substance by metabolism within the cell [106,107]. Thus, therapeutics that are inhibitors or substrates of both could be expected to lead to drug–drug interactions, resulting in increased levels of the co-administered drugs circulating in plasma.

4. CONCLUSIONS

Transporters are critical for the movement of hydrophilic and hydrophobic drugs across membranes. In clinical studies, transporters have been shown to be important in the oral absorption and tissue distribution of a number of very successful marketed drugs. The discovery and development of new agents will benefit from attention paid to the role of transporters in enhancing and/or limiting the biodistribution of compounds to target tissues. Incorporation of transport properties into structural activity relationships should aid in the design and development of new therapeutics with improved efficacy.

REFERENCES

[1] W. Sadee, V. Drubbisch and G. L. Amidon, *Pharm. Res.*, 1995, **12**, 1823.
[2] W. Sadee, R. C. Graul and A. Y. Lee, in *Classification of membrane transporters, in Membrane Transporters as Drug Targets* (eds G. Amidon and W. Sadee), Kluwer Academic/Plenum, New York, 1999, p. 29.
[3] A. H. Dantzig, *Adv. Drug Del. Rev.*, 1997, **23**, 63.

[4] D. P. Rajan, R. Kekuda, W. Huang, L. D. Devoe, F. H. Leibach, P. D. Prasad and V. Ganapathy, *Biochem. Biophys. Acta.*, 2000, **1463**, 6.
[5] V. Ganapathy, Y. Miyamoto and F. H. Leibach, *Beitrage Zu Infusionstherapie und Klinische Ernahrung*, 1987, **17**, 54.
[6] A. H. Dantzig, J. Hoskins, L. B. Tabas, S. Bright, R. L. Shepard, I. L. Jenkins, D. C. Duckworth, R. Sportsman, D. MacKensen, P. R. Rosteck and P. L. Skatrud, *Science*, 1994, **264**, 430.
[7] C. Landowski, D. Sun, D. Foster, S. Menon, J. Barnett, L. Welage, C. Ramachandran and G. Amidon, *J. Pharmacol. Exp. Ther.*, 2003, **306**, 778.
[8] http://www.gene.ucl.ac.uk/nomenclature/genefamily.shtml.
[9] W. Kong, K. Engel and J. Wang, *Curr. Drug Metab.*, 2004, **5**, 63.
[10] M. Huang, Y. Wang, S. B. Cogut, B. S. Mitchell and L. M. Graves, *J. Pharmacol. Exp. Ther.*, 2003, **304**, 753.
[11] L. M. Mangravite, G. Xiao and K. M. Giacomini, *Am. J. Physiol. Renal Physiol.*, 2003, **284**, F902.
[12] H. Uchino, Y. Kanai, D. K. Kim, M. F. Wempe, A. Chairoungdua, E. Morimoto, M. W. Anders and H. Endou, *Mol. Pharmacol.*, 2002, **61**, 729.
[13] M. J. Pinho, P. Gomes, M. P. Serrao, M. J. Bonifacio and P. Soares-da-Silva, *Hypertension*, 2003, **42**, 613.
[14] M. Brandsch, I. Knutter and F. H. Leibach, *Eur. J. Pharm. Sci.*, 2004, **21**, 53.
[15] T. Zhu, X. Chen, A. Steel, M. Hediger and D. Smith, *Pharm. Res.*, 2000, **17** (5), 526.
[16] T. Terada and K. Inui, *Curr. Drug Metab.*, 2004, **5**, 85.
[17] H. Daniel and G. Kottra, *Pflugers Archiv. Eur. J. Physiol.*, 2004, **447**, 610.
[18] S. Galic, H. P. Schneider, A. Broer, J. W. Deitmer and S. Broer, *Biochem. J.*, 2003, **376**, 413.
[19] Y. H. Li, M. Tanno, T. Itoh and H. Yamada, *Intern. J. Pharm.*, 1999, **191**, 151.
[20] R. Daberkow, B. White, R. Cederberg, J. Griffin and J. Zempleni, *J. Nutr.*, 2003, **133**, 2703.
[21] R. Menon and W. Barr, *Biopharm. Drug Dispos.*, 2002, **23**, 317.
[22] H. Lennernas, *Clin. Pharmacokinet.*, 2003, **42**, 1141.
[23] K. Iseki, M. Sugawara, K. Sato, I. Naasani, T. Hayakawa, M. Kobayashi and K. Miyazaki, *J. Pharmacol. Exp. Ther.*, 1999, **289**, 66.
[24] Y. Nozaki, H. Kusuhara, H. Endou and Y. Sugiyama, *J. Pharmacol. Exp. Ther.*, 2004, **309**, 226.
[25] H. Naggar, Y.-J. Fei, V. Ganapathy and S. B. Smith, *Exp. Eye Res.*, 2003, **77**, 687.
[26] T. Li, T. Tomimatsu, K. Ito and T. Horie, *Life Sci.*, 2003, **73**, 2631.
[27] L. Matherly and D. Goldman, *Vitam. Horm.*, 2003, **66**, 403.
[28] V. Ganapathy, S. Smith and P. Prasad, *Pflügers Archiv. Eur. J. Physiol.*, 2004, **447**, 641.
[29] I. Goldman and R. Zhao, *Semin. Oncol.*, 2002, **6**, 3.
[30] T. Nozawa, S. Sugiura, M. Nakajima, A. Goto, T. Yokoi, J. Nezu, A. Tsuji and I. Tamai, *Drug Met. Disp.*, 2004, **32**, 291.
[31] Y. Nishizato, I. Ieiri, H. Suzuki, M. Kimura, K. Kawabata, T. Hirota, H. Takane, S. Irie, H. Kusuhara and Y. Urasaki, *Clin. Pharmacol. Ther.*, 2003, **73**, 554.
[32] O. Briz, M. Serrano, R. MacIas, J. Gonzalez-Gallego and J. Marin, *Biochem. J.*, 2003, **371**, 897.
[33] M. Dean, Y. Hamon and G. Chimini, *J. Lipid Res.*, 2001, **42**, 1007.
[34] G. Leonard, T. Fojo and S. Bates, *Oncologist*, 2003, **8**, 411.
[35] L. M. S. Chan, S. Lowes and B. H. Hirst, *Eur. J. Pharm. Sci.*, 2004, **21**, 25.
[36] G. Kruh and M. Belinsky, *Oncogene*, 2003, **22**, 7537.
[37] P. A. Rea, *J. Exp. Bot.*, 1999, **50**, 895.
[38] S. Hoffmeyer, O. Burk, O. von Richter, H. P. Arnold, J. Brockmoller, A. Johne, I. Cascorbi, T. Gerloff, I. Roots, M. Eichelbaum and U. Brinkmann, *Proc. Natl Acad. Sci. USA*, 2000, **97**, 3473.
[39] R. Kerb, S. Hoffmeyer and U. Brinkmann, *Pharmacogenomics*, 2001, **2**, 1.
[40] S. Saito, A. Iida, A. Sekine, Y. Miura, C. Ogawa, S. Kawauchi, S. Higuchi and Y. Nakamura, *J. Human Gen.*, 2002, **47**, 147.
[41] A. Siddiqui, R. Kerb, M. Weale, U. Brinkmann, A. Smith, D. Goldstein, N. Wood and S. Sisodiya, *N. Engl. J. Med.*, 2003, **348**, 1480.

[42] Y. Imai, M. Nakane, K. Kage, S. Tsukahara, E. Ishikawa, T. Tsuruo, Y. Miki and Y. Sugimoto, *Mol. Cancer Ther.*, 2002, **1**, 611.
[43] http://nutrigene.4t.com/humanabc.htm.
[44] S. Tsuruoka, K. Ishibashi, H. Yamamoto, M. Wakaumi, M. Suzuki, G. Schwartz, M. Imai and A. Fujimura, *Biochem. Biophys. Res. Commun.*, 2002, **298**, 41.
[45] J. M. Ford and W. N. Hait, *Pharmacol. Rev.*, 1990, **42**, 155.
[46] H. Dai, P. Marbach, M. Lemaire, M. Hayes and W. F. Elmquist, *J. Pharmacol. Exp. Ther.*, 2003, **304**, 1085.
[47] M. Cvetkovic, B. Leake, M. Fromm, G. Wilkinson and R. Kim, *Drug Met. Dispos.*, 1999, **27**, 866.
[48] V. Lecureur, D. Sun, E. Schuetz and J. Schuetz, *Hepatology*, 2000, **28**, 1477.
[49] V. Lecureur, D. Sun, P. Hargrove, E. G. Schuetz, R. B. Kim, L.-B. Lan and J. D. Schuetz, *Mol. Pharmacol.*, 2000, **57**, 24.
[50] A. C.G. Haimeur, R. G. Deeley and S. P. Cole, *Curr. Drug Metab.*, 2004, **5**, 21.
[51] D. Keppler, T. Kamisako, I. Leier, Y. Cui, A. T. Nies, H. Tsujii and J. Konig, *Adv. Enzyme Reg.*, 2000, **40**, 339.
[52] M. T. Huisman, J. W. Smit, K. Crommentuyn, N. Zelcer, H. Wiltshire, J. H. Beijenen and A. H. Schinkel, *AIDS*, 2002, **16**, 2295.
[53] S. E. Pratt, R. Emerick, L. Horwitz, M. Gallery, A. Lesoon, S. Jink, V. J. Chen and A. H. Dantzig, *AACR*, 2002, **43**.
[54] Y. Kiuchi, H. Suzuki, T. Hirohashi, C. A. Tyson and Y. Sugiyama, *FEBS Lett.*, 1998, **433**, 149.
[55] G. Kruh, H. Zeng, P. Rea, G. Liu, Z. Chen, K. Lee and M. Belinsky, *J. Bioenerg. Biomemb.*, 2001, **33**, 493.
[56] J. Sampath, M. Adachi, S. Hatse, L. Naesens, J. Balzarini, R. Flatley, L. Matherly and J. Schuetz, *AAPS PharmSci.*, 2002, **4**, E14.
[57] J. D. Schuetz, M. C. Connelly, D. X. Sun, S. G. Paibir, P. M. Flynn, R. V. Srinivas, A. Kumar and A. Fridland, *Nat. Med.*, 1999, **5**, 1048.
[58] P. Wielinga, G. Reid, E. Challa, I. van der Heijden, L. van Deemter, M. de Haas, C. Mol, A. Kuil, E. Groeneveld, J. Schuetz, C. Brouwer, R. De Abreu, J. Wijnholds, J. Beijnen and P. Borst, *Mol. Pharmacol.*, 2002, **62**, 1321.
[59] P. Wielinga, I. van der Heijden, G. Reid, J. Beijnen, J. Wijnholds and P. Borst, *J. Biol. Chem.*, 2003, **278**, 17664.
[60] G. Jedlitschky, B. Burchell and D. Keppler, *J. Biol. Chem.*, 2000, **275**, 30069.
[61] Y. Guo, E. Kotova, Z. Chen, K. Lee, E. Hopper-Borge, M. Belinsky and G. Kruh, *J. Biol. Chem.*, 2003, **278**, 29509.
[62] T. Bera, S. Lee, G. Salvatore, B. Lee and I. Pastan, *Mol. Med.*, 2001, **7**, 509.
[63] S. E. Bates, R. Robey, K. Miyake, K. Rao, D. D. Ross and T. Litman, *J. Bioenerg. Biomemb.*, 2001, **33**, 503.
[64] T. Janvilisri, H. Venter, S. Shahi, G. Reuter, L. Balakrishnan and H. W. van Veen, *J. Biol. Chem.*, 2003, **278**, 20645.
[65] J. W. Jonker, M. Marije Buitelaar, E. Wagenaar, M. A. van der Valk, G. L. Scheffer, R. J. Scheper, T. Plösch, F. Kuipers, R. P. J. Oude Elferink, H. Rosing, J. H. Beijnen and A. H. Schinkel, *Proc. Natl Acad. Sci. USA*, 2002, **99**, 15649–15654.
[66] X. Wang, T. Furukawa, T. Nitanda, M. Okamoto, Y. Sugimoto, S. Akiyama and M. Baba, *Mol. Pharmacol.*, 2003, **63**, 65.
[67] R. Valdes, M. Ortega, F. Casado, A. Felipe, A. Gil, A. Sanchez-Pozo and M. Pastor-Anglada, *Gastroenterology*, 2000, **119**, 1623.
[68] S. A. Adibi, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2003, **285**, G779.
[69] R. H. Erickson, J. R. Gum, Jr, M. M. Lindstrom, D. McKean and Y. S. Kim, *Biochem. Biophys. Res. Commun.*, 1995, **216**, 249.
[70] A. Ayrton and P. Morgan, *Xenobiotica*, 2001, **31**, 469.
[71] T. Bergan, *Drugs*, 1987, **34**, 89.
[72] C. Nightingale, D. S. Greene and R. Quintiliani, *J. Pharm. Sci.*, 1975, **64**, 1899.
[73] J. Meerum Terwogt, M. Malingre, J. Beijnen, W. ten Bokkel Huinink, H. Rosing, F. Koopman, O. van Tellingen, M. Swart and J. Schellens, *Clin. Cancer Res.*, 1999, **5**, 3379.

[74] H. Spahn-Langguth, G. Baktir, A. Radschuweit, A. Okyar, B. Terhaag, P. Ader, A. Hanafy and P. Langguth, *Int. J. Clin. Pharm. Ther.*, 1998, **36**, 16.
[75] H. Kast, B. Goodwin, P. Tarr, S. Jones, A. Anisfeld, C. Stoltz, P. Tontonoz, S. Kliewer, T. Willson and P. Edwards, *J. Biol. Chem.*, 2002, **277**, 2908.
[76] L. Payen, L. Sparfel, A. Courtois, L. Vernhet, A. Guillouzo and O. Fardel, *Cell Biol. Toxicol.*, 2002, **18**, 221.
[77] G. Kullak-Ublick and M. Becker, *Drug Metab. Rev.*, 2003, **35**, 305.
[78] K. Westphal, A. Weinbrenner, M. Zschiesche, G. Franke, M. Knoke, R. Oertel, P. Fritz, O. von Richter, R. Warzok, T. Hachenberg, H. Kauffmann, D. Schrenk, B. Terhaag, H. Kroemer and W. Siegmund, *Clin. Pharmacol. Ther.*, 2000, **68**, 345.
[79] B. Greiner, M. Eichelbaum, P. Fritz, H. P. Kreichgauer, O. von Richter, J. Zundler and H. K. Kroemer, *J. Clin. Invest.*, 1999, **104**, 147.
[80] C. M. Kruijtzer, J. H. Beijnen, H. Rosing, W. W. Bokkel Huinink, M. Schot, R. C. Jewell, E. M. Paul and J. H. Schellens, *J. Clin. Oncol.*, 2002, **20**, 2943.
[81] K. Inui, S. Masuda and H. Saito, *Kidney Int.*, 2000, **58**, 944.
[82] J. W. Massarella, L. A. Nazareno, S. Passe and B. Min, *Pharm. Res.*, 1996, **13**, 449.
[83] A. Somogyi, *Clin. Exp. Pharmacol. Physiol.*, 1996, **23**, 986.
[84] T. Sekine, S. H. Cha, Y. Kanai and H. Endou, *Clin. Exp. Nephrol.*, 1999, **3**.
[85] S. Khamdang, M. Takeda, E. Babu, R. Noshiro, M. Onozato, A. Tojo, A. Enomoto, X.-L. Huang, S. Narikawa, N. Anzai, P. Piyachaturawat and H. Endou, *Eur. J. Pharmacol.*, 2003, **465**, 1.
[86] H. Sun, H. Dai, N. Shaik and W. Elmquist, *Adv. Drug Deliv. Rev.*, 2003, **55**, 83.
[87] H. Cooray, C. Blackmore, L. Maskell and M. Barrand, *NeuroReport*, 2002, **13**, 2059.
[88] G. Fricker and D. Miller, *Pharmacology*, 2004, **70**, 169.
[89] W. M. Pardridge, *Diabetologia*, 1981, **20** (suppl.), 246.
[90] E. G. Chikhale, K. Y. Ng, P. S. Burton and R. T. Borchardt, *Pharm. Res.*, 1994, **11**, 412.
[91] E. G. Chikhale, P. S. Burton and R. T. Borchardt, *J. Pharmacol. Exp. Ther.*, 1995, **273**, 298.
[92] V. A. Levin, *J. Med. Chem.*, 1980, **23**, 682.
[93] F. Thiebaut, T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan and M. C. Willingham, *J. Histochem. Cytochem.*, 1989, **37**, 159.
[94] C. Cordon-Cardo, J. P. O'Brien, D. Casals, L. Rittman-Grauer, J. L. Biedler, M. R. Melamed and J. R. Bertino, *Proc. Natl Acad. Sci. USA*, 1989, **86**, 695.
[95] K. Mahar Doan, J. Humphreys, L. Webster, S. Wring, L. Shampine, C. Serabjit-Singh, K. Adkison and J. Polli, *J. Pharmacol. Exp. Ther.*, 2002, **303**, 1029.
[96] E. Kemper, A. van Zandbergen, C. Cleypool, H. Mos, W. Boogerd, J. Beijnen and O. van Tellingen, *Clin. Cancer Res.*, 2003, **9**, 2849.
[97] E. Kemper, C. Cleypool, W. Boogerd, J. Beijnen and O. Van Tellingen, Cancer Chemother. Pharmacol., 2003.
[98] A. J. Sadeque, C. Wandel, H. He, S. Shah and A. J. Wood, *Clin. Pharmacol. Ther.*, 2000, **68**, 231.
[99] A. H. Schinkel, E. Wangenaar, C. A. Mol and L. van Deemter, *J. Clin. Invest.*, 1996, **97**, 2517.
[100] P. Borst and R. Elferink, *Annu. Rev. Biochem.*, 2002, **71**, 537.
[101] G. Jedlitschky and D. Keppler, *Vitam. Horm.*, 2002, **64**, 153.
[102] E. G. Schuetz, S. Strom, K. Yasuda, V. Lecureur, M. Assem, C. Brimer, J. Lamba, R. B. Kim, V. Ramachandran, B. J. Komoroski, R. Venkataramanan, H. Cai, C. J. Sinal, F. J. Gonzalez and J. D. Schuetz, *J. Biol. Chem.*, 2001, **276**, 39411.
[103] R. Kim, *Eur. J. Clin. Inv.*, 2003, **33**, 1.
[104] T. Nozawa, M. Nakajima, I. Tamai, K. Noda, J. Nezu, Y. Sai, A. Tsuji and T. Yokoi, *J. Pharmacol. Exp. Ther.*, 2002, **302**, 804.
[105] A. Hedman, B. Angelin, A. Arvidsson, O. Beck, R. Dahlqvist, B. Nilsson, M. Olsson and K. Schenck-Gustafsson, *Clin. Pharmacol. Ther.*, 1991, **49**, 256.
[106] E. G. Schuetz, A. H. Schimkel, M. V. Relling and J. D. Schuetz, *Proc. Natl Acad. Sci. USA*, 1996, **93**, 4001.
[107] E. Schuetz, L. Lan, K. Yasuda, R. Kim, T. Kocarek, J. Schuetz and S. Strom, *Mol. Pharmacol.*, 2002, **62**, 439.

The Hit-to-Lead Process in Drug Discovery

Paul Gillespie and Robert A. Goodnow Jr.

Hoffmann-La Roche, Inc., Nutley, NJ 07110, USA

Contents

1. INTRODUCTION

Despite technological advances such as high throughput screening (HTS) and combinatorial chemistry, the number of drug launches has fallen steadily during the past several years [1]. The ten to fifteen year discovery and development cycle makes it necessary for most companies to work on multiple projects simultaneously; therefore, drug discovery requires a regular supply of leads to drive this process. Of the few published studies concerning pre-clinical attrition rates going from target validation to clinical candidate selection, estimates of rates for successfully finding a lead and for optimizing that lead are reported as 60 and 63%, respectively [2]. The way in which research and development organizations are willing to take a family of lead molecules into clinical development is one of two reasons cited for higher success rates in delivering compounds for launch; selecting compounds for clinical studies according to stricter, more aggressive quality criteria is also cited as a success factor [3]. Both of these factors speak to the opportunity to enhance the success rates in drug discovery by providing more and better leads. To this end, the emergence of a new phase in drug discovery, commonly called the hit-to-lead process, has been the target of increased attention and thus, merits detailed definition and exemplification.

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2. DEFINITION OF THE HIT-TO-LEAD PROCESS

Most leads are identified by assaying natural products, historical compound collections, combinatorial chemistry libraries, and compounds synthesized according to knowledge-based approaches such as study of literature and patents. Often compounds are identified through an HTS assay protocol, the predominant means to identify potential leads in a random manner. Depending on the biological target, the assay of hundreds of thousands of single compounds usually produces some number of primary hits. The process of primary hit filtration and assessment is known as hit-to-lead [4–10]. In the hit-to-lead process, an early prospecting of the potential of a compound series in question is conducted to assess the chance of success in lead optimization and beyond. Experience has shown that of the many primary hits identified from a high throughput screen, only a handful are likely to advance to the resource-intensive lead optimization phase.

Almost all hit-to-lead programs begin with SAR studies directed toward increasing the potency of the hit and understanding the SAR landscape around it. It makes sense to increase potency since many screening hits have activity in the single-digit micromolar range and this is generally not a sufficient level of potency for a human therapeutic. Moreover, increasing potency is often accompanied by reduction in the severity of some issues associated with a high dose, such as solubility, selectivity, toxicity, permeability, etc. Many recently published papers covering hit-to-lead work focus on SAR studies and improvements in potency [11–24]. In some cases, crystal structures are used to direct structural modification of the hit [25,26].

In other cases, liabilities are identified early on, and overcoming these liabilities becomes the focus of the hit-to-lead efforts, often in combination with efforts to improve the potency of the hits. For example, in order to minimize possible unwanted side-effects, selectivity may be an important issue and several publications have appeared describing improvements in this area [27–33]. In addition to selectivity, some other issues that have received recent attention in hit-to-lead publications are anticipated toxicity [34]; metabolism [35]; and a combination of poor solubility, high lipophilicity and lack of functional activity [36].

Some publications describe a series of analogues where different issues are addressed by different modifications of the hit. For example, some compounds may show that potency increases are possible while other compounds show that oral bioavailability is achievable in the series. The challenge in such cases is to show that it is possible to identify a molecule in which all issues are satisfactorily resolved in the same molecule. Recent publications have described cases of improvements in potency and bioavailability [37]; potency, selectivity and water solubility [38]; or oral bioavailability.

Unlike lead optimization, the focus of hit-to-lead efforts is not to identify the best compound, but rather to provide data around a series of compounds that rationalizes the decision to advance a given series of compounds into the lead optimization phase. An early identification of liabilities significant enough to disqualify a series of compounds for further work is of greatest value before the expenditure of lead optimization effort and resources on that series.

The process of primary hit filtration, assessment, and prospecting of the potential for a tractable lead series is central to the hit-to-lead process. The hit-to-lead process is composed of the general stages of hit assessment, hit validation, and identification of the best hits. The goal of any hit-to-lead effort is the identification of an optimal lead series and its liabilities for transition to lead optimization efforts. Although the lead series criteria are specific to a given project, one can organize the common thinking about this process and some of its criteria as shown in Fig. 1. Of the reasons that a compound may fail during lead optimization, several may be generalized and may be used as a basis for consideration of the suitability of any molecule for a target. For example, recent reports have described methods to identify so-called frequent hitters, molecules that appear active in several HTS assays unrelated as members of a single protein family [39]. Because attempts to optimize frequent hitters as leads are nearly always futile, these molecules should be eliminated rapidly from consideration.

For a given molecule, failure to meet some hit-to-lead criteria does not necessarily eliminate the molecule from further consideration as much as it helps to direct on synthesis of other molecules to understand the potential to remediate the problem. Successful practices for the identification of good leads from HTS data are an efficient means to increase the chances for success in the lead optimization phase.

Assessing hits	Validating hits	Identification of high quality hits	"A Good Lead"
Structure & purity confirmed	Activity confirmed with powder sample	Resolution & assay of chiral isomers	Elucidation of kinetics, mode of action
Not a frequent hitter	Feasible chemistry for analog synthesis	Synthesis amenable to HTC	NMR or X-ray of structure-target complex
Minimum toxicity alerts	Potency < 10 μM	Plausible SAR in 50-100 analogs	Potency < 1 μM
Minimum Lipinski Rules violations	Appropriate target selectivity	No Lipinski Rules violation	Encouraging preliminary PK
Solubility, permeability, logP calculated	Solubility, permeability, logD measured	Determination of metabolic liabilities	Low hERG channel binding liability
	Intellectual property issues assessed	LogD: 0 – 3	Aq. solubility > 100 μg / mL
		PAMPA: high	Low Cyp450, PGP liabilities

Fig. 1. Stages and general criteria for the hit-to-lead process.

As encouraging information is accumulated, the molecule or series of molecules in question progresses from the status of an HTS primary hit to *bona fide* lead series.

3. PUBLISHED EXAMPLES OF HIT-TO-LEAD STUDIES

The most clearly defined milestones in the drug discovery process are project initiation and entry-into-humans. Intermediate milestones are defined differently in different organizations and are often not described in publications. Consequently, the identification of papers dealing with the hit-to-lead process is somewhat subjective. We have included in this review papers dealing with the early stages of drug discovery, and some may actually include elements of lead optimization.

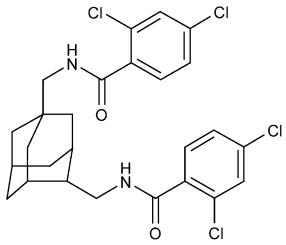
3.1. P2X7 receptor antagonists

Elaboration of an HTS hit with respect to a specific target criteria was recently reported for antagonists of the P2X7 receptor, a ligand-gated ion channel involved in the mediation of inflammatory and immune processes [40]. Extensive and specific criteria for the lead target profile were reported (Table 1) and were used to evaluate the nature of initial hits and the progress of hit-to-lead efforts. Emphasis on finding molecules with good metabolic stability is evident in this example, but many of these properties would define good leads for other targets as well.

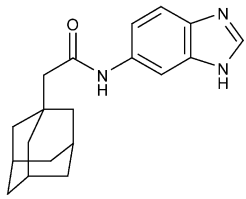
Through HTS, the structure **1** was identified and evaluated against the criteria above. The goals of the hit-to-lead effort were to maintain inhibitory potency, to understand the preliminary SAR, to improve clearance values and to reduce the compound's molecular weight and *c* log *P*. During the course of the SAR investigation, it was found that a single aromatic substituent was sufficient for significant potency. This change gave a significant reduction in molecular weight. SAR study of aryl ring substitution through preparation of various analogs also identified the possibility of introducing heterocylic fused rings (**2**). These improvements in the molecule along with acceptable pharmacokinetic properties in the rat made it possible to advance the molecule to lead optimization (LO) efforts.

Table 1

Property	Target	Hit 1	Lead 2
Inhibitory potency (pA_2)	>7.0	6.9	7.4
Rat hepatocyte clearance ($\mu L/min/10^6$ cells)	<14		5
Human liver microsomal clearance ($\mu L/min/mg$)	<23		21
Rat iv clearance (mL/min/kg)	<35		47
Rat Vss (L/kg)	>0.5		2
$T_{1/2}$ (h)	>0.5		1
Molecular weight (Da)	<450	540	309
c log *P*	<3.0	6.2	



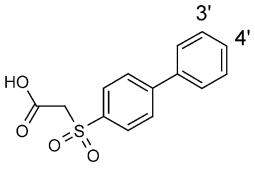
1
pIC_{50} = 6.9



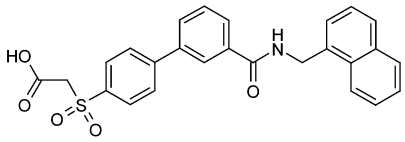
2
pIC_{50} = 7.0

3.2. HPV6 E1 helicase inhibitor

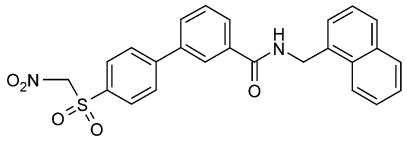
The hit-to-lead process for an HTS hit **3** active against human papillomavirus HPV6 E1 helicase was divided into two steps: initial prospecting of gross SAR through a few modifications followed by a more detailed analysis of the most permissive areas [41]. The first step identified the importance of the sulfonyl acetic acid moiety. Specifically, substitutions with carboxylate isosteres such as tetrazoles, phosphonates, or sulfates were not tolerated. Further, changes in the oxidation state of sulfur as well as the length of the alkyl linker between sulfone and carboxylate could not be changed without significant loss in potency. In the second step, the distal aromatic ring was derivatized through a carboxylate functionality, providing compounds with single digit nanomolar inhibitory potency such as compound **4**.



3 HTS hit
HPV6 IC_{50} 2 μM
MW 276



4
HPV6 I_{50} 0.0043 μM
MW 473



5
HPV6 IC_{50} 0.63 μM
MW 474
Moderately permeable (Caco-2)

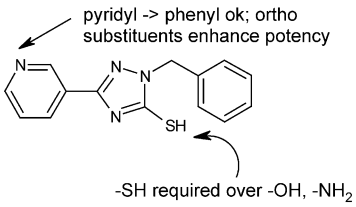
This two-step SAR analysis confirmed that active compounds could be derived for this series. This analysis also highlighted two key liabilities: the potential instability of

the sulfonylacetic acid moiety and the absence of cell-based activity likely due to poor cellular permeability. These two problems were addressed by substitution of the sulfonylacetic acid moiety with several potential isosteres. It was found that substitution of the carboxyl group with nitro (compound **5**) provided a moderately permeable compound (Caco-2 assay result) but with a greater than 100-fold loss in potency.

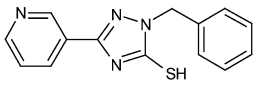
3.3. CXCR2 receptor antagonists

The results for a hit-to-lead investigation were reported for a molecule **6** identified in an HTS campaign for inhibitors of the CXCR2 receptor, of interest for treatment of immune and inflammatory disease conditions [42]. A profile for the desired lead for this target was extensively defined and serves as useful benchmarks to evaluate the initial hit and progress towards identifying a lead. Preliminary SAR studies indicated that the pyridyl group could be substituted by a phenyl but that modification of the thiol was not tolerated. It is theorized that the acidity of the thiol group (p*K*a ~6.5) is an important feature of these types of inhibitors. Ortho-substitutions of the phenyl ring were shown to increase inhibitory potency; mono- and di-chlorination of the benzyl ring were found to enhance inhibitory potency. A pharmacokinetic study of the HTS hit indicated moderate clearance and a long half-life in the rat. The results of the hit-to-lead work identified molecule **7** having more than 100-fold greater inhibitory potency. Although the molecule was more lipophilic than both the original hit and the target lipophilicity, the molecule had good bioavailability in a rat pharmacokinetic study. Having achieved good results for lead-like potency and DMPK profile, further improvements in potency will be the goal of the lead optimization phase.

Property	Goal	Hit 6	Lead 7
CXCR2 (IC_{50}) (binding, μM)	<0.1	4.6	0.028
Whole cell assay (FLIPR, μM)	<0.1	2.4	0.048
Rat hepatocyte clearance (μL/min/10^6 cells)	<14	19	26
Human liver microsome clearance (μL/min/mg)	<23	13	14
Rat iv clearance (mL/min/kg)	<35	12	12
Rat iv Vss (L/kg)	>0.5	1.3	8
Rat iv $T_{1/2}$ (h)	>0.5	3.4	9
Rat po F (%)	>10		61
Plasma protein binding (%)	<99.5		99.0
Solubility (μg/mL)	>10		20
MW (Da)	<450	268	336
c log *P*	<3.0	3.4	4.3
log *D*	<3.0		3.2



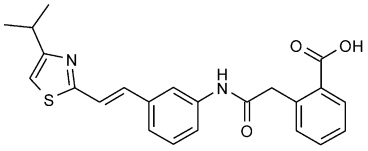
6 CXCR2-R IC_{50} = 4.6 μM
MW = 286



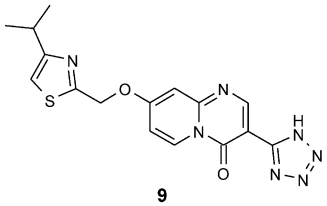
7 CXCR2-R IC_{50} = 28 nM
MW = 370

3.4. MexAB-OprM efflux pump inhibitors

One liability that has received some attention is protein binding. Compound **8** is an effective *in vitro* potentiator of the effect of antibacterial quinolones such as levofloxacin, through inhibition of an efflux pump [43]. However, it has limited *in vivo* activity attributed to its high protein binding. The scaffold was modified to a pyridopyrimidine in an attempt to reduce protein binding. Although compound **9** has *in vitro* activity about 10-fold lower than **8**, the desired reduction in protein binding has been achieved, and most significantly, the compound shows activity *in vivo*.



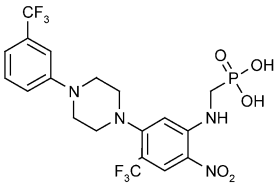
8
MPC_8 < 0.63 μg/mL
Protein Binding > 99.9%



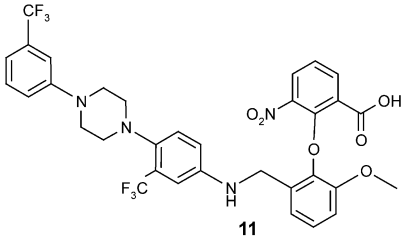
9
MPC_8 5 μg/mL
Protein Binding 88.4%

3.5. PAI-1 inhibitors

Compound **10** is a micromolar inhibitor of plasminogen inhibitor-1 (PAI-1) with poor oral bioavailability which is attributed at least in part to the phosphonic acid [44]. It was found that compound **11** gave a combination of slightly better potency with much better pharmacokinetic properties in rats (for example, 43% oral bioavailability).



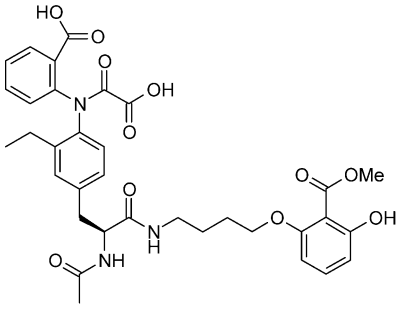
10
IC_{50} 1.8 μM
Poor oral bioavailability



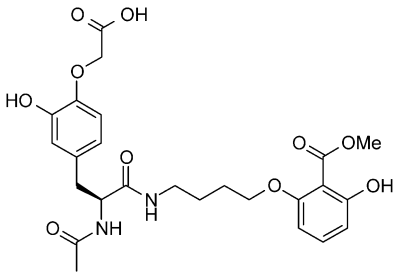
11
IC_{50} 0.5 μM
F 43% (rat)

3.6. PTP1B inhibitors

Often, high-throughput screening hits have poor permeability, and some publications address improvements in this area, sometimes in combination with improvements in oral bioavailability [45]. In some cases, a high price in terms of potency is paid to overcome a permeability problem. For example, compound **12** is a potent inhibitor of protein tyrosine phosphatase 1B (PTP1B) and it shows 3.6-fold selectivity over the closely related T cell phosphatase [46]. However, **12** shows poor permeability in the Caco2 system, and this is attributed to the two negative charges it bears at physiological pH. A replacement was sought for the phosphotyrosine mimetic and it was found that an o-hydroxy-phenoxy-acetic acid gave a compound with good permeability and increased selectivity over the T cell phosphatase. However, the improvement in permeability has come at a price in terms of potency: **13** has a K_i of 9 μM against PTP1B, compared to a K_i of 0.018 μM for **12**.



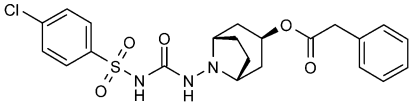
12
Ki 0.018 μM
Papp < 1 x 10^{-6} cm/s



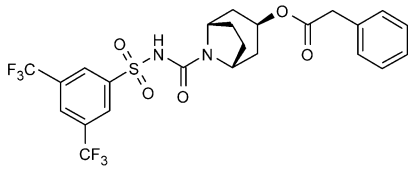
13
Ki 9 μM
Papp > 10 x 10^{-6} cm/s

3.7. Steroid sulfatase inhibitors

In some cases, the screening hit suffers from poor synthetic tractability which would make the lead optimization process slow and difficult, and which could cause cost-of-goods issues in manufacturing if the project were successful. Compound **14** is an inhibitor of human steroid sulfatase but it contains a complex hydrazine substructure which presents synthetic challenges [47]. It was found that removal of one nitrogen from the structure gave a more synthetically tractable series where improvements in potency over the original hit were possible (see for example compound **15**).



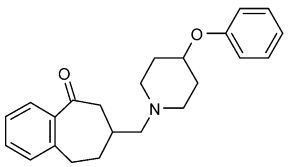
14
Ki 0.89 μM



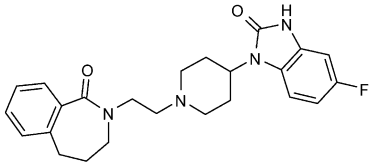
15
Ki 0.076 μM

3.8. 5-HT$_7$ receptor antagonists

Another example of improving synthetic tractability comes in a series of 5-HT$_7$ receptor antagonists [48]. The initial hit (compound **16**) contains a beta-substituted ketone which is synthetically challenging. With the help of molecular modeling based on a receptor homology structure, it was found that the position of attachment of the sidechain could be moved so that it was alpha to the ketone carbonyl, facilitating synthesis but retaining the activity of the compounds. Furthermore, it was found that the ketone could be replaced with an amide, removing the chiral center and making the series even more synthetically accessible. These modifications led to the identification of compound **17**, SB-691673, as a potent and selective 5-HT$_7$ receptor antagonist.



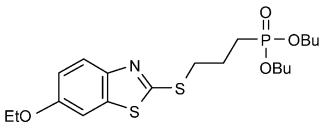
16
pKi 7.73



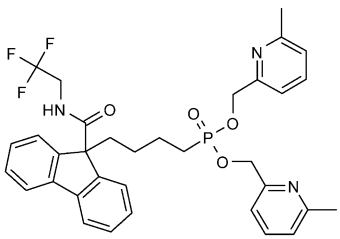
17
pKi 8.65

3.9. MTP inhibitors

One recent publication describes a case where optimization of a screening hit led to a significant increase in potency, oral activity, and *in vivo* efficacy. A high-throughput screen was used to identify compound **18** as an inhibitor of micro-somal triglyceride transfer protein (MTP) [49]. A combination of scaffold-hopping and SAR studies led to the identification of **19** which is 400-fold more potent than **18** in the *in vitro* assay, and which showed efficacy in lowering cholesterol when dosed orally in hamsters.



