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CORRECTION-

In Volume 35 of Annual Reports in Medicinal Chemistry, the first author was inadvertently omitted from chapter 6. We apologize for this error. The correct heading for this chapter is as follows:

Chapter 6. Recent Developments in Antitussive Therapy

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This chapter has been updated, and appears in this Volume as Chapter 4.

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 that are expected to provide the basis for entirely new future therapies.

Volume 36 retains the familiar format of previous volumes, this year with 30 chapters. Sections I–IV are disease-oriented and generally report on specific medicinal agents with updates from Volume 35 on antitussive therapy, anticoagulants, antibacterials, and antiretroviral therapies. As in past volumes, annual updates have been limited to the most active areas of research in favor of specifically focused 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 bioinformatics, protein structure prediction, proteonomics, and therapeutic antibodies. Chapters in Section VI, Topics in Drug Design and Discovery, reflect the current focus on mechanism-directed drug discovery and newer technologies. These include chapters on aspartyl protease inhibitors, ADME by computer, PET ligands for assessing receptor occupancy *in vivo,* and strategies for analytical characterization and profiling of compound libraries.

Volume 36 concludes with To Market, To Market — 2000, a chapter on NCE and NBE introductions worldwide in 2000 and chapters on new developments in animal health care and the impact of intellectual property issues on the pharmaceutical industry. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in Volumes 1–35 of this series.

Volume 36 of *Annual Reports in Medicinal Chemistry* was assembled with the superb editorial assistance of Ms. Sylvie Duchesne, Ms. Nadège Pingray, and Ms. Lisa Bausch and I thank them for their hard work. I have continued to work with innovative and enthusiastic section editors and my sincere thanks goes to them again this year. I hope that you will enjoy and profit from reading this volume.

Annette M. Doherty
Fresnes, France
May, 2001

SECTION I. CNS AGENTS

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Chapter 1: Same Brain, New Decade: Challenges in CNS Drug Discovery in the Postgenomic, Proteomic Era

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Introduction. The brain is a highly complex organ that mediates conceptual thought, cognition, volition, self-consciousness and emotion (1,2). As "the interpreter and responder to environmental challenges", the brain, working via the peripheral nervous system, processes information and controls behavioral responses via "systems replete with specialized circuits, parallel pathways, and redundant mechanisms to protect the individual, thus ensuring propagation of the genome and survival of the species (3,4). Accordingly, brain dysfunction resulting from genetic, environmental and/or aging factors has a major negative impact on the quality of life and individual survival.

CNS COMPLEXITY AND DRUG DISCOVERY

The human brain contains approximately 100 billion neurons and expresses greater than 60% of known human genes. In comparison, the nervous system of the threadworm, C. elegans, a model system for studying genomic function, has a mere 302 neurons, 3 x 10^{-6} % the number in the human brain. Neurons in C. elegans are interconnected via 600 electrical and 5000 chemical synapses (5). The C. elegans genome codes for approximately 1000 G-protein coupled receptors (GPCRs), 90 ligand-gated ion channels, 80 potassium-selective ion channels and 228 nuclear receptors providing a virtually infinite number of postgenomic molecular substrates through which neuronal function can be regulated (6,7). Understanding this complexity at the level of the human brain and postgenomic interactions between genes and their products (epigenetics; 8) is a key challenge in understanding human CNS disease pathophysiology and in designing new drugs that are safer and more efficacious to treat these diseases.

The characterization of simpler systems like C. elegans, has prompted a shift away from an increasingly reductionistic approach to the study of the brain and nervous system, focused almost exclusively on molecular function at the synaptic level, to a renewed appreciation of the hierarchical complexity (gene, synapse, pathway, phenotype) of the nervous system (9) and the need to: a) integrate structure with function at the tissue and whole animal level; and b) integrate and iterate animal studies with emerging clinical research at both the systems and compound levels. Given emerging knowledge regarding the intrinsic complexity of the human brain, the success resulting from serendipity in the last 40 years is impressive and has provided a number of highly effective CNS drugs, the majority of which act synaptically, either mimicking (agonists), facilitating (transmitter uptake blockers, allosteric modulators) or antagonizing the effects of endogenous neurotransmitters and neuromodulators (10,11). Not only neurons but also glial cells - astrocytes, microglia, etc. are potential drug targets.

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The function of the brain is extremely dynamic. Neonate, adult and aged brains are morphologically and phenotypically very distinct. Diseased brain can be very different from the 'normal', healthy brain. Transmitters and receptors present during development disappear in adult brain, while receptors change in number and function due to disease and nervous system trauma. Mechanisms to sustain brain homeostasis, including trophic factor maintenance of neuronal viability, are negatively impacted by aging, leading to an accumulation of environmental insults. Brain function is influenced by hormones, via the hypothalamic-pituitary axis (HPA; 3) and by peripheral e.g. cardiovascular/ vascular, system function.

Global sales of CNS drugs in 1999, including pain, exceeded $50 billion, approximately 15% of the total global drug sales. This market will continue to grow as the aging population increases (by 2030, the number of Americans 65 or older will double; 11) and life-style factors, including stress and information overload resulting from a breakdown in societal support systems for the individual (3,12,13).

The Decade of the Brain initiative of the 1990s was intended to "enhance the awareness of the benefits to be derived from brain research" and thus elucidate the cause(s) of CNS disorders, enabling development of effective treatments (14). Despite this and newer drug discovery technologies, both chemical and biological, that include draft sequences of the human genome (15), the discovery and timely development of CNS drugs remains one the most challenging in pharmaceutical research (11). Factors contributing to this include: the inherent complexity of the brain; a paucity of knowledge regarding function at the molecular level - especially in disease states; animal models with limited predictive value; a lack of robust, quantitative diagnostic tools to track disease occurrence and progression; imprecise physician diagnoses of symptomatically related psychiatric disease states that frequently involve ethnic and cultural factors; and challenges in defining efficacy in human trials due to high placebo responses (16,17).

CNS drug discovery to date has thus been highly iterative in nature, building on established mechanism(s) of action of clinically effective compounds with improvements in tolerance and/or safety. The successful antidepressant SSRIs (selective serotonin reuptake inhibitors), e.g., fluoxetine, are mechanistically similar to the traditional tricyclic antidepressants (TCAs), e.g. impramine. In addition, the reason for the superior efficacy of the 'atypical' antipsychotic medication, clozapine – the mechanism(s) of action of which has been repeatedly redefined as new CNS receptors have been identified – as compared with other dopamine (DA) receptor antagonists, has not been elucidated.

An additional complication in developing new CNS drugs is that drugs currently used for one indication can be used to treat different CNS disorders - e.g. valproate for epilepsy, bipolar affective disorder (BPAD), migraine (18) and dementia-associated agitation, and anticonvulsants, e.g. gabapentin, that modulate neuronal firing are used for the treatment of neuropathic pain and, potentially, BPAD.

Diagnosis and classification of CNS disorders relies on two key reference works: the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) and the International Classification of Diseases - Revision 10 (ICD-10), the European equivalent of DSM-IV. While invaluable tools, their use is confounded by ethnic -, societal - and gender- related differences in patient diagnosis. CNS diseases also have a high incidence of co-morbidities - depression is associated with chronic pain and excessive stress and alcoholism with depression, anxiety and cognitive impairment. The neuronal loss associated with stroke leads to cognitive dysfunction, mood disorder(s) and

TABLE 1: TRENDS IN PSYCHIATRIC DRUG TREATMENT

Disease State	Current Approaches	Experimental Approaches
Schizophrenia	DA receptor blockers – haloperidol, clozapine, chlorpromazine, risperidone, olanzepine	DA: Clozapine-like agents, partial agonists, D4 receptor antagonists. NMDA receptor/glycine modulators - D-serine, serine racemase α-7 nicotinic receptor agonists $5HT_{2A}$ inverse agonists – AC 90179 Neurokinin-3 and cholecystokinin$_1$ antagonists
Depression	TCAs - impramine, amitriptyline: Monoamine oxidase inhibitors (MAOIs) - tranylcypromine SSRIs- citalopram, fluoxetine SNRIs (5HT/ NE reuptake inhibitors) - venlafaxine	Improved monoamine uptake inhibitors $5HT_{1A}$ receptor ligands NK-1 receptor antagonists Corticotropin releasing factor (CRF) receptor antagonists
Bipolar Affective Disorder	Lithium, valproic acid, carbamazepine	Antiepileptics: pregabalin, topiramate etc. Valproate analogs - TV-1901 etc.
Anxiety: panic disorder, OCD (obsessive-compulsive disorder) GAD (generalized anxiety disorder), PTSD (postraumatic stress disorder), acute stress disorder	Benzodiazepines (BZs)-diazepam, clonazepam $5HT_{1A}$ partial agonists-buspirone SSRIs	Newer BZs: pagaclone, deramciclane $5HT_{1A}$ agonists: lesopitron, S-15535 Orphanin FQ receptor agonists – Ro 64-6198 CRF receptor antagonists
Attention deficit hyperactivity disorder (ADHD)	Psychostimulants – methylphenidate d-amphetamine	α4β2- nicotinic receptor agonists – ABT-089 Histamine H_3 antagonists - GT 2331 Monoamine uptake blockers - atomoxetine
Compulsive/addictive disorders : cocaine, amphetamine, heroin, alcohol, nicotine (smoking) addiction Recreational drug use Cannabinoid, PCP Compulsive disorders: Gambling, sexual behavior, eating (obesity, anorexia, bulimia)	Methadone, LAAM Naloxone Disulfiram Acamprosate Nicotine patches Buproprion Phenylpropanolamine Sibutramine Orlistat PPARγ antagonists - troglitazone	DA transport blockers - RTI -113 D1 receptor ligands - DAS-431, CEE 03-310 Cocaine vaccine (TA-CD) and catalytic antibodies - mAb 15A10 Obesity: Leptin modulators, CART, GLP1, amylin, galanin, neuropeptide Y, α- MSH, famoxin, fatty acid synthase (FAS) inhibitors, orexin, melanocortin - 4 (MC-4)/SLC-1 and SOCS3 antagonists
Sleep disorders: sleep pattern disruption (jet lag) Insomnia, narcolepsy	Hypnotics- Secobarbital, triazolam, estazolam Modafinal, Melatonin	Agomelatine, Adenosine agonists H_3 agonists – SCH 50971 Orexin agonists, NBI 34060
Sexual disorders Erectile dysfunction/ Female sexual dysfunction	Sildenafil Apomorphine	IC-531, BAY 38-9456 DA agonists

dementia; and the loss in cognitive function occurring in Alzheimer's disease (AD) leads to aggression, anxiety and depression. The overlap in symptoms between diagnostically distinct disease states and the high co-morbidity with other distinct CNS disorders makes clinical experimentation an absolute necessity in defining the utility of CNS drugs. The area is historically replete with compounds advancing to the clinic for one indication and being found to be useful for another (10).

TARGET DYNAMICS

Ongoing research, basic and applied, has continued to identify a number of new approaches to the treatment of CNS disorders that are currently to or through clinical trial validation. These are shown alongside existing approaches in Tables 1 and 2; some are incremental improvements on existing mechanisms (D4 antagonists for schizophrenia; valproate analogs) while others (D-serine for schizophrenia (19); caspase inhibitors for neurodegenerative disorders; vaccines for AD and stroke (20,21)) are highly novel approaches. In many instances however, the challenge in CNS drug R & D is in improving (reducing) the side effect liabilities for compounds active at a known CNS drug target (e.g. D2 receptor) to allow higher levels of drug to be administered. This can be accomplished by understanding the mechanism(s) by which known compounds produce their side effects and by then 'tuning out' this property or adding additional properties to newer compounds to overcome side effects. Side effects, while never a trivial issue, are of increasing importance, especially in CNS disorders requiring chronic therapy in a young population that is, apart from their disease-related disability, relatively healthy. For example, antipsychotics show a class-related phenomenon of QT-syndrome prolongation that can result in ventricular tachycardia, heart block and fatalities. This has been a major factor in the comparative lack of new drug approvals for this class (22).

A less obvious instance of potential side effect liability results from approaching the process of compound identification using a highly reductionistic molecular approach that lacks an integrated, pharmacological framework. The logic, a priori, is that by identifying a compound interacting with high affinity at a defined molecular target, it will lack interactions with other molecular targets. Leptin, the 167mer secreted from adipocytes, acts via leptin receptors to reduce food intake and was thought to represent a promising anorectic agent (23) However, acting via the hypothalamus, leptin also inhibits bone formation, an effect that would limit the chronic use of the peptide in obesity (24).

EXPLOITING THE GENOME OF THE BRAIN

A large number of chromosomal loci containing susceptibility genes potentially involved in disease etiology as well as gene candidates for schizophrenia, BPAD, etc. have been identified (25,26). Validation of these is based on epidemiological data showing a significant genetic contribution to disease etiology. Interactions between more than one susceptibility gene and environmental risk factors (the "envirome"; 27) clearly contribute to disease incidence, the norm of reaction factor - indicating that biology – and human behavior - cannot be classified simply in terms of DNA sequences (28). In schizophrenia, concordance rates between monozygotic and dizygotic twins are 50% and 15% with an overall heritability of 68% (29). Focusing on disease genes within chromosomal regions implicated through genetic linkage analysis (using DNA from affected family pedigrees) requires a case control study design involving large cohorts (200-500 of patients and controls) derived from ethnically homogeneous populations matched for age and sex. The quality of the case histories is crucial in assuring the validity of diagnosis and in identifying ethnically unmatched individuals who contribute to stratification effects. The identification of putative disease-associated genes in an initial population should be replicated in additional populations. However, gene association studies often fail to replicate due to locus or genetic heterogeneity or simply because of the poor quality of the collection. With the sequencing of the human genome and identification of more than 2.5 million single nucleotide polymorphisms (SNPs; 30), phenotypic traits will be increasingly correlated with genetic variability with the

TABLE 2: TRENDS IN NEUROLOGICAL DRUG TREATMENT

Disease State	Current Approaches	Experimental Approaches
Dementias AD; early onset familial AD (EOFAD), vascular dementia, dementia with Lewy bodies (DLB), dementia associated with Parkinson's disease, AIDS and age-related dementia. Picks' dementia, frontotemporal, substance-induced and alcohol dementia	Cholinergic replacement: Donepezil, rivastigmine Galanthamine etc. Nootropics: piractetam, aniracetam idebenone	Inhibitors of oxidative stress: MAO-B inhibitors - rasagiline; free radical scavengers: ARL-16556 Nicotinic and DA D1/D5 agonists. COX-2 inhibitors – rofecoxib HMG CoA reductase inhibitors – simvistatin Trophic factor replacement /neuronal growth stimulators – BDNF, AIT-082, kinase signaling pathways Amyloid vaccine - AN 1792/Betabloc BACE1 inhibitors - L-685,458 Caspase inhibitors – IDN-6556
Parkinson's disease (PD)	DA replacement: L-dopa, pramipexole cabergoline, piribedil, pergolide, ropinerole	Neuroimmunophilins, GPI-1337 Adenosine A_{2A} agonists - KW 6002, SCH 58261 Inhibitors of oxidative stress: Rasagiline - Caspase inhibitors
Epilepsy	Phenytoin, carbamazepine, valproate, ethosuximide, Phenobarbital Felbamate, lamotrigine, gabapentin, tiagabine, Vigabatrin	Leviracetam . Valproate analogs: TV-1901, NPS 1776, ABS-103, DP-VPA etc. Sodium channel modulators: GW 273293, Co 102862 Calcium antagonists: zonisamide, retigabine, PNU 156654E GABA modulators: losigamone, pregabalin, Co 15279, rufinamide. Glutamate receptor antagonists: Talampanel , TV 141, PNU 191779E
Stroke	tissue plasminogen activator, tPA	NMDA/ glycine site modulators: D-serine, licostinel, MDL 105518, GV 224029 NR1 vaccine (AAVNMDAR1) NAALADase inhibitors: 2-PMPA Caspase-3 inhibitors $P2X_7$ receptor antagonists $P2Y_{12}$ antagonists: AR-C 69931MX
Spinal cord injury	Steroids	NR2B NMDA antagonist - CP-101,606
Multiple sclerosis	Interferon Immunosupressants methylprednisolone prednisone methotrexate azathioprine	Clabridine, mitoxantrone, paclitaxel, sulfasalazine, lenercept (sTNFR-IgG p55) ISIS 107248 - antisense Cannabinoid receptor agonists: R (+)-WIN 55,212, methanadamide
Pain	Opioids NSAIDs COX-2 inhibitors	$\alpha4\beta2$ nicotinic agonists – ABT-594 NMDA receptor antagonists Neurokinin-1 antagonists Vanilloid receptor modulators $P2X_3$ receptor antagonists $GABA_B$ activated Kirs – gabapentin Voltage-gated Na^+ channels Growth factors – NGF

information generated used to substantiate emerging findings that genes associated with one CNS disease may also be associated with other distinct disease states e.g. disease A involves interactions between genes X, Y and Z (plus the "envirome"; 27) while disease B involves genes X, S and T. Currently used symptomatic approaches to disease diagnosis may be replaced with patient genotyping followed by the use of compounds targeted towards the products of the individual disease

associated genes for disease treatment. The evolution of cognitive neuropsychiatry/psychopathology is an aspect of this trend (31). Individual genetic information can also be used as a pharmacogenomics approach to clinical trials to assess individual responses to drug efficacy, metabolism and toxicity (32).

It has been widely anticipated that identification of disease - associated genes and generation of drug-induced genomic 'fingerprints" using microarray technology in combination with transgenic animal models and human and animal brain imaging using positron emission tomography (PET), SPECT, and functional nuclear magnetic imaging (fMRI) will together reveal a host of unique drug discovery targets. In doing so, this will enhance understanding of disease etiology and drug targets (33-36). Transgenic animal models may be more predictive of the human disease state than classical models and may aid in both identifying the desired properties of new generations of CNS medications and also impact clinical trial design to improve the transition of compounds from animals to humans. The challenges in developing such models are however, far from straightforward (37).

A more high throughput approach to identifying disease-associated genes involves differential display methodologies using DNA microarrays to produce gene expression signatures (GESs). By comparing brains from diseased individuals with controls, disease - associated alterations in gene expression can be measured. This method can also be used to gather information to assess the importance of candidate genes identified in patient populations as well as their protein products and associated proteins. However, the paucity of well-preserved and adequately documented human brain collections limits this approach. There is also the possibility that any observed changes may be drug related and thus lead to false positives. GESs can also be generated by treating animals, tissues and/or cells with a known drug at a dose reflecting its therapeutic plasma level to see whether there are similarities in the genetic "fingerprint" between structurally dissimilar and even mechanistically different drugs that are used to treat the same CNS disease. New compounds can then be screened via microarrays to see whether they have profiles similar to that of the reference compound(s). For instance, generation of dose, temporal and tissue specific GESs for atypical antipsychotics like clozapine could be used to identify newer DA receptor blockers with a similar genetic profile for animal testing without actually knowing the function of the protein expressed by the gene. This approach can also be used to examine side effect profiles of known drugs and new drug candidates.

Other differential display approaches involve experimental manipulation of animals or cell lines. This approach has worked successfully in generating a GES for aging and metabolism where much of the biochemistry/proteomics had been established (38). Protein differential display is potentially more valuable from a physiological perspective than RNA expression although there are still challenges relating expressed protein quantity to function (39).

AD is the most studied CNS disease from a genetic perspective. While its cause remains unknown, there is general agreement that amyloid-β (Aβ), a 40 - 42mer proteolytically generated from amyloid precursor protein (APP) that is associated with senile plaques in AD brains is involved in the disease (40). Whether this is the cause or result of the AD process has not yet been established. Approximately 10% of AD cases show early onset with a familial distribution consistent with an autosomal dominant inheritance. However, the majority of late onset AD cases are likely to be sporadic (41). Four genes have been identified that show an inherited susceptibility to AD. Three of these, presenilin -1 and -2 (PS-I and PS-II) and APP, are highly penetrant and were identified through genetic linkage studies of family pedigrees (42). The fourth, the cholesterol binding protein, apolipoprotein E (ApoE) was detected by association analysis and represents a risk factor to disease susceptibility

(43). The ApoE IV allele was associated with an increased risk of developing AD, although in contrast to mutations on chromosomes 1(PS-2), 14 (PS-1) and 21 (APP), was not an invariant disease predictor. Treatment of cell lines and guinea pigs with the HMG CoA reductase inhibitor, simvistatin, markedly lowers plasma levels of Aβ peptide (44). A linkage region on chromosome 10q may contain susceptibility genes for late onset AD (45).

Despite progress in the genetics of AD, current medicinal chemistry efforts are based on inhibiting the production of Aβ (46,47). Other approaches include the use of anti-inflammatory agents, lipid-lowering statins and vaccines (20, 44, 48).

The identification of drug-associated genes is not always straightforward. Administration of clinical doses of Li^+, a frontline treatment for BPAD, to rats, led to the identification of a "novel lithium regulated gene" (NLRG) postulated to be involved in BPAD (49). NLRG was homologous to a family of prokaryotic nitrogen permease regulators. It had higher expression in the heart, liver and testes than brain suggesting that it might be more related to the side effects of Li^+ than the latter's actions as a psychoactive drug. A similar approach identified a novel human diphosphoinositol polyphosphate phosphohydrolase (hDIPP2) isoform that was increased in frontal cortex by Li^+, but not by valproate or carbamazepine, treatment (50).

Once a disease - or drug - associated gene has been identified, it is not always clear how it can be used as a drug discovery target. The motif of the protein product may not correspond to a typical CNS, or any other, drug target and may be part of a pathway associated with the target disease. It is then necessary to use the protein partner trapping/ "bait and prey' technologies, e.g., two-hybrid arrays, fluorescence resonance energy transfer (FRET) techniques, that are part of the evolving proteomics approach to drug discovery, to identify more facile targets (51). Another approach to exploiting the genome for CNS drug discovery is that of orphan receptor or target mining (52). By identifying motifs on the genome common to known drug targets, e.g. GPCRs, LGICs, new homologs can be identified that lack a known ligand. Cloning the receptor and identifying the natural ligand can validate its potential role. A successful example of this approach is the orphanin/FQ (O/FQ) receptor, discovered in the mid 1990s as an orphan member of the opioid GPCR family. Following identification of the peptide O/FQ as the endogenous ligand, brain localization of the receptor indicated a role in anxiety leading to the identification of the novel, non-peptide anxiolytic, Ro 64-6198 (53).

ANIMAL MODELS – TRANSGENIC MICE AND MUTATED FLIES

Animal models of CNS diseases, like those of other human diseases (e.g. hypertension, cancer) are surrogates rather than models of the human disease state. The spontaneously hypertensive rat (SHR), a model of hypertension has added little to the understanding of the molecular lesions in this cardiac disorder. Similarly, use of ocular angiogenesis models has only a theoretical relationship with the ability of compounds to inhibit or reverse the growth of cancer metastases. Nonetheless, animal models remain a critical link between *in vitro*, mechanistic compound characterization and transition to the clinic. The majority of currently used animal models of CNS disorders were developed using drugs, the therapeutic utility of which was determined in the clinic and were invariably mechanism/drug rather than disease specific. Even with the many refinements introduced over the past three decades (54), the models available often result in the identification of newer compounds acting via the same mechanisms as the compounds used to develop the models. Thus, these compounds, to varying degrees, have many of the same liabilities as the original compounds. The predictability of animal models is uncertain. The NK-1 receptor antagonist, MK-869 had robust effects in pain models,

but lacked any analgesic activity in the clinic. It was subsequently found to have antidepressant activity in animal models and initial Phase II trials (55).

Classical behavioral despair models (Porsolt swim test, tail suspension) remain reliable methods for identifying antidepressants. Newer paradigms, e.g. chronic mild stress, rat Flinders Sensitive Line (FSL), guinea - pig vocalization models have proven useful in identifying atypical antidepressants with mechanisms distinct from classical monoamine potentiators e.g. TCAs, SSRIs. (55-57)

Transgenic mouse models of CNS disorders have provided mixed results. NMDA receptor subunit 2B (NR2B) over expression in the Doogie mouse enhances NMDA receptor activation, facilitating synaptic transmission, resulting in superior learning and memory performance (58). In contrast, models of neurodegenerative disorders, specifically AD, have been less facile and it is only recently that transgenic models have been developed that show both the histological and behavioral deficits associated with AD (59). The fruit fly, Drosophila, has been used to develop models of CNS disease states e.g. ethanol ingestion e.g. cheapdate (60). α-Synuclein transfection into Drosophila results in a novel PD model (61).

CLINICAL TRIAL CHALLENGES

In addition to high placebo responses more than 50% of antidepressant trials fail due to a lack of difference between placebo and active drug (16,17). An inability to demonstrate clinical efficacy for a new compound may result from poor pharmacokinetics and/or limited human bioavailability in the compound selected or evaluation at an inappropriate dose, either on the wrong part of the 'U-shaped' dose response curve typical of CNS active agents or at too low a dose to avoid side effect liabilities. The increasing trend in determining the plasma levels of a compound associated with efficacy in an animal model and extrapolating these to achieve similar levels in humans rather than using a dose - to dose - extrapolation markedly improves the understanding of why a compound failed to work in humans. The ineffectiveness in a clinical trial of a compound with less than 5% bioavailability in humans, a half life in the order of minutes when given systemically and plasma levels well below those assessed as producing efficacy in an animal model is far from conclusive proof of failure of a mechanistic hypothesis. An additional complication is the relationship of plasma concentrations of a compound to those in the brain and accessibility to the brain through the blood brain barrier. Brain levels can be grossly assessed by measuring ex vivo receptor binding as a surrogate of brain residence or by using brain-imaging techniques (35). These have become increasingly important in assessing brain levels of a new drug as measured by in vivo receptor occupancy and relating these to efficacy measured in behavioral paradigms (62).

FUTURE DIRECTIONS

Improved success in CNS drug discovery and development depends on four key factors: i) an improved understanding of the etiology and molecular pathophysiology of CNS diseases; ii) development of animal models that provide some reasonable degree of predictivity of the efficacy and side- effect liabilities of compounds being advanced to the clinic; iii) improved diagnostic procedures for CNS disorders and iv) design and execution of clinical trials that are a direct continuum of preclinical research and are sufficiently flexible and rationally conceived to enhance understanding of the molecular targets selected and their relevance to the human disease. In the latter context, it will be important to provide sufficient resources, comparable to those being invested in genomic approaches to target discovery to drive this critical part of the drug discovery process (63). Indeed, CNS drug discovery must be viewed as extending from the identification of the first

lead molecule active at the selected target until the time that the code on the first double blind, placebo controlled, Phase IIa trial is broken. The use of Phase I/II bridging studies may also facilitate the process (64)

The need for safe and effective medications to treat CNS disorders is evident not only in the context of the limitations of existing medications but also in regard to the major unmet medical need associated with the increasingly aged population. Effective medications for the treatment of the various addictive behaviors including substance abuse, for AD and other dementias and for stroke would significantly reduce the burden of disease cost to society and add immeasurably to the individual quality of life, to that of immediate caregivers and to society as a whole.

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Chapter 2. Potassium Channel Modulators for the Treatment of CNS Disorders.

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Introduction - Investigation of the role of potassium channels in the treatment of human disease continues to be a growing field of research. The ability of K^+ channels to regulate membrane potential accords them a central role in varied cellular processes that govern excitability, action potential characteristics, stimulus-secretion coupling, cell volume regulation and epithelial electrolyte transport. Attention from medicinal chemists to K^+ channels as drug targets has grown with the realization that a variety of K^+ channel modulators offer significant therapeutic opportunities in cardiac, smooth muscle, neuronal, immune and secretory systems. Progressive improvements in molecular biology have enabled regular cloning of potassium channels of interest, and defined families of these channels have facilitated a comprehensive understanding of their function. Importantly, many families of increasingly selective small molecules have emerged as tools for target validation and clinical proof of principle. Many reviews have appeared summarizing the therapeutic potential of these channels (1-3). The scope of this report is to update the key advances in potassium channel biology which lend themselves to a more expedient identification of agents for the treatment of CNS disorders, emphasizing developments in medicinal chemistry from potassium channels where modulators would have considerable clinical potential.

We have focused on recent developments in key areas of potassium channel biology according to specific subtypes. Modulators of calcium-activated potassium channels for CNS indications such as neuroprotection, depression or epilepsy are described. Medicinal chemistry has evolved considerably in this area, and the first reports of efficacy data have emerged from late-stage clinical trials. Cognitive processes associated with voltage-gated channels such as Kv1.1 and M-type channels are also detailed. Although this is a newer target for CNS indications, effects on these channels have been disclosed from detailed *in vitro* evaluation of several compounds whose pharmacology was presumably well established. Lastly we describe the role of K_{ATP} channels for the treatment of conditions such as epilepsy or pain. As the role of K_{ATP} openers in neuronal signaling becomes better defined, this long-established cardiovascular target may also become an attractive area for study pursuant to CNS indications.

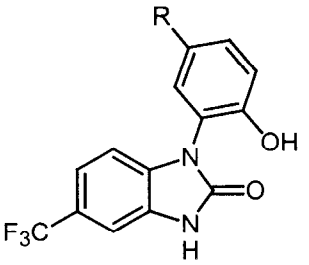
BK_{Ca} Channel Openers - Calcium-activated K^+ channels are regulated by membrane depolarization and changes in intracellular Ca^{2+} levels. The high conductance or maxi-K (BK_{Ca}) channels are activated by an increase in intracellular calcium concentration and membrane depolarization. These channels are sensitive to blockade by iberiotoxin and charybdotoxin. Cloning of multiple splice variants of the pore-forming α subunit (*mSlo*, *hSlo* from initially cloned *Drosophila slowpoke* (*dSlo*) Ca^{2+}-activated K^+ channel) and multiple β subunits have recently generated considerable diversity within the BK_{Ca} family. This, together with the widespread distribution of BK_{Ca} channels throughout the CNS and in peripheral tissues, offers opportunities for discovering novel therapeutic agents as well as significant challenges in the form of tissue and organ specificity.

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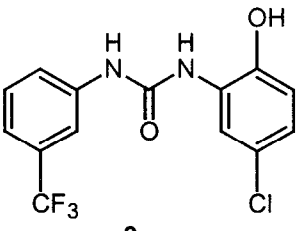
Therapeutic applications for BK_{Ca} channel openers have focused on stroke, epilepsy, and bladder overactivity although there is evidence for utility in the treatment of other CNS conditions including psychoses (4,5). In general, the design of selective BK_{Ca} openers has focused on neuroprotection secondary to ischemic stroke. CNS damage after ischemic stroke arises from several sources after reduction of blood flow to the brain tissue. Short-term impairment evolves from the increase in intracellular Ca^{2+} which potentiates cytotoxic events such as excess enzyme induction and mitochondrial damage (6). The role of a BK_{Ca} opener in treating this disorder involves cellular membrane hyperpolarization via increased BK_{Ca}-mediated efflux of K^+, thereby reducing intracellular Ca^{2+} concentration with concomitant protection of central neuronal tissues from further damage.

In general, activation of BK_{Ca} currents has been assessed by determining the ability of a single concentration of the test compound to increase cloned mammalian (*mSlo* or *hSlo*) currents in *Xenopus* oocytes or cell lines, which limits strict comparison of compound potencies. Furthermore, selectivity of available openers across various ion channel types and more importantly, evaluation versus the cloned BK_{Ca} channels containing diverse β subunit combinations remains to be investigated.

Early BK_{Ca} channel openers were relatively weak agents, or they were known to possess ancillary pharmacology which limited their utility as therapeutic agents or as probes to validate the relevance of BK_{Ca} channels *in vivo* (4,5,7).



1 R = Cl
2 R = CF_3

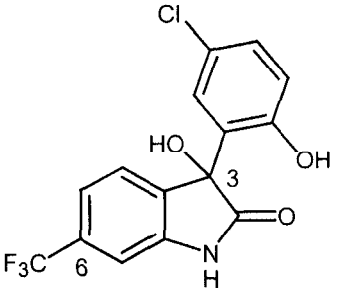


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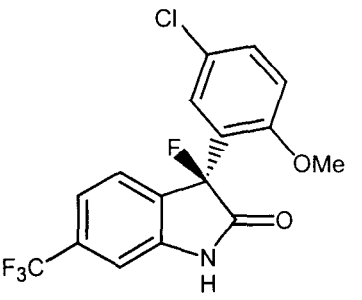
A number of different structural classes of BK_{Ca} openers have appeared in the literature. Benzimidazolone analogs such as NS-4 (**1**) and NS-1619 (**2**) stimulate BK_{Ca} activity leading to membrane hyperpolarization. Trifluoromethyl analog **2** activates BK_{Ca} currents at 10-30 μM in vascular and nonvascular smooth muscle although over similar concentration ranges, the compound also inhibits delayed rectifiers and Ca^{2+} currents. Diphenylurea analog **3** enhances BK_{Ca} activity by shifting current activation to more negative potentials at micromolar concentrations.

The majority of newer small molecule BK_{Ca} openers possess some common structural features. One such structural motif is the presence of two aromatic rings linked via a spacer unit that is either a heterocycle or a urea. In the case of the heterocyclic spacer it can sometimes be found fused to one of the aromatic rings as in the case of **2**. Also present on one of the aromatic rings of many BK_{Ca} openers are the 5-halo-2-hydroxy or 5-halo-2-methoxy substitution patterns. Many of the newer BK_{Ca} openers that have appeared continue to fit this general description of the pharmacophore with novel modifications of earlier BK_{Ca} structures.

Novel aryl oxindole BK_{Ca} channel openers have been reported for the treatment of stroke, and clinical data have begun to emerge for the first of these BK_{Ca} channel openers (8). Structurally these bear a close resemblance to the benzimidazolones with the primary exception being the replacement of nitrogen with carbon at the 3 position of the core heterocycle. The (-) enantiomer of compound **4** has been shown to increase BK_{Ca} currents in the *mSlo* channel expressed in *Xenopus* oocytes by 41% over control at 20 μM. In the same study, the reference oxindole derivative, **1** activated BK_{Ca} currents 32% at 20 μM. Some stereodifferentiation may have been seen in this series as the (+) enantiomer activated currents only 24% at 20 μM.



4



5

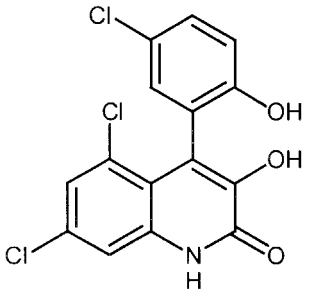
The related 3-fluoro analog BMS-204352 (MaxiPost™) Compound **5** is reported to be neuroprotective and it reduced infarct size in two preclinical rat stroke models (9,10). Efficacy was observed over a dose range of 10 ng/kg to 3 mg/kg. Interestingly, at doses greater than 1 mg/kg, **5** displayed an inverted-U dose response relationship in this rat model.

This fluoro-oxindole also reduced electrically evoked hippocampal field potentials when administered iv at 30 ng/kg. **5** had no effects on heart rate and mean arterial pressure (MAP) in conscious dogs at doses up to 3 mg/kg iv, and unlike the 3-hydroxy derivative **4**, **5** quickly enters the rat brain after iv administration with a brain: plasma ratio of 9.6 (9). *In vitro*, the compound is a potent and efficacious BK_{Ca} channel opener using clonal cell lines of human and rat BK_{Ca} α, and in hippocampal slices it was effective (IC_{50} = 352 nM) at reducing glutamate release (9).

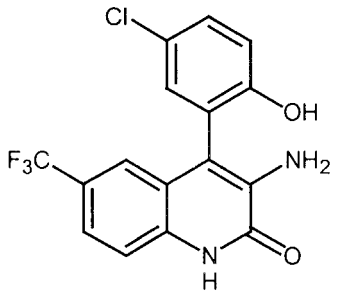
The racemate of **5** activated BK_{Ca} currents with a profile similar to compound **4** in cloned *Slo* channels (11). The 3-fluoro substitution of **5** was introduced to improve metabolic stability. A number of other analogs lacking the 3-fluoro or 3-hydroxy groups showed even greater potentiation of BK_{Ca} currents in vitro suggesting these substitutions are not absolute requirements. Replacement of the 6-CF_3 group with 6-iodo, 6-phenyl or a fused phenyl also provided active analogs.

Initial clinical data in healthy subjects showed that **5** was well tolerated up to doses of 0.3-0.4 mg/kg iv, where postural hypotension became a limiting factor (12). At lower doses no effects on psychomotor performance or cardiovascular function were noted, and pharmacokinetic data indicate a high clearance (~1 l/min), widespread distribution (~550 l), and an attractive $t_{1/2}$ (16-20 h) in these studies (12). In suspected acute stroke patients **5** was also well tolerated with no clinically significant adverse events. Currently **5** is undergoing late-stage clinical trials for stroke (8). Although results from the first trial indicated no significant degree of efficacy *vs* placebo, a second study is underway to rigorously evaluate the neuroprotective potential of this novel BK_{Ca} opener (13).

Newer families of BK_{Ca} openers have appeared based on the oxindole series. The 3-hydroxy and 3-amino-4-aryl-quinolin-2(1H)one structures such as **6** and **7** have been disclosed as novel chemotypes with potent BK_{Ca} opening activity *in vitro*



6

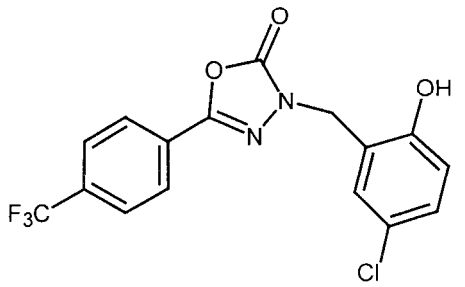


7

and *in vivo* (14,15). Within this series, the trend continues wherein the 5-halo-2-hydroxy substitution pattern is present on one of the aromatic rings.

The majority of analogs for which data was disclosed possess the 5-chloro-2-hydroxyphenyl or 5-chloro-2-methoxyphenyl groups. Compound **6** activated currents by more than 50% at 20 μM in *mSlo* or *hSlo* channels. Compound **7** similarly activated BK currents more than 50% at 20 μM. *In vivo,* this latter compound reduced infarct volume by 14% when dosed (0.001 mg/kg, iv) in a focal stroke model in rats (15).

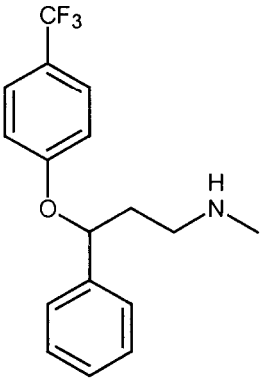
The 1,3,4-oxadiazolone compound **8** exemplifies another novel structural series of BK_{Ca} modulators (16). Once again the 5-halo-2-hydroxyphenyl moiety is present, in this case linked to the second aromatic ring via the oxadiazolone ring. This compound is reported to potentiate BK_{Ca} currents by 26% at 1 μM. *In vivo* **8** reduced infarct volume by 18% in a focal stroke model in Wistar rats at a dose of 10 μg/kg, administered iv. Similarly, in an equivalent model in spontaneously hypertensive rats, infarct size was reduced by 14% at a dose of 10 mg/kg (ip). A number of structural analogs of this compound have also been claimed to activate BK_{Ca} currents *in vitro*. All of those analogs with reported data again possess the 5-halo-2-hydroxyphenyl group with variations in the spacer heterocycle or additional aromatic ring substituents (16).



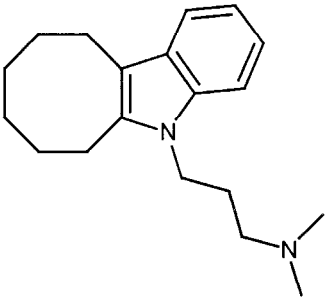
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$K_v1.1$ - The voltage-gated $K_v1.1$ channel has been associated with cognitive and nociceptive processes as well as mood regulation and epilepsy. Knockdown of $K_v1.1$ with an antisense oligonucleotide (icv) has been shown to impair memory in mice and rats (17). More recently, the dual $K_v1.1/K_v1.3$ blocker kaliotoxin (icv) was demonstrated to improve learning performance in rats (18). Support for the involvement of $K_v1.1$ in depression comes from a recent animal study where mice given a $K_v1.1$ antisense oligonucleotide (icv) exhibited dose dependent behavior that was consistent with a depressed state (19).

It has been known for some time that the tricyclic antidepressants amitriptyline and imipramine are able to block neuronal K^+ currents by approximately 50% at 10 μM (20). More recent studies with other antidepressants like the selective serotonin reuptake inhibitor (SSRI) fluoxetine **9** and the atypical antidepressant iprindole **10** have shown that they block K_v currents from rat cerebellar granule neurons with IC_{50} values of 15 μM and approximately 30 μM, respectively (21). Fluoxetine was also found to inhibit K^+ currents in oocytes (IC_{50} = 600-700 μM) and Chinese hamster



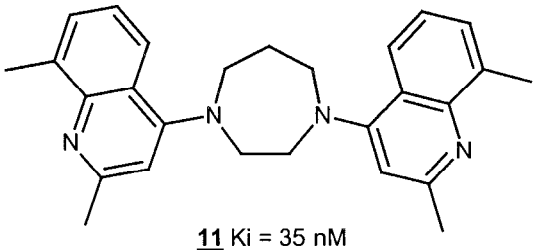
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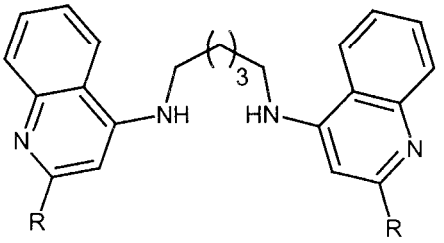
10

ovary (CHO) cells (IC_{50} = 55 μM) expressing recombinant $K_v1.1$ (22,23). The potency of fluoxetine to inhibit $K_v1.1$ is within the reported therapeutic brain concentrations of 5 to 70 μM, suggesting that this activity may contribute to either the therapeutic effect or side effects of the drug (23,24).

SK_{Ca} Channels - The small conductance Ca^{2+}-activated potassium channel (SK_{Ca}) is found on sympathetic neurons, and it has been implicated in CNS disorders such as depression and cognitive impairment. Three cloned subtypes of the SK_{Ca} channel have been identified, SK1, SK2 and SK3. The SK1 and SK2 subtypes are believed to be important in diseases involving loss of learning and memory as in Alzheimer's disease. Recent results with the SK blocker apamin (icv) found it reduced immobility comparable to the antidepressants imipramine and amitriptyline in the mouse forced swim test, a model of depression (19). This antidepressant-like effect occurred in the absence of any effects on motor coordination.



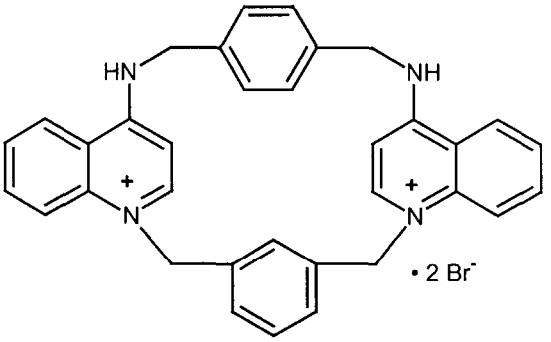
11 Ki = 35 nM



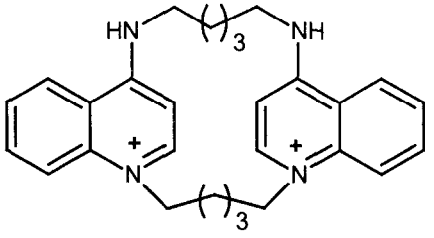
12 R = Et, Ki = 17 nM
13 R = Pr, Ki = 13 nM

Two series of novel bisquinoline analogs of the SK_{Ca} blocker dequalinium with the potential for cognition enhancing and antidepressant properties were recently disclosed (25,26). Compounds **11-13** displaced ^{125}I-apamin with K_i values ranging from 13 to 35 nM.

The results of continued SAR studies around the potent SK_{Ca} channel blocker UCL 1684 (**14**) (IC_{50} = 3 nM) have recently appeared in the literature (27). SK_{Ca} blocking activity in rat superior cervical ganglion (SCG) neurons is reduced 10-60 fold by changes in the position of attachment of the phenyl groups linking the two quinolinium groups. Likewise, larger biphenyl-type linkers lowered the potency, although substantial variation in the nature of the larger linkers was tolerated with potencies consistently in the range of 100-400 nM. Molecular modeling studies were performed to identify key differences in the low energy conformations of **14** and analogs that could account for the differences in activities observed. The most potent compound, UCL 1684, possessed two distinct low energy conformations whereas the least potent analog could exist in as many as nine conformational minima. In the course of these SAR investigations, the aromatic linker of **14** could be replaced with an alkyl linker, leading to the discovery of UCL 1848 (**15**) (SCG IC_{50} = 2 nM). Linking alkyl groups between 3 and 10 carbons were studied and the five carbon chain of **15** was found to be optimal in this series (28). Given the potent activity observed with either aromatic or alkyl linkers it was postulated that the linker groups do not engage in a specific interaction with the channel protein, but rather serve only to orient the quinolinium groups. Compound **15** was also found to be a highly potent blocker at the cloned SK2 subtype expressed in HEK 293 cells (IC_{50}~0.1 nM).