18
IC$_{50}$ 4.5 μM



19
IC$_{50}$ 0.011 μM

4. SUMMARY AND CONCLUSIONS

Despite different work practices among drug discovery organizations, it appears that there are features of the hit-to-lead process that can be generalized. In order to avoid

beginning resource intensive lead optimization efforts with leads that are not good starting points, hit-to-lead efforts are focused on an early assessment and prospecting of early hits. Hit-to-lead efforts usually focus on developing a preliminary SAR and identification of liabilities for a compound class. As demonstrated by the examples given, relatively simply changes can provide extensive information about the potential for optimization of an initial hit.

REFERENCES

[1] J. Drews, *Drug Discovery Today*, 2003, **8**.
[2] D. Brown and G. Superti-Furga, *Drug Discovery Today*, 2003, **8**, 1067.
[3] *Parexel's Pharmaceutical R&D Statistical Sourcebook*, 2001, p. 139.
[4] W. F. Michne, *Pharm. News*, 1996, **3**, 19.
[5] S. Campbell and E. Differding, *Chim. Nouv.*, 2001, **19**, 3347.
[6] A. Tropsha and C. H. Reynolds, *J. Mol. Graph. Model*, 2002, **20**, 427.
[7] A. D. Baxter and P. M. Lockey, *Drug Disc. World*, 2001, **2**, 9.
[8] S. J. Teague, A. M. Davis, P. D. Leeson and T. Oprea, *Angew. Chem. Int. Ed. Engl.*, 1999, **38**, 3743.
[9] A. Alanine, M. Nettekoven, E. Roberts and A. W. Thomas, *Comb. Chem. High Throughput Screen*, 2003, **6**, 51.
[10] K. H. Bleicher, H. J. Bohm, K. Muller and A. I. Alanine, *Nature Rev. Drug Disc.*, 2003, **2**, 369.
[11] S. X. Cai, B. Nguyen, S. Jia, J. Herich, J. Guastella, S. Reddy, B. Tseng, J. Drewe and S. Kasibhatla, *J. Med. Chem.*, 2003, **46**, 2474.
[12] J. Wityak, J. Das, R. V. Moquin, Z. Shen, J. Lin, P. Chen, A. M. Doweyko, S. Pitt, S. Pang, D. R. Shen, Q. Fang, H. F. de Fex, G. L. Schieven, S. B. Kanner and J. C. Barrish, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4007.
[13] I. Churcher, K. Ashton, J. W. Butcher, E. E. Clarke, T. Harrison, H. D. Lewis, A. P. Owens, M. R. Teall, S. Williams and J. D. J. Wrigley, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 179.
[14] N. J. Green, J. Xiang, J. Chen, L. Chen, A. M. Davies, D. Erbe, S. Tam and J. F. Tobin, *Bioorg. Med. Chem.*, 2003, **11**, 2991.
[15] J. Witherington, V. Bordas, A. Gaiba, N. S. Garton, A. Naylor, A. D. Rawlings, B. P. Slingsby, D. G. Smith, A. K. Takle, R. W. Ward, J. Witherington, V. Bordas, A. Gaiba, N. S. Garton, A. Naylor, A. D. Rawlings, B. P. Slingsby, D. G. Smith, A. K. Takle and R. W. Ward, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3055.
[16] P. L. Beaulieu, M. Bos, Y. Bousquet, G. Fazal, J. Gauthier, J. Gillard, S. Goulet, S. LaPlante, M.-A. Poupart, S. Lefebvre, G. McKercher, C. Pellerin, V. Austel and G. Kukolj, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 119.
[17] J. A. Jablonowski, C. A. Grice, W. Chai, C. A. Dvorak, J. D. Venable, A. K. Kwok, K. S. Ly, J. Wei, S. M. Baker, P. J. Desai, W. Jiang, S. J. Wilson, R. L. Thurmond, L. Karlsson, J. P. Edwards, T. W. Lovenberg and N. I. Carruthers, *J. Med. Chem.*, 2003, **46**, 3957.
[18] K. G. Rosauer, A. K. Ogawa, C. A. Willoughby, K. P. Ellsworth, W. M. Geissler, R. W. Myers, Q. Deng, K. T. Chapman, G. Harris and D. E. Moller, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4385.
[19] Z. Ma, S. Djuric, A. S. Florjancic, H. Yong and Y. Gu, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 267.
[20] J. G. Kettle, A. W. Faull, A. J. Barker, D. H. Davies and M. A. Stone, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 405.
[21] M. E. McDonnell, S.-P. Zhang, N. Nasser, A. E. Dubin and S. L. Dax, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 531.
[22] A. Alanine, L. Anselm, L. Steward, S. Thomi, W. Vifian and M. D. Groaning, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 817.

[23] K. S. Atwal, S. Ahmad, C. Z. Ding, P. D. Stein, J. Lloyd, L. G. Hamann, D. W. Green, F. N. Ferrara, P. Wang, W. L. Rogers, L. M. Doweyko, A. V. Miller, S. N. Bisaha, J. B. Schmidt, L. Li, K. J. Yost, H.-J. Lan and C. S. Madsen, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1027.
[24] R. D. Clark, A. Jahangir, D. Severance, R. Salazar, T. Chang, D. Chang, M. F. Jett, S. Smith and K. Bley, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1053.
[25] M. Anderson, J. F. Beattie, G. A. Breault, J. Breed, K. F. Byth, J. D. Culshaw, R. P. A. Ellston, S. Green, C. A. Minshull, R. A. Norman, R. A. Pauptit, J. Stanway, A. P. Thomas and P. J. Jewsbury, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3021.
[26] J. F. Beattie, G. A. Breault, R. P. A. Ellston, S. Green, P. J. Jewsbury, C. J. Midgley, R. T. Naven, C. A. Minshull, R. A. Pauptit, J. A. Tucker and J. E. Pease, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2955.
[27] J. Renaud, S. F. Bischoff, T. Buhl, P. Floersheim, B. Fournier, C. Halleux, J. Kallen, H. Keller, J.-M. Schlaeppi and W. Stark, *J. Med. Chem.*, 2003, **46**, 2945.
[28] P. Vianello, P. Cozzi, A. Galvani, M. Meroni, M. Varasi, D. Volpi and T. Bandiera, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 657.
[29] J. Finn, K. Mattia, M. Morytko, S. Ram, Y. Yang, X. Wu, E. Mak, P. Gallant and D. Keith, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2231.
[30] H. Masaki, Y. Mizuno, A. Tatui, A. Murakami, Y. Koide, S. Satoh and A. Takahashi, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4085.
[31] S. Ebdrup, L. G. Sorensen, O. H. Olsen and P. Jacobsen, *J. Med. Chem.*, 2004, **47**, 400.
[32] J. Witherington, V. Bordas, A. Gaiba, A. Naylor, A. D. Rawlings, B. P. Slingsby, D. G. Smith, A. K. Takle and R. W. Ward, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3059.
[33] K.-K. Ho, K. C. Appell, J. J. Baldwin, A. C. Bohnstedt, G. Dong, T. Guo, R. Horlick, K. R. Islam, S. G. Kultgen, C. M. Masterson, E. McDonald, K. McMillan, J. R. Morphy, Z. Rankovic, H. Sundaram and M. Webb, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 545.
[34] A. Perez-Medrano, S. A. Buckner, M. J. Coghlan, R. J. Gregg, M. Gopalakrishnan, M. E. Kort, J. K. Lynch, V. E. Scott, J. P. Sullivan, K. L. Whiteaker and W. A. Carroll, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 397.
[35] K. Pan, M. K. Scott, D. H. S. Lee, L. J. Fitzpatrick, J. J. Crooke, R. A. Rivero, D. I. Rosenthal, A. H. Vaidya, B. Zhao and A. B. Reitz, *Bioorg. Med. Chem.*, 2003, **11**, 185.
[36] Y.-L. Chou, D. D. Davey, K. A. Eagen, B. D. Griedel, R. Karanjawala, G. B. Phillips, K. L. Sacchi, K. J. Shaw, S. C. Wu, D. Lentz, A. M. Liang, L. Trinh, M. M. Morrissey and M. J. Kochanny, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 507.
[37] T. I. Richardson, P. L. Ornstein, K. Briner, M. J. Fisher, R. T. Backer, C. K. Biggers, M. P. Clay, P. J. Emmerson, L. W. Hertel, H. M. Hsiung, S. Husain, S. D. Kahl, J. A. Lee, T. D. Lindstrom, M. J. Martinelli, J. P. Mayer, J. T. Mullaney, T. P. O'Brien, J. M. Pawlak, K. D. Revell, J. Shah, J. M. Zgombick, R. J. Herr, A. Melekhov, P. B. Sampson and C. R. King, *J. Med. Chem.*, 2004, **47**, 744.
[38] M. Hirose, S.-I. Egashira, Y. Goto, T. Hashihayata, N. Ohtake, H. Iwaasa, M. Hata, T. Fukami, A. Kanatani and K. Yamada, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4497.
[39] O. Roche, P. Schneider, J. Zuegge, W. Guba, M. Kansy, A. Alanine, K. Bleicher, F. Danel, E.-M. Gutknecht, M. Rogers-Evans, W. Neidhart, H. Stalder, M. Dillon, E. Sjoegren, N. Fotouhi, P. Gillespie, R. Goodnow, W. Harris, P. Jones, M. Taniguchi, S. Tsujii, W. von der Saal, G. Zimmermann and G. Schneider, *J. Med. Chem.*, 2002, **45**, 137.
[40] A. Baxter, J. Bent, K. Bowers, M. Braddock, S. Brough, M. Fagura, M. Lawson, T. McInally, M. Mortimore, M. Robertson, R. Weaver and P. Webborn, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4047.
[41] A.-M. Faucher, P. W. White, C. Brochu, C. Grand-Maître, J. Rancourt and G. Fazal, *J. Med. Chem.*, 2004, **47**, 18.
[42] A. Baxter, C. Bennion, J. Bent, K. Boden, S. Brough, A. Cooper, E. Kinchin, N. Kindon, T. McInally, M. Mortimore, B. Roberts and J. Unitt, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2625.
[43] K. Nakayama, Y. Ishida, M. Ohtsuka, H. Kawato, K.-i. Yoshida, Y. Yokomizo, T. Ohta, K. Hoshino, T. Otani, Y. Kurosaka, K. Yoshida, H. Ishida, V. J. Lee, T. E. Renau and W. J. Watkins, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4205.

[44] B. Ye, Y.-L. Chou, R. Karanjawala, W. Lee, S.-F. Lu, K. J. Shaw, S. Jones, D. Lentz, A. Liang, J.-L. Tseng, Q. Wu and Z. Zhao, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 761.
[45] M. S. S. Palanki, P. E. Erdman, M. Ren, M. Suto, B. L. Bennett, A. Manning, L. Ransone, C. Spooner, S. Desai, A. Ow, R. Totsuka, P. Tsao and W. Toriumi, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 4077.
[46] Z. Xin, G. Liu, C. Abad-Zapatero, Z. Pei, B. G. Szczepankiewicz, X. Li, T. Zhang, C. W. Hutchins, P. J. Hajduk, S. J. Ballaron, M. A. Stashko, T. H. Lubben, J. M. Trevillyan and M. R. Jirousek, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3947.
[47] P. Nussbaumer, D. Geyl, A. Horvath, P. Lehr, B. Wolff and A. Billich, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3673.
[48] I. T. Forbes, D. G. Cooper, E. K. Dodds, S. E. Douglas, A. D. Gribble, R. J. Ife, A. P. Lightfoot, M. Meeson, L. P. Campbell, T. Coleman, G. J. Riley and D. R. Thomas, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1055.
[49] D. R. Magnin, S. A. Biller, J. Wetterau, J. A. Robl, J. K. Dickson, P. Taunk, T. W. Harrity, R. M. Lawrence, C.-Q. Sun, T. Wang, J. Logan, O. Fryszman, F. Connolly, K. Jolibois and L. Kunselman, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1337.

Prodrug Strategies in the Design of Nucleoside and Nucleotide Antiviral Therapeutics

Richard L. Mackman and Tomas Cihlar

Gilead Sciences, 333 Lakeside Drive, Foster City, CA 94404, USA

Contents

1. INTRODUCTION

The past decade has seen significant advances in the development of small molecule nucleoside and nucleotide drugs for the treatment of viral diseases. Currently there are almost 20 drugs that fall into this category [1]. The active species for the polymerase target is the triphosphate (or triphosphate mimic) nucleotide, **4**, which is generated through a series of enzyme mediated phosphorylations from the nucleoside precursor **1** [2]. The triphosphate nucleotide either acts as a competitive inhibitor with the natural nucleotides, or as a substrate, becoming incorporated into the growing polynucleotide chain thereby modifying its properties. Extensive research efforts in the past decade have been directed toward the development of nucleoside therapeutics, analogs of **1** that are anabolically converted to the triphosphate species. There have been two major problems encountered in this approach. First, the nucleosides typically have low log *D*, stability, solubility and permeability properties that can limit oral bioavailability or tissue penetration, and second, the enzymes responsible for anabolism may not recognize the nucleoside or nucleotide precursors. To solve these problems, prodrugs of nucleosides **1**, and also prodrugs of the phosphate species **2**–**4** (or their phosphonate analogs) have been developed. Several recently approved drugs are the products of these research efforts (e.g. tenofovir disoproxil, abacavir, adefovir dipivoxil, famciclovir, valaciclovir). This review will explore some of the prodrug strategies that have been reported in the literature. Several reviews that overlap with the scope of this review can also be consulted for further information [3–5].

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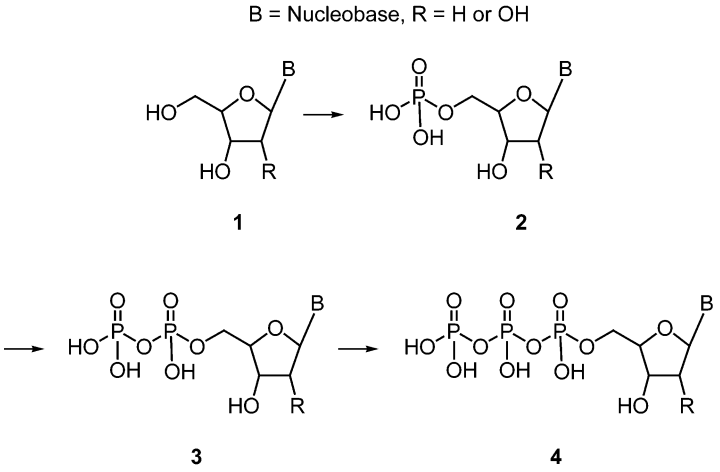
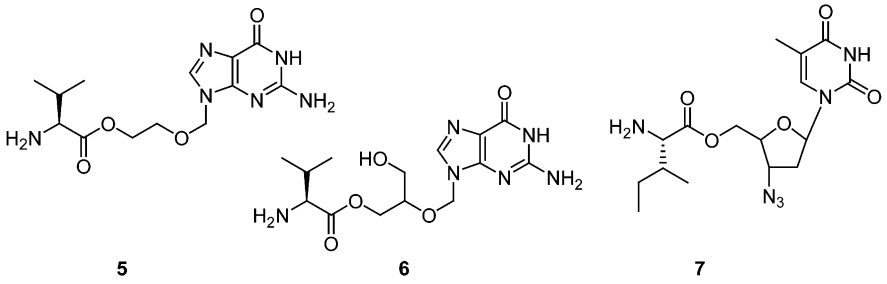


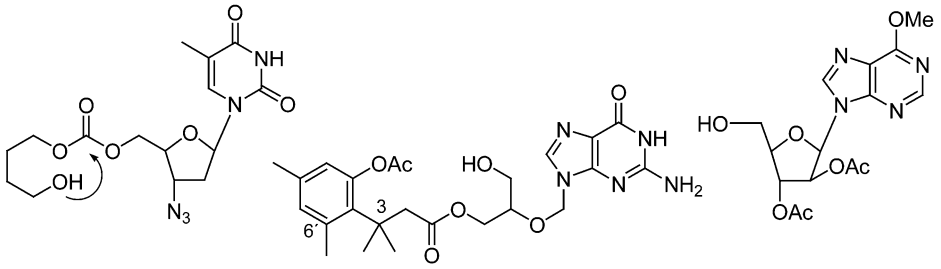
2. PRODRUGS OF NUCLEOSIDES – THE RIBOSE RING

The dominant prodrug strategy adopted on the ribose ring involves appending carboxyl groups, e.g. esters, carbonates and carbamates, to the ribose hydroxyl groups, in order to improve drug exposure and oral bioavailability. The prodrug can block unwanted metabolic pathways, increase log *D*, and improve permeability. Additionally, some prodrugs, e.g. amino acid esters, can take advantage of *in vivo* transporter systems. Peptidases, esterases and other hydrolases in the host catalyze the release of the parent molecule. The L-valine ester prodrug valaciclovir, **5**, has a 3-5 fold improved oral bioavailability compared to acyclovir (aciclovir) [6]. This allows for a more convenient administration of acyclovir for treating herpes zoster (VZV) and genital herpes (HSV) [7].



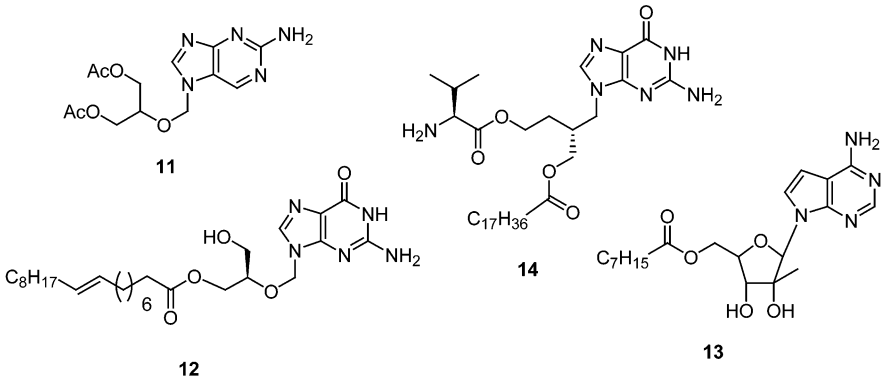
It has been determined that **5** is a substrate for the intestinal and renal peptide transporters, PEPT1 and PEPT2 [8]. A biphenyl hydrolase-like protein has been isolated from human intestinal cells and shown to activate valaciclovir to acyclovir [9]. Valganciclovir **6**, a peptide analog of ganciclovir, is also recognized by the same

peptide transporters and improves oral bioavailability ⩾ 10-fold compared to ganciclovir [10–12]. Valganciclovir is approved for the treatment of CMV retinitis. Dipeptide derivatives of acyclovir, designed to target the peptide transporters, have also been reported [13]. Increasing the level and duration of AZT exposure in the CNS system, where latent reservoirs of HIV virus exist, has been an intense area of HIV research. The isoleucine ester of AZT, **7**, displayed similar *in vitro* activity to AZT, improved toxicity in H9 cells, and in a rabbit model, resulted in greater exposure to AZT in the brain compared to administration of AZT [14,15]. Many carboxyl based prodrug strategies on AZT including steroid esters, carbohydrate esters, saturated and unsaturated fatty acid esters, and dihydropyridine esters have been reviewed [4]. Recently, a novel prodrug approach on AZT, involving an intramolecular cyclic rearrangement of a carbonate prodrug, **8**, has been reported [16,17]. A 30- to 50-fold improvement in the *in vitro* HIV activity and reduced toxicity, compared to AZT, was noted.



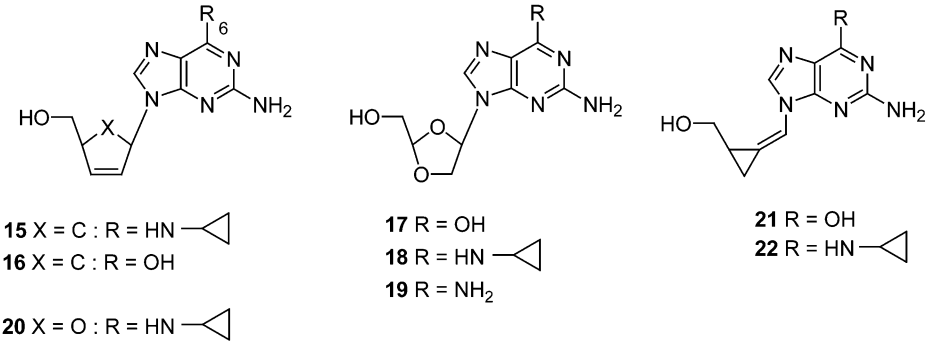
8 **9** **10**

Another group utilized an approach referred to as the ‘trimethyl lock’ on ganciclovir, analog **9**, and reported a 4-fold increase in oral bioavailability [18]. Cleavage of the acetate ester is followed by intramolecular lactonization promoted by the 3,3-dimethyl and 6′-methyl (hence ‘trimethyl’) groups, to release ganciclovir. Di- and tri-ester prodrugs of the varcella zoster virus agent, 6-methoxypurine arabinoside, have been reported [19]. The bis-acetate prodrug, **10**, displayed the optimal balance of bioavailability and solubility. Hoe-961, **11**, is an orally active acetate ester prodrug of S2242, a novel N7-substituted anti-viral agent for treatment of HSV [20]. Lipid esters that take advantage of a high lipophilicity to promote penetration across membranes have been widely explored. The elaidic acid ester prodrug of ganciclovir, **12** (P-4018), has been shown to be more efficacious than ganciclovir in an animal model of HSV infection [21]. Simple ester prodrugs, e.g. **13**, of a novel pyrrolopyrimidine HCV polymerase inhibitor have recently been reported in the patent literature [22]. Valomaciclovir stearate **14** (ABT-606/MIV-606) is an example of a double prodrug of H2G (omaciclovir) for the treatment of varicella zoster virus infections. The combination of the valine and stearyl esters leads to a 5.5–10% bioavailability improvement in rats compared to H2G [23].



3. PRODRUGS OF NUCLEOSIDES – THE NUCLEOBASE

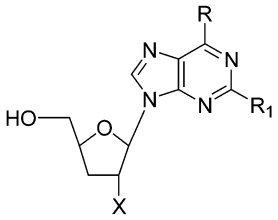
The HIV drug abacavir **15** (1592U89), is a nucleobase prodrug of carbovir **16** [24,25]. A number of different C-6 amino substituents were explored to improve the oral bioavailability of **16**, resulting in the discovery of the C-6 cyclopropylamine prodrug **15** [24]. Abacavir is first phosphorylated by adenosine phosphotransferase and then converted by a cytosolic deaminase to carbovir monophosphate [26]. A small portion ($<2\%$) of **15** is probably directly converted to **16** by the action of adenosine deaminase prior to the initial phosphorylation. Since abacavir is not efficiently converted by adenosine deaminase in plasma, cleavage to carbovir is predominantly intracellular. Adenosine deaminase has been utilized in many other nucleobase prodrug strategies (see below) and several reports profiling the substrate specificity of this enzyme are available [27–32]. Spurred by the success of abacavir, two C-6 aminopurine prodrugs of DXG, **17**, are under clinical investigation for the treatment of HIV. SPD-756, **18**, is a C-6 cyclopropylamine prodrug of **17**, and DAPD, **19**, a soluble adenosine deaminase activated prodrug of **17** [33–35].

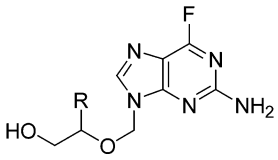


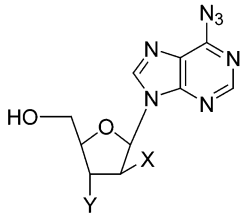
Recently, anti-HIV activity for the C-6 cyclopropyl amine prodrug of D4G, **20**, has been reported. This compound exhibits greater stability, lipophilicity and solubility

than D4G [36]. Similarly, C-6 amino prodrug groups were investigated on synguanol **21** (QYL-438), a novel cyclopropylmethylene nucleoside active against CMV [37]. The C-6 cyclopropyl amine analog, **22** (QYL-769), displayed potency against ganciclovir resistant isolates [37].

The HIV inhibitor didanosine (ddI), **23**, can be considered to be a nucleobase prodrug since it leads to the generation of ddA triphosphate [38]. DdI is first converted to ddI monophosphate, and then converted to ddA monophosphate by adenylosuccinate synthetase/lyase before subsequent phosphorylation to ddA triphosphate [39,40]. DdA, **24**, can also be rapidly converted to ddI *in vivo* [39].







23 R = OH : R_1 = X = H
24 R = NH_2 : R_1 = X = H
25 R = F : R_1 = X = H
26 R = F : R_1 = NH_2 : X = H
27 R = Cl : R_1 = X = H
28 R = OH : R_1 = H : X = F
29 R = X = F : R_1 = H
32 R = OC_6H_{13} : R1 = X = H

30 R= H
31 R = CH_2OH

33 X = F, Y = H
34 X = Y = OH

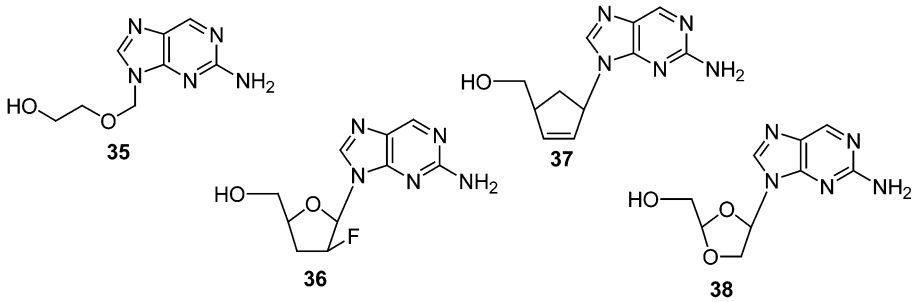
DdI, like AZT, has been the focus of research efforts aimed to improve its CNS penetration. C-6 halo purines can be converted to C-6 hydroxyl purines (inosine or guanine) by adenosine deaminase, and have the advantage of increased lipophilicity which can facilitate greater penetration into the CNS. Early work on C-6 halo prodrugs of the HIV agents, ddI and ddG, indicated that C-6 fluoro prodrugs, **25** and **26** respectively, provide optimal antiviral activity [41,42]. Lipophilicity ($\log P$) was increased by >1 log unit and a correlation between anti-HIV activity and adenosine deaminase activity was indicated [42]. 6-Chloro ddI, **27**, is less lipophilic than its C-6 iodine or bromine counterparts, yet provides a superior exposure to **23** in rat brain tissue due to greater adenosine deaminase conversion [43]. This result indicates that a balance between lipophilicity and conversion by adenosine deaminase is necessary. A similar approach has been applied to 2′F-ddI, **28**, a more acid stable analog of **23**. The C-6 fluoro analog, **29**, had the highest rate of adenosine deaminase conversion and the best anti-HIV activity [44]. The C-6 fluoro prodrug group strategy has also been used to improve solubility and oral bioavailability of acyclovir and ganciclovir, prodrugs **30** and **31** respectively [45].

A series of C-6 alkoxy prodrugs of **23** have been evaluated for their anti HIV activity and conversion by adenosine deaminase [46]. Small alkoxy groups exhibited low HIV activity and relatively weak conversion by adenosine deaminase to **23**. Interestingly

the most active analog, hexyloxy substituted, **32**, was not a good substrate for adenosine deaminase. In this example, it is proposed that mono phosphorylation of **32** is followed by AMP deaminase mediated conversion to ddI monophosphate [46].

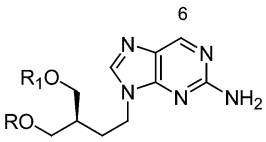
A recent approach to nucleobase prodrugs, designed to improve CNS penetration, utilizes C-6 azido groups that can be reduced by the P450 NADPH reductase system. C-6 azido analog of 2′F-*ara*ddI, **33**, was shown to be converted *in vivo* to 2′F-*ara*ddI [47]. However, poor oral bioavailability coupled with the low levels of deaminase activity in the brain led to undetectable brain exposure of 2′F-*ara*ddI. In contrast, *in vivo* administration of C-6 azido prodrug **34**, resulted in an extended *ara*-A half life and significant exposure of *ara*-A in the brain tissue, thereby validating the potential of this approach [48].

One of the earliest examples of a nucleobase prodrug is desiciclovir, **35** (A515U, 515U74), a prodrug of acyclovir that displayed improved water solubility and oral bioavailability compared to acyclovir [49]. Prodrug **35** is a substrate for xanthine oxidase which oxidises the C-6 carbon to generate acyclovir. In human trials, **35** resulted in a high acyclovir plasma AUC comparable to that obtained with i.v. administered acyclovir, and much improved over oral acyclovir [50]. Once again, this nucleobase approach was applied to 2′F-*ara*ddI to improve CNS penetration. Oral dosing of **36** in mice led to significantly higher levels of 2′F-*ara*ddI in the brain tissue compared to 2′F-*ara*ddI [51]. Xanthine oxidase mediated conversion of **36** to 2′F-*ara*ddI was demonstrated *in vitro* [51]. Attempts to improve the oral bioavailability of carbovir by using the C-6 deoxy 2-aminopurine prodrug **37**, or oral bioavailability of DXG, by using the C-6 deoxy 2-aminopurine dioxolone **38**, have also been reported [52,53]. The latter was found to give higher brain levels of DXG than the parent non-prodrug [53].

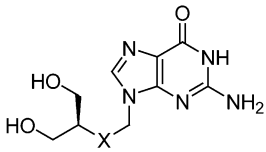


Significant success with C-6 deoxy nucleobase prodrugs has been achieved in combination with other prodrug motifs (double prodrugs). Famciclovir, **39** (BRL 42810), is a double prodrug of the HSV agent penciclovir, **40**, and is approved for treatment of HSV infections [54]. In rats, famciclovir was found to have superior oral bioavailability (41%) compared to the single 6-deoxy penciclovir prodrug, **41** (9%). 6-Deoxy penciclovir was shown to be converted to penciclovir by xanthine oxidase *in vitro* [55]. Recently, carbonate prodrugs, e.g. **42**, produced slightly greater urine levels of penciclovir **40** in mice compared to famciclovir [56]. Hoe-602, **43**, is a double prodrug of ganciclovir, **44**, that improves ganciclovir exposure in rhesus monkeys [57]. Another

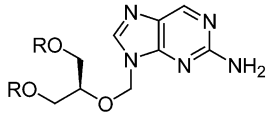
prodrug of ganciclovir, **45** (841U83) bearing a bis-pivaloyl ester produced a 2-fold improvement in rat oral bioavailability over 6-deoxy ganciclovir **46** and a 7-fold improvement over **44** [58].



39 R = R_1 = Ac
41 R = R_1 = H
42 R = $CO_2{}^{i}Pr$: R_1 = H

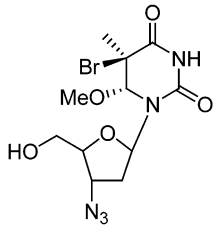


40 X = CH_2
44 X = O



43 R = ^{i}Pr
45 R = $CO^{t}Bu$
46 R = H

There are relatively few examples of pyrimidine nucleobase prodrugs compared to purine prodrugs. However, some efforts to increase lipophilicity and CNS penetration of the HIV drugs AZT, D4T and FLT, through the design of 5-halo-6-alkoxy substituted 5,6-didehydropyrimidine prodrugs have been described [59–63]. AZT prodrug, **47**, and D4T prodrug, **48** both display anti-HIV activity comparable to AZT and D4T respectively [61,63]. Both prodrugs can be converted in the presence of glutathione or mouse liver homogenate to their respective parent drugs [61,63]. *In vivo* mice studies indicated a rapid conversion of **47** to AZT and 2–4 fold greater exposure to AZT in the brain tissue following IV administration [62]. The same approach has also been applied to the development of prodrugs for the anti-herpes agent EDU [64]. The 5-Br,5-ethyl,6-methoxy analog, **49**, exhibited equipotent *in vitro* activity against HSV-1 and HSV-2 compared to EDU. 5-Iodo,2-pyrimidinone-2-deoxy ribose **50** also has activity toward HSV-1 and HSV-2 viruses [65]. Aldehyde oxidase has been discovered to possibly carry out the conversion of **50** to 5IU, **51** [65]. Since aldehyde oxidase is enriched in the liver this could lend itself to a specific liver targeting prodrug strategy.



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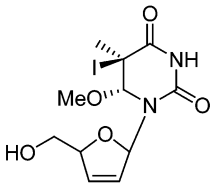

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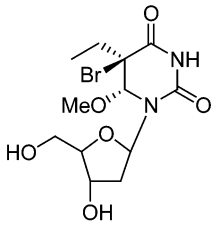

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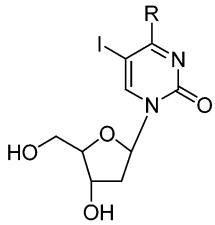


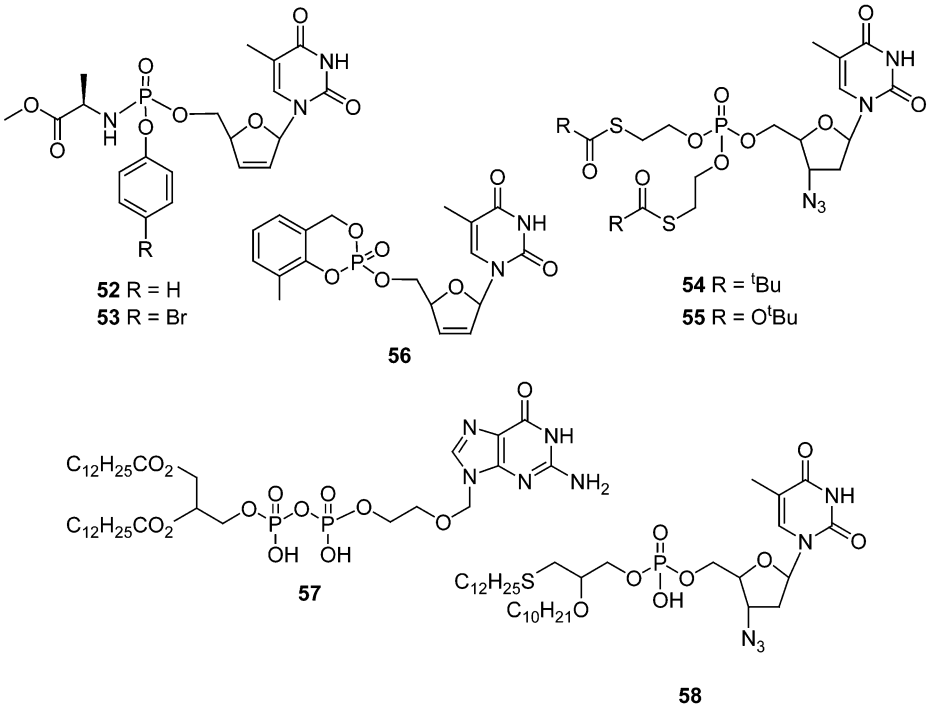
50 R = H
51 R = OH

4. PRODRUGS OF NUCLEOSIDE MONOPHOSPHATE (NMP) ANALOGS

In order to bypass the often limiting first phosphorylation step in the activation of nucleoside analogs, various prodrugs of their monophosphates have been explored with phosphoramidate [66], bis(S-acylthioethyl) (SATE) ester [67], and cyclosaligenyl

(cycloSal) prodrugs [68] showing the most promising results. Phenyl methyl-alaninyl phosphoramidate of d4T, **52** (So324), is a more potent inhibitor of HIV-1 than d4T and retains its activity in TK-deficient cells, proving the intracellular delivery of d4T-MP [69]. Modifications of the amino acid [70,71], carboxyester [72] and phosphoester [73] of **52** did not yield more active prodrugs with the exception of **53** (stampidine) that shows a favorable toxicity profile and antiviral activity in various animal models [74–77]. Currently, **53** is being considered as an anti-HIV microbicide [78]. Phosphoramidates with improved antiviral potency relative to their parent nucleosides have been successfully prepared from ddA [79], d4A [79], iso-ddA [80], abacavir [81] and other nucleosides [82,66]. However, this approach failed to improve the activity of AZT [83], 3TC [84] and ACV [85], presumably due to inefficient intracellular hydrolysis of their phosphoramidate prodrugs or limited catabolic stability of the delivered NMP analogs.

Applying the bis(SATE) approach to ddA, iso-ddA, and L-ddA dramatically improved their anti-HIV potency [86–88]. Although various bis(SATE) prodrugs were prepared from AZT [89], no improvement in antiviral activity was achieved. Following oral administration in mice, bis(tBu-SATE)-AZTMP, **54**, delivered AZT, but not the intact prodrug into systemic circulation [90]. In contrast, the bis(hydroxy-tBu-SATE) of AZTMP, **55**, was more stable and showed improved anti-HIV activity and permeability [91]. Bis(SATE) prodrugs were also prepared from acyclovir, ddC, 3TC and adenallene nucleosides [92–94]. Although the potential of these prodrugs remains to be fully explored *in vivo*, mutagenic activity and toxicity of the episulfide by-product spontaneously released during the activation of these prodrugs, may limit their long term therapeutic applications [95].

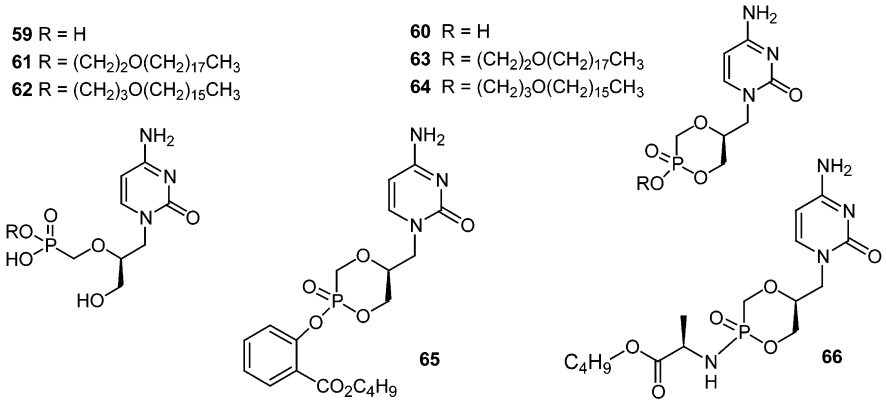


Unlike phosphoramidate and bis(SATE) prodrugs that are activated through enzymatic hydrolysis [96,87], the design of cycloSal prodrugs relies on the release of NMP through sequential chemical hydrolysis of the two cyclosaligenyl esters [68]. Aromatic ring substitutions and phosphorous stereochemistry both affect the rate of hydrolysis and the anti-HIV activity of these prodrugs [97]. Although **56** is only 2-fold more active than d4T, it retains its activity in TK-deficient cells [97]. Analogous cycloSal monophosphate prodrugs of ddA, d4A, 2′F-ddA showed 10- to 500-fold improved anti-HIV-1 activity relative to the parent nucleosides [98,99].