14

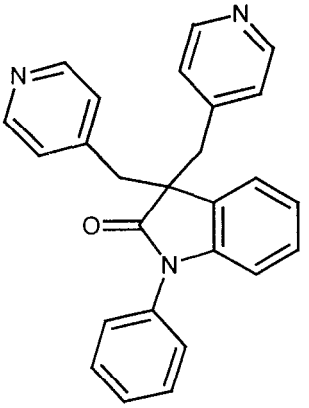


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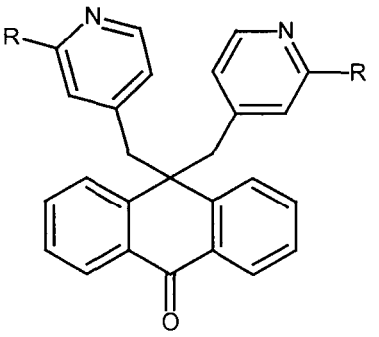
KCNQ Channels - Although modulators of various voltage-gated K^+ channels have earlier been claimed as cognition enhancers, recent attention has been focused on blockers of the M-type K^+ channel. These channels are derived by combinations of the KCNQ3 subunit with KCNQ2, KCNQ4 or KCNQ5 which play critical roles in regulating neuronal excitability (29). Mutations in KCNQ channel subunits lead to benign familial neonatal convulsions, a generalized form of epilepsy and autonomic dominant progressive hearing loss (3).

Compounds developed as cognition enhancers such as linopirdine (**16**) and XE-991 (**17**) are blockers of M channels which have been associated with enhancement of transmitter release by these drugs (30). Increased acetylcholine release in rat brain and improvements in animal models of learning and memory have been observed with **16**. This compound inhibits M current in hippocampal neurons with an IC_{50} value of 2.4 μM. At higher concentrations (>10 μM) it also blocks calcium-

activated and other voltage-dependent currents (31). Although clinical data with **16** remains inconclusive, analogs such as **17** with superior pharmacological and pharmacodynamic properties have emerged as orally active agents with potential for treatment of cognitive deficits.



16

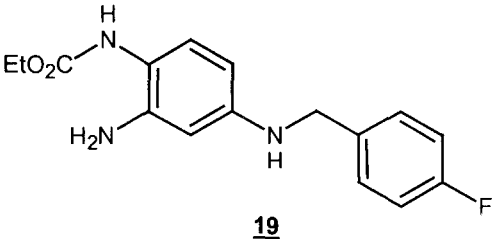


17 R = H
18 R = F

Further modifications led to the identification of DMP-543 (**18**), a compound at least 10 times more potent in releasing ACh from hippocampus with an improved half life and a brain: plasma distribution compared to **16** (32). The unsubstituted bispyridine **17** inhibited cloned KCNQ2 and KCNQ2+KCNQ3 channels with IC_{50} values of 0.7 and 0.6 μM respectively, comparable to those required for inhibition of M current in sympathetic ganglia neurons (33). On the other hand, heteromers derived from KCNQ1/minK that underlie cardiac I_{Ks} current, are 14-18 fold less sensitive to **17** blockade compared to either KCNQ1 alone or neuronal KCNQ2/3 combination demonstrating selectivity for this class of compounds for neurotransmitter release over cardiac function (34).

Genetic evidence linking mutations in certain KCNQ family members to benign familial neonatal convulsions has prompted examination of openers of these K^+ channels as antiepileptic agents. Retigabine, an anticonvulsant, **19** has been shown to activate human KCNQ2 and KCNQ2/3 channels expressed in CHO cells in a partially **16** sensitive manner suggesting that M-channel activation may contribute to the pharmacology observed for this compound (35).

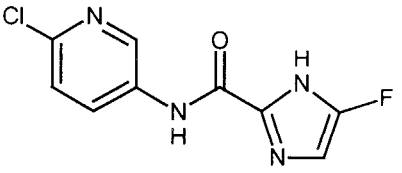
Retigabine has also been shown to be neuroprotective in *in vivo* models of cerebral or carotid occlusion (36). Although the lack of selectivity of retigabine limits current understanding of the role of M channels in disease processes, selective KCNQ openers could be beneficial in diseases involving hyperexcitability.



19

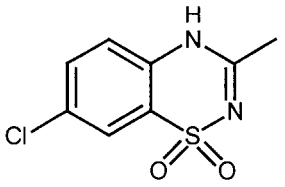
KCNQ channels have also emerged as an attractive target for pain. KCNQ2/3 mRNA is expressed in human dorsal root ganglion cells and openers have been shown to hyperpolarize the membrane potential suggesting a possible role of these currents in pain processing. This is supported by *in vivo* data showing that KCNQ

openers are analgesic in animal models of pain such as the formalin and hot plate tests. In addition to possessing anxiolytic properties (37). Several benzanilide analogs such as **20** are claimed as KCNQ channel openers following evaluation in $^{86}Rb^+$ efflux assays in KCNQ2-transfected CHO-K1 cells. Detailed data describing the potency and / or selectivity of these agents are not reported (38).

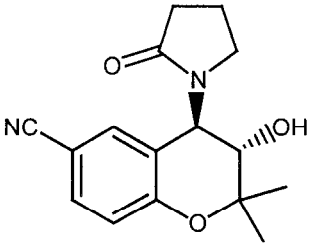


20

K_{ATP} Channels - Openers of various neuronal K^+ channels have long been proposed for stabilizing cellular hyperexcitability. Initial studies showed that openers of K_{ATP} channels such as cromakalim have neuroprotective effects in transient global forebrain ischemia in rats (39). Diazoxide (**21**) and (-)-cromakalim (**22**) have also been shown to protect hippocampal neurons against oxidative injury induced by amyloid β-peptide (Aβ) *in vitro* (40). Recent studies showing that transgenic overexpression of SUR1 alone in the forebrain significantly protects mice from seizures and neuronal damage without interfering with locomotor or cognitive function supports the notion that potent and selective openers of neuronal K_{ATP} channels that penetrate the blood brain barrier in pharmacologically relevant concentrations may be viable antiepileptic and/or neuroprotective agents (41).



21



22

K_{ATP} channel activation has also been implicated in antinociception evoked by various neurotransmitter receptors. Cromakalim differentially enhanced antinociception induced by agonists of receptors coupled to pertussis toxin-sensitive G proteins. In mice, icv administration of cromakalim dose-dependently potentiated the antinociceptive effects of clonidine, morphine and phenylisopropyladenosine in a gliquidone-sensitive manner. Under these conditions, cromakalim did not modify antinociceptive effects of κ-opioid receptor and γ-amino butyric acid agonists (42). This shows that opening of K_{ATP} channels may play a role in the antinociception evoked by α2, μ opioid and adenosine A1 receptors, but not those evoked by $GABA_B$ and κ-opioid receptors.

Conclusion - Potassium channels are increasingly being identified as molecular targets for any number of pathophysiologic disease states. These channels are critical to neurotransmission, and small alterations in their function result in remarkable changes in membrane excitability and neuronal function. Significant progress has been made in linking many CNS disorders to K^+ channel modulation. The biology of potassium channels has evolved to the stage where the impact of specific ion channels can evaluated at the molecular level and progress has been made to determine whether these effects are relevant to various aspects of neuronal modulation. Compounds altering currents of large conductance calcium-activated K^+

channels (BK_{Ca}) are the best characterized, yet there remains opportunity for improvements in potency and subtype selectivity across the α/β combinations in the family of BK_{Ca}s. Considerable efforts have focused on such improvements, resulting in an array of new structures currently under characterization. Openers of small conductance calcium-activated channels (SK_{Ca}) are in early stage evaluation, yet *in vivo* studies provide some validation of ongoing medicinal chemistry efforts. Voltage-gated channels have also shown *in vivo* activity in models of CNS function, with a number of modulators appearing from well-established structure-activity relationships. Altered function of M-type channels have provided *in vivo* activity which has prompted further study, and a number of novel structures have emerged from this relatively new area of research. Conversely the well-established medicinal chemistry of K_{ATP} channel modulators emanating from cardiovascular research should provide new opportunities for CNS applications as a deeper understanding of K_{ATP}-mediated neuronal processes develops.

Despite considerable scientific progress, the ever present need for ion channel selectivity and target validation remains a key hurdle. Ion channel targets mandate subtype selectivity, yet it is unclear whether such *in vitro* discrimination will result in superior tissue and organ specificity. Clearly a number of CNS-related targets have such potential, yet key questions remain with respect to elucidating neuronal K^+ channel properties. For the treatment of CNS disorders, there is limited clinical data, however the considerable progress in the study of neuronal K^+ channel modulation will likely result in continued clinical evaluation of candidate neuromodulators.

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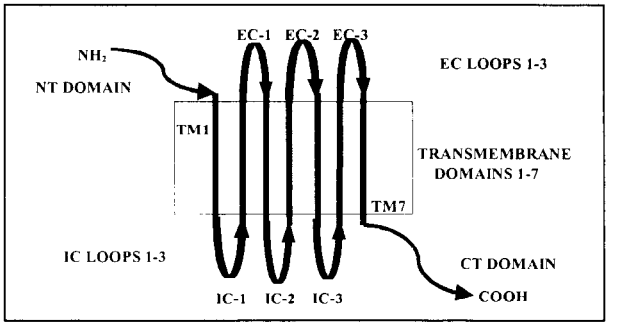
Chapter 3. New Developments in the Study of Corticotropin Releasing Factor.

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Introduction - Corticotropin releasing factor (CRF) or hormone (CRH) is one of several neurohormones synthesized by specific hypothalamic nuclei in the brain and released into the portal system, which bathes the anterior pituitary. Here the peptide activates the transcription of the pro-opiomelanocortin gene resulting in release of ACTH and β-endorphin from anterior pituitary cells. The fundamental role of CRF is to prepare the organism for an appropriate response to various stressors such as physical trauma, insults to the immune system and social interactions. CRF also has marked CNS effects by acting at higher centers in the brain, particularly cortical regions where there is a widespread distribution of CRF neurons. It is the hyper- or hyposensitivity of the system that can lead to human pathologies. Since this topic was last reviewed in Annual Reports (1), several other detailed accounts have appeared covering receptor structure and function (2), small molecule receptor antagonists and peptide ligands (3-5) and therapeutic implications including depression (6,7), stress-induced gastrointestinal dysfunction (8), drug addiction (9), pain (10) and eating disorders (11). This update covers research that was published from mid to late 1999 onwards in the areas listed above.

CRF RECEPTOR STRUCTURE AND FUNCTION

It is well established that CRF exerts its actions by interaction with one of two distinct subtypes of G-protein coupled receptors (GPCRs), each being encoded by separate genes, and which fall into the second category ('Class B') of this gene superfamily. A cDNA encoding a third receptor has been isolated from catfish pituitary and urophysis and displays CRF_1-like behavior both structurally and in response to peptide agonists (12). This raises the possibility that mammalian genomes may also yield genes for as yet undeciphered receptor subtypes. Various forms of these two CRF receptors arise from different splice modifications and also differ both in their anatomical location and their response to peptide ligands (13). Other than $CRF_{1\beta}$ (a 29 amino acid insert into IC1, see Figure) which had both reduced affinity and efficacy in its interaction with CRF and $CRF_{1\gamma}$ (in which the exon encoding the mid-region of the N-terminus has been deleted), another variant ('$CRF_{1\delta}$') has been identified in human pregnant myometrium and fetal membranes arising from an exon deletion normally encoding a 14 amino acid sequence towards the C-terminus of the putative 7th helical region (14). Although binding of CRF to this variant remained essentially unchanged when compared to $CRF_{1\alpha}$, the receptor was only poorly coupled to G_s so that CRF behaved as a weak, partial agonist. Whilst there is uncertainty about the physiological significance of these splice variants, these data provide insight into functional domains of CRF_1. Thus, residues downstream from the 7th helical domain are important for signal transduction whereas the N-terminal domain may be more concerned with formation of the first 'collision complex' between ligand and receptor. Also, for G_s-linked GPCRs in general, agonist-dependent desensitization has been shown to



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involve phosphorylation of residues, typically Ser or Thr, in the C-terminal domain and/or IC3 – a process which for $hCRF_1$ seems to be driven by a G-protein receptor kinase (GRK3) rather than PKA or calmodulin pathway (15).

Splice variants of CRF_2 previously reported include $CRF_{2\beta}$ and $CRF_{2\gamma}$ which have the N-terminal 34 residues of $CRF_{2\alpha}$ replaced by a new 54 or 20 residue sequence respectively. Human $CRF_{2\beta}$ was shown to prefer urocortin binding to sauvagine (5-fold) and then CRF (30-fold) suggesting that urocortin may be the natural agonist. The role of the receptor N-terminus is again apparent given that urocortin bound 10 times more tightly to the $CRF_{2\beta}$ variant. More recently, a severely truncated form of $CRF_{2\alpha}$, $CRF_{2\alpha}$-tr, from rat amygdala, thalamus and hypothalamus where levels of mRNA for CRF_1 and $CRF_{2\alpha}$ are undetectable, was shown to be composed of the N-terminal 236 amino acids terminating mid-way up the fourth transmembrane domain (16). This receptor bound rCRF with identical affinity to $CRF_{2\alpha}$ but not sauvagine or urocortin and did not cause accumulation of cAMP. For CRF_2, this points to a dominant role for the N-terminal region and EC1 in binding CRF (again, not activation) to the receptor and suggests that the preferred ligand, urocortin, has additional productive interactions with other extracellular elements. Antidepressant drugs such as imipramine increased the expression of $CRF_{2\alpha}$-tr and thus the number of binding sites for CRF in the amygdala suggesting that these receptors may regulate the release of endogenous CRF as a contribution to the mechanism of imipramine action (17). In summary, it may be concluded that the NT domain together with the EC1 form the initial ligand recognition site, whereas the CT together with the IC3 are the key elements required for signal transduction. At least for CRF_2, EC2 and EC3 probably provide additional binding sites for urocortin supporting the concept that the family of natural CRF peptide agonists may have different, albeit overlapping, binding sites.

Earlier studies with chimeric constructs of CRF_1 and $CRF_{2\alpha}$ (the later having the His185 variant located at the junction of EC1 and TM3) had shown that two regions of the receptor are important for r/hCRF binding to CRF_1 – the junction of the second EC loop with TM5 and, secondly, the first EC loop. However, it has now been shown that the most abundant form of $CRF_{2\alpha}$ has Arg185 (in common with CRF_1) which has a higher affinity for the CRF peptides, sauvagine and urotensin (3- and 9-fold respectively over (H185)-$CRF_{2\alpha}$ (18).

To determine the amino acid residues in the N-terminal domain of CRF_1 receptors responsible for binding to the CRF peptides, mutant receptors were constructed by replacing specific residues in the $hCRF_1$ with amino acids from the corresponding positions in the N-terminal domain of another class B receptor, human vasoactive intestinal peptide (VIP) receptor type-2 (19). Two regions in the NT domain of $hCRF_1$, one mapped to residues 43 – 50 and the second from residues 76 – 84, were found to be important for binding of CRF peptide agonists and antagonists as well as activation by CRF peptide agonists. By replacing the putative EC3 of $rCRF_1$ with the corresponding loops from either the PACAP or glucagon receptors, it was shown that this region is involved in both binding and activation (20). Accordingly, both mutant receptors failed to bind oCRF with high affinity (K_D ~ 2 μM) and both were uncoupled from cAMP production. Furthermore, the amino acid residues in the EC3 domain responsible for receptor activation were tentatively identified as Tyr^{346}, Phe^{347} and Asn^{348} in CRF_1. The mutant $rCRF_1$ receptor with all three amino acids changed to Ala displayed reduced binding (K_D = 64 nM) and oCRF behaved as a weak partial agonist (EC_{50} = 32 nM; 15% efficacy; c.f. with wild type receptor, EC_{50} = 0.3 nM).

The strategy based on construction of chimeric receptors derived from the promiscuous $hCRF_1$ and the ligand selective Xenopus (x) CRF_1 and $h/xCRF_2$ was used to elucidate the ligand-binding domains residing in CRF_2 (21). Chimeric

receptors, in which the N-terminal domain of either $hCRF_2$ or $xCRF_2$ replaced the NT domain of $hCRF_1$, bound all CRF peptides non-selectively with high affinity, whereas chimeric receptors, in which the NT domain of either the $hCRF_{2\alpha}$ or $xCRF_2$ replaced the NT domain of $xCRF_1$, bound the CRF peptides with significantly lower affinity. Chimeric receptors, incorporating the NT domain of $xCRF_1$ linked to either $hCRF_2$ or $xCRF_2$, showed a similar pharmacological profile as the two parent CRF_2 proteins, indicating that the peptide ligand-selective domains in CRF_2 reside in EC1, EC2 and EC3.

For CRF peptide agonists, the essential function of the N-terminal domain in the CRF receptor is to capture the CRF peptide ligand at its C-terminal region, followed by proper orientation of the ligand's N-terminal region to activate the receptor at the EC domains in the seven transmembrane region. This concept was validated by preparation of a constitutively activated $hCRF_1$ involving a tethered CRF peptide reminiscent of the thrombin receptor system (22). Thus a CRF_1 chimera, in which the N-terminal domain (approximately) corresponding to residues 1 – 111 of $hCRF_1$ was replaced by the N-terminal r/hCRF(1-16) sequence, had a 25-fold increase in 'basal' levels of cAMP production and a 20-fold increase over wild type receptors where the basal levels were determined in the presence of 10 μM of the non-peptide, CRF_1 selective antagonist, antalarmin (1, below). As would be expected from a model which predicts a different binding site for peptide (NT and EC domains) and non-peptide (TM regions predominantly) antagonists, the peptide antagonist, astressin (cyclo(30-33)[D-Phe^{12}, $Nle^{21,28}$, Glu^{30}, Lys^{33}]h/rCRF(12-41)NH_2) did not inhibit constitutive activation – astressin clearly was not able to compete with the intramolecular tethered ligand. Furthermore, when the same leucine at position 8 of CRF that is critical for potency at the wild type receptor (Leu8Ala is 300-fold less potent than CRF) is mutated to alanine in a tethered peptide, the resulting protein is no longer constitutively active even though the receptor itself could still be activated with urocortin.

In common with other GPCRs, there are two conserved cysteines in the first and second EC loops (C188 and C258 in CRF_1) which, for rhodopsin forms a critical disulphide bond (C110-C187) clearly visible in the crystal structure (23). Assuming that rhodopsin is a good homology model for GPCR's in general, this S-S link has a profound effect on the accessibility of the helical domains to small molecule ligands since it places the EC2 at the extracellular surface and partially penetrates the helical bundle. This same disulfide bridge in the CRF_1 may be implied from both single and double mutational studies (24). High affinity CRF binding was not observed in the C188S, C258A or C188S/C258A mutants although the double mutant remained fully coupled to G_s possibly suggesting that a reorganization of disulphide bonds is a necessary step towards receptor activation. Although not definitive, it was also proposed that the pattern of disulphide bonds for the CRF_1 is C44-C102, C68-C87 and C188-C258. The NT domain of group B GPCR's contains 6 cysteine residues and the role of each of the segments flanked by these cysteines was studied by forming chimeric receptors between mCRF and rPTH (homologue scanning mutagenesis). It was shown that the region towards TM1 (C68 to G109) is required for high affinity agonist binding with C68-C87 and C102-E109 being particularly important for sauvagine although not CRF. In contrast, the C87-C102 region was required for receptor activation (25).

All variants of CRF receptors are known to signal through G_s as the predominant G-protein. However, in transfected cells, $CRF_{1\alpha}$ stably expressed in HEK293 cells was shown to activate other multiple G-proteins (G_i, $G_{q/11}$ and G_o) and hence two distinct signalling pathways – PLC and adenylate cyclase (14). In a more native system, rat cortical membranes, similar results were obtained by measuring the incorporation of the non-hydrolysable GTP analogue, [α-^{32}P]-GTP-γ-azidoanilide, into G-proteins upon receptor activation with either CRF or urocortin (26).

CRF RECEPTOR PEPTIDE LIGAND STRUCTURE-ACTIVITY

From over 15 years of studies with CRF peptide analogs, it can be concluded that [1] deletion of residues from the N-terminus of CRF resulted in a gradual loss of potency but with maintenance of full efficacy until deletion of leucine 8 so that CRF(9-41) behaves as an antagonist; [2] the aminated C-terminus of CRF is essential for binding; [3] α-helix inducing residues introduced into the 8-32 region potentiated binding affinity. Thus the accepted model for CRF binding implies the presence of a central helical region that separates the unstructured activation domain (the N-terminus) from the C-terminus apparently obligatory for initial receptor recognition. Alanine scanning experiments revealed that the side-chain functional groups of the 'linker' region had little effect on activity (27). Introduction of C^{α}-methyl residues into astressin afforded astressin-B (cyclo(30-33)[D-Phe^{12}, Nle^{21,38}, (C^{α}-Me)Leu^{27,40}, Glu^{30}, Lys^{33}]Ac-h/rCRF(9-41)NH_2), the most potent antagonist for CRF_1 reported to date (28).

Whilst CRF displays 10-100 fold selectivity for CRF_1 over CRF_2 and there are numerous, potent CRF_1-selective (non-peptide) antagonists, the reverse had not been achieved. The discovery of a 38 amino acid mouse 'urocortin-II' having exquisite selectivity for CRF_2 (K_i = 0.66 nM; EC_{50} (cAMP) = 0.14 nM) has re-asserted the view that the endogenous ligand for the CRF_2 receptor may be urocortin-like; preliminary evidence for a human analog also exists (29). By searching GenBank databases for sequence motifs with primary and secondary structures shared by the CRF peptide family, two other human CRF_2 specific ligands, 'stresscopin and stresscopin-related peptide', have been identified which were shown to display typical CRF_2-like behaviours in rats (suppresion of food intake, delayed gastric emptying) but failed to activate CRF_1 receptors since there was no effect on ACTH release from cultured rat anterior pituitary cells (30).

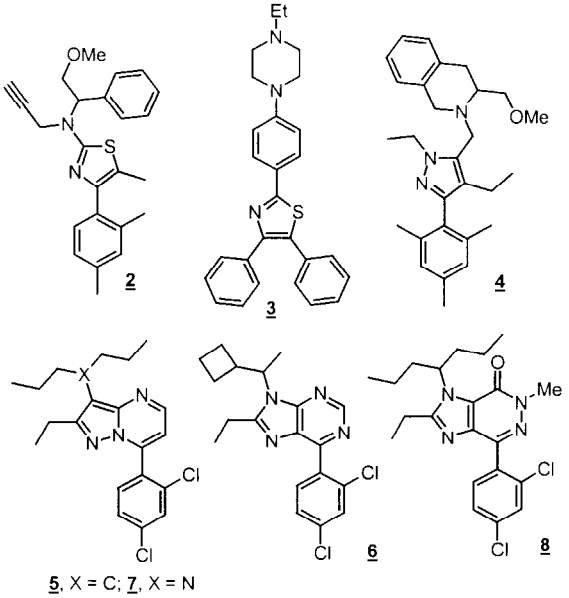
Truncation of the first ten N-terminal residues of sauvagine combined with substitutions at the 11th and 12th positions gave [D-Phe^{11},His^{12}]sauvagine(11-40)NH_2, called antisauvagine-30 (ASV-30), exhibiting a 100-fold selectivity for $CRF_{2\beta}$ over CRF_1 (31). This peptide has been radiolabeled and displayed saturable, high affinity ($hCRF_2$ K_D = 125 pM) binding and was apparently even more selective (1000 – 10,000-fold) for $hCRF_2$ the difference being ascribed to the binding protocols used (32). In competition binding studies with the $hCRF_{2\alpha}$ receptor, hCRF displaced labeled CRF and antisauvagine-30 with different affinities (K_i = 42 and 300 nM, respectively) (33).

NON PEPTIDE LIGANDS FOR CRF RECEPTORS

The design and synthesis of low molecular weight (< 500) non-peptide ligands for the CRF receptors continues to be a very active area of research. Much more is known about the requirements for small molecules to bind and antagonize the function of CRF_1 than that of CRF_2. Non-peptide ligands for the CRF_1 receptor are comprised of a putative hydrogen bond acceptor (HBA) in a core aromatic heterocycle that is flanked by a branched alkyl group and a 2,4-disubstituted aromatic substituent from pharmacophore analysis of the known antagonists using Catalyst (2, 34, 35). The 2-substituent on the aromatic ring appears to force the aromatic group out of plane relative to the heterocyclic core. Tri-subsitituted thiazoles constituted some of the first heterocyclic CRF_1 antagonists known (1). A new report (36) suggests that a new set of substituents attached to the 5-membered heterocyclic thiazole core also provide high affinity antagonists of the CRF_1 receptor as exemplified by compound **2**. The branched alkyl substituents may not be restricted to the previously reported bis-cyclopropyl groups and the aromatic region of the molecule remains the same. Thiazoles **3** also are claimed as CRF_2 antagonists in a patent but, unfortunately, no

specific biological activity is described (37). The relationship between the thiazole structure and CRF_2 antagonist activity is clearly different from the thiazole CRF_1 antagonists.

Other 5-membered ring heterocycles like pyrazoles and imidazoles also play the role of core HBA in CRF_1 antagonists. A recent patent describing pyrazoles **4** specifically claims tetrahydroisoquinoline substituents in the branched alkyl region of the molecule (38). This basic side chain may provide much needed increases in aqueous solubility that has been traditionally lacking in the CRF_1 antagonists reported to date. The aromatic side chain and the HBA moiety are separated by one atom analogous to the thiazoles. There are several bicyclic pyrazoles and imidazoles reported that display a two atom spacing relationship. Pyrazole **5** and imidazole **6** are high affinity ligands for the CRF_1 receptor with K_i values of 1.2 nM and 0.93 nM respectively (39, 40). Replacing X = nitrogen in **7** (12 nM) with X = carbon (1.2 nM) to give **5** at the branch point results in a 10 fold increase in activity (39). A wide variety of heterocycles also serve as templates as illustrated in compound **8**, a 2.5 nM antagonist of CRF_1 (41).



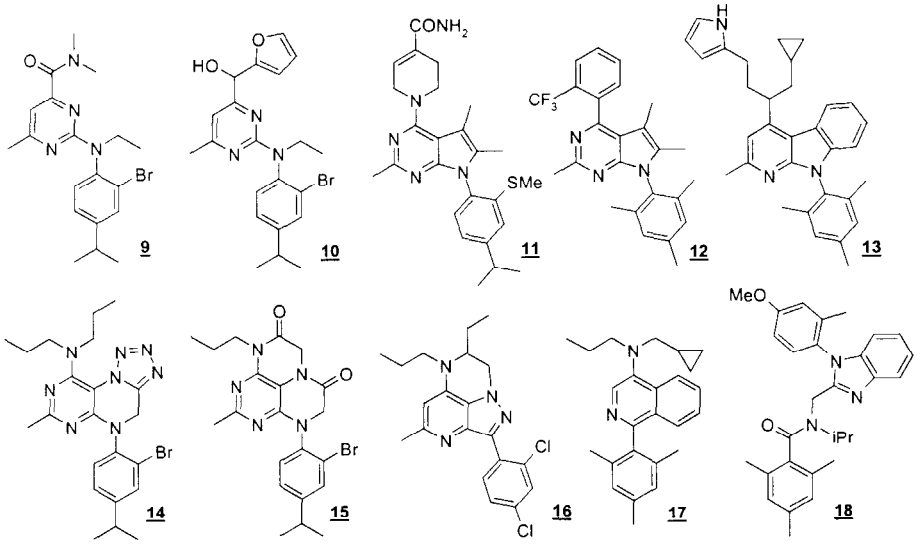
2 **3** **4**

5, X = C; **7**, X = N **6** **8**

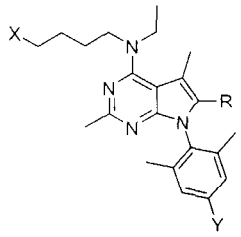
Six-membered ring heterocycles have been the most studied core groups in the area of small molecule antagonists of CRF_1. A survey of the structural requirements in the branched alkyl region of pyrimidine antagonists revealed that much less lipophilic substitents might be tolerated in the branched alkyl region (42). Compound **9**, which incorporates a carboxamide group, and **10** which contains a furylcarbinol moiety are only 10-fold less active than the highest affinity small molecule ligands reported. Another carboxamide containing side chain was part of an intriguing report from a patent that claims **11** as a CRF antagonist but without disclosing specific biological activity (43). Aromatic groups can also be tolerated in the branched alkyl region (44), as exemplified by compound **12** (K_i = 9.7 nM). Tricyclic pyridine and pyrimidine antagonists have also been reported in the patent literature of late. These new antagonists can be further subdivided into fused tricyclic cores and conformationally constrained side chain templates (45 - 47). The compounds **13** and **14** typify the fused tricyclic core series and the flexibility of the branched alkyl side chains is limited in **15** and **16**. One feature consistent in the pyrimidine and pyridine classes of CRF_1 antagonists, in contrast to 5-membered ring heterocycles, is the optimal substitution of

a methyl group adjacent to the hydrogen bond acceptor nitrogen with a two-atom spacer between the aromatic moiety.

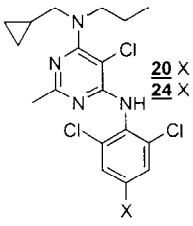
Analogous to the 5-membered ring heterocycles, pyridine based core structures, such as **17**, with one-atom spacers between the key nitrogen and the aromatic side chain have been reported to be CRF_1 antagonists (48). New non-aromatic cores that could also serve as the HBA moiety include the acyclic amide **18** claimed as a CRF_1 antagonist in a patent that also maintains the one atom spacer relationship between the aromatic and oxygen HBA (49).



In addition to the basic understanding of the CRF_1 binding SAR there has also been some recent work in the synthesis of potential positron emission tomography (PET) and single photon emission computed tomography (SPECT) CRF_1 ligands for non-invasive clinical neuroimaging. Fluorinated and iodinated analogs of the three most utilized pharmacological tools **1**, CP-154526 and NBI-27914 (compounds **19** and **20**, respectively) have been reported (50-52). The fluoro analogs **21** and **22** as well as iodo analogs **23** and **24** are all high affinity ligands. The use of all of these compounds *in vivo* was limited by their high lipophilicity, limited solubility and poor bioavailability. Radiotracer **22** was evaluated *in vivo* but due to the limited brain exposure of the compound, studies were limited only to imaging of the pituitary. Imaging studies showed an enlarged pituitary in rats with chronic peripheral administration of CRF (51). These observations are analogous to those seen in depressed patients through the use of magnetic resonance imaging (MRI).



1 X = H, Y = R = Me (Antalarmin)
19 X = R = H, Y = Me (CP-154526)
21 X = F, Y = R = Me
22 X = F, Y = Me, R = H
23 X = R = H, Y = I



20 X = Cl (NBI-27914)
24 X = I

PHARMACOLOGICAL STUDIES

Anxiety and Depression – Central administration of CRF produces anxiety-like behavior in animals and antagonists of the CRF receptors have reversed these effects (6,7). Astressin reversed the CRF induced anxiety-like behavioral effects as measured in the plus maze paradigm and reversed the anxiogenic effects of social

stress (53, 54). Strangely, astressin had no effect in reducing the CRF induced increases in locomotor activity analogous to the small molecule CRF_1 selective antagonist **19** (54) unlike the effects observed with CRF(9-41) and [D-Phe^{12}]CRF(12-41). Peptide antagonists have also been utilized to reduce the abnormally fearful behavior of the offspring of pregnant rats that were exposed to the stress of daily handling and saline injections and the anxiogenic effects of the C-type natriuretic peptide, an important neuromodulatory peptide within the CNS (55, 56).

Selective small molecule CRF_1 antagonists have also reversed the anxiogenic effects of CRF, implicating CRF_1 as the key receptor mediating these effects. Analogous to the peptide antagonists, **19** blocked ultrasonic vocalizations of rats exposed to stress and neonatal separation and blocked interferon-α (IFN-α) depression-like behavior as measured by the tail suspension test in mice (57 - 59). Depression is a common side effect observed in IFN-α treated hepatitis C patients which is reversed with antidepressent therapy. Chronic administration of **19** (3.2 mg / kg / day) for 10 days in rodents resulted in a decrease of anxiety-associated behavior in the defensive withdrawal model whereas acute treatment had no effect. Increased expression of CRF (mRNA) in the hypothalamic paraventricular nucleus was observed in the high (32 mg / kg) chronic dose, which is opposite to the postmortem analysis of depressed humans where CRF is increased (60). Oral administration of **19** also inhibited anxiety-associated behaviors in the intruder paradigm in primates providing evidence that CRF_1 antagonists may be useful in treating anxiety and depression (61).

Since earlier work had shown that CRF_1 null mice displayed anxiolytic-like effects, $CRF_2^{-/-}$ mice were also studied in anxiety paradigms such as the elevated plus maze (62). The mutant mice were hypersensitive to stress, displayed increased anxiety-like behavior and had decreased food intake upon food deprivation, the later possibly a marker of an increased anxiety state following deprivation (63). Whilst iv infusion of urocortin into wild type mice produced a depressor response, $CRF_2^{-/-}$ animals showed no measurable change. In other experiments, male, but not female, mutant mice exhibited enhanced anxious behavior which correlated with a reduction in Creb phosphorylation leading to the proposal that CRF_2 mediates a central anxiolytic response to oppose the anxiogenic effect of CRF_1 (64).

Stress-Induced Gastrointestinal Dysfunction – Adverse events in life such as family death, marital stress and physical or sexual abuse have been reported more frequently in irritable bowel syndrome (IBS) patients than the general population. Similar to various stressors, CRF inhibits gastric emptying while accelerating colonic motility (8). Stress and central or peripherally administered CRF stimulated defecation (65, 66) can be blocked with astressin, icv **20** or ip **19**. Intracisternal (ic) administration of astressin blocked ic CRF delay of gastric emptying while ic **20** had no effect. Intravenous **1** also did not attenuate CRF induced gastric emptying (67). It appears that CRF_1 mediates the stress associated increase in colonic motility of and CRF_2 mediates the associated delay of gastric emptying suggesting that CRF_1 and CRF_2 antagonists may prove to be novel treatments of IBS.

Drug Addiction – There is evidence that CRF may play a role in the stress-induced relapse of drug abuse as well as the anxiety-like behaviors observed during acute drug withdrawal (9). Chronic cocaine administration in rats is anxiogenic and icv [D-Phe^{12}]CRF(12-41) attenuates the behavior, as characterized by the defensive burying paradigm (68). The compound **19** alleviated the symptoms associated with opiate withdrawal after chronic morphine, and blocked drug seeking behavior as measured through cocaine self administration (69, 70). Stress-induced relapse of alcohol seeking and opiate relapse was diminished by **19** but not antisauvigine-30 suggesting a CRF_1 role (71, 72).

Eating Disorders – Stress decreases food intake and weight gain and these effects are reproduced with centrally administered CRF (11). These effects are inhibited by antisauvigine-30 and CRF(9-41), which also alleviates the stress-like effects are also seen with the brain anorectic agent, CART (68, 73 - 74). In contrast, the small molecule CRF_1 antagonist **20** had no effect CRF induced decreases in body weight suggesting a role for CRF_2 (73). Urocortin is a 30-fold selective CRF_2 agonist whose effects on food intake can also be blocked by antisauvigine-30. Antisauvagine-30 had no effect on the associated changes in tissue weights and serum chemistry seen with central CRF administration implying that CRF_2 mediates only the anorexic, not the metabolic effects, of CRF (71). Central administration of urocortin-II has supported earlier observations in CRF_1 knock out mice that intimated CRF_2 activation may be only responsible for later stage (beyond 6 hours) suppression of food intake and not the early stage anorexic effects or the effects on metabolism that CRF_1 activation provides (52, 75).

CLINICAL OPPORTUNITIES FOR CRF

From SAR studies in the pyrazolo[1,5-a]pyrimidine series emerged NBI-30775 (R121919) as a potent (CRF_1 K_i = 2.8 nM), selective (CRF_2 K_i = > 2000 nM) CRF_1 receptor antagonist which inhibits CRF-induced cAMP accumulation (IC_{50} = 26 nM) and CRF-stimulated ACTH release from cultured rat anterior pituitary cells (EC_{50} = 28 nM) and displayed an excellent pharmacokinetic profile in rat following oral administration (35,76). The effect of R121919 was also studied in the elevated plus maze in two complementary rat breeds that exhibit high- and low-anxiety related behavior (77). The compound reduced anxiety-related behavior in the former group in a dose dependent manner but was without effect in the low anxiety group suggesting that CRF antagonists should be measurably active in clinical situations where there is an exaggerated stress response. Based on this and other data, the compound was selected for clinical development and, in an open label study with 20 depressed patients, a significant reduction in depression and anxiety scores was observed (78).

Conclusions - Identification of urocortin-II, antisauvagine-30 and stresscopin should help elucidate the relative contributions of CRF_2 to the CRF system. There continues to be advances in the understanding of the receptor structure and the SAR of small molecule CRF_1 antagonists but completely novel structures away from the current, well-mapped area are still awaited. Unlike the CRF_1 receptor, CRF_2 appears to be reticent to small molecule intervention. Recent research has added to the understanding of the role of CRF in disease areas like drug addiction, eating disorders and IBS with some encouraging results in the clinical utility of CRF_1 antagonists in the treatment of anxiety and depression.

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SECTION II. CARDIOVASCULAR AND PULMONARY DISEASES

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

Chapter 4. Recent Developments in Antitussive Therapy

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Introduction - Cough is a forceful defensive reflex maneuver that leads to expulsion of irritants, fluids, mucus or foreign material from the respiratory tract. Specifically, the reflex triggers a complex, multiphasic motor pattern characterized by sequential coordination of large increases in motor output to an array of inspiratory and expiratory skeletal muscles. This highly coordinated musculoskeletal activation process consists of three sequential phases, namely deep inspiration, compression (i.e. contraction against a closed glottis) and vigorous expulsion. The expulsion is ultimately attained through the combined forceful contraction of thoracic, abdominal and diaphragm muscles through the generation of rapid airflow (1, 2). While generally beneficial, cough is a prominent pathophysiological feature associated with many airway and lung diseases such as asthma, upper respiratory viral and bacterial infections, post-nasal drip syndrome, gastroesophageal reflux disease, pulmonary neoplasm, chronic bronchitis and chronic obstructive pulmonary disease (1, 3). Treatment may be complicated by the determination whether cough is mainly productive (sputum generating) or non-productive (dry cough). Chronic persistent non-productive cough (i.e. cough greater than three weeks duration) occurs in a post-viral airway disease setting in about 30% of the patients with acute respiratory infection. Furthermore, uncontrolled chronic cough is known to lead to significant morbidity and is associated with rib fractures, ruptured abdominal muscles, pneumothorax, marked decrement in the amount and quality of sleep, and loss of consciousness (1).

Drugs used for the treatment of cough are among the most widely used prescription and OTC drugs in the world (4). In the prescription market in 1999, sales of antitussives in the USA, Canada and Europe totaled approximately $750 million (5). The pharmacotherapy of cough is broadly divided into two major categories based on their purported site of action. These include drugs that act by a central mechanism to block the frequency (and/or amplitude) of the efferent cough motor output (e.g. opiates) or act in the periphery to inhibit the generation of the tussigenic sensory impulses (e.g. local anesthetics) (6, 7). Current antitussive therapy is dominated by the use of older drugs such as dextromethorphan and codeine. These drugs however carry significant side effect liabilities including among others, sedation, abuse liability and respiratory depression. Thus, because persistent cough is underserved by current therapeutic options available, there is an increasing need for safe and efficacious antitussive alternative(s) to existing medications. This report highlights some of the advances in the development of novel antitussives, and briefly reviews their chemistry, mechanism and site of action.

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NEURAL REGULATION OF THE COUGH REFLEX

Afferent Mechanisms of the Cough Reflex - Coughing is elicited by stimulation of specialized sensory nerves in the airways. A variety of different stimuli including airway inflammation, chemical irritants (e.g. capsaicin) and mechanical stimuli can trigger coughing in humans and animals. The polymodal sensory nerves that are prominently involved in neural regulation of cough are myelinated Aδ rapidly adapting receptors (RAR) and unmyelinated bronchial and pulmonary C-fibers (8). Their nerve endings appear to be prominently localized within the epithelial layer of the trachea and lower airways (2). The afferents transmitting the tussigenic impulses are carried by the vagus nerve to the nucleus tractus solitarius (NTS) in the medulla oblongata.

The unmyelinated C-fiber afferents contain neuropeptides of the tachykinin family, such as substance P and neurokinin A (NKA) as well as calcitonin gene related peptide (CGRP), which are synthesized in the nodose and jugular nerve cell bodies and undergo retrograde transport to the nerve terminals in the airways. In humans, the major subgroup of afferent nerves involved in the generation of cough is located primarily throughout the trachea and intra- and extrapulmonary bronchi. In addition to relaying impulse information to the CNS cough center, these nerves exert a local pro-inflammatory response due to the release of tachykinins from the peripheral nerve terminals. This neurogenic inflammatory response is characterized by smooth muscle contraction, edema and stimulation of neighboring RARs leading to amplification of the cough response (2, 8).

Efferent Mechanisms of the Cough Reflex - Tussigenic sensory impulses, in the form of propagating action potentials conducting up the axon, reach the NTS in the lower brainstem. When a critical threshold is exceeded, a cough response is produced. The "brainstem cough center", or central organization of the neural substrates involved in the coordination of the cough motor response, are poorly defined and refer primarily to the functional neural network in the medulla oblongata. The large forces generated during the expiratory motor phase of coughing collapse bronchi, which in turn increases the shear forces that promote clearance of airway material.

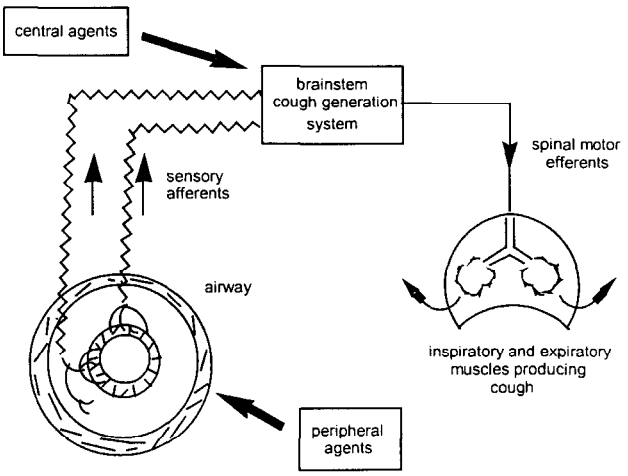
SITE OF ACTION OF ANTITUSSIVE DRUGS

Central Site of Action - Centrally active antitussive agents act preferentially by depressing the cough center at the level of the lower brainstem without affecting peripheral sensory or motor endplate effector responses, Figure 1 (6, 7). The μ opiate receptor agonists codeine and dextromethorphan, which are considered among the most effective antitussive agents available, are prototype centrally active antitussive agents. Whereas these agents are generally effective against cough of various etiologies, they also have prominent dose-dependent sedative and respiratory side effects that often limit their clinical utility.

Peripheral Site of Action – Antitussive agents may also act in the periphery to inhibit the sensory impulses that lead to the activation of the cough reflex, Figure 1. Recently there has been a strong interest in developing broadly efficacious antitussive drugs that act exclusively by a peripheral action in order to minimize the potential for undesirable side effects and liabilities characteristic of centrally active antitussive drugs such as codeine. One challenging aspect to the development of peripherally acting antitussives is the requirement that any novel agent display a high degree of selectivity for pulmonary afferents over other sensory afferents so as to avoid potential side effects related to nonspecific sensory function impairment. To date, however, most of the peripherally active antitussives display limited efficacy

and/or poor oral bioavailability. In general, peripherally active agents act by decreasing the sensitivity of airway cough receptors to tussigenic stimuli leading to an inhibition of pulmonary vagal discharge. When applied topically, local anesthetic antitussives, such as benzonatate, are generally considered to block voltage-dependent Na^+ channels, which in turn inhibits the generation and transmission of impulses to the CNS, leading to an attenuation of the cough response. Unfortunately, the local anesthetics are of minimal clinical value because their use is limited to topical delivery in lozenge form, and they have marginal efficacy at tolerated doses, short duration of action, and dose dependent potential for CNS-related side effects.