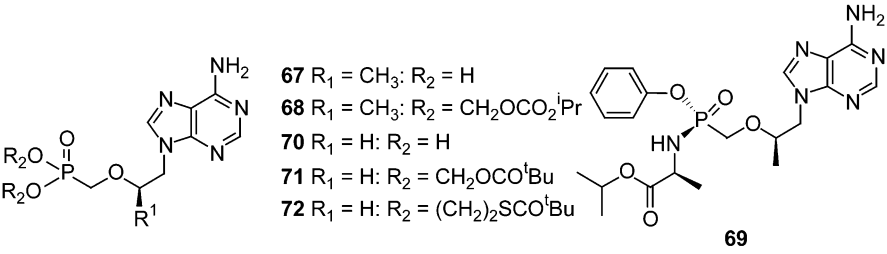
Phospholipid prodrugs represent yet another approach for the intracellular delivery of NMPs. Acyclovir diphosphate dimyristoylglycerol, **57**, retains its activity against ACV-resistant TK-deficient HSV strains and shows improved efficacy in a rabbit HSV retinitis model [100,101]. Nucleoside diphosphate diglyceride hydrolase is likely responsible for the release of NMPs in this class of prodrugs [102]. Unlike parent ACV, 1-octadecyl-*sn*-glycero-3-phospho-ACV inhibited HBV *in vitro* and exhibited 100% oral absorption in mice [103]. Thioether lipid prodrug of AZT-MP, **58** (fozivudine), displayed clinical efficacy in HIV-infected patients with potentially improved tolerability due to reduced systemic exposure to AZT [104]. However, as shown with other monophosphate prodrugs of AZT, **58** may only efficiently deliver AZT and not AZT-MP into cells. A broader spectrum of antiviral NMP prodrugs has been reviewed [5].

5. PRODRUGS OF ACYCLIC NUCLEOSIDE PHOSPHONATES (ANPs)

ANPs are enzymatically stable NMP analogs requiring only two-step activation to their diphosphates in order to act as inhibitors of viral polymerases [105]. Cidofovir, **59** (HPMPC), has been licensed for the treatment of CMV retinitis and has potential for the management of papillomavirus infections/malignancies and poxvirus outbreaks [106–108]. The limiting toxicity of **59** is nephrotoxicity due to its accumulation in kidney *via* the hOAT1 transporter [109]. The cyclic prodrug **60** (cHPMPC) shows the same antiviral activity and accumulates to a lesser extent in kidney [110,111]. Consequently, it exhibits reduced nephrotoxicity due to its weaker interaction with hOAT1 [109,110]. Prodrug **60** is relatively stable in plasma, but cellular cCMP-hydrolase quickly converts it to **59** [110,112]. Similarly, the cyclic prodrug of ganciclovir phosphonate, is a potent CMV inhibitor with reduced renal accumulation and toxicity compared to the parent ANP [113]. Due to their negative charge, free ANPs exhibit only limited oral bioavailability [114,115]. Therefore, ANP prodrugs with various phosphonate-masking groups have been explored. Alkoxyalkyl esters **61–64** substantially enhanced the activity of cidofovir and cHPMPC against herpes viruses and pox viruses [116,117]. Following oral administration, **61**, **62** and some other ether lipid prodrugs of **59** showed good bioavailability, anti-pox virus efficacy in animal models, and reduced renal accumulation [118,119]. Aryl prodrug **65** enhanced the oral bioavailability of **60** in dogs to $>40\%$, but no improvement in activity against pox viruses was observed [120,121]. In contrast, amidate, **66** improved the antiviral activity of **60** by >5-fold [121].

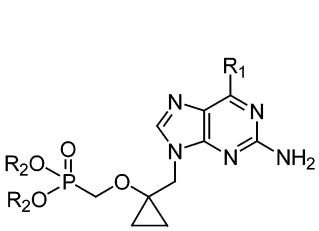


Tenofovir, **67** (PMPA) is an ANP with potent activity against HIV and HBV and a favorable resistance and toxicity profiles [105,122,123]. Among a variety of tenofovir prodrugs, bis(POC)PMPA, **68**, (tenofovir disoproxil) showed efficient intracellular conversion to tenofovir-DP and up to 100-fold enhanced *in vitro* anti-HIV activity compared to tenofovir [124,125]. Bioavailability of tenofovir in humans following the oral administration of **68** approaches 40% [126]. Prodrug **68** was recently licensed for the treatment of HIV-infected patients and its combination with lamivudine and efavirenz is among the most potent antiretroviral regimens in treatment-naïve patients [107]. Alaninyl amidate, **69** (GS-7340) has increased plasma stability relative to **68**, but is quickly converted to tenofovir in target cells [127]. Consequently, **69** substantially enhances *in vivo* delivery of tenofovir into T-lymphocytes and lymphatic tissues [128]. Phase I evaluation of **69** monotherapy in HIV-infected patients is in progress. Adefovir, **70** (PMEA) exhibits a broad-spectrum activity against herpes viruses, HBV, and HIV [105]. Bis(POM)PMEA, **71** (adefovir dipivoxil) showed optimal activity and oral bioavailability [129–131] and was subsequently licensed for the treatment of HBV infection including lamivudine-resistant viruses [132,133]. Bis(SATE) prodrug, **72** of adefovir has comparable anti-HIV activity to **71** and appears to be more resistant against chemical and enzymatic hydrolysis [134]. Among other adefovir prodrugs, methyl-alaninyl amidate improves anti-HIV activity of adefovir by 30-fold [135].

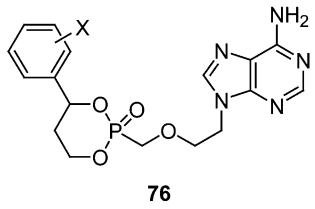


Bis(POM) prodrug **73** (LB80380) is currently being evaluated for the treatment of HBV infection [136]. In blood, **73** is hydrolyzed to **74**, which then undergoes intracellular conversion to a guanine analog **75** [136]. Furthermore, **73** showed promising

activity in the woodchuck HBV model and promising oral bioavailability (15–60%) in various animal species [137,138]. MB6866, **76** (Hepavir B) is a cyclic diester prodrug of adefovir specifically activated by liver CYP450-mediated oxidative metabolism [139]. Preclinical evaluation of **76** indicated preferential liver targeting and reduced kidney accumulation of adefovir. Clinical evaluation of **76** in HBV-infected patients is ongoing [140].



73 R_1 = H: R_2 = CH_2OCO^tBu
74 R_1 = H: R_2 = H
75 R_1 = OH: R_2 = H



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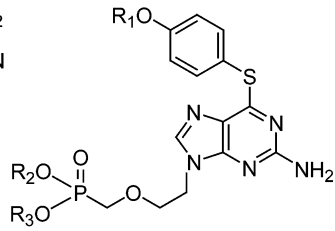


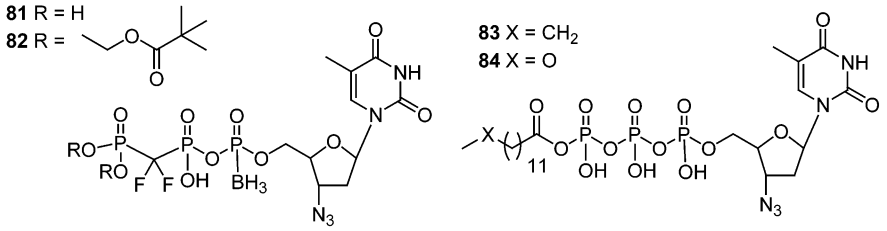
77 R_1 = CH_3: R_2 = R_3 = CH_2CF_3
78 R_1 = CH_3: R_2 = R_3 = H
79 R_1 = CH_3: R_2 = CH_2CF_3: R_3 = H
80 R_1 = H: R_2 = CH_2CF_3: R_3 = H

LY582563, **77** (MCC-478) shows potent *in vitro* anti-HBV activity (EC_{50} = 30 nM) but no HIV-1 activity [141,142]. Unlike **70**, the activity of **77** is compromised in the presence of lamivudine-resistance mutations [141]. Effect of various 6-arylthio substitutions on the activity, toxicity and PK parameters of this class of prodrugs has been evaluated [143]. Compounds **78**, **79** and **80** are the main metabolites of **77** but no data is available indicating that the diphosphate metabolite of **78** is the active species inhibiting HBV DNA polymerase [142]. In phase I studies, **77** demonstrated clinical efficacy in HBV-infected patients [144].

6. PRODRUGS OF NUCLEOSIDE DIPHOSPHATES (NDP) AND TRIPHOSPHATES (NTP)

Similar to the NMPs, delivery of NDP and NTP drugs to target cells bypasses some or all of the activating phosphorylation steps. However, chemical and enzymatic breakdown of the NDP and NTP phosphate bonds complicate this approach in addition to the high polarity of these species. Despite these drawbacks the development of chemically and enzymatically stable triphosphate mimic prodrugs of AZT have been reported [145]. Stabilization was achieved by replacement of the β-γ oxygen with a difluoromethylene group, and the α-P hydroxyl with a borane group thus generating a 'triphosphate' mimic, **81** (AZT-P3M). Inhibition of HIV RT by **81** is comparable to AZT triphosphate. Among the prodrugs designed to mask the phosphonate diacids, the γ-bis(POM) prodrug **82**, exhibited 5-fold greater potency toward HIV-1 than AZT and an improved resistance profile, most likely due to the presence of α-borano moiety that has been shown to favorably affect the activity against drug resistant HIV RT [146]. Lipophilic acyl prodrugs of AZT tri-phosphates, e.g. **83** and **84** have been explored [147]. The acyl group is selectively hydrolyzed in buffer, by virtue of the increased

lability of the acyl phosphate bond compared to the phosphodiester bond. However, in cell culture experiments, there was no significant improvement in antiviral activity which was attributed to the instability of the compound in culture media [147].



7. CONCLUSION

The recent success of new antiviral agents, noteably, famciclovir, valaciclovir, valganciclovir, tenofovir disoproxil, adefovir dipivoxil and abacavir, exemplifies the substantial progress that has been made in the development of nucleotide and nucleoside prodrugs. It is expected that continued research in this field will lead to further novel antiviral prodrugs that possess optimal tissue specificity and oral bioavailability properties.

REFERENCES

[1] E. De Clercq, *J. Clin. Virol.*, 2001, **22**, 73.
[2] D. S. Stein and K. H. P. Moore, *Pharmacotherapy*, 2001, **21**, 11.
[3] J. P. Krise and V. J. Stella, *Adv. Drug. Del. Rev.*, 1996, **19**, 287.
[4] K. Parang, L. I. Wiebe and E. E. Knaus, *Curr. Med. Chem.*, 2000, **7**, 995.
[5] T. Calogeropoulou, A. Detsi, E. Lekkas and M. Koufaki, *Curr. Top. Med. Chem.*, 2003, **3**, 1467.
[6] C. M. Perry and D. Faulds, *Drugs*, 1996, **52**, 754.
[7] D. Ormrod, L. J. Scott and C. M. Perry, *Drugs*, 2000, **59**, 839.
[8] M. E. Ganapathy, W. Huang, H. Wang, V. Ganapathy and F. H. Leibach, *Biochem. Biophys. Res. Commun.*, 1998, **246**, 470.
[9] I. Kim, X.-Y. Chu, S. Kim, C. J. Provoda, K.-D. Lee and G. L. Amidon, *J. Biol. Chem.*, 2003, **278**, 25348.
[10] M. Curran and S. Noble, *Drugs*, 2001, **61**, 1145.
[11] P. Reusser, *Expert Opin. Invest. Drugs*, 2001, **10**, 1745.
[12] M. Sugawara, W. Huang, Y.-J. Fei, F. H. Leibach, V. Ganapathy and M. E. Ganapathy, *J. Pharm. Sci.*, 2000, **89**, 781.
[13] A. E. Thomsen, G. M. Friedrichsen, A. H. Sørenson, R. Andersen, C. U. Nielsen, B. Brodin, M. Begtrup, S. Frokjaer and B. Steffansen, *J. Controlled Release*, 2003, **86**, 279.
[14] S. K. Aggarwal, S. R. Gogu, S. R. S. Rangan and K. C. Agrawal, *J. Med. Chem.*, 1990, **33**, 1505.
[15] R. H. Lupia, N. Ferencz, J. J. L. Lertora, S. K. Aggarwal, W. J. George and K. C. Agrawal, *Antimicrob. Agents Chemother.*, 1993, **37**, 818.
[16] P. Vlieghe, T. Clerc, C. Pannecouque, M. Witvrouw, E. De Clercq, J.-P. Salles and J.-L. Kraus, *J. Med. Chem.*, 2001, **44**, 3014.

[17] P. Vlieghe, F. Bihel, T. Clerc, C. Pannecouque, M. Witvrouw, E. De Clercq, J.-P. Salles, J.-C. Chermann and J.-L. Kraus, *J. Med. Chem.*, 2001, **44**, 777.
[18] M. P. Dillon, H. Cai and H. Maag, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 1653.
[19] L. A. Jones, A. R. Moorman, S. D. Chamberlain, P. de Miranda, D. J. Reynolds, C. L. Burns and T. A. Krenitsky, *J. Med. Chem.*, 1992, **35**, 56.
[20] D. F. Smee, A. C. Morrison, K. W. Bailey and R. W. Sidwell, 41st Intersci. Conf. Antimicrob. Agents Chemother., Chicago, IL, Dec 16–19th, 2001, Abstract 1594.
[21] G. Andrei, R. Snoeck, J. Neyts, M. L. Sandvold, F. Myhren and E. De Clercq, *Antiviral Res.*, 2000, **45**, 157.
[22] B. Bhat, S. S. Carroll, A. B. Eldrup, M. Maccoss, D. B. Olsen and T. P. Prakash, *PCT Patent Appl.* 007512 A2, 2004.
[23] N. G. Johansson, P. Englehart, M. Högberg, B. Lindborg, K. Linden, L. Ståhle and X.-X. Zhou, *Antiviral Res.*, 1998, **37**, A76, Abstract 125.
[24] S. M. Daluge, S. S. Good, M. B. Faletto, W. H. Miller, M. H. St Clair, L. R. Boone, M. Tisdale, N. R. Parry, J. E. Reardon, *et al.*, *Antimicrob. Agents Chemother.*, 1997, **41**, 1082.
[25] R. Vince and M. Hua, *J. Med. Chem.*, 1990, **33**, 17.
[26] M. B. Faletto, W. H. Miller, E. P. Garvey, M. H. St Clair, S. M. Daluge and S. S. Good, *Antimicrob. Agents Chemother.*, 1997, **41**, 1099.
[27] M. H. Maguire and M. K. Sim, *Eur. J. Biochem.*, 1971, **23**, 22.
[28] S. Frederiksen, *Arch. Biochem. Biophys.*, 1966, **113**, 383.
[29] R. Wolfenden, *J. Am. Chem. Soc.*, 1966, **88**, 3157.
[30] B. M. Chassy and R. J. Suhadolnik, *J. Biol. Chem.*, 1967, **242**, 3655.
[31] H. P. Baer, G. I. Drummond and J. Gillis, *Arch. Biochem. Biophys.*, 1968, **123**, 172.
[32] L. N. Simon, R. J. Bauer, R. L. Tolman and R. K. Robins, *Biochemistry*, 1970, **9**, 573.
[33] P. A. Furman, J. Jeffrey, L. L. Kiefer, J. Y. Feng, K. S. Anderson, K. Borroto-Esoda, E. Hill, W. C. Copeland, C. K. Chu, J.-P. Sommadossi, *et al.*, *Antimicrob. Agents Chemother.*, 2001, **45**, 158.
[34] J. P. Mewshaw, F. T. Myrick, D. A. C. S. Wakefield, B. J. Hooper, J. L. Harris, B. McCreedy and K. Borroto-Esoda, *J. Acq. Immune Defic. Syndr.*, 2002, **29**, 11.
[35] N. Rioux, R. Michaud, R. Bethell and B. A. Hamelin, World Congress of Pharmacology, July 7–12, 2002, Abstract 136.7.
[36] A. S. Ray, Z. Yang, C. K. Chu and K. S. Anderson, *Antimicrob. Agents Chemother.*, 2002, **46**, 887.
[37] R. J. Rybak, C. B. Hartline, Y.-L. Qiu, J. Zemlicka, E. Harden, G. Marshall, J.-P. Sommadossi and E. R. Kern, *Antimicrob. Agents Chemother.*, 2000, **44**, 1506.
[38] D. Faulds and R. N. Brogden, *Drugs*, 1992, **44**, 94.
[39] G. Ahluwalia, D. A. Cooney, H. Mitsuya, A. Fridland, K. P. Flora, Z. Hao, M. Dalal, S. Broder and D. G. Johns, *Biochem. Pharmacol.*, 1987, **36**, 3797.
[40] M. A. Johnson, G. Ahluwalia, M. C. Connelly, D. A. Cooney, S. Broder, D. G. Johns and A. Fridland, *J. Biol. Chem.*, 1988, **263**, 15354.
[41] T. Shirasaka, K. Murakami, H. Ford, Jr., J. A. Kelly, H. Yoshioka, E. Kojima, S. Aoki, S. Broder and H. Mitsuya, *Proc. Natl Acad. Sci. USA*, 1990, **87**, 9426.
[42] K. Murakami, T. Shirasaka, H. Yoshioka, E. Kojima, S. Aoki, H. Ford, Jr., J. S. Driscoll, J. A. Kelley and H. Mitsuya, *J. Med. Chem.*, 1991, **34**, 1606.
[43] M. E. Morgan, S.-C. Chi, K. Murakami, H. Mitsuya and B. D. Anderson, *Antimicrob. Agents Chemother.*, 1992, **36**, 2156.
[44] H. Ford, Jr., M. A. Siddiqui, J. S. Driscoll, V. E. Marquez, J. A. Kelley, H. Mitsuya and T. Shirasaka, *J. Med. Chem.*, 1995, **38**, 1189.
[45] D.-K. Kim, H.-K. Kim and Y.-B. Chae, *Bioorg. Med. Chem. Lett.*, 1994, **4**, 1309.
[46] C. L. Burns, M. H. St Clair, L. W. Frick, T. Spector, D. R. Averett, M. L. English, T. J. Holmes, T. A. Krenitsky and G. W. Koszalka, *J. Med. Chem.*, 1993, **36**, 378.
[47] T. Koudriakova, K. K. Manouilov, K. Shanmuganathan, L. P. Kotra, F. D. Boudinot, E. Cretton-Scott, J.-P. Sommadossi, R. F. Schinazi and C. K. Chu, *J. Med. Chem.*, 1996, **39**, 4676.
[48] L. P. Kotra, K. K. Manouilov, E. Cretton-Scott, J.-P. Sommadossi, F. D. Boudinot, R. F. Schinazi and C. K. Chu, *J. Med. Chem.*, 1996, **39**, 5202.

[49] T. A. Krenitsky, W. W. Hall, P. De Miranda, L. M. Beauchamp, H. J. Schaeffer and P. D. Whiteman, *Proc. Natl Acad. Sci. USA*, 1984, **81**, 3209.
[50] P. J. Rees, P. Selby, H. G. Prentice, P. D. Whitman and D. M. Grant, *J. Antimicrob. Chemother.*, 1986, **18** (suppl. B), 215.
[51] K. Shanmuganathan, T. Koudriakova, S. Nampalli, J. Du, J. M. Gallo, R. F. Schinazi and K. Chu, *J. Med. Chem.*, 1994, **37**, 821.
[52] R. Vince, J. Brownell and S. A. Beers, *Nucleosides Nucleotides*, 1995, **14**, 39.
[53] H. Chen, F. D. Boudinot, C. K. Chu, H. M. McClure and R. F. Schinazi, *Antimicrob. Agents Chemother.*, 1996, **40**, 2332.
[54] R. A. V. Hodge, D. Sutton, M. R. Boyd, M. R. Harnden and R. L. Jarvest, *Antimicrob. Agents Chemother.*, 1989, **33**, 1765.
[55] M. R. Harnden, R. L. Jarvest, M. R. Boyd, D. Sutton and R. A. V. Hodge, *J. Med. Chem.*, 1989, **32**, 1738.
[56] D.-K. Kim, N. Lee, Y.-W. Kim, K. Chang, J.-S. Kim, G.-J. Im, W.-S. Choi, I. Jung, T-S. Kim, Y.-Y. Hwang, *et al.*, *J. Med. Chem.*, 1998, **41**, 3435.
[57] I. Winkler, E. Winkelmann, T. Scholl, M. Rösner, G. Jähne and M. Helsberg, *Antiviral Res.*, 1990, **14**, 61.
[58] H. C. Krasny, L. Beauchamp, T. A. Krenitsky and P. De Miranda, *Drug Metab. Dispos.*, 1995, **23**, 1242.
[59] L. I. Wiebe, E. E. Knaus, A. M. Cheraghali, R. Kumar, K. W. Morin and L. Wang, *Nucleosides Nucleotides*, 1995, **14**, 501.
[60] R. Kumar, L. Wang, L. I. Wiebe and E. E. Knaus, *J. Med. Chem.*, 1994, **37**, 3554.
[61] R. Kumar, L. Wang, L. I. Wiebe and E. E. Knaus, *J. Med. Chem.*, 1994, **37**, 4297.
[62] L. Wang, K. W. Morin, R. Kumar, M. Cheraghali, K. G. Todd, G. B. Baker, E. E. Knaus and L. I. Wiebe, *J. Med. Chem.*, 1996, **39**, 826.
[63] R. Kumar, L. Wang, L. I. Wiebe and E. E. Knaus, *Nucleosides Nucleotides*, 1996, **15**, 265.
[64] A. M. Cheraghali, R. Kumar, L. Wang, E. E. Knaus and L. I. Wiebe, *Biochem. Pharmacol.*, 1994, **47**, 1615.
[65] C.-N. Chang, S.-L. Doong and Y.-C. Cheng, *Biochem. Pharmacol.*, 1992, **43**, 2269.
[66] J. Zemlicka, *Biochim. Biophys. Acta*, 2002, **1587**, 276.
[67] G. Gosselin, J.-L. Girardet, C. Périgaud, S. Benzaria, I. Lefebvre, N. Schlienger, A. Pompon and J.-L. Imbach, *Acta Biochim. Pol.*, 1996, **43**, 196.
[68] C. Meier, *Mini Rev. Med. Chem.*, 2002, **2**, 219.
[69] J. Balzarini, A. Karlsson, S. Aquaro, C.-F. Perno, D. Cahard, L. Naesens, E. De Clercq and C. McGuigan, *Proc. Natl Acad. Sci. USA*, 1996, **93**, 7295.
[70] C. McGuigan, H.-W. Tsang, D. Cahard, K. Turner, S. Velazquez, A. Salgado, L. Bidois, L. Naesens, E. De Clercq and J. Balzarini, *Antiviral Res.*, 1997, **35**, 195.
[71] C. McGuigan, L. Bidois, A. Hiouni, C. Ballatore, E. De Clercq and J. Balzarini, *Antiviral Chem. Chemother.*, 2000, **11**, 111.
[72] C. McGuigan, P. W. Sutton, D. Cahard, K. Turner, G. O'Leary, Y. Wang, M. Gumbleton, E. De Clercq and J. Balzarini, *Antiviral Chem. Chemother.*, 1998, **9**, 473.
[73] C. McGuigan, D. Cahard, H. M. Sheeka, E. De Clercq and J. Balzarini, *J. Med. Chem.*, 1996, **39**, 1748.
[74] F. M. Uckun, S. Pendergrass, T. K. Venkatachalam, S. Qazi and D. Richman, *Antimicrob. Agents Chemother.*, 2002, **46**, 3613.
[75] F. M. Uckun, S. Qazi, S. Pendergrass, E. Lisowski, B. Waurzyniak, C.-L. Chen and T. K. Venkatachalam, *Antimicrob. Agents Chemother.*, 2002, **46**, 3428.
[76] F. M. Uckun, C.-L. Chen, P. Samuel, S. Pendergrass, T. K. Venkatachalam, B. Waurzyniak and S. Qazi, *Antimicrob. Agents Chemother.*, 2003, **47**, 1233.
[77] F. M. Uckun, A. S. Petkevich, A. O. Vassilev, H. E. Tibbles and L. Titov, *BMC Infect. Dis.*, 2004, **4**, 1.
[78] O. J. D'Cruz, P. Samuel, B. Waurzyniak and F. M. Uckun, *Biol. Reprod.*, 2003, **69**, 1843.
[79] J. Balzarini, J. Kruining, O. Wedgwood, C. Pannecouque, S. Aquaro, C.-F. Perno, L. Naesens, M. Witvrouw, R. Heijtink, E. De Clercq and C. McGuigan, *FEBS Lett.*, 1997, **410**, 324.

[80] P. Franchetti, L. Cappellacci, M. Grifantini, L. Messini, G. A. Sheikha, A. G. Loi, E. Tramontano, A. de Montis, M. G. Spiga and P. La Colla, *J. Med. Chem.*, 1994, **37**, 3534.
[81] A. Brancale, S. Srinivasan, A. Hassan-Abdallah, Y. Wang, R. Pathirana, A. Siddiqui, S. Harris, C. McGuigan, E. De Clercq, J. Balzarini, *et al.*, *Antiviral Res.*, 2003, **57**, A46.
[82] K. S. Gudmundsson, S. M. Daluge, L. C. Johnson, R. Jansen, R. Hazen, L. D. Condreay and C. McGuigan, *Nucleosides Nucleotides Nucl. Acids*, 2003, **22**, 1953.
[83] C. McGuigan, R. N. Pathirana, N. Mahmood, K. G. Devine and A. J. Hay, *Antiviral Res.*, 1992, **17**, 311.
[84] J. Balzarini, O. Wedgwood, J. Kruining, H. Pelemans, R. Heijtink, E. De Clercq and C. McGuigan, *Biochem. Biophys. Res. Commun.*, 1996, **225**, 363.
[85] C. McGuigan, M. J. Slater, N. R. Parry, A. Perry and S. Harris, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 645.
[86] C. Périgaud, A.-M. Aubertin, S. Benzaria, H. Pélicano, J.-L. Girardet, G. Maury, G. Gosselin, A. Kirn and J.-L. Imbach, *Biochem. Pharmacol.*, 1994, **48**, 11.
[87] G. Valette, A. Pompon, J.-L. Girardet, L. Cappellacci, P. Franchetti, M. Grifantini, P. La Colla, A. G. Loi, C. Périgaud, G. Gosselin and J.-L. Imbach, *J. Med. Chem.*, 1996, **39**, 1981.
[88] L. Placidi, A. Faraj, A. G. Loi, C. Pierra, D. Egron, E. Cretton-Scott, G. Gosselin, C. Périgaud, L. T. Martin, R. F. Schinazi, *et al.*, *Antiviral Chem. Chemother.*, 2001, **12**, 99.
[89] I. Lefebvre, C. Périgaud, A. Pompon, A.-M. Aubertin, J.-L. Girardet, A. Kirn, G. Gosselin and J.-L. Imbach, *J. Med. Chem.*, 1995, **38**, 3941.
[90] X. Tan, F. D. Boudinot, C. K. Chu, D. Egron, C. Perigaud, G. Gosselin and J.-L. Imbach, *Antiviral Chem. Chemother.*, 2000, **11**, 203.
[91] M. Shafiee, S. Deferme, A.-L. Villard, D. Egron, G. Gosselin, J.-L. Imbach, T. Lioux, A. Pompon, S. Varray, A.-M. Aubertin, *et al.*, *J. Pharm. Sci.*, 2001, **90**, 448.
[92] C. Périgaud, G. Gosselin, J.-L. Girardet, B. E. Korba and J.-L. Imbach, *Antiviral Res.*, 1999, **40**, 167.
[93] B. Groschel, N. Himmel, J. Cinatl, C. Périgaud, G. Gosselin, J.-L. Imbach, H. W. Doerr and J. Cinatl, Jr., *Nucleosides Nucleotides*, 1999, **18**, 921.
[94] A. Egron, C. Périgaud, G. Gosselin, A.-M. Aubertin, H. Gatanaga, H. Mitsuya, J. Zemlicka and J.-L. Imbach, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 265.
[95] R. J. Jones and N. Bischofberger, *Antiviral Res.*, 1995, **27**, 1.
[96] D. Saboulard, L. Naesens, D. Cahard, A. Salgado, R. Pathirana, S. Velazquez, C. McGuigan, E. De Clercq and J. Balzarini, *Mol. Pharmacol.*, 1999, **56**, 693.
[97] C. Meier, M. Lorey, E. De Clercq and J. Balzarini, *J. Med. Chem.*, 1998, **41**, 1417.
[98] C. Meier, T. Knispel, E. De Clercq and J. Balzarini, *J. Med. Chem.*, 1999, **42**, 1604.
[99] C. Meier, T. Knispel, V. E. Marquez, M. A. Siddiqui, E. De Clercq and J. Balzarini, *J. Med. Chem.*, 1999, **42**, 1615.
[100] K. Y. Hostetler, S. Parker, C. N. Sridhar, M. J. Martin, J.-L. Li, L. M. Stuhmiller, G. M. T. van Wijk, H. van den Bosch, M. F. Gardner, K. A. Aldern, *et al.*, *Proc. Natl Acad. Sci. USA*, 1993, **90**, 11835.
[101] I. Taskintuna, A. S. Banker, M. Flores-Aguilar, G. Bergeron-Lynn, K. A. Aldern, K. Y. Hostetler and W. R. Freeman, *Retina*, 1997, **17**, 57.
[102] G. M. T. van Wijk, K. Y. Hostetler and H. van den Bosch, *Biochim. Biophys. Acta*, 1991, **1084**, 307.
[103] K. Y. Hostetler, J. R. Beadle, G. D. Kini, M. F. Gardner, K. N. Wright, T. H. Wu and B. A. Korba, *Biochem. Pharmacol.*, 1997, **53**, 1815.
[104] P. M. Girard, P. S. Pegram, B. Diquet, R. Anderson, F. Raffi, R. Tubiana, D. Sereni and D. Boerner, *J. Acq. Immune Defic. Syndr.*, 2000, **23**, 227.
[105] L. Naesens, R. Snoeck, G. Andrei, J. Balzarini, J. Neyts and E. De Clercq, *Antiviral Chem. Chemother.*, 1997, **8**, 1.
[106] E. De Clercq, *Clin. Microbiol. Rev.*, 2003, **16**, 569.
[107] E. De Clercq, *Expert. Rev. Anti-Infect. Ther.*, 2003, **1**, 21.
[108] R. O. Baker, M. Bray and J. W. Huggins, *Antiviral Res.*, 2003, **57**, 13.
[109] E. S. Ho, D. C. Lin, D. B. Mendel and T. Cihlar, *J. Am. Soc. Nephrol.*, 2000, **11**, 383.

[110] N. Bischofberger, M. J. M. Hitchcock, M. S. Chen, D. B. Barkhimer, K. C. Cundy, K. M. Kent, S. A. Lacy, W. A. Lee, Z.-H. Li, D. B. Mendel, *et al.*, *Antimicrob. Agents Chemother.*, 1994, **38**, 2387.
[111] K. C. Cundy, A. M. Bidgood, G. Lynch, J. P. Shaw, L. Griffin and W. A. Lee, *Drug Metab. Dispos.*, 1996, **24**, 745.
[112] D. B. Mendel, T. Cihlar, K. Moon and M. S. Chen, *Antimicrob. Agents Chemother.*, 1997, **41**, 641.
[113] D. F. Smee and E. J. Reist, *Antimicrob. Agents Chemother.*, 1996, **40**, 1964.
[114] M. Wachsman, B. G. Petty, K. C. Cundy, H. S. Jaffe, P. E. Fisher, A. Pastelak and P. S. Lietman, *Antiviral Res.*, 1996, **29**, 153.
[115] K. C. Cundy, P. Barditch-Crovo, B. G. Petty, A. Ruby, M. Redpath, H. S. Jaffe and P. S. Lietman, *Antimicrob. Agents Chemother.*, 1999, **43**, 271.
[116] J. R. Beadle, C. Hartline, K. A. Aldern, N. Rodriguez, E. Harden, E. R. Kern and K. Y. Hostetler, *Antimicrob. Agents Chemother.*, 2002, **46**, 2381.
[117] E. R. Kern, C. Hartline, E. Harden, K. Keith, N. Rodriguez, J. R. Beadle and K. Y. Hostetler, *Antimicrob. Agents Chemother.*, 2002, **46**, 991.
[118] S. L. Ciesla, J. Trahan, W. B. Wan, J. R. Beadle, K. A. Aldern, G. R. Painter and K. Y. Hostetler, *Antiviral Res.*, 2003, **59**, 163.
[119] D. C. Quenelle, D. J. Collins, W. B. Wan, J. R. Beadle, K. Y. Hostetler and E. R. Kern, *Antimicrob. Agents Chemother.*, 2004, **48**, 404.
[120] R. Oliyai, M. N. Arimilli, R. J. Jones and W. A. Lee, *Nucleosides Nucleotides Nucl. Acids*, 2001, **20**, 1411.
[121] K. A. Keith, M. J. M. Hitchcock, W. A. Lee, A. Holý and E. R. Kern, *Antimicrob. Agents Chemother.*, 2003, **47**, 2193.
[122] P. R. Harrigan, M. D. Miller, P. McKenna, Z. L. Brumme and B. A. Larder, *Antimicrob. Agents Chemother.*, 2002, **46**, 1067.
[123] G. Birkus, M. J. M. Hitchcock and T. Cihlar, *Antimicrob. Agents Chemother.*, 2002, **46**, 716.
[124] M. N. Arimilli, C. U. Kim, J. Dougherty, A. Mulato, R. Oliyai, J. P. Shaw, K. C. Cundy and N. Bischofberger, *Antiviral Chem. Chemother.*, 1997, **8**, 557.
[125] B. L. Robbins, R. V. Srinivas, C. Kim, N. Bischofberger and A. Fridland, *Antimicrob. Agents Chemother.*, 1998, **42**, 612.
[126] P. Barditch-Crovo, S. G. Deeks, A. Collier, S. Safrin, D. F. Coakley, M. Miller, B. P. Kearney, R. L. Coleman, P. D. Lamy, J. O. Kahn, *et al.*, *Antimicrob. Agents Chemother.*, 2001, **45**, 2733.
[127] E. J. Eisenberg, G. X. He and W. A. Lee, *Nucleosides Nucleotides Nucl. Acids*, 2001, **20**, 1091.
[128] W. Lee, G. He, A. Mulato, W. Delaney, E. Eisenberg, T. Cihlar, S. Xiong, M. D. Miller, S. Gill, R. Shibata, C. Gibbs, 9th Conference on Retroviruses, Opportunistic Inf., Seattle, WA, Feb. 24–28, 2002, Abstract 384-T.
[129] J. E. Starrett, Jr., D. R. Tortolani, M. J. M. Hitchcock, J. C. Martin and M. M. Mansuri, *Antiviral Res.*, 1992, **19**, 267.
[130] J. E. Starrett, Jr., D. R. Tortolani, J. Russell, M. J. M. Hitchcock, V. Whiterock, J. C. Martin and M. M. Mansuri, *J. Med. Chem.*, 1994, **37**, 1857.
[131] M. N. Amirilli, J. P. Dougherty, K. C. Cundy and N. Bischofberger, in *Adv. Antiviral Drug Design*, (eds E. De Clercq), Elsevier, Amsterdam, 1999, 3, pp. 69–91.
[132] R. B. Qaqish, K. A. Mattes and D. J. Ritchie, *Clin. Ther.*, 2003, **25**, 3084.
[133] T. M. Dando and G. L. Plosker, *Drugs*, 2003, **63**, 2215.
[134] S. Benzaria, H. Pélicano, R. Johnson, G. Maury, J.-L. Imbach, A.-M. Aubertin, G. Obert and G. Gosselin, *J. Med. Chem.*, 1996, **39**, 4958.
[135] C. Ballatore, C. McGuigan, E. De Clercq and J. Balzarini, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1053.
[136] J. Kim, W. Y. Cho, Y. G. Cho, G. W. Kim, M. K. Lee, T. G. Kim, C. M. Kim, M. J. Han, J. Choi, J. Hwang, *et al*., 42nd Intersci. Conf. Antimicrob. Agents Chemother., San Diego, CA, Sep. 27–31, 2002, Abstract F-1690.

[137] S. H. Kim, E. Choi, H. K. Jung, S. H. Noh, K. H. Chung, O. H. Choi, S. C. Bu, S. H. Lee, M. Park, J. Kim and S. Lee, 42nd Intersci. Conf. Antimicrob. Agents Chemother., San Diego, CA, Sep. 27–31, 2002, Abstract F-1693.
[138] J. Kim, H. R. Choi, Y. Z. Kim, S. H. Lee, S. Lee and I. C. Kim, *Antiviral Res.*, 2003, **57**, A91.
[139] M. D. Erion, T. J. Colby, K. R. Reddy, D. A. MacKenna, S. H. Boyer, J. M. Fujitaki, D. L. Linemeyer, D. A. Bullough and P. D. van Poelje, *Hepatology*, 2002, **36**, 301A.
[140] Z. Hong, 16th Intl. Conference on Antiviral Res., Pre-conference satelite symposium "Clinical Update on Antiviral Drugs", Savannah, GA, Apr. 27–May 1, 2003.
[141] S. K. Ono-Nita, N. Kato, Y. Shiratori, F. J. Carrilho and M. Omata, *Antimicrob. Agents Chemother.*, 2002, **46**, 2602.
[142] N. Kamiya, A. Kubota, Y. Iwase, K. Sekiya, M. Ubasawa and S. Yuasa, *Antimicrob. Agents Chemother.*, 2002, **46**, 2872.
[143] K. Sekiya, H. Takashima, N. Ueda, N. Kamiya, S. Yuasa, Y. Fujimura and M. Ubasawa, *J. Med. Chem.*, 2002, **45**, 3138.
[144] S. D. Wise, K. W. Soon, S. L. Lowe, C. H. Teng, A. X. Pan and J. McGill, *Gastroenterology*, 2002, **122** (S1), Abstract T1360.
[145] J. M. Leeds, M. E. Ariza, G. Wang, M. Prhavc, N. Boyle, V. Rajwanshi, F. Chen, P. Fagan, T. Bruice, J. Brooks, *et al*., 43rd Intersci. Conf. Antimicrob. Agents Chemother., Chicago, IL, Sep. 14–17, 2003, Abstract F-1836.
[146] P. Meyer, B. Schneider, S. Sarfati, D. Deville-Bonne, C. Guerreiro, J. Boretto, J. Janin, M. Veron and B. Canard, *EMBO J.*, 2000, **19**, 3520.
[147] D. Bonnaffé, B. Dupraz, J. Ughetto-Monfrin, A. Namane, Y. Henin and T. H. Dinh, *J. Org. Chem.*, 1996, **61**, 895.

Pre-Clinical Assessment of Drug-Induced QT Interval Prolongation. Current Issues and Impact on Drug Discovery

Bernard Fermini and Anthony A. Fossa

Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340, USA

Contents

1. INTRODUCTION

Over the past decade, a number of non-cardiovascular drugs have had their label revised, or have been withdrawn from the market, because of unexpected post-marketing reports of sudden cardiac death associated with a prolongation of the QT interval on surface electrocardiograms (ECG), and an increased risk of developing a rare polymorphic ventricular tachyarrhythmia called torsades de pointes (TdP) (Table 1). Although a direct link between prolongation of the QT interval and the onset of arrhythmias remains to be demonstrated with certainty, it is currently assumed that even a small increase in the QT interval is associated with some risk of developing TdP. As a result, the issue of drug-induced QT prolongation has become the subject of intense regulatory review. QT prolongation is now the leading cause for the withdrawal of approved drugs from the market, and represents a major hurdle for the development process of a myriad of potentially new drugs. Consequently, it has become imperative for the pharmaceutical industry to implement strategies to address this critical issue. Various assays and models are being considered by both regulators and the drug industry to identify, as early as possible in the discovery process, new chemical entities that have the propensity to affect the QT interval in an effort to avoid future health concerns for patients, as well as commercial fallout from adverse labeling and costly withdrawals from the market place.

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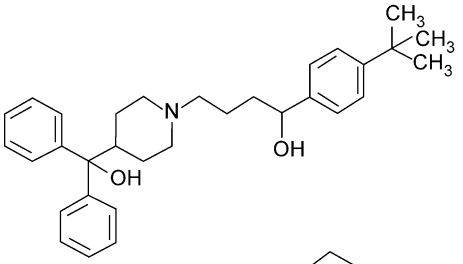
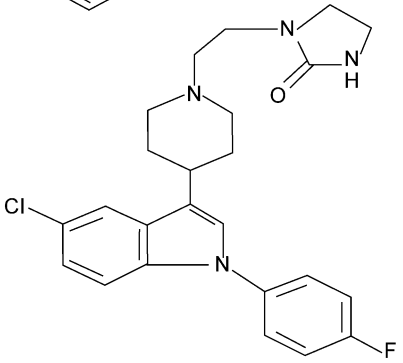
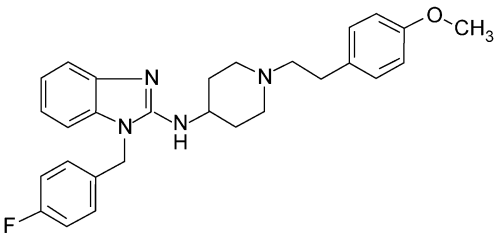
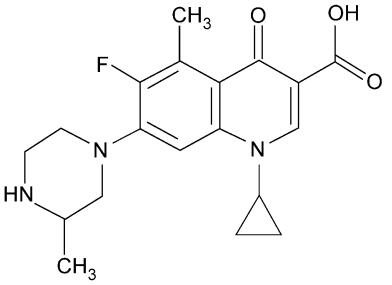
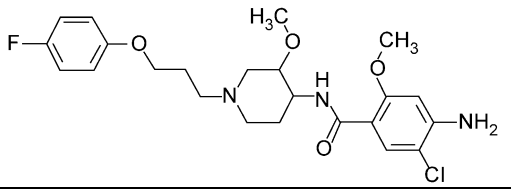
Table 1. Drugs withdrawn for the US market

Drug	Structure	Class	HERG IC_{50} (nM)	Date withdrawn
Terfenadine		Antihistamine	204	Feb 1998
Sertindole		Antipsychotic	14	Dec 1998

(Continued)

Table 1. Continued

Drug	Structure	Class	HERG IC_{50} (nM)	Date withdrawn
Astemizole		Antihistamine	0.9	June 1999
Grepafloxacin		Antibiotic	50,000	Nov 1999
Cisapride		GI prokinetic	6.5	July 2000

This chapter addresses the issue of drug-induced QT prolongation, and examines its impact on drug-development programs in the pharmaceutical industry.

2. ELECTROPHYSIOLOGICAL MECHANISMS UNDERLYING QT PROLONGATION

The surface ECG is a reflection of the sum of action potentials generated from all the cells in the heart. Action potentials result from the opening and closing of membrane-spanning proteins that form ion channels in the membrane. A sequential change in the inward and outward flow of positive ions through these selective channels determines the complex morphology and duration of cardiac action potentials (Fig. 1). Rapid entry of sodium ions initiates the depolarization of the ventricles (phase 0), followed by a rapid repolarization though transiently activating and inactivating outward potassium channels (phase 1). This is followed by a plateau phase (phase 2), mainly determined by the entry of calcium ions through L-type calcium channels, and a repolarization (phase 3) phase resulting from the inactivation of calcium channels, and the increase in net outward potassium currents carried mainly by the slow (I_{Ks}) and rapid (I_{Kr}) components of the delayed rectifier potassium channels. In humans, I_{Kr} appears to play a significant role in determining ventricular action potential duration (APD) and repolarization, as congenital mutations of this channel are associated with a decrease in current amplitude and prolongation the QT interval [1]. Clinically, cardiac repolarization is assessed by measuring the QT interval on

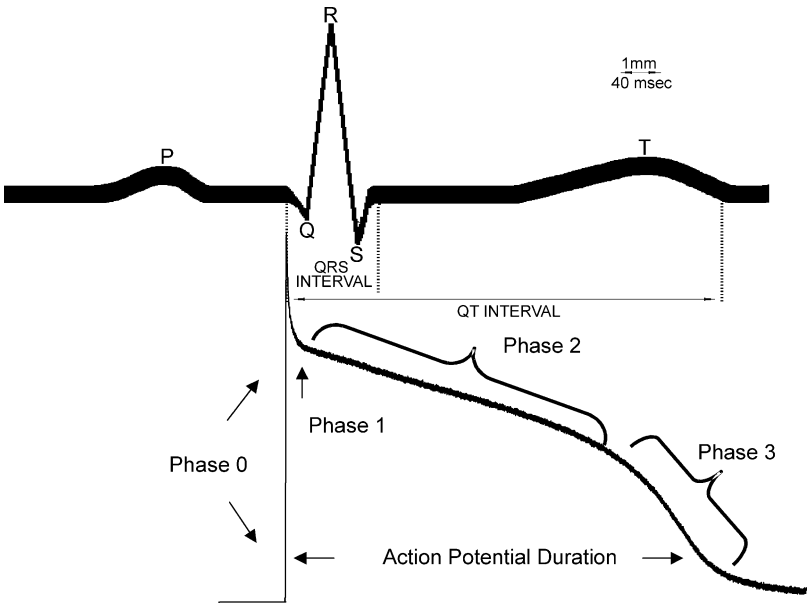


Fig. 1.

the ECG, which is defined as the duration between the beginning of the QRS complex and the end of the T wave, and is a reflection of ventricular APD (Fig. 1). When corrected for individual heart rate, the QT interval is defined as 'corrected' QT, or QTc. Normal upper limit for QTc is approximately 430 ms for men, and 450 ms for women. Drugs that prolong the APD or the QT interval may do so by interacting with one or several ion channels. Excessive APD prolongation (typically in Purkinje fibers and/or midmyocardial cells) increases the propensity of developing spontaneous oscillations of the membrane potential that can give rise to one or more premature responses called early after-depolarizations (EADs). When generated in the presence of transmural heterogeneity in ventricular repolarization, EADs are believed to contribute to the generation of extrasystoles that can trigger TdP [2].

3. QT PROLONGATION AND TORSADES DE POINTES

Prolongation of the QT interval in patients is usually observed in response to an underlying medical condition, or in association with drug treatment, or else in patients afflicted with one of the congenital long-QT syndromes. Genetic studies have identified at least six genes that, if mutated, result in ion channel malfunction that can cause the long-QT syndrome. Studies of one of these genes (KCNH2), the human ether-à-go-go-related gene (HERG at the LQT2 locus), which encodes the cardiac potassium channel I_{Kr}, has provided invaluable information in understanding the mechanisms underlying drug-induced QT prolongation [3,4]. In the large majority of cases, drugs that prolong the QT interval and cause TdP inhibit HERG or I_{Kr} at therapeutic, or supra therapeutic concentrations. Wide ranges of chemical structures developed against many different molecular targets have been shown to inhibit the HERG channel, relative to other voltage-dependent K^+ channels. It is believed that most of the drugs that inhibit HERG current bind to a site located in the intracellular region of the pore cavity of the channel. Recent studies have revealed that the pore of HERG channels is large, which makes it more likely to trap small molecules of different classes, or compounds that are too large to block other potassium channels [5]. Moreover, unlike other potassium channels, HERG channels inactivate rapidly, effectively trapping drug molecules in the vestibule of the channel, leading to an increase in drug concentration near the pore [6]. Using homology modeling of the HERG channel, based on the crystallographic structure of two bacterial potassium channels (KcsA and MthK) representing the closed and open states of the channels, investigators were able to show that the HERG channel also contains two aromatic residues in each S6 helix (Tyr652 and Phe656), which undoubtedly contributes to allowing many different chemical structures to dock and bind to the inner mouth of the channel [5,7]. Such residues are not found in other potassium channels that are less susceptible to drug inhibition. More recently, ligand-based in silico models of the HERG channel have been published, and should help us further our understanding of how drug molecules interact with this channel [8,9].

Although prolongation of the QT interval can lead to life-threatening arrhythmias, in reality the incidence of such arrhythmias is rare. For example, the antibiotic agent grepafloxacin was removed from the US market in November 1999. Out of over

2.7 million prescriptions filled for this drug, there were seven cardiovascular events related to fatalities, with three documented cases of TdP [10]. Currently, more than fifty drugs have been reported to prolong the QT interval and/or induce TdP (see http://www.arizonacert.org). Among the most common are the three antiarrhythmic agents, Ibutilide (rate of TdP: 2–6%), sotalol (rate of 1.8–4.8%) and quinidine (rate 2–8.8%), as well as the prokinetic agent, cisapride (rate of 1 per 120,000 patients) and the antihistamine, terfenadine (rate of 8%). Of these, grepafloxacin, terfenadine and cisapride have been removed from the US market, as well as others such as astemizole (antihistamine) and sertindole (antipsychotic). All of these drugs have been reported to inhibit HERG current amplitude [11–15]. Hence, early identification of compounds that have the propensity to become pro-arrhythmic poses a challenge to physicians, the pharmaceutical industry and regulators. Taking into account the tens of millions of patients exposed these drugs; it remains that no more than a few dozen cases of TdP have been reported. This raises two important issues: (1) is QT prolongation a valid predictor of TdP and (2) why do only a small proportion of individuals experience TdP while the majority appears unaffected by drugs that produce QT interval prolongation?

3.1. QT prolongation as a surrogate marker for TdP

Clinicians and regulators use the QT interval as a surrogate marker for the prediction of adverse effects such as TdP. Rightfully or not, it is currently assumed that even small changes in the QT interval indicate some risk of TdP, and there is presently no well-established threshold below which a prolonged QT interval is believed to be harmless. Yet, not all drugs that prolong the QT interval cause TdP. For example, the calcium channel blocker verapamil, used for the treatment of hypertension, has been shown to prolong the QT interval in a manner that is correlated to its plasma concentration [16], but there are few described cases of verapamil-induced TdP [17]. Also, the antiarrhythmic agent, amiodarone, consistently prolongs the QT interval to more than 500 ms, but rarely causes TdP [18]. Because both verapamil and amiodarone inhibit ion channels other than I_{Kr}, it has been proposed that such mechanisms may preclude the generation of arrhythmias by either mitigating the prolongation of the action potential expected for I_{Kr} inhibition, or reducing the risk of developing EADs by affecting L-type calcium channels and/or the regulation of intracellular calcium levels. Nonetheless, the overall risk of arrhythmia does appear to increase with increasing QTc. Data on QTc intervals in case reports of TdP on a number of cardiac and non cardiac drugs indicate that a QTc interval of >500 ms was most commonly observed before the TdP event. In support of this argument, recent studies looking at the risk stratification of patients with the congenital Long-QT syndrome, LQT2, indicate that the risk of developing a cardiac event before the age of 40 years increases among patients with QTc intervals over 500 ms [19,20]. Finally, available data suggest that in individual subjects, an increase of 60 ms in peak or maximum QTc interval over baseline is also predictive of a potential risk [21]. Therefore, although the relationship between QT interval prolongation and TdP is imperfect, it will continue to be used as a surrogate marker, until a better clinical alternative is identified.

3.2. Risk factors for drug-induced arrhythmias

Many different factors that influence cardiac repolarization and the duration of the QT interval have been associated with an increased risk of drug-induced arrhythmias. These include age (very young and elderly), gender (female > male), heart rate (bradycardia), cardiac disorders, electrolyte imbalance, disease states (hepatic and renal), and concomitant medication. When present, such factors may contribute to exacerbate the actions of a drug that prolongs the QT interval, and predispose to an increase risk of developing TdP. In support of this idea of individual predisposition, a study of patients prescribed a Class III antiarrhythmic agent showed that those who developed TdP had more drug-induced QT prolongation than those who did not [22]. Moreover, the degree of QTc prolongation in the group with TdP was unrelated to drug dosage, suggesting that those who developed TdP showed an abnormal response to the drug. In some patients, the susceptibility has a genetic basis. Current data suggests that as many as 10–15 percent of the individuals that experience drug-induced TdP may carry mutations associated with the long QT-syndrome that may compromise cardiac repolarization, and aggravate the effects of a drug that is otherwise safe in the absence of such background factors [23]. These data suggest that the simultaneous combination of risk factors and genetics may be required to confer increased risk of drug-induced TdP in a certain population of patients. It is hoped that in the future, genetic research will play an important role in identifying populations at higher risk, so they can be treated accordingly when requiring therapies leading to an increased risk of developing TdP.

4. REGULATORY PERSPECTIVES

In response to increased reports of post-marketing QT prolongation and cardiac adverse events by non-cardiovascular drugs, the Committee for Proprietary Medicinal Products (CPMP) in 1997 issued a 'Points to Consider' document entitled 'The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products' [24]. This document outlined a series of experimental non-clinical and clinical models for assessing the potential for QT prolongation by non-cardiovascular agents. The non-clinical approaches emphasized *in vitro* electrophysiological studies examining action potentials in isolated cardiac tissues like Purkinje fibers and papillary muscle, whereas the *in vivo* approach focused on large animal assessment effects on blood pressure, heart rate, and more robust measurements of electrocardiogram intervals. The clinical guidance also focused on more intense assessments of electrocardiogram intervals obtained from a larger patient population earlier in development. Specific methods and magnitude of changes were noted in the document, thus opening the debate on the scientific basis and relevance of all these recommendations.

Given the tremendous economic and medical consequence of this issue, the International Conference on Harmonization (ICH) recently established an expert working group (EWG) to draft guidance recommending the incorporation of pre-clinical models predictive of QT prolongation and proarrhythmia in the development process of new drugs [25]. The resultant draft guidance document entitled 'Safety Pharmacology Studies For Assessing The Potential For Delayed Ventricular Repolarization

(QT Interval Prolongation) By Human Pharmaceuticals S7B' was signed as a Step 2 draft document in February 2002 (see http://www.ich.org). At Step 2, a consensus draft document is transmitted by the ICH to the regulatory authorities of the three ICH regions (EU, Japan, USA), for internal and external consultation. The S7B document advocates a testing strategy that incorporates (1) an assay evaluating the block of repolarizing currents such as HERG or I_{Kr}; (2) a repolarization assay that evaluates changes in APD in an integrated cardiac tissue preparation such as Purkinje fibers; and (3) an assay evaluating changes in the QT interval in an *in vivo* preparation. The implication of such guidelines is that any drug that blocks repolarizing currents and prolongs the cardiac APD in the *in vitro* models or prolongs the QT interval or elicits arrhythmias in the *in vivo* model is considered to pose a risk to human. Yet, the absence of findings in these assays is not considered to preclude a potential risk to humans. The Food and Drug Administration (FDA), as an organization, has not issued a written statement or documents on QT prolongation. However, it is supporting a study by the International Life Sciences Institute and Health and Environmental Sciences Institute (ILSI/HESI), evaluating the sensitivity and specificity of the *in vitro* and *in vivo* assays proposed by the ICH S7B. It is also collaborating with Georgetown University in evaluating several drugs known to cause TdP in the HERG assay. Results from this initiative have not yet been published.

5. PRE-CLINICAL MODELS TO ASSESS THE PRO-ARRHYTHMIC POTENTIAL OF NON-CARDIOVASCULAR DRUGS

There has been much debate within the pharmaceutical industry as to the most predictive *in vitro* and *in vivo* models for predicting the risk of TdP in clinical use. A survey of the current practice in the pharmaceutical industry for assessing the potential of QT prolongation by non-cardiovascular drugs showed that there was a wide diversity in the testing methodologies used [26]. A more recent study examined the relative value of pre-clinical cardiac electrophysiology data for predicting the risk of TdP in clinical use [27]. This study also introduced the notion of a safety margin between the IC_{50} for inhibition of the HERG channel, and the maximal effective therapeutic plasma concentration of a drug attained during clinical use. Finally, it proposed a pre-clinical screening scheme with the purpose of acquiring important information on any new compound before it enters clinical evaluation. These assays are similar to that described in the S7B document, and their utility, advantages and disadvantages are now discussed.

5.1. *In vitro* assays

As mentioned previously, two functional assays are regularly used to evaluate the potential of a drug to delay cardiac repolarization. One assay looks at the effects of drugs on the action potential of cardiac tissues obtained from the myocardium of animal species demonstrating an ionic profile similar to that of the human heart (including dog, rabbit, ferret, swine and guinea pig). In this assay, a fine tipped electrode impales a single myocyte within the exposed surface of the preparation, and records action potentials (AP). Changes in the morphology or duration of the AP will reflect pharmacological

effects of the drug studied on native cardiac channels. One added advantage of this approach is that the experimental conditions can be manipulated to simulate predisposing factors for QT prolongation and TdP. This assay is also useful because it allows drug effects on native cardiac channels to be studied, and can be used to detect effects mediated through pumps or exchange mechanisms, as well as ion channels other than the I_{Kr} channel. On the other hand, metabolites of a drug cannot be generated, and need to be tested in a separate series of experiences. Moreover, this assay is labor intensive, has a very low throughput, and failure to see APD prolongation in this model cannot exclude pro-arrhythmic toxicity or the risk of TdP in humans [28].

The other approach evaluates the effects of drugs on HERG current expressed in heterologous expression systems, or on native I_{Kr} current recorded from isolated cardiac myocytes, using the voltage clamp technique. This technique uses a single microelectrode to voltage-clamp the electrical potential difference across the cell membrane, while measuring the current carried by ions flowing through ion channels expressed in the cell membrane. No other method can provide such high quality and physiologically relevant data of precise and detailed activity of ion channel function. However, the predictive value of studies performed using this technique will depend on several factors including: selectivity of the compounds for HERG/I_{Kr} over other ion channels, concentration range studied, experimental conditions and protocols used. One of the major disadvantages of this assay is that it is technically difficult, and is low throughput. But this situation may be about to change, as high throughput planar patch-clamp technology is now emerging, and commercially available systems pledge to increase the rate of data acquisition dramatically to screen thousands of compounds per day [29].

A recent study investigated the utility of these two *in vitro* assays, and compared matched concentrations of a set of 10 structurally diverse drugs on APD changes in canine Purkinje fibers, and HERG channels stably expressed in human embryonic kidney cells [30]. The study highlighted the difficulty of assessing the potential pro-arrhythmic risk of drugs on the basis of concentration-dependent effects using these two assays. Moreover, it showed that, overall, the extent of HERG inhibition was poorly correlated with APD prolongation, consistent with the idea of additional drug effects on non-HERG channels. While HERG inhibition detected many of the drugs linked to QT prolongation and TdP, it was not fully predictive of pro-arrhythmic risk, as compounds, like verapamil, elicited significant HERG block with little effect on APD. Taken together, these results suggest that the HERG assay may occasionally oversimplify the drug effect on the repolarization process and that neither assay alone can adequately predict the pro-arrhythmic risk of drugs.

5.2. *In vivo* assays

In this assay, the ECG effects of a drug candidate are monitored in either conscious or anesthetized guinea pigs, rabbits, dogs or monkeys. Based on the survey of current practices, the dog seems to be the most popular species for *in vivo* studies. This seems to be justified mostly by the fact that the heart rate range is closer to humans than smaller animals, and by the similarities of the ionic makeup of Purkinje fibers and ventricular action potentials. The preferred methodology is the use of unrestrained animals

implanted with telemetry systems so that autonomic tone and stress influences are minimized. The heart rate, blood pressure, and ECG are continuously recorded over a range of escalating doses in order to detect the occurrence of any arrhythmias or QT interval prolongation [31]. However, the conscious dog model is relatively expensive, and is not suitable to screen a large number of compounds. Alternatively, the effects of compounds on the ECG and monophasic action potentials can be studied in the anesthetized state. This model is often used when the drug candidate that interferes with the determination of the pharmacological effect on the QT interval produces adverse behavior or dose-limiting emesis. One major advantage of the anesthetized model is that ventricular electrical pacing can be used to control the heart rate, thus eliminating the need to correct the QT interval for heart rate. One of the disadvantages to using anesthetized animals is that they cannot be used repeatedly. Furthermore, because parenteral routes of administration are necessary, the metabolic profile may not necessarily reflect the clinical profile when normally given orally in the conscious state (i.e., no first-pass metabolism through the liver). Another limitation is that most anesthetics interfere with cardiac conduction and may interact with the drug candidate, which can complicate the interpretation of the results. Although this assay can provide much information about the cardiac safety of a drug candidate, including the potential effects of any active metabolites, it has a low throughput and is extremely labor intensive.

Clearly, no single pre-clinical assay has an absolute predictive value of clinical outcome. Therefore, the use of several complimentary models is required to identify the potential hazard and assess the potential risk of QT interval prolongation and pro-arrhythmia of new pharmaceuticals. Finally, it should be emphasized that no matter how careful, extensive and complementary, the pre-clinical assessment process of a drug candidate for cardiac safety will always be superseded by clinical data.

6. IMPACT ON DRUG DISCOVERY AND DEVELOPMENT

Early identification of the risk of new drugs, or their metabolites, to induce QT prolongation has become a major goal for the pharmaceutical industry, and an integral part of the development process of every new drug. One of the real challenges is to develop new drugs that have minimal activity, at clinically relevant concentrations, on repolarizing currents such as HERG/I_{Kr} over their respective therapeutic target. It is likely that pre-clinical studies such as those proposed in the S7B guideline and well-planned clinical programs will reduce the number of pro-arrhythmic drugs from reaching the market. But in the process, millions of dollars in added research and development costs are at stake. Once established that a drug has the potential to prolong the QTc interval at clinically relevant concentrations, a company has to evaluate the approvability, labeling implications and consequent commercial fallout of such a drug against the benefit of continuing development. Given these high hurdles, it is likely that several potential new drugs will see their development either stopped, or greatly delayed. On the other hand, attempting to develop drugs that are entirely without effects on HERG, I_{Kr} or the QT interval may paralyze the drug discovery and development process, and also lead to the reduction in the number of novel drugs urgently needed for the treatment of diseases

for which there are currently few available options. Ultimately, a thorough assessment of the risk a new drug carries for adverse pro-arrhythmic events against its overall benefit will weigh heavily in its approvability, especially if it offers clear advantages over marketed products, or is used for the treatment for life-threatening conditions.

There is no doubt that pre-clinical assessment of compounds has improved our ability to detect non-cardiovascular drugs that have the propensity to prolong the QT interval. The next challenge remains in developing appropriate models that will unfailingly determine the pro-arrhythmic potential and clinical outcome of such drugs.

REFERENCES

[1] D. M. Roden and J. R. Balser, *Cardiovasc. Res.*, 1999, **44**, 242.
[2] C. Antzelevitch and W. Shimizu, *Curr. Opin. Cardiol.*, 2002, **17**, 43.
[3] M. T. Keating and M. C. Sanguinetti, *Cell*, 2001, **104**, 569.
[4] M. E. Curran, I. Splawski, K. W. Timothy, G. M. Vincent, E. D. Green and M. T. Keating, *Cell*, 1995, **80**, 795.
[5] J. S. Mitcheson, J. Chen, M. Lin, C. Culberson and M. C. Sanguinetti, *Proc. Natl Acad. Sci. USA*, 2000, **97**, 12329.
[6] J. S. Mitcheson, J. Chen and M. C. Sanguinetti, *J. Gen. Physiol.*, 2000, **115**, 229.
[7] Y. Jiang, A. Lee, J. Chen, M. Cadena, B. T. Chait and R. MacKinon, *Nature*, 2002, **417**, 515.
[8] S. Ekins, W. J. Crumb, R. D. Sarazan, J. H. Wikel and S. A. Wrighton, *J. Pharmacol. Exp. Ther.*, 2002, **301**, 427.
[9] E. Cavalli, F. Poluzzi, F. DePonti and M. Recanatini, *J. Med. Chem.*, 2002, **45**, 3844.
[10] E. Rubenstein and J. Camm, *J. Antimicrob. Chemother.*, 2002, **49**, 593.
[11] W. J. Crumb, *J. Pharmacol. Exp. Ther.*, 2000, **292**, 262.
[12] D. Rampe, M. K. Murawsky, J. Grau and E. W. Lewis, *J. Pharmacol. Exp. Ther.*, 1998, **286**, 286.
[13] Z. Zhou, V. R. Vorperian, Q. Gong, S. Zhang and C. T. January, *J. Cardiovasc. Electrophysiol.*, 1999, **10**, 836.
[14] J. Kang, L. Wang, X. L. Chen, D. J. Triggle and D. Rampe, *Mol. Pharmacol.*, 2001, **59**, 122.
[15] S. Mohammad, Z. Zhou, Q. Gong and C. T. January, *Am. J. Physiol.*, 1997, **273**, H2534.
[16] M. DeCicco, *Crit. Care Med.*, 1999, **27**, 332.
[17] S. L. Winters, P. Scheitzer, J. Kupersmith and J. A. Gomes, *J. Am. Coll. Cardiol.*, 1985, **6**, 257.
[18] R. lazzara, *Ann. Intern. Med.*, 1989, **111**, 549.
[19] S. G. Priori, P. J. Schwartz, C. Napolitano, R. Bloise, E. Ronchetti, M. Grillo, A. Vicentini, C. Spazzolini, J. Nastoli, G. Botelli, R. Folli and D. Cappelleti, *N. Engl. J. Med.*, 2003, **348**, 1866.
[20] W. Zareba, A. J. Moss, P. J. Schwartz, M. Vincent, J. L. Robinson, S. G. Priori, J. Benhorin, E. H. Locati, J. A. Tobin, M. T. Keating, M. H. Lehmann and W. J. Hall, *N. Engl. J. Med.*, 1998, **339**, 960.
[21] R. R. Shaw, *Fundam. Clin. Pharmacol.*, 2003, **16**, 147.
[22] D. S. Ravin and J. W. Levenson, *Ann. Pharmacother.*, 1997, **31**, 867.
[23] P. Yang, H. Kanki, B. Drolet, T. Yang, J. Wei, P. C. Viswanathan, S. H. Honloser, W. Shimizu, P. J. Schartz, M. Stanton, K. T. Murray, K. Norris, A. L. George and D. M. Roden, *Circulation*, 2002, **105**, 1943.
[24] Committee for Proprietary Medicinal Product (CPMP). Points to Consider: The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products. The European Agency for the Evaluation of Medicinal Products, London, England. Human Medicines Evaluation Unit, 1997.
[25] Safety pharmacology studies for assessing the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals. ICH S7B. The European Agency for the Evaluation of Medicinal Products CPMP/ICH/423/02, February, 2003.

[26] T. G. Hammong, L. Carlsson, A. S. Davies, W. G. Lynch, I. MacKenzie, W. S. Redfern, A. T. Sullivan and A. J. Cam, *Cardiovasc. Res.*, 2001, **49**, 741.
[27] W. S. Redfern, L. Carlsson, A. S. Davis, W. G. Lynch, I. MacKenzie, S. Palethorpe, P. K. S. Siegl, I. Strang, A. T. Sullivan, R. Wallis, A. J. Camm and T. G. Hammond, *Cardiovasc. Res.*, 2003, **58**, 32.
[28] H. Masumiya, M. Saito, M. Ito, T. Matsuda, K. Noguchi, N. Iida-Tanaka, H. Tanaka and K. Shigenobu, *Biol. Pharm. Bull.*, 2004, **27**, 131.
[29] L. Kiss, P. B. Bennett, V. N. Uebele, K. S. Koblan, S. A. Kane, B. Neagle and K. Schroeder, *Assay Drug Develop. Tech.*, 2003, **1**, 127.
[30] R. L. Martin, J. S. McDermott, H. J. Salmen, J. Palmatier, B. F. Cox and G. A. Gintant, *J. Cardiovasc. Pharmacol.*, 2004, **43**, 369.
[31] P. Champeroux, E. Martel, C. Vannier, V. Blanc, J. Y. Leguennec, J. Fowler and S. Richard, *Therapie*, 2000, **55**, 101.
[32] B. Fermini and A. A. Fossa, *Nat. Rev. Drug Discov.*, 2003, **2**, 439.

Section 7
Trends and Perspectives

Editor: Annette M. Doherty
Pfizer Global Research & Development, Sandwich Laboratories
Sandwich,
United Kingdom

To Market, To Market—2003

Shridhar Hegde, Jeffery Carter,

Pfizer Global Research and Development, St. Louis, MO 63017, USA

Contents

1. INTRODUCTION

The number of new molecular entities and biologics entering the drug market in 2003 was lower than in 2002 (27 vs 33), with a clear trend toward increasing contribution of the biotechnology sector relative to previous years [1–5]. From the 18 NCEs and 9 NBEs introduced last year, there were eight first-in-class therapies, representing an increase compared with 2002 and largely attributed to the emergence of new biologics. The US was

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again the most active market with 18 new product launches, followed by Europe and Japan with 7 and 2, respectively. Of the major pharmaceutical companies, GlaxoSmithKline and Eli Lilly had the most productive year with a marketing or co-marketing role on three new launches each, followed by Pfizer, Merck, Novartis, Sankyo and Bristol–Myers Squibb, each launching two new products. Eli Lilly and Novartis were at the origin of three new substances each; however, a significant majority of the new entities originated from smaller drug companies, suggesting a boost in licensing activity by major drug firms.

As in the two previous years, the antiinfective domain continued to be the most prolific therapeutic area with the introduction five new entities. Four new AIDS drugs were launched in 2003, most notably Roche/Trimeris's HIV fusion inhibitor, Fuzeon® (enfuvirtide), a first-in-class HIV therapy. Enfuvirtide acts by preventing HIV from fusing with and entering CD4 T cells whereas the existing therapies target post-entry events in the viral life cycle. Other new HIV drugs included a reverse transcriptase inhibitor, Emtriva™ (emtricitabine), and two HIV protease inhibitors, Lexiva™ (fosamprenavir) and Reyataz™ (atazanavir). Daptomycin (Cubicin™) was introduced last year as a new fast-acting antibiotic for once-daily treatment of infections caused by Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA) and methicillin-succeptible *S. aureus* (MSSA). MRSA now accounts for nearly 60% of all staphylococcal infections in US hospitals.

The cardiovascular sector had four new drugs entering the market in 2003, equaling the productivity in 2002. Two new second-generation statins, often referred to as superstatins, were introduced last year adding to the list of five previously marketed drugs from this class of hypolipidemic agents. Crestor™ (rosuvastatin) entered its first market in Canada and Livalo® (pitavastatin) was launched in Japan. Two new antihypertensives, Calblock® (azelnidipine), a gradual-onset, long-acting calcium antagonist, and Inspra™ (eplerenone), a selective aldosterone blocker, were also introduced last year. Eplerenone is additionally indicated to improve survival among patients with clinical evidence of congestive heart failure after an acute myocardial infarction.

The anticancer field saw the introduction of three new drugs, including Velcade® (bortezomib), the first proteasome inhibitor for the treatment of multiple myeloma, and Erbitux® (cetuximab), the first monoclonal antibody targeting epidermal growth factor receptor (EGFR) for the treatment of irinotecan-refractory colorectal cancer. Bexxar® (tositumomab), a ^{131}I radiolabeled antibody, was launched for the treatment of rituximab-refractory non-Hodgkin's lymphoma, making it the second radioimmunotherapy agent on the market for this indication. In addition, two new antiemetics were marketed for the treatment of chemotherapy-induced nausea and vomiting: Emend® (aprepitant), a neurokinin NK-1 receptor antagonist, and Aloxi® (palonosetron hydrochloride), a selective 5-HT3-receptor antagonist.

In the field of allergic and respiratory diseases, two new entities reached the market last year. Rupafin® (rupatidine fumarate), a dual mechanism drug with non-sedating histamine H_1 antagonist activity and platelet-activating factor antagonist activity, was launched for the treatment of perennial and seasonal allergic rhinitis in patients aged 12 years or older. Xolair® (omalizumab), the first humanized anti-IgE antibody, was introduced as a new type of treatment for allergic asthma.

In the area of endocrine and metabolic diseases, three new drugs appeared on the market. Somavert® (pegvisomant), a PEGylated recombinant human growth hormone

antagonist, was launched last year as a novel treatment for acromegaly. Aldurazyme® (laronidase), a recombinant form of α-L-iduronidase, was launched as an enzyme replacement therapy for the treatment of mucopolysaccharidosis I. In addition, Zavesca™ (miglustat), a glucosylceramide synthase inhibitor, was introduced as the first orally active treatment for type 1 Gaucher's disease.

Following the success of Viagra® (sildenafil) in the treatment of male erectile dysfunction (ED), two new phosphodiesterase type 5 (PDE5) inhibitors were launched in 2003: Cialis™ (tadalafil), a long-acting drug with high selectivity for PDE5, and Levitra™ (vardenafil), a potent PDE5 inhibitor with slightly faster on-set of action than Viagra®.

The CNS area was represented by the launch of Strattera™ (atomoxetine) for the once- or twice-daily treatment of attention deficit/hyperactivity disorder (ADHD). Atomoxetine is postulated to be a selective inhibitor of norepinephrine reuptake and it is the first approved treatment for this order that is not a stimulant.

Two new biologic immunosuppressive agents, Amevive® (alefacept), a recombinant fusion protein, and Raptiva™ (efalizumab), a humanized recombinant monoclonal antibody, were launched in 2003 as psoriasis therapies, representing the first advance in treatment for the condition in many years. Myfortic®, an enteric-coated tablet containing mycophenolic acid, was launched as an oral immunosuppressant for use in the prevention of kidney rejection during transplantation.

A significant NBE introduced last year was Humira™ (adalimumab), a novel disease-modifying antirheumatic drug (DMARD) developed by Abbott. Adalimumab is the first fully humanized monoclonal antibody that binds to human TNF-α and it is the third biologic DMARD on the market with this mechanism of action. The other notable NBE launched in 2003 was FluMist™ (influenza virus live), the first influenza vaccine delivered as a nasal spray, marking a significant innovation in the vaccination field.

Two other biologics were launched in 2003 but they are not considered new entities. Aralast™ brand of the alpha1-proteinase inhibitor (A1PI) was marketed for the augmentation therapy of hereditary emphysema caused by A1PI deficiency, making it the second brand on the market in addition to Prolastin® by Bayer. Advate, a recombinant antihemophilic factor (rAHF), was introduced for the treatment of bleeding episodes in patients with hemophilia A. While structurally similar to the previously marketed rAHF molecule Recombinate, Advate is produced without the addition of plasma proteins or albumin in the cell culture process, purification or final formulation, thus eliminating the risk of infection by pathogens contained in these additives.

2. ADALIMUMAB (RHEUMATOID ARTHRITIS) [6–10]

Country of origin	US
Originator	Cambridge antibody technology
First introduction	US
Introduced by	Abbott

(Continued)

Trade name	Humira
CAS registry no.	331731-18-1
Class	Recombinant humanized antibody
Type	IgG1 monoclonal anti-TNF
Molecular weight	148 kDa
Expression system	CHO cell line
Manufacturer	Abbott

Adalimumab is the first fully human neutralizing IgG1 monoclonal antibody specific for TNF-alpha and is the third TNF sequestrant marketed. It was launched in US, UK and Germany for the treatment of rheumatoid arthritis. It prevents TNF binding to p55 and p75 cell surface TNF receptors thereby decreasing leukocyte migration and acute phase reactants such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and levels of serum IL-6, MMP-1 and MMP-3. Adalimumab was developed starting with a phage-display derived murine antibody, followed by replacement of both heavy and light chains with human forms and further optimization to yield the final humanized form. In receptor binding studies, adalimumab exhibits an IC_{50} between 7.8×10^{-11} and 15.6×10^{-11} M with a K_d of 1×10^{-10} M. It also binds to pro-TNFalpha on cell membranes mediating complement-dependent toxicity and to the Fc receptor mediating antibody-dependent cytotoxicity. In a human-TNF transgenic polyarthritis mouse model, adalimumab was efficacious based upon both clinical and histological readouts. Patients responding inadequately to methotrexate were co-administered methotrexate and adalimumab, which resulted in improved ACR scores in a 52-week study (ACR20: 59%; ACR50: 42%; ACR70; 23%). Radiographic analysis at six months showed decreased progression of structural joint damage (96% $>$ placebo) and that joint-space narrowing stabilizes after six months. Adalimumab has an ED_{50} of 0.3 to 0.5 mg/kg and is dosed subcutaneously once every two weeks (0.8 mL containing 40 mg). The Vd_{ss} ranged from 0.063 to 0.76 L/kg consistent with being highly localized within the vasculature. It is slowly cleared with clearance values ranging from 0.18 to 0.27 mL/min and with a terminal half-life of about 12 days. As is the case with other TNF-alpha sequestrants, injection-site irritation is the most common side effect. The risk of developing opportunistic infection, especially tuberculosis, has been noted with TNF sequestrant biologics, which has led to screening of patients to identify those at risk.