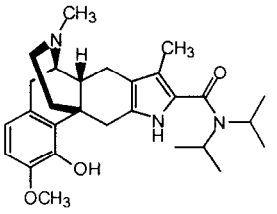
Figure 1



CENTRALLY ACTING AGENTS

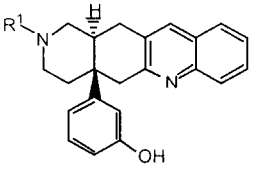
Several classes of drugs inhibit the cough reflex primarily by a central mechanism in humans and experimental animals. Included among these are opioids, $GABA_B$ agonists, and neurokinin antagonists (6, 9-11).

Opioids - Studies detailing the selective δ opioid receptor agonist SB 227122 (**1**) have recently been described (12). Compound **1** binds to the human δ receptor with high affinity (K_i = 6.9 nM) while its activity at the μ and κ opioid receptors is significantly weaker (K_i = 2030 nM and >5000 nM respectively). In vivo, **1** dose-dependently inhibited citric acid induced cough in the guinea pig with an ED_{50} = 7.3 mg/kg when administered parenterally. This compares favorably with the activity of codeine (a μ receptor agonist) and BRL 52974 (a κ receptor agonist) in the same model (ED_{50} = 5.2 and 5.3 mg/kg respectively). Activation of the μ and κ receptors is associated with many of the side effects seen with the opioid antitussives such as respiratory depression, constipation and dependence (μ receptor), and diuresis and sedation (κ receptors). Therefore, it may be expected that a selective δ receptor agonist might be devoid of such side effects.



1

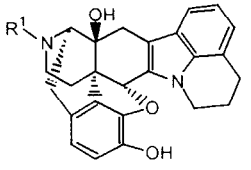
Several patent applications have claimed a variety of opioid-like compounds for the treatment of cough e.g. octahydroisoquinolines of general structure **2** (13-16). In a capsaicin-induced cough model in the mouse, maximum antitussive activity of **2** was obtained with a small hydrophobic group ($R^1 = CH_3$) (13).



R^1	Dose (mg/kg, s.c.)	% Inhibition
CH_3	1	72
H	10	34
CH_2cyclopropyl	1	30
CH_2CH_2Ph	1	44

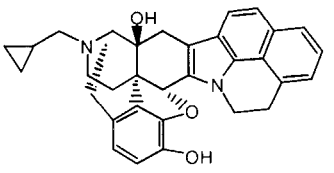
2

As mentioned previously, selective δ agonists may offer a therapeutic advantage over nonselective opioid agonists due to their potentially improved side-effect profile. A series of selective δ agonists exemplified by **3** were recently described (14). Larger hydrophobic groups present as R^1 appear to impart greater binding affinity for this receptor than the smaller methyl group implying the presence of a hydrophobic pocket. Among compounds that were assayed for in vivo antitussive activity, using the capsaicin-induced cough model in the rat, compounds **4** and **5** were the most potent (14).

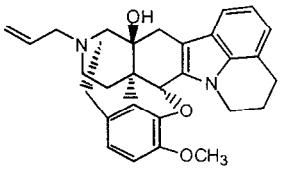


R^1	K_e (nM)
$CH_2CHCH_2CH_2$	0.05
CH_3	0.21
CH_2CH_2Ph	0.06
CH_2CH_2-2-thienyl	0.03

3

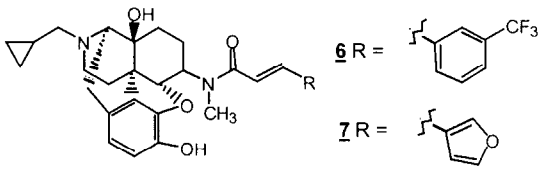


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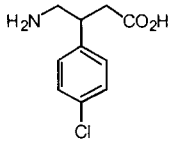
5

Analogs of compound **4** in which the indole group was replaced with an amide moiety have also been described (15, 16). It appears from the binding data that these compounds are, in general, more selective for the κ receptor than for the μ or δ receptors. The antitussive activity of two representative examples, compounds **6** and **7**, was 2.6 μg/kg and 3.7 μg/kg, respectively, determined in the tracheal stimulated antitussive model.



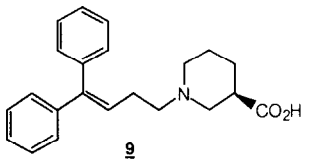
While the presence of functional μ opioid receptors has been reported on peripheral afferent terminals in the airways (17), it does not appear that they contribute significantly to the clinical antitussive action of μ opioid agonists since aerosol administration of codeine, morphine or BW 443C, a peptide opioid agonist, failed to reduce capsaicin-induced cough in human subjects (18).

$GABA_B$ Agonists - GABA (γ-aminobutyric acid) is an inhibitory neurotransmitter present in both the peripheral and central nervous systems. It binds to two different receptors termed $GABA_A$ and $GABA_B$. Experimental evidence indicates that activation of central $GABA_B$ receptors can influence the cough reflex. For example, the $GABA_B$ agonists baclofen (**8**) potently inhibits capsaicin-induced cough in the guinea pig by a central mechanism, ED_{50} = 0.04 mg/kg, s.c. (9, 19). This level of activity compared favorably with codeine and dextromethorphan in this model. The ability of baclofen (**8**) to increase the cough threshold against capsaicin was further demonstrated in a double-blind, placebo-controlled study in healthy human volunteers. Pretreatment with baclofen (10 mg, 3 times/day) for fourteen days suppressed capsaicin-induced cough (20).



8

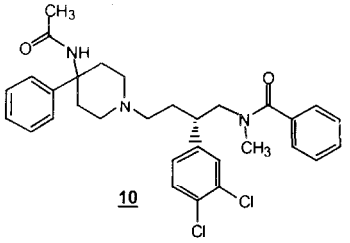
The activity of GABA is modulated by its rapid uptake by high-affinity presynaptic transporters. Therefore, an alternative approach to activation of the GABA receptor would be to administer a compound that could inhibit the reuptake of GABA. The use of compounds that inhibit the uptake of GABA have been claimed for the treatment of cough (21). Among a number of compounds specified in this application, compound **9** was reported to inhibit citric acid induced cough in the guinea pig by 42% (10 mg/kg, p.o.).



9

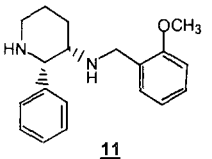
Neurokinin Antagonists - Activation of the NK_1 and NK_2 receptors by endogenous tachykinins has been associated with a variety of pathological conditions affecting the lung and bronchi including plasma extravasation, bronchoconstriction and cough (22). Several studies in a number of different animal models have also demonstrated that antagonists of the NK_2 receptor can modulate the cough reflex by a central action (11). For example, SR 48968 (**10**), a non-peptide NK_2 receptor antagonist, was shown to inhibit cough in a dose dependent manner in the citric acid induced cough model in the guinea pig (ED_{50} = 0.1 mg/kg, i.p.) (23). By comparison, under similar conditions codeine was significantly less active (ED_{50} = 8 mg/kg, p.o.). The activity of SR 48968 was apparently not mediated via an opioid

mechanism since naloxone, a nonspecific opioid antagonist that blocks the antitussive effect of codeine, had no effect.



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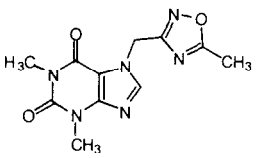
The antitussive activity of selective NK_1 antagonists is still an area of active investigation (11, 24). The NK_1 antagonist CP 99,994 (**11**) has been shown to inhibit cough in both the guinea pig and cat by a central mechanism (24). However, the NK_1 antagonist SR 140333 was not active in the citric acid-induced cough model in the guinea pig (25).



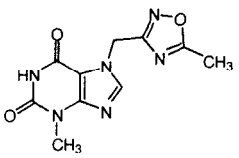
11

In addition to their central activity, neurokinin antagonists can also act at peripheral sites. NK_1 and NK_2 antagonists have been shown to inhibit the pro-inflammatory, bronchoconstrictor and RAR stimulation (tussigenic Aδ fibers) activity of sensory tachykinins by a blocking action at the level of the effector cell.

Miscellaneous - CH-170 (**12**) is a xanthine derivative that was in preclinical development as a potential antitussive (26). In pharmacological studies, **12** demonstrated antitussive activity weaker than that of codeine in several in vivo models e.g. the citric acid induced cough model [ED_{50} (CH-170) = 22.2 mpk, ED_{50} (codeine) = 12.2 mpk]. Development of **12** was suspended when it was discovered that the demethylated metabolite, CH-13584 (**13**), was a more potent antitussive. In several in vivo studies, **13** showed similar activity to both codeine and dextromethorphan as an antitussive [ED_{50} in the 4-8 mg/kg range, p.o.] and displayed mucolytic activity (27).



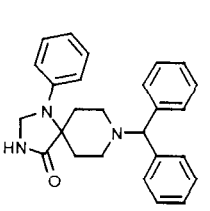
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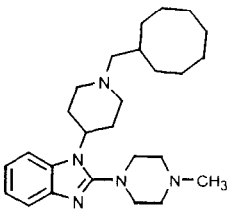
13

A number of studies were undertaken to determine the mechanism of action of **13** but these led to no firm conclusions. It is clear from these studies that **13** does not behave like theophylline although it structurally quite similar (28), nor does it exert its antitussive activity via an opioid mechanism since it does not show appreciable binding to any of the opioid receptors. However, the antitussive activity of CH-13584 is blocked by the non-specific opioid antagonist naloxone. This implies that **13** may act by facilitating the release of endogenous opioids which then exert an antitussive effect (29).

Selective activation of ORL-1 receptors located on sensory neuronal circuitry responsible for cough generation in the CNS, and possibly in the periphery (**30**), have most recently been reported to inhibit irritant cough in experimental animals (31). Selective agonists of the nociceptin ORL-1 receptor, that display weak μ binding activity, have been claimed for the treatment of cough (32). Representative ORL-1 agonists include **14**, which displayed a K_I = 18 nM in a nociceptin binding assay (32), and **15** (33).



14

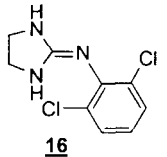


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PERIPHERALLY ACTING AGENTS

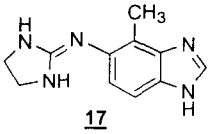
Other classes of drugs appear to inhibit the cough primarily by a peripheral mechanism. Representatives among these antitussive agents are α_2-adrenoceptor agonists, potassium channel openers and $GABA_B$ agonists.

α_2-Adrenoceptor Agonists - α_2-Adrenoceptors are present on nerves that innervate the airways and smooth muscle. Like GABA, activation of these receptors has been shown to cause inhibition of sensory nerve transmission that in turn can modulate the cough reflex. Inhaled clonidine (**16**), a non-specific α_2-adrenoceptor agonist, caused a concentration dependent inhibition of citric acid induced cough in the guinea pig at a dose of 10-1000 μM (34). However, in contrast to its effect in the guinea pig, inhaled clonidine had no effect on capsaicin-induced cough in healthy human volunteers indicating a difference in the innervation of the airways of the two species.



16

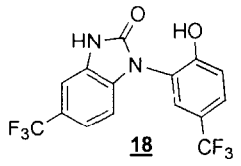
A series of bicyclic heteroaromatic analogs of clonidine has been reported to be α_2-adrenoceptor agonists useful for the treatment of a number of diseases including cough (35). Compound **17**, a typical example of the general structure, was reported to be more selective for α_2-adrenoceptors over α_1 in a binding assay and to display agonist activity similar to clonidine. In vivo, **17** was orally active and did not produce centrally mediated effects at doses that were peripherally effective (36).



17

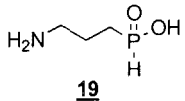
Potassium Channel Openers - Sensory nerves play an important role in airway disease by mediating central reflexes such as cough and local axon reflexes which

result in the release of neuropeptides such as Substance P and NKA. NS1619 (**17**), a Ca^{+2} activated potassium channel opener (BK_{Ca}), inhibits the activity of sensory fiber mediated reflexes in guinea pig airways (37). In conscious guinea pigs, cough due to citric acid inhalation was reduced by 60% in animals that were previously exposed to **18** (300 μM). Selective BK_{Ca} channel openers may represent a new class of antitussive agents that act by reducing peripheral cough reflexes.



18

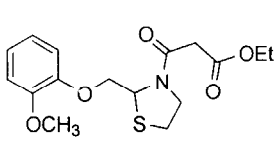
$GABA_B$ Agonists - A peripheral site of action to inhibit the efferent function of sensory afferents may also contribute to the antitussive activity of $GABA_B$ agonists. Specifically, 3-aminopropylphosphinic acid (3-APPI, **19**), a $GABA_B$ agonist that does not cross the blood-brain-barrier, inhibits tachykinergic-mediated airway pathophysiological responses such as bronchospasm, vagally-induced airway microvascular leakage and cough (inhibition of capsaicin-induced cough in guinea pigs, ED_{50} = 0.36 mg/kg, s.c.) in experimental animals by attenuating the release of pro-inflammatory neurogenic neuropeptides from pulmonary afferent nerve terminals (9, 19).



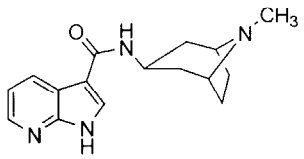
19

Miscellaneous - Moguisteine (**20**) is a non-narcotic antitussive agent currently in Phase II clinical trials (38). Although its mechanism of action is not known, it is thought to act by decreasing the sensitivity of pulmonary RARs in the lung. It was equipotent to codeine in several animal models of cough. In eight volunteers, acetylene induced coughing was suppressed by 61% after treatment with moguisteine (50 mg tablet, p.o.).

DF-1012 (**21**) has demonstrated potent, long-acting and orally effective antitussive activity in the guinea pig in several different cough models (39). It is currently in Phase II clinical trials.



20



21

Conclusion - Cough is a serious symptom of a wide range of pathophysiological conditions. Current therapies rely heavily on the use of centrally acting agents such as codeine and dextromethorphan and to a lesser extent on the peripherally acting local anesthetics. While codeine is an effective antitussive agent, it is plagued by a number of unwanted side effects such as sedation, respiratory depression, tolerance, and abuse potential. Dextromethorphan is generally less effective than codeine and still suffers from some of the same liabilities. The ideal antitussive should possess the following pharmacodynamic and pharmacokinetic properties: efficacy equivalent or better than codeine, oral activity, a pharmacokinetic profile that supports once daily dosing, no GI side effects, no sedative or respiratory

depression liability, and no tolerance or abuse liability. It remains to be determined whether any of the new approaches to antitussive therapy will demonstrate a significant therapeutic advance over existing therapies.

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Chapter 5. Phosphodiesterase 4 Inhibitors

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Introduction - cAMP and cGMP are ubiquitous second messengers which mediate biological responses to a variety of hormones, neurotransmitters, autocoids and drugs (1). Increased concentration of these cyclic nucleotides results in activation of protein kinase A (PKA) and protein kinase G (PKG). These kinases phosphorylate a variety of substrates, including transcription factors and ion channels, which regulate a myriad of physiological functions, including the activation state of a variety of cell types. Phosphodiesterase (PDE) enzymes are responsible for the inactivation of cAMP and cGMP by hydrolysis to AMP and GMP, respectively. Inhibition of PDE enzymes results in an accumulation of cAMP and/or cGMP, which has many biological consequences including smooth muscle relaxation and reduction in inflammatory cell activity. The classification of PDE enzymes into seven isozyme families, on the basis of substrate affinity and specificity, regulatory characteristics and inhibitor profile, was described several years ago (2-4). Recently this classification has been extended to include PDE8, PDE9, PDE10 and PDE11 (5).

PDE4 is the predominant isozyme present in inflammatory cells and selective PDE4 inhibitors are a popular target for novel anti-inflammatory drugs (6). The distribution of PDE4 in inflammatory cells and airway smooth muscle, and the effects of selective PDE4 inhibitors *in vitro*, have been comprehensively reviewed (7,8). The therapeutic potential of PDE4 inhibitors in a variety of diseases has been reviewed recently (8,9). First generation PDE4 inhibitors, typified by rolipram, suffered from dose-limiting side effects including nausea, emesis and gastrointestinal disturbances, which severely restricted their therapeutic utility. Second generation compounds such as Ariflo™ have been identified with reduced side effect liability, and the clinical development of such compounds has been reviewed recently (10). Two strategies for minimizing side effects have been pursued; selectivity for the catalytic site over the high affinity rolipram binding site (HPDE) and selectivity for a specific PDE4 subtype over the other subtypes (6,8-10).

In this chapter we will focus on progress which has occurred during the last three years, with an emphasis on new medicinal chemistry research in the area and the current status of compounds which are undergoing clinical evaluation.

PDE4 INHIBITORS UNDER CLINICAL EVALUATION

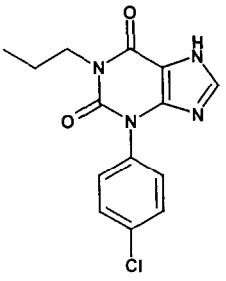
Table 1 summarizes the reported status of all of those PDE4 inhibitors currently in clinical development. A number of new compounds have entered development over the last 3 years, but very few of them have reported data beyond early Phase I safety and tolerability studies. Respiratory disease is still the main therapeutic focus for this class of drugs in the clinic. However, extensions into diseases such as IBD and atopic dermatitis are now actively being pursued (9).

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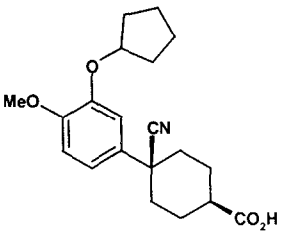
Table 1 – Status of PDE4 Inhibitors in Clinical Development

Drug	Compound Number	Company	Indication	Status
Arofylline	1	Almirall	Asthma	Phase III
Cilomilast	2	GSK	Asthma	Phase II
			COPD	Phase III
Roflumilast	3	Byk Gulden	COPD/Asthma	Phase III
V-11294A	5	Napp	Asthma	Phase II
CDC-801	6	Celgene	Crohns Disease	Phase II
BAY 19-8004	7	Bayer	Asthma/COPD	Phase II
Cipamfylline	8	Leo	Atopic Dermatitis	Phase II
SCH-351591	12	Schering Plough	Asthma	Phase I
PD 189659	10	Pfizer	Asthma	Phase I

Arofylline (LAS 31025) 1, a xanthine derivative, has been in development since 1996, but very little clinical data has been published. Data from a small Phase II study in mild asthmatics has provided encouragement. In patients with at least 15% forced expiratory volume (FEV_1) reversibility, the onset and duration of bronchodilator activity of Arofylline at 4, 10 and 20 mg doses was evaluated against placebo in a cross over, randomised, double blind trial. The compound showed significant bronchodilator effects from 1-8h post dose with the 10 mg and 20 mg dose, with no serious adverse reactions (11). Also, a larger 232 patient, 6 week double blind, randomized, placebo controlled trial, studying moderate asthmatics has been reported using a 90 mg/day slow release formulation. By the end of the study, peak expiratory flow rate (PEFR) increased significantly in the treated group from 377 to 405l/s. The patients also suffered fewer asthma exacerbation episodes than the placebo group, and no serious side effects were detected (12) .



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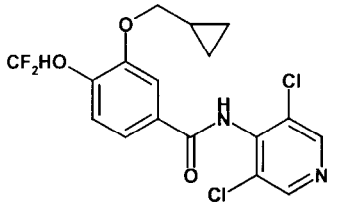
Cilomilast (Ariflo) 2 is reported to be in Phase II for asthma and Phase III for COPD (chronic obstructive pulmonary disease) (13). Phase I data illustrated that the compound was well tolerated up to 15 mg bid in a 9 day study in healthy male volunteers (14,15). No drug related effects were observed in haematology, clinical chemistry, urinalysis or cardiovascular tests (15). Other studies in man have confirmed the high bioavailability of the compound (96%) and its linear pharmacokinetics (13,16). Cilomilast principally undergoes hydroxylation, dealkylation and glucuronidation, none of which are mediated by P450 enzymes (17).

In Phase II trials, Cilomilast (10 mg bid) showed efficacy in exercise-induced asthma and a dose of 15 mg has been reported to improve respiratory function in patients with asthma who are not adequately controlled by inhaled corticosteroids (19). Treatment with Cilomilast for 6 weeks resulted in a 160ml improvement in trough FEV_1 when compared to placebo (18,19). Preliminary (6 week) data from a Phase III trial

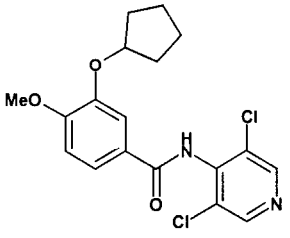
involving over 300 patients with asthma showed that the compound (15 mg bid) markedly improved FEV_1 compared to placebo (19).

Results from a Phase II trial in patients with COPD have demonstrated that Cilomilast (15mg bid for 6 weeks) increases FEV_1 and forced vital capacity (FVC) by 11% and 7% over baseline respectively (20). In addition, consistent improvements relative to placebo were observed in excertional dyspnea, rescue bronchodilator use and resting post-exercise SaO_2. Cilomilast was safe and well tolerated in this study. Volunteer studies have also shown that there is no interaction between Cilomilast and the absorption of either digitoxin or warfarin, two common drugs prescribed to COPD patients (21,22).

Roflumilast **3**, a close analogue of the RPR PDE4 inhibitor Piclamilast (RP 73401) **4**, is reported to be in multinational Phase III clinical trials for asthma and COPD, although very little data has been reported on the compound. Early Phase II data in exercise induced asthmatics (28 day, 16 patients) have shown that Roflumilast is significantly superior to placebo. The compound was also shown to be safe and well tolerated. In the same study, blood samples were taken and inhibition of LPS induced TNFα release was determined as a surrogate marker of inhibition of inflammatory cell activation and a 21% reduction in TNFα levels was observed (23,24).

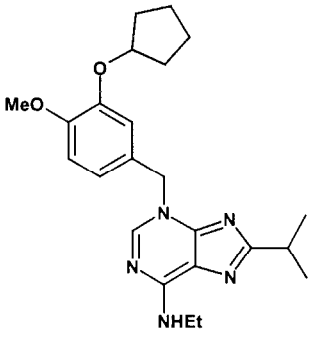


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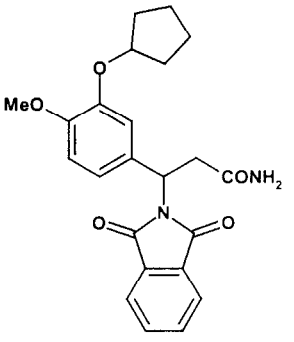


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Napp is developing a rolipram derivative, V-11294A **5**, for the treatment of asthma. In initial Phase I trials, the compound inhibited the *ex vivo* production of TNFα in whole blood at doses that did not elicit gastrointestinal effects (25). In addition, the compound has a bioavailability of over 50% and a half-life of 9.7 hours (26).



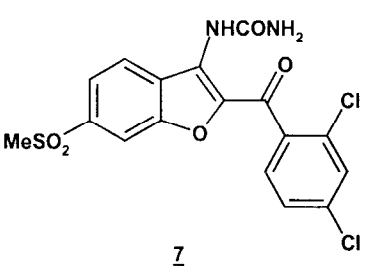
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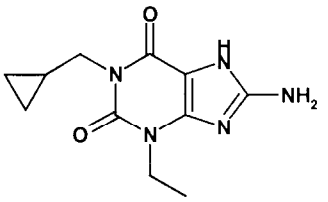
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CDC-801 **6** is Celgene's lead compound in a series of thalidomide analogues that have been termed SelCIDs™ (Selective Cytokine Inhibitory Drugs). CDC-801 was found to be well tolerated and non-emetic in Phase I studies (50-1000 mg). Similar

results were obtained in a 7 day multiple dose Phase I study in 18 healthy male volunteers (27) and the compound is now undergoing Phase II studies in Crohn's disease (28). It has been developed primarily to improve the TNFα inhibitory activity of thalidomide, for the treatment of autoimmune diseases, rather than to enhance the PDE4 inhibitory activity, and is a relatively modest inhibitor of both (PDE4 IC_{50} (U937 cells) 1.1μM; TNFα production IC_{50} (monocytes) 2.5μM) (29).



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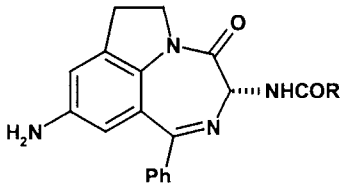


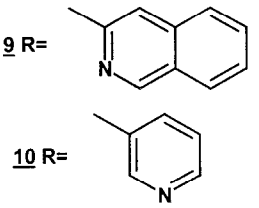
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Bayer is developing BAY-19-8004 **7**, a benzofuran derivative, for the potential treatment of asthma and COPD. The compound is reported to be currently in Phase II clinical trials (30).

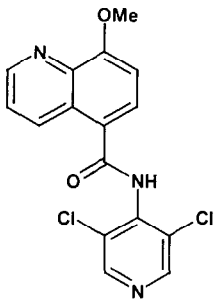
Cipamfylline **8**, is a xanthine derivative that was initially developed by SKB. Initial Phase I studies showed that the pharmacokinetics of the compound were not affected by food, but it was not progressed further (31). More recently, the compound has been licensed to Leo Pharmaceuticals and is currently undergoing Phase II studies for atopic dermatitis (32).

Recent data from Parke Davis on their benzodiazepine series of PDE4 inhibitors has shown a change in emphasis. The original development candidate, CI-1018 **9**, has been discontinued due to teratogenic effects in preclinical toxicology studies (33). This has now been replaced by a structural analogue, PD 189659 (CI-1044) **10**, which is a modestly potent PDE4 inhibitor (IC_{50} 800nM), but has high bioavailability. This compound is currently undergoing Phase I clinical trials (33).

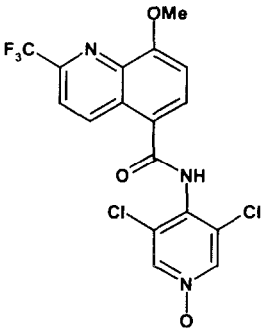




Schering Plough has announced that SCH-351591 is currently in Phase I studies (34). It is a compound that originated at Chiroscience, as D4396 and has been described as related to, but more potent than their original development candidate, D4418 **11**. Although the structure has not been disclosed, a recent patent application from Chiroscience, that discloses pharmacokinetic data on the N-oxide **12** suggests that this may be D4396 (6,35).



11



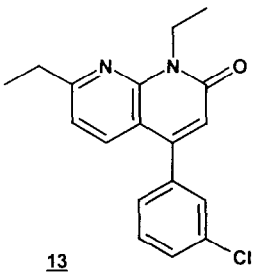
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EVALUATION OF PDE4 INHIBITORS IN ANIMAL MODELS

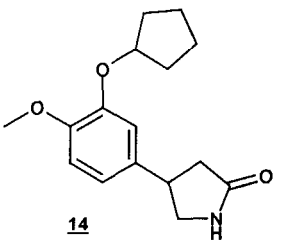
Respiratory Disorders - The efficacy of PDE4 inhibitors in animal models of asthma and COPD is well documented. A large number of structurally diverse, selective PDE4 inhibitors have demonstrated their ability to inhibit bronchoconstriction and airway hyper-responsiveness, eosinophil infiltration and local cytokine recruitment in a variety of models involving a range of stimuli. Efficacy has been shown in mice, rats, guinea pigs, dogs and monkeys, and the therapeutic potential of PDE4 inhibitors in asthma, allergic rhinitis and COPD has been extensively reviewed (7-9,36-39). The activity of recently disclosed PDE4 inhibitors in these models will be discussed in the Medicinal Chemistry Developments section in this chapter.

Recently, a ferret eosinophilia model has been reported, which enables the comparison of efficacy and emesis to be made in the same species (40). YM976 **13** was shown to suppress eosinophil accumulation in a dose-dependent manner, and an ED_{50} of 1.2 mg/kg po was determined. At 10 mg/kg po, no emesis was observed, indicating that YM976 possesses a good separation between the efficacious dose and the emetic dose.

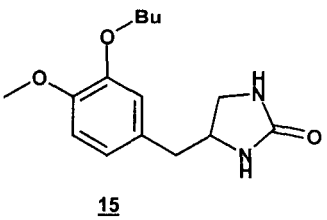
A potential role for PDE4 inhibitors in the treatment of exacerbations of asthma triggered by viral infection has been proposed, based on the ability of rolipram **14** and Ro-20-1724 **15** to reduce both airway hyper-responsiveness and eosinophilia following RSV infection in mice (41).



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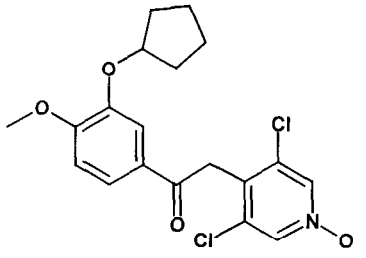
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Skin Diseases - The rationale for the use of PDE4 inhibitors in the treatment of atopic dermatitis and psoriasis has been discussed (9). There are few reports of the use of PDE4 inhibitors in animal models of either atopic dermatitis or psoriasis, but some preliminary clinical studies have been carried out (9). Recently, the effects of RP 73401 **4** on an allergic skin reaction in mice were reported (42). Following challenge by administration of dinitrochlorobenzene or toluenediisocyanate, a reduction in ear

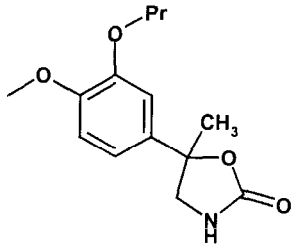
thickness was achieved by dosing with RP 73401 either topically or ip. SB-207499 **2** has also been reported to be effective in a murine model of allergic dermatitis, dosed either po or iv (43).

The efficacy of arofylline **1** in dogs with atopic dermatitis has been reported (44). Forty atopic dogs were studied for four weeks following oral administration of arofylline (1 mg/kg bid), prednisone or a combination of arofylline and prednisone. Arofylline was as effective as prednisone in controlling pruritus and was also effective in controlling skin lesions. However, a large number of the dogs treated with arofylline vomited and some were withdrawn from the study. In general, the vomiting subsided or disappeared after the first few days (44). The use of topical administration, or a more selective PDE4 inhibitor, would be expected to overcome the dose limiting side effect observed in this study.

Rheumatoid Arthritis - *In vitro* and *in vivo* evidence suggesting that PDE4 inhibitors would be expected to be beneficial in the treatment of rheumatoid arthritis has been summarised recently (9,45) . PDE4 inhibitors have been found to be efficacious in several animal models of arthritis (45). Rolipram **14** has been shown to exhibit anti-inflammatory effects in carrageenan-induced paw oedema and the adjuvant arthritis model. It has also been shown to ameliorate collagen II-induced (CIA) arthritis in mice (45). RPR 73401 **4** and RPR 109026 **16** have shown excellent anti-arthritic activity in the CIA model and the Streptococcal cell wall (SCW)-induced arthritis model (45). A recent study in the adjuvant arthritis model demonstrated that rolipram abrogated oedema formation and significantly inhibited hyperalgesia. Inhibition of cellular influx and inhibition of bone and cartilage destruction were also achieved (46). The efficacy of rolipram in the SCW arthritis model has also been documented recently (47).



16



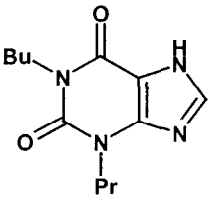
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Multiple Sclerosis - Immune cell activation and induction of the secretion of inflammatory cytokines, particularly TNFα, are implicated in multiple sclerosis. The ability of PDE4 inhibitors to modulate these events suggests a role for the use of PDE4 inhibitors in this disease. This is substantiated by the activity of PDE4 inhibitors, particularly rolipram **14**, in experimental auto-immune encephalomyelitis (EAE), an animal model of multiple sclerosis. A recent review summarises the results obtained with pentoxifylline, ibudilast, rolipram and BBB022A in EAE models in several animal species (48). In addition, the efficacy of mesopram **17** in a variety of EAE models in rats and mice has been described recently (49). EAE in Lewis rats was completely suppressed by mesopram, and this was accompanied by a reduction in inflammatory lesions in the spinal cord and brain. Mesopram ameliorated clinical symptoms in a chronic EAE model in SJL mice and in a relapsing-remitting EAE model in SWXJ mice.

IBD - The role for PDE4 inhibitors in IBD relies on the ability of such compounds to inhibit the production of TNFα. To date, there are only a few reports concerning the use of PDE4 inhibitors in IBD models. In a dextran sulphate induced model of colitis in

the rat, oral administration of rolipram **14** and arofylline **1** resulted in amelioration of bleeding and a reduction in inflammatory markers (50). Rolipram has also been shown to be efficacious in the TNBS colitis model in rats, ameliorating the course of disease and preventing late collagen deposition (51). Recently, the effects of rolipram in dextran sulphate induced colitis in mice have been described (52). Rolipram reduced the clinical score, partially reversed the reduction of colon length and improved the histological score. Suppression of colonic tissue TNFα concentrations was also observed.

Osteoporosis - Osteoporosis is characterised by low bone mass and loss of skeletal architecture, which leads to bone fracture. Accelerated bone resorption by osteoclasts and reduced bone formation by osteoblasts probably both contribute to pathological events associated with the disease. Agents which influence one or both of these processes would be expected to offer benefit in the treatment of osteoporosis. Several cytokines, including TNFα, are thought to promote bone resorption by osteoclasts, which implies that agents which are able to suppress the production of these cytokines could have a role in reducing bone loss. In addition, elevation of cAMP in osteoblasts has been shown to enhance their ability to form bone, suggesting that agents which are able to elevate cAMP levels should have the potential to increase bone mass. Since PDE4 inhibitors are able to inhibit the production of TNFα and are also able to elevate cAMP levels, a therapeutic effect in osteoporosis would be predicted (53-55). Several recent studies provide evidence to support this hypothesis. The effectiveness of XT-44 **18** in three osteopenia models has been described (53). In Walker 256/s tumour-bearing mice, oral administration of XT-44 inhibited the decrease in bone mineral density. In the sciatic neurectomised rat model oral administration of XT-44 was able to recover bone mineral density. Finally, in ovariectomized rats an increase in bone mineral density was observed following oral administration of XT-44. The mechanism by which XT-44 exerts these effects has been discussed, but is not entirely clear (54). Recently, the effects of rolipram **14** and pentoxifylline in normal mice were investigated. Both compounds were able to increase significantly both cortical and cancellous bone mass, predominantly by the acceleration of bone formation (55). It has been suggested that the disease modifying effects of PDE4 inhibitors in animal models of rheumatoid arthritis are related to their ability to suppress osteopenia (45).



18

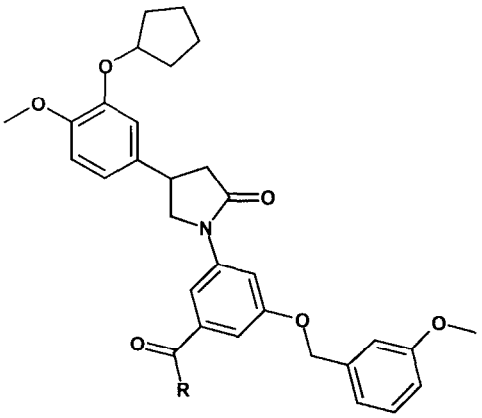
Miscellaneous Diseases - A role for PDE4 in the modulation of a variety of biological and pathobiological processes in the kidney has been proposed (56). Thus, PDE4 inhibitors could have utility in disorders such as glomerulonephritis, renal transplantation and acute renal failure. Rolipram **14** has been shown to be effective in the treatment of experimental crescentic glomerulonephritis in rats (57). Severe crescentic glomerulonephritis was induced by a single injection of anti-glomerular basement membrane antiserum. This model is reported to be TNF dependent (57). Systemic administration of rolipram prevented glomerular injury during the early stages of experimental crescentic glomerulonephritis and reduced TNFα levels in the glomeruli and renal tubules. Rolipram was able to reduce the severity of the disease when dosed 4 days after the induction of nephritis, when leukocyte infiltration was maximal and there was already evidence of glomerular injury (57). Support for the use

of PDE4 inhibitors in acute renal failure is provided by a study which describes the effect of Ro 20-1724 **15** in a model of multiple organ dysfunction syndrome (MODS) (58). MODS was induced by ip injection of zymosan and Ro 20-1724 was administered simultaneously by mini-infusion pump. Chronic treatment with Ro 20-1724 protected renal function and mesenteric perfusion during MODS.

The potential for the use of PDE4 inhibitors in a variety of CNS disorders, including depression, Parkinson's disease and learning and memory impairment was reviewed in 1999 (9). A more recent publication provides further evidence for the use of rolipram in memory deficit (59).

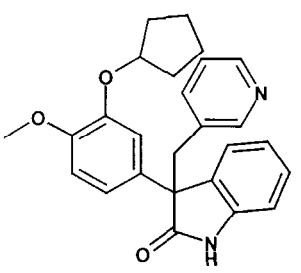
Medicinal Chemistry Developments - The continued search for novel PDE4 inhibitors has resulted in a large number of publications in the last three years reporting structure-activity relationships. Rolipram analogues have continued to receive a considerable amount of attention and many of these are summarised below. The other major structural classes are the nitroquazones and the xanthines, which have been reviewed recently (36). A discussion of PDE4 inhibitors disclosed only in the patent literature is beyond the scope of this chapter, but readers are directed to a recent review (6).

A series of N-aryl rolipram derivatives has been investigated with the aim of improving PDE4 potency while maintaining selectivity over PDE3 (60,61). The compounds were designed to be administered by inhalation, and two selected examples **19** and **20** inhibited airway inflammation in a Brown Norway rat model when dosed by the intratracheal route (60). Two related series of rolipram analogues have been described, in which the effect of quaternary substitution has been evaluated (62,63). Oxindoles such as **21** have moderate activity for PDE4 (IC_{50} 0.4μM) and exhibit some selectivity for catalytic activity over the HPDE (K_i 2.6μM) (62). Compounds such as **22**, lacking the fused phenyl ring, were found to have improved potency (PDE4 IC_{50} 6.3nM) but generally to exhibit poor selectivity over the HPDE (K_i 7.5nM) (63). Although designed as conformationally constrained analogues of RP 73401 (64), indanes represented by **23** can also be seen to have structural similarities to the rolipram analogues described above. Compound **23** was evaluated in a murine endotoxemia model, by oral dosing, and the ED_{50} determined to be 15 mg/kg. No emesis was observed in 4 dogs dosed iv with 3 mg/kg of compound **23** (64).

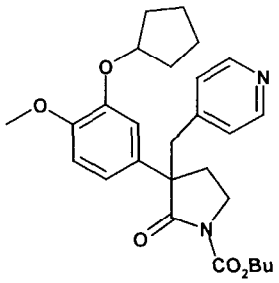


19 R=OH

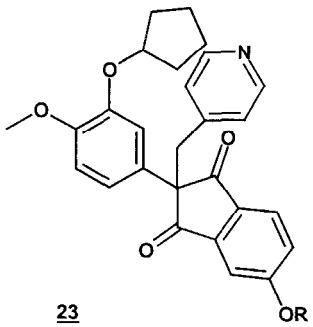
20 R=NHNMe$_2$



21

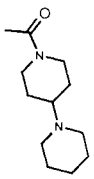


22

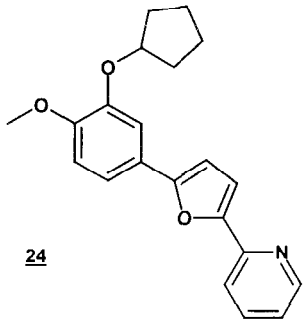


23

R =



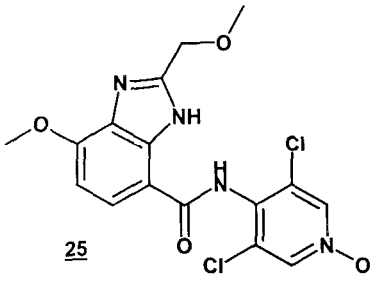
Substituted furans have recently been described as PDE4 inhibitors, and significant potency enhancement was achieved by the incorporation of the catechol unit found in rolipram analogues (65). Compound **24** was selected for further evaluation, but was found to cause emesis in ferrets when dosed orally at 10 mg/kg. A related series of substituted thiophenes has also been described (66).



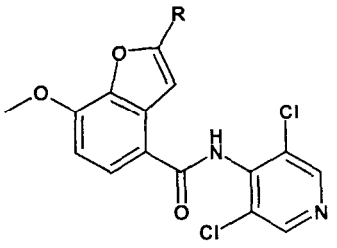
24

Modification of the catechol unit to provide bicyclic systems has been described by several groups and two distinct classes are evident. In one class a methoxy substituent is retained and the compounds can be visualised as cyclization of the oxygen from the cyclopentyloxy substituent onto the phenyl ring. In the second class, the additional ring can be viewed as replacing both catechol oxygens. The first class is exemplified by series of benzimidazoles and benzofurans. Benzimidazoles, represented by **25**, have been shown to be potent PDE4 inhibitors (67). Compound **25** has a bioavailability of 87% in the mouse and inhibits SCW induced arthritis in rats with an ED_{50} of 5 mg/kg bid. Several benzofuran analogues were evaluated in a murine endotoxeamia model, and the most potent compound was **26**, with an ED_{50} 20 mg/kg po (68). In a separate publication, benzofuran **27** was shown to inhibit eosinophilia in a guinea pig model when dosed orally at 0.5 mg/kg, and to be devoid of side effects in

ferrets at an oral dose of 10 mg/kg (69). Analogues in which both oxygens have been replaced by a heterocyclic ring include a series of indoles (70). Although potent PDE4 inhibitors were discovered, the compounds demonstrated very disappointing results *in vivo*. For example, compound **28** inhibits PDE4 with an IC_{50} of 12nM, but inhibits TNFα production only by 30% when dosed orally at 30 mg/kg to balb/c mice. Poor oral absorption is likely to be responsible for the lack of *in vivo* activity. Improvement in oral absorption was achieved by incorporation of the pyridine-N-oxide (71). Compound **29** inhibits TNFα release in a murine endotoxemia model with an ED_{50} of 7.1 mg/kg, and it also inhibited SCW induced arthritis in a rat model with an ED_{50} of 23 mg/kg.

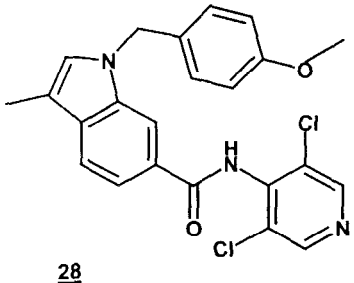


25

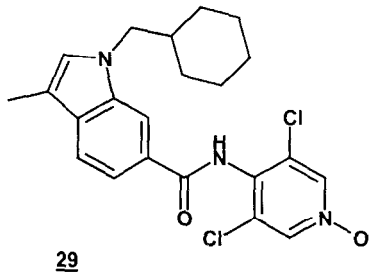


26 R = ^{n}Bu

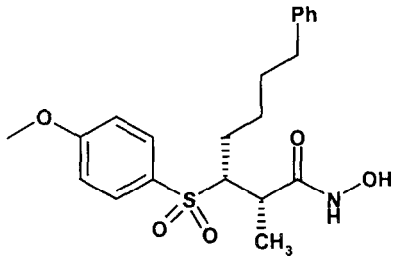
27 R = $COCH_3$



28



29

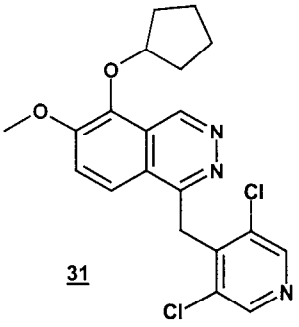


30

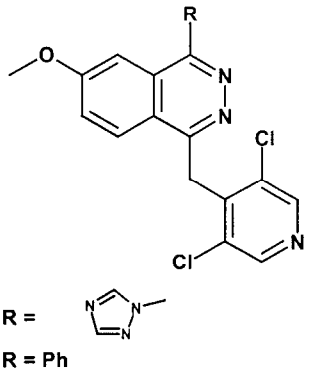
Simultaneous inhibition of PDE4 and selected MMP enzymes has been reported for a series of arylsulphonylhydroxamic acids (72). Although the incorporation of a catechol unit is optimal for PDE4 activity, this is detrimental for MMP activity. The optimal balance between PDE4 and MMP activity was achieved with compound **30**.

A novel series of phthalazine derivatives has been described recently (73). These compounds were designed as conformationally constrained analogues of RP 73401 in which the carbonyl function is replaced by the π-bond of an aryl ring. Compound **31** is a potent inhibitor of PDE4 (IC_{50} 53nM) with selectivity for the catalytic site over the HPDE site (K_i 149nM). When dosed iv in dogs at 10μmol/kg, no emesis was observed.