3. ALEFACEPT (PLAQUE PSORIASIS) [11–15]

Country of origin	US
Originator	Biogen
First introduction	US
Introduced by	Biogen

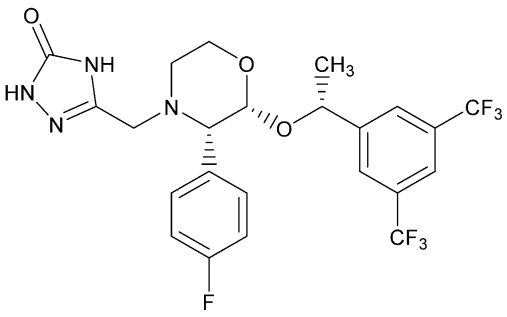
(Continued)

Trade name	Amevive
CAS registry no.	222535-22-0
Class	Recombinant protein
Type	Human LGA3-IgG1 fusion
Molecular weight	91.4 kDa
Expression system	CHO cells
Manufacturer	Biogen

Alefacept is a recombinant human LFA3-Ig-G1 fusion protein that blocks the T-cell CD2 receptor preventing T-cell proliferation, a key mechanism in psoriasis. It was launched in the US for the treatment of chronic plaque psoriasis. It is a dimeric fusion protein consisting of the LFA-3 (leukocyte function antigen-3) and Fc (hinge, CH2 and CH3) portion of human IgG1 and has been produced in Chinese hamster ovary (CHO) and NS-0 myeloma cell lines. In transgenic mice expressing human CD2, alefacept demonstrated efficacy in inhibiting antigen and mitogen-induced T-cell response. In the baboon cardiac-graft survival model alefacept, dosed 3 mpk intravenously for 12 days and treatment initiated two days before graft surgery, increased the mean survival of the transplanted hearts from 10.6 days (placebo) to 18.0 days (treatment group). In all clinical trials, improvement in PASI scores were noted and a long-lasting effect was noted. Thus this agent does not require chronic, maintenance dosing in all patients. Clinical studies for repeat courses (second twelve weeks) showed additional improvement. It has a half-life of 12 days, is dosed once weekly by injection either subcutaneously or intramuscularly and is approved for 12-week regimen. Side effects limit the use of current treatments such as phototherapy, cyclosporin, and methotrexate. Thus far, alefacept has been well tolerated.

4. APREPITANT (ANTIEMETIC) [16–19]

Country of origin	US
Originator	Merck
First introduction	US
Introduced by	Merck
Trade name	Emend
CAS registry no.	170729-80-3
Molecular weight	534.44

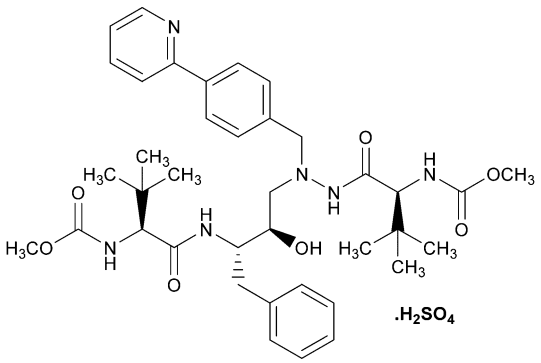


Aprepitant, a substance P (neurokinin-1 [NK-1]) receptor antagonist used for the treatment of chemotherapy-induced nausea and vomiting, was launched in the US and

was later approved in the European Union. It is a non-peptide analog having a trisubstituted morpholine with three chiral centers. Two syntheses have been described. In six steps *p*-fluorophenylacetic acid is converted to 4-benzyl-3-*p*-fluorophenyl-2-oxomorpholine with a resolution step setting the S-stereochemistry. This intermediate is converted in six steps to aprepitant, with two of the steps utilizing a chiral induction strategy to set the new centers based upon the chiral 2-oxomorpholine intermediate. SAR efforts leading to aprepitant included engineering in potency for NK-1, decreasing affinity for L-type calcium ion channels, most importantly by decreasing the basicity of the core heterocycle. *In vitro*, it binds with very high affinity (90 pM) to the hNK1 in transfected CHO cells. It is described as an inverse agonist of hNK-1 receptor, with slow dissociation rate under some conditions. In ferrets dosed orally or intravenously prior to emetogen challenge (cisplatin, apomorphine or morphine), retching and vomiting was reduced. Its antiemetic effect is enhanced with the dosing of dexamethasone and it is effective against both the acute and delayed phase of cisplatin-induced emesis. Cisplatin-induced emesis clinical studies showed that aprepitant (125 mg p.o.) in combination with ondansetron (32 mg i.v.) and dexamethasone (20 mg p.o.) therapeutically followed by repeat dosing (days 2–5) of aprepitant (80 mg) dexamethasone (20 mg) provided acute (8 h) and delayed phase (days 2–7) no vomiting rates of 83 and 70%, respectively. L-758298, a prodrug of aprepitant, was not as effective as ondansetron (32 mg i.v.) in reducing acute phase vomiting, but was superior in reducing vomiting in the delayed phase. The terminal half-life range of aprepitant is 9–13 h and the bioavailability is about 65%. It is highly protein bound (95%) and has a Vdss of 70 L. It is a moderate CYP3A4 inhibitor, thus several drugs cleared by CYP3A4 should not be used concurrently. It is also an inducer of CYP2C9 thus potentially modulating the PK of drugs cleared by CYP2C9. Most side effects were mild to moderate, with fatigue, asthenia, diarrhea, and hiccups.

5. ATAZANAVIR (ANTIVIRAL) [20–24]

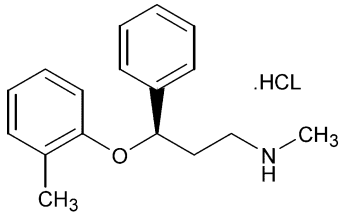
Country of origin	US
Originator	Novartis
First introduction	US
Introduced by	BMS
Trade name	Reyataz
CAS registry no.	198904-31-3
Molecular weight	705 (parent)



Atazanavir is an inhibitor of human immunodeficiency virus type 1 (HIV-1) protease, an enzyme that is essential for the processing of Gag and Gag-Pol polyproteins into structural and enzymatic proteins required for viral replication. It has a similar pharmacophore motif to the other six widely marketed HIV protease inhibitors, most of which are based upon a hydroxyethylamine template. Uniquely, it possesses an aza-peptide motif but maintains many similar pharmacophore elements including lipophilic moieties that presumably bind to S_2, S_1, S'_1, and S'_2 positions. Atazanavir is pseudo-symmetric about the central template, incorporating D-*tert*-Leucine at both termini. This compound is synthesized in about seven steps, with a key coupling of the chiral epoxide (derived from phenylalanine and imparting one chiral center) and *N*-*tert*-boc-*N*′-(4-[2-pyridyl]benzyl)hydrazine. Removal of both *tert*-Boc groups and double acylation with methoxycarbonyl-*tert*-Leucine provides the product. Another synthesis of atazanavir entails ten steps and utilizes α-(*tert*-boc-amino)phenylpropanal as a chiral intermediate. It is a potent inhibitor of indinavir-resistant and saquinavir-resistant strains of HIV-1 ($IC_{50} = 0.03–0.1$ and $0.04–0.1$ μM, respectively). In 300 patients who had failed previous treatment, atazanavir (400 mg once daily) was compared to lopinavir (400 mg twice daily) and ritonavir (100 mg); both arms additionally receiving two non-reverse transcriptase inhibitors. After 24 weeks, HIV RNA levels of <400 copies/mL were noted in 61% of patients receiving atazanavir and 81% of those taking lopinavir/ritonavir. After 96 weeks of therapy with atazanavir, HIV RNA copy levels were found to be <400 and <50 in 80 and 58% of patients, respectively. A study of the cross-resistance profile relative to other protease inhibitors using a panel of 551 clinical isolates (without prior atazanavir exposure but with cross-resistance to one or two other protease inhibitors; the majority had resistance to nelfinavir) showed that greater than 80% retained susceptibility to atazanavir. All of the resistant isolates from patients taking atazanavir had an I50 L substitution. The recommended dosage of atazanavir is 400 mg once daily. It has a mean half-life range of 7.9–6.5 h with about 60% bioavailability and moderate plasma protein binding (86% albumin and 89% alpha-1-acid glycoprotein (AAG)). Atazanavir was well tolerated in clinical studies and it displayed minimal lipid modulation when tested in combination with two non-reverse transcriptase inhibitors. Atazanavir had no effect on total cholesterol, low-density lipoprotein, and triglyceride levels when compared with other protease inhibitors that caused sustained elevations in these lipid levels.

6. ATOMOXETINE (ATTENTION DEFICIT HYPERACTIVITY DISORDER) [25–28]

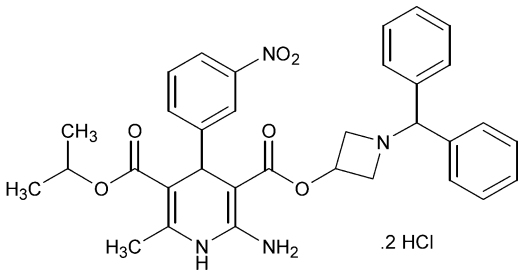
Country of origin	US
Originator	Eli Lilly & Co
First introduction	US
Introduced by	Eli Lilly & Co
Trade name	Strattera
CAS registry no.	082248-59-7
Molecular weight	291.82



Atomoxetine is the first non-stimulant marketed for the treatment of attention deficit hyperactivity disorder (ADHD). It is the R-stereoisomer of the racemate tomoxetine and is a selective and potent norepinephrine uptake inhibitor ($K_i = 0.7-1.9$ nM) that is devoid of binding to monoamine receptor. It also has little effect on dopamine and serotonin reuptake or acetylcholine, H1 histamine, alpha1 or alpha1-adrenergic or dopamine receptors. It is prepared from racemic 1-phenylbut-3-en-1-ol via a selective enzymatic acylation leaving the desired S-stereoisomer as the alcohol. This alcohol is converted via a Mitsunobu reaction with *ortho*-cresol to the corresponding ether with isomeric R-configuration. Ozonolysis and reduction steps provided the terminal alcohol that is mesylated and displaced with methylamine. Its selectivity for norepinephrine relative to dopamine inhibition was demonstrated *in vivo* preclinically. In a two-lever (two condition) discriminative stimulus effect study in squirrel monkeys, tomoxetine and other norepinephrine uptake inhibitors substituted for cocaine under low-dose training conditions, whereas dopamine uptake inhibitors substituted for cocaine in both low and high-dose conditions. In clinical ADHD studies in adolescents, it was significantly different from placebo in 1.2 and 1.8 mpk/day dosing. In the clinical study in adults using the CAARS scale a 95 mg/day dose provided greater than 30% improvement in total scores. Atomoxetine is about 63% orally bioavailable, is highly protein bound (98%, primarily to albumin) and has a half-life of about 5.2 h. It is metabolized by CYP2D6 resulting in differential clearance for poor metabolizers (half-life of 19 h with a 10 times higher AUC) relative to extensive metabolizers. The total daily dose for children, adolescents and adults is a maximum of 100 mg/day. Common side effects in children and adults include nausea, decreased appetite, and dizziness. Adults may also have insomnia.

7. AZELNIDIPINE (ANTIHYPERTENSIVE) [29–31]

Country of origin	Japan
Originator	Sankyo
First introduction	Japan
Introduced by	Sankyo
Trade name	Calblock
CAS registry no.	116574-11-9
Molecular weight	582.66

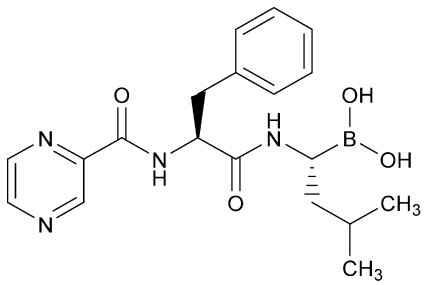


Azelnidipine, a member of the 1,4-dihydropyridine class of L-type calcium channel blockers with a slow onset profile, was marketed in Japan for the treatment of hypertension. Azelnidipine is synthesized via the condensation of *iso*-propyl 2-(3-nitrobenzylidene)acetoacetate with (1-diphenylmethylazetidin-3-yl)-3,3-diamino acrylate. The diamino acrylate intermediate is prepared from the cyanoacetic ester

by sequential treatment with HCl and ammonia. In receptor binding studies using porcine heart membrane fractions, azelnidipine exhibits an IC_{50} of 3.1 nM and an apparent K_i of 2.1 nM. Its tight binding and slow onset are correlated with its high lipophilicity. A slow onset is also noted *in vitro* in a rat aortic strip contraction assay, and this effect continues after removal of the drug from the bath solution. In the conscious spontaneously hypertensive rat (SHR) model of hypertension, it was more potent that nicardipine and also had a more gradual onset and long-lasting effect. This effect was noted both when dosed orally or intravenously. When SHR dosed at 1 or 3 mpk/day for 15 weeks, a sustained reduction in systolic blood pressure was noted (19 and 43 mmHg reduction, respectively). Cardiac output was increased and total peripheral resistance was decreased in each group. Clinical studies of patients with mild-to-moderate hypertension have shown that long-term treatment with azelnidipine provided a sustained decrease in blood pressure (mean reduction systolic /diastolic: 27.8/16.6 mmHg). It similarly controlled blood pressure, as did amlodipine at 24 h. It possesses a gradual onset of activity with plasma levels increasing before the antihypotensive effect is attained. After plasma levels drop, the pharmacodynamic effect is sustained. In clinical studies, azelnidipine did not show reflex tachycardia, a common side effect of this class. Most common side effects were facial flushing and headache, similar to other dihydropyridines. Azelnidipine is dosed orally once daily (8–16 mg), is rapidly absorbed in a dose-dependent fashion, and has a mean terminal half-life of 19.2 h (8 mg dosage p.o. for seven days). Uniquely, it possesses a 2-amino function associated with a longer half-life than related agents wherein this moiety is a methyl. The very highly lipophilic 3-carboxylic ester side-chain is purported to contribute to the gradual onset of activity and prolonged pharmacodynamic effect, unlike other drugs in this class. This compound exhibits a much less pronounced first-pass metabolic effect than nicardipine.

8. BORTEZOMIB (ANTICANCER) [32–37]

Country of origin	US
Originator	Millenium (LeukoSite, Proscript)
First introduction	US
Introduced by	Millenium
Trade name	Velcade
CAS registry no.	179324-69-7
Molecular weight	384



Bortezomib, a potent ubiquitin proteasome (26S) inhibitor ($K_i = 0.6$ nM), was launched in the US as a treatment for multiple myeloma. This proteasome is required for

the proteolytic degradation of the majority of cellular proteins and is present in all cells. It is required for the control of inflammatory processes, cell cycle regulation and gene expression and is a novel target in cancer treatment. Bortezomib is a *N*-acyl-pseudo-dipeptidyl boronic acid and formulated as a mannitol ester. Aldehyde containing peptides are also proteasome inhibitors, but lack chiral stability (racemization) and selectivity against other proteases including cysteine proteases. Replacement of the aldehyde moiety by a boronic acid avoids these shortcomings and provides some measure of selective proteasome inhibition relative to many other serine proteases. It is prepared by coupling the pinanediol ester of leucine boronic acid with *N*-*tert*-Boc-phenylalanine utilizing *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as the coupling agent. The *tert*-Boc protecting group is then cleaved from the dipeptide and pyrazinecarboxylic acid is coupled to form the terminal amide. Hydrolysis of the boronate ester is accomplished via a two-phase transesterification procedure using isobutyl boronic acid and aqueous extraction. In a study of patients who had received at least two prior therapies and demonstrated disease progression on their most recent therapy, about twenty eight percent showed a response to bortezomib. The response lasted a median time of one year. Another trial in 54 patients with relapsed multiple myeloma showed similar responses. It is dosed intravenously at an exposure of 1.3 mg/m^2/dose twice weekly for two weeks followed by a 10-day drug-holiday. At therapeutic doses, the plasma drug levels were reported to drop to near detection limits within minutes of intravenous dosing. Based upon an *ex vivo* proteasome activity assay using blood cells, the pharmacodynamic half-life ranged from 9 to 15 h in patients with advanced malignancies.

9. CETUXIMAB (ANTICANCER) [38–40]

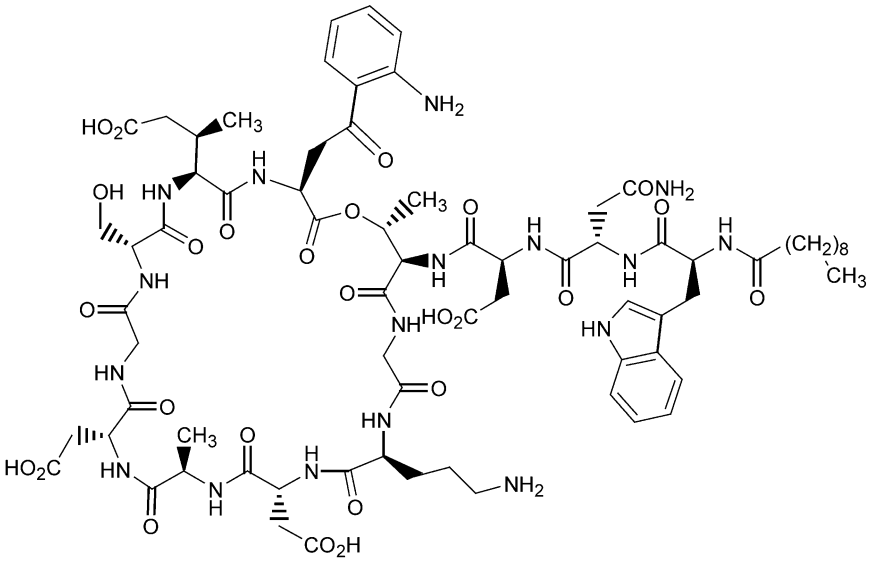
Country of origin	US
Originator	ImClone
First introduction	Switzerland
Introduced by	Merck/BMS
Trade name	Erbitux
CAS registry no.	205923-56-4
Class	Monoclonal antibody
Type	murine anti-EGFR
Molecular weight	152 kDa
Expression system	murine myeloma cell culture
Manufacturer	ImClone

Cetuximab, a human/mouse chimeric monoclonal antibody that blocks the EGFR, was launched last year for use in combination with irinotecan in the treatment of

patients with colorectal cancer who no longer respond to standard chemotherapy treatment with irinotecan. Cetuximab is obtained by chimerization of M225, a murine anti-EGFR antibody; specifically heavy and light chains of the murine antibody are cloned and adapted for expression with constant regions of the human kappa light chain and human gamma1 heavy chain. It is produced by mammalian (suspension) cells in serum-free medium, purified by protein A affinity chromatography, ion-exchange chromatography, and gel filtration. Cetuximab binds specifically to EGFR on both normal and tumor cells. Overexpression of the human EGFR is detected in many cancers, including those of the colon and rectum. The binding of cetuximab to EGFR prevents growth factors from binding to the receptor, thereby inhibiting cell growth and inducing apoptosis. The therapeutic regimen of cetuximab consists of an initial loading dose of 400 mg/m^2 administered as a 120-min IV infusion, followed by weekly maintenance dose of 250 mg/m^2 infused over 60 min. The steady-state plasma concentrations of cetuximab are reached by the third weekly infusion and the mean elimination half-life is 114 h. Cetuximab is eliminated by binding to EGFRs in various tissues, followed by internalization of the antibody-EGFR complex. Systemic clearance of the antibody is saturated at higher doses, and this appears to correlate with saturation of EGFR binding. In a multicenter clinical trial in more than 300 patients with advanced metastatic colorectal cancer, combination therapy with cetuximab and irinotecan produced response in more than half of the patients, shrinking tumors in 23% and stopping tumor growth in an additional 33% of the patients. The most common adverse reactions associated with cetuximab were acneform rash, asthenia/malaise, fever, nausea, abdominal pain, constipation and vomiting. Serious adverse events such as infusion reaction, fever, sepsis, kidney failure, dehydration and diarrhea were experienced by $<10\%$ of the patients.

10. DAPTOMYCIN (ANTIBIOTIC) [41–44]

Country of origin	US
Originator	Lilly
First introduction	US
Introduced by	Cubist
Trade name	Cubicin
CAS registry no.	103060-53-2
Class	Recombinant peptide
Type	Antimicrobial peptide
Molecular weight	1621
Expression system	Streptomyces roseporus
Manufacturer	Abbott



Daptomycin is the first entry of a new class of cyclic lipopeptide antibiotics that disrupts multiple aspects of bacterial membrane function including disruption of membrane potential and amino acid transport, inhibition of lipoteichoic acid synthesis and inhibition of peptidoglycan synthesis. It is indicated for the treatment of complicated skin and skin-structure infections (cSSSI) caused by a range of Gram-positive bacteria. This is distinct from previous classes of antibiotics that inhibit bacterial cell wall biosynthesis, bacterial DNA replication, and folate coenzyme biosynthesis. Due to this unique mechanism, cross-resistance has not been noted with any other class of antibiotics. It is produced by the fermentation of Streptomyces roseporus. The fatty acid side chain is a key determinant of acute toxicity, with the ten-carbon chain least acutely toxic to mice. Daptomycin has shown efficacy in a variety of animal models versus several Gram-positive infectious agents including methicillin-susceptible S aureus, vancomycin-resistant E faecalis, spyogenes and S pneumoniae. It retains *in vitro* activity against methicillin, vancomycin and linezolid-resistant strains including Staphylococcus aureus (MRSA and VRSA), which is the leading cause of hospital-acquired infections (nosocomial infections (Nis)). The MIC values against Gram-positive pathogens are relatively low, ranging from 0.06 to 2.0 μg/mL. In two clinical studies treating patients (ca. 1090 for both arms of both studies) with complicated skin and soft tissue infections (cSSTIs) in which gram-positive pathogens were suspected and parenteral antibiotics were required, daptomycin provided similar clinical success rates as compared to standard therapy with vancomycin or semisynthetic penicillins such as cloxacillin, oxacillin, or flucloxacillin. The daptomycin treated group showed more rapid improvement as noted by scoring on day three or four and also had a shorter duration of treatment versus the standard therapy group (7 vs 8 days). It is dosed once daily (4 mg/kg/day) by intravenous infusion and has a half-life of 8.1 h. It is primarily cleared renally and thus requires dosing adjustments for those with severe renal insufficiency (CLCR $<$30 mL/min). Clinical safety of daptomycin is similar to other antibiotics.

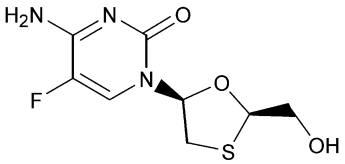
11. EFALIZUMAB (PSORIASIS) [45–48]

Country of origin	US
Originator	XOMA
First introduction	US
Introduced by	Genentech/XOMA
Trade name	Raptiva
CAS registry no.	214745-43-4
Class	Recombinant monoclonal antibody
Type	Humanized IgG1, anti-CD11
Molecular weight	~150 kDa
Expression system	CHO cells
Manufacturer	Genentech

Efalizumab, a humanized monoclonal antibody marketed for the treatment of psoriasis, is a full-length IgG1 antibody developed through a murine anti-human CD11a mAb. It is produced in a Chinese hamster ovary mammalian cell expression system in a nutrient medium containing the antibiotic gentamicin. Psoriasis is a disease mediated through inflammatory cells (primarily T-cells expressing CD4 or CD8 markers) and keratinocytes. CD11a is the alpha-chain LFA-1 (leukocyte function associate antigen; integrin family). It is expressed on the surface of T-lymphocytes and it binds to the intercellular cell adhesion molecules (ICAM-1, -2 and -3) on endothelial cells, monocytes, keratinocytes, fibroblasts, and activated lymphocytes. By blocking LFA-1 binding the ability of T cells to adhere, migrate and be activated is blunted. Studies in chimpanzee and murine animal models demonstrated that efalizumab down regulates the expression of LFA-1 on lymphocytes, prevents contact dermatitis to 2,4-dinitrofluorobenzene, increases skin and heart transplant survival. In the collagen-induced arthritis model it delays onset and decreases the severity of the arthritic condition. In a study of 498 patients, efalizumab treatment of 1 or 2 mg/kg/wk for 12 weeks provided, respectively, a 39 or 27% Psoriasis Area and Severity Score (PASI) improvement of $\geq$75%. By comparison, placebo provided a 2% improvement. It was further demonstrated that a second 12-week course could provide additional improvement. As dose increases, clearance decreases (dose 0.1 mg/kg: 322 ml/day/kg; dose 10 mg/kg: 6.6 mL/day/kg) with data suggesting saturation of clearance above 10 μg/mL. A pharmacokinetic model positively correlates number of circulating cells expressing CD11a with relative clearance. This blockade decreases the CD11a expressed on circulating lymphocytes to about 25% of their baseline levels in patients with plaque psoriasis. Efalizumab is formulated as a once-weekly subcutaneous injectable, dosed at 0.7 to 1 mg/kg/week. Although mild adverse events were noted such as headache, pain chills, nausea, and fever, these events generally decreased after one or two doses. The overall rate of infections was only 3% higher than the placebo-arm and no depletion of T-cells was noted.

12. EMTRICITABINE (ANTIVIRAL) [49–52]

Country of origin	US
Originator	Emory University
First introduction	US
Introduced by	Gilead (formerly Triangle)
Trade name	Emtriva
CAS registry no.	143491-57-0
Molecular weight	247.25



Emtricitabine is a synthetic nucleoside inhibitor of HIV-1 reverse transcriptase. Its mechanism of action entails the phosphorylation of the oxathiolane carbinol function by cellular enzymes to form the corresponding 5′-triphosphate, which competes with the endogenous 2′-deoxycytidine 5′-triphosphate substrate. The *in vitro* activity of emtricitabine ranges from IC_{50} of 0.00013 to 0.64 μM against lymphoblastoid cell lines, MAGI-CCR5 cell lines, and peripheral blood mononuclear cells. Emtricitabine is prepared in about 16 steps from L-gulose, from which the O–C–S carbon stereochemistry is derived in the formation of substituted key 4-*O*-Acetyl-1,3-oxathiolane intermediate. This intermediate is coupled with *N*-benzoyl-*O*-trimethylsilyl-5-fluoro-cytosine in the presence of trimethylsilyl triflate. The resulting anomeric mixture is separated by silica chromatography and subjected to two deprotection steps. Emtricitabine resistant isolates (M184V/I) were cross-resistant to lamivudine (des-fluoro version of emtricitabine) and zalcitabine but remained sensitive to abacavir, didanosine, stavudine, tenofovir, zidovudine, and non-nucleoside reverse transcriptase inhibitors (delavirdine, efavirenz, and nevirapine). In a 48-week clinical trial using emtricitabine with didanosine and efavirin versus stavudine, didanosine and efavirenz produced 81 versus 68% responder rate respectively. In one study, 37.5% of treatment naïve patients that were not achieving successful viral levels were found to exhibit a reduced potency toward emtricitabine. This resistance was due to M184V/I mutation in the HIV reverse transcriptase gene. Recommended dosing of emtricitabine is 200 mg (capsule) once per day. It is 93% bio-available and has a plasma half-life about 10 h, with 86% renal clearance. Side effects associated with emtricitabine treatment were generally mild to moderate and included headache, diarrhea, nausea, and rash. A generally mild and asymptomatic hyperpigmentation of the palms and/or soles was also observed.

13. ENFUVIRTIDE (ANTIVIRAL) [53–57]

Country of origin	US
Originator	Duke University

(Continued)

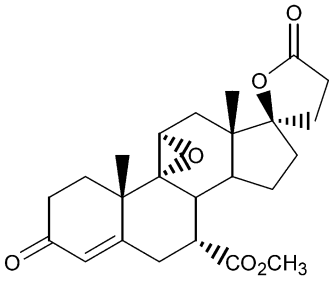
First introduction	US
Introduced by	Roche/Trimeris
Trade name	Fuzeon
CAS registry no.	159519-65-0
Molecular weight	4492

N-acetyl-L-tyrosyl-L-threonyl-L-seryl-L-leucyl-L-isoleucyl-L-histidyl-L-seryl-L-leucyl-L-isoleucyl-L-glutamyl-L-glutamyl-L-seryl-L-glutaminyl-L-asparaginyl-L-glutaminyl-L-glutaminyl-L-glutamy-L-lysyl-L-asparaginyl-L-glutamyl-L-glutaminyl-L-glutamyl-L-leucyl-L-leucyl-L-glutamyl-L-leucyl-L-aspartyl-L-lysyl-L-tryptophyl-L-alanyl-L-seryl-L-leucyl-L-tryptophyl-L-asparaginyl-L-tryptophyl-L-phenylalaninamide

Enfuvirtide is the first of a new class of HIV therapeutics that interferes with the entry of HIV-1 by inhibiting fusion of viral and cellular membranes. It binds to the first heptad-repeat (HR1) in ghe gp41 subunit of the viral envelope glycoprotein and prevents the conformational changes required for the fusion with cell membranes, thus representing a new mechanism. It is a potent inhibitor of this binding with an IC_{50} in laboratory and primary isolates (HIV-1 clades A–G) ranging from 4 to 280 nM (18–1260 ng/mL), but has virtually no activity against HIV-2. Due to its unique mechanism, it does not show cross-resistance to NRTI, NNRTI and PIs. It is prepared by chemical synthesis, using a combination of solution and solid phase steps. The solid phase steps utilize fmoc protection and the acid sensitive 2-chlorotrityl chloride resin to produce peptides of lengths ranging from nine to sixteen amino acids. These amino acids are subsequently coupled to form the full-length peptide followed by side-chain deprotection and column chromatography. In the HuPBMC-SCID mouse model of HIV-1 infection enfuvirtide showed a dose-dependent decrease in viral load. At doses of 200 mg/kg/day, it decreased the RNA levels to 8.2 copies/1 million cells compared to 17 million copies/1 million cells in the saline-treated group. In a clinical study involving 78 heavily pretreated patients (plasma HIV RNA >5000 copies/mL), enfuvirtide dosing of 12.5–200 mg/day by b.i.d. subcutaneous injection resulted in a dose-dependent decrease in viral load (plasma HIV RNA 0.3–1.6 log 10 copies/mL). In a combination drug study where all patients received an optimized retroviral regimen with or without enfuvirtide, it was found that, at week 24, the enfuvirtide group had a 4.5 fold-relative drop in HIV RNA copies/mL relative to the standard treatment group. Subjects received 3–5 antiretroviral agents with or without enfuvirtide. In clinical trials, HIV-1 isolates from 185 patients using enfuvirtide in a cocktail with other antiretroviral agents showed 4 to 422-fold decrease in sensitivity to this drug. These more resistant types had changes in the gp41 amino acids 36–45. Enfuvirtide is dosed twice per day (90 mg) by subcutaneous injection and has an elimination half-life of about 3.8 h. It is highly plasma protein bound (92%) and has a Vdss is 5.5 L. Local injection site irritation (98% had at least one reaction), diarrhea (26.8%), nausea (20.1%), and fatigue (16.1%) were the most common adverse events.

14. EPLERENONE (ANTIHYPERTENSIVE) [58–60]

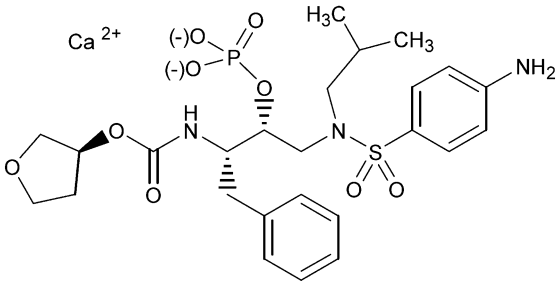
Country of origin	US
Originator	Ciba-Geigy (Novartis)
First introduction	US
Introduced by	Pfizer (Pharmacia)
Trade name	Inspra
CAS registry no.	107724-20-9
Molecular weight	414.50



Eplerenone derives its antihypertensive effect by blocking the binding of aldosterone at the mineralocorticoid receptor (MR). The drug, which was previously approved only for the oral treatment of hypertension, is now indicated to improve survival of stable patients with left ventricular systolic dysfunction (ejection fraction $<40\%$) and clinical evidence congestive heart failure (CHF) after an acute myocardial infarction. Aldosterone is a key hormone in the renin-angiotensin-aldosterone system (RAAS), which is of critical importance in the development and progression of hypertension, cardiac remodeling and other cardiovascular diseases. The purpose of RAAS is to control sodium, potassium, and fluid volume balance. Aldosterone binds to MRs in both epithelial (e.g. kidney) and nonepithelial (e.g. heart, blood vessels, and brain) tissues and increases blood pressure through induction of sodium reabsorption and possibly other mechanisms. The actions of aldosterone can be blocked by spironolactone (Aldactone®), a relatively nonselective MR antagonist that has been used in clinical practice for many years. Eplerenone, a structural analog of spironolactone, is a highly selective MR antagonist, with significantly lower affinity for other nuclear receptors. It can be prepared by several related ways, with the key step being the introduction of 11-α-hydroxy group on the steroid scaffold via microbiological conversion. The presence of the 11-α-hydroxy group permits the derivation of the epoxy functionality found in eplerenone. Following oral administration, eplerenone is well absorbed and reaches peak plasma concentrations in $\sim$2 h. The bioavailability of eplerenone is 98% and it is cleared predominantly by CYP3A4 metabolism, with an elimination half-life of 4–6 h. Steady state is reached within two days. Eplerenone therapy is typically initiated with 25 mg once daily oral dosing and, if tolerated by the patient, titrated to 50 mg once daily. In a clinical study, eplerenone significantly reduced deaths in congestive heart failure patients after a heart attack, above and beyond standard therapy, including ACE inhibitors and β-blockers. The trial in more than 6600 hospitalized patients demonstrated a 15% reduction in the risk of death for eplerenone compared with placebo, in addition to standard treatment. The most commonly reported adverse events associated with eplerenone are hyperkalemia and increased creatine.

15. FOSAMPRENAVIR (ANTIVIRAL) [61,62]

Country of origin	US
Originator	Vertex
First introduction	US
Introduced by	Vertex/GSK
Trade name	Lexiva
CAS registry no.	226700-81-8
Molecular weight	623.67



Fosamprenavir, a prodrug of the HIV protease inhibitor amprenavir, is indicated for the oral treatment of HIV infection in adults in combination with other antiretroviral agents. Although amprenavir has excellent antiviral potency and good tolerability, its water-solubility is poor (0.04 mg/ml). As a result, the formulation of the agent includes a high percentage of organic excipients to facilitate gastric dissolution, which limits the amount of active drug that can be formulated per capsule. Fosamprenavir is a highly soluble phosphate ester of amprenavir. It allows more convenient dosing and reduction in pill counts as compared to amprenavir. Fosamprenavir is readily prepared in two steps starting from a key intermediate used in the synthesis of amprenavir, by phosphorylating a hydroxyl group and subsequently reducing a *p*-nitrophenyl to a *p*-aminophenyl group. Fosamprenavir has little or no antiviral activity *in vitro*. After oral administration, it is rapidly and almost completely hydrolyzed by phosphatases in the gut epithelium to amprenavir prior to reaching systemic circulation. The time to reach peak plasma concentration of amprenavir is approximately 2.5 h and the plasma elimination half-life is approximately 7.7 h. Amprenavir is metabolized in the liver by CYP3A4 and $>90\%$ of the dose is excreted as metabolites in urine and feces. In most patients, fosamprenavir is administered at daily doses of 700–1400 mg in conjunction with ritonavir. Monotherapy with fosamprenavir is only recommended in antiretroviral therapy-naïve patients and the dosing regimen is 1400 mg twice daily. The most common adverse events experienced with fosamprenavir are diarrhea, nausea, vomiting, headache and rash.

16. INFLUENZA VIRUS (LIVE) (ANTIVIRAL VACCINE) [63–65]

Country of origin	US
Originator	MedImmune
First introduction	US
Introduced by	MedImmune/Wyeth
Trade name	FluMist

(Continued)

Class	Influenza virus
Type	AH1N1 + AH3N2 + B
Expression system	Embryonic poultry cells
Manufacturer	MedImmune

FluMist™, launched last year by MedImmune and Wyeth, is a live attenuated influenza virus (LAIV) vaccine and it is a cold-adapted trivalent formulation. It is the first nasally administered vaccine indicated for providing active immunity against select influenza A and B strains in healthy people aged 5–49 years. FluMist™ is not indicated for the treatment of influenza or prevention of variant strains. Local administration of the vaccine mist to the interior nare yields serum IgG antibody levels sufficient to provide protection against influenza infection. In addition, intranasal delivery promotes the production of mucosal IgA antibodies and local T-cell formation. The cold-adapted formulation allows it to grow in the lower temperatures of nasal passages and not in the lungs where influenza develops. After initial vaccination with FluMist™, protective antibody levels are generally achieved in approximately 2 weeks and can persist for a period of six months or longer. In clinical trials, a single dose of the vaccine in children over the age of 15 months demonstrated 89% efficacy against developing culture-confirmed influenza illness. In healthy adults aged 18–64 years, vaccination significantly reduced severe febrile illness by 18.8% and upper respiratory illnesses by 23.6%. FluMist™ is well tolerated in children and adults. Since the vaccine contains live attenuated virus the adverse events generally mimic mild flu-like symptoms such as cough, rhinorhea, sore throat and headache. FluMist™ is contraindicated in persons with biological or drug-induced immunodeficiency. Each year, influenza vaccines are standardized and consist of two types of influenza A strains and one type of influenza B strain, as determined by worldwide surveillance and mutation patterns. For 2003–2004, the intranasal LAIV vaccine contains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2) and B/Hong Kong/330/221 antigens. The antigens are derived from wild type and master donor virus strains replicated in embryonic poultry cells.