In contrast, RP 73401 caused emesis when dosed at 1μmol/kg iv. The possibility of replacing the cyclopentyloxy moiety by a substituent at the 4-position of the phthalazine nucleus was demonstrated by the synthesis of several potent PDE4 inhibitors (74). Compounds **32** and **33** showed efficacy *in vivo* when dosed at 30μmol/kg ip in a guinea pig eosinophilia model. Bioavailability in the rat was determined to be 27% for both compounds. No emesis was observed in dogs with these compounds at a dose of 30 μmol/kg iv.



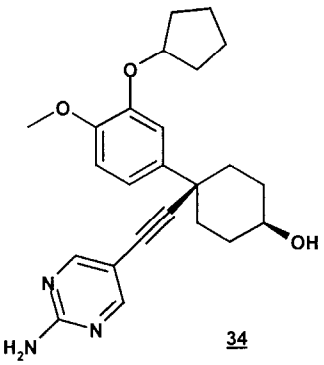
31



32 R =

33 R = Ph

SB 222618, **34**, has been described as a PDE4 inhibitor with potential in the treatment of inflammatory diseases such as asthma. This compound is clearly structurally related to Ariflo™, and a method for its synthesis has been described, although no biological data have been reported (75).

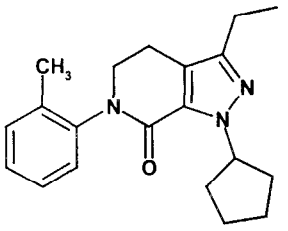


34

The structure activity relationships of a series of tetrahydro[1,4]diazepinoindoles related to CI-1018 have been described recently (76,77) . Several potent PDE4 inhibitors with selectivity over the HPDE were described. The most promising compound was CI-1044, **10**, which was found to inhibit antigen induced eosinophilia in Brown Norway rats with EC_{50} 3.2 mg/kg po and to inhibit TNFα production in Wistar rats with EC_{50} 2.8 mg/kg po. CI-1044 did not cause emesis in ferrets when dosed at 40 mg/kg iv. These compounds were reported to be devoid of selectivity for any PDE4 subtype. Thus, the observed *in vivo* selectivity for efficacy versus emesis is unlikely to be linked to subtype selectivity. The authors conclude that PDE4 subtype selectivity is not the most appropriate strategy for the identification of non-emetic compounds.

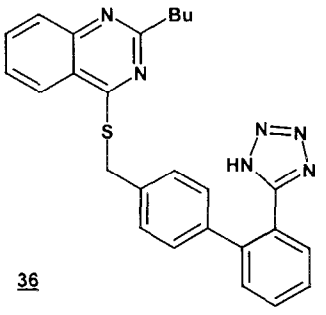
A number of structurally distinct series of PDE4 inhibitors have been identified by HTS screening of corporate compound collections, including a series of pyrazolpyridines. Optimisation of the substituents at the 1-, 3- and 6-positions resulted

in the discovery of CP-220,629 **35**, which is a potent PDE4 inhibitor. CP-220,629 was active in the antigen-induced airway obstruction model in guinea pigs with an ED_{50} of 2 mg/kg po, and it also demonstrated efficacy in atopic monkeys challenged with antigen. Ferrets dosed with CP-220,629 at 1 mg/kg sc showed no adverse effects (78).

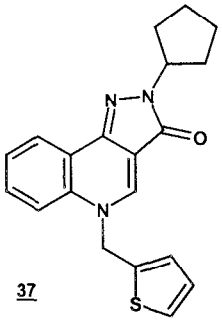


35

Several series of PDE4 inhibitors have been developed using a pharmacophore based on nitroquazone to direct screening of corporate and commercially available compound collections (79-81) . The angiotensin-II antagonist losartan was found to be a weak PDE4 inhibitor and an optimisation process was undertaken. Some improvement in potency was achieved, but the most potent compound in the series **36**, possessed only low micromolar activity (79). More potent compounds were discovered in a second series, and compound **37** also demonstrated activity *in vivo* in a guinea pig eosinophilia model when dosed orally at 10 mg/kg. No emesis was observed following dosing to dogs at 3 mg/kg iv (80).



36



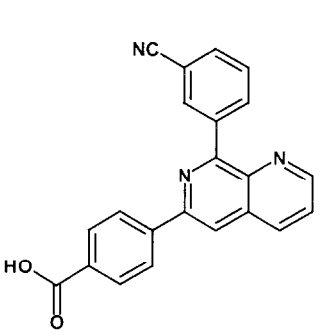
37

OTHER DEVELOPMENTS

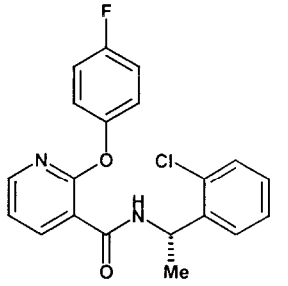
PDE4 Crystal Structure - Most of the work described in this review has taken place without a clear understanding of the way in which inhibitors bind to the enzyme. This has been due to the fact that until recently, a crystal structure of any of the PDE4 isoforms in the presence and/or absence of an inhibitor, has not been achieved. A group from Glaxo Wellcome have now achieved this, successfully crystallizing the catalytic domain of human PDE4B to a resolution of 1.77A (82). Docking studies with cAMP provide a retrospective understanding of why certain residues, known to be important from mutation studies, are key to the binding of the substrate and the mechanistic integrity of the enzyme. This crystal structure, as a representative of this class of enzyme, should further assist the design of specific inhibitors of the PDE4 enzymes and may provide further insights into the design of PDE4 isoform selective inhibitors.

Subtype Selective Inhibitors - Originally, attempts to identify selective PDE4 inhibitors focussed on enhancing selectivity for PDE4 relative to other PDE enzymes, such as

PDE3. More recently, as it became apparent that there were 4 isoforms of PDE4 and a number of splice variants of these different forms, attention has been focused on optimizing selectivity for these different isoforms. Both Pfizer and Novartis have recently claimed compounds with selectivity for the PDE4D isoform (83,84). The nicotinamide derivative **39** is reported to possess 100 fold selectivity for the inhibition of PDE4D relative to other PDE4 isoforms (83,85). A series of PDE4D selective compounds, structurally related to nitroquazone, has been reported (86). Compounds such as **38** exhibit low nanomolar activity as PDE4D inhibitors with 50 fold selectivity over the other PDE4 subtypes. Selectivity for catalytic activity over inhibition of the HPDE was also observed. *In vivo* activity was assessed in a rat antigen-induced eosinophilia model, and compound **38** demonstrated activity following oral dosing at 1 mg/kg. No data was reported concerning the emetic effects of compounds of this series.



38



39

PDE4D Knockout Mice - In order to try and clarify the role of the different PDE4 isoforms in disease, a significant amount of work is being undertaken to generate mice that are deficient in the various isoforms. The first of these to be reported is for mice deficient in PDE4D (87), and these mice have been shown to have an unexpected impairment of muscarinic acetylcholine receptor function. Thus, methacholine fails to evoke bronchoconstriction in the null mice when compared with wildtype animals, whereas the same response evoked by 5HT was unaffected, indicating that PDE4D disruption does not impair smooth muscle functional responses *per se*.

Also, sensitization of the null mice to ovalbumin does not lead to the expected airway hyper-reactivity after methacholine challenge, as seen in the wildtype animals. Another intriguing finding is the development of pulmonary inflammation, characterized by increased numbers of eosinophils, lymphocytes and neutrophils in the bronchoalveolar lavage fluid, in ovalbumin challenged, sensitized null mice. This illustrates that inhibition of PDE4 isoforms other than PDE4D must play a critical role in abrogating pulmonary inflammation. These data are supported by the cellular data generated by workers at SKB (88). They have shown that PDE4A or PDE4B and not PDE4D is the functionally important target for suppression of human inflammatory cell function. By correlating the PDE4 inhibitory activity in monocyte assays, they have shown that functional activity is correlated to PDE4A or PDE4B but not PDE4D. Clearly, similar studies in other key inflammatory cells with both isoform selective inhibitors and isoform selective knockout mice should clarify which isoform is the preferred target.

Conclusions - Over a decade has passed since the publication of the first information highlighting the potential of PDE4 inhibitors for the treatment of asthma. A large number of structurally diverse compounds have been synthesised and many have shown very impressive activity in animal models of respiratory disease. However, most

of the early development compounds have failed to provide good oral efficacy in man at doses that did not show the nausea and vomiting side effects associated with this class of drug. The most exciting development over the last three years has been the demonstration of efficacy by Cilomilast in Phase II/III clinical trials in asthma and COPD patients (11,12). These results illustrate that preclinical studies aimed at trying to delineate the efficacy and side effect potential of PDE4 inhibitors can be used to provide compounds with an enhanced therapeutic index in man. It is possible that the use of rational approaches to improve the therapeutic index, based on our vastly improved knowledge of the molecular biology and biochemistry of PDE4, will provide even better clinical candidates than Cilomilast. In part, the answer to this question may reside with some of the early development candidates described in this review. Whatever the difficulties, PDE4 inhibition remains a highly active field of drug research in which there is justifiable optimism for a successful commercial outcome.

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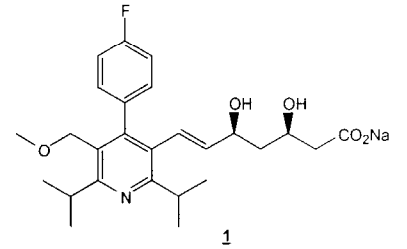
Chapter 6. Recent Advances in the Science and Treatment of Atherosclerosis

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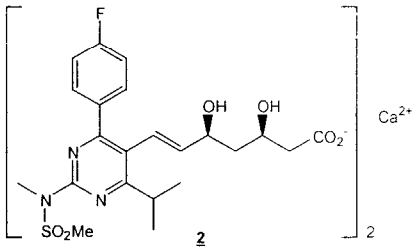
Introduction – Coronary Heart Disease (CHD) has been for some time the leading cause of death in the western world. In 1998 in the United States, some 459,841 deaths were directly attributed to CHD (1). In addition, CHD is a mentioned causal factor in the U.S. in approximately 600,000 deaths overall annually. Atherosclerosis is a process that leads to a complex disease state that is typically characterized by a thickening of the artery wall. It is responsible for many deaths involving heart attack and stroke. Further, it is estimated that it accounts for nearly three-fourths of all deaths from cardiovascular disease. Projected costs of heart disease in the U.S. for 2001 are approximately 194 billion dollars. Risk factors associated with a high incidence of CHD have been delineated from clinical studies and extensive epidemiological studies (2). Prominent among these risk factors is a high level of low density lipoprotein cholesterol (LDL-C) and much of the drug discovery efforts have been focused on lowering this key risk factor. Complementary strategies have also emerged focusing on raising high density lipoprotein cholesterol (HDL-C) or inhibiting arterial cell wall processes that lead to atherosclerosis. Recent reviews have appeared describing new results in lipid lowering strategies (3,4). This article will describe recent pharmacological developments in treating atherosclerosis and review recent discoveries relevant to cholesterol metabolism.

LDL-C LOWERING AGENTS

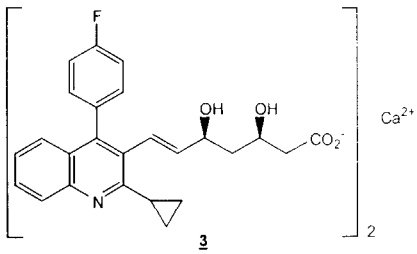
Cholesterol Biosynthesis Inhibitors – The most clinically successful cholesterol lowering agents are the HMG-CoA reductase inhibitors (HMGRI) lovastatin, pravastatin, simvastatin, fluvastatin and atorvastatin. The principal effect of these drugs certainly is lowering LDL-C, however, secondary effects of these drugs have been the focus of recent research (5). Second generation HMGRIs have recently been reported to have superior potency and efficacy in lowering LDL-C in humans with hypercholesterolaemia. Cerivastatin, **1**, inhibits microsomal rat liver HMG-CoA reductase activity with a K_i of 1.3 nM (lovastatin has a K_i = 150 nM in the same assay) (6). Cerivastatin has been shown to reduce serum LDL-C 33.4 to 44.0% in hypercholesterolaemic patients receiving 0.4 to 0.8 mg/day. These patients also showed improvements in their HDL-C (+3.2 to 8.7%) and triglyceride profiles (–10.4 to –18.4%).



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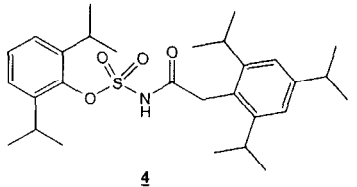
Rosuvastatin, ZD-4522 or **2**, inhibits rat liver HMG-CoA reductase with an IC_{50} of 11 nM (7). Rosuvastatin inhibited

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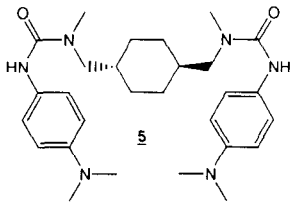
cholesterol biosynthesis in isolated rat liver hepatocytes with an IC_{50} of 1.12 nM (pravastatin has an IC_{50} of 198 nM in the same assay). In addition, **2** reduced plasma cholesterol levels 26% in beagle dogs treated with 3 mg/kg/day for 14 days. Similarly, **2** reduced plasma cholesterol levels 22% in cynomologous monkeys dosed at 12.5 mg/kg compared with a 19% reduction by pravastatin at 50 mg/kg. Rosuvastatin lowered LDL-C 43% in double-blind dose ranging clinical trial at a dose of 2.5 mg/day. Comparable results were seen in the control side of the study using atorvastatin at 10 mg. In that study, **2** lowered total cholesterol by 31% and triglycerides 11%, and raised HDL-C 9% at the same dose (8). This compound also shows no indication of metabolism by cytochrome P450 (CYP) 3A4 and hence low tendency for drug-drug interaction via this pathway.

Pitavastatin (itavastatin, nisvastatin, NK-104 or **3**) is a potent inhibitor of rat liver microsomal HMG-CoA reductase (IC_{50} = 6.8 nM) and in guinea pigs, **3** greatly reduced total plasma cholesterol and triglycerides at a 1 mg/kg dose. Pitavastatin also lowers triglycerides in a rat liver perfusion assay suggesting a novel secondary activity of the molecule that directs free fatty acids to oxidative pathways rather than esterification. Pitavastatin lowers total cholesterol and LDL-C 31% and 40%, respectively, in familial hypercholesterolaemic patients given 2 mg/day for eight weeks. Increasing the dosage to 4 mg/day for another eight weeks resulted in total cholesterol lowering of 37% and LDL-C lowering of 48% (9). Pitavastatin had no effect on HDL-C, but significantly lowered triglycerides (-23%) at the 4 mg/day dose. Pitavastatin was safe and well tolerated in this study and has little CYP metabolism (10). This new class of HMGRIs looks very promising; however, clinical experience with the agents is still young.

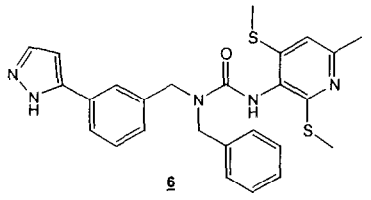
Acyl-CoA: Cholesterol *O*-Acyl Transferase (ACAT) Inhibitors – Despite clinical trials that have failed to show significant human efficacy, work continues in the area of inhibiting ACAT as a means of affecting cholesterol absorption and atherosclerosis. Avasimibe (CI-1011 or **4**) has been shown to have pharmacological effects beyond intestinal ACAT inhibition in inhibiting arterial cell wall plaque formation in New Zealand white rabbits fed a high fat, high cholesterol diet (11). In this study, avasimibe was dosed at 25 mg/kg after exposure of rabbits to high fat, high cholesterol diet for 9 weeks, followed by 6 weeks of a high fat diet. No effect on total plasma cholesterol exposure was observed. Avasimibe significantly reduced the



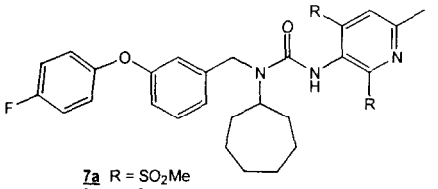
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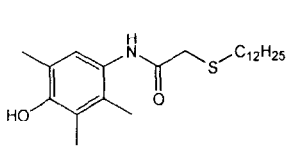
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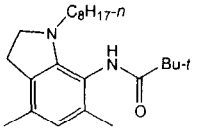
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7a R = SO_2Me
7b R = CH_3



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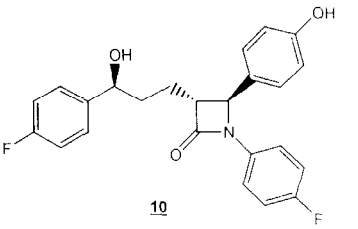


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formation of thoracic aortic lesions (-41%), aortic arch cross-sectional lesion area (-35%), and monocyte-macrophage area (-27%). The reduction in macrophage recruitment to the lesion area also resulted in a significant reduction in latent and active matrix metalloproteinase (MMP) activity in the regions affected. MMPs have been suggested to be destabilizing to established lesions in atherosclerotic plaque, and lesion development has been correlated with expression levels of several MMP isozymes: MMP-1, MMP-3, MMP-7 and MMP-9 (12,13). Avasimibe also affects bile acid synthesis by upregulating 7-α-hydroxylase activity and by increasing free cholesterol availability for the bile acid pathway (14). Avasimibe has been shown to lower VLDL-C and triglyceride levels in patients with combined hyperlipidemia at a dose of 50-500 mg/day. In this study, avasimibe did not effect LDL-C, HDL-C or total cholesterol or the marker proteins apolipoprotein B or apolipoprotein A1 (15, 16).

Several other ACAT inhibitors have also been reported to have interesting biological properties. NTE-122 (**5**) inhibits whole-cell ACAT activity in CaCo-2 cells with an IC_{50} of 4.7 nM (17). It was shown that **5** is an orally active ACAT inhibitor that blocked intestinal cholesterol absorption in rats at 1 mg/kg. Another potent series of ACAT inhibitors is typified by FR 186054, **6** (18, 19). FR 186054 inhibits rabbit intestinal microsomal ACAT with an IC_{50} of 99 nM and inhibits cholesterol absorption in cholesterol-fed rats when administered in the diet (ED_{50} of 46 mg/kg). Particular attention was paid to toxicological issues with this series and FR 186054 displayed no adrenal toxicity in dogs exposed to a single dose (10 mg/kg). Further work in this program identified FR 190809 (**7a**) and FR 186485 (**7b**) as potent ACAT inhibitors with minimal adrenal toxicological potential (20).

A potent systemic ACAT inhibitor, F 12511 (**8**) inhibits rabbit intestinal microsomal ACAT with an IC_{50} of 41 nM. F 12511 inhibits ACAT activity in HEPG2, CaCo-2, and macrophagic THP-1cells with IC_{50} values of 3, 7, and 71 nM, respectively (21). F 12511 is highly potent and efficacious at inhibiting cholesterol absorption in cholesterol-fed animal models in rats, guinea pigs and rabbits (ED_{30} for lowering plasma cholesterol levels in guinea pigs was 8 μg/kg). Atherosclerotic lesion formation was also decreased in rabbits treated with **8** in a dose related manner. F 12511 also has no adrenal toxicological effects in cultured human cells (22). Indolinyl amide **9** is a modest ACAT inhibitor (IC_{50} = 90 nM) but has potent anti-peroxidative activity in an LDL oxidation model. *In vivo* studies revealed that **9** has significant oral bioavailability in dogs at 10 mg/kg and lowers total plasma cholesterol in both rats (10 mg/kg/day) and hamsters (20 mg/kg/day) (23).

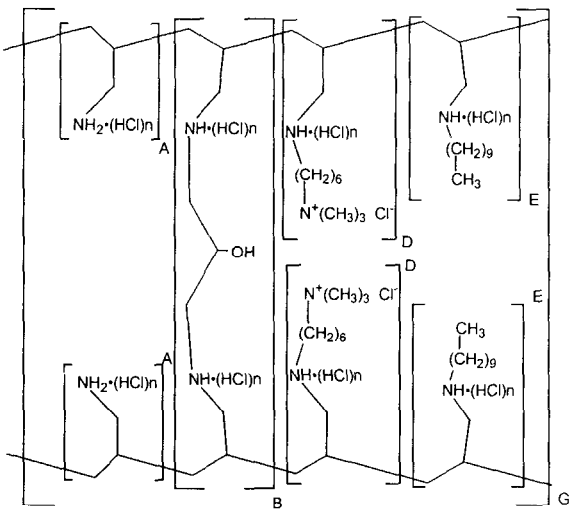


10

Cholesterol Absorption Inhibitors – One of the most promising new therapies for inhibiting cholesterol absorption is ezetimibe (Sch 58235, **10**). In rhesus monkeys on a high-fat, high-cholesterol diet, **10** inhibited cholesterol absorption with an ED_{50} of 0.5 μg/kg (24, 25). **10** is 80-times more potent at inhibiting cholesterol absorption in rhesus monkeys than in a similar assay in hamsters. It displays synergistic lowering of LDL-C when given in conjunction with statins in (26). In a randomized, double-blind, placebo-controlled trial in hypercholesterolaemic humans on an AHA step-1 diet, ezetimibe lowered LDL-C from 10-19% in doses from 0.25 to 10 mg once a day. Reductions were observed after the first week of dosing and maximized at two weeks. HDL-C levels were slightly raised in these studies (27). Ezetimibe and simvastatin given in combination (10 mg each) resulted in 52% reductions in LDL-C (28). The synergy seen with **10** and statins stems from the complementary nature of their activities. Statins not only inhibit cholesterol biosynthesis, but also upregulate clearance mechanisms (LDL expression levels, etc.). A large percentage of the

cholesterol scavenged by the LDL receptor in the liver travels to the gall bladder and is eliminated to the intestine with bile. Much of this cholesterol is reabsorbed from the intestines and therefore indistinguishable from dietary cholesterol. **10** blocks the reabsorption of all intestinal cholesterol, both biliary and dietary sources. The molecular mechanism by which this drug works is the subject of significant effort. Recent advances in the understanding of cholesterol homeostasis and metabolism as detailed below underscore the importance of investigations in this area.

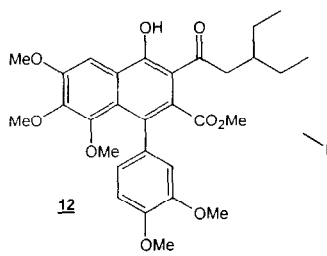
Bile Acid Sequesterants – Clinical experience has been poor with this class of non-systemic LDL-C lowering agents due to limiting side effect profiles and poor patient compliance. These agents (e.g., cholestyramine or cholestipol) irreversibly bind bile acids in the intestine and prevent reabsorption. The effect of this strategy is to upregulate both the synthesis of bile acids from cholesterol and LDL receptor activity. Frequently these agents are given in combination with a statin and additive effects are seen. A new non-absorbed bile acid sequestering resin, colesavelam hydrochloride (Cholestagel™, WelChol™, **11**) has been designed to be a hydrogel, absorbing large amounts of water in addition to the bile acids. The effect is to produce a non-absorbed resin that has significantly lower incidence of gastrointestinal side effects and subsequently higher patient compliance. Colesavelam hydrochloride lowered LDL-C by 19% in hypercholesterolaemic patients at total daily doses greater than 3 g (29, 30). Colesevelam is also additive in its effects on lowering LDL-C with statins (31). Colesevelam hydrochloride was granted marketing approval in May 2000 by the US FDA. Cholestimide (cholebine or MCI-196) is another new bile acid sequestering resin that has been shown to reduce LDL-C in patients (-22%) while raising HDL-C by 8% based on a 3 g/day dose (32). Side effects including constipation and flatulence occurred in 23% of patients.



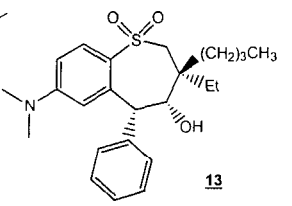
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A = Primary Amines
B = Cross-linked Amines
D = Quaternary Ammonium Alkylated Amines
E = Decylated Amines
n = Fraction of Protonated Amines
G = Extended Polymeric Framework

Ileal Na+/Bile Acid Cotransporter (IBAT) Inhibitors – IBAT is the active bile acid reabsorption transporter in the ileum, which is responsible for the enterohepatic circulation of bile acids. Inhibition of IBAT should result in similar LDL-C lowering effects seen with bile acid sequestering resins. The IBAT inhibitor S-8921 (**12**) has been shown to increase fecal bile acid excretion, increase hepatic LDL receptor expression and reduce plasma cholesterol levels in rabbits (33). SC-990 (**13**) is a nanomolar IBAT inhibitor that has

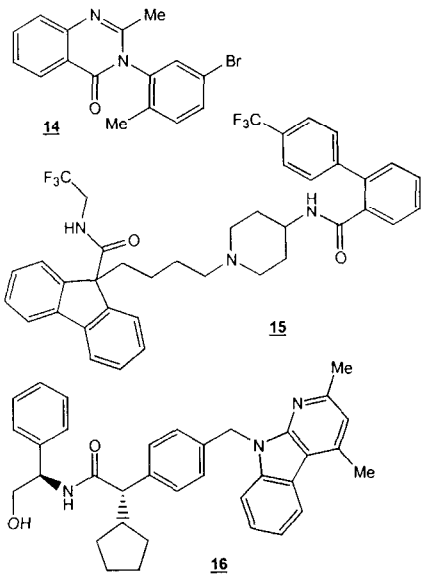


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advanced to clinical trials. There have been no reports describing the potential diarrhea that is expected to occur when the large intestine is exposed to an excess level of free bile acids (34).



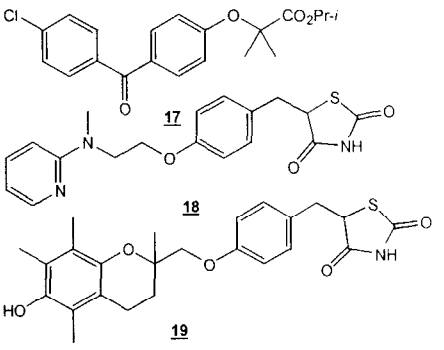
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Microsomal Triglyceride Transfer Protein (MTP) Inhibitors – MTP is involved it transporting lipids, including triglycerides and cholesteryl esters, into chylomicrons in the intestine and VLDL in the liver. CP-10447 (**14**) inhibits the assembly and production of VLDL from hepatocytes (35). The consequence of this inhibition is to reduce apolipoprotein B100 production. Inhibition of MTP therefore decreases plasma VLDL levels. MTP inhibition in enterocytes also inhibits chylomicron production resulting in reduced absorption of triglycerides and cholesterol in the intestines. The MTP inhibitor BMS 201038 (**15**) is a potent inhibitor of MTP with a K_i of 0.5 nM and was reported to decrease plasma cholesterol levels by 90% and triglycerides more than 49% in hamsters and Watanabe rabbits which lack functional LDL receptors (36). The use of (**15**) in combination with other cholesterol lowering agents has recently been patented. (37). The MTP inhibitor Bay 13-9952 (implitapide, **16**) has progressed to clinical trials and was reported to reduce LDL cholesterol by 58% at 160 mg/day and to result in an almost total inhibition of postprandial plasma triglyceride response following a high fat meal (38, 39). Bay 13-9952 at 160 mg/day was associated with gastrointestinal side effects (nausea and diarrhea) (38). Lipid malabsorption and fatty liver have also been noted after treatment with MTP inhibitors.

NUCLEAR RECEPTOR BASED LIPID MEDIATORS



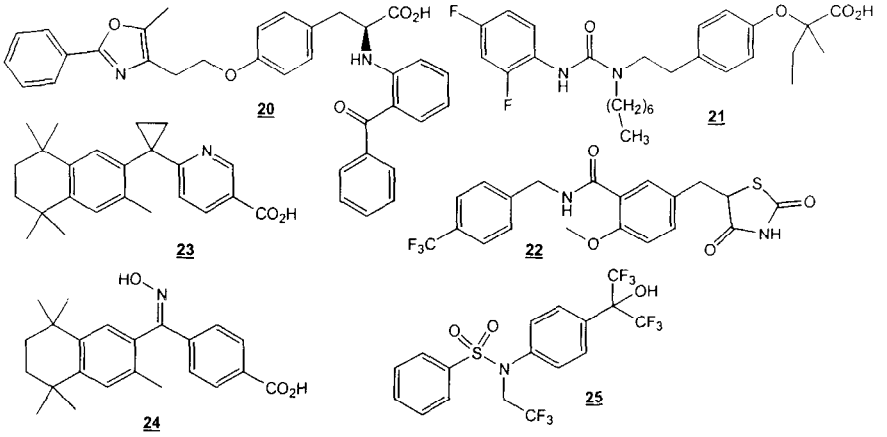
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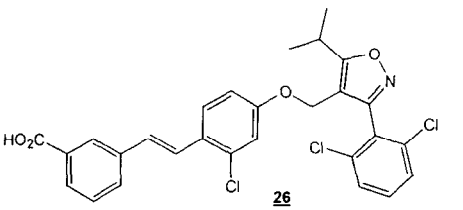
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Nuclear Hormone receptors There are several nuclear hormone receptors which are involved in the regulation of lipid metabolism. The liver X receptors (LXR), retinoid X receptors (RXR), and farnesoid X receptors (FXR) are activated by oxysterols, retinoids, and bile acids, respectively. RXR also forms heterodimers with peroxisome proliferator-activated receptors alpha, delta, and gamma (PPARs), with LXR alpha and beta, and FXR. All of these nuclear receptors have been targeted for drug development (40). Fibrates (e.g. fenofibrate, **17**) are agonists of PPAR alpha, which results in the induction of a series of genes leading to a reduction in plasma triglyceride levels and an increase in HDL-cholesterol. The glitazones (e.g. rosiglitazone **18** and troglitazone **19**) are agonists of PPAR gamma, which improve insulin sensitivity and are used as oral hypoglycemic agents. A high affinity PPAR gamma agonist, GI262570, **20** (pK_i = 8.9), has been reported to have potent

antihyperglycemic effects as well as antihyperlipidemic activity (41). PPAR gamma agonists have also been reported to have direct anti-atherosclerosis activity (42). GW2331 (**21**) and KRP-297 (**22**) are investigational compounds which act as agonists of both PPAR alpha and gamma (43, 44). These compounds would potentially improve insulin sensitivity, as well as reduce plasma triglycerides and increase HDL-C. Since RXR forms a heterodimer with the PPARs, RXR agonists, LG100268 (**23**) and LG100364 (**24**), have also been investigated as anti-diabetic and HDL-C increasing agents (45, 43).



Adenosine triphosphate-binding cassette (ABC) transporters are a large family of proteins that mediate the ATP dependent transmembrane transport of a variety of substrates including lipids and xenobiotics. RXR and LXR agonists have recently been shown to have an effect on cholesterol absorption and the expression of ABCs. ABCA1 functions to transport cellular cholesterol and phospholipids to apolipoprotein A1 to form HDL particles. Mutations in ABCA1 result in Tangier disease, which is characterized by the absence of HDL and defective cellular cholesterol efflux (46). ABCA1 expression is regulated by the nuclear receptor LXR/RXR. Agonists of LXR (TO901317, **25**) and RXR (LG100268) have been shown to induce ABCA1 expression, resulting in an increase in cellular cholesterol efflux (47, 48, 49). These agonists also caused a reduction in cholesterol absorption, presumably by pumping intestinal enterocyte cholesterol back into the lumen of the intestine through ABCA1 (47). The RXR agonist, LG100268, was also shown to reduce cholesterol absorption partially by decreasing bile acid production through RXR/FXR repression of CYP7A1 activity. GW4064, **26**, is a non-steroidal specific FXR agonist (EC_{50} = 15 nM) which significantly lowers triglycerides in a dose dependent manner (50). This compound could selectively influence bile acid production and thus affect cholesterol metabolism.

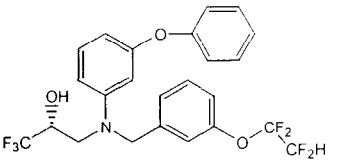


With the discovery of ABCA1 and ABCG1 as transporters involved in cellular cholesterol efflux and ABCG5/8 as the transporter mutated in sitosterolemics (51,52), the ABC transporter family of proteins is rapidly expanding and will be an active area of future atherosclerosis drug discovery research (46). Agents that stimulate ABCA1 and ABCG5/G8 activity may result in the inhibition of cholesterol absorption. Reverse cholesterol transport may be enhanced with agents that

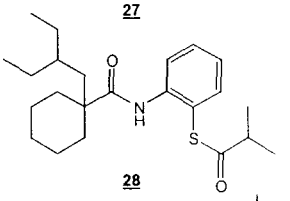
stimulate ABCG1 and ABCA1, which are involved in cellular cholesterol efflux. ABCA1 is involved in the movement of cellular cholesterol and phospholipids to apolipoprotein A1 to form HDL particles. An additional receptor involved in HDL cholesterol movement is the scavenger receptor class B type 1 (SR-B1). SR-B1 has been identified as the HDL receptor that is responsible for the selective uptake of HDL cholesterol by the liver and steroidogenic cells (53). SR-B1 enhances the clearance of HDL cholesterol and the delivery of the cholesterol to bile for excretion. This reverse cholesterol transport pathway has been demonstrated in transgenic mice overexpressing hepatic SR-B1, and in SR-B1 knockout mice, with an increase and decrease in biliary cholesterol, respectively (54). Agents designed to stimulate the activity or expression of SR-B1 may enhance reverse cholesterol transport and inhibit atherogenesis (55, 56).

OTHER LIPOPROTEIN MEDIATORS

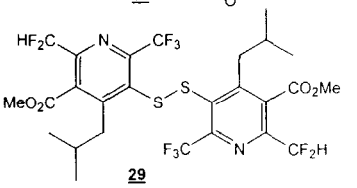
Cholesterol Ester Transfer Protein (CETP) Inhibitors - CETP is a plasma glycoprotein that mediates the exchange of cholesteryl ester in HDL for triglyceride in very low density lipoprotein (VLDL) particles. Inhibition of this process is believed to elevate HDL-C levels and be antiatherogenic. Several compounds have been reported to be potent CETP inhibitors and have effects on cellular lipids. SC-795 (**27**) is a potent CETP inhibitor with an IC_{50} of 20nM in buffer and 600nM in the presence of human serum (57). SC-795 inhibits cholesteryl ester – triglyceride exchange by binding to CETP reversibly and stereospecifically competitive with the lipid binding site (58). JTT-705 (**28**) has an IC_{50} of 9 μM against CETP and is reported to attenuate atherosclerosis in rabbits. JTT-705 increases HDL and decreases levels of non-HDL-C in the reported models (59). SC-71952 (**29**) is a 1 μM inhibitor of CETP which first binds reversibly to a lipophilic binding site on CETP, then more slowly captured by a resident cystein in an irreversible manner to inactivate CETP (60). Significant elevation of HDL levels in humans has been suggested for this class of molecules, however published data remains to be disclosed.



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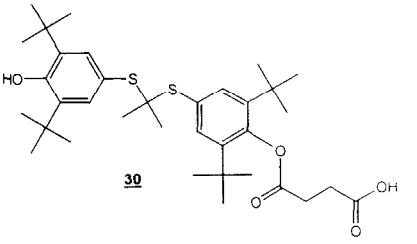


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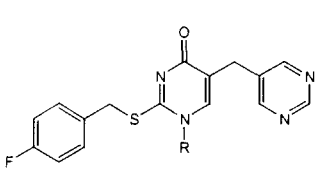
Vascular Protectants - The compound AGI-1067, **30**, has been reported to inhibit atherosclerosis in apo E and LDL receptor knockout mice (61, 62). AGI-1067 is an anti-inflammatory, lipid-lowering compound, that has been shown to selectively inhibit cytokine induced vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemotactic protein 1 (MCP-1) expression in vitro and in vivo. AGI-1067 lowers plasma cholesterol levels in cholesterol-fed mice and apo E knockout mice. In LDL receptor knockout mice, AGI-1067 inhibited atherosclerosis without lowering plasma cholesterol levels. These results indicate that AGI-1067 lowers plasma cholesterol through enhanced LDL receptor mediated lipoprotein clearance and that this compound has a direct anti-atherosclerosis vascular protectant activity. AGI-1067 has progressed to clinical trials and may be



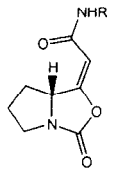
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useful in the treatment of atherosclerosis and restenosis following vascular interventions.

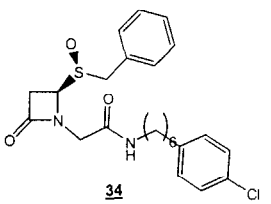
Oxidized lipids are thought to play a role in the inflammatory signaling and the deposition of LDL cholesterol in atherosclerotic plaques. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is primarily associated with LDL and has been shown to hydrolyze phospholipids from oxidized LDL. The inhibition of Lp-PLA_2 may inhibit the arterial deposition of pro-inflammatory oxidized fatty acids and lysophosphatidylcholine and inhibit atherogenesis. Orally active Lp-PLA_2 inhibitors pyrimidinones **31** and **32** have been shown to inhibit plasma PLA_2 activity following oral administration to Watanabe (WHHL) rabbits (63). Bicyclic enol carbamates (e.g.



31 R = (E) $(CH_2)_3CONH(CH_2)_8CH{=}CHC_8H_{17}$
32 R = (Z) $(CH_2)_3CONH(CH_2)_8CH{=}CHC_8H_{17}$



33 R = (R) $C_{16}H_{31}$



34

33) are potent as PLA_2 inhibitors with IC_{50}'s as low as 12 nM (64). Azetidinone SB-222657 (**34**) is also a potent inhibitor of PLA_2 and has been used as a tool to investigate phospholipid metabolism that occurs with during the oxidative metabolism of lipoproteins (65). SB-222657 is able to prevent the hydrolysis of oxidized phosphatidylcholines without influencing the kinetics of LDL oxidation under a variety of conditions.

SUMMARY AND FUTURE DIRECTIONS

The current pharmacological focus for clinical treatment of atherosclerosis continues to be lowering of serum LDL-C. In this regard, the statins have really proven their worth in managing this disease. The combination of these lipid lowering agents with next generation lipid modulating drugs show great promise for the future. Cholesterol absorption agents, novel bile acid sequesterants, and new methods for elevating anti-atherosclerotic HDL-C levels may have a very real impact in patient care in the immediate future. More long term prospects may come from the discoveries of the nuclear hormone receptors, the ABC transporters, and their intricate biological interplay that seems to have profound effects on cellular steroidal balance.

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Chapter 7. Progress in the Treatment of Pulmonary Disease in Cystic Fibrosis

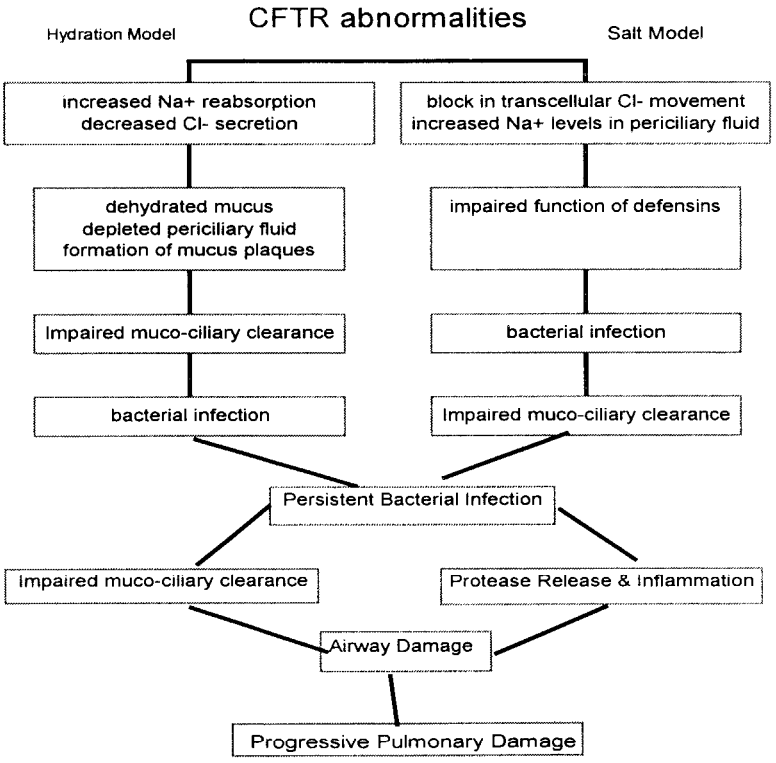
Pallav L Shah MD, MRCP
Royal Brompton Hospital, Sydney Street, London SW3 6NP, UK

Introduction - Cystic fibrosis is a genetic disease that affects one in 2500 live births. A basic defect in chloride transport leads to impaired clearance of airway secretions and a susceptibility to bacterial infection. Once infection is established, there is a vicious cycle that leads to progressive inflammation and infection, which in turn leads to progressive pulmonary damage. Although cystic fibrosis is a multisystem disorder, pulmonary disease is the main cause of morbidity, and respiratory failure remains the main cause of death.

PATHOPHYSIOLOGY

Cystic fibrosis is caused by a defect of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1-3). This gene codes for a transmembrane protein, which functions

Figure 1. The current concept of the pathogenesis of cystic fibrosis lung disease



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as an ion channel. Defects in the protein cause chloride impermeability and excessive sodium reabsorption. There are two divergent models (Figure 1) of how CFTR abnormalities causes abnormalities in host defence and mucus hydration (4). The mucus hydration/isotonic model suggests that CFTR abnormalities increases sodium transport and impairs chloride secretion (5). There is increased fluid absorption from the airways leading to dehydrated secretions. The depleted periciliary fluid impairs ciliary transport and the dehydrated mucus forms large immobile plaques. This in turn leads to a susceptibility to infection and subsequent establishment of the infective and inflammatory cycle.

The salt model suggests that there is a block in transcellular chloride movement leading to elevated levels of sodium in the airway surface liquid (6). The greater sodium levels neutralize the function of the human defensins (7). This accounts for the susceptibility to bacterial infections, especially Pseudomonas. The combination of early infection and poor clearance of airway secretions allows the establishment of a vicious cycle of infection and inflammation.

Chronic endobronchial infection induces a potent neutrophil host immune response, but the neutrophils are unable to clear the organisms. In the early phase of infection, Pseudomonal toxins such as Pseudomonas elastase and alkaline protease interfere with phagocytosis and specific humoral and cellular immune response of the lung. Species of *P. aeruginosa* produce a mucoid exopolysaccharide coat, which evades the neutrophil defense by forming microcolonies in a biofilm. Bacterial persistence promotes a strong neutrophil dominated inflammatory response in cystic fibrosis, which is self perpetuating.

The hydration model suggests that mucus retention precedes infection, whereas the salt model suggests that infection precedes the cycle of mucus retention and inflammation. With both scenarios, once established, the cycle of infection and inflammation is self-perpetuating, and leads to lung destruction, and accounts for the inexorable decline in pulmonary function and early mortality.

GENE THERAPY

There have been considerable improvements in molecular biology techniques, which has enabled gene therapy to reach the stage of human clinical trials. The vector types that were used in the initial clinical trials were adenovirus vectors, adeno-associated virus vectors, and liposomal delivery systems. The clinical studies initially tested the principle of gene therapy in the nose, which is an easily accessible site. These studies suggested transfection in 50% of treated subjects with some functional correction in less than a third of cases (8-17). Pulmonary delivery is more challenging, and requires delivery of the drugs to the appropriate part of the lung, penetration of the thick mucus barrier, into the cell and into the nucleus of the target cell. Studies to date demonstrate delivery to 30% of treated subjects and partial correction in some of these individuals (12, 16-17). A Phase II study which delivered aerosolized liposomal DNA complexes to the lung demonstrated a partial correction (about 25%) of the ion transport defect (18), which probably represents a transfection efficiency of about 1%. Although the current studies have had low transfection rates, the evidence suggests that only 5% of normal CFTR is required to correct the ion transport defect (19). Treatment was generally well tolerated apart from a self-limiting influenza-like illness.

The key limitations that have been observed with all the clinical trials of gene therapy for cystic fibrosis are; 1) low transfection rates, 2) short duration of expression and 3) host inflammatory response. The mucus barrier is one factor that limits effective gene transfection, and studies have shown that the use of mucolytics improves transfection (20). The inflammatory load in airway secretions is another factor, and effective inhibition of the neutrophil protease load of bronchoalveolar lavage fluid from patients with cystic fibrosis has

been shown to improve transfection efficiency (21). Retroviral vectors are now being considered for gene transfer, as it may lead to more stable integration into the host DNA and hence stable integration (22). Animal studies have shown that the recombinant Sendai virus and the retroviral vector, Filovirus-pseudotyped lentivirus vector, produces more effective gene transfection (23,24).