17. LARONIDASE (MUCOPOLYSACCARIDOSIS I) [66,67]

Country of origin	US
Originator	BioMarin
First introduction	US
Introduced by	BioMarin/Genzyme
Trade name	Aldurazyme
CAS registry no.	210589-09-6
Class	Recombinant protein
Type	Glycosidase

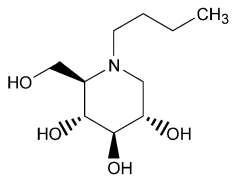
(Continued)

Molecular weight	83 kDa
Expression system	CHO Cells
Manufacturer	BioMarin

Mucopolysaccharidosis I (MPS I) is a rare genetic lysosomal storage disease caused by the deficiency of a-L-iduronidase, an enzyme required for the catabolism of dermatan sulfate and heparin sulfate. The deficiency blocks the degradation of these mucopolysaccharides, which accumulate in a variety of tissues including liver, spleen, heart and connective tissues. The clinical manifestations of MPS I can include progressive developmental delay, airways obstruction, hepatosplenomegaly, severe joint restriction and cardiovascular disease. There are three subtypes of MPS I depending on its clinical severity: Hurler's syndrome (severe), Hurler-Scheie syndrome (moderate), and Scheie syndrome (mild). Among the existing therapies, bone marrow transplantation has been the only effective option for Hurler's syndrome. Laronidase was launched last year as an enzyme replacement therapy for the treatment of patients with Hurler and Hurler-Scheie syndromes and patients with the Scheie syndrome who have moderate to severe symptoms. It is a recombinant form of the human a-L-iduronidase produced by overexpression in a Chinese hamster ovary cell line. The recommended dosage regimen of laronidase is 0.58 mg/kg of body weight administered once weekly as an intravenous infusion. The efficacy of laronidase was demonstrated in a 26-week, double-blind, placebo-controlled clinical trial by measuring improvement in pulmonary function and endurance. The laronidase-treated patients showed a mean increase of 4.0% in predicted forced vital capacity (FVC) and a mean increase of 38.0 m in the distance walked in 6 min as compared with placebo-treated patients. Reductions in liver size and in urinary glycosaminoglycan excretion were also observed. The most common adverse events associated with laronidase were upper respiratory tract infection, rash and injection site reaction.

18. MIGLUSTAT (GAUCHER'S DISEASE) [68–70]

Country of origin	US
Originator	G.D. Searle (Pfizer)
First introduction	UK
Introduced by	Actelion
Trade name	Zavesca
CAS registry no.	72599-27-0
Molecular weight	219.28

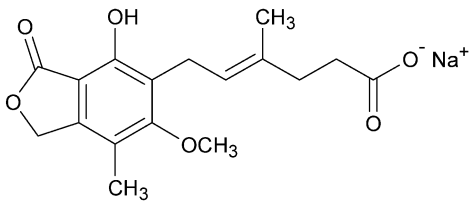


Miglustat is an *N*-alkylated iminosugar, launched as an oral treatment for mild to moderate type 1 Gaucher's disease in adult patients for whom enzyme replacement therapy is not a therapeutic option. It is readily synthesized from D-glucose in three

steps by first converting to *N*-butylglucamine via reductive amination with butylamine, followed by a microbial oxidation to an aminofuranose intermediate and subsequent reductive cyclization. Type 1 Gaucher's disease is a metabolic disorder caused by the lysosomal accumulation of certain glycosphingolipids (GSLs) as a result of deficiency in their degradation. Enlargement of the liver and spleen, low blood platelet and bone lesions are among the key symptoms of this disease. Miglustat acts by inhibiting glucosylceramide synthase, a glucosyl transferase enzyme in the biosynthesis of most GSLs, which results in the lowering of GSLs to a level that can be effectively cleared. Up to 50% reduction in liver and splenocyte GSL levels are achieved in mice by long-term administration of Miglustat (600–1800 mg/kg/day for 118 days). Miglustat, dosed at 50 and 100 mg in Gaucher patients, exhibits dose proportionate pharmacokinetics ($t_{max} = 2.5$ h, $t_{1/2} = 6$ to 7 h) and >90% oral bioavailability. Steady-state plasma levels are reached after 4–6 weeks of treatment. Miglustat is not significantly metabolized in humans and the major route of excretion is renal. In clinical trials, efficacy was demonstrated by significant reductions in liver and spleen volumes (12 and 19%, respectively) at 12 months and increase in hemoglobin and platelet count (0.91 g/dL and 13.6×10^9/l, respectively) at 24 months. Miglustat is generally well tolerated by patients and the most common side effects are diarrhea and weight loss.

19. MYCOPHENOLATE SODIUM (IMMUNOSUPPRESSANT) [71,72]

Country of origin	Switzerland
Originator	Novartis
First introduction	Switzerland
Introduced by	Novartis
Trade name	Myfortic
CAS registry no.	37415-62-6
Molecular weight	342.32



Mycophenolate sodium, an immunosuppressive agent, was launched last year in Switzerland as an oral treatment in combination with Neoral® and corticosteroids for the prevention of acute transplant rejection in adult patients receiving allogeneic renal transplantation. In contrast to the previously marketed product mycophenolate mofetil (MMF, CellCept®), which is a prodrug and must be converted to mycophenolic acid (MPA) *in vivo*, Myfortic® contains MPA itself as the active ingredient. Myfortic® was designed to enhance the therapeutic efficacy of MPA through increased tolerability relative to systemic exposure. Unlike MMF, which is absorbed in the stomach, the enteric-coated formulation of MPA sodium is mainly absorbed in the small intestine, thus protecting the upper GI tract from the side effects of MPA. MPA is an inhibitor of inosine monophosphate dehydrogenase (IMDPH), a vital enzyme in the de novo pathway of purine biosynthesis. Proliferating lymphocytes rely principally on this pathway for purine production,

thus rendering them succeptible to depletion of purine bases by MPA. Inhibition of IMPDH by MPA results in the inhibition of both T- and B-lymphocyte proliferation upon antigen challenge and facilitates the prevention of acute graft rejection. Following oral administration of MPA sodium, the t_{max} of MPA is 1.5–2 h, with a mean absolute bioavailability of 71% and a mean half-life of 11.7 h. MPA is primarily metabolized in the liver by glucuronidation and excreted mainly in the urine as the metabolite. The recommended dosage regimen of MPA sodium is 720 mg twice daily, which provides equimolar amounts of MPA compared with MMF 1000 mg twice daily. In two major clinical trials in 748 patients, Myfortic® was demonstrated to be a highly potent and well-tolerated immunosuppressant for new renal transplant patients. A trend was seen towards fewer dose reductions due to GI intolerability and less serious infections relative to other MPA drugs. The most common adverse events associated with MPA treatment are diarrhea and leukopenia.

20. OMALIZUMAB (ALLERGIC ASTHMA) [73–75]

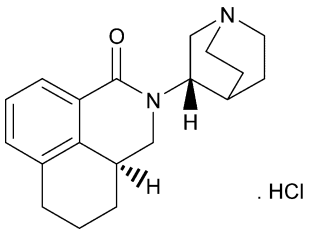
Country of origin	US
Originator	Genentech
First introduction	US
Introduced by	Genentech/Novartis
Trade name	Xolair
CAS registry no.	242138-07-04
Class	Humanized monoclonal antibody
Type	murine anti-IgE
Molecular weight	149 kDa
Expression system	CHO Cells
Manufacturer	Genentech

Omalizumab is a recombinant humanized construct of murine IgG1k monoclonal antibody introduced last year for the treatment of allergic asthma. It forms complexes with free, circulating serum IgE, which results in the inhibition of binding of IgE to the high-affinity IgE-receptor (FCεRI) on the surface of mast cells and basophils. Reduction in surface-bound IgE on FcεRI-bearing cells limits the degree of release of mediators of the allergic response. Omalizumab is produced by a Chinese hamster ovary cell suspension culture in a nutrient medium containing the antibiotic gentamicin. The recommended dosage is 150–375 mg administered subcutaneously every 2 or 4 weeks. Omalizumab has an average absolute bioavailability of 62% and an average terminal half-life of 26 days. Following a single SC dose, omalizumab is absorbed slowly, reaching peak serum concentrations after 7–8 days. However, serum levels of free IgE begin to decline in a dose-dependent manner within an hour after the first injection and typically lead to >96% reduction in free IgE concentrations. The omalizumab-IgE complexes have a longer

half-life and are eliminated more slowly than free IgE. After 16 weeks of dosing, total serum IgE (free plus bound IgE) is five times higher than pretreatment levels. Clearance of the omalizumab-IgE complexes occurs via the Fcγ receptors reticuloendothelial system. The efficacy and safety of omalizumab in the treatment of inhaled corticosteroid-dependent (ICS) asthma was evaluated in a 28-week double-blinded, placebo-controlled clinical study, which entailed co-administration of ICS for 16 weeks, followed by a gradual reduction in ICS dose over 12 weeks. A significant reduction in steroid dose with fewer exacerbations during steroid withdrawal phase was noted and more subjects receiving omalizumab were able to discontinue their ICS than in the placebo group (39.6 vs 19.1%, respectively; $p < 0.001$). Omalizumab was well tolerated and the most common adverse effects were arthralgia, generalized pain, leg pain, and injection-site reactions.

21. PALONOSETRON (ANTIEMETIC) [76–79]

Country of origin	US
Originator	Syntex (Roche Bioscience)
First introduction	US
Introduced by	MGI Pharma/Helsinn
Trade name	Aloxi
CAS registry no.	135729-62-3
Molecular weight	332.88



Palonosetron is a novel 5-HT3 receptor antagonist launched last year as an injectable agent for the prevention of acute and delayed nausea and vomiting associated with cancer chemotherapy. It has a much longer half-life (~40 h) than the other currently available 5-HT3 antagonists, which provides efficacy advantages in the prevention of delayed nausea and vomiting that typically occurs after 24 h and up to six days post chemotherapy administration. Palonosetron was developed as a conformationally restricted analog of the previously known 5HT3 antagonists tropisetron and granisetron. It is synthesized in four steps starting with the condensation of 1,8-naphthalic anhydride and (*S*)-3-aminoquinuclidine to produce the corresponding imide. The subsequent steps include catalytic hydrogenation of one of the aromatic rings of the imide intermediate, selective reduction of one of the carbonyls to a hydroxyl group, dehydration to an olefin and catalytic hydrogenation. The recommended dosage of palonosteron is 0.25 mg, administered as a single intravenous dose approximately 30 min before the start of chemotherapy. Palonosetron exhibits dose-proportional pharmacokinetics and it is moderately bound to plasma proteins (62%). Fifty percent of the dose is metabolized in the liver, and 40% is excreted unchanged in the urine. In comparative clinical studies with two other 5-HT3 receptor antagonists, palonosetron is shown to be as effective as dolasetron and more effective than ondansetron in controlling acute nausea and vomiting and superior to both in the control of delayed nausea and vomiting. Efficacy of

palonosetron has been demonstrated in patients receiving initial and repeat courses of both moderately and highly emetogenic chemotherapy, including cisplatin, cyclophosphamide and dacarbazine. At 0.25 and 0.75 mg doses, palonosetron is well tolerated and the most occurring adverse events are headache, diarrhea, fatigue, abdominal pain and insomnia.

22. PEGVISOMANT (ACROMEGALY) [80–82]

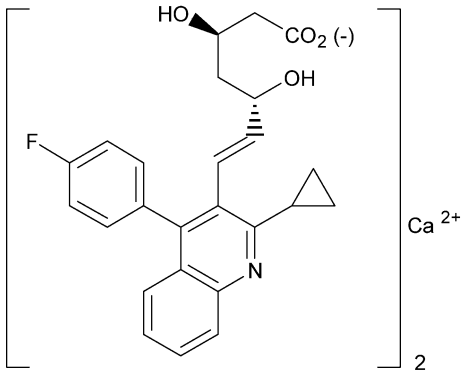
Country of origin	US
Originator	Sensus (Pfizer)
First introduction	US
Introduced by	Pfizer
Trade name	Somavert
CAS registry no.	218620-50-9
Class	PEGylated recombinant protein
Type	Human growth hormone
Molecular weight	40–50 kDa
Expression system	*E. coli*
Manufacturer	Abbott

Acromegaly is a rare debilitating endocrine disease caused by the excessive secretion of growth hormone (GH) and increased production of insulin-like growth factor I (IGF-I) in middle-aged adults. In over 90% of acromegaly patients, excess of GH is associated with a benign pituitary adenoma. The clinical manifestations of uncontrolled acromegaly include soft-tissue swelling, joint pain, nerve entrapment, glucose intolerance, hypertension, and cardiac disease. Patients with acromegaly have increased morbidity and mortality relative to the general population. The currently available treatment options for acromegaly are surgical removal of the adenoma, radiation therapy, and drug therapy with dopamine antagonists or somatostatin analogues. Pegvisomant, launched last year as a new treatment for acromegaly, is a modified form of human GH that acts as a highly selective GH receptor antagonist. It is a PEGylated form of a recombinant human GH antagonist (B2036). Pegvisomant selectively binds to GH receptors on cell surfaces, where it blocks the binding of endogenous GH, and thus interferes with GH signal transduction. This leads to a decrease in serum concentrations of IGF-I. A 12-week randomized, double-blind, placebo-controlled clinical study found that daily treatment of acromegaly patients with 10, 15, and 20 mg of pegvisomant resulted in normalized IGF-I concentrations in 54, 81, and 89% of patients, respectively, compared to 10% in the placebo group. An open-label extension study in which pegvisomant was individually titrated demonstrated that 12-month or longer treatment resulted in normalized IGF-I concentrations in 97% of patients. The recommended dosage regimen of pegvisomant consists of a 40 mg loading dose, followed by 10 mg daily dose, administered subcutaneously. The t_{max} of pegvisomant following SC

administration is 33–77 h, with a bioavailability of 57% and $t_{1/2}$ of six days. Pegvisomant is generally well tolerated and the most common side effects include injection-site reactions, diarrhea, nausea, chest pain and flu-like symptoms.

23. PITAVASTATIN (HYPOCHOLESTEROLEMIC) [83–86]

Country of origin	Japan
Originator	Nissan
First introduction	Japan
Introduced by	Sankyo/Kowa
Trade name	Livalo
CAS registry no.	147526-32-7
Molecular weight	880.98

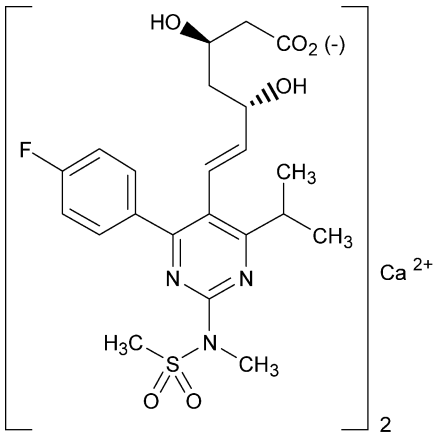


Pitavastatin, launched last year for the treatment of hypercholesterolemia, belongs to the family of second-generation statins, also referred to as superstatins due to their improved efficacy as cholesterol lowering agents. Like other statins, pitavastatin reduces plasma cholesterol levels by competitively inhibiting HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis in the liver. It is a more potent inhibitor of HMG-CoA reductase than the previously marketed statins and has the potential benefit of not undergoing significant metabolism by CYP3A4. Pitavastatin is synthesized in a multi-step sequence, including the key step of introducing the dihydroxyheptenoate side chain by cross-coupling of a 3-iodoquinoline intermediate with an alkenylborane reagent. Unlike rosuvastatin, the other statin introduced last year, pitavastatin has a high oral bioavailability (~80%). Plasma protein binding is also high for pitavastatin (>95%), and regardless of the dosing, the highest tissue levels are found in the liver, its target organ. After oral administration, the peak plasma concentration is reached at ~0.8 h and the mean elimination half-life is ~11 h. Pitavastatin is only minimally metabolized, mainly by CYP2C8 and CYP2C9, and the predominant route of elimination of the parent drug and its metabolites is by means of bile excretion followed by elimination in the feces. In clinical studies, oral doses of 2–4 mg/day of pitavastatin produced dose-dependent reduction in LDL-cholesterol levels by 40–48% from baseline in patients with heterozygous familial hypercholesterolemia. In a 12-week double-blind comparative study, pitavastatin (2 mg/day) was more effective than pravastatin (10 mg/day) in reducing LDL-cholesterol levels (38 and 18%, respectively); however, both agents

produced similar increases in HDL-cholesterol (~9%). The drug was well tolerated and the adverse reactions were mild and transient.

24. ROSUVASTATIN (HYPOCHOLESTEROLEMIC) [87–90]

Country of origin	Japan
Originator	Shinogi
First introduction	Netherlands
Introduced by	AstraZeneca
Trade name	Crestor
CAS registry no.	147098-20-2
Molecular weight	1001.15

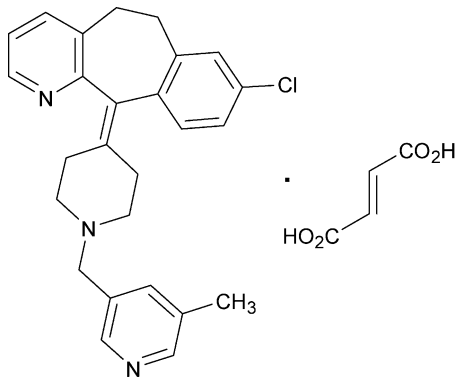


Rosuvastatin, one of the two new statins launched last year for the treatment of hypercholesterolemia, has high hepato-selectivity and more potent inhibitory effect on HMG-CoA reductase than the previously marketed statins. In rat hepatocytes, it inhibits cholesterol biosynthesis with an IC_{50} of 1.12 nM, which is ~100-fold higher potency than pravastatin. Rosuvastatin is synthesized in a 12-step sequence, entailing the construction of a pyrimidinyl aldehyde intermediate in eight steps and subsequent introduction of the dihydroxyheptenoate side chain via Wittig reaction with a β-ketophosphorane reagent and stereoselective carbonyl reduction of the resultant enone. Pharmacokinetic properties of rosuvastatin in humans, dosed at 5–80 mg, are approximately linear with dose. Following oral administration, rosuvastatin is rapidly absorbed with an oral bioavailability of ~20% and t_{max} of ~3 h. It has a prolonged duration of action, with terminal $t_{1/2}$ of ~20 h, compatible with once-daily dosing. In humans, rosuvastatin is minimally metabolized through CYP2C9 and CYP2C19, with little or no metabolism via the CYP3A4. Approximately 90% of the administered oral dose is eliminated in the feces (92% as the parent compound) and the rest in the urine. Rosuvastatin is considered a "superstatin" due to its ability, at well-tolerated doses, to lower LDL cholesterol and triglycerides to a much greater extent than first generation statins. In patients with hypercholesterolemia, rosuvastatin treatment at doses of 5 and 10 mg/day over 12-week period resulted in 40–43% reduction of LDL-cholesterol levels, 12–13% increase in HDL-cholesterol, and 17–19% reduction in triglycerides. In comparison, the efficacy range of LDL-cholesterol reductions by atorvastatin (10 mg/day), pravastatin (20 mg/day), and simvastatin (20 mg/day) was 28–35%. Rosuvastatin is a well-tolerated drug at doses of 1–20 mg

and the most common side effects at these doses are headache, myalgia, pain and pharyngitis, which are consistent with those previously reported for statin therapy.

25. RUPATADINE FUMARATE (ANTIALLERGIC) [91–93]

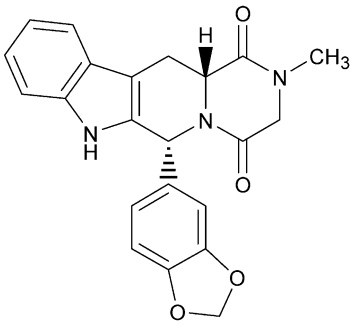
Country of origin	Spain
Originator	Uriach
First introduction	Spain
Introduced by	Uriach
Trade name	Rupafin
CAS registry no.	182349-12-8
Molecular weight	532.04



Rupatadine fumarate, a novel antiallergic drug with a dual mechanism of action, was introduced last year in Spain as an oral treatment for perennial and seasonal rhinitis. Rupatadine acts as non-sedating histamine H1 receptor antagonist and platelet-activating factor (PAF) antagonist. Its K_i^{app} values against [^{3}H]WEB-2086 binding to rabbit platelet membrane PAF receptors and [^{3}H]pyralimine binding to guinea pig cerebellum membrane H1 histamine receptors are 0.55 and 0.10 μM, respectively. It has a rapid onset of action, with patients experiencing relief of symptoms within 2 h, and its long duration of action (>24 h) permits once-daily dosing. Rupatidine is prepared in a 6-step convergent synthesis, with the key steps involving the Grignard reaction of a *N*-alkyl-4-chloropiperdine with a benzocycloheptapyridinone intermediate, followed by dehydration. Rupatadine is rapidly absorbed after oral administration. The time to reach maximum plasma concentration is 0.75–1 h and the mean half-life in healthy volunteers is ~6 h. It is extensively metabolized, mainly by CYP3A4, and the major elimination route for the drug is biliary excretion. In comparative clinical trials, rupatadine 10 mg once daily was as effective as certizine 10 mg in short-term studies (2–4 weeks duration), but provided a better profile of CNS side effects. In comparison with ebastine 10 mg and loratadine 10 mg, rupatadine showed a superior relief of rhinitis symptoms at the same dose. Rupatadine was well tolerated in clinical trials and, at the recommended daily dose of 10 mg, was free of the sedative effects associated with first-generation antihistamines. In addition, there were no significant differences in the overall incidence of adverse events in rupatidine-treated patients and those treated with placebo or standard reference products.

26. TADALAFIL (MALE SEXUAL DYSFUNCTION) [94–98]

Country of origin	US
Originator	Lilly/ICOS
First introduction	UK, Germany
Introduced by	Lilly/ICOS
Trade name	Cialis
CAS registry no.	171596-29-5
Molecular weight	389.41



Tadalafil is one of the two new PDE5 inhibitors launched last year for the oral treatment of male erectile dysfunction. Tadalafil is a β-carboline derivative and it is structurally distinct from vardenafil (Levitra®) and sildenafil (Viagra®), both of which are PDE5 inhibitors based on a fused pyrimidine core structure. Tadalafil is synthesized in three steps starting from D-tryptophan methyl ester, by first condensing with piperonal in a Pictet-Spengler cyclization reaction to form the tetrahydro-β-carboline derivative, which is followed by chloroacetylation of the piperidine ring nitrogen and cyclization with methylamine. Tadalafil is a potent and highly selective inhibitor of PDE5 ($IC_{50} = 1$ nm). It shows >10,000-fold selectivity for PDE5 versus PDE1, 2, 3, 4, 7, 8 and 9, and >700-fold selectivity versus PDE6. Typically administered at 10 and 20 mg doses, tadalafil is rapidly absorbed and has a t_{max} of 2 h, which is slightly longer than those of sildenafil (1 h) and vardenafil (0.75 h). Clinically, all of these agents appear to have efficacy for many men within 30–60 min. However, tadalafil distinguishes itself from other PDE5 inhibitors in terms of significantly longer duration of action. The half-life of tadalafil dosed at 20 mg is 17.5 h as compared with 3.8 h for sildenafil (100 mg) and 4.7 h for vardenafil (20 mg). In clinical studies, significant rates of response were reported up to 36 h following drug ingestion. Tadalafil is predominantly metabolized in the liver by CYP3A4 to entities that are not active against PDE5 and excreted mainly as metabolites in the feces and the urine. The pharmacokinetics of tadalafil are unaffected by factors such as intake of food and alcohol, age, the presence of diabetes, and mild or moderate hepatic insufficiency. The most common drug-related adverse events are headache, back pain, dyspepsia, and myalgia. At 10 and 20 mg doses, Tadalafil does not have a significant effect on blood pressure and heart rate and does not result in increased instances of myocardial infarction. Rare reports of prolonged erections greater than 4 h and priapism have been noted with the use of tadalafil. Priapism, if not treated properly, can result in irreversible damage to the erectile tissue. Patients who have an erection lasting greater than 4 h are advised to seek emergency medical attention. Tadalafil has a modest synergistic effect

on the nitrate-induced reduction in blood pressure and, as with sildenafil and vardenafil, it is contraindicated for use in patients on nitrate therapy. In diabetic patients, improvement of erectile function by tadalafil is irrespective of the type of diabetes and the type of diabetic therapy.

27. TOSITUMOMAB (ANTICANCER) [99,100]

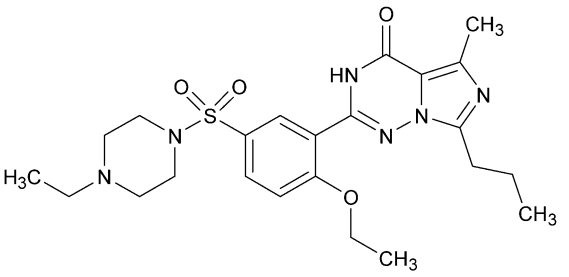
Country of origin	US
Originator	Corixa
First introduction	US
Introduced by	GSK/Corixa
Trade name	Bexxar
CAS registry no.	192391-48-3
Class	Monoclonal antibody
Type	murine anti-CD20 radioimmunoconjugate
Molecular weight	150 kDa
Expression system	murine/human hybridoma cell line
Manufacturer	Corixa

Tositumomab was launched last year as a new radioimmunotherapeutic antibody for the treatment of B-cell non-Hodgkin's lymphoma (NHL). It is specifically indicated for patients with CD20-positive, follicular NHL, with or without transformation, whose disease is refractory to rituximab and has relapsed following chemotherapy. Tositumomab is an anti-CD20 murine IgG_{2a} lambda monoclonal antibody produced in an antibiotic-free culture of mammalian cells. The therapeutic regimen of the drug is composed of tositumomab and ^{131}I-tositumomab. This dual-action therapy combines the tumor-targeting ability of a cytotoxic monoclonal antibody and the therapeutic potential of radiation with patient-specific dosing. The binding of tositumomab to CD20 antigen on NHL cells initiates an immune response to the cancer and the radiolabeled antibody, ^{131}I-tositumomab, delivers a dose of radiation directly to tumor cells. Iodine-131 has a physical half-life of eight days. It emits β particles and γ rays, but the primary antitumor effect results from the β particles. In contrast to the external beam radiation, ^{131}I-tositumomab delivers radiation to the tumor at a continuous low dose rate. The dose rate initially increases, as radiolabelled antibodies accumulate at the tumor site, with a maximal tumor dose rate of <0.10 Gy/h. The dose rate then decreases with physical decay of the radioisotope and clearance from the body. The tositumomab and ^{131}I-tositumomab therapeutic regimen is dosed in two steps: a dosimetric step followed 7–14 days later by a therapeutic step. The dosimetric step consists of successive infusions of tositumomab (450 mg) and ^{131}I-tositumomab (5.0 mCi, 35 mg). The therapeutic step consists of 450 mg tositumomab infusion, followed by ^{131}I-tositumomab as calculated to patient-specific activity. Since non-tumor localization of ^{131}I is primarily in the thyroid, the patients are also treated with a thyro-protective agent such as potassium iodide throughout the therapeutic course. In a clinical study in patients who had an average

of four prior chemotherapies and who did not respond to or relapsed from rituximab therapy, overall response to tositumomab and ^{131}I-tositumomab treatment was achieved in 63% of the patients and complete response was achieved in 29% of the patients. The duration of response in this category of patients was 25 months. The most common adverse reactions associated tositumomab regimens are severe or life-threatening cytopenia, infections, hemorrhage, allergic reactions, secondary leukemia, and myelodysplasia.

28. VARDENAFIL (MALE SEXUAL DYSFUNCTION) [101–104]

Country of origin	Germany
Originator	Bayer AG
First introduction	Germany
Introduced by	Bayer/GSK
Trade name	Levitra
CAS registry no.	224785-90-4
Molecular weight	488.61



Vardenafil is a new PDE5 inhibitor launched last year for oral treatment of male erectile dysfunction and it has significant structural similarity with sildenafil (Viagra®), which was the first PDE5 inhibitor introduced in 1998 for this indication. Vardenafil is synthesized in three steps starting with a cyclization reaction of 2-ethyoxybenzamidine with 2-butyramidopropionic acid and ethoxyallyl chloride to construct the imidazotriazine ring system, followed by sulfonation to the corresponding sulfonyl chloride and subsequent condensation with 1-ethylpiperazine. The potency of PDE5 inhibition by vardenafil ($IC_{50} = 0.7$ nM) is ~10 times greater than that of sildenafil ($IC_{50} = 6.6$ nM). Vardenafil is typically administered in single doses of 10 and 20 mg. The time to reach maximum plasma concentration is 0.75 h, which is slightly shorter than those of sildenafil ($t_{max} = 1.16$ h) and tadalafil ($t_{max} = 2$ h), and the half-life is 4–5 h. Although it is almost completely absorbed following oral administration, the mean absolute bioavailability of a 10 mg dose is ~15%, resulting from extensive first pass metabolism. Vardenafil is metabolized in the liver primarily by CYP3A4 and is eliminated mainly in feces. In clinical studies, 10–20 mg doses of vardenafil was well tolerated and efficacious in patients with ED of various severities, including subjects with comorbidities such as diabetes mellitus or hypertension or hyperlipidemia. The side-effect profile of vardenafil is similar to that of sildenafil, with headache, flushing, dyspepsia and nasal congestion being the most common adverse events. Vardenafil has systemic vasodilatory properties, which can cause transient decrease in supine blood pressure; however, it does not appear to translate into clinical effects. The mean maximum decreases in supine systolic blood pressure following 20 and 40 mg vardenafil were 6.9 and 4.3 mmHg, respectively, when

compared to placebo. However, single and multiple oral doses of vardenafil up to 40 mg produced no clinically relevant changes in the ECGs of normal male volunteers.

REFERENCES

[1] The collection of new therapeutic entities first launched in 2003 originated from the following sources: (a) CIPSLINE, Prous database; (b) Iddb, Current Drugs database; (c) IMS R&D Focus; (d) Adis Business Intelligence R&D Insight; (e) Pharmaprojects.
[2] A. I. Graul, *Drug News Perspect.*, 2004, **17**, 43.
[3] C. Boyer-Joubert, E. Lorthiois and F. Moreau, *Ann. Rep. Med. Chem.*, 2003, **38**, 347.
[4] P. Bernardelli, B. Gaudilliere and F. Vergne, *Ann. Rep. Med. Chem.*, 2002, **37**, 257.
[5] B. Gaudilliere, P. Bernardelli and P. Berna, *Ann. Rep. Med. Chem.*, 2001, **36**, 293.
[6] P. Emery, *Drugs Today*, 2003, **39** (Suppl. B), 17.
[7] L. A. Edwards, *Formulary*, 2003, **38**, 272.
[8] M. H. Weisman, L. W. Moreland, D. E. Furst, M. E. Weinblatt, E. C. Keystone, H. E. Paulus, L. S. Teoh, R. B. Velagapudi, P. A. Noertersheuser, G. R. Granneman, S. A. Fischkoff and E. K. Chartash, *Clin. Ther.*, 2003, **25**, 1700.
[9] K. P. Machold and J. S. Smolen, *Exp. Opin. Biol. Ther.*, 2003, **3**, 351.
[10] M. E. Weinblatt, E. C. Keystone, D. E. Furst, L. W. Moreland, M. H. Weisman, C. A. Birbara, L. A. Teoh, S. A. Fischkoff and E. K. Chartash, *Arthritis Rheum.*, 2003, **48**, 3.
[11] S. J. Bashir and H. I. Maibach, *Curr. Opin. Invest. Drugs*, 2001, **2**, 631.
[12] A. D. Ormerod, *Curr. Opin. Invest. Drugs*, 2003, **4**, 608.
[13] A. K. Vaishnaw and C. N. TenHoor, *J. Pharmacokinet. Pharmacodyn.*, 2003, **29**, 415.
[14] L. A. Sorbera, L. Revel and R. Fernandez, *Drugs Future*, 2001, **26**, 527.
[15] R. J. Kaplon, P. S. Hochman, R. E. Michler, P. A. Kwiatkowski, N. M. Edwards, C. L. Berger, H. Xu, W. Meier, B. P. Wallner, P. Chisholm and C. C. Marboe, *Transplantation*, 1996, **61**, 356.
[16] J. J. Hale, S. G. Mills, M. MacGoss, P. E. Finke, M. A. Cascieri, S. Sadowski, E. Ber, G. G. Chicchi, M. Kurtz, J. Metzger, G. Eiermann, *et al.*, *J. Med. Chem.*, 1998, **41**, 4607.
[17] L. A. Sorbera, J. Castaňer, M. Bayes and J. Silvestre, *Drugs Future*, 2002, **27**, 211.
[18] S. P. Chawla, R. J. Gralla, P. J. Hesketh, S. M. Grunberg, M. E. Elmer and K. J. Horgan, *Proc. Am. Soc. Clin. Oncol.*, 2001, **19**, 1759.
[19] S. P. Chawla, S. M. Grunberg, R. J. Gralla, P. J. Hesketh, C. Rittenberg, M. E. Elmer, C. Schmidt, A. Taylor, A. D. Carides, J. K. Evans and K. J. Horgan, *Cancer*, 2003, **97**, 2290.
[20] D. R. Goldsmith and C. M. Perry, *Drugs*, 2003, **63**, 1679.
[21] G. Bold, A. Fassler, H. G. Capraro, R. Cozens, T. Klimkait, J. Lazdins, J. Mestan, B. Poncioni, J. Rosel, D. Stover, M. Tintelnot Blomley, *et al.*, *J. Med. Chem.*, 1998, **41**, 3387.
[22] P. J. Piliero, *Exp. Opin. Invest. Drugs*, 2002, **11**, 1295.
[23] X. Rabasseda, J. Silvestre and J. Castaňer, *Drugs Future*, 1999, **24**, 375.
[24] B. S. Robinson, K. A. Riccardi, Y. F. Gong, Q. Guo, D. A. Stock, W. S. Blair, B. J. Terry, C. A. Deminie, F. Djang, R. J. Colonno and P. F. Lin, *Antimicrob. Agents Chemother.*, 2000, **44**, 2093.
[25] F. Bracher and T. Litz, *Bioorg. Med. Chem.*, 1996, **4**, 877–880.
[26] D. T. Wong, P. G. Threlkeld, K. L. Best and F. P. Bymaster, *J. Pharm. Exp. Ther.*, 1982, **222**, 61.
[27] R. D. Spealman, *J. Pharm. Exp. Ther.*, 1995, **275**, 53.
[28] S. L. Baldinger and M. W. Yogman, *Formulary*, 2003, **38**, 85.
[29] K. Wellington and L. J. Scott, *Drugs*, 2003, **63**, 2613.
[30] T. Kobayashi, T. Inoue, S. Nishino, Y. Fujihara, K. Oizumi and T. Kimura, *Chem. Pharm. Bull.*, 1995, **43**, 797.
[31] H. Koike, M. Yoshimoto and H. Nishino, EP 266922 A1 & B1, 1988.
[32] J. Adams, M. Behnke, S. Chen, A. A. Cruickshank, L. R. Dick, L. Grenier, J. M. Klunder, Y. T. Ma, L. Plamondon and R. L. Stein, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 333.
[33] E. S. Lightcap, T. A. McCormack, C. S. Pien, V. Chau, J. Adams and P. J. Elliott, *Clin. Chem.*, 2000, **46**, 673.

[34] J. Adams, *Semin. Oncol.*, 2001, **28**, 613.
[35] T. Hideshima, P. Richardson, D. Chauhan, V. J. Palombella, P. J. Elliott, J. Adams and K. C. Anderson, *Cancer Res.*, 2001, **61**, 3071.
[36] P. J. Elliott and J. S. Ross, *Am. J. Clin. Pathol.*, 2001, **116**, 637.
[37] A. F. Kisselev and A. L. Goldberg, *Chem. Biol.*, 2001, **8**, 739.
[38] M. S. Kies and P. M. Harari, *Curr. Opin. Invest. Drugs*, 2002, **3**, 1385.
[39] A. Etessami and J. Bourhis, *Drugs Future*, 2000, **25**, 895.
[40] N. A. Reynolds and A. J. Wagstaff, *Drugs*, 2004, **64**, 109.
[41] H. K. Sun, J. L. Kuti and D. P. Nicolau, *Formulary*, 2003, **38**, 634.
[42] A. Raja, J. LaBonte, J. Lebbos and P. Kirkpatrick, *Nat. Rev. Drug Discov.*, 2003, **2**, 942.
[43] F. P. Tally and M. F. DeBruin, *J. Antimicrob. Chemother.*, 2000, **46**, 523.
[44] M. J. Rybak, E. Hershberger, T. Moldovan and R. G. Grucz, *Antimicrob. Agents Chemother.*, 2000, **44**, 1062.
[45] R. L. Dedrick, P. Walicke and M. Garovoy, *Transpl. Immunol.*, 2002, **9**, 181.
[46] A. Gottlieb, J. G. Krueger, R. Bright, M. Ling, M. Lebwohl, S. Kang, S. Feldman, M. Spellman, K. Wittkowski, A. J. Ochs, P. Jardieu, *et al.*, *J. Am. Acad. Dermatol.*, 2000, **42**, 428.
[47] R. J. Bauer, R. L. Dedrick, M. L. White, M. J. Murray and M. R. Garovoy, *J. Pharmacokinet. Biopharm.*, 1999, **27**, 397.
[48] M. Rybak, E. Hershberger, T. Moldovan and R. G. Grucz, *Antimicrob. Agents Chemother.*, 2000, **44**, 1062.
[49] L. M. Bang and L. J. Scott, *Drugs*, 2003, **63**, 2413.
[50] L. S. Jeong, R. F. Schinazi, J. W. Beach, H. O. Kim, S. Nampalli, K. Shanmuganathan, A. J. Alves, A. McMillan, C. K. Chu and R. Mathis, *J. Med. Chem.*, 1993, **36**, 181.
[51] R. F. Schinazi, A. McMillan, D. Cannon, R. Mathis, R. M. Lloyd, A. Peck, J. P. Sommadossi, M. St Clair, J. Wilson, P. A. Furman, G. Painter and W. B. Choi, *Antimicrob. Agents Chemother.*, 1992, **36**, 2423.
[52] G. R. Painter, D. C. Liotta, M. Almond, D. Cleary and J. Soria, *WO* 2000009494 A1, 2000.
[53] S. Jiang, K. Lin, N. Strick and A. R. Neurath, *Biochem. Biophys. Res. Commun.*, 1993, **195**, 533.
[54] N. Cammack, *Curr. Opin. Infect. Dis.*, 2001, **14**, 13.
[55] C. Wild, T. Oas, C. McDanal, D. Bolognesi and T. Matthews, *Proc. Nat. Acad. Sci.*, 1992, **89**, 10537.
[56] T. J. Ketas, I. Frank, P. J. Klasse, B. M. Sullivan, J. P. Gardner, C. Splenlehauer, M. Nesin, W. C. Olson, J. P. Moore and M. Pope, *J. Virol.*, 2003, **77**, 2762.
[57] K. A. Nagashima, D. A. Thompson, S. I. Rosenfield, P. J. Maddon, T. Dragic and W. C. Olson, *J. Infect. Dis.*, 2001, **183**, 1121.
[58] J. Martin and H. Krum, *Curr. Opin. Invest. Drugs*, 2001, **2**, 521.
[59] E. G. McMahon, *Curr. Opin. Pharmacol.*, 2001, **1**, 190.
[60] X. Rabasseda, J. Silvestre and J. Castañer, *Drugs Future*, 1999, **24**, 488.
[61] L. A. Sorbera, L. Martin, J. Castañer and R. M. Castañer, *Drugs Future*, 2001, **26**, 224.
[62] A. H. Corbett and A. D. M. Kashuba, *Curr. Opin. Invest. Drugs*, 2002, **3**, 384.
[63] R. Belshe, P. Mendelman, J. Treanor, J. King, W. Gruber, P. Piedra, D. Bernstein, F. G. Hayden, K. Kotloff, K. Zangwill, D. Iacuzio and M. Wolff, *N. Engl. J. Med.*, 1998, **338**, 1405.
[64] K. L. Nichol, P. M. Mendelman, K. P. Mallon, L. A. Jackson, G. J. Gorse, R. B. Belshe, W. P. Glezen and J. Wittes, *JAMA*, 1999, **282**, 137.
[65] J. J. Treanor, K. Kotloff, R. F. Betts, R. Belshe, F. Newman, D. Iacuzio, J. Wittes and M. Bryant, *Vaccine*, 2000, **18**, 899.
[66] J. A. McIntyre, *Drugs Future*, 2003, **28**, 432.
[67] E. D. Kakkis, J. Muenzer, G. E. Tiller, L. Waber, J. Belmont, M. Passage, B. Izikowski, J. Phillips, R. Doroshow, I. Walot, R. Hoft and E. Neufeld, *N. Engl. J. Med.*, 2001, **344**, 182.
[68] L. A. Sorbera, J. Castañer and M. Bayes, *Drugs Future*, 2003, **28**, 229.
[69] R. H. Lachmann, *Curr. Opin. Invest. Drugs*, 2003, **4**, 472.
[70] G. M. Pastores and N. L. Barnett, *Expert Opin. Invest. Drugs*, 2003, **12**, 273.

[71] S. Gabardi, J. L. Tran and M. R. Clarkson, *Ann. Pharmacother.*, 2003, **37**, 1685.
[72] A. I. Graul and A. M. Collins, *Drugs Future*, 2003, **28**, 273.
[73] M. Cullell-Young, M. Bayes and P. A. Leeson, *Drugs Future*, 2001, **27**, 537.
[74] W. Busse, J. Corren, B. Q. Lanier, M. McAlary, A. Fowler-Taylor, G. D. Cioppa, A. van As and N. Gupta, *J. Allergy Clin. Immunol.*, 2001, **108**, 184.
[75] J. Corren, T. Casale, Y. Deniz and M. Ashby, *J. Allergy Clin. Immunol.*, 2003, **111**, 87.
[76] G. Stacher, *Curr. Opin. Invest. Drugs*, 2002, **3**, 1502.
[77] M. V. DiVall and R. J. Cersosimo, *Formulary*, 2003, **38**, 414.
[78] B. A. Kowalczyk and C. A. Dvorak, *Synthesis*, 1996, **7**, 816.
[79] A. Graul and J. Castaňer, *Drugs Future*, 1996, **21**, 906.
[80] P. M. Stewart, *Eur. J. Endocrinol.*, 2003, **148**, S27.
[81] P. J. Trainer, W. M. Drake, L. Katznelson, P. U. Freda, V. Herman-Bonert, A. J. van der Lely, E. V. Dimaraki, P. M. Stewart, K. E. Friend, M. L. Vance, G. M. Besser and J. A. Scarlett, *N. Engl. J. Med.*, 2001, **344**, 182.
[82] X. Rabasseda and P. Leeson, *Drugs Future*, 1999, **24**, 969.
[83] P. Iglesias and J. J. Diez, *Expert Opin. Invest. Drugs*, 2003, **12**, 1777.
[84] K. Kajinami, H. Mabuchi and Y. Saito, *Expert Opin. Invest. Drugs*, 2000, **9**, 2653.
[85] N. A. Flores, *Curr. Opin. Invest. Drugs*, 2002, **3**, 1334.
[86] W. L. Isley, *Drugs Today*, 2001, **37**, 587.
[87] A. Graul and J. Castaňer, *Drugs Future*, 1999, **24**, 511.
[88] Information Update, *Drugs Future* 2003, **28**, 617.
[89] C. M. White, *J. Clin. Pharmacol.*, 2002, **42**, 963.
[90] M. H. Davidson, *Expert Opin. Invest. Drugs*, 2002, **11**, 125.
[91] I. Izquierdo, M. Merlos and J. Garcia-Rafanell, *Drugs Today*, 2003, **39**, 451.
[92] J. Garcia-Rafanell, J. Uriach and Cia, *Drugs Future*, 1996, **21**, 1032.
[93] E. Carceller, M. Merlos, M. Giral, D. Balsa, C. Almansa, J. Bartroli, J. Garcia-Rafanell and J. Forn, *J. Med. Chem.*, 1994, **37**, 2697.
[94] L. A. Sorbera, L. Martin, P. A. Leeson and J. Castaňer, *Drugs Future*, 2001, **26**, 15.
[95] D. P. Rotella, *Curr. Opin. Invest. Drugs*, 2003, **4**, 60.
[96] J. Kuan and G. Brock, *Expert Opin. Invest. Drugs*, 2002, **11**, 1605.
[97] S. Uckert, C. G. Stief and U. Jonas, *Expert Opin. Invest. Drugs*, 2003, **12**, 1521.
[98] R. C. Rosen and J. B. Costis, *Am. J. Cardiol.*, 2003, **92**, 9.
[99] B. Cheson, *Curr. Opin. Invest. Drugs*, 2002, **3**, 165.
[100] C. R. Culy and H. M. Lamb, *BioDrugs*, 2000, **14**, 195.
[101] C. I. Coleman, J. M. Carabino, C. M. Vergara and F. Wang, *Formulary*, 2003, **38**, 131.
[102] J. M. Young, *Expert Opin. Invest. Drugs*, 2002, **11**, 1487.
[103] F. Sommer and U. Engelmann, *Curr. Opin. Invest. Drugs*, 2002, **3**, 607.
[104] L. A. Sorbera, L. Martin, X. Rabasseda and J. Castaňer, *Drugs Future*, 2001, **26**, 141.

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
abacavir sulfate	antiviral	1999	35, 333
acarbose	antidiabetic	1990	26, 297
aceclofenac	antiinflammatory	1992	28, 325
acemannan	wound healing agent	2001	37, 259
acetohydroxamic acid	hypoammonuric	1983	19, 313
acetorphan	antidiarrheal	1993	29, 332
acipimox	hypolipidemic	1985	21, 323
acitretin	antipsoriatic	1989	25, 309
acrivastine	antihistamine	1988	24, 295
actarit	antirheumatic	1994	30, 296
adalimumab	rheumatoid arthritis	2003	39, 339
adamantanium bromide	antiseptic	1984	20, 315
adefovir dipivoxil	antiviral	2002	38, 348
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
agalsidase alfa	fabry's disease	2001	37, 259
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical antiinflammatory	1985	21, 323
alefacept	plaque psoriasis	2003	39, 340
alemtuzumab	anticancer	2001	37, 260
alendronate sodium	osteoporosis	1993	29, 332
alfentanil HCl	analgesic	1983	19, 314
alfuzosin HCl	antihypertensive	1988	24, 296
alglucerase	enzyme	1991	27, 321
alitretinoin	anticancer	1999	35, 333
alminoprofen	analgesic	1983	19, 314
almotriptan	antimigraine	2000	36, 295
anakinra	antiarthritic	2001	37, 261
alosetron hydrochloride	irritable bowel syndrome	2000	36, 295
alpha-1 antitrypsin	protease inhibitor	1988	24, 297
alpidem	anxiolytic	1991	27, 322
alpiropride	antimigraine	1988	24, 296
alteplase	thrombolytic	1987	23, 326
amfenac sodium	antiinflammatory	1986	22, 315
amifostine	cytoprotective	1995	31, 338
aminoprofen	topical antiinflammatory	1990	26, 298
amisulpride	antipsychotic	1986	22, 316
amlexanox	antiasthmatic	1987	23, 327
amlodipine besylate	antihypertensive	1990	26, 298
amorolfine HCl	topical antifungal	1991	27, 322
amosulalol	antihypertensive	1988	24, 297
ampiroxicam	antiinflammatory	1994	30, 296

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
amprenavir	antiviral	1999	35, 334
amrinone	cardiotonic	1983	19, 314
amrubicin HCl	antineoplastic	2002	38, 349
amsacrine	antineoplastic	1987	23, 327
amtolmetin guacil	antiinflammatory	1993	29, 332
anagrelide HCl	hematological	1997	33, 328
anastrozole	antineoplastic	1995	31, 338
angiotensin II	anticancer adjuvant	1994	30, 296
aniracetam	cognition enhancer	1993	29, 333
anti-digoxin polyclonal antibody	antidote	2002	38, 350
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
aprepitant	antiemetic	2003	39, 341
APSAC	thrombolytic	1987	23, 328
aranidipine	antihypertensive	1996	32, 306
arbekacin	antibiotic	1990	26, 298
argatroban	antithromobotic	1990	26, 299
arglabin	anticancer	1999	35, 335
aripiprazole	neuroleptic	2002	38, 350
arotinolol HCl	antihypertensive	1986	22, 316
arteether	antimalarial	2000	36, 296
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
atazanavir	antiviral	2003	39, 342
atomoxetine	attention deficit hyperactivity disorder	2003	39, 343
atorvastatin calcium	dyslipidemia	1997	33, 328
atosiban	preterm labor	2000	36, 297
atovaquone	antiparasitic	1992	28, 328
auranofin	chrysotherapeutic	1983	19, 314
azelnidipine	antihypertensive	2003	39, 344
azelaic acid	antiacne	1989	25, 310
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
balofloxacin	antibacterial	2002	38, 351
balsalazide disodium	ulcerative colitis	1997	33, 329
bambuterol	bronchodilator	1990	26, 299
barnidipine HCl	antihypertensive	1992	28, 328
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benazepril HCl	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
benidipine HCl	antihypertensive	1991	27, 322

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
beraprost sodium	platelet aggreg. inhibitor	1992	28, 326
betamethasone butyrate prospinate	topical antiinflammatory	1994	30, 297
betaxolol HCl	antihypertensive	1983	19, 315
betotastine besilate	antiallergic	2000	36, 297
bevantolol HCl	antihypertensive	1987	23, 328
bexarotene	anticancer	2000	36, 298
biapenem	antibacterial	2002	38, 351
bicalutamide	antineoplastic	1995	31, 338
bifemelane HCl	nootropic	1987	23, 329
bimatoprost	antiglaucoma	2001	37, 261
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisantrene HCl	antineoplastic	1990	26, 300
bisoprolol fumarate	antihypertensive	1986	22, 317
bivalirudin	antithrombotic	2000	36, 298
bopindolol	antihypertensive	1985	21, 324
bortezomib	anticancer	2003	39, 345
bosentan	antihypertensive	2001	37, 262
brimonidine	antiglaucoma	1996	32, 306
brinzolamide	antiglaucoma	1998	34, 318
brodimoprin	antibiotic	1993	29, 333
bromfenac sodium	NSAID	1997	33, 329
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budipine	antiParkinsonian	1997	33, 330
budralazine	antihypertensive	1983	19, 315
bulaquine	antimalarial	2000	36, 299
bunazosin HCl	antihypertensive	1985	21, 324
bupropion HCl	antidepressant	1989	25, 310
buserelin acetate	hormone	1984	20, 316
buspirone HCl	anxiolytic	1985	21, 324
butenafine HCl	topical antifungal	1992	28, 327
butibufen	antiinflammatory	1992	28, 327
butoconazole	topical antifungal	1986	22, 318
butoctamide	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cabergoline	antiprolactin	1993	29, 334
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	hypertensive	1988	24, 298
calcipotriol	antipsoriatic	1991	27, 323
camostat mesylate	antineoplastic	1985	21, 325
candesartan cilexetil	antihypertension	1997	33, 330
capecitabine	antineoplastic	1998	34, 319
carboplatin	antibiotic	1986	22, 318
carperitide	congestive heart failure	1995	31, 339

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
caspofungin acetate	antifungal	2001	37, 263
cefbuperazone sodium	antibiotic	1985	21, 325
cefcapene pivoxil	antibiotic	1997	33, 330
cefdinir	antibiotic	1991	27, 323
cefditoren pivoxil	oral cephalosporin	1994	30, 297
cefepime	antibiotic	1993	29, 334
cefetamet pivoxil HCl	antibiotic	1992	28, 327
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefoselis	antibiotic	1998	34, 319
cefotetan disodium	antibiotic	1984	20, 317
cefotiam hexetil HCl	antibiotic	1991	27, 324
cefozopran HCl	injectable cephalosporin	1995	31, 339
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
cefpirome sulfate	antibiotic	1992	28, 328
cefpodoxime proxetil	antibiotic	1989	25, 310
cefprozil	antibiotic	1992	28, 328
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
ceftibuten	antibiotic	1992	28, 329
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celecoxib	antiarthritic	1999	35, 335
celiprolol HCl	antihypertensive	1983	19, 317
centchroman	antiestrogen	1991	27, 324
centoxin	immunomodulator	1991	27, 325
cerivastatin	dyslipidemia	1997	33, 331
cetirizine HCl	antihistamine	1987	23, 331
cetrorelix	female infertility	1999	35, 336
cetuximab	anticancer	2003	39, 346
cevimeline hydrochloride	anti-xerostomia	2000	36, 299
chenodiol	anticholelithogenic	1983	19, 317
CHF-1301	antiparkinsonian	1999	35, 336
choline alfoscerate	nootropic	1990	26, 300
cibenzoline	antiarrhythmic	1985	21, 325
cicletanine	antihypertensive	1988	24, 299
cidofovir	antiviral	1996	32, 306
cilazapril	antihypertensive	1990	26, 301
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
cinildipine	antihypertensive	1995	31, 339

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cinitapride	gastroprokinetic	1990	26, 301
cinolazepam	hypnotic	1993	29, 334
ciprofibrate	hypolipidemic	1985	21, 328
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
cisatracurium besilate	muscle relaxant	1995	31, 340
citalopram	antidepressant	1989	25, 311
cladribine	antineoplastic	1993	29, 335
clarithromycin	antibiotic	1990	26, 302
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
clopidogrel hydrogensulfate	antithrombotic	1998	34, 320
cloricromen	antithrombotic	1991	27, 325
clospipramine HCl	neuroleptic	1991	27, 325
colesevelam hydrochloride	hypolipidemic	2000	36, 300
colestimide	hypolipidaemic	1999	35, 337
colforsin daropate HCl	cardiotonic	1999	35, 337
crotelidae polyvalent immune fab	antidote	2001	37, 263
cyclosporine	immunosuppressant	1983	19, 317
cytarabine ocfosfate	antineoplastic	1993	29, 335
dalfopristin	antibiotic	1999	35, 338
dapiprazole HCl	antiglaucoma	1987	23, 332
daptomycin	antibiotic	2003	39, 347
defeiprone	iron chelator	1995	31, 340
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
delapril	antihypertensive	1989	25, 311
delavirdine mesylate	antiviral	1997	33, 331
denileukin diftitox	anticancer	1999	35, 338
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
desloratadine	antihistamine	2001	37, 264
dexfenfluramine	antiobesity	1997	33, 332
dexibuprofen	antiinflammatory	1994	30, 298
dexmedetomidine hydrochloride	sedative	2000	36, 301
dexmethylphenidate HCl	psychostimulant	2002	38, 352
dexrazoxane	cardioprotective	1992	28, 330
dezocine	analgesic	1991	27, 326
diacerein	antirheumatic	1985	21, 326
didanosine	antiviral	1991	27, 326
dilevalol	antihypertensive	1989	25, 311
dirithromycin	antibiotic	1993	29, 336
disodium pamidronate	calcium regulator	1989	25, 312
divistyramine	hypocholesterolemic	1984	20, 317
docarpamine	cardiostimulant	1994	30, 298

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
docetaxel	antineoplastic	1995	31, 341
dofetilide	antiarrhythmic	2000	36, 301
dolasetron mesylate	antiemetic	1998	34, 321
donepezil HCl	anti-Alzheimer	1997	33, 332
dopexamine	cardiostimulant	1989	25, 312
dornase alfa	cystic fibrosis	1994	30, 298
dorzolamide HCL	antiglaucoma	1995	31, 341
dosmalfate	antiulcer	2000	36, 302
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxercalciferol	vitamin D prohormone	1999	35, 339
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
drospirenone	contraceptive	2000	36, 302
drotrecogin alfa	antisepsis	2001	37, 265
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
dutasteride	5α reductase inhibitor	2002	38, 353
duteplase	anticougulant	1995	31, 342
ebastine	antihistamine	1990	26, 302
ebrotidine	antiulcer	1997	33, 333
ecabet sodium	antiulcerative	1993	29, 336
edaravone	neuroprotective	2001	37, 265
efalizumab	psoriasis	2003	39, 349
efavirenz	antiviral	1998	34, 321
efonidipine	antihypertensive	1994	30, 299
egualen sodium	antiulcer	2000	36, 303
eletriptan	antimigraine	2001	37, 266
emedastine difumarate	antiallergic/antiasthmatic	1993	29, 336
emorfazone	analgesic	1984	20, 317
emtricitabine	antiviral	2003	39, 350
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
enfuvirtide	antiviral	2003	39, 350
enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
entacapone	antiparkinsonian	1998	34, 322
epalrestat	antidiabetic	1992	28, 330
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epinastine	antiallergic	1994	30, 299
epirubicin HCl	antineoplastic	1984	20, 318

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
eplerenone	antihypertensive	2003	39, 352
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eprosartan	antihypertensive	1997	33, 333
eptazocine HBr	analgesic	1987	23, 334
eptilfibatide	antithrombotic	1999	35, 340
erdosteine	expectorant	1995	31, 342
ertapenem sodium	antibacterial	2002	38, 353
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
escitalopram oxolate	antidepressant	2002	38, 354
esmolol HCl	antiarrhythmic	1987	23, 334
esomeprazole magnesium	gastric antisecretory	2000	36, 303
ethyl icosapentate	antithrombotic	1990	26, 303
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
etoricoxibe	antiarthritic/analgesic	2002	38, 355
exemestane	anticancer	2000	36, 304
exifone	nootropic	1988	24, 302
ezetimibe	hypolipidemic	2002	38, 355
factor VIIa	haemophilia	1996	32, 307
factor VIII	hemostatic	1992	28, 330
fadrozole HCl	antineoplastic	1995	31, 342
falecalcitriol	vitamin D	2001	37, 266
famciclovir	antiviral	1994	30, 300
famotidine	antiulcer	1985	21, 327
fasudil HCl	neuroprotective	1995	31, 343
felbamate	antiepileptic	1993	29, 337
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenoldopam mesylate	antihypertensive	1998	34, 322
fenticonazole nitrate	antifungal	1987	23, 334
fexofenadine	antiallergic	1996	32, 307
filgrastim	immunostimulant	1991	27, 327
finasteride	5α-reductase inhibitor	1992	28, 331
fisalamine	intestinal antiinflammatory	1984	20, 318
fleroxacin	antibacterial	1992	28, 331
flomoxef sodium	antibiotic	1988	24, 302
flosequinan	cardiostimulant	1992	28, 331
fluconazole	antifungal	1988	24, 303
fludarabine phosphate	antineoplastic	1991	27, 327
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flurithromycin ethylsuccinate	antibiotic	1997	33, 333
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
fluticasone propionate	antiinflammatory	1990	26, 303
flutoprazepam	anxiolytic	1986	22, 320
flutrimazole	topical antifungal	1995	31, 343
flutropium bromide	antitussive	1988	24, 303
fluvastatin	hypolipaemic	1994	30, 300
fluvoxamine maleate	antidepressant	1983	19, 319
follitropin alfa	fertility enhancer	1996	32, 307
follitropin beta	fertility enhancer	1996	32, 308
fomepizole	antidote	1998	34, 323
fomivirsen sodium	antiviral	1998	34, 323
fondaparinux sodium	antithrombotic	2002	38, 356
formestane	antineoplastic	1993	29, 337
formoterol fumarate	bronchodilator	1986	22, 321
fosamprenavir	antiviral	2003	39, 353
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosinopril sodium	antihypertensive	1991	27, 328
fosphenytoin sodium	antiepileptic	1996	32, 308
fotemustine	antineoplastic	1989	25, 313
fropenam	antibiotic	1997	33, 334
frovatriptan	antimigraine	2002	38, 357
fudosteine	expectorant	2001	37, 267
fulveristrant	anticancer	2002	38, 357
gabapentin	antiepileptic	1993	29, 338
gadoversetamide	MRI contrast agent	2000	36, 304
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303
ganirelix acetate	female infertility	2000	36, 305
gatilfloxacin	antibiotic	1999	35, 340
gefitinib	antineoplastic	2002	38, 358
gemcitabine HCl	antineoplastic	1995	31, 344
gemeprost	abortifacient	1983	19, 319
gemtuzumab ozogamicin	anticancer	2000	36, 306
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
glatiramer acetate	Multiple Sclerosis	1997	33, 334
glimepiride	antidiabetic	1995	31, 344
glucagon, rDNA	hypoglycemia	1993	29, 338
GMDP	immunostimulant	1996	32, 308
goserelin	hormone	1987	23, 336
granisetron HCl	antiemetic	1991	27, 329
guanadrel sulfate	antihypertensive	1983	19, 319
gusperimus	immunosuppressant	1994	30, 300
halobetasol propionate	topical antiinflammatory	1991	27, 329
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
histrelin	precocious puberty	1993	29, 338

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320
ibandronic acid	osteoporosis	1996	32, 309
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
ibutilide fumarate	antiarrhythmic	1996	32, 309
ibritunomab tiuxetan	anticancer	2002	38, 359
idarubicin HCl	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
iloprost	platelet aggreg. inhibitor	1992	28, 332
imatinib mesylate	antineoplastic	2001	37, 267
imidapril HCl	antihypertensive	1993	29, 339
imiglucerase	Gaucher's disease	1994	30, 301
imipenem/cilastatin	antibiotic	1985	21, 328
imiquimod	antiviral	1997	33, 335
incadronic acid	osteoporosis	1997	33, 335
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indinavir sulfate	antiviral	1996	32, 310
indobufen	antithrombotic	1984	20, 319
influenza virus (live)	antiviral vaccine	2003	39, 353
insulin lispro	antidiabetic	1996	32, 310
interferon alfacon-1	antiviral	1997	33, 336
interferon gamma-1b	immunostimulant	1991	27, 329
interferon, gamma	antiinflammatory	1989	25, 314
interferon, gamma-1α	antineoplastic	1992	28, 332
interferon, β − 1a	multiple sclerosis	1996	32, 311
interferon, β-1b	multiple sclerosis	1993	29, 339
interleukin-2	antineoplastic	1989	25, 314
ioflupane	diagnosis CNS	2000	36, 306
ipriflavone	calcium regulator	1989	25, 314
irbesartan	antihypertensive	1997	33, 336
irinotecan	antineoplastic	1994	30, 301
irsogladine	antiulcer	1989	25, 315
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam	antiinflammatory	1983	19, 320
isradipine	antihypertensive	1989	25, 315
itopride HCl	gastroprokinetic	1995	31, 344
itraconazole	antifungal	1988	24, 305
ivermectin	antiparasitic	1987	23, 336
ketanserin	antihypertensive	1985	21, 328
ketorolac tromethamine	analgesic	1990	26, 304
kinetin	skin photodamage/ dermatologic	1999	35, 341
lacidipine	antihypertensive	1991	27, 330
lafutidine	gastric antisecretory	2000	36, 307
lamivudine	antiviral	1995	31, 345

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
lamotrigine	anticonvulsant	1990	26, 304
landiolol	antiarrhythmic	2002	38, 360
lanoconazole	antifungal	1994	30, 302
lanreotide acetate	acromegaly	1995	31, 345
lansoprazole	antiulcer	1992	28, 332
laronidase	mucopolysaccaridosis I	2003	39, 354
latanoprost	antiglaucoma	1996	32, 311
lefunomide	antiarthritic	1998	34, 324
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
lepirudin	anticoagulant	1997	33, 336
lercanidipine	antihyperintensive	1997	33, 337
letrazole	anticancer	1996	32, 311
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levalbuterol HCl	antiasthmatic	1999	35, 341
levetiracetam	antiepileptic	2000	36, 307
levobunolol HCl	antiglaucoma	1985	21, 328
levobupivacaine hydrochloride	local anesthetic	2000	36, 308
levocabastine HCl	antihistamine	1991	27, 330
levocetirizine	antihistamine	2001	37, 268
levodropropizine	antitussive	1988	24, 305
levofloxacin	antibiotic	1993	29, 340
levosimendan	heart failure	2000	36, 308
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
linezolid	antibiotic	2000	36, 309
liranaftate	topical antifungal	2000	36, 309
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lodoxamide tromethamine	antiallergic ophthalmic	1992	28, 333
lomefloxacin	antibiotic	1989	25, 315
lomerizine HCl	antimigraine	1999	35, 342
lonidamine	antineoplastic	1987	23, 337
lopinavir	antiviral	2000	36, 310
loprazolam mesylate	hypnotic	1983	19, 321
loprinone HCl	cardiostimulant	1996	32, 312
loracarbef	antibiotic	1992	28, 333
loratadine	antihistamine	1988	24, 306
lornoxicam	NSAID	1997	33, 337
losartan	antihypertensive	1994	30, 302
loteprednol etabonate	antiallergic ophthalmic	1998	34; 324
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
Lyme disease	vaccine	1999	35, 342
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatoprotective	1985	21, 329
manidipine HCl	antihypertensive	1990	26, 304

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
masoprocol	topical antineoplastic	1992	28, 333
maxacalcitol	vitamin D	2000	36, 310
mebefradil HCl	antihypertensive	1997	33, 338
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
meloxicam	antiarthritic	1996	32, 312
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
meropenem	carbapenem antibiotic	1994	30, 303
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
micafungin	antifungal	2002	38, 360
mifepristone	abortifacient	1988	24, 306
miglitol	antidiabetic	1998	34, 325
miglustat	gaucher's disease	2003	39, 355
milnacipran	antidepressant	1997	33, 338
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303
misoprostol	antiulcer	1985	21, 329
mitoxantrone HCl	antineoplastic	1984	20, 321
mivacurium chloride	muscle relaxant	1992	28, 334
mivotilate	hepatoprotectant	1999	35, 343
mizolastine	antihistamine	1998	34, 325
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
modafinil	idiopathic hypersomnia	1994	30, 303
moexipril HCl	antihypertensive	1995	31, 346
mofezolac	analgesic	1994	30, 304
mometasone furoate	topical antiinflammatory	1987	23, 338
montelukast sodium	antiasthma	1998	34, 328
moricizine HCl	antiarrhythmic	1990	26, 305
mosapride citrate	gastroprokinetic	1998	34, 326
moxifloxacin HCL	antibiotic	1999	35, 343
moxonidine	antihypertensive	1991	27, 330
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
mycophenolate mofetil	immunosuppressant	1995	31, 346
mycophenolate sodium	immunosuppressant	2003	39, 356
nabumetone	antiinflammatory	1985	21, 330
nadifloxacin	topical antibiotic	1993	29, 340
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
naftifine HCl	antifungal	1984	20, 321
naftopidil	dysuria	1999	35, 344
nalmefene HCl	dependence treatment	1995	31, 347
naltrexone HCl	narcotic antagonist	1984	20, 322
naratriptan HCl	antimigraine	1997	33, 339
nartograstim	leukopenia	1994	30, 304
nateglinide	antidiabetic	1999	35, 344
nazasetron	antiemetic	1994	30, 305
nebivolol	antihypertensive	1997	33, 339
nedaplatin	antineoplastic	1995	31, 347
nedocromil sodium	antiallergic	1986	22, 324
nefazodone	antidepressant	1994	30, 305
neflinavir mesylate	antiviral	1997	33, 340
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
neridronic acide	calcium regulator	2002	38, 361
nesiritide	congestive heart failure	2001	37, 269
neticonazole HCl	topical antifungal	1993	29, 341
nevirapine	antiviral	1996	32, 313
nicorandil	coronary vasodilator	1984	20, 322
nifekalant HCl	antiarrythmic	1999	35, 344
nilutamide	antineoplastic	1987	23, 338
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
nipradilol	antihypertensive	1988	24, 307
nisoldipine	antihypertensive	1990	26, 306
nitisinone	antityrosinaemia	2002	38, 361
nitrefazole	alcohol deterrent	1983	19, 322
nitrendipine	hypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norelgestromin	contraceptive	2002	38, 362
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
OCT-43	anticancer	1999	35, 345
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
olanzapine	neuroleptic	1996	32, 313
olimesartan Medoxomil	antihypertensive	2002	38, 363
olopatadine HCl	antiallergic	1997	33, 340
omalizumab	allergic asthma	2003	39, 357
omeprazole	antiulcer	1988	24, 308
ondansetron HCl	antiemetic	1990	26, 306
OP-1	osteoinductor	2001	37, 269
orlistat	antiobesity	1998	34, 327
ornoprostil	antiulcer	1987	23, 339

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
osalazine sodium	intestinal antinflamm.	1986	22, 324
oseltamivir phosphate	antiviral	1999	35, 346
oxaliplatin	anticancer	1996	32, 313
oxaprozin	antiinflammatory	1983	19, 322
oxcarbazepine	anticonvulsant	1990	26, 307
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
paclitaxal	antineoplastic	1993	29, 342
palonosetron	antiemetic	2003	39, 358
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
pantoprazole sodium	antiulcer	1995	30, 306
parecoxib sodium	analgesic	2002	38, 364
paricalcitol	vitamin D	1998	34, 327
parnaparin sodium	anticoagulant	1993	29, 342
paroxetine	antidepressant	1991	27, 331
pazufloxacin	antibacterial	2002	38, 364
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pegaspargase	antineoplastic	1994	30, 306
pegvisomant	acromegaly	2003	39, 359
pemirolast potassium	antiasthmatic	1991	27, 331
penciclovir	antiviral	1996	32, 314
pentostatin	antineoplastic	1992	28, 334
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
perospirone HCL	neuroleptic	2001	37, 270
picotamide	antithrombotic	1987	23, 340
pidotimod	immunostimulant	1993	29, 343
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide HCl	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pimecrolimus	immunosuppressant	2002	38, 365
pimobendan	heart failure	1994	30, 307
pinacidil	antihypertensive	1987	23, 340
pioglitazone HCL	antidiabetic	1999	35, 346
pirarubicin	antineoplastic	1988	24, 309
pirmenol	antiarrhythmic	1994	30, 307
piroxicam cinnamate	antiinflammatory	1988	24, 309
pitavastatin	hypocholesterolemic	2003	39, 360
pivagabine	antidepressant	1997	33, 341
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
pramipexole HCl	antiParkinsonian	1997	33, 341
pramiracetam H_2SO_4	cognition enhancer	1993	29, 343
pranlukast	antiasthmatic	1995	31, 347

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
prezatide copper acetate	vulnery	1996	32, 314
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propagermanium	antiviral	1994	30, 308
propentofylline propionate	cerebral vasodilator	1988	24, 310
propiverine HCl	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
prulifloxacin	antibacterial	2002	38, 366
pumactant	lung surfactant	1994	30, 308
quazepam	hypnotic	1985	21, 332
quetiapine fumarate	neuroleptic	1997	33, 341
quinagolide	hyperprolactinemia	1994	30, 309
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
quinupristin	antibiotic	1999	35, 338
rabeprazole sodium	gastric antisecretory	1998	34, 328
raloxifene HCl	osteoporosis	1998	34, 328
raltitrexed	anticancer	1996	32, 315
ramatroban	antiallergic	2000	36, 311
ramipril	antihypertensive	1989	25, 317
ramosetron	antiemetic	1996	32, 315
ranimustine	antineoplastic	1987	23, 341
ranitidine bismuth citrate	antiulcer	1995	31, 348
rapacuronium bromide	muscle relaxant	1999	35, 347
rebamipide	antiulcer	1990	26, 308
reboxetine	antidepressant	1997	33, 342
remifentanil HCl	analgesic	1996	32, 316
remoxipride HCl	antipsychotic	1990	26, 308
repaglinide	antidiabetic	1998	34, 329
repirinast	antiallergic	1987	23, 341
reteplase	fibrinolytic	1996	32, 316
reviparin sodium	anticoagulant	1993	29, 344
rifabutin	antibacterial	1992	28, 335
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
riluzole	neuroprotective	1996	32, 316
rimantadine HCl	antiviral	1987	23, 342
rimexolone	antiinflammatory	1995	31, 348
risedronate sodium	osteoporosis	1998	34, 330
risperidone	neuroleptic	1993	29, 344
ritonavir	antiviral	1996	32, 317
rivastigmin	anti-Alzheimer	1997	33, 342

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
rizatriptan benzoate	antimigraine	1998	34, 330
rocuronium bromide	neuromuscular blocker	1994	30, 309
rofecoxib	antiarthritic	1999	35, 347
rokitamycin	antibiotic	1986	22, 325
romurtide	immunostimulant	1991	27, 332
ronafibrate	hypolipidemic	1986	22, 326
ropinirole HCl	antiParkinsonian	1996	32, 317
ropivacaine	anesthetic	1996	32, 318
rosaprostol	antiulcer	1985	21, 332
rosiglitazone maleate	antidiabetic	1999	35, 348
rosuvastatin	hypocholesterolemic	2003	39, 361
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin HCl	antibacterial	1992	28, 335
rupatadine fumarate	antiallergic	2003	39, 362
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin HCl	hyperphenylalaninemia	1992	28, 336
saquinavir mesvlate	antiviral	1995	31, 349
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 328
seratrodast	antiasthmatic	1995	31, 349
sertaconazole nitrate	topical antifungal	1992	28, 336
sertindole	neuroleptic	1996	32, 318
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline HCl	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
sibutramine	antiobesity	1998	34, 331
sildenafil citrate	male sexual dysfunction	1998	34, 331
simvastatin	hypocholesterolemic	1988	24, 311
sivelestat	anti-inflammatory	2002	38, 366
SKI-2053R	anticancer	1999	35, 348
sobuzoxane	antineoplastic	1994	30, 310
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatomedin-1	growth hormone insensitivity	1994	30, 310
somatotropin	growth hormone	1994	30, 310
somatropin	hormone	1987	23, 343
sorivudine	antiviral	1993	29, 345
sparfloxacin	antibiotic	1993	29, 345
spirapril HCl	antihypertensive	1995	31, 349
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
succimer	chelator	1991	27, 333

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
sufentanil	analgesic	1983	19, 323
sulbactam sodium	β-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
suplatast tosilate	antiallergic	1995	31, 350
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
tacalcitol	topical antipsoriatic	1993	29, 346
tacrine HCl	Alzheimer's disease	1993	29, 346
tacrolimus	immunosuppressant	1993	29, 347
tadalafil	male sexual dysfunction	2003	39, 363
talipexole	antiParkinsonian	1996	32, 318
taltirelin	CNS stimulant	2000	36, 311
tamsulosin HCl	antiprostatic hypertrophy	1993	29, 347
tandospirone	anxiolytic	1996	32, 319
tasonermin	anticancer	1999	35, 349
tazanolast	antiallergic	1990	26, 309
tazarotene	antipsoriasis	1997	33, 343
tazobactam sodium	β-lactamase inhibitor	1992	28, 336
tegaserod maleate	irritable bowel syndrome	2001	37, 270
teicoplanin	antibacterial	1988	24, 311
telithromycin	antibiotic	2001	37, 271
telmesteine	mucolytic	1992	28, 337
telmisartan	antihypertensive	1999	35, 349
temafloxacin HCl	antibacterial	1991	27, 334
temocapril	antihypertensive	1994	30, 311
temocillin disodium	antibiotic	1984	20, 323
temoporphin	antineoplastic/ photosensitizer	2002	38, 367
temozolomide	anticancer	1999	35, 349
tenofovir disoproxil fumarate	antiviral	2001	37, 271
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terbinafine HCl	antifungal	1991	27, 334
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333
tiagabine	antiepileptic	1996	32, 319
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
tilisolol HCl	antihypertensive	1992	28, 337
tiludronate disodium	Paget's disease	1995	31, 350
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
tiopronin	urolithiasis	1989	25, 318
tiotropium bromide	bronchodilator	2002	38, 368
tiquizium bromide	antispasmodic	1984	20, 324
tiracizine HCl	antiarrhythmic	1990	26, 310
tirilazad mesylate	subarachnoid hemorrhage	1995	31, 351
tirofiban HCl	antithrombotic	1998	34, 332
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
tolcapone	antiParkinsonian	1997	33, 343
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
topiramate	antiepileptic	1995	31, 351
topotecan HCl	anticancer	1996	32, 320
torasemide	diuretic	1993	29, 348
toremifene	antineoplastic	1989	25, 319
tositumomab	anticancer	2003	39, 364
tosufloxacin tosylate	antibacterial	1990	26, 310
trandolapril	antihypertensive	1993	29, 348
travoprost	antiglaucoma	2001	37, 272
treprostinil sodium	antihypertensive	2002	38, 368
tretinoin tocoferil	antiulcer	1993	29, 348
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
trimegestone	progestogen	2001	37, 273
trimetrexate glucuronate	*Pneumocystis carinii* pneumonia	1994	30, 312
troglitazone	antidiabetic	1997	33, 344
tropisetron	antiemetic	1992	28, 337
trovafloxacin mesylate	antibiotic	1998	34, 332
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
unoprostone isopropyl ester	antiglaucoma	1994	30, 312
valaciclovir HCl	antiviral	1995	31, 352
vadecoxib	antiarthritic	2002	38, 369
vaglancirclovir HCL	antiviral	2001	37, 273
valrubicin	anticancer	1999	35, 350
valsartan	antihypertensive	1996	32, 320
vardenafil	male sexual dysfunction	2003	39, 365
venlafaxine	antidepressant	1994	30, 312
verteporfin	photosensitizer	2000	36, 312
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
voglibose	antidiabetic	1994	30, 313
voriconazole	antifungal	2002	38, 370
xamoterol fumarate	cardiotonic	1988	24, 312
zafirlukast	antiasthma	1996	32, 321
zalcitabine	antiviral	1992	28, 338

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
zaleplon	hypnotic	1999	35, 351
zaltoprofen	antiinflammatory	1993	29, 349
zanamivir	antiviral	1999	35, 352
zidovudine	antiviral	1987	23, 345
zileuton	antiasthma	1997	33, 344
zinostatin stimalamer	antineoplastic	1994	30, 313
ziprasidone hydrochloride	neuroleptic	2000	36, 312
zofenopril calcium	antihypertensive	2000	36, 313
zoledronate disodium	hypercalcemia	2000	36, 314
zolpidem hemitartrate	hypnotic	1988	24, 313
zomitriptan	antimigraine	1997	33, 345
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