MODULATING CFTR PROCESSING

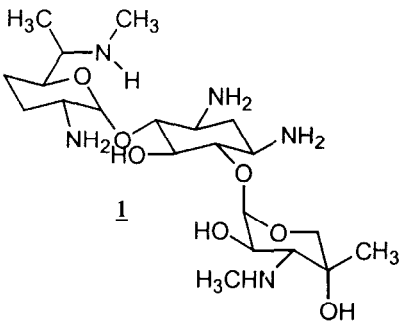
Regulation of CFTR Synthesis - There are five main classes of mutations of the cystic fibrosis gene (Table 1). The abnormalities are due to either failure of synthesis of the protein, due to problems in transcription or translation, defective intra-cellular processing, block in regulation, altered conductance or reduced synthesis. There are a number of different strategies to overcome these blocks.

Table 1. Modulation of CFTR processing with possible interventions.

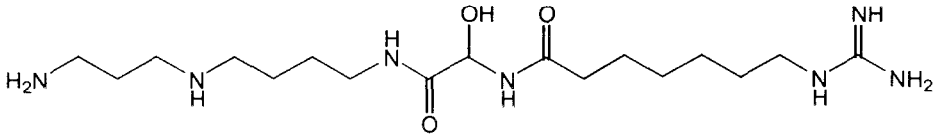
Site of Action	Mechanism of genetic defect	Drug therapy	Effect of drug therapy
DNA	Stop codons (e.g. G542X, R553X)	aminoglycosides	Translation misreading allowing production of RNA
RNA		Phenylbutyrate	Increased transcription of ΔF508
Protein Maturation	Degradation of product in endopalsmic reticulum	Growth at lower temp Glycerol Deoxyspergualin Phenylbutyrate	Improved trafficking
Function	Reduced or absent activity	CPX, DAX Genistein Phosphatase inhibitors Nucleotide phosphodiesterase inhibitors	Activation of CFTR

Approximately 5% of the CFTR mutations are premature stop codon mutations (e.g. G542X, R553X). These may be overcome by the topical application of aminoglycosides which cause translation misreadings and hence may overcome the premature stop codon mutations (25). Low concentrations of the synthetic aminoglycoside G-418 in cell culture studies suppressed the premature stop codons and resulted in full length functional CFTR when expressed (26). The CFTR was expressed on the apical cell surface with restoration of the chloride channel activity. Nine cystic fibrosis patients have been treated with topical gentamicin (**1**) for 14 days. An improvement in nasal potential differences was observed in 7 of the 9 patients (27). Although these mutations only account for a small number of patients with cystic fibrosis this strategy shows some promise with other synthetic aminoglycosides in development.

Intracellular processing of CFTR - The ΔF_{508} mutation is the most common mutation, and is found in about 70% of patients with cystic fibrosis. There is defective processing of the mutant protein, which is retained and degraded in the endoplasmic reticulum. Trafficking of the CFTR protein may be facilitated by compounds such as deoxyspergualin (**2**) which bind with cellular chaperones such as heat shock proteins and may allow trafficking of the mutant CFTR (28). Treatments that are now under clinical development are discussed below.

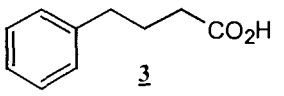


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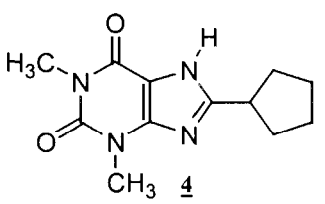
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Phenylbutyrate - Phenylbutyrate (**3**) upregulates expression of ΔF_{508} CFTR (29). It may also downregulate the heat shock cognate protein (Hsc 70) which has a role in lysosomal degradation of intracellular proteins. The net effect is that increased amounts of ΔF_{508} CFTR is able to escape the endoplasmic reticulum quality control mechanisms, which is then expressed at the apical surface and functions as a chloride channel. A pilot double-blind placebo controlled study with 19 g of oral **3** demonstrated safety and partial efficacy . No change in sweat chloride values were observed (30). A dose ranging study with 24 cystic fibrosis patients with 20g, 30g and 40g of **3** is currently underway. Further studies include a randomized double-blind crossover trial with four weeks in each treatment period.

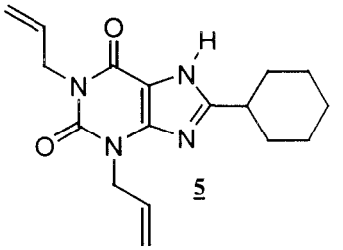


3

CPX (8-cyclopentyl-1, 3-dipropylxanthine) - CPX (8-cyclopentyl-1, 3-dipropylxanthine, **4**) improves CFTR trafficking and stimulate chloride ion transport in CF patients with the ΔF_{508} mutation (31). Although CPX is an A_1-adenosine receptor antagonist, it appears to act by directly binding to the nucleotide binding fold 1 of CFTR (32), and activates the CFTR channel (33). It's affinity for ΔF_{508} CFTR is higher than for wild type CFTR. The activity of CPX does not appear to be mediated by antagonism of A_1-adenosine receptor as analogues with a similar affinity for these receptors were not as effective at activating chloride efflux. Furthermore, activity for CPX has been demonstrated in cystic fibrosis cells that lack classical A_1-adenosine receptors. CPX probably binds to CFTR close to the ΔF_{508} locus, and induces a local conformational change that affects its protein-lipid interactions, and hence may improve trafficking, maturation and expression of CFTR at the apical surface.



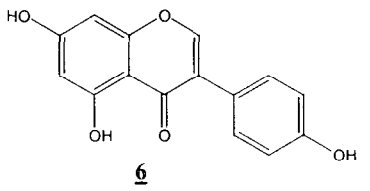
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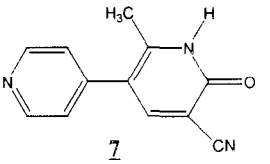
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The key advantages of CPX are low toxicity, need for low tissue concentrations for activity and oral administration. Hence CPX it has the potential to improve both pulmonary and gastrointestinal manifestations. A phase I randomized double-blind placebo controlled study of CPX in patients with mild cystic fibrosis enrolled 37 patients with a forced expiratory volume in one second (FEV_1) greater then 60% predicted. This single dose study evaluated the basic safety and pharmacokinetics of the product (34). However, multiple doses over several days are required before a clinical effect is observed. SciClone Pharmaceuticals have initiated a multicenter dose ranging Phase II study in patients with mild to moderate disease. A second generation molecule DAX (**5**) has even greater affinity for CFTR and is also currently under development (33).

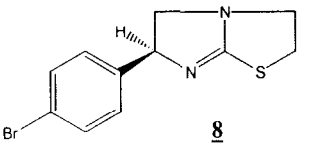
Activation & Regulation of Mutant CFTR - CFTR can be activated by protein kinase A dependent mechanisms. Genistein (**6**) is a novel substance that is currently under investigation which may act through this mechanism (35). Complimentary strategies include activation and regulation of CFTR function by phosphatase inhibitors and the use of specific classes of nucleotide phosphodiesterase inhibitors (36,37). A number of compounds are under investigation in the cystic fibrosis murine models. Milrinone (**7**) a specific type III phosphodiesterase inhibitor has been reported to induce chloride secretion *in vitro*, but human studies have not shown any significant changes (38,39). Bromotetramsole (**8**) has been shown to stimulate CFTR activity by inhibiting a membrane associated protein phosphatase (purified protein phosphatase type 2C) (35).



6



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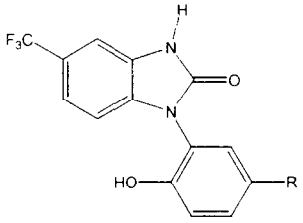


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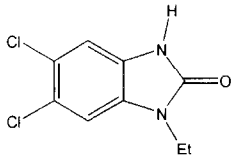
Benzimidazolones cause sustained chloride secretion and probably act by direct stimulation of CFTR (40). Substances that are under current investigation include NS004 (**9**), NS1619 (**10**) and DCEBIO (**11**). Other classes of compounds, which have been found to activate CFTR in vitro by high throughput screening assays are the 7,8-benzoflavones and fused pyrazolo heterocycles. The most potent in each group respectively are UCCF-029 (**12**) and UCCF-180 (**13**) (41,42).

CFTR REPLACEMENT

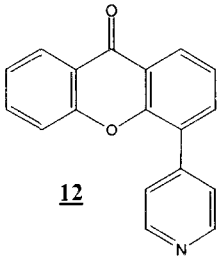
Recombinant CFTR has been produced in cell lines, and also in the milk of certain animals using transgenic expression. This makes protein replacement therapy a possibility in the future. Murine studies have shown some incorporation into the nasal epithelia and correction of the nasal potential difference (43).



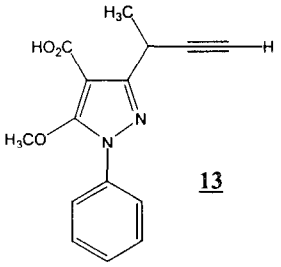
9 (R = Cl)
10 (R = CF_3)



11



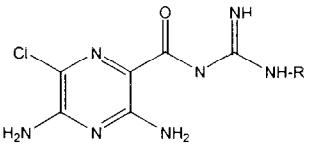
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13

MODULATION OF NON CFTR ION CHANNELS

Trials with amiloride (**14**), a sodium channel blocker, have produced variable results and the latest study treated 137 patients with either placebo or **14** for six months, but failed to show any clinical benefit (44). The short duration of action of the drug and only partial correction of the ion transport abnormality, may account for the lack of efficacy. Benzamil (**15**) is an analogue of **14** with a longer duration of action. Administration to the nasal epithelium has confirmed the more prolonged duration of action in patients with cystic fibrosis (45). A randomized placebo controlled study in 10 cystic fibrosis patients treated with either **14**, **15** or saline nasal spray demonstrated similar degree of improvements in nasal potential differences with either **14** or **15**. However, a significantly longer duration of activity was seen for **15** (4.3 hrs compared to 0.6 hrs with **14**) (46).



14 (R = -H)
15 (R = $-CH_2Ph$)

Activation of Alternative Chloride Channels - Uridine triphosphate (UTP, **16**) is a chloride secretagogue which acts via purinergic (P_2 receptors). It improves mucus hydration and improves mucociliary clearance (47-49). It has been used alone and in combination with **14** in Phase I trials and is now entering Phase II studies. Duramycin (**17**) is a 19 amino acid polycyclic peptide, which also has chloride secretagogue properties with the

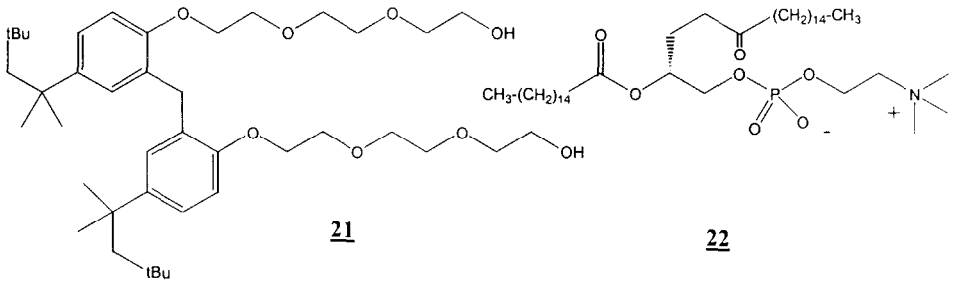
advantages of a long half-life and low systemic absorption. Normal volunteers demonstrated evidence of chloride secretion following nasal administration and studies in patients with cystic fibrosis are due to commence. Other compounds with chloride secretagogue properties under investigation are the benzamidazolones and have been discussed earlier. Some of the benzamidazolones also induce chloride secretion by stimulating the hIK1 potassium channel. A group of synthetic channel forming peptides also induce chloride secretion (50). The peptide (C-K4-M2GlyR) was shown to induce chloride secretion and further activation was induced by benzimidazolones.

ALTERATION OF THE PHYSICAL PROPERTIES OF AIRWAYS SECRETIONS

The success of dornase alfa in the treatment of patients with cystic fibrosis has renewed interest in mucolytics. The rationale is that improvements in viscoelasticity or adhesive properties of sputum may improve the clearance of airway secretions. This in turn may improve pulmonary function and possibly retard progression of pulmonary disease. The products of disintegrating neutrophils contribute to the abnormal rheology and surface properties of cystic fibrosis sputum. The deoxyribonucleic acid (DNA) from the disintegrating cells polymerizes and forms highly viscoelastic complexes with mucous glycoproteins. Dornase alfa (**18**) was developed to reduce the viscoelasticity of cystic fibrosis sputum and initial clinical studies have been encouraging. Longer term studies suggest sustained benefit in terms of reduction of respiratory exacerbations and a possible reduction in the rate of decline of pulmonary function (51).

Filamentous actin is another product of the inflammatory cascade observed in cystic fibrosis and these polymers interact with mucous glycoproteins and other inflammatory products to further exacerbate the abnormal rheology of cystic fibrosis sputum. Gelsolin (**19**) is a polypeptide that cleaves actin polymers to smaller units. A dose dependent reduction in sputum viscoelasticity has been demonstrated *in vitro*. A pilot study with **19** suggested that treatment was safe but there was only a slight improvement in pulmonary function (unpublished data).

N-acetylcysteine lysinate (Nacystelyn®, **20**) is a new mucolytic which in two acute studies in patients with cystic fibrosis has demonstrated improvements in the rheological properties of sputum. A cross over study in twelve patients with cystic fibrosis treated for two weeks demonstrated a reduction in sputum viscoelasticity. No effect on pulmonary function was observed. An imbalance in the lipid composition of cystic fibrosis mucus is an additional cause of the enhanced adhesion of cystic fibrosis sputum. Tyloxapol (**21**) and the surfactant Exosurf® (**22**) have been used in Phase I studies but no further development in patients with cystic fibrosis has been reported.



21 **22**

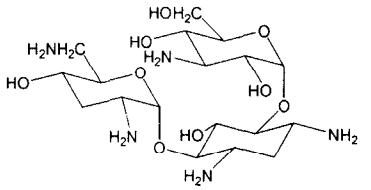
Hypertonic saline acts as a mucokinetic agent and an osmotic agent, which may reduce viscoelasticity of mucus by reducing the intermolecular bonds. This agent which has been used empirically in the past is undergoing formal assessment and studies thus far show a

dose dependent increase in mucociliary clearance. A randomized open label study with hypertonic saline has demonstrated improvements in pulmonary function. A cross over study with hypertonic saline and dornase alfa has shown comparable effects on pulmonary function with both treatments. However, individual response is heterogeneous and unpredictable. Furthermore some patients respond to dornase alfa but not to hypertonic saline and *vice versa*.

The response to hypertonic saline has in turn stimulated interest in osmotic agents such as mannitol (**23**). In a pilot study **23** improved mucociliary clearance in patients with cystic fibrosis (52). Dextran is another agent, which reduces the viscoelasticity of cystic fibrosis sputum *in vitro*, and reduces colonisation of the airways by pseudomonas (53). *In vivo* studies in dogs have shown improvements in both mucus viscoelasticity and tracheal mucus transport rates (54).

CONTROL OF ENDOBRONCHIAL INFECTION

Bactericidal Agents - Oral, inhaled and intravenous antibiotics remain the mainstay of treatment to control the degree of bacterial infection in patients with cystic fibrosis. However, penetration of antibiotics into the endobronchial secretions is poor. Inhaled antibiotics were initially shown to be effective in improving pulmonary function and reducing respiratory exacerbations in the 1980's (55). A strategy of high dose inhaled tobramycin (**24**, Tobi®) has recently been introduced to ensure peak drug concentration in sputum were bactericidal for sensitive isolates (400 to 1000 μm per g of sputum). Cyclical treatment with inhaled dextran improves pulmonary function and reduces respiratory exacerbations (56). However, studies have not shown that this strategy is superior to current doses and preparations of inhaled antibiotics.



24

Cationic antimicrobial peptides with bactericidal properties including anti-pseudomonal activity even to antibiotic resistant strains of *P. aeruginosa* are under development (57),(58), (59). The peptides are also thought to be less likely to select for resistant strains and act synergestically with antibiotics. They are essentially salt insensitive and should not be affected by the abnormal cystic fibrosis airway surface liquid. They bind lipopolysaccharide endotoxin and hence may also reduce the tumour necrosis factor α (TNFα) response of host immune cells. The Demeter peptide D2A21 (**25**) and recombinant versions (RBPI21, **26**) of the human bactericidal/permeability increasing protein (BPI) are also due to enter clinical trials for cystic fibrosis (60, 61).

Phe-Ala-Lys-Lys-Phe-Ala-Lys-Lys-Phe-Lys-Lys-Phe-Ala-Lys-Lys-Phe-Ala-Lys-Phe-Ala-Phe-Ala-Phe

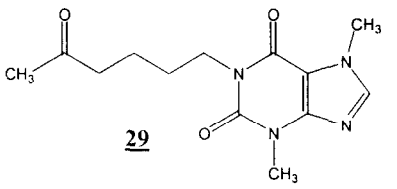
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Anti-colonization Strategies - New strategies are being explored to inhibit or reduce colonization with *Pseudomonas*. In the majority of patients with cystic fibrosis, antibodies produced against *P. aeruginosa* are non-opsonophagocytic and therefore ineffective in

combating the infection or inhibiting colonisation of the airways. Pooled immunoglobulin from a group of individuals who produced opsonic antibodies has been used in the evaluation of passive immunization. The main disadvantages of this treatment is the potential risk of infection with viruses or agents that have still not been characterised and to a certain extent even contamination of the products with known viruses. An oral Pseudomonas vaccine (Pseudostat, **27**) has completed Phase II studies in patients with cystic fibrosis. In these studies placebo or **27** was administered orally on the first three days of first three months and changes in pulmonary function and frequency of exacerbation's were monitored for six months. The recent development of an intranasal *P. aeruginosa* hybrid outer membrane protein F-1 vaccine (**28**) may provide the breakthrough required (62). The vaccine has been shown to produce a significant mucosal immunological response and hence adequate production of IgA antibodies, which will be important for patients with cystic fibrosis. It also has the advantages of cross protection for all pseudomonas serotypes and free form contamination by LPS which would induce a proinflammtory cytokine response.

One possible strategy of preventing Pseudomonas colonization is to inhibit *P aeruginosa* adherence to the respiratory tract and or enhance phagocytosis of *P. aeruginosa* by host defense cells. Dextran has been found to interfere with adhesion of *P. aeruginosa* and *Burkholderia cepacia* to respiratory epithelial cells in a non-specific manner (63). In a murine model, pre-treatment with aerosol dextran reduced the risk of developing pneumonia in mice inoculated with intranasal *P. aeruginosa* (64).

Anti-inflammatory & Antiprotease Therapy - In the past, oral steroids have been used in attempts to try and control the inflammation associated with CF but the side effects outweighed the benefits. Trials with inhaled steroids have not documented any conclusive benefits. High dose non-steroidal anti-inflammatory drugs (NSAID's) have been shown to ameliorate neutrophil influx in a murine models of sepsis. A four year study of ibuprofen in patients with cystic fibrosis has shown that rates of decline in lung function was reduced in younger patients. Pentoxifylline (**29**) is another inhibitor of neutrophil chemotaxis and degranulation undergoing a multicenter study in patients with cystic fibrosis.

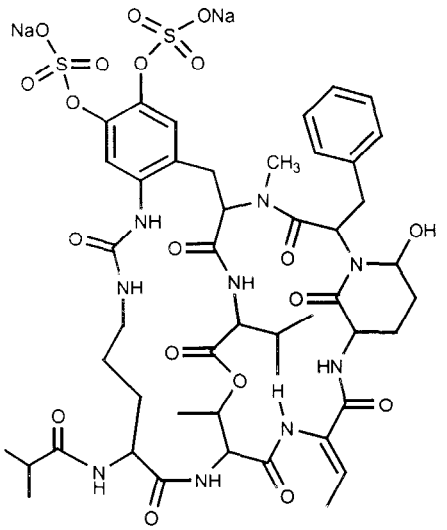


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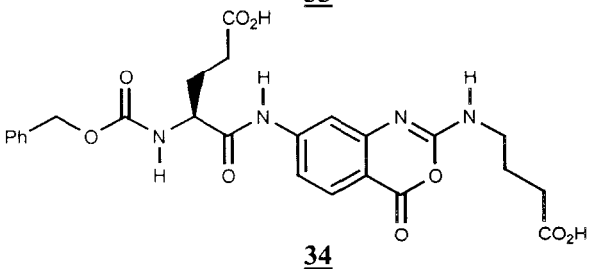
Large amounts of neutrophil elastase and other proteases are released by neutrophils due to ineffective phagocytosis and apoptosis. Neutrophil elastase is responsible for much of the pulmonary damage that occurs. The protease/antiprotease balance is overwhelmed in patients with cystic fibrosis due to the enormous protease burden. The problem is exaggerated by the cleavage of antiproteases by neutrophil elastase and perpetuation of the detrimental inflammatory response. Antiprotease therapy represents a crucial strategy in the treatment of cystic fibrosis. Previous studies with plasma derived alpha-1-antitrypsin (Prolastin®, **30**) have demonstrated small reductions in neutrophil elastase activity (65,66). The transgenic form of alpha-1-antitrypsin (**31**) produced in the milk of sheep and recombinant secretory leukoprotease inhibitor (**32**) are being evaluated in clinical trials in patients with cystic fibrosis.

The impact of current antiproteases is going to be limited in patients with established pulmonary disease due to the heavy protease burden. Current inhibitors act on a one to one binding and therefore large volumes of inhibitor would be required to completely mop up the

proteases. A number of novel synthetic antiproteases are under development which may have a greater impact. The trifluoromethyl ketone-based elastase inhibitors have been shown to reduce elastase activity without increasing vulnerability to infection (67). The synthetic elastase inhibitors FR134043 (**33**) and TEI-8362 (**34**) have been shown to have beneficial in vitro activity (68,69).



33



34

Conclusion - A number of strategies for the treatment of pulmonary disease in cystic fibrosis are being developed. Treatments aimed at correcting the ion transport defect by genetic therapy or modulating the function of mutant CFTR or by activating other channels are likely to be more effective in younger patients before airway infection and inflammation are well established. In contrast, anti-inflammatory and antibacterial treatments are aimed at those with established chronic bronchopulmonary sepsis. However, even anti-inflammatory treatments may be more effective if used early in the natural history of the disease. Treatments such as dornase alfa or hypertonic saline are also useful, as they enhance clearance of airway secretions. Airway clearance should underpin any treatment for cystic fibrosis, as this will reduce bacterial, inflammatory and protease load in the airways by regularly flushing away the secretions. There is unlikely to be a single answer to the treatment of cystic fibrosis, and the best outcome is likely to require the targeting of multiple sites, and hence the use of combination treatment.

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Chapter 8. Anticoagulants: Inhibitors of Thrombin and Factor Xa

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Introduction - Thrombin and factor Xa (fXa), members of the trypsin family of serine proteases, are pivotal components of the blood coagulation cascade. Thrombin is the final enzyme of the cascade. Its primary actions are to cleave fibrinogen to release fibrin and to activate platelets via the thrombin receptor and, since polymerized fibrin and activated platelets are the principal components of blood clots, thrombin inhibition will ameliorate coagulation. Thrombin inhibitors have the additional effect of indirectly slowing thrombin generation by attenuating the positive feedback effects of thrombin. FXa, a structurally closely related enzyme, as a complex with factor Va and calcium ions on a phospholipid surface (referred to as prothrombinase), activates prothrombin to give thrombin. Thus inhibitors of fXa exert an anticoagulant effect by directly slowing the generation of thrombin. This article will review the literature on covalent and non-covalent thrombin and fXa inhibitors which published during the year 2000 and it is intended to complement the recent updates of comprehensive reviews of fXa inhibitors and non-covalent inhibitors thrombin and fXa (1-4).

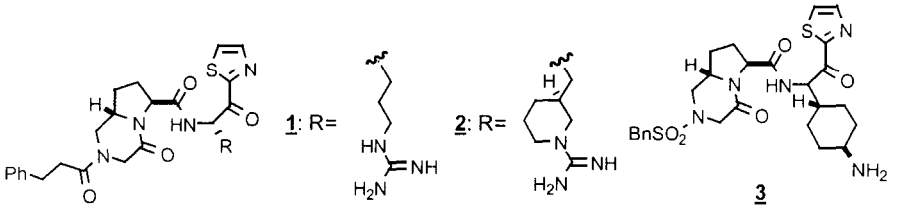
Heparin (and its derivatives) and coumadin (warfarin) are the standard anticoagulants prescribed for the treatment and prevention of deep vein thrombosis and for the prophylaxis of cardiogenic thromboembolism. They have been in clinical use for over half a century, but both have serious drawbacks which limit their safe and efficacious use. Heparin, a rapidly acting anticoagulant, is administered by injection. It is a complex mixture of sulfated glucosaminoglycans of molecular weight range 3-30 KDa, components of which inhibit the blood coagulation cascade by catalyzing the binding of antithrombin (antithrombin III) to thrombin and factor Xa. Low Molecular Weight Heparin (LMWH) is more specific in its actions and it selectively catalyzes the binding of antithrombin to factor Xa. Many recent studies with LMWH have shown it to be safer and easier to use than unfractionated heparin. It is administered by injection however, efficacy being achieved with a fixed dose given subcutaneously once or twice a day. The progress in the development of heparin mimetics will not be reviewed here (5).

Coumadin is an orally active anticoagulant prescribed for longer term therapy. It is a vitamin K antagonist which inhibits the post-translational modification of a number of enzymes in the coagulation cascade including prothrombin and factors VII, IX and X. Since its anticoagulant action depends on the rate of enzyme turnover, coumadin has a slow onset of action and it takes several days to reach an equilibrium. Typically coumadin therapy is initiated concomitantly with heparin. The latter is carefully withdrawn as the actions of coumadin reach an equilibrium. Even when treatment with coumadin is in a steady state, it is sensitive to changes in vitamin K intake in the diet and it has many drug interactions. Consequently, careful monitoring of the patient's blood coagulation parameters is necessary to minimize the bleeding risk. Thus it has been recognized for some time that direct thrombin or fXa inhibitors which have a rapid onset of action and predictable pharmacodynamics would be safer and easier to use alternatives to coumadin.

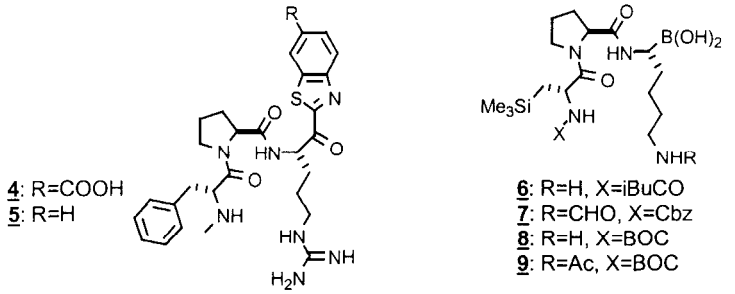
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THROMBIN INHIBITORS

Covalent Peptide Derivatives - In the past year, a few research groups have published work on covalent thrombin inhibitors related to the small molecule synthetic tripeptide arginine aldehyde derivatives. A rationale for the improvement in selectivity for thrombin over trypsin and fXa, on changing the P1 group from an arginine to an amidinopiperidine in thiazolo ketone derivatives **1** and **2**, was reported (6). After detailed analysis of the crystal structures of **1** and **2** bound to both thrombin and trypsin, it was concluded that the improved selectivity of the amidinopiperidine **2** was due to its interaction with residue 192. In thrombin this is a glutamic acid, whereas in trypsin (and fXa) it is a glutamine. Since a similar difference in binding to fXa is seen with these two inhibitors, the authors suggest that this effect might be general for other inhibitors of trypsin-like serine proteases. The efficacy of a similar bicyclic lactam derived thrombin inhibitor **3** was also described (7). Deletion of the serine trap and addition of a second phenyl group at P3 to compensate for the loss of potency gave a compound with similar properties to **3**, but with faster binding kinetics (see Non-Covalent section below). This series, both covalent and non-covalent, had poor oral bioavailability in the rat.



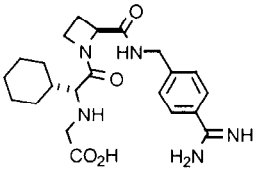
The X-ray crystal structure of benzothiazole carboxylic acid derivative RWJ-51438 (**4**) bound to thrombin was reported (8). It showed the heterocycle occupying the S1' site with an edge to face contact with the indole of Trp-60D similar to the bound structure of parent compound RWJ-50353 (**5**). However the carboxyl group of **4** is able to form an additional salt bridge to the side chain of Lys-60F. Compound **5** shows dose dependent efficacy after iv administration in a canine arterial thrombosis model and in a rabbit deep vein thrombosis model (9). The APTT returned to baseline 30 minutes post dose in the dog indicating rapid clearance of **5** from the plasma.



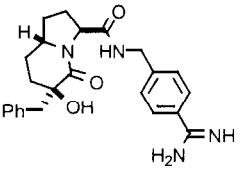
Crystal structures of boronic acid derivatives **6** and **7** were reported (10). Compound **6** binds in the expected fashion with the amino side chain stretching into S1 to form a salt bridge with Asp-189. In contrast, the formamide side chain of compound **7** reaches out of the pocket, making a pair of hydrogen bonds to Gly-219. Interestingly, amine **8** and acetamide **9** have similar Ki's and the authors attribute this to additional hydrophobic interactions between the N-terminal Boc group and the amide methyl group, (**9** adopting a pseudomacrocyclic binding conformation), which compensates for the loss of the salt bridge to Asp189 in **8**, assuming they bind like **6** and **7** respectively).

Non-Covalent Peptide Derivatives - Members of this class are the farthest advanced in development as orally available anticoagulants. Melagatran (**10**) and its oral prodrug H 376/95 are well advanced in clinical trials and preliminary results from the METHRO II dose-response study comparing them with the LMWH daltaparin as thromboembolic prophylaxis after total hip or total knee replacement were reported (11). The patients received either subcutaneous daltaparin or subcutaneous melagatran followed by H 376/95 bid. Dose dependent efficacy was seen with melagatran/H 376/95 with superior efficacy to daltaparin at the highest dose.

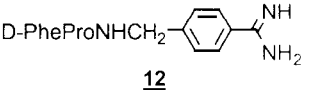
There have been a couple of reports on modifications to the P2 region of this inhibitor class. Use of cyclopentane and cyclopentene dicarboxamide proline isosteres for the P2 residue in melagatran led to a pronounced drop in potency as the amide link between P2 and P3 results in unfavorable interactions with the enzyme (12). The synthesis of a constrained indolizidinone mimic **11** of known D-Phe-Pro derivative **12** was reported, although **11** proved less potent than the peptide (13).



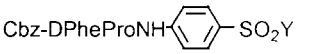
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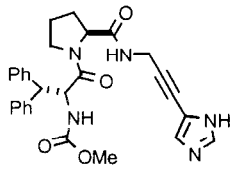
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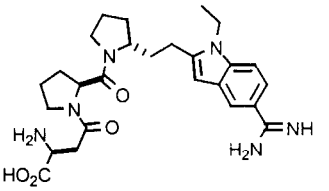
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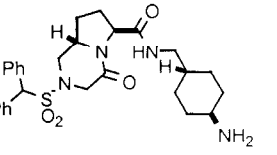
13: Y= $NC(NH_2)_2$
14: Y= $NC(NH_2)OMe$
15: Y= $NHNC(NH_2)_2$



16



17

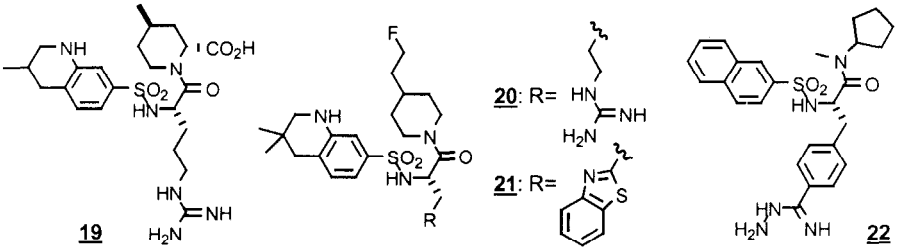


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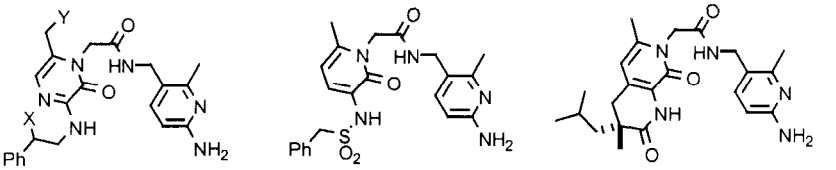
Other groups have continued the search for less basic P1 groups. In an extensive SAR investigation of new, moderately basic sulfonylguanidines, sulfonylaminoguanidines and sulfonyl-O-methylisoureas P1 groups, peptide derivatives **13**, **14** and **15** were reported to be roughly equipotent with Ki's from 11 to 16 nM (14). A series of mildly basic P1 heterocyclic derivatives in which the P1 group was linked to P2 using a propargyl amide were reported (15). Imidazole was the best P1 group examined and compound **16** was orally bioavailable in rats (F=58%), although it showed only moderate efficacy in a rat model of venous thrombosis.

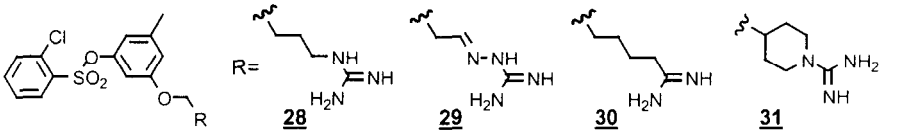
There have been other reports on the efficacy of peptide derived inhibitors. BSF 208791, a 'D-Phe-Pro-Arg type' inhibitor (no structure given) was shown to be efficacious in a rabbit jugular vein thrombosis model after oral administration (16) and AT-1362 (**17**) was shown to be efficacious in a rat venous thrombosis model after oral administration (17), although the oral bioavailability was modest (F=11%). The efficacy of bicyclic lactam derived thrombin inhibitors such as **18**, related to covalent inhibitor **3** (see Covalent section above) in rat models of arterial and venous thrombosis was described (7).

N-α-Tosylarginine Methyl Ester Derived Thrombin Inhibitors - A class of non-covalent thrombin inhibitors including NAPAP, argatroban (**19**) and napsagatran have their origins in the simple arginine derivative N-α-tosylarginine methyl ester (TAME). Argatroban is approved for marketing in the U.S. as an iv administered treatment for coronary heart disease in patients who develop heparin induced thrombocytopenia. Work on developing orally bioavailable argatroban analogs with less polar replacements for the guanidine side chain was reported (18,19). For example replacing the guanidine of CGH728 (**20**, Ki=6nM) with a benzothiazole gave CGH752 (**21**, Ki=26nM)(18). Further preclinical evaluation of CI-1028 (LB30057, **22**) demonstrated dose dependent efficacy after oral administration in a dog model of arterial and venous thrombosis (19).



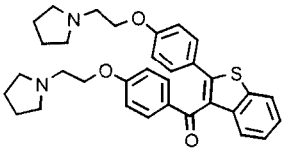
Pyridinone and Pyrazinone Acetamide Peptidomimetics - Further work on the pyridinone and pyrazinone acetamide class of thrombin inhibitors has been reported. A multi-kilogram synthesis of the core of orally bioavailable pyrazinone acetamide inhibitor L-375,378 (**23**) was developed (20) and in preclinical work on **23**, hydroxymethyl metabolite **24** was isolated from rats (21). A quantitative method for the simultaneous detection of **23**, **24** and benzylic alcohol metabolite **25** in plasma was developed (22). Finally, the first examples of conformationally constrained bicyclic analogs of pyridinone acetamide thrombin inhibitor L-374,087 (**26**) were reported (23). Fused lactam **27**, for example, is potent (Ki=0.24 nM) and is well absorbed in dogs after oral administration and has a half life of 90 minutes.



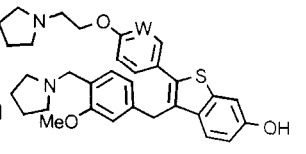


1,3,5-Trisubstituted Benzene Derivatives - One segment of a class of thrombin inhibitors which do not form the usual hydrogen bond array to the thrombin peptide backbone is made up of 1,3,5-Trisubstituted benzene derivatives. In recent work on this series, the guanidine group of sulfonate **28** was replaced with a less basic amidinohydrazone in order to improve the oral activity of the series (24). Compound **29** is orally bioavailable in dogs (F=23%) and rabbits (F=60%) and it is slightly more potent than **28**. X-ray crystallographic analysis of **28** and **29** bound in the active site

show their binding modes are similar. Replacement of the guanidine of **28** with amidine derivatives was less successful, the best being **30** which is five-fold less active than **28** (25). The SAR about the P2 and P3 groups of amidinopiperidine **31** showed that methyl is clearly optimal in P2 and that changes to the *ortho*-substituent in P3 is better tolerated than substitution at the *meta* or *para*-positions (26).



32

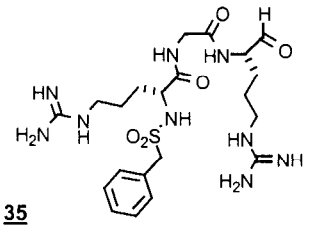


33: W=CH
34: W=N

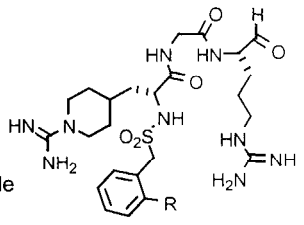
2,3-Disubstituted Benzothiophenes - Benzothiophene derivatives derived from screening lead **32** comprise another series of thrombin inhibitors which have reduced capacity for hydrogen bonding to the enzyme. A detailed account of the investigations in to the SAR of this series was reported (27). Compound **32** was optimized by contracting the link to the pyrrolidine that binds in the distal pocket of the enzyme. Compounds **33** and **34** were found to be moderately efficacious in a rat model of thrombosis (27). However, there was evidence of extensive partitioning into tissues *in vivo* with these compounds and this was used to explain the drop in efficacy compared to a peptide aldehyde of similar potency. With a single methylene, morpholine was an equipotent replacement for pyrrolidine (28). A full description of the X-ray crystal structure of four members of the benzothiophene series, including **32**, was reported (29)(see reference 30 for the preliminary communication). Finally, examination of a number of heterocyclic replacements for the benzothiophene showed that 1,2-disubstituted indole is preferred over azaindole and benzimidazole (31).

FACTOR Xa INHIBITORS

Substrate Peptide Derived Covalent Inhibitors - Two groups have reported the development of potent covalent inhibitors of fXa which are derived from small peptide substrates of the enzyme. In an exploration of the SAR at the P2-4 positions of arginine aldehyde based inhibitors, glycine was preferred over proline at P2 since it had improved selectivity versus thrombin (32). D-Arg was the best P3 group compared to a variety of other straight chain basic amines, and benzyl sulfonamide was the best P4 group examined. Compound **35** was equipotent (IC_{50}) against fXa



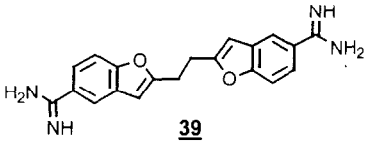
35



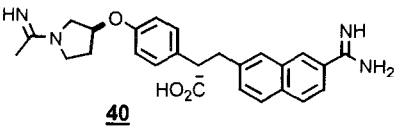
36: R=H
37: R=CO_2Me
38: R=CO_2H

and prothrombinase, and was 3300 fold selective versus thrombin. Separately it was reported that the activity of **35** was 4 fold greater against fXa than prothrombinase (33). A series of constrained arginine P3 derivatives were prepared and it was found that D-configured 3 and 4-amidinopiperidinyl alanine and 4-amidinopiperidinyl glycine were all potent D-Arg replacements (33). After iv dosing *in vivo* (species not given) compound **36** caused a substantial 'blood pressure/MAP effect' which was attenuated in the dosing of ester **37** and abolished with the corresponding acid **38**.

Bis-Arylamidine Related Inhibitors - There is a growing body of work on fXa inhibitors which draws on the initial studies on bis-(amidinoaryl) inhibitors of fXa or thrombin such as **39**. Optimization of the basic ends of **39** and inclusion of a carboxylic acid substituent on the central ethylene link gave DX-9065a (**40**, Ki=41 nM). Results from Phase I clinical trial with **40** as an iv agent have been reviewed (5). It was reported that **40** is cleared intact after iv administration in man, principally in the urine, with a half life of almost 7 hours (34).

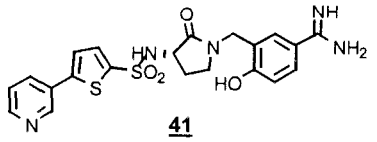


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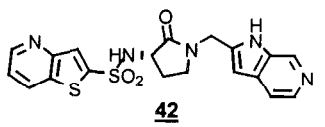


40

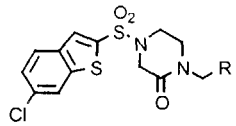
RPR 130737 (**41**), a 3-aminopyrrolidinone, was extensively characterized in vitro and is a potent and selective fXa inhibitor (35). In order to improve the oral activity of this series, libraries of 4-aminopyridine and azaindole derivatives were prepared as less basic replacements for the benzamidine (36). The X-ray crystal structure of one of these, 6-azaindole **42** (Ki=18 nM), bound to fXa was reported (37). The thienopyridine occupies the aryl binding site, the pyrrolidinone carbonyl oxygen atom forms a hydrogen bond to the NH of Gly-219 and the azaindole occupies S1, binding to the carboxylate of Asp-189 via a water bridge. An additional hydrogen bond is formed from the azaindole NH to the carbonyl of Gly-219. This same paper describes the crystal structures of aminoisoquinoline analogs of **42** bound to fXa and a thienopyridine analog of **41** bound to trypsin. In a related series, a piperazinone isomer of the 3-aminopyrrolidinone was used as the central scaffold to give potent achiral compound **43**, Ki=1.3 nM (38). In order to find a less basic P1 derivative in this series, a variety of heterocycles were attached to the piperazinone scaffold via a



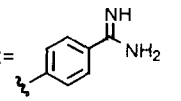
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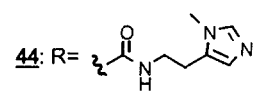
42



43: R=

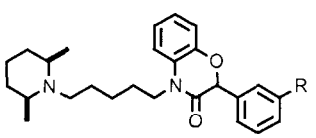


44: R=

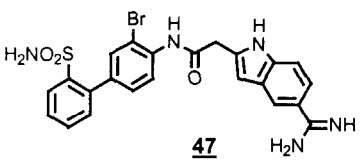


glycine residue. Of these, imidazole **44** (Ki=12 nM) was the most potent.

Screening lead **45** (IC_{50}=27 μM) was optimized at P1 using DX-9065a as a guide (39). Benzamidine **46** (IC_{50}=3 nM) was efficacious after iv administration in a rabbit venous thrombosis model and in a dog model of arterial and venous thrombosis. However, compound **46** had poor oral bioavailability in rats and dogs with a half life of less than 30 min. DX-9065a was also used as the starting point to prepare a series of inhibitors with amidino benzimidazole or indole P1 groups linked to a benzamidine aryl binding site element (40). Use of an amide link to a biphenylsulfonamide replacement for the benzamidine gave low nanomolar compounds such as SE 170 (**47**). Compound **47** was efficacious in a rabbit arterio-venous shunt thrombosis model. In another line of investigation, 3,5,5-trisubstituted

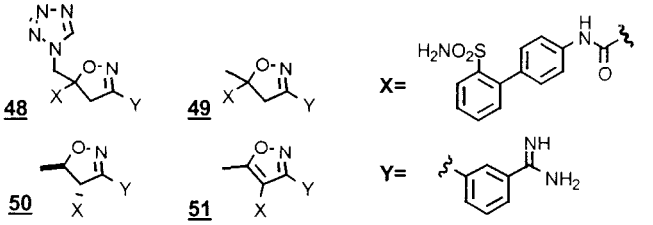


45: R=H
46: R=$CNHNH_2$

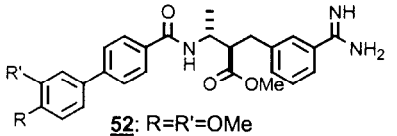


47

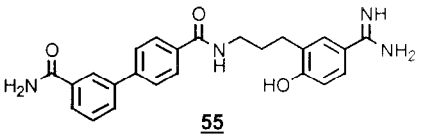
isoxazoline SK549 (**48**) was shown to be efficacious in the rabbit a-v shunt model (41) and in a rabbit carotid arterial thrombosis model (42). Compound **48** also had an antithrombotic effect after intraduodenal administration (41). Isomerization of closely related 3,5,5-trisubstituted isoxazoline **49** to the (trans)-3,4,5 isomer **50** improved the potency six-fold (43). In a further development, isoxazole **51** (SA862) was 12-fold more potent than **50** and was efficacious in the rabbit a-v shunt model.