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GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19 (319)
mifepristone		1988	24 (306)
lanreotide acetate	ACROMEGALY	1995	31 (345)
pegvisomant		2003	39 (359)
nitrefazole	ALCOHOL DETERRENT	1983	19 (322)
omalizumab	ALLERGIC ASTHMA	2003	39 (357)
tacrine HCl	ALZHEIMER'S DISEASE	1993	29 (346)
quinfamide	AMEBICIDE	1984	20 (322)
tibolone	ANABOLIC	1988	24 (312)
mepixanox	ANALEPTIC	1984	20 (320)
alfentanil HCl	ANALGESIC	1983	19 (314)
alminoprofen		1983	19 (314)
dezocine		1991	27 (326)
emorfazone		1984	20 (317)
eptazocine HBr		1987	23 (334)
etoricoxib		2002	38 (355)
flupirtine maleate		1985	21 (328)
fosfosal		1984	20 (319)
ketorolac tromethamine		1990	26 (304)
meptazinol HCl		1983	19 (321)
mofezolac		1994	30 (304)
parecoxib sodium		2002	38 (364)
propacetamol HCl		1986	22 (325)
remifentanil HCl		1996	32 (316)
sufentanil		1983	19 (323)
suprofen		1983	19 (324)
desflurane	ANESTHETIC	1992	28 (329)
propofol		1986	22 (325)
ropivacaine		1996	32 (318)
sevoflurane		1990	26 (309)
levobupivacaine hydrochloride	ANESTHETIC, LOCAL	2000	36 (308)
azelaic acid	ANTIACNE	1989	25 (310)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
betotastine besilate	ANTIALLERGIC	2000	36 (297)
emedastine difumarate		1993	29 (336)
epinastine		1994	30 (299)
fexofenadine		1996	32 (307)
nedocromil sodium		1986	22 (324)
olopatadine hydrochloride		1997	33 (340)
ramatroban		2000	36 (311)
repirinast		1987	23 (341)
suplatast tosilate		1995	31 (350)
tazanolast		1990	26 (309)
lodoxamide tromethamine	ANTIALLERGIC	1992	28 (333)
rupatadine fumarate		2003	39 (362)
loteprednol etabonate	OPHTHALMIC	1998	34 (324)
donepezil hydrochloride	ANTI-ALZHEIMERS	1997	33 (332)
rivastigmin		1997	33 (342)
gallopamil HCl	ANTIANGINAL	1983	19 (319)
cibenzoline	ANTIARRHYTHMIC	1985	21 (325)
dofetilide		2000	36 (301)
encainide HCl		1987	23 (333)
esmolol HCl		1987	23 (334)
ibutilide fumarate		1996	32 (309)
landiolol		2002	38 (360)
moricizine hydrochloride		1990	26 (305)
nifekalant HCl		1999	35 (344)
pilsicainide hydrochloride		1991	27 (332)
pirmenol		1994	30 (307)
tiracizine hydrochloride		1990	26 (310)
anakinra	ANTIARTHRITIC	2001	37 (261)
celecoxib		1999	35 (335)
etoricoxib		2002	38 (355)
meloxicam		1996	32 (312)
leflunomide		1998	34 (324)
rofecoxib		1999	35 (347)
valdecoxib		2002	38 (369)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
amlexanox	ANTIASTHMATIC	1987	23 (327)
emedastine difumarate		1993	29 (336)
ibudilast		1989	25 (313)
levalbuterol HCl		1999	35 (341)
montelukast sodium		1998	34 (328)
pemirolast potassium		1991	27 (331)
seratrodast		1995	31 (349)
zafirlukast		1996	32 (321)
zileuton		1997	33 (344)
balofloxacin	ANTIBACTERIAL	2002	38 (351)
biapenem		2002	38 (351)
ciprofloxacin		1986	22 (318)
enoxacin		1986	22 (320)
ertapenem sodium		2002	38 (353)
fleroxacin		1992	28 (331)
norfloxacin		1983	19 (322)
ofloxacin		1985	21 (331)
pazufloxacin		2002	38 (364)
pefloxacin mesylate		1985	21 (331)
pranlukast		1995	31 (347)
prulifloxacin		2002	38 (366)
rifabutin		1992	28 (335)
rifapentine		1988	24 (310)
rufloxacin hydrochloride		1992	28 (335)
teicoplanin		1988	24 (311)
temafloxacin hydrochloride		1991	27 (334)
tosufloxacin tosylate		1990	26 (310)
arbekacin	ANTIBIOTIC	1990	26 (298)
aspoxicillin		1987	23 (328)
astromycin sulfate		1985	21 (324)
azithromycin		1988	24 (298)
aztreonam		1984	20 (315)
brodimoprin		1993	29 (333)
carboplatin		1986	22 (318)
carumonam		1988	24 (298)
cefbuperazone sodium		1985	21 (325)
cefcapene pivoxil hydrochloride		1997	33 (330)
cefdinir		1991	27 (323)
cefepime		1993	29 (334)
cefetamet pivoxil hydrochloride		1992	28 (327)
cefixime		1987	23 (329)
cefmenoxime HCl		1983	19 (316)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefminox sodium		1987	23 (330)
cefodizime sodium		1990	26 (300)
cefonicid sodium		1984	20 (316)
ceforanide		1984	20 (317)
cefoselis		1998	34 (319)
cefotetan disodium		1984	20 (317)
cefotiam hexetil hydrochloride		1991	27 (324)
cefpimizole		1987	23 (330)
cefpiramide sodium		1985	21 (325)
cefpirome sulfate		1992	28 (328)
cefpodoxime proxetil		1989	25 (310)
cefprozil		1992	28 (328)
ceftazidime		1983	19 (316)
cefteram pivoxil		1987	23 (330)
ceftibuten		1992	28 (329)
cefuroxime axetil		1987	23 (331)
cefuzonam sodium		1987	23 (331)
clarithromycin		1990	26 (302)
dalfopristin		1999	35 (338)
daptomycin		2003	39 (347)
dirithromycin		1993	29 (336)
erythromycin acistrate		1988	24 (301)
flomoxef sodium		1988	24 (302)
flurithromycin ethylsuccinate		1997	33 (333)
fropenam		1997	33 (334)
gatifloxacin		1999	35 (340)
imipenem/cilastatin		1985	21 (328)
isepamicin		1988	24 (305)
lenampicillin HCl		1987	23 (336)
levofloxacin		1993	29 (340)
linezolid		2000	36 (309)
lomefloxacin		1989	25 (315)
loracarbef		1992	28 (333)
miokamycin		1985	21 (329)
moxifloxacin HCl		1999	35 (343)
quinupristin		1999	35 (338)
rifaximin		1985	21 (332)
rifaximin		1987	23 (341)
rokitamycin		1986	22 (325)
RV-11		1989	25 (318)
sparfloxacin		1993	29 (345)
sultamycillin tosylate		1987	23 (343)
telithromycin		2001	37 (271)
temocillin disodium		1984	20 (323)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
trovafloxacin mesylate		1998	34 (332)
meropenem	ANTIBIOTIC, CARBAPENEM	1994	30 (303)
panipenem/ betamipron		1994	30 (305)
mupirocin	ANTIBIOTIC, TOPICAL	1985	21 (330)
nadifloxacin		1993	29 (340)
alemtuzumab	ANTICANCER	2001	37 (260)
alitretinoin		1999	35 (333)
arglabin		1999	35 (335)
bexarotene		2000	36 (298)
bortezomib		2003	39 (345)
cetuximab		2003	39 (346)
denileukin diftitox		1999	35 (338)
exemestane		2000	36 (304)
fulvestrant		2002	38 (357)
gemtuzumab ozogamicin		2000	36 (306)
ibritumomab tiuxetan		2002	38 (359)
letrazole		1996	32 (311)
OCT-43		1999	35 (345)
oxaliplatin		1996	32 (313)
raltitrexed		1996	32 (315)
SKI-2053R		1999	35 (348)
tasonermin		1999	35 (349)
temozolomide		1999	35 (350)
topotecan HCl		1996	32 (320)
tositumomab		2003	39 (364)
valrubicin		1999	35 (350)
angiotensin II	ANTICANCER ADJUVANT	1994	30 (296)
chenodiol	ANTICHOLELITHOGENIC	1983	19 (317)
duteplase	ANTICOAGULANT	1995	31 (342)
lepirudin		1997	33 (336)
parnaparin sodium		1993	29 (342)
reviparin sodium		1993	29 (344)
lamotrigine	ANTICONVULSANT	1990	26 (304)
oxcarbazepine		1990	26 (307)
progabide		1985	21 (331)
vigabatrin		1989	25 (319)
zonisamide		1989	25 (320)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
bupropion HCl	ANTIDEPRESSANT	1989	25 (310)
citalopram		1989	25 (311)
escitalopram oxalate		2002	38 (354)
fluoxetine HCl		1986	22 (320)
fluvoxamine maleate		1983	19 (319)
indalpine		1983	19 (320)
medifoxamine fumarate		1986	22 (323)
metapramine		1984	20 (320)
milnacipran		1997	33 (338)
mirtazapine		1994	30 (303)
moclobemide		1990	26 (305)
nefazodone		1994	30 (305)
paroxetine		1991	27 (331)
pivagabine		1997	33 (341)
reboxetine		1997	33 (342)
setiptiline		1989	25 (318)
sertraline hydrochloride		1990	26 (309)
tianeptine sodium		1983	19 (324)
toloxatone		1984	20 (324)
venlafaxine		1994	30 (312)
acarbose	ANTIDIABETIC	1990	26 (297)
epalrestat		1992	28 (330)
glimepiride		1995	31 (344)
insulin lispro		1996	32 (310)
miglitol		1998	34 (325)
nateglinide		1999	35 (344)
pioglitazone HCl		1999	35 (346)
repaglinide		1998	34 (329)
rosiglitazone maleate		1999	35 (347)
tolrestat		1989	25 (319)
troglitazone		1997	33 (344)
voglibose		1994	30 (313)
acetorphan	ANTIDIARRHEAL	1993	29 (332)
anti-digoxin polyclonal antibody	ANTIDOTE	2002	38 (350)
crotelidae polyvalent immune fab		2001	37 (263)
fomepizole		1998	34 (323)
aprepitant	ANTIEMETIC	2003	39 (341)
dolasetron mesylate		1998	34 (321)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
granisetron hydrochloride		1991	27 (329)
ondansetron hydrochloride		1990	26 (306)
nazasetron		1994	30 (305)
palonosetron		2003	39 (358)
ramosetron		1996	32 (315)
tropisetron		1992	28 (337)
felbamate	ANTIEPILEPTIC	1993	29 (337)
fosphenytoin sodium		1996	32 (308)
gabapentin		1993	29 (338)
levetiracetam		2000	36 (307)
tiagabine		1996	32 (320)
topiramate		1995	31 (351)
centchroman	ANTIESTROGEN	1991	27 (324)
caspofungin acetate	ANTIFUNGAL	2001	37 (263)
fenticonazole nitrate		1987	23 (334)
fluconazole		1988	24 (303)
itraconazole		1988	24 (305)
lanoconazole		1994	30 (302)
micafungin		2002	38 (360)
naftifine HCl		1984	20 (321)
oxiconazole nitrate		1983	19 (322)
terbinafine hydrochloride		1991	27 (334)
terconazole		1983	19 (324)
tioconazole		1983	19 (324)
voriconazole		2002	38 (370)
amorolfine hydrochloride	ANTIFUNGAL, TOPICAL	1991	27 (322)
butenafine hydrochloride		1992	28 (327)
butoconazole		1986	22 (318)
cloconazole HCl		1986	22 (318)
liranaftate		2000	36 (309)
flutrimazole		1995	31 (343)
neticonazole HCl		1993	29 (341)
sertaconazole nitrate		1992	28 (336)
sulconizole nitrate		1985	21 (332)
apraclonidine HCl	ANTIGLAUCOMA	1988	24 (297)
befunolol HCl		1983	19 (315)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
bimatroprost		2001	37 (261)
brimonidine		1996	32 (306)
brinzolamide		1998	34 (318)
dapiprazole HCl		1987	23 (332)
dorzolamide HCl		1995	31 (341)
latanoprost		1996	32 (311)
levobunolol HCl		1985	21 (328)
travoprost		2001	37 (272)
unoprostone isopropyl ester		1994	30 (312)
acrivastine	ANTIHISTAMINE	1988	24 (295)
astemizole		1983	19 (314)
azelastine HCl		1986	22 (316)
cetirizine HCl		1987	23 (331)
desloratadine		2001	37 (264)
ebastine		1990	26 (302)
levocabastine hydrochloride		1991	27 (330)
levocetirizine		2001	37 (268)
loratadine		1988	24 (306)
mizolastine		1998	34 (325)
setastine HCl		1987	23 (342)
alacepril	ANTIHYPERTENSIVE	1988	24 (296)
alfuzosin HCl		1988	24 (296)
amlodipine besylate		1990	26 (298)
amosulalol		1988	24 (297)
aranidipine		1996	32 (306)
arotinolol HCl		1986	22 (316)
azelnidipine		2003	39 (344)
barnidipine hydrochloride		1992	28 (326)
benazepril hydrochloride		1990	26 (299)
benidipine hydrochloride		1991	27 (322)
betaxolol HCl		1983	19 (315)
bevantolol HCl		1987	23 (328)
bisoprolol fumarate		1986	22 (317)
bopindolol		1985	21 (324)
bosentan		2001	37 (262)
budralazine		1983	19 (315)
bunazosin HCl		1985	21 (324)
candesartan cilexetil		1997	33 (330)
carvedilol		1991	27 (323)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
celiprolol HCl		1983	19 (317)
cicletanine		1988	24 (299)
cilazapril		1990	26 (301)
cinildipine		1995	31 (339)
delapril		1989	25 (311)
dilevalol		1989	25 (311)
doxazosin mesylate		1988	24 (300)
efonidipine		1994	30 (299)
enalapril maleate		1984	20 (317)
enalaprilat		1987	23 (332)
eplerenone		2003	39 (352)
eprosartan		1997	33 (333)
felodipine		1988	24 (302)
fenoldopam mesylate		1998	34 (322)
fosinopril sodium		1991	27 (328)
guanadrel sulfate		1983	19 (319)
imidapril HCl		1993	29 (339)
irbesartan		1997	33 (336)
isradipine		1989	25 (315)
ketanserin		1985	21 (328)
lacidipine		1991	27 (330)
lercanidipine		1997	33 (337)
lisinopril		1987	23 (337)
losartan		1994	30 (302)
manidipine hydrochloride		1990	26 (304)
mebefradil hydrochloride		1997	33 (338)
moexipril HCl		1995	31 (346)
moxonidine		1991	27 (330)
nebivolol		1997	33 (339)
nilvadipine		1989	25 (316)
nipradilol		1988	24 (307)
nisoldipine		1990	26 (306)
olmesartan medoxomil		2002	38 (363)
perindopril		1988	24 (309)
pinacidil		1987	23 (340)
quinapril		1989	25 (317)
ramipril		1989	25 (317)
rilmenidine		1988	24 (310)
spirapril HCl		1995	31 (349)
telmisartan		1999	35 (349)
temocapril		1994	30 (311)
terazosin HCl		1984	20 (323)
tertatolol HCl		1987	23 (344)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
tiamenidine HCl		1988	24 (311)
tilisolol hydrochloride		1992	28 (337)
trandolapril		1993	29 (348)
treprostinil sodium		2002	38 (368)
trimazosin HCl		1985	21 (333)
valsartan		1996	32 (320)
zofenopril calcium		2000	36 (313)
aceclofenac	ANTIINFLAMMATORY	1992	28 (325)
AF-2259		1987	23 (325)
amfenac sodium		1986	22 (315)
ampiroxicam		1994	30 (296)
amtolmetin guacil		1993	29 (332)
butibufen		1992	28 (327)
deflazacort		1986	22 (319)
dexibuprofen		1994	30 (298)
droxicam		1990	26 (302)
etodolac		1985	21 (327)
flunoxaprofen		1987	23 (335)
fluticasone propionate		1990	26 (303)
interferon, gamma		1989	25 (314)
isofezolac		1984	20 (319)
isoxicam		1983	19 (320)
lobenzarit sodium		1986	22 (322)
loxoprofen sodium		1986	22 (322)
nabumetone		1985	21 (330)
nimesulide		1985	21 (330)
oxaprozin		1983	19 (322)
piroxicam cinnamate		1988	24 (309)
rimexolone		1995	31 (348)
sivelestat		2002	38 (366)
tenoxicam		1987	23 (344)
zaltoprofen		1993	29 (349)
fisalamine	ANTIINFLAMMATORY, INTESTINAL	1984	20 (318)
osalazine sodium		1986	22 (32)
alclometasone dipropionate	ANTIINFLAMMATORY, TOPICAL	1985	21 (323)
aminoprofen		1990	26 (298)
betamethasone butyrate propionate		1994	30 (297)
butyl flufenamate		1983	19 (316)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
deprodone propionate		1992	28 (329)
felbinac		1986	22 (320)
halobetasol propionate		1991	27 (329)
halometasone		1983	19 (320)
hydrocortisone aceponate		1988	24 (304)
hydrocortisone butyrate propionate		1983	19 (320)
mometasone furoate		1987	23 (338)
piketoprofen		1984	20 (322)
pimaprofen		1984	20 (322)
prednicarbate		1986	22 (325)
pravastatin	ANTILIPIDEMIC	1989	25 (316)
arteether	ANTIMALARIAL	2000	36 (296)
artemisinin		1987	23 (327)
bulaquine		2000	36 (299)
halofantrine		1988	24 (304)
mefloquine HCl		1985	21 (329)
almotriptan	ANTIMIGRAINE	2000	36 (295)
alpiropride		1988	24 (296)
eletriptan		2001	37 (266)
frovatriptan		2002	38 (357)
lomerizine HCl		1999	35 (342)
naratriptan hydrochloride		1997	33 (339)
rizatriptan benzoate		1998	34 (330)
sumatriptan succinate		1991	27 (333)
zolmitriptan		1997	33 (345)
dronabinol	ANTINAUSEANT	1986	22 (319)
amrubicin HCl	ANTINEOPLASTIC	2002	38 (349)
amsacrine		1987	23 (327)
anastrozole		1995	31 (338)
bicalutamide		1995	31 (338)
bisantrene hydrochloride		1990	26 (300)
camostat mesylate		1985	21 (325)
capecitabine		1998	34 (319)
cladribine		1993	29 (335)
cytarabine ocfosfate		1993	29 (335)
docetaxel		1995	31 (341)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
doxifluridine		1987	23 (332)
enocitabine		1983	19 (318)
epirubicin HCl		1984	20 (318)
fadrozole HCl		1995	31 (342)
fludarabine phosphate		1991	27 (327)
flutamide		1983	19 (318)
formestane		1993	29 (337)
fotemustine		1989	25 (313)
geftimib		2002	38 (358)
gemcitabine HCl		1995	31 (344)
idarubicin hydrochloride		1990	26 (303)
imatinib mesylate		2001	37 (267)
interferon gamma-1α		1992	28 (332)
interleukin-2		1989	25 (314)
irinotecan		1994	30 (301)
lonidamine		1987	23 (337)
mitoxantrone HCl		1984	20 (321)
nedaplatin		1995	31 (347)
nilutamide		1987	23 (338)
paclitaxal		1993	29 (342)
pegaspargase		1994	30 (306)
pentostatin		1992	28 (334)
pirarubicin		1988	24 (309)
ranimustine		1987	23 (341)
sobuzoxane		1994	30 (310)
temoporphin		2002	38 (367)
toremifene		1989	25 (319)
vinorelbine		1989	25 (320)
zinostatin stimalamer		1994	30 (313)
porfimer sodium	ANTINEOPLASTIC ADJUVANT	1993	29 (343)
masoprocol	ANTINEOPLASTIC, TOPICAL	1992	28 (333)
miltefosine		1993	29 (340)
dexfenfluramine	ANTIOBESITY	1997	33 (332)
orlistat		1998	34 (327)
sibutramine		1998	34 (331)
atovaquone	ANTIPARASITIC	1992	28 (326)
ivermectin		1987	23 (336)
budipine	ANTIPARKINSONIAN	1997	33 (330)
CHF-1301		1999	35 (336)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
droxidopa		1989	25 (312)
entacapone		1998	34 (322)
pergolide mesylate		1988	24 (308)
pramipexole hydrochloride		1997	33 (341)
ropinirole HCl		1996	32 (317)
talipexole		1996	32 (318)
tolcapone		1997	33 (343)
lidamidine HCl	ANTIPERISTALTIC	1984	20 (320)
gestrinone	ANTIPROGESTOGEN	1986	22 (321)
cabergoline	ANTIPROLACTIN	1993	29 (334)
tamsulosin HCl	ANTIPROSTATIC HYPERTROPHY	1993	29 (347)
acitretin	ANTIPSORIATIC	1989	25 (309)
calcipotriol		1991	27 (323)
tazarotene		1997	33 (343)
tacalcitol	ANTIPSORIATIC, TOPICAL	1993	29 (346)
amisulpride	ANTIPSYCHOTIC	1986	22 (316)
remoxipride hydrochloride		1990	26 (308)
zuclopenthixol acetate		1987	23 (345)
actarit	ANTIRHEUMATIC	1994	30 (296)
diacerein		1985	21 (326)
octreotide	ANTISECRETORY	1988	24 (307)
adamantanium bromide	ANTISEPTIC	1984	20 (315)
drotecogin alfa	ANTISEPSIS	2001	37 (265)
cimetropium bromide	ANTISPASMODIC	1985	21 (326)
tiquizium bromide		1984	20 (324)
tiropramide HCl		1983	19 (324)
argatroban	ANTITHROMBOTIC	1990	26 (299)
bivalirudin		2000	36 (298)
defibrotide		1986	22 (319)
cilostazol		1988	24 (299)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
clopidogrel hydrogensulfate		1998	34 (320)
cloricromen		1991	27 (325)
enoxaparin		1987	23 (333)
eptifibatide		1999	35 (340)
ethyl icosapentate		1990	26 (303)
fondaparinux sodium		2002	38 (356)
indobufen		1984	20 (319)
limaprost		1988	24 (306)
ozagrel sodium		1988	24 (308)
picotamide		1987	23 (340)
tirofiban hydrochloride		1998	34 (332)
flutropium bromide	ANTITUSSIVE	1988	24 (303)
levodropropizine		1988	24 (305)
nitisinone	ANTITYROSINAEMIA	2002	38 (361)
benexate HCl	ANTIULCER	1987	23 (328)
dosmalfate		2000	36 (302)
ebrotidine		1997	33 (333)
ecabet sodium		1993	29 (336)
egualen sodium		2000	36 (303)
enprostil		1985	21 (327)
famotidine		1985	21 (327)
irsogladine		1989	25 (315)
lansoprazole		1992	28 (332)
misoprostol		1985	21 (329)
nizatidine		1987	23 (339)
omeprazole		1988	24 (308)
ornoprostil		1987	23 (339)
pantoprazole sodium		1994	30 (306)
plaunotol		1987	23 (340)
polaprezinc		1994	30 (307)
ranitidine bismuth citrate		1995	31 (348)
rebamipide		1990	26 (308)
rosaprostol		1985	21 (332)
roxatidine acetate HCl		1986	22 (328)
roxithromycin		1987	23 (342)
sofalcone		1984	20 (323)
spizofurone		1987	23 (343)
teprenone		1984	20 (323)
tretinoin tocoferil		1993	29 (348)
troxipide		1986	22 (327)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
abacavir sulfate	ANTIVIRAL	1999	35 (333)
adefovir dipivoxil		2002	38 (348)
amprenavir		1999	35 (334)
atazanavir		2003	39 (342)
cidofovir		1996	32 (306)
delavirdine mesylate		1997	33 (331)
didanosine		1991	27 (326)
efavirenz		1998	34 (321)
emtricitabine		2003	39 (350)
enfuvirtide		2003	39 (350)
famciclovir		1994	30 (300)
fomivirsen sodium		1998	34 (323)
fosamprenavir		2003	39 (353)
foscarnet sodium		1989	25 (313)
ganciclovir		1988	24 (303)
imiquimod		1997	33 (335)
indinavir sulfate		1996	32 (310)
interferon alfacon-1		1997	33 (336)
lamivudine		1995	31 (345)
lopinavir		2000	36 (310)
neflinavir mesylate		1997	33 (340)
nevirapine		1996	32 (313)
oseltamivir phosphate		1999	35 (346)
penciclovir		1996	32 (314)
propagermanium		1994	30 (308)
rimantadine HCl		1987	23 (342)
ritonavir		1996	32 (317)
saquinavir mesylate		1995	31 (349)
sorivudine		1993	29 (345)
stavudine		1994	30 (311)
tenofovir disoproxil fumarate		2001	37 (271)
valaciclovir HCl		1995	31 (352)
zalcitabine		1992	28 (338)
zanamivir		1999	35 (352)
zidovudine		1987	23 (345)
influenza virus	ANTIVIRAL VACCINE	2003	39 (353)
cevimeline hydrochloride	ANTI-XEROSTOMIA	2000	36 (299)
alpidem	ANXIOLYTIC	1991	27 (322)
buspirone HCl		1985	21 (324)
etizolam		1984	20 (318)
flutazolam		1984	20 (318)
flutoprazepam		1986	22 (320)
metaclazepam		1987	23 (338)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
mexazolam		1984	20 (321)
tandospirone		1996	32 (319)
atomoxetine	ATTENTION DEFICIT HYPERACTIVITY DISORDER	2003	39 (343)
flumazenil	BENZODIAZEPINE ANTAG.	1987	23 (335)
bambuterol	BRONCHODILATOR	1990	26 (299)
doxofylline		1985	21 (327)
formoterol fumarate		1986	22 (321)
mabuterol HCl		1986	22 (323)
oxitropium bromide		1983	19 (323)
salmeterol hydroxynaphthoate		1990	26 (308)
tiotropium bromide		2002	38 (368)
APD	CALCIUM REGULATOR	1987	23 (326)
clodronate disodium		1986	22 (319)
disodium pamidronate		1989	25 (312)
gallium nitrate		1991	27 (328)
ipriflavone		1989	25 (314)
neridronic acid		2002	38 (361)
dexrazoxane	CARDIOPROTECTIVE	1992	28 (330)
bucladesine sodium	CARDIOSTIMULANT	1984	20 (316)
denopamine		1988	24 (300)
docarpamine		1994	30 (298)
dopexamine		1989	25 (312)
enoximone		1988	24 (301)
flosequinan		1992	28 (331)
ibopamine HCl		1984	20 (319)
loprinone hydrochloride		1996	32 (312)
milrinone		1989	25 (316)
vesnarinone		1990	26 (310)
amrinone	CARDIOTONIC	1983	19 (314)
colforsin daropate HCL		1999	35 (337)
xamoterol fumarate		1988	24 (312)
cefozopran HCL	CEPHALOSPORIN, INJECTABLE	1995	31 (339)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefditoren pivoxil	CEPHALOSPORIN, ORAL	1994	30 (297)
brovincamine fumarate	CEREBRAL VASODILATOR	1986	22 (317)
nimodipine		1985	21 (330)
propentofylline		1988	24 (310)
succimer	CHELATOR	1991	27 (333)
trientine HCl		1986	22 (327)
fenbuprol	CHOLERETIC	1983	19 (318)
auranofin	CHRYSOTHERAPEUTIC	1983	19 (314)
taltirelin	CNS STIMULANT	2000	36 (311)
aniracetam	COGNITION ENHANCER	1993	29 (333)
pramiracetam H_2SO_4		1993	29 (343)
carperitide	CONGESTIVE HEART FAILURE	1995	31 (339)
nesiritide		2001	37 (269)
drospirenone	CONTRACEPTIVE	2000	36 (302)
norelgestromin		2002	38 (362)
nicorandil	CORONARY VASODILATOR	1984	20 (322)
dornase alfa	CYSTIC FIBROSIS	1994	30 (298)
neltenexine		1993	29 (341)
amifostine	CYTOPROTECTIVE	1995	31 (338)
nalmefene HCL	DEPENDENCE TREATMENT	1995	31 (347)
ioflupane	DIAGNOSIS CNS	2000	36 (306)
azosemide	DIURETIC	1986	22 (316)
muzolimine		1983	19 (321)
torasemide		1993	29 (348)
atorvastatin calcium	DYSLIPIDEMIA	1997	33 (328)
cerivastatin		1997	33 (331)
naftopidil	DYSURIA	1999	35 (343)
alglucerase	ENZYME	1991	27 (321)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
erdosteine	EXPECTORANT	1995	31 (342)
fudosteine		2001	37 (267)
agalsidase alfa	FABRY'S DISEASE	2001	37 (259)
cetrorelix	FEMALE INFERTILITY	1999	35 (336)
ganirelix acetate		2000	36 (305)
follitropin alfa	FERTILITY ENHANCER	1996	32 (307)
follitropin beta		1996	32 (308)
reteplase	FIBRINOLYTIC	1996	32 (316)
esomeprazole magnesium	GASTRIC ANTISECRETORY	2000	36 (303)
lafutidine		2000	36 (307)
rabeprazole sodium		1998	34 (328)
cinitapride	GASTROPROKINETIC	1990	26 (301)
cisapride		1988	24 (299)
itopride HCL		1995	31 (344)
mosapride citrate		1998	34 (326)
imiglucerase	GAUCHER'S DISEASE	1994	30 (301)
miglustat		2003	39 (355)
somatotropin	GROWTH HORMONE	1994	30 (310)
somatomedin-1	GROWTH HORMONE INSENSITIVITY	1994	30 (310)
factor VIIa	HAEMOPHILIA	1996	32 (307)
levosimendan	HEART FAILURE	2000	36 (308)
pimobendan		1994	30 (307)
anagrelide hydrochloride	HEMATOLOGIC	1997	33 (328)
erythropoietin	HEMATOPOETIC	1988	24 (301)
factor VIII	HEMOSTATIC	1992	28 (330)
malotilate	HEPATOPROTECTIVE	1985	21 (329)
mivotilate		1999	35 (343)
buserelin acetate	HORMONE	1984	20 (316)
goserelin		1987	23 (336)
leuprolide acetate		1984	20 (319)
nafarelin acetate		1990	26 (306)
somatropin		1987	23 (343)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
zoledronate disodium	HYPERCALCEMIA	2000	36 (314)
sapropterin hydrochloride	HYPERPHENYL-ALANINEMIA	1992	28 (336)
quinagolide	HYPERPROLACTINEMIA	1994	30 (309)
cadralazine	HYPERTENSIVE	1988	24 (298)
nitrendipine		1985	21 (331)
binfonazole	HYPNOTIC	1983	19 (315)
brotizolam		1983	19 (315)
butoctamide		1984	20 (316)
cinolazepam		1993	29 (334)
doxefazepam		1985	21 (326)
loprazolam mesylate		1983	19 (321)
quazepam		1985	21 (332)
rilmazafone		1989	25 (317)
zaleplon		1999	35 (351)
zolpidem hemitartrate		1988	24 (313)
zopiclone		1986	22 (327)
acetohydroxamic acid	HYPOAMMONURIC	1983	19 (313)
sodium cellulose PO4	HYPOCALCIURIC	1983	19 (323)
divistyramine	HYPOCHOLESTEROLEMIC	1984	20 (317)
lovastatin		1987	23 (337)
melinamide		1984	20 (320)
pitavastatin		2003	39 (360)
rosuvastatin		2003	39 (361)
simvastatin		1988	24 (311)
glucagon, rDNA	HYPOGLYCEMIA	1993	29 (338)
acipimox	HYPOLIPIDEMIC	1985	21 (323)
beclobrate		1986	22 (317)
binifibrate		1986	22 (317)
ciprofibrate		1985	21 (326)
colesevelam hydrochloride		2000	36 (300)
colestimide		1999	35 (337)
ezetimibe		2002	38 (355)
fluvastatin		1994	30 (300)
meglutol		1983	19 (321)
ronafibrate		1986	22 (326)
modafinil	IDIOPATHIC HYPERSOMNIA	1994	30 (3030)
bucillamine	IMMUNOMODULATOR	1987	23 (329)
centoxin		1991	27 (325)
thymopentin		1985	21 (333)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
filgrastim	IMMUNOSTIMULANT	1991	27 (327)
GMDP		1996	32 (308)
interferon gamma-1b		1991	27 (329)
lentinan		1986	22 (322)
pegademase bovine		1990	26 (307)
pidotimod		1993	29 (343)
romurtide		1991	27 (332)
sargramostim		1991	27 (332)
schizophyllan		1985	22 (326)
ubenimex		1987	23 (345)
cyclosporine	IMMUNOSUPPRESSANT	1983	19 (317)
gusperimus		1994	30 (300)
mizoribine		1984	20 (321)
muromonab-CD3		1986	22 (323)
mycophenolate sodium		2003	39 (356)
mycophenolate mofetil		1995	31 (346)
pimecrolimus		2002	38 (365)
tacrolimus		1993	29 (347)
defeiprone	IRON CHELATOR	1995	31 (340)
alosetron hydrochloride	IRRITABLE BOWEL SYNDROME	2000	36 (295)
tegasedor maleate		2001	37 (270)
sulbactam sodium	β-LACTAMASE INHIBITOR	1986	22 (326)
tazobactam sodium		1992	28 (336)
nartograstim	LEUKOPENIA	1994	30 (304)
pumactant	LUNG SURFACTANT	1994	30 (308)
sildenafil citrate	MALE SEXUAL DYSFUNCTION	1998	34 (331)
tadalafil		2003	39 (363)
vardenafil		2003	39 (365)
gadoversetamide	MRI CONTRAST AGENT	2000	36 (304)
telmesteine	MUCOLYTIC	1992	28 (337)
laronidase	MUCOPOLYSACCARIDOSIS	2003	39 (354)
interferon $\beta - 1$a	MULTIPLE SCLEROSIS	1996	32 (311)
interferon β-1b		1993	29 (339)
glatiramer acetate		1997	33 (334)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
afloqualone	MUSCLE RELAXANT	1983	19 (313)
cisatracurium besilate		1995	31 (340)
doxacurium chloride		1991	27 (326)
eperisone HCl		1983	19 (318)
mivacurium chloride		1992	28 (334)
rapacuronium bromide		1999	35 (347)
tizanidine		1984	20 (324)
naltrexone HCl	NARCOTIC ANTAGONIST	1984	20 (322)
tinazoline	NASAL DECONGESTANT	1988	24 (312)
aripiprazole	NEUROLEPTIC	2002	38 (350)
clospipramine hydrochloride		1991	27 (325)
nemonapride		1991	27 (331)
olanzapine		1996	32 (313)
perospirone hydrochloride		2001	37 (270)
quetiapine fumarate		1997	33 (341)
risperidone		1993	29 (344)
sertindole		1996	32 (318)
timiperone		1984	20 (323)
ziprasidone hydrochloride		2000	36 (312)
rocuronium bromide	NEUROMUSCULAR BLOCKER	1994	30 (309)
edaravone	NEUROPROTECTIVE	1995	37 (265)
fasudil HCL		1995	31 (343)
riluzole		1996	32 (317)
bifemelane HCl	NOOTROPIC	1987	23 (329)
choline alfoscerate		1990	26 (300)
exifone		1988	24 (302)
idebenone		1986	22 (321)
indeloxazine HCl		1988	24 (304)
levacecarnine HCl		1986	22 (322)
nizofenzone fumarate		1988	24 (307)
oxiracetam		1987	23 (339)

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bromfenac sodium	NSAID	1997	33 (329)
lornoxicam		1997	33 (337)
OP-1	OSTEOINDUCTOR	2001	37 (269)
alendronate sodium	OSTEOPOROSIS	1993	29 (332)
ibandronic acid		1996	32 (309)
incadronic acid		1997	33 (335)
raloxifene hydrochloride		1998	34 (328)
risedronate sodium		1998	34 (330)
tiludronate disodium	PAGET'S DISEASE	1995	31 (350)
temoporphin	PHOTOSENSITIZER	2002	38 (367)
verteporfin		2000	36 (312)
beraprost sodium	PLATELET AGGREG. INHIBITOR	1992	28 (326)
epoprostenol sodium		1983	19 (318)
iloprost		1992	28 (332)
sarpogrelate HCl	PLATELET ANTIAGGREGANT	1993	29 (344)
trimetrexate glucuronate	*PNEUMOCYSTIS CARINII* PNEUMONIA	1994	30 (312)
histrelin	PRECOCIOUS PUBERTY	1993	29 (338)
atosiban	PRETERM LABOR	2000	36 (297)
gestodene	PROGESTOGEN	1987	23 (335)
nomegestrol acetate		1986	22 (324)
norgestimate		1986	22 (324)
promegestrone		1983	19 (323)
trimegestone		2001	37 (273)
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24 (297)
nafamostat mesylate		1986	22 (323)
adrafinil	PSYCHOSTIMULANT	1986	22 (315)
dexmethylphenidate HCl		2002	38 (352)
dutasteride		2002	38 (353)
alefacept	PSORIASIS	2003	39 (340)
efalizumab		2003	39 (349)

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
finasteride	5α-REDUCTASE INHIBITOR	1992	28 (331)
surfactant TA	RESPIRATORY SURFACTANT	1987	23 (344)
Adalimumab	RHEUMATOID ARTHRITIS	2003	39 (339)
dexmedetomidine hydrochloride	SEDATIVE	2000	36 (301)
kinetin	SKIN PHOTODAMAGE/ DERMATOLOGIC	1999	35 (341)
tirilazad mesylate	SUBARACHNOID HEMORRHAGE	1995	31 (351)
APSAC	THROMBOLYTIC	1987	23 (326)
alteplase		1987	23 (326)
balsalazide disodium	ULCERATIVE COLITIS	1997	33 (329)
tiopronin	UROLITHIASIS	1989	25 (318)
propiverine hydrochloride	UROLOGIC	1992	28 (335)
Lyme disease	VACCINE	1999	35 (342)
clobenoside	VASOPROTECTIVE	1988	24 (300)
falecalcitriol	VITAMIN D	2001	37 (266)
maxacalcitol		2000	36 (310)
paricalcitol		1998	34 (327)
doxercalciferol	VITAMIN D PROHORMONE	1999	35 (339)
prezatide copper acetate	VULNERARY	1996	32 (314)
acemannan	WOUND HEALING AGENT	2001	37 (257)
cadexomer iodine		1983	19 (316)
epidermal growth factor		1987	23 (333)