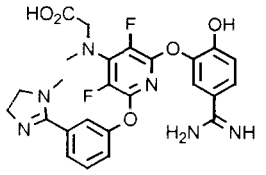
In another biaryl series derived from beta-amido ester screening leads, it was reported that RPR 208566 (**52**), was efficacious in a rat arterial thrombosis model (44). The X-ray crystal structures of a related member of the series, RPR 128515 (**53**), bound to fXa and to trypsin were reported (37). The two enzyme bound structures are similar, with the benzamidine forming a salt bridge with Asp-189 and the biphenyl occupying the aryl binding site. The fXa structure shows the amide forming two, opposed hydrogen bonds; from the carbonyl to Gly-219 and from the NH to an ordered water which itself hydrogen bonds to the OH of Tyr-99. A simplified, achiral link between the benzamidine S1 binding group and the lipophilic biaryl of inhibitor **54** (Ki=5 nM) was investigated (45). Use of the *para*-hydroxybenzamidine S1 binding group and a primary carboxamide substituent on the biaryl (compound **55**, Ki=7 nM) compensated for the loss in affinity for the enzyme upon deletion of the propyl chain substituents. Compound **55** was reported to have excellent selectivity against thrombin and trypsin.



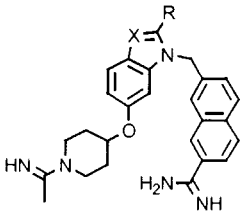
52: R=R'=OMe
53: R=H, R'=CH_2NH_2
54: R=R'=H



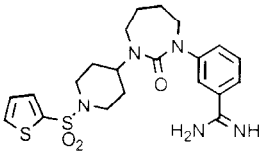
55



56



57: X=CH, R=CO_2Me
58: X=N, R=iPr

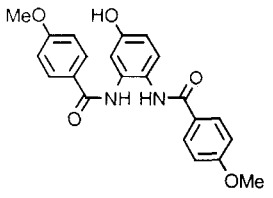


59

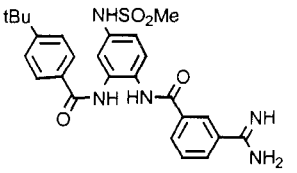
BABCH Derived Inhibitors - The X-ray crystal structure of orally bioavailable bis-amidine compound ZK-807834 (CI-1031, **56**) bound to fXa (46) was reported. The benzamidine binds in the S1 pocket and the imidazoline binds in the aryl binding site. The hydroxy substituent (pKa 6.8) hydrogen bonds to the side chain of Ser-195. Compound **56** was found to be efficacious in rabbit models of arterial and venous thrombosis (47). Use of an indole core in place of the pyridine and optimization of the basic ends of the molecule led to potent naphthamidine **57** (48). The aqueous solubility and potency was improved by switching to a benzimidazole template (compound **58** for example) (49). In a related, cyclic urea series, optimization of the

substituent which binds in the aryl binding pocket led to thiophenesulfonamide **59**, a compound which was efficacious in the rabbit a-v shunt thrombosis model (50).

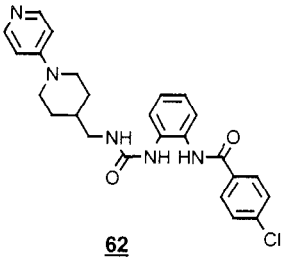
1,2-Phenylenediamine Derived Inhibitors - High throughput screening of a combinatorial library identified diamide **60** as a lead fXa inhibitor (51). Initial SAR investigation suggested that the 3-6 positions on the central aromatic ring were solvent exposed when bound in the active site, with one benzamide occupying S1 and the other S4 (51, 52). Incorporation of a benzamidine S1 binding element and a more lipophilic S4 binding group gave **61**, a potent compound (2xAPTT=720 nM) which was efficacious in rat and rabbit a-v shunt models of thrombosis (53). On the other hand, lengthening the link to the S4 binding group and use of a 4-piperidinylpyridine group gave an efficacious non-benzamidine containing inhibitor **62** which had similar efficacy to **61** in the rabbit a-v shunt model (54).



60



61



62

CONCLUSION

A number of groups continue to publish on reversible, covalent inhibitors of thrombin or fXa. However, the majority of the publications over the past year describe work on non-covalent inhibitors. Similar issues in inhibitor design are seen with both enzymes and the well established principle of incorporating mildly basic or non-basic arginine mimics in thrombin inhibitors to improve the oral bioavailability is now being extensively applied in the fXa field. The use of X-ray crystallography to determine the structure of thrombin/inhibitor complexes is routine. The same cannot be said for fXa yet, although the number of published co-crystal structures has grown sharply over the past year, and trypsin continues to be used as a fXa surrogate, when all else fails.

The development of orally active, direct inhibitors of thrombin and fXa as safer and easier to use alternatives to warfarin is a very active area research. There is no doubt as to the potential clinical efficacy of such compounds. The question remains as to which class of compound would have a lower bleeding risk in a given therapeutic setting.

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SECTION III. CANCER AND INFECTIOUS DISEASES

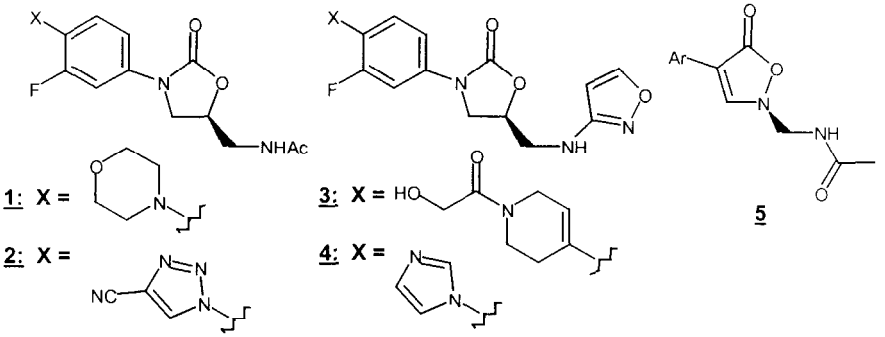
Editor: Jacob J. Plattner, Chiron Corporation
Emeryville, CA

Chapter 9. Recent Developments in Antibacterial Research

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Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT 06492

Introduction - Significant effort continues to be devoted to the research, development and marketing of novel antibacterial agents. The driving force for much of this effort is the threat imposed by increasing problems with resistance to known classes of agents in clinical use (1-7). Many promising compounds are being identified from previously described classes. In addition, new approaches based on adjunct therapy and novel target inhibition have emerged, in part due to the impact that genomics is starting to have on the antibacterial research field.

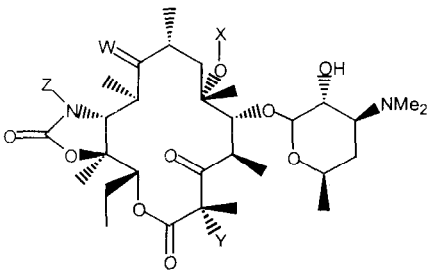
Oxazolidinones - Linezolid (**1**) was approved by the US FDA in April 2000 for treatment of patients with infections caused by Gram-positive bacteria (8-10). Approved indications include nosocomial pneumonia, community-acquired pneumonia, complicated and uncomplicated skin and skin structure infections and vancomycin-resistant enterococcus infections caused by methicillin resistant *Staphylococcus aureus* (MRSA). A review has appeared on patent activity in the oxazolidinone area (11). Activity continues to be vigorous in the search for improved oxazolidinone derivatives. Analogues bearing heterocyclic side-chains have shown a broadened spectrum of activity. For example, **2** has an MIC of 2 μg/mL against *Haemophilus influenzae* compared with 16 μg/mL for linezolid (12). Triazole **2** is also more potent than linezolid against Gram-positive pathogens. Analogues with novel amides in place of the N-acetate and novel substituents in place of the morpholine have been discovered through application of combinatorial chemistry (13-17). At best, these derivatives have activity comparable to linezolid. Analogues with novel heterocyclic replacements for the C-5 N-acetate group have potent *in vitro* and *in vivo* activity (18-20). Both **3** and **4** have excellent activity against Gram-positive organisms and good potency in a mouse thigh infection model. C-5 O-linked heterocyclic derivatives showed good *in vitro* antibacterial activity, but were generally less potent *in vivo* than the C-5 N-linked heterocyclic analogues (18-20). Isoxazolinones of type **5** have been reported as novel core analogues of oxazolidinones (21).

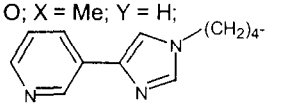


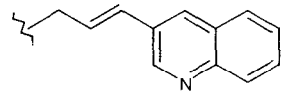
Macrolides - Ketolide derivatives of macrolides continue to dominate the macrolide development arena. Telithromycin (**6**) completed Phase III clinical trials in late 1999, and filing for US FDA approval occurred in December 1999 (22). Approval has been voluntarily delayed until 2001 for unspecified reasons. Telithromycin has broad-

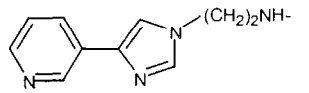
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spectrum activity, including activity against erythromycin-resistant Gram-positive pathogens (22, 23). ABT-773 (**7**), which bears a 6-*O*-substituted side chain and a C11-C12 carbamate, is a novel ketolide antibiotic in Phase II trials (24, 25). ABT-773 has been reported to have broad-spectrum *in vitro* activity. ABT-773 is designed to cover *erm*-containing macrolide-resistant *Streptococcus pneumoniae* through a combination of tighter ribosomal binding, accumulation at a faster rate in bacteria than erythromycin, ability to bind methylated ribosomes, and ability to accumulate in bacteria with an efflux-resistant phenotype. The animal pharmacological profile and Phase I clinical data suggests that ABT-773 is a safe BID (or QD) ketolide. CP-654743 (**8**) is a novel C-2-fluoro-ketolide with *in vitro* activity against typical respiratory pathogens and *in vivo* efficacy comparable to telithromycin (26, 27). Patent activity in the macrolide field has been recently reviewed (28).

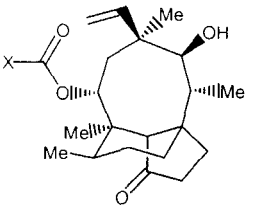


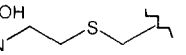
6: W = O; X = Me; Y = H;
Z = 

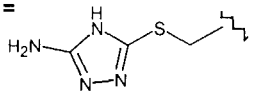
7: W = O; Y = H; Z = H;
X = 

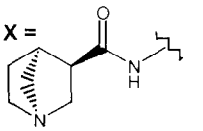
8: W = N-OMe; X = Me; Y = F;
Z = 

Pleuromutilins - The pleuromutilins are an older antibiotic class that have recently been reinvestigated for their potential as antibacterial agents (29). Pleuromutilins selectively inhibit bacterial protein synthesis by binding to the 70S subunit of the bacterial ribosome; inhibition of protein synthesis occurs at an early point in the elongation cycle. Pleuromutilin (**9**) itself was first isolated in the 1950s. In the 1970s, tiamulin (**10**) was identified for use as therapeutic agent for veterinary use and azamulin (**11**) was investigated for human therapy. Azamulin has excellent potency against Gram-positive and some Gram-negative bacteria, however development was discontinued after Phase I trials. The main liabilities in the pleuromutilin class are susceptibility to metabolism, moderate bioavailability, and potent cytochrome P450 inhibition for some derivatives (e.g., **10** is a nanomolar inhibitor of the 3A4 CyP isozyme). SAR studies have shown that the C-14 position is most amenable to modification with retention of biological activity. Semi-synthetic analogue work in the 1970s showed that S-linked derivatives had excellent potency. More recently, carbamoyl-linked C-14 analogues have shown promising activity and improved bio-availability (30, 31). SB-264128 (**12**) has MICs of 0.25 μg/mL against *S. pneumoniae* and *H. influenzae*, along with good oral efficacy in respiratory infection models. Carbamate **12** was unsuitable for development due to myocardial effects in animals and an inadequate therapeutic ratio (29). However, the myocardial effect is not found in closely related analogues, indicating that the toxicity is not class-related.



9: X = CH_2OH
10: X = 

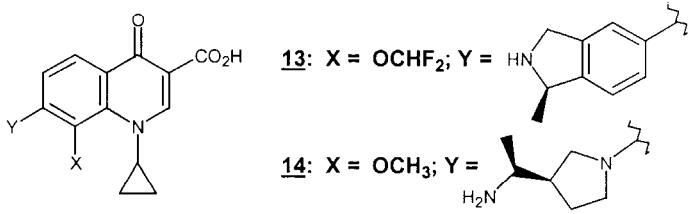
11: X = 

12: X = 

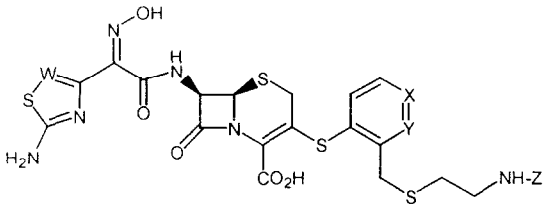
Quinolones - Several excellent updates on novel quinolones were published (32, 33). Trovafloxacin, which was approved by the FDA and launched in early 1998, was determined to be associated with liver toxicity and was restricted in use in 1999. Moxifloxacin (formerly known as Bay 12-8039), a broad spectrum 8-methoxy fluoroquinolone, gained US FDA approval in 1999 for oral treatment of sinusitis, acute bacterial exacerbation of chronic bronchitis, and community-acquired pneumonia (34, 35). Gatifloxacin, another broad spectrum 8-methoxy fluoroquinolone, gained US FDA approval for 8 indications (community-acquired pneumonia, acute bacterial exacerbation of chronic bronchitis, acute sinusitus, uncomplicated urinary tract infections, complicated urinary tract infections, pyelonephritis, and uncomplicated gonococcal infections) and was launched in December, 1999 in oral and iv forms (35-37). Gemifloxacin (SB-265805, LB-20304a) completed clinical trials and was submitted for US FDA approval in late 1999, but was issued a non-approval letter in December, 2000 (34).

BMS-284756 (**13**), the first developmental candidate lacking the classical C-6 fluorine substituent, is in Phase II/III trials. This novel quinolone is extremely active *in vitro* against Gram-positive pathogens including penicillin-sensitive and -resistant *S. pneumoniae* (38-42), and most staphylococci (41-43). BMS-284756 is active *in vivo* in various animal models and *in vitro* pharmacokinetic/pharmacodynamic models (44-48), is predicted to have lower arthrotoxicity than other quinolones (49), and has excellent anti-anaerobic activity *in vitro* (50). BMS-284756 has dual target inhibition activity, reduced frequency of resistance of emergence of mutants *in vitro*, activity against quinolone-resistant Gram-positive mutants (43, 51-54), and selectivity against its human homologue topoisomerase (52, 55). BMS-284756 has an excellent safety profile based on Phase I safety data reports (56, 57).

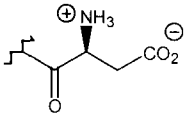
Quinolone derivatives that lack fluorine anywhere in the molecule have been reported (non-fluorinated quinolones or NFQs). Compounds such as **14** have been reported to have varying *in vitro* activities against clinical pathogens, but overall *in vitro* antibacterial activities matching those of their fluorinated counterparts (58-63). **14** exhibits broad-spectrum *in vitro* activity (58-60) and targets both DNA gyrase and topoisomerase IV (61). NFQs maintain *in vitro* activity in the presence of many mutations that confer resistance to fluoroquinolones (62). In addition to targeting DNA synthesis, NFQs inhibit RNA synthesis more than other quinolones (63).



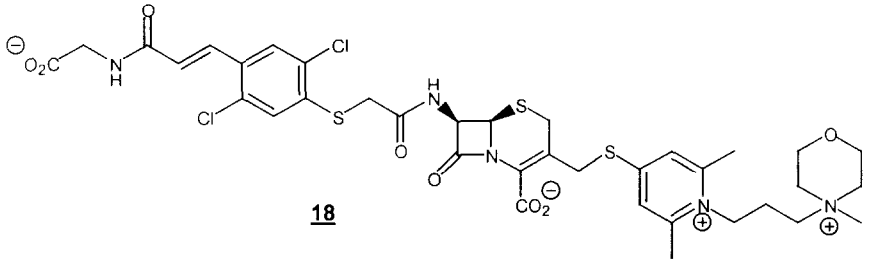
Cephalosporins - MC-02,479 (**15**) is a novel parenteral cephalosporin derivative that is currently in Phase I clinical trials (64, 65). This compound has good activity against a range of Gram-positive bacteria, including MRSA (MIC_{90} = 2 μg/mL). Efforts to further optimize this compound have focused on improvements in solubility (66-68) and pharmacokinetic properties (69-72). MC-04,546 (**16**) was identified as a candidate with greater serum stability, lower clearance, and improved *in vivo* potency relative to **15** (69-72). Although the water solubility of **16** was good (~4 mg/mL at pH 7), significant improvements were obtained by attaching a prodrug moiety, as exemplified by the aspartyl amide **17**, which has a water solubility greater than 20 mg/mL at neutral pH (73-75). Prodrug **17** was rapidly cleaved to **16** *in vivo* and proved efficacious in murine infection models.



15: W = C-Cl; X = N; Y = CH; Z = H
16: W = N; X = CH; Y = N; Z = H
17: W = N; X = CH; Y = N;
Z =

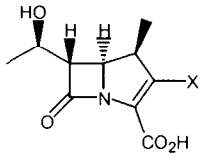


BMS-247243 (**18**) is a novel parenteral cephalosporin derivative with excellent activity against Gram-positive bacteria (76-82). BMS-247243 has good potency against MRSA, with an MIC_{90} of 4 μg/mL. This compound was identified from a series of analogues bearing lipophilic substituents at the C-7 position. Introduction of the additional carboxylate on the C-7 side-chain gave improved solubility and safety in preclinical testing. BMS-247243 is efficacious in a variety of infection models.

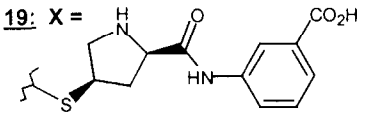


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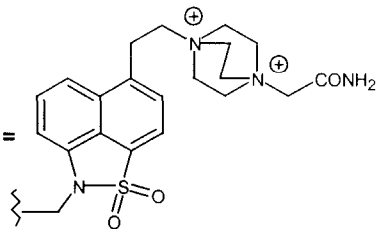
Carbapenems - Research efforts in the carbapenem field have focused both on improvements of parenteral agents and prodrugs for oral use (83). Among the most advanced parenteral carbapenems in development is MK-826 (**19**), a broad-spectrum parenteral derivative with a significant pharmacokinetic advantage over other carbapenems in that it can be administered once daily (83, 84). The application for US FDA approval of MK-826 was submitted in late 2000. Efforts to confer anti-MRSA activity to the carbapenem class have resulted in the identification of L-786,392 (**20**), which bears unique methylenesulfonamide substituent with a remote bis-quaternary group at the C-2 position (85-87). Earlier derivatives in this series that lacked the releasable substituent were believed to cause immune-based toxicity due to non-specific protein acylation, followed by immune recognition and response to the appended hapten.



19: X =



20: X =



Miscellaneous Agents - A number of complex natural products and semi-synthetic natural products are progressing in clinical and preclinical studies as parenteral agents for the treatment of serious Gram-positive infections, particularly those caused by resistant organisms. Daptomycin (Cidecin), a bactericidal lipopeptide, is in Phase III clinical trials (88, 89). NDA regulatory filing is expected in 2002. The safety profile for daptomycin has been optimized by use of once-daily dosing. The glycopeptide LY-333328 (Oritavancin) has also advanced to Phase III trials (90, 91).

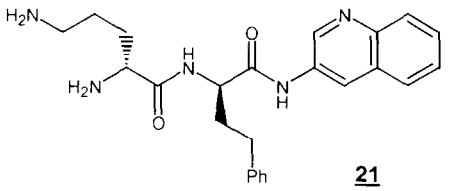
A noteworthy feature of LY-333328 is its activity against vancomycin- and teicoplanin-resistant enterococci. BI-397 (V-glycopeptide) is a semi-synthetic glycopeptide in clinical development (92, 93). BI-397 has been well tolerated in Phase I trials and has shown an exceptionally long terminal half-life of 174 h in humans. Ziracin, an oligosaccharide antibacterial agent, reached Phase III clinical trials before being discontinued due to a lack of a sufficient safety window (94, 95). The thiazolyl peptide GE-2270 has been the subject of preclinical work to improve solubility while retaining the excellent Gram-positive activity of the parent compound (96, 97). Analogues with solubility improved by 100-fold or more were reported; unfortunately these derivatives were less potent than GE-2270. Synthetic analogues of the glycolipid moenomycin have been studied in an attempt to identify novel agents with antibacterial activity and potential for oral administration (98-100). Through a combinatorial library approach, disaccharides bearing novel side-chains were identified as potent inhibitors of cell-wall biosynthesis with moderate antibacterial activity. The glycylcycline GAR-936, which is a 9-t-butylglycylamido of minocycline, is in Phase II clinical trials (101, 102).

GAR-936 has broad-spectrum activity, including activity against tetracycline-resistant bacteria and multiply-resistant Gram-positive organisms. Based on laboratory studies and clinical trial data, resistance development is expected to be slow. GAR-936 has a half-life of 36 h in humans.

ADJUNCT THERAPY AGENTS

β-Lactamase Inhibitors - Development of new β-lactamase inhibitors is of great interest due to the increasing importance of serine- and metallo-β-lactamases in causing resistance to β-lactam antibiotics (103-105). Classical β-lactamase inhibitors such as clavulinic acid, sulbactam and tazobactam are primarily active against class A serine-based β-lactamases, but lack activity against class C serine-based and class D metallo enzymes. 7-Alkylidene cephem sulfones have been reported to inhibit both class A and C enzymes with IC_{50}s in the low nanomolar range (106, 107). A series of mono-bactam derivatives that rapidly acylate the active site serine of class A enzymes have been described (108, 109). These compounds do not appear to acylate class C β-lactamases, but are still competitive inhibitors of these enzymes. Non-β-lactam inhibitors of class A and C β-lactamases have been reported, including rhodanine-based class C β-lactamase inhibitors and boronate-based dual class A/C inhibitors (110, 111). In the area of metallo-β-lactamase inhibition, thiazolidine and proline mercaptocarboxylates and succinic acid derived inhibitors have been reported(112, 113).

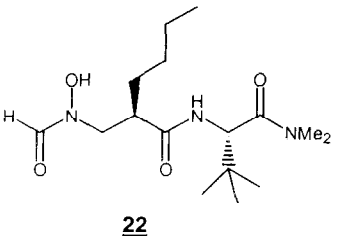
Efflux Pump Inhibitors - Efflux pumps play a critical role in resistance development for many classes of antibiotics (114). The potential for adjunct therapy with efflux inhibitors has been validated with the demonstration of potentiation of antimicrobial activity using bacterial and fungal efflux inhibitors (115-119). *In vivo* potentiation has been demonstrated for levofloxacin activity with the bacterial efflux inhibitor MC-02,595 (**21**) in the treatment of infections caused by *Pseudomonas aeruginosa* strains having various Mex pump expression levels and a *gyr*A mutation (120). Several new patents have issued in support of microbial efflux pump inhibitors (121-125). Efforts continue industry-wide to identify both broad spectrum efflux pump inhibitors and narrow spectrum (microorganism and/or drug-specific) efflux pump inhibitors such as those shown to potentiate levofloxacin activity against *P. aeruginosa* (126-128). A series of Nor A efflux pump inhibitors have been reported as being more potent and less toxic than reserpine, potentiating the anti-*S. aureus* activity of ciprofloxacin (129). A compound that reverses efflux-mediated tetracycline resistance, primarily in Gram-negative bacteria has been reported (130).



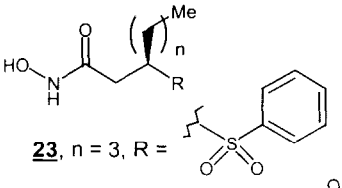
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INHIBITORS OF NOVEL TARGETS

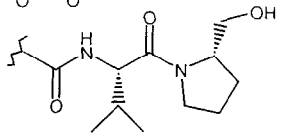
Efforts in microbial genomics are beginning to impact the discovery of novel antimicrobials (131), particularly in the search for small molecule, non-natural product based agents. A bacterial target that has attracted a great deal of attention is peptide deformylase (PDF), a metalloenzyme involved in removal of a formyl group from growing polypeptides (132). Several groups have reported hydroxamic acid derivatives as potent PDF inhibitors, including **22**, **23**, and **24** (133-135). Other classes of PDF inhibitors have been reported (136-140). **22** has an IC_{50} of 7 nM against PDF, along with bacteriostatic activity against Gram-positive and Gram-negative bacteria (MICs = 4-32 μg/mL), and oral activity in a murine systemic S. *aureus* infection model. A potential concern with PDF as a target is that formylation is not essential for initiation of protein synthesis in all bacteria (141). Resistance to inhibition occurs rapidly as a result of mutations in the formyltransferase gene, although bacteria bearing these mutations are reported to have a substantially reduced growth rate (142).



22



23, n = 3, R =



24, n = 4, R =

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Chapter 10. New Therapies for Parasitic Infection

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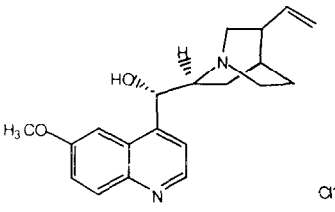
Introduction - There is no doubt that significant advancements in antiinfective therapy have improved the quality of life in highly developed nations. However, in underdeveloped countries, there exist major infectious diseases that account for a large portion of global morbidity. Some of these diseases have the potential to become a threat to those living in North America. Tuberculosis claims an estimated 2 million lives each year, and drug-resistant strains originally found in New York and Russia are now being identified in other locations. Malaria, African trypanosomiasis and leishmaniasis accounted for an additional 1,210,000 deaths in 1999, and estimates suggest that these numbers are rising rapidly (1). Malaria, trypanosomiasis and leishmaniasis are the focus of this chapter, but many other life-threatening parasitic diseases exist. Current therapies for parasitic infection are inadequate, especially in light of the emergence of drug-resistant parasitic strains. Many of the drugs currently used are toxic or non-efficacious, and there are no effective treatments for some parasitic diseases. Drug discovery efforts against the diseases mentioned above are limited, either because infected persons in underdeveloped areas cannot afford even a single course of therapy, or because the infected population is too small to justify the required research expenditures. Efforts to fight parasitic diseases in Third World nations are often hampered by economic issues and political turmoil. This virtually assures that the world's most impoverished people will continue to bear the major burden of parasitic disease. Clearly, there is a need for new antiinfective agents that are potent, non-toxic and inexpensive to manufacture. This report describes recent progress towards identifying suitable agents to treat parasitic diseases.

MALARIA

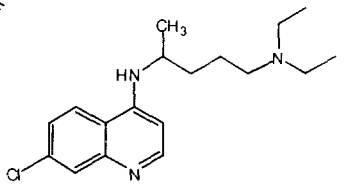
Background - Malaria is still one of the world's most deadly diseases, and affects approximately 400 million people worldwide. In Africa alone, more than one million children under the age of 5 die from malaria each year (2,3). Human infection can be caused by four distinct species of the protozoon *Plasmodium*, but *P. vivax* and *P. falciparum* account for more than 95% of malaria cases. Nearly all deaths caused by malaria are due to infection by *P. falciparum* (3,4). Malaria is transmitted by the bite of the female anopheles mosquito, at which time sporozoites of *P. falciparum* are discharged into the puncture wound. Sporozoites are then carried to the liver, where they enter hepatic mesenchymal cells and begin to grow. Lysis of the hepatocyte then releases the merozoite form of *P. falciparum,* which invades host red blood cells (RBC's), feeding on hemoglobin during the erythrocytic portion of its life cycle (5). In the RBC, the parasite expresses a number of polypeptide products that are exported to the surface of the RBC, rendering it antigenic. These peptides are products of the *var*, *rif*, and *clag* genes located on chromosome 2 and 3 of the *P. falciparum* genome, and allow the infected RBC's to adhere to vessel walls, and to accumulate in specific organs such as the brain (2,3). In order to escape the host immune system, the parasite regularly exchanges these peptides in a process called antigenic variation. Although *P. falciparum* has a complex biochemistry and life cycle, recent research has revealed a number of potential new drug targets (4).

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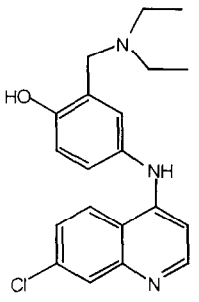
Synthetic Analogues Related to Quinine and Chloroquine. The cinchona alkaloid quinine, **1**, was the first compound to exhibit significant antimalarial activity. Subsequent studies produced the synthetic analogue chloroquine, **2**, which was initially an excellent treatment for malaria. However, the evolution of chloroquine-resistant strains of *P. falciparum* have rendered this drug virtually useless in certain areas of the world (6). To address this resistance, a number of Mannich bases of chloroquine were synthesized, producing the active quinoline analogues amodiaquine, **3** and tebuquine, **4**. These analogues are significantly more active against *Plasmodia*, but unfortunately, chronic toxicity limits their use. Raynes et al. have described a



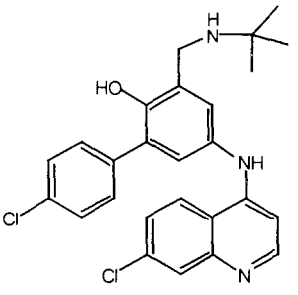
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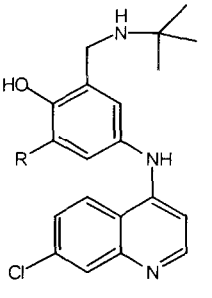
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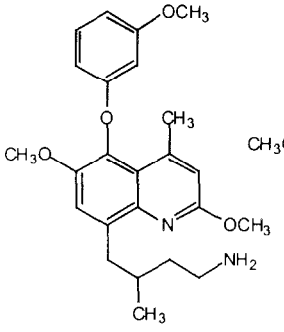


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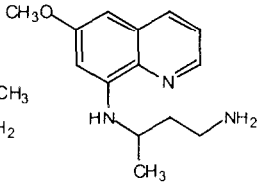


5 (R = propyl)
6 (R = isopropyl)

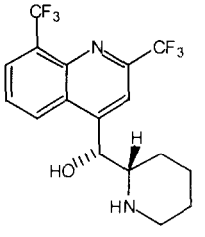
series of derivatives of **4** in which the diethylamino moiety has been replaced by a tert-butylamino group, a modification which reduces cross-resistance (6). The 5'-substituted analogues **5** and **6**, proved to be the most effective antimalarials in



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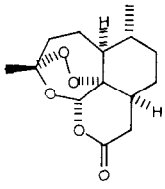
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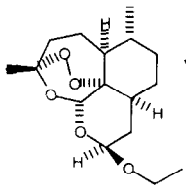
9

vitro, with IC_{50} values of 0.98 and 1.24 nM against *P. falciparum*, and favorable cross-resistance profiles. Other quinolines of interest include tafenoquine **7**, which is a second generation agent related to primoquine, **8** and mefoquine, **9**. Tafenoquine, also known as WR 238605, appears to be an effective prophylactic agent for the prevention of *P. falciparum* malaria (7).

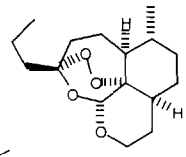
Artemisinin and Its Analogues. Perhaps the most promising advance in the treatment of malaria is the discovery of artemisinin, **10**, which is also known by the Chinese name qinghaosu, and the related compound arteether, **11**. These analogues are 1,2,4-trioxosesquiterpenes that produce oxidative stress in *P. falciparum*, and they are reduced by the organism in an Fe(II)-dependent process to



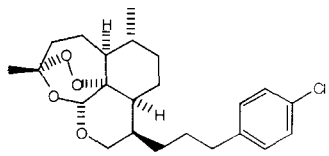
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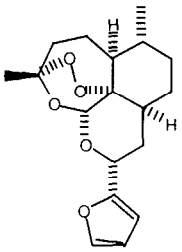


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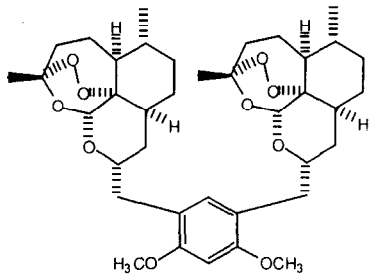


13

produce cytotoxic radical intermediates (8). Artemisinin itself is a potent antimalarial, with an IC_{50} of 7.3 nM against *P. falciparum*. In the 10-deoxyartemisinin series, replacement of the 3-methyl group with an n-propyl moiety, as in **12**, produced a 7-fold increase in activity with respect to artemisinin, while substitution at the 9 position produced a 70-fold increase (compound **13**) (9). Artemisinin and its derivatives have limited oral bioavailability, and are hydrolytically unstable, problems which have been addressed by the synthesis of analogues related to **14** and **15**, in which the 10 position has been functionalized. The semisynthetic analogue **14** is hydrolytically stable, and retains an IC_{50} value of 1.4 nM (10). It is also orally available, and is more potent *in vivo* than artemisinin.

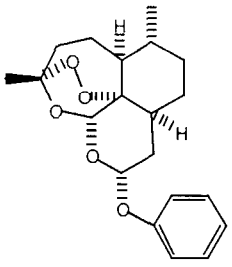


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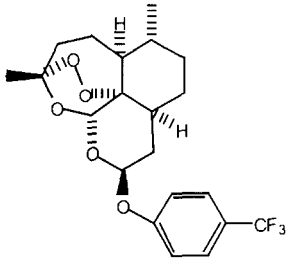


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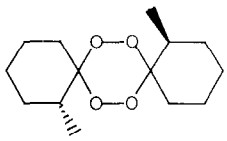
Artemisinin-derived trioxane dimers such as **15** are also stable to hydrolysis; **15** has an IC_{50} of 1.3 nM against cultured *P. falciparum* (11). In a series of C10-phenoxy analogues of artemisinin, excellent activity was retained, and the analogues were hydrolytically stable (12). Interestingly, the most active



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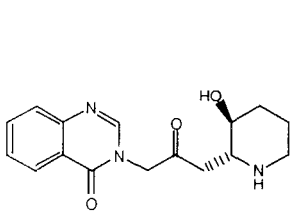


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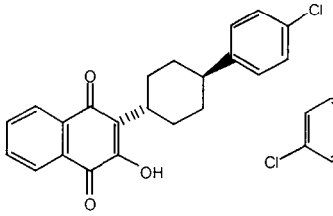
analogues were **16**, a C10α derivative, and **17**, a C10β derivative, with IC_{50} values of 2.61 and 3.90 nM, respectively. The promising antimalarial activity of artemisinin and its derivatives prompted the evaluation of dispiro-1,2,4,5-tetraoxanes such as **18**, WR148999. Compound **18** possesses antimalarial activity comparable to

artemisinin, but also shares the characteristics of being hydrolytically unstable and poorly bioavailable by oral administration (13).

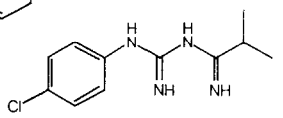
Miscellaneous Antimalarial Agents. A number of synthetic agents and natural product derivatives have been shown to possess antimalarial activity. The herbal natural product (+)-febrifugine, **19** (Chinese name: chang shan) and its derivatives possess significant antimalarial activity (14). Promising cure rates in humans have been attained by using atovaquone, **20** and proguanil, **21** in combination (15). The recently discovered phenanthrene halofantrine, **22**, has also shown significant antimalarial activity *in vitro* (7), and bisquinoline heteroalkanediamines such as **23** (IC_{50} = 1.2 nM) are effective against *P. falciparum in vitro* and *in vivo* (16). Potential



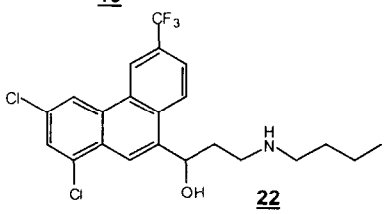
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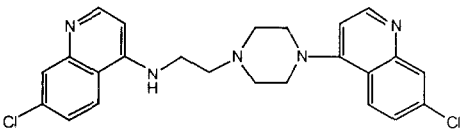
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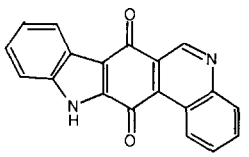
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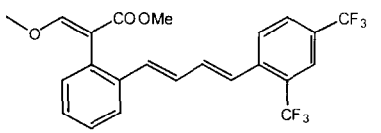
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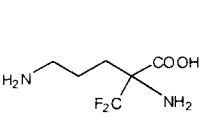
25

new pharmacophores for antimalarial lead optimization are typified by calothrixins such as **24**, which exhibit nanomolar IC_{50} values (17) and phenyl β-methoxyacrylates such as **25**, which has an *in vitro* IC_{50} of 0.06 ng/mL, and an *in vivo* effect at 100 mg/kg (18).

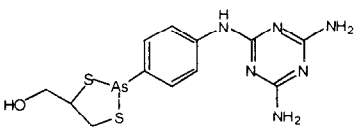
AFRICAN TRYPANOSOMIASIS AND CHAGAS' DISEASE

Background - Trypanosomiasis is caused by several members of the family *Trypanosomatidae* including *Trypanosoma brucei brucei* (a disease that affects livestock but not humans), *T. brucei gambiense* (West African trypanosomiasis) and *Trypanosoma brucei rhodesiense* (East African trypanosomiasis). In some areas where African sleeping sickness was virtually eradicated, the destruction of the health care system by war has allowed the disease to reappear in epidemic proportion. A variant of the African disease, known as Chagas' Disease, is caused by *T. cruzi*, and is endemic to South America. In 1999, more than 87,000 people died from trypanosomiasis, and the World Health Organization now estimates that 300,000 new cases are diagnosed per year. Like many parasitic organisms the cellular biochemistry of trypanosomes has not been fully elucidated. *T. b. gambiense* and *T. b. rhodesiense* are both transmitted by the bite of the tsetse fly, following which the flagellated trypomastigote form of the organism develops in the blood and lymphhatic system of the host. The first signs of early stage disease appear in the

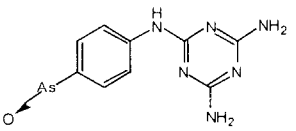
lymph nodes, however, late stage disease (initiated by invasion of the CNS) can only be confirmed by lumbar puncture. Early stage disease is usually treated effectively with suramin or pentamidine. However, diagnosis of early stage trypanosomiasis is difficult, especially in rural areas, and as such many patients progress to late-stage disease before seeking treatment. There are only two effective treatments for late-stage trypanosomiasis. The ornithine decarboxylase inhibitor eflornithine, **26**, has been shown to be curative in end stage infections caused by *T. b. gambiense*, but is ineffective against *T. b. rhodesiense* (19). Eflornithine is expensive to produce, and thus its availability in impoverished nations is limited. End stage trypanosomiasis is treated with melarsoprol, **27**, which is converted to the active metabolite melarsen oxide, **28** (20). Serious side effects include a 10% incidence of encephalopathy which is fatal 3-5% of patients. The situation is further complicated by the emergence of arsenic-resistant strains of *T. b. gambiense* and *T. b. rhodesiense*. *T. cruzi* is more difficult to treat, since this form of trypanosome is intracellular, and drugs used for the disease must pass through mammalian membranes and the blood-brain barrier to be effective. Benznidazole and nifurtimox have been used for early stage Chagas disease, but are ineffective in the progressive, chronic infection that ultimately causes cardiac failure. Nonetheless, trypanosomes possess a number of parasite-specific targets for drug design, which could facilitate the discovery of low cost, effective agents for trypanosomiasis. A comprehensive review of agents used to treat trypanosomiasis has recently been published (21).



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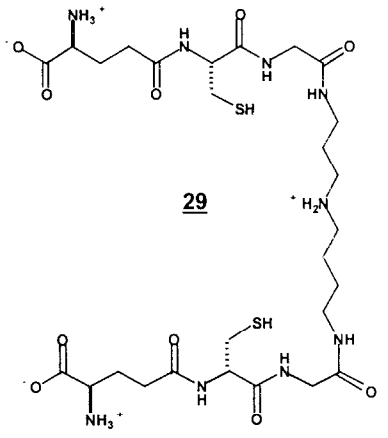


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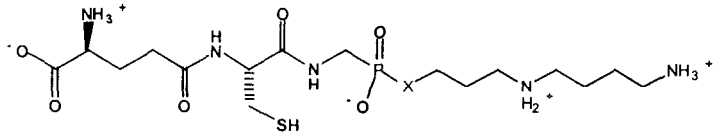
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Trypanosomal Biochemistry. Polyamine metabolism in African trypanosomes is similar to mammalian polyamine metabolism, in that the organism synthesizes putrescine and spermidine from ornithine (22), and trypanosomal forms of ornithine decarboxylase and spermidine synthase have been identified. However, these organisms do not produce spermine, but instead convert spermidine and two molecules of host-derived glutathione into trypanothione, **29**, which is used to protect the organism against oxidative stress. The arsenical drugs mentioned above act by forming a covalent complex with trypanothione, thus inactivating it and exposing the organism to oxidative damage. The formation of trypanothione is mediated by two ATP-dependent enzymes, glutathionylspermidine synthetase (GSPS) and trypanothione synthetase (TS). In addition, oxidized trypanothione must be reconverted to its reduced form by a third enzyme unique to the parasite, trypanothione reductase.



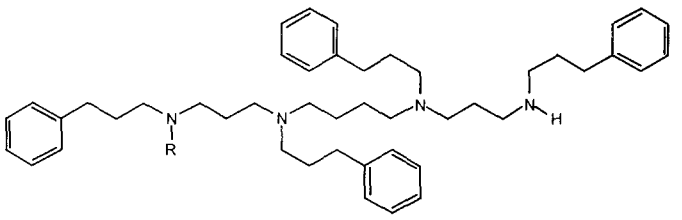
29

Agents Targeted to the Polyamine Pathway. A number of novel agents have been developed which target the trypanosomal polyamine biosynthetic pathway. The phosphate-based transition state analogues **30** and **31** were designed to mimic the tetrahedral transition state of GSPS (23, 24).These analogues inhibited GSPS from *Escherichia coli* with K_i values of 6.0 and 3.2 μM, respectively. In addition, **31** was



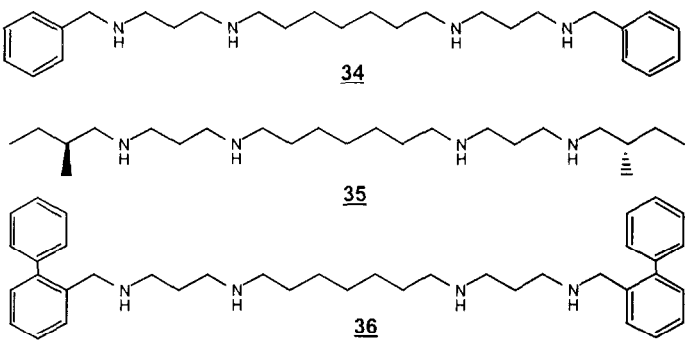
30 (X = O)
31 (X = CH_2)

shown to be a slow binding inhibitor of the enzyme, and formed an E-I* complex with a 410-fold higher affinity than the collisional E-I complex. A number of N-(3-phenylpropyl) substituted spermine analogues were recently reported that act as potent inhibitors of trypanothione reductase (25). Compounds **32** and **33** were the most potent inhibitors, with K_I values of 0.61 and 0.15 mM against trypanothione reductase from *T cruzi*. Compound **32** showed significant



32 (R = H)
33 (R = $CH_2CH_2CH_2$-Ph

antitrypanosomal activity *in vitro*, with IC_{50} values between 0.12 and 0.16 μM against 4 strains of trypanosomal clinical isolates, including the arsenic-resistant K 243-As-10-3 variant. An extended series of polyamine analogues based on the lead structure MDL 27695, 34, has recently been reported. Compound **34** was shown to possess significant antimalarial and antileishmanial activity *in vitro* and *in vivo*, and produced cures in murine models of malaria and leishmania (26 - 29). In 1996, **34** was found to possess antitrypanosomal activity as well (30), prompting the synthesis of the series of analogues mentioned above. Compounds **35** and **36** were both effective *in vitro* against the LAB 110, K 243, K 269 and K 243 As-10-3 strains of trypanosome (31, 32). The most impressive activity was found in the case of **35**, which inhibited the



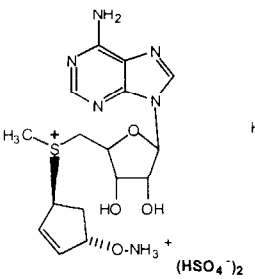
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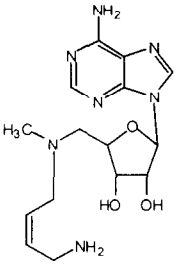
36

growth of K 243 with an IC_{50} of 40 nM, the same IC_{50} as melarsen oxide. Further, **35** inhibited growth in the K 243 As-10-3 arsenic resistant strain (IC_{50} = 165 nM), against which melarsoprol is inactive.

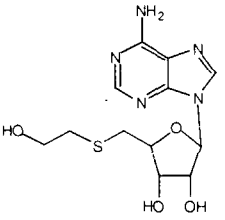
Trypanocides That Utilize a Unique Parasitic Nucleotide Transporter It has recently been shown that trypanosomes do not synthesize purines, but instead import them using two unusual adenosine transporters, P1 and P2 (33). The P1 transporter is responsible for importing adenosine and inosine, while the P2 receptor accepts a



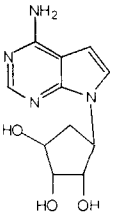
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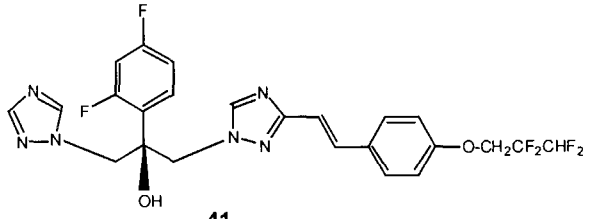
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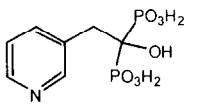
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number of substrates, including adenine, adenosine, suramin, pentamidine and arsenical drugs like melarsoprol, **27**. The P2 transporter is missing in arsenic-resistant strains. A number of nucleoside analogues have been synthesized that appear to act as substrates for the P1 or P2 transporter, that act by as yet unknown mechanisms. The *trans*-1S,4S isomer of **37** is an irreversible inactivator of mammalian and bacterial S-adenosylmethionine decarboxylase, and also acts as a trypanocide with an IC_{50} of 0.9 μM (34). Another inhibitor of this enzyme, AbeAdo **38**, was found to be a substrate for the P2 transporter in *T. b. rhodesiense*, and exhibited a nanomolar IC_{50} value. It was also curative for *T. b. rhodesiense* infections in mice (35). However, this drug has never been developed for therapeutic use. Nucleoside analogues related to HETA, **39** enter trypanosomes via the P2 and S-adenosylmethionine transport systems (36). HETA is concentrated 10-80-fold in trypanosomes, and kills the parasite by reducing protein methylation, producing *in vivo* in cures against a number of strains (37) The (+)-isomer of 7-deaza-noraristeromycin, **40**, appears to utilize the trypanosomal P1 system, and shows activity against *T. b. brucei* (IC_{50} = 0.16 μM) and the arsenic resistant K 243 As-10-3 strain (IC_{50} = 5.3 μM) (38).

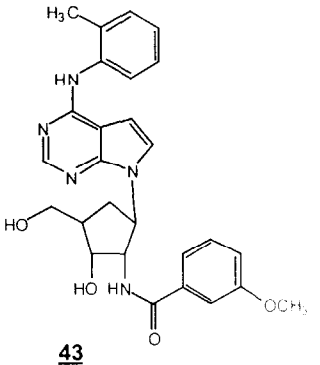
Miscellaneous antitrypanosomal agents. The *bis*-triazole D0870, **41**, inhibits the growth of *T. cruzi* epimastigotes (IC_{50} = 0.1 μM), and induces radical cures in murine models of the infection (MIC = 1-3 mM) (39). A series of *bis*-phosphonates related to risedronate, **42**, have also been shown to be effective against *T. brucei* and *T. cruzi*, and also showed low micromolar activity against *Leishmania donovani, P. falciparum* and *Toxoplasma gondii* (40). Both **41** and **42** appear to act by disrupting



41



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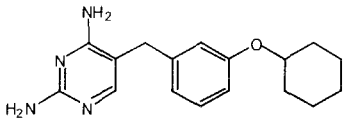


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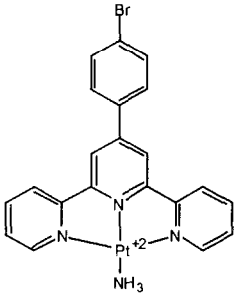
parasitic sterol biosynthesis. Finally, N^6-substituted adenosines related to **43** act as tight-binding inhibitors of trypanosomal glyceraldehyde-3-phosphate dehydrogenase, and inhibit growth of *T. brucei* and *T. cruzi* with an IC_{50} of 30 μM (41).

LEISHMANIASIS

Background Leishmaniasis can be caused by one of several strains of the Trypanosomatid *Leishmania*. The disease can be divided into three catagories: cutaneous, mucosal and visceral leishmaniasis. The first two catagories are diseases which, although potentially disfiguring, are generally not life threatening. However, visceral leishmaniasis, also called kala-azar, produces life threatening systemic infection if left untreated (42). Visceral leishmaniasis is caused by organisms of the *L. donovani* complex (*L. donovani, L. infantum* and *L. chagasi*). The promastigote form develops in the hindgut of 30 species of phlebotamine sandflies, and is transmitted to the host during a blood meal. The organism develops in the skin cells, spleen, liver or bone marrow of the host; patients experience anemia and cachexia, and ultimately succumb to the parasite. Although the disease is endemic to rural South America and Africa, it is encroaching on urban areas, and is also a risk for travelers in affected areas (e.g. Gulf War veterans).



44



45

Chemotherapy for Leishmaniasis. Because leishmania are biochemically similar to trypanosomes, many of the antitrypanosomals mentioned above are also effective against *L. donovani*. This is not universally true, however, due to the differences in tissue distribution between the parasites. The standard treatment for visceral leishmaniasis is potassium stibogluconate, a moderately toxic antimonial for which resistance is increasing. Pentamidine is also marginally effective. *L. donovani* has a nucleotide importer analogous to the trypanosomal P1 protein, but no analogues have been targeted to the transporter to date. The parasite also contains a trypanothione pathway, but the analogues above have not been evaluated against leishmania. The polyamine **34** has been shown to be an effective agent *in vitro* and *in vivo* (26, 27), and *bis*-phosphonates such as **42** are effective *in vitro* (40). Novel approaches include the specific dihydrofolate reductase inhibitor **44** (IC_{50} = 6 μM)(43), and (terpyridine)-platinum(II) complexes such as **45**, which are effective against *L. donovani* (100% inhibition at 1 μM), *T. cruzi* (65% inhibition at 1 μM) and *T. b. brucei* (100% inhibition at 30 nM)(44). Characterization of leishmanial membrane transporters (45) should result in identification of new targets for selective drug design. As parasitic biochemical processes are better understood, new targets for drug design will emerge. Although hampered by geographical and economic factors, there is hope that effective, non-toxic cures for parasitic infection will be found.

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Chapter 11. Growth Factor Receptor Kinases in Cancer

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Introduction – The search for new cancer chemotherapeutic agents continues to be an active area of research at many pharmaceutical and biopharmaceutical companies. The last ten years has seen a growth in the area of research around inhibitors of serine/threonine and tyrosine kinases as potential cancer therapeutics. Determining the role that these key signaling enzymes play in the proliferation and spread of cancers has been critical to the identification of new biological targets with new mechanisms of action.

Protein tyrosine kinases (PTKs) utilize adenosine triphosphate (ATP) to phosphorylate specific tyrosine residues within the sequence of functional proteins. In so doing, PTKs mediate the transmission of mitogenic signals and numerous other cellular events. One particular group of enzymes that has attracted a significant amount of interest has been the growth factor receptor tyrosine kinases (1, 2), specifically, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). The topic of this chapter will be ATP-competitive inhibitors of these tyrosine kinases. Other topics such as monoclonol antibodies (mAbs), soluble receptors and ribozymes will not be covered here; however, lead references are provided (3-6).

EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

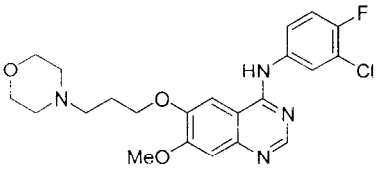
Intense interest has been placed on inhibitors of the EGFR (erb-B1), due to the involvement of growth factors in this gene family in the development and progression of cancer. These growth factors bind to the extracellular domain of the EGFR resulting in receptor dimerization and subsequent activation of the intracellular tyrosine kinase domain by the process of autophosphorylation on several, key tyrosine residues. Various transducing molecules bind to the newly formed high affinity binding sites (7). This cascade can be used to explain the activities of the other receptor tyrosine kinases (PDGFR, FGFR and VEGFR) to be discussed in this chapter.

Overexpression of members of the EGF family, such as transforming growth factor-alpha (TGF-α), and/or EGFR has been linked to poor prognosis in several human tumor types (8). For example, in breast cancer ~30% of tumors overexpress EGFR (erb-B1) or HER-2 (erb-B2) and 90% overexpress HER-3 (erb-B3). In patients with tumors that overexpress the erb-B receptors, poorer prognosis and decreased survival is observed compared to patients with tumors that are erb-B receptor negative (9, 10). Cancer cell lines that exhibit resistance to several, traditional cytotoxic agents have been found to also overexpress EGFR (11). Blockade of the TGF-α/EGFR autocrine pathway was seen as a potentially useful new therapeutic modality in the treatment of cancer (12-14). Towards this end, several ATP-competitive compounds that inhibit the ligand-induced autophosphorylation of the EGFR have been explored (15-20). Many of these agents have been tested *in vitro* and in preclinical tumor models in mice. The quinazoline nucleus has served as the template for many of these compounds.

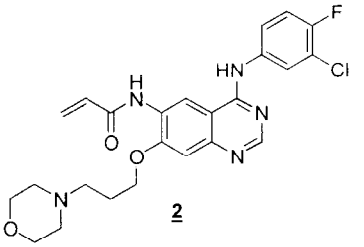
ZD-1839 (**1**) is a selective EGFR antagonist (IC_{50} = 23 nM) that has entered clinical trials (21, 22). ZD-1839 is a reversible, ATP-competitive inhibitor with a K_i = 2.1 nM against purified EGFRTK and is >80-fold selective for EGFR *versus* unrelated

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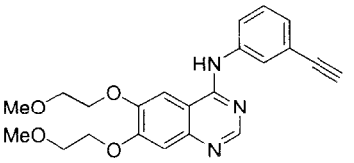
kinases. It has been demonstrated *in vivo* in A431 human epidermoid carcinoma tumor models in nude mice that **1**, dosed at 10 mg/kg/day p.o., reduced tumor growth rates by 50%. At 200 mg/kg/day, ZD-1839 caused tumor regression of 1.5 gram tumors to undetectable sizes within 2 weeks. Tumor growth was suppressed even after 4 months, as long as treatment was continued; however, if treatment with **1** was discontinued, regrowth usually occurred. Similar cytostatic effects were seen in several other human tumor xenograft models in mice, including A549 lung, LoVo colon and DU145 prostate carcinomas (2, 23).



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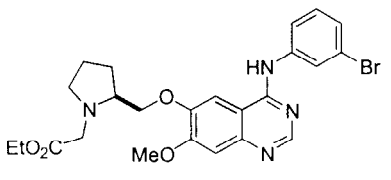
In combination studies with cytotoxic agents (cisplatin, paclitaxel, doxorubicin, etoposide, topotecan and raltitrexed), all doses of **1** exhibited supra-additive, growth inhibitory effects when tested in OVCAR-3 ovarian, GEO colon, MCF-10A Ha-*ras* and ZR-75-1 breast carcinoma mouse models. All of these cell lines are known to express functional EGFR and secrete high levels of TGF-α (24). It has been shown that there is a functional link between EGF receptor activation and several of the molecular mechanisms that are involved in angiogenesis, tumor cell invasion and metastasis (25). Blockade of the EGFR in several human cancer xenografts modulated the inhibition of the production of several angiogenic factors including TFG-α, VEGF, bFGF and interleukin-8 (26-30).

Clinically, **1** appears to be well tolerated. Phase I studies with **1** at dose levels of 150, 225, 300, 400, 600 and 800 mg/day p.o. in patients with incurable, solid, malignant tumors known to express EGFR, produced mild, drug-related side effects such as acneiform skin rash and diarrhea. Pharmacokinetic parameters have been reported and are dose proportional in patients following oral administration of 150-800 mg/day of **1**. The C_{max} ranged from 145-1902 ng/mL (n = 19) and the AUC_{0-24} ranged from 3102-39263 ng·hr/mL (n = 17) (22, 31). ZD-1839 is currently in Phase II/III trials in patients with non-small cell lung cancer (NSCLC) (22). The trial design is with 250 or 500 mg/day p.o. doses of **1** in combination with standard regimens of gemcitabine/cisplatin or paclitaxel/carboplatin. The primary endpoint is an increase in overall survival in patients receiving **1** compared with those receiving placebo. Secondary endpoints include improvement in disease-related symptoms, quality of life, safety and correlations between EGFR expression and survival. Patients with advanced NSCLC who have failed one or two previous chemotherapy regimens will be treated once daily with either 250 or 500 mg/kg doses of **1**. The primary endpoints are

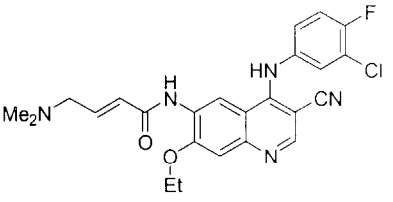
objective tumor response rate and disease-related symptom improvement, while secondary objectives include tolerability and quality of life assessments (22).

A related quinazoline, CI-1033 (**2**), has also entered the clinic (32). This compound differs from most ATP-competitive kinase inhibitors in that it is an irreversible inhibitor of the enzyme (2, 33). The thiol of Cys-773 of the EGF receptor adds to the acrylamide functionality of **2** in a Michael fashion. *In vitro*, **2** inhibits EGFR, erb-B2 and erb-B4 with IC_{50}s = 1.5 nM, 5 nM and 10 nM, respectively and exhibits >100-fold selectivity *versus* related tyrosine kinases (33, 34). The rationale for the inhibition of all of the members of the erb-B family of RTKs is based on the finding that heterodimers of these receptors are more transforming than are the homodimers. CI-1033 also inhibits erb-B2 autophosphorylation in MDA-MB-453 cells with an IC_{50} = 9 nM (33). In A431 tumor xenografts, **2** showed potent antitumor activity at an optimal dose of 5 mg/kg/day p.o. Tumor growth was delayed by more than 50 days at this dose compared to controls. Preliminary toxicity in mice showed <10% weight loss in the treated animals at 18 mg/kg/day (33). In combination experiments with ionizing radiation, the exposure of erb-B positive breast cancer cells to ionizing radiation resulted in activation and autophosphorylation of the erb-B receptors. Treatment with **2** sensitized these cells to radiation resulting in a synergistic interaction (35). In the clinic, assessment of the pharmacokinetics of **2** following an oral dose in 40 patients indicated that the AUC was linear with dose and the compound had a 3-4 hour elimination half-life. Mechanism related toxicities were observed, mainly skin rash and diarrhea. One area of concern with **2** is that pharmacodynamic studies indicate that CI-1033 can abolish the activity of some cytotoxic compounds, which could complicate combination studies with standard chemotherapeutic regimens (34).

OSI-774 (**3**), one of the simplest quinazoline-based ATP-competitive EGF receptor inhibitors, has also entered clinical trials (36). This compound exhibits 50% inhibition of EGFR at 2 nM with >1000-fold selectivity *versus* $pp60^{c\text{-}src}$, $pp145^{c\text{-}abl}$, insulin receptor and IGF-1 tyrosine kinases. Against EGF-dependent mitogenesis and proliferation in HN5 head and neck tumor cells, **3** has an IC_{50} = 20 nM (2, 37). OSI-774 is orally bioavailable and is 95% plasma protein-bound in mouse plasma (38). *In vivo* studies on the tumor growth of HN5 xenografts in mice showed that **3** inhibited tumor growth by 50% at a dose of 7 mg/kg/day p.o. and 100% at 50 mg/kg/day (37). Similar results were found in an A431 human epidermoid carcinoma model with tumor stasis being achieved at a dose of 100 mg/kg/day p.o. (37). OSI-774 is well tolerated in athymic mice at 400 mg/kg/day, but animal deaths were observed at 800 mg/kg/day after 8 days of dosing (38). In Phase I clinical trials it was determined that a dose of 150 mg/kg was safe and tolerable with dose-limiting toxicities including acneiform rash and diarrhea (39). In a Phase II trial in patients with advanced squamous cell carcinoma of the head and neck, OSI-774 was evaluated as a single agent at a dose of 150 mg/kg/day p.o. Of the 113 patients, 78 were evaluable with 10 showing a partial response, 23 with stable disease and 45 with progressive disease. Dose reduction was required in 24 patients mainly due to skin rash (40).



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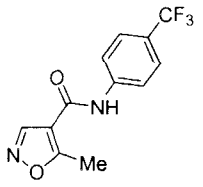
There have been several other compounds reported in the literature for the inhibition of EGFR autophosphorylation (41-44, 47, 48). However, the development

status of these compounds has not been released. A series of quinazolines related to **1** (**4** and IC_{50} = 15-20 nM) and pyrimidopyrimidines have been reported in the patent literature (47, 48). Cyano quinolines have also been reported to be inhibitors of EGFR (44). Compound **5** has been reported as having advanced into the clinic (45, 46). A pharmacophore model has also been proposed and has been used as a tool for the discovery of other compounds that show EGFR inhibition (49, 50).

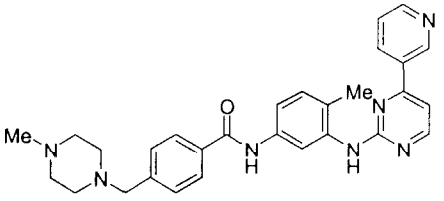
PLATELET-DERIVED GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

Platelet-derived growth factor (PDGF), a major mitogen for fibroblasts, smooth muscle cells and glial cells, has been implicated in the proliferation of numerous tumor types. Overexpression of the PDGF receptor (PDGFR) has also been linked to cancer cell growth in gliomas, anaplastic astrocytomas, and lung, prostate, head and neck, and breast tumors. Coexpression of PDGF and PDGFR is frequently found. This autocrine loop continuously stimulates cancer cell growth. The PDGFR has also been linked to angiogenesis. PDGFR is required for the growth of pericytes, small cells that support new microvessel formation. Therefore, disruption of PDGFR-mediated signaling may have direct and indirect effects on tumor growth and metastasis (2, 51, 52). Several selective PDGFR inhibitors have been reported and selected compounds have entered clinical trials.

SU-101 (**6**), an isoxazole with an IC_{50} = 500 nM against PDGFR-β, showed antiproliferative effects on SKOV-3T and PA-1 cells *in vitro* with IC_{50}s in the 40 μM range (53). Examination of **6** in a Calu-6 lung tumor xenograft model in athymic mice showed that the compound alone at a dose of 5 mg/kg/day i.p. inhibited tumor growth by 17% when compared to controls. In an SF763 glioblastoma (PDGFR-expressing) tumor xenograft model, **6** when dosed at 20 mg/kg/day i.p. inhibited tumor growth by 68% when compared to controls (54). In humans, **6** undergoes ring-opening metabolism to yield a long-lived metabolite that is inactive against PDGFR-β. The $t_{1/2}$ for this conversion is 1.7 hours, while the elimination half-life for the metabolite is 19 days (54). SU-101 was evaluated in a Phase II trial in patients with relapsed glioblastoma multiforme, a very aggressive brain tumor, and showed no added benefit over procarbazine (55). This failure, coupled with the metabolism issues, halted further investigation of this compound.



6



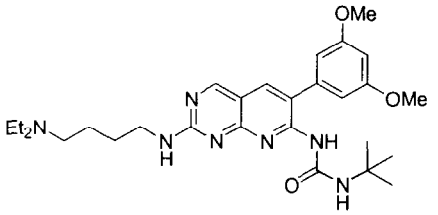
7

Another compound that is receiving a great deal of attention is STI-571 (**7**), a 50 nM PDGFR and 38 nM v-Abl inhibitor (structural studies on a variant of **7** complexed with the catalytic domain of Abelson tyrosine kinase have been reported). STI-571 is >1000-fold selective against PDGFR and v-Abl *versus* EGFR, c-src, PKA, PKC_{α} and PKC_{δ}. When dosed orally, **7** has shown good activity in animal models of leukemia (from its v-Abl activity), as well as anti-leukemia activity in the clinic (the anti-leukemic activity of **7** will not be elaborated upon in this review, however, lead references are provided) (58-60). STI-571 is also under investigation for the treatment of glioblastomas, thus exploiting **7**'s kinase inhibitory profile (i.e. PDGFR activity). In PDGFR-expressing U343 and U87 human glioblastoma intracranial models in nude

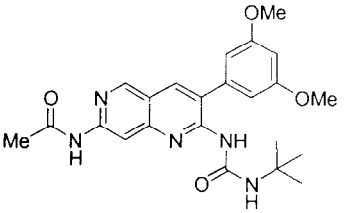
mice, **7**, at either a twice a day dose of 25 mg/kg p.o. or a single daily dose of 50 mg/kg p.o., prolonged survival by 10-16 days (61).

FIBROBLAST GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

In normal tissues, fibroblast growth factors (FGFs) are mitogenic toward a variety of cells, including mesenchymal, neuronal and epithelial cells. This family of growth factors is involved in regulation of cell growth and differentiation, embryogenesis and angiogenesis. Overexpression of FGFs and/or the FGFRs has been linked to several pathologies, including tumorigenesis, psoriasis, rheumatoid arthritis and diabetic retinopathy (62). The interest in FGFR has been stimulated primarily by its involvement in angiogenesis and the necessity of this process to tumor growth and metastasis. Elucidation of the mechanism through which FGFs and FGFRs function in the angiogenic process points more toward their involvement with the maintenance of tumor angiogenesis than its initiation (VEGF is thought to be responsible for the initiation of tumor angiogenesis) (63). Unlike the EGFR and PDGFR inhibitors, inhibitors of fibroblast growth factor receptor tyrosine kinase (FGFR) act on non-cancerous cells. The risk of tumor resistance to these agents is, therefore, limited. However, it has been shown that several tumor types overexpress FGF and/or FGFR, and disruption of this autocrine loop would represent another mode of tumor growth inhibition (62, 63).



8



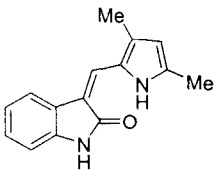
9

Selective FGFR inhibitors have been reported (64-70) and the X-ray co-crystal structure of two classes of compounds have been solved with the ATP-binding kinase domain (65, 66). One such compound, PD-173074 (**8**), has an IC_{50} = 9 nM against FGFR-1 and is a potent inhibitor of bFGF-stimulated HUVEC proliferation with an IC_{50} = 7 nM (65). PD-173074 was evaluated in a mouse model of angiogenesis. A bFGF/heparin –impregnated Matrigel plug was implanted in mice followed by treatment with **8** at 100 mg/kg/day for 7 days. Nearly 100% inhibition of angiogenesis was observed in this experiment (64). In a murine mammary 16c tumor model, treatment with photodynamic therapy, followed by a 30 mg/kg/day p.o. dose of **8** for 28 days resulted in complete tumor growth inhibition, although 11-12 days after secession of drug treatment, the tumors grew at the same rate as controls (64). A recently reported analog, **9**, had an IC_{50} = 25 nM against FGFR-1 and exhibited efficacy in a murine mammary 16c tumor model at doses ranging from 80-200 mg/kg p.o. (70). However, this compound proved ineffective in M5076 reticulum cell sarcoma and Lewis lung carcinoma models in mice.

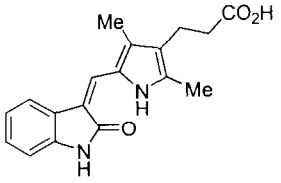
VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS

The search for antiangiogenic agents for the treatment of cancer has been an area of intense research for the last decade. Several approaches have been investigated leading to several clinical trials of new chemotherapeutics (71). One such area has been the investigation of inhibitors of vascular endothelial growth factors and their receptors. Most notably, ATP-competitive inhibitors of VEGFR-2 (KDR) have

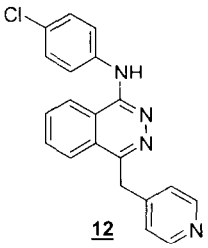
undoubtedly attracted the most attention throughout the pharmaceutical industry. VEGF is a specific mitogen for vascular endothelial cells, cells that serve as the basis for the body's vascular network (72). The receptors for VEGF are almost exclusively expressed on endothelial cells, allowing for cellular specificity (73). Also, it has been found that many tumor types overexpress VEGF and/or VEGFR (74-76), thus providing an autocrine or paracrine loop for their continued survival. Release of VEGF by tumor cells stimulates the surrounding endothelia of the existing vasculature, thereby recruiting new blood vessels to be formed from those nearby. Without the process of angiogenesis, the tumor would be starved of nutrients and would become necrotic. Angiogenesis also allows the tumor cells to metastasize, thus, inhibiting angiogenesis could have a benefit in slowing primary tumor growth and minimizing metastasis. Another benefit is that the target tissue is the host vasculature, thus resistance to VEGFR inhibitors by the tumor cells should not be an issue. VEGF and VEGFR have also been implicated in other disorders, such as diabetic retinopathy, psoriasis and rheumatoid arthritis. More structural diversity has been encountered in the search for agents to inhibit VEGFR than the other kinases mentioned in this review.



10



11



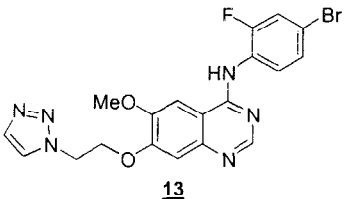
12

One of the earliest compounds to be reported as a specific inhibitor of VEGFR was SU-5416 (**10**), a pyrroloindolinone. This compound inhibits VEGFR-2 (KDR) autophosphorylation with an IC_{50} = 200 nM. SU-5416 also inhibits Flt-1, PDGFR-β and c-Kit with IC_{50}s = 8 nM, 680 nM and 400 nM, respectively (77). The inhibition of human umbilical vascular endothelial cell (HUVEC) proliferation was also observed (IC_{50} = 40 nM) (78). Studies in Balb/c *nu/nu* mouse tumor models showed that **10** had substantial inhibitory activity on primary tumor growth at a dose of 25 mg/kg/day i.p. (85% , 62%, 52%, 32% and 71% inhibition against A375, A431, Calu-6, 3T3HER2 and 488G2M2 cell lines, respectively) (78). In a subcutaneous NF-1 neurogenic sarcoma xenograft model in NOD-SCID mice, **10** inhibited tumor growth by 54% at a dose of 25 mg/kg/day i.p. by day 8 of treatment. Vessel density in the tumors was also decreased by 49% relative to controls (79). In order to evaluate the effect of **10** on tumor metastasis, Balb/c mice underwent splenic injection of CT-26 colon cancer cells to generate metastases. Treatment with SU-5416 (12 mg/kg/day i.p.) resulted in a 32% decrease in liver weight, 48% fewer surface metastases and a 42% decrease in vessel count, relative to controls (80). In Phase I clinical trials, 69 patients with advanced malignancies were treated with **10** by i.v. administration (81). The drug was generally well tolerated, producing mild-to-moderate toxicities such as headache and nausea. Seven patients were on treatment for >6 months, including two who were treated for >18 months, with no evidence of cumulative toxicities, providing preliminary evidence that SU-5416 could be used as a chronic therapy. The drug has since entered into broad Phase I/II and Phase II/III clinical testing as both a single-agent therapy and in combination with cytotoxic chemotherapy (81).

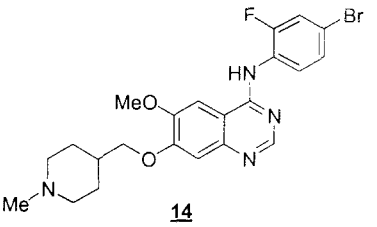
An orally bioavailable analog of **10** has also been reported. SU-6668 (**11**), is a broad-spectrum inhibitor of VEGFR-2 and FGFR-1 *trans*-phosphorylation and PDGFR-β autophosphorylation, with K_is = 2.1 μM, 1.2 μM and 8 nM, respectively. Compound **11** also inhibits VEGF-driven mitogenesis of HUVECs (IC_{50} = 340 nM) (82). Balb/c

mice with splenic injection of CT-26 colon cancer cell were treated with **11** at a dose of 200 mg/kg/day p.o. Relative to controls, treated mice showed a 55% decrease in metastases, a 36% decrease in microvessel formation and a 36% decrease in liver weight (80). Similarly, an A431 s.c. tumor xenograft model in athymic mice was used to assess the effects of **11** on primary tumor growth (82). In this model, **11** at an oral dose of 200 mg/kg/day showed 100% growth inhibition of CT-26 tumors. SU-6668 was also able to regress established CT-26 tumors that had reached 200, 400 and 800 mm^3 at a dose of 200 mg/kg/day p.o. Complete regression of the tumors was observed in 20 of 39 mice. For animals whose tumors had not completely responded after the first treatment, a second round of therapy achieved complete regression. SU-6668 has also shown substantial tumor growth inhibition against Colo205, H460, C6 and SKOV3TP5 cancers at 200 mg/kg/day p.o. (98%, 94%, 81% and 75%, respectively).

A series of aminophthalazines has been reported recently (77). Compound **12** (PTK-787/ZK222584), has entered Phase II trials for the treatment of cancer. The SAR in this series has identified the 4-pyridylmethyl substituent as being essential for activity. Substituted anilines in the 1-position of the phthalazine provided the most potent compounds with **12** providing a high level of activity against VEGFR-2 (IC_{50} = 37 nM) with a high level of selectity (>1000-fold *versus* non-class III RTKs). The proliferation of HUVECs is potently inhibited by **12** as well (IC_{50} = 17 nM) (77). It is noteworthy that **12** is less potent against Flk, the mouse homolog to KDR, with an IC_{50} = 270 nM (83). Preliminary pharmacokinetics (PK) in mice given a single 50 mg/kg oral dose of **12** indicated that the compound is rapidly and efficiently absorbed (t_{max} = 15 min., C_{max} = 32 μM) and >1 μM concentrations of the parent compound in plasma after 8 hours (77). *In vivo* inhibition of tumor growth following oral administration of **12** has been reported for a variety of tumor cell lines (83-85). The growth of DU145 and CWR-22 prostate carcinomas was inhibited 70% (50 mg/kg p.o. b.i.d.) and 100% (50 mg/kg p.o. q.d.), respectively (83). To assess the effects of **12** on tumor growth and metastasis, mice were implanted with renal carcinoma (RENCA). At an oral dose of 50 mg/kg/day, **12** showed a 67% decrease in primary tumor growth, a 78% reduction in lung metastases and an 87% reduction in lymph node metastases after 21 days of treatment (84). Intraperitoneal implantation of SKOV-3 human ovarian carcinoma cells into nude mice followed by oral treatment with **12** at 50 mg/kg/day inhibited tumor growth by 50%, doubled survival time and decreased the formation of ascites by 90% (85). Further work with the phthalazine series has provided alternative scaffolds that also inhibit VEGF receptor kinases in the submicromolar range. 1-Anilino-2-isoquinolines and anthranilamides have been reported as potent Flt-1 inhibitors with IC_{50}s in the submicromolar to subnanomolar range (86-88).



13



14

Quinazolines, as demonstrated by earlier examples in this review, have served as scaffolds for the design of potent, specific inhibitors of protein tyrosine kinases. One such series has provided ZD-4190 (**13**), a 30 nM inhibitor of VEGFR-2 with >1000-fold selectivity against other kinases (89). Compound **13** also inhibits VEGF-stimulated HUVEC proliferation (IC_{50} = 50 nM). Following a single oral dose of 100 mg/kg in mice, concentrations of parent drug were quite high (C_{6h} = 45 μM) even after 24 hours (C_{24h} = 13 μM). Oral administration of **13** (100 mg/kg/day) to mice bearing Calu-6, PC-

3, MDA-MB-231 and SKOV-3 tumors resulted in 75%, 88%, 83% and 95% inhibition of tumor growth, respectively (91, 92). Recent reports have indicated that further development of **13** has been discontinued and ZD-6474 (**14**) has advanced into clinic trials (91-93). ZD-6474 is reportedly >1400-fold more soluble than ZD-4190 at pH 7.4. *In vitro*, **14** potently inhibits KDR (IC_{50} = 40 nM), while being a weak inhibitor of Flt-1, EGFR and FGFR-1 (IC_{50}s = 1.6 μM, 500 nM and 3.6 μM, respectively). The proliferation of HUVECs is also inhibited by ZD-6474 (IC_{50} = 60 nM). When dosed orally at 100 mg/kg/day, ZD-6474 inhibited tumor growth from 92-100% in Calu-6 colon, MDA-MB-231 breast, SKOV-3 ovarian, A431 vulval, A549 lung and PC-3 prostate cancer models in athymic mice. Regression of PC-3 tumors of various sizes was seen after treatment with **14** at 100 mg/kg/day; while the growth of Calu-6 tumors of various sizes was completely inhibited at the same dose (92, 93).

Conclusion – The number of kinases expressed throughout the body will make it difficult to determine the inhibitory profile of any compound designed to inhibit specific members of this class of enzymes. Animal models of efficacy and longer-term toxicity studies may help to determine if the inhibition of multiple kinases leads to unacceptable side effects. If these findings establish that high specificity and selectivity are not required, it will help pave the way for the discovery and development of additional kinase inhibitors for the potential treatment of proliferative disorders. The long-term health benefits of these compounds in the treatment of cancer are unclear, however, it is encouraging that several agents have entered, and continue to progress through, clinical trials.

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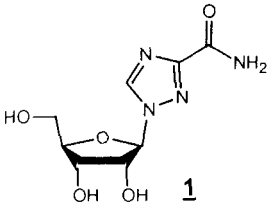
Chapter 12. Non-HIV Antiviral Agents

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Introduction – This review will attempt to update the latest advances in the design and development of agents for non-HIV infections (1). The first section will present information regarding the targets and agents aimed toward improving hepatitis therapy. The following sections will cover the agents studied for use against viral respiratory diseases such as influenza, respiratory syncytial virus and rhinovirus. Finally, the last sections will describe agents in development for treating herpes, cytomegalovirus and related viruses.

HEPATITIS VIRUSES

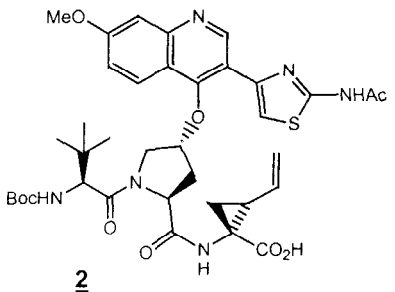
Hepatitis C virus (HCV) – Interferon-alpha (IFN-α) co-administered with ribavirin (**1**), has resulted in an improved therapy for treating HCV, with sustained virologic response rates increased to ~40% (compared to <20% for IFN-α monotherapy) (2). This method has been less effective for treating patients previously unresponsive to IFN-α monotherapy (3,4), and has shown some side effects (5). Use of pegylated IFN-α may lead to less frequent dosing due to improved half-life and clearance, thus resulting in fewer side effects, and has demonstrated a 36% sustained virological response rate (2).



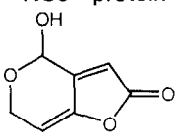
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Medicinal chemistry efforts directed towards several different viral target proteins have recently been reviewed (6,7), and some of the latest advances will be described. New reports of viable cell-based assays for HCV replication hold promise for more detailed investigation of these target proteins and their inhibitors (8,9).

NS3 serine protease inhibitors (10): Structural features of the enzyme complexed with peptide inhibitors have been determined by NMR (11,12) and x-ray crystallography (13). Continued efforts focused on reducing the size of peptide inhibitors have resulted in a potent and highly specific tripeptide inhibitor (**2**, IC_{50} = 15 nM) (14). Results from a truncation study of product-based peptides suggest the importance of a full-length NS3 protein in accurately determining the activity of smaller peptide inhibitors (15). Recent reports of small-molecule nonpeptide inhibitors include the bicyclic hemiketal lactone SCH-351633 (**3**, IC_{50} = 25 μM) (16).



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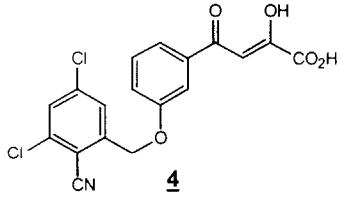


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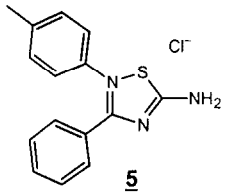
NS5B RNA polymerase inhibitors (17): Reports on the x-ray crystal structure for this enzyme indicate a unique active site different from other known DNA and RNA polymerases (18). The enzyme is not sensitive to many known nucleotide polymerase inhibitors (19). SAR for a series of ketoacids revealed a potent inhibitor of NS5B (**4**, IC_{50} = 56 nM) (20).

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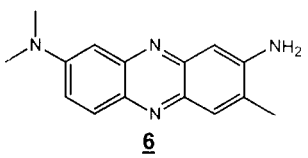
Helicase/ATPase inhibitors: Few small molecule inhibitors have been described (6). Recent examples include a substituted thiadiazole (**5**, IC_{50} = 25-50 μM) (21) and, interestingly, ribavirin 5'-triphosphate (IC_{50} = 40 μM) (22).



4



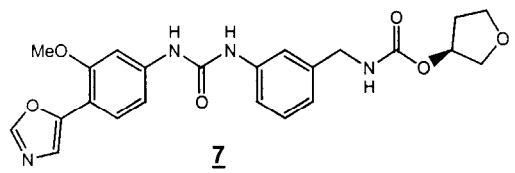
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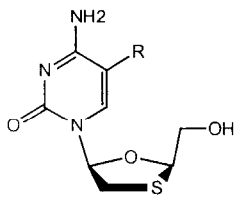
Translation (IRES) inhibitors: The secondary structure in the 5'-NTR (non-translatable region) of the viral RNA constitutes the IRES (internal ribosome entry site), which is required to initiate protein translation (23). Phenazine derivate **6** has been reported as an inhibitor of IRES controlled translation *in vitro* (IC_{50} = 0.02 μg/mL) (24). Other approaches being investigated clinically (Phase II) include a 20 base antisense oligodeoxynucleotide (ISIS-14803) and a catalytic ribozyme, heptazyme (LY-466700) (7). The host cellular protein *La* has been reported to play a critical role in HCV translation (25), and may represent a novel drug target.

Other targets and approaches to therapy: The cellular receptor CD81 has been reported to bind the HCV envelope protein E2 and may represent a novel target for HCV therapy (26). A new approach for treating HCV infection is the inosine monophosphate dehydrogenase (IMPDH) inhibitor VX-497 (**7**), presently in phase II clinical trials (7). This compound is reported to effect lymphocyte migration and proliferation and could potentially demonstrate both antiviral and anti-inflammatory activities (27). Ketoprofen, an anti-inflammatory compound, was found to have similar effectiveness as ribavirin when co-administered with IFN-α (28).

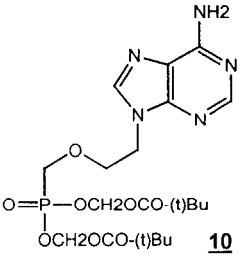


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Hepatitis B virus (HBV) – The discovery of potent, orally active nucleoside inhibitors of HBV RT has greatly improved treatment options for HBV infected patients (29). Lamivudine (**8**) has demonstrated superior efficacy to IFN-α therapy (30), and has been reported to improve liver function in patients with HBV-related liver disease (31). Unfortunately, the rate of sustained response following therapy is low, and the development of resistant mutants has been observed following long-term treatment (32). Other promising agents are under clinical investigation, alone and in combination with **8** and/or IFN-α (33). Phase I/II studies of emtricitabine (**9**) have demonstrated similar potency to lamivudine, but **9** is not active against lamivudine resistant virus (34). Adefovir dipivoxil (**10**) is a potent inhibitor of lamivudine resistant virus *in vitro* (34), and is presently in Phase III clinical trials.

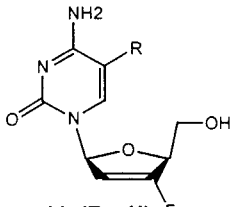


8 (R = H)
9 (R = F)

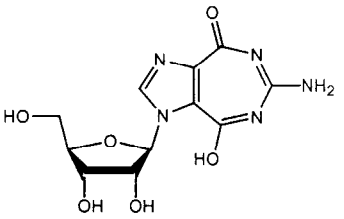


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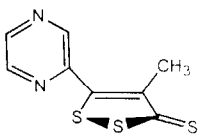
Several novel nucleosides with potent HBV activity *in vitro* have recently been reported (33), including the 2'-fluoro-2',3'-unsaturated analogs **11** and **12** (EC_{50} = 0.18, 0.22 μM, respectively) (35) and ring-expanded analog **13** (EC_{50} = 0.17 μM) (36).



11 (R = H)
12 (R = F)



13



14

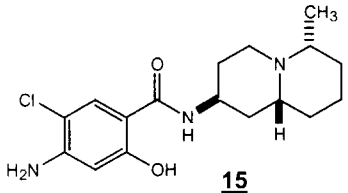
Other HBV antivirals under investigation include oltipraz (**14**), which represents a novel class of inhibitors of HBV transcription (37). Antisense RNAs derived from the HBV genome have been reported to inhibit HBV replication in hepatocellular carcinoma cells, and are promising as antiviral agents and novel tools for studying HBV transcription (33).

RESPIRATORY VIRUSES

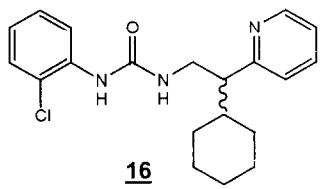
Influenza virus – There are two surface glycoproteins on the influenza virus, which serve as therapeutic targets: 1) hemagglutinen(HA), and 2) neuraminidase(NA). The former enzyme controls the initial binding and endocytosis events of the attacking virus onto the host cell while the latter is the key enzyme in release of the mature virions following infection and replication. Early influenza therapy with amantidine and rimantidine was poorly effective and selective only for the A strains (9 subtypes reported) of influenza, not the B strains (1 subtype).

Hemagglutinen inhibitors: A recent series of anti-influenza compounds were described as being hemagglutinen fusion inhibitors (38). These quinolizidines, typified by **15**, were believed to induce a change in the conformation of hemagglutinen thus blocking the fusion process (39). Although they exhibited activity against influenza A in cell culture, these analogs were selective for H1 and H2 subtypes but were relatively inactive against the H3 subtype. The phenolic group on the salicylamide ring was found to be important for activity since the corresponding OMe and H replacements were inactive, and the 2,5-disubstitution was found to be the preferred pattern on this ring leading to BMY-27709 (**15**) as a candidate compound.

Another smaller series of compounds which lack broad influenza activity were preliminarily reported as being aryl alkyl ureas (**16**). The best analogs showed potent activity (IC_{50} = 0.02 ug/mL) against influenza A (H1N1) but not against H2, H3 subtypes or influenza B (40).



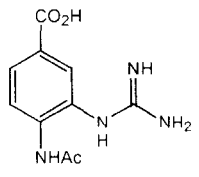
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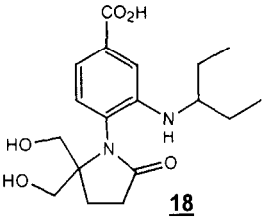
16

Neuraminidase inhibitors: Non-vaccine therapy toward treating the influenza virus (flu) continues to gain attention with the release of several drugs which target the neuraminidase enzyme. This enzyme utilizes sialidase activity to release virion particles from the surface of an infected cell. Structurally, all neuraminidase inhibitors incorporate a carboxylic acid group, an N-acetyl function on the opposite side of the molecule, a ring structure to position these residues, and for most, a basic amine/guanidine moiety.

Using a molecular modeling approach based upon the x-ray structure of the neuraminidase enzyme, a series of benzoic acid NA inhibitors was designed (41). The initial lead was the *N*-acetyl guanidine compound, BANA-113 (**17**). Optimization led to the N-3-pentyl analog **18** which exhibited potent N9-subtype enzyme inhibitory (Ki = 48 nM). This series was also selective for influenza A, in some cases by 1000-fold. These results made clear the effect of movements of residue Glu278 on the A/B selectivity of this class. This residue and the salt bridge made to a nearby Arg residue (Arg 226) in influenza A neuraminidase twist the protein backbone in B strains, thus requiring a different conformation for optimal fit. By appropriate design of the polar sidechain one can decrease the differences in the A/B potency.



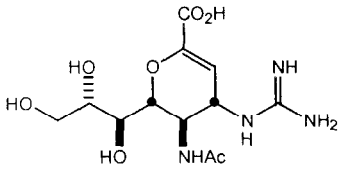
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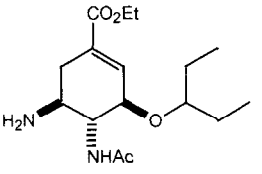
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The first major neuraminidase inhibitor released was (**19**) GG-167 (zanamavir) (42). This compound exhibits a broad range of activity against both influenza A and B strains although it is more active versus A. It is administered via an inhaler, which, though not as convenient as an oral drug, delivers the agent to the primary site of infection, the lungs, thus avoiding potential side effects.

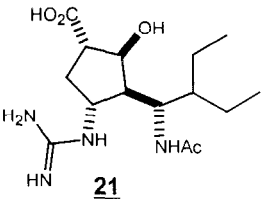
A second drug targeting neuraminidase, GS-4104 (oseltamir) (**20**), was released in the fall of 1999 and has the advantage of being orally active (43). This drug is administered as an ethyl ester prodrug whose higher bioavailability (35% as prodrug ester in rats vs. 4% for parent acid) greatly increased its efficacy. Both zanamivir and oseltamir are based upon the structure of sialic acid and are competitive inhibitors which bind to the well-defined sialic acid binding pocket in neuraminidase.



19

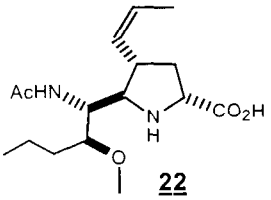


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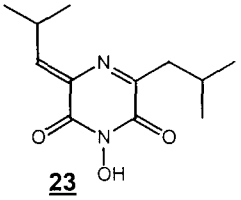
A third influenza drug, RWJ 270201 (**21**), was reported to be in Phase III (44). It is also a sialic acid analog but incorporates a cyclopentane core and has been shown to be as active as zanamivir and oseltamir with the range of antiviral activity against influenza A strains being from 0.2-1 μM and against B from 0.5-5 μM in cell culture.



22

One exception to the above-mentioned structural requirements for NA inhibitors appeared last year in a pyrrolidine inhibitor (**22**). In place of the typically polar amino/guanidino group, a propenyl group was present (45). This series exhibited very potent activity against both influenza A and B, though has not yet reached the clinic.

Endonuclease inhibitors: Another biological target in the influenza virus is the viral transcriptase enzyme, which plays a key role in endonuclease activity during the viral replication process (46). A naturally occurring 2,6-diketopiperazine compound, flutimide (**23**), was identified as a potent inhibitor of this enzyme and shows potent inhibition of both influenza A



23

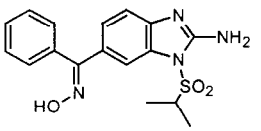
and B in cell culture. The limited SAR describes the N-hydroxy group as being required for activity and that aryl substitution of either sidechain improved activity.

Respiratory Syncytial Virus (RSV) – Respiratory syncytial virus is a disease which typically affects the lower respiratory tract of children from 6 weeks to two years in age. Those individuals who are particularly high-risk candidates are: 1) infants born prematurely or with congenital heart defects, 2) those suffering from bronchopulmonary dysplasia, or 3) those patients with immune-compromising illnesses (47). Until recently, the only approved drug for treatment was ribavirin (**1**) dosed as an intranasal aerosol or via oral administration in conjunction with IFN-α.

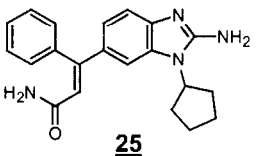
A recently reported agent, VX-497 (**6**), is believed to target inositol monophosphate dehydrogenase and function as an uncompetitive inhibitor (48). This compound has been in clinical trials for psoriasis and HCV, but was found to be more active than ribavirin for RSV therapy *in vitro*. The next stage of this compound's development will include studying effects in combination with IFN-α.

Human Rhinovirus – The picornavirus family comprises both enterovirus and rhinovirus classes. These viruses are characterized as having a viral capsid and single plus-strand RNA, and human rhinovirus (HRV) is considered the single, most prevalent causative agent of the common cold (49). While considered the most common agent, HRV actually accounts for only 35% of these infections with the remainder caused by a variety of corona-, adeno-, paramyxo-, and respiratory syncytial viruses. Much of the SAR of the rhinoviral research programs are based upon well-documented cell culture assays since no dependable animal models, other than primates, have been reported.

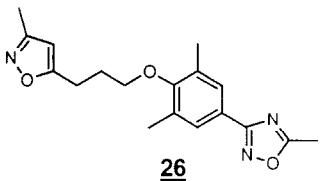
Viral replication inhibitors such as enviroxime (**24**) and its analogs have been synthesized and modified without the benefit of guidance provided by mechanism of action studies (50). Due in large part to the oximino group, this series was shown to suffer rapid glucuronidative inactivation. By modifying the oxime group and the propylsulfonamide group to a vinyl carboxamide and an N-cyclopentyl group, respectively, as in **25**, it was shown that this series can be orally administered with retention of potency and reduction of the extent of metabolic inactivation seen in the parent drug (51).



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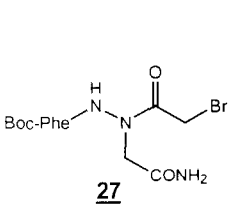


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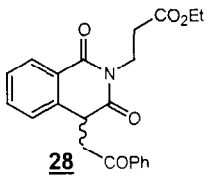
One of the major capsid proteins in picornaviruses, VP-1, serves as the target for the antiviral agent VP 63843 (pleconaril) (**26**). This agent binds to a hydrophobic cleft in the protein, thus blocking further viral processing and uncoating. Pleconaril is presently in Phase III clinical trials as both an anti-rhinoviral and anti-enteroviral agent. Oral bioavailability in man approaches 70%, and this drug has shown an excellent safety profile (49). Pleconaril has shown good *in vivo* efficacy in mice for which an enteroviral model is available, and these positive results have now been evidenced in clinical trials for chronic meningoencephalitis and viral meningitis. This agent serves as a benchmark to which all other rhinoviral agents are compared.

Posttranslational processing of the rhinovirus polyprotein involves proteolytic activity by the 3C and the 2A proteinases. Since these processes are necessary for the production of essential proteins involved with viral replication, they serve as ideal targets for antiviral therapy (52). Compounds such as bromomethylketone hydrazides

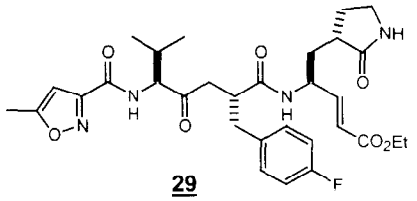
(27) form covalent complexes with the 3CP enzyme, in this case via the displacement of the α-bromo atom by active site cysteine (53,54).



27



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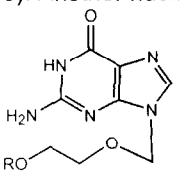
Another series known as the homophthalimides (28) were designed as 3CP inhibitors and found to also exhibit excellent activity versus the 2A protease by formation of covalent drug-enzyme complexes via addition of the cysteine to the heterocyclic ring (55). Potency values for analog 28 (LY 353352) were in the range of 4-60 μM for various 2A serotypes and antiviral activity (IC_{50} = 15.8 μM) was exhibited in H1-HeLa cells in an HRV-14 infection.

The most successful agent reported in this class is AG-7088 (29), also shown to be an irreversible inhibitor of 3CP (52). It has shown good potency in 48 3C serotypes (56) with an average EC_{50} = 23 μM. The planned route of administration is intranasal, and unlike some of the other agents it can be administered 14-26 h post infection. In a separate study, 29 (<0.1 μM) reduces the levels of IL-6 and IL-8, which may allow for more rapid patient recovery by reduction of the inflammatory symptoms (57).

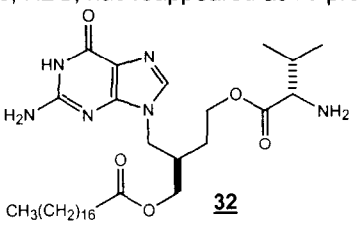
HERPES AND RELATED VIRUSES

Herpes Simplex Virus (HSV) – Herpes simplex virus causes relatively common orofacial or genital infections that occur via two serotypes, HSV-1 and HSV-2. Herpes virus contains a protein capsid core of double stranded DNA. The most common infection, genital herpes, currently infects an estimated 10-50 million people. These infections are initially acute, with symptoms of lethargy and fever followed by a period of latency. During the latent phase, the infection can be reactivated by any of a number of immune-compromising events. Recently, the concept of asymptomatic viral shedding for HSV-2 has been used as an explanation for how this disease can be spread without awareness of the carrier (58). This study implies an even higher percentage of HSV carriers in the population.

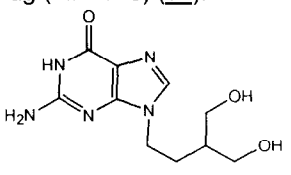
Acyclovir (ACV) (30) is the most common agent used for herpes virus infection (59). This agent is known to be phosphorylated 106 times faster by HSV thymidine kinase than by the host enzymes, and the corresponding triphosphate product has a greater affinity for the HSV DNA polymerase. A recently developed *L*-valyl prodrug of acyclovir, valaciclovir (31) has shown 5-fold improved bioavailability when taken qd (60). Another nucleoside, H2G, has reappeared as its prodrug (MIV-606) (32).



30 R = H
31 R = (*L*)-valine ester



32

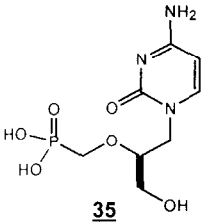


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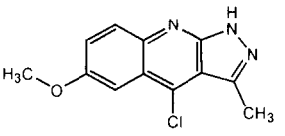
Since many of the agents utilized for this disease are nucleosides and nucleoside analogs, most find use in other related herpes therapy such as cytomegalovirus (CMV) and varicella-zoster virus. Like many of the other nucleoside analogs, penciclovir (33),

one of the first topical agents to exhibit activity during early or late stage disease, is being studied via a series of prodrug forms to increase the poor oral bioavailability.

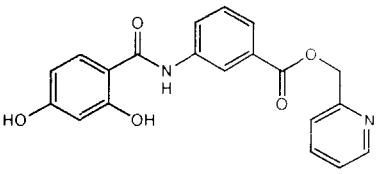
Another set of related viruses, human herpesvirus 6 and 7 (HHV-6, HHV-7), has been studied regarding their sensitivity to a variety of these nucleoside and non-nucleoside agents. It was shown that cidofovir (CDF) (**35**), an oxymethylphosphonate analog, showed better activity than ACV against both HHV-6 and HHV-7. Interestingly, some differences were noted in selectivity of HHV-6 vs. -7 to these agents inferring a difference in the targeted viral enzymes (61).



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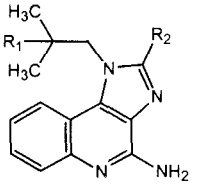
37

The tricyclic derivative, SCH-43478 (**36**) has exhibited potent activity in cell culture assays comparable to acyclovir (59). Although sc administration of this compound showed equivalent efficacy to acyclovir in a guinea pig lesion model, it was not developed as a clinical candidate due to poor oral bioavailability. A recent patent describes a set of compounds typified by **37** whose anti-HSV activity was attributed to inhibition of the HSV-1 DNA helicase-primase (62). This target has recently been identified and differentiated from any host cell DNA primase activity (63).

The FDA has recently approved a 10% docosanol (**38**) cream formulation (Abreva) as the first available OTC cold sore medicine. This drug has been shown to interact indirectly with the virus by modifying the cell membrane in such a way as to inhibit the virus envelope/cell membrane fusion process, thus blocking viral entry (64).

$CH_3(CH_2)_{20}$-CH_2-OH

38



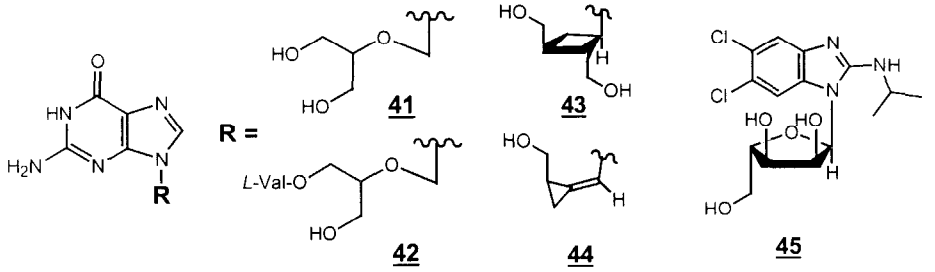
39 R_1 = H; R_2 = H
40 R_1 = OH; R_2 = CH_2OEt

A series of anti-herpes agents were initially found to be immunomodulators via potent induction of interferon-alpha and other cytokines in rodents (65). The topical antiviral activity of the imidazo-quinolines imiquimod (**39**) and resiquimod (**40**) has since been realized, with the latter being launched as a topical product for genital warts and lesions related to human papilloma virus.

Cytomegalovirus (CMV) – Another opportunistic virus is cytomegalovirus, a member of the herpesvirus group also having double strand DNA as its genome. While CMV is carried as a latent infection in the majority of the population, it causes morbidity and mortality in immune-compromised individuals such as arise in neonates, elderly, organ transplant, and HIV patients (66). Over 30% of the latter patients experience CMV retinitis from ocular manifestations of the disease.

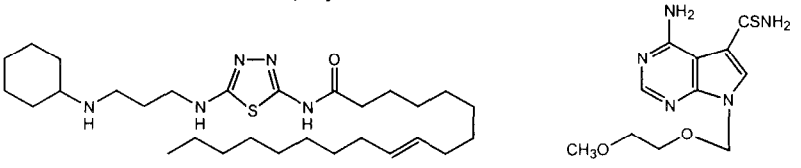
Several nucleosides and nucleoside-like agents have been approved for CMV therapy as DNA synthesis inhibitors. A recent entry to this series of agents is cidofovir (**35**), an agent previously described for its HHV-6,-7 activity. This compound was approved for CMV retinitis in 1996 and is currently in clinical study both as monotherapy and in combination with ganciclovir (GCV) (**41**). CDF exhibits greater

potency than GCV and much improved pharmacokinetics (67). Due to its structure, CDF does not require viral phosphorylation to monophosphate prior to cellular phosphorylations to the active triphosphate. The increased stability of this pseudo-triphosphate is reflected in its half life of 17 hours; however, these persistent blood levels may be the cause of the dose-limiting nephrotoxicity apparent in most patients. Furthermore, the poor oral bioavailability requires IV administration. In spite of these problems, CDF appears to be the most promising agent currently available.



One of the earlier agents initially administered IV, (GCV) (**41**), has now been approved as an oral formulation, and is currently being studied in combination regimens with other related agents (66). Recently, a valyl ester prodrug, valganciclovir (**42**), has entered Phase III trials and has been shown to exhibit 60% oral bioavailability (68). The development of the cyclobutyl analog, lobucavir (**43**) has been suspended due to potential toxicity. A nucleoside analog in preclinical development is (**44**) QYL-438 (synguanol) (69). Maribavir (**45**), which shows excellent potency and is presently in Phase I/II study, is known to be a DNA synthesis inhibitor but is not phosphorylated in cells, and shows no inhibition of reverse transcriptase or DNA polymerase (70).

A novel series of 1,3,4-thiadiazole anti-CMV agents have recently been patented (71). These compounds were tested in a CMV polymerase assay and analogs such as **46** showed 100% inhibition of the polymerase.



Another new series of agents showing anti-CMV activity have recently been reported. These include the dihydroisoquinoline analogs (72), and the pyrrolopyrimidine analogs of toyocamycin (**47**) (73). The latter series was found to inhibit CMV via a novel mode of action that involved targeting the immediate-early protein (IE-1) in the viral replication process. This occurs prior to when nucleoside agents such as GCV elicit their effects (74). A second series of compounds exhibiting this effect on IE proteins are the antisense agents, typified by fomivirsen (**48**). This agent is a 20-mer phosphorothiolate oligonucleotide designed against the IE1 and IE2 protein in CMV, which gained FDA approval for use against CMV retinitis in 1998. These oligos have the disadvantage of being administered IV but are very selective for the target sequence and show little toxicity.

48 **d(P-thio)(G-C-G-T-T-T-G-C-T-C-T-T-C-T-T-C-T-T-G-C-G)**

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Chapter 13. *In Vitro* and *In Vivo* Approaches to Studying Antiretroviral Therapy (ART)-Induced Metabolic Complications

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Introduction – HIV protease inhibitors (PIs), as a component of highly active antiretroviral therapy (HAART), are effective antiretroviral agents in the treatment of HIV/AIDS (1). These agents inhibit the HIV-1 aspartyl protease, an enzyme essential for virion maturation and release and have played a major role in reducing the morbidity and mortality associated previously with HIV infection (2-4). Despite their effectiveness, long-term therapy with the PIs has been associated with a syndrome or syndromes of peripheral fat wasting, fat redistribution and alterations of glucose and lipid metabolism following treatment (5-12). However, not all patients treated with PIs develop these metabolic complications and in those that do, the degree to which these adverse events manifest themselves, varies (9-12). Identification of factors that influence an individual's predisposition to acquiring any or all of these adverse complications remains elusive. Nonetheless, the appearance of these abnormalities following HAART suggests that lipid metabolism in adipocytes and hepatocytes represents an ideal focal point for studies in order to ascertain their underlying cause. Thus, since the first report of HAART-associated metabolic complications, a number of groups have turned their attention to the adipocyte and various aspects of its physiology in response to treatment with PIs. Employing *in vitro* cell-culture or *in vivo* animal model systems, these studies have taken a reductionist approach to look at the effects of PIs in isolation, away from the compounding influences of the other components of the HAART regimen. As a consequence, insights into how alterations of fat metabolism, in response to PI therapy, may lead to the development of the metabolic complications, have been obtained. This review discusses these findings and their implications for designing more efficacious antiretroviral therapies with reduced potential for causing metabolic complications in subjects treated with HAART.

Background – Although HAART has been key to prolonging life for HIV-infected individuals, long-term therapy in a number of patients has been associated with the development of adverse metabolic complications (5-12). Four main elements of the HIV Lipodystrophy Syndrome (HLS) have been identified and include complications of fat wasting (lipoatrophy), fat accumulation/redistribution (lipohypertrophy), and various disturbances of lipid and glucose metabolism. Lipoatrophy invariably involves loss of peripheral subcutaneous fat from the face and extremities whereas lipohypertrophy involves increases in visceral fat leading to central (truncal) obesity as well as redistribution to the dorsocervical fat pad (buffalo hump) and focal lipomatous growths in the subcutaneous compartment of the head and extremities (13). Disturbances of lipid and glucose metabolism result in increased serum levels of cholesterol (CH), triglyceride (TG), fatty acid (FA), insulin and glucose which can lead to insulin resistance and ultimately, diabetes (11,14,15).

The existence of various metabolic abnormalities arising in a significant number of patients following antiretroviral therapy was first recognized following the introduction of PIs and as a result, were attributed initially to the PI arm of HAART (5-12). However, upon further analysis by multiple groups, independent associations between the appearance of the metabolic complications and various factors indicated the underlying cause of HLS to be multi-faceted and not solely the result of PI usage (16-18). Numerous factors, including various aspects of HIV disease itself as well as the patient's past and current drug regimen, are now recognized as being involved in the

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development of these abnormalities. Thus, all elements of HAART including nucleoside reverse-transcriptase inhibitors (NRTIs), non-nucleoside reverse-transcriptase inhibitors (NNRTIs), and PIs have been shown to be culpable to varying degrees (13,16-18). However, a major emphasis has been placed on determining how PI usage contributes to the observed adverse reactions, in part, due to the major role that PIs play in reducing the morbidity and mortality associated with HIV infection.

In recent years, a greater understanding of and appreciation for the complexity of adipose (fat) tissue metabolism has been obtained which demonstrates that this tissue has functions well beyond the mere storage of excess fuel reserves (19-21). Indeed, adipose tissue possesses an endocrine function and serves as an active participant in the metabolic process (20,21). One of the molecules secreted by adipocytes, leptin, serves as a "lipid sensor" to regulate satiety and energy expenditure (22-24). In addition, evidence suggests a role for leptin in control of adipose tissue distribution and mass (25,26). Functionally distinct depots of adipose tissue exist throughout the body with differential regulation between the depots. For example, subcutaneous fat is more responsive than visceral fat to signaling mediated by the heterodimer nuclear receptor complex formed by the peroxisome proliferator activated receptor γ (PPARγ) and the retinoid X receptor (RXR) (27). The PPARγ/RXR pair is known to be a potent mediator of adipogenesis, a process by which mature adipocytes are formed from immature precursor cells (19,28). The fact that the various metabolic abnormalities observed following HAART are correlated closely with alterations of fat and glucose metabolism has brought studies in this arena to the forefront, in attempts to elucidate how and why these complications occur.

In Vitro APPROACHES

Adipocyte Biology – Recognizing the fundamental importance of fat metabolism, a number of studies on the effects of PIs on this process have focused on determining whether or not these agents can affect adipocyte differentiation. An initial report to address this issue in cell culture investigated the effects of ritonavir (RTV) and indinavir (IDV) on the differentiation of the murine preadipocyte cell line, 3T3-L1 (29). In culture, the differentiation of these fibroblast-like cells into mature adipocytes, a process known as adipogenesis, requires the combined action of insulin, glucocorticoids such as dexamethasone, and a cAMP-raising agent such as isobutylmethylxanthine (IBMX). Culture conditions were chosen such that either inhibitory or stimulatory effects of the PIs on differentiation could be observed. As assessed by positive staining of lipid droplets with Oil Red O as well as extraction and measurement of cellular TG, both RTV and IDV increased adipogenesis significantly (29). While the mechanism for the increased differentiation observed was not determined, the possibility of a release of a protease-mediated inhibition of adipogenesis by the PIs was suggested (29).

In marked contrast to these results a second report, likewise using the differentiation of 3T3-L1 cells in culture as a marker of adipogenesis, demonstrated a significant impairment of this process by several PIs in a dose-wise fashion (30). Employing Oil Red O staining, measurements of TG concentration, and gene expression studies, adipogenesis was inhibited in the presence of either IDV, RTV, amprenavir (APV), or nelfinavir (NFV). Relative to IDV or APV, RTV and NFV had the greatest effects on inhibition of TG accumulation and expression of genes for FA-binding protein (aP2), lipoprotein lipase (LPL), and Adipo Q, marker genes for adipogenesis (28,31,32). However, the PIs did not inhibit transcriptional regulation of PPARγ in transfected cells, suggesting that inhibition of adipogenesis was not the result of a direct effect of PIs on PPARγ activation.

A comparison of the cell culture conditions between these two reports shows them to be very similar, making it unclear as to why such disparate results were

obtained. In partial agreement with the second report (30), a study using saquinavir (SQV) and IDV showed these PIs inhibit differentiation of human preadipocytes (33). In this study, SQV had a greater inhibitory effect than IDV; however, neither PI was shown capable of altering expression of aP2, even when potent stimulators of both PPARγ and RXR were present in culture (33). Once again it was concluded that PI-mediated effects on adipogenesis were occurring via a PPARγ/RXR-independent mechanism.

Unlike murine 3T3-L1 cells or human preadipocytes, which are committed to undergo differentiation into adipocytes, murine C3H10T1/2 cells are pluripotent mesenchymal stem cells not committed to adipocyte differentiation, yet have the capacity to do so when appropriately induced (34). Also, while insulin, glucocorticoids and IBMX induce adipogenesis of 3T3-L1 cells (35), insulin and PPARγ/RXR agonists induce adipogenesis of C3H10T1/2 cells (36). Furthermore, whereas 3T3-L1 cells express a phenotype similar to white adipose tissue (35), C3H10T1/2 cells express a phenotype similar to brown adipose tissue (36). In a study examining the affects of PIs in C3H10T1/2 cells, NFV, RTV and SQV reduced TG accumulation, adipogenesis and expression of the adipose markers, aP2, adipsin (complement factor D) and LPL (37). Histological analysis showed NFV, RTV and SQV decreased Oil Red O-staining of cytoplasmic fat droplets and that this occurred in the presence of the RXR agonist, LGD1069, indicating the inhibitory effects were not due to an absence of RXR ligand. Furthermore, in mature adipocytes NFV, RTV and SQV increased acute lipolysis, the hydrolysis of stored TG into glycerol and FA. In contrast, APV and IDV had little effect on lipolysis, adipogenesis, or expression of aP2, adipsin and LPL in these cells (37). Although SQV inhibited ligand-binding to PPARγ to a minor degree, none of the other PIs bound to the nuclear receptors RXR or PPARγ, suggesting that inhibition of adipogenesis was not due to antagonism of ligand binding to this heterodimer pair. Nonetheless, the finding that NFV, RTV, and SQV inhibited expression of gene products (aP2, adipsin and LPL) under transcriptional control by PPARγ/RXR indicates that in some cases PIs may have an indirect effect on PPARγ/RXR signaling. Impaired PPARγ/RXR signaling and increased lipolysis could result in reduced differentiation of peripheral adipocytes and storage of fat. The results of these studies suggest that the effects of PIs on fat metabolism may vary between the PIs, cell types and fat depots.

More recently, two additional studies have addressed further the effects of PIs on adipogenesis in 3T3-L1 cells (38,39) and as with earlier reports (29,30), seemingly contradictory results were obtained. In one study, RTV enhanced significantly 3T3-L1 preadipocyte differentiation as well as transiently raised protein expression of the mature form of adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein 1c (ADD-1/SREBP-1c) (38). ADD-1/SREBP-1c is a transcription factor that promotes CH biosynthesis and adipocyte differentiation (40,41). In this instance, a 30% increase in cellular TG mass was observed (38). On the other hand, employing similar culture conditions, IDV, NFV, RTV and SQV were each shown to inhibit either preadipocyte differentiation or promote adipocyte cell death to varying degrees with NFV eliciting both effects to the greatest extent (39). Furthermore, the expression of the mature form of ADD-1/SREBP-1c was reduced markedly by NFV treatment. In addition to lowered levels of TG and ADD-1/SREBP-1c expression, NFV also reduced protein expression of CCAAT/enhancer-binding protein α (C/EBPα), an additional transcription factor regulating adipogenesis (42,43), as well as PPARγ and aP2. It was concluded that the reduced expression of these proteins in response to NFV was directly related to the degree of inhibition of adipogenesis observed (39). Furthermore, while treatment of mature adipocytes with either NFV, RTV or SQV resulted in loss of cellular TG, effects were once again greatest with NFV and the NFV-mediated effects on cell viability were limited to mature adipocytes only and not observed in preadipocytes (39). Again, the reason for the discrepancy

between these two reports in unclear. Perhaps, in part, it is due to subtle differences in culture conditions not yet identified. For instance, as discussed further below, differences in amount of exogenous factors, such as retinoids, may lie at the heart of such disparate results. However, perhaps the principal reason for such results lies in the emerging realization that the effects of the PIs are not all the same as distinct differences between the various members of this class to impart a spectrum of adverse events has been recognized as described above (37,39).

Insulin-stimulated glucose transport into muscle and fat is a rate-limiting step in whole body glucose disposal after a meal. To determine the effects of PIs on glucose transport, studies were done that examined the effects of IDV on glucose transporters in 3T3-L1 adipocytes and transfected Xenopus oocytes (44). IDV inhibited glucose disposal by 63% at 100 μM in murine 3T3-L1 adipocytes. IDV had no effect on Glut1 and reduced Glut4 activity by 45% in Xenopus oocytes expressing either protein, suggesting that IDV may selectively inhibit the glucose transport function of Glut4 *in vitro*. Although it was hypothesized that this contributes to the insulin resistance in HIV-infected subjects, the effects of PIs on glucose transport *in vivo* have not been tested. However, IDV decreases plasma insulin levels and the insulin resistance index and increases plasma glucose levels in AKR/J mice (45). This suggests IDV affects pathways other than Glut4 which are involved in glucose metabolism *in vivo*, such as insulin secretion.

Insights into a potential mechanism of action for at least one of the PIs, IDV, come from published reports showing that IDV and various retinoic acid derivatives share common toxicities including nail, skin, and hair defects (12,46,47). These reports suggest that IDV and retinoids may exert their effects through similar molecular mechanisms involving elements of the retinoid-signaling pathway. Pharmacological effects of retinoids are, in part, due to their ability to bind and transactivate heterodimers consisting of retinoid A receptors (RARs) and RXRs (48-51). At low concentrations, all trans-retinoic acid (ATRA) is selective for RAR but activates RXR at high concentrations. Whereas activation of the RAR/RXR heterodimer inhibits adipogenesis, activation of the PPARγ/RXR heterodimer stimulates adipogenesis (36,52). Studies were performed to test this hypothesis *in vitro* by examining the effects of PIs on retinoid signaling in C3H10T1/2 (53). Cells were cultured in the presence of either APV, IDV, NFV, RTV or SQV and synthetic ATRA and metabolic responsiveness assessed by measuring the activity of a retinoid-regulated protein, alkaline phosphatase (ALP) (53). Of the PIs tested, only IDV stimulated ATRA-dependent ALP activity in a dose-dependent fashion and altered stem cell morphology consistent with development along an osteoblast-like lineage. While ATRA inhibits adipogenesis in these pluripotent cells, it stimulates expression of osteoblast genes, such as ALP (36,52). IDV's effects were observed only in the presence of ATRA. Co-culture with the retinoic acid receptor RAR-antagonist, AGN 193109, inhibited the synergistic effects of IDV and ATRA, implying IDV stimulates RAR signaling selectively. Significantly, when effects of IDV on adipogenesis in C3H10T1/2 cells were reassessed following co-culture with IDV and increasing amounts of retinoic acid, marked inhibition of lipid accumulation was observed as reported previously for NFV, RTV, and SQV (37).

A further ramification of these findings is related to the fact that IDV had differential effects on adipogenesis using either low (25 nM) or high (400 nM) concentrations of ATRA in the presence of the PPARγ agonist, BRL49653, and insulin (53). Specifically, IDV inhibited lipid accumulation in the presence of low concentrations of ATRA, but stimulated fat accumulation in the presence of high concentrations of ATRA. The RAR antagonist AGN 193109 did not inhibit fat accumulation in the presence of 400 nM ATRA and IDV, indicating the effect was not mediated by RAR, but rather by RXR. Consequently, these findings support the notion

that altered retinoid signaling promotes some of the adverse reactions associated with IDV therapy, in particular, altered lipid metabolism. Furthermore, differential responsiveness of stem cells to low or high concentrations of ATRA is consistent with the idea that retinoids have pharmacologically distinct effects on RAR and RXR (36,48-52). Lastly, these results may, in part, explain some of the discrepancies reported for the 3T3-L1 cell line and effects of PIs on lipid metabolism discussed above if differences in actual retinoid concentrations were evident (29,30,38,39).

Hepatocyte Biology - Not all *in vitro* approaches to study the effects of PIs on lipid metabolism have focused on stem cells, preadipocytes and adipocytes. For instance, the liver plays a central role in lipoprotein metabolism and metabolic abnormalities in hepatocytes can result in deleterious consequences for the host. In spite of this, reports addressing the effects of PIs on hepatocytes are rare (54,55). One study, however, employed the human hepatoma cell line, HepG2, to determine the potential for PIs to affect fat metabolism in cultured hepatocytes (54). In this study, cells were incubated with varying concentrations of PIs in the presence of [^{14}C]-acetate and intracellular TG, FA and CH content quantified from cell lysates of PI-treated and untreated cells. NFV and Lopinavir (LPV) produced a significant concentration-dependent increase in intracellular TG synthesis (136% and 146%, respectively) at 10 μM (54). Likewise, RTV and SQV also increased significantly intracellular TG (82% and 96%, respectively) whereas APV and IDV had no effect. None of the PIs affected FA synthesis although RTV increased CH synthesis by 48%. Similar results were obtained in studies employing primary rat hepatocytes where 10 μM RTV increased CH synthesis by 56±17% and RTV and NFV increased TG synthesis (167±73% and 102±52%, respectively). These results should be contrasted with those described above in adipocytes, where NFV, RTV, and SQV, but not APV or IDV (in the absence of retinoids), reduced TG synthesis and stimulated lipolysis (37). In a second paper, also employing HepG2 cells, IDV was shown to impair insulin signaling suggesting a possible association with the development of the metabolic complications (55). It remains to be determined whether or not this may alter the well known ability of insulin to suppress hepatic gluconeogenesis.

The opposing effects of PIs on different cell types could help explain the loss of fat from one compartment (subcutaneous adipose tissue) with subsequent repartitioning elsewhere (liver). Increased TG synthesis in the liver by select PIs could result in increased levels of TG-laden VLDL and chylomicron particles leading to dyslipidemia and insulin resistance. However, as in adipocytes, these observations show that select PIs affect distinct metabolic pathways which could account for the differential effects of PIs observed in the clinical setting (9,11,56).

When HepG2 cells were treated with NFV (10 μM) in the absence or presence of the RXR agonist, LG100268 (100 nM), or the RXR homodimer antagonist, LG100754 (100 nM), differential effects on TG synthesis resulted (54). Whereas LG100754 had no effect on TG synthesis, LG100268 or NFV increased TG synthesis significantly (72% and 145%, respectively). In cells exposed to both drugs simultaneously, cellular TG synthesis increased 335% relative to controls. Other PIs mixed with LG100268 caused similar but less pronounced effects. Furthermore, NFV and LG100268 caused differential effects on expression of mRNA for diacylglycerol acyl transferase (DGAT) and fatty acid synthase (FAS), two essential enzymes in the synthesis of TG and FA, (54). Thus, NFV increased expression of DGAT mRNA (47%, $P=0.024$) to a greater extent than FAS mRNA ($P=0.9$, relative to controls) whereas LG100268 increased expression of FAS mRNA (57%, $P<0.001$) but not DGAT mRNA ($P=0.26$, relative to controls). A combination of NFV and LG100268 did not increase DGAT expression significantly compared to either agent alone. However, FAS expression increased significantly (134%, $P<0.001$) in cells treated simultaneously with both drugs, suggesting PIs and LG100268 affect distinct molecular mechanisms. As with the gene

expression studies in adipocytes mentioned above, studies such as these will be vital in elucidating the mechanisms of action of the PIs on inducing the various metabolic abnormalities manifested. These results suggest that PIs may cause dyslipidemia by altering expression of genes involved in lipid synthesis in adipocytes (29,30,37-39,53) and hepatocytes (54).

In Vivo APPROACHES

Multiple genetic and environmental components influence susceptibility to metabolic diseases. For example, diet and physical activity affect the risk of developing obesity and genetic analysis reveals that Pima Indians are more susceptible to obesity than Caucasians (57). The observation that not all subjects treated with HAART develop the same metabolic complications supports the hypothesis that susceptibility to metabolic diseases varies with environment, duration of therapy, and/or genetics.

Whereas select PIs may cause hyperlipidemia in humans, serum TG levels decrease in Wistar rats treated with RTV and in SWR/J mice treated with SQV or NFV (58,59). This difference in clinical and rodent studies may be due to genetic or species-specific effects or environmental factors, such as HIV infection or diet. For example, serum FA levels decrease in obesity resistant SWR/J mice but increase in obesity prone AKR/J mice treated with NFV, indicating that genetic susceptibility to obesity influences the dyslipidemia associated with NFV (59). Similarly, serum glucose levels decrease in SWR/J mice but increase in AKR/J mice fed a low fat diet and treated with IDV or NFV (59). However, RTV treatment had no significant effect on serum glucose in Wistar rats (58). Since genotypes and responses to PIs may vary between individuals in the clinic and various strains of rodents, multiple animal models are needed to understand the metabolic pathways affected by HAART.

Alterations in dietary fat and carbohydrate or fasting can also influence the effects of PIs on metabolism. IDV and NFV increased blood urea nitrogen and TG levels whereas SQV increased CH levels in AKR/J mice fed a high fat, low carbohydrate diet (45). APV did not increase plasma lipid levels in these mice (45). The effects of IDV, NFV and SQV were not observed in AKR/J mice fed a low fat, high carbohydrate diet. IDV and NFV treated mice fed the latter diet had greater serum glucose levels, weight gain and fat accumulation; IDV treated mice had lower insulin; and SQV treated mice had lower CH than placebo treated mice. Thus, high fat, low carbohydrate diet increases PI-mediated hyperlipidemia while low fat, high carbohydrate diet increases PI-mediated hyperglycemia in AKR/J mice. Consistent with these observations, NFV increases serum TG in fed but not fasted AKR/J mice, whereas RTV increases glucose in fasted but not fed AKR/J mice (54).

A comparison between AKR/J mice fed either low or high fat diets reveals IDV treatment increases FA, pancreatic lipase, bilirubin and alkaline phosphatase in mice fed either diet (45). NFV also increases serum FA and glycerol levels in AKR/J mice fed each diet (45). In both fasted and fed AKR/J mice, RTV treatment increases serum TG levels (54). A comparison between SWR/J and AKR/J mice reveals NFV and IDV treatment increases pancreatic lipase in both strains of mice (59). In contrast to IDV and NFV, there was little effect of SQV and APV on FA, lipase, bilirubin or alkaline phosphatase in either mouse strain (59). Taken together, these observations indicate some PI-associated affects are unaltered by diet, fasting or genetic background.

The different effects of PIs in rodents and *in vitro* indicate that each PI has unique pharmacological properties, some which are influenced by environmental or genetic

factors. For example, the level of serum TG levels in AKR/J mice fed a high fat diet and treated with equal amounts of PIs follows the ranking RTV>NFV=IDV>SQV=APV (45,54,59). Several clinical reports also indicate there is variability between the PIs in their effects on hyperlipidemia. In a study of 93 patients, RTV therapy caused hyperlipidemia more than NFV or IDV therapy (56). In another study of 67 patients, NFV-treated patients had the highest median total CH, whereas RTV-treated patients had the greatest TG level (11). Similarly, patients receiving RTV or RTV/SQV had greater serum lipid levels than patients receiving IDV (5,9). These results indicate that each PI influences a distinct metabolic pathway, perhaps accounting for the differences seen for these drugs *in vivo*. Although the reasons for these differences remain unknown, they could result from differences in drug metabolism, protein-binding, or cell/ tissue penetration.

While the molecular target for PI-induced dyslipidemia is unclear, *in vivo* studies indicate the PIs stimulate biochemical pathways involved in lipid uptake and TG synthesis. Treatment of AKR/J mice with IDV or NFV stimulates pancreatic lipase activity, an enzyme that hydrolyzes diglycerides in the gut and contributes to increased dietary fat absorption (45). In the clinic, post-heparin lipase activity in plasma and removal of remnant lipoproteins are unaffected by RTV, indicating that abnormal lipoprotein lipase and TG clearance do not contribute to hyperlipidemia (60). Consistent with this hypothesis, treatment of AKR/J mice with RTV and NFV increases TG levels by 200-300% after injection with Triton WR-1339, an inhibitor of TG clearance (54). The observation that TG plasma levels increase in the absence of TG clearance indicates these PIs stimulate TG synthesis *in vivo*.

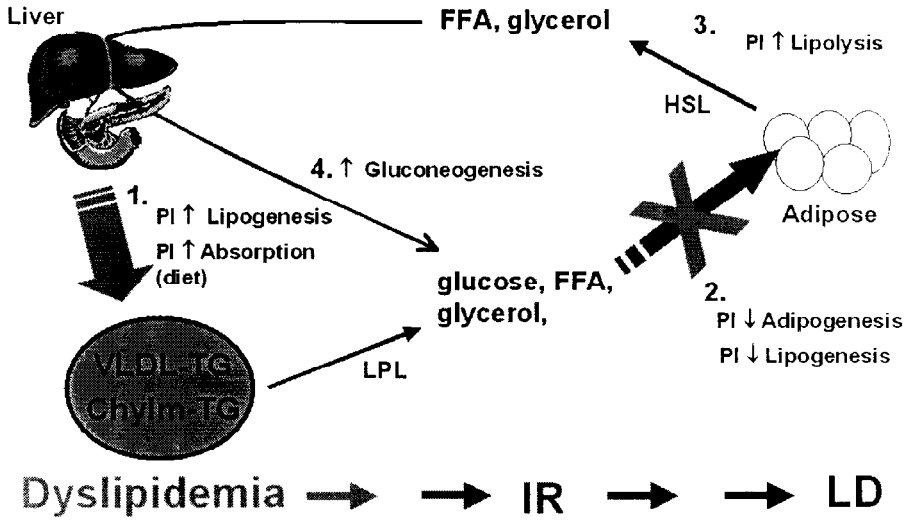


Figure 1. A model for PI-associated metabolic complications. 1. Increased hepatic lipogenesis and increased absorption of dietary fat due to increased pancreatic lipase may lead to elevated levels of both VLDL- and chylomicron-triglycerides (TG), respectively. 2. Reduced expression of lipoprotein lipase (LPL) and adipsin (complement factor D) as well as decreased levels of adipogenesis and lipogenesis in subcutaneous adipose tissue results in less fat storage in this tissue. 3. Increased lipolysis of stored fat in subcutaneous adipose tissue via hormone-sensitive lipase (HSL) further reduces the extent of fat storage. 4. Free fatty acids (FFA) and glycerol, the products of lipolysis, re-circulate through the liver. FFA are used as fuel or re-incorporated into VLDL-/chylomicron-TG while glucose is produced from glycerol via hepatic gluconeogenesis. As the syndrome progresses, serum lipids and glucose increase while adipose tissue decreases, potentially giving rise to syndromes of dyslipidemia, insulin resistance (IR) and lipodystrophy (LD).

Conclusion – Fat metabolism represents a focal point in attempts to identify mechanisms underlying the appearance of metabolic complications following HAART. *In vitro* effects of PIs on the differentiation of adipocytes and lipid metabolism in adipocytes and hepatocytes demonstrate differences in the ability of the various PIs to affect these processes in diverse cell types. Results of *in vivo* studies show that both genetic and environmental factors play a major role in determining the nature and extent of the complications arising. Based on the experimental results described herein, a model to illustrate how these complications may arise is presented in Figure 1. Careful analysis of the effects of infection and individual PI and NRTI on fat and carbohydrate metabolism may improve the ability to predict, prevent or treat side effects associated with HAART. Further studies on the mechanisms of action for each PI and their abilities to cause metabolic changes in the clinic are needed. The results of these studies may allow safer and more effective therapy for AIDS to be used in the future.

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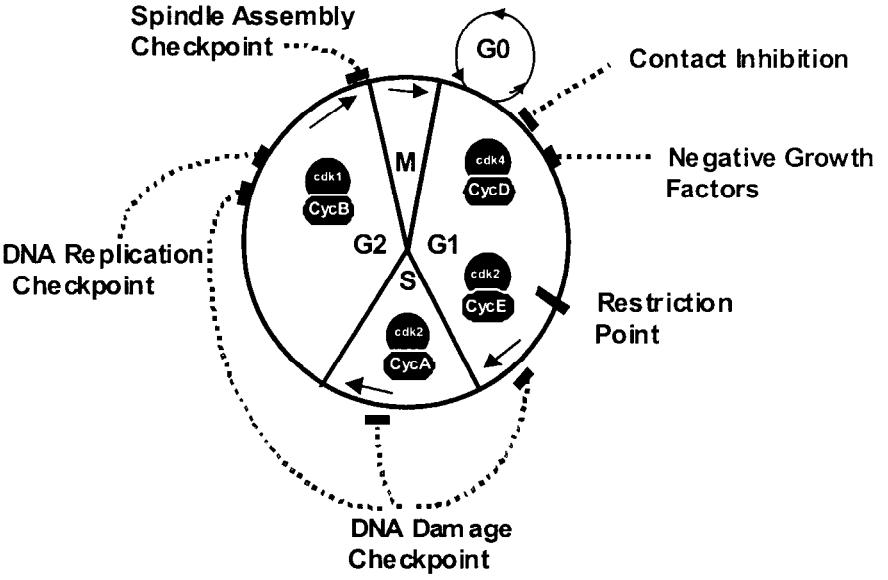
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Chapter 14. Cell Cycle Kinases and Checkpoint Regulation in Cancer

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Introduction - The cell cycle incorporates the co-ordination of a variety of cellular events for the purpose of accurate replication of the genome and cytokinesis (1). Cell cycle progression is regulated primarily at the G1/S and G2/M phase transitions by a series of "checkpoints". These checkpoints serve to maintain the proper sequence of cell cycle events and allow the cell to respond to insults or to proliferative signals (2,3). Activation of a checkpoint results in a pause in cell cycle progression to allow for the choice between the alternate pathways leading to cytokinesis, differentiation, quiescence and cell death; the loss of proper checkpoint control in cancer cells contributes to tumorigenesis (4,5). Given that checkpoints by their very nature negatively regulate proliferation, it is reasonable to assume that small molecule inhibitors that target these pathways would be effective modulators of cell proliferation. The observation that checkpoint control is implemented through the regulation of cyclin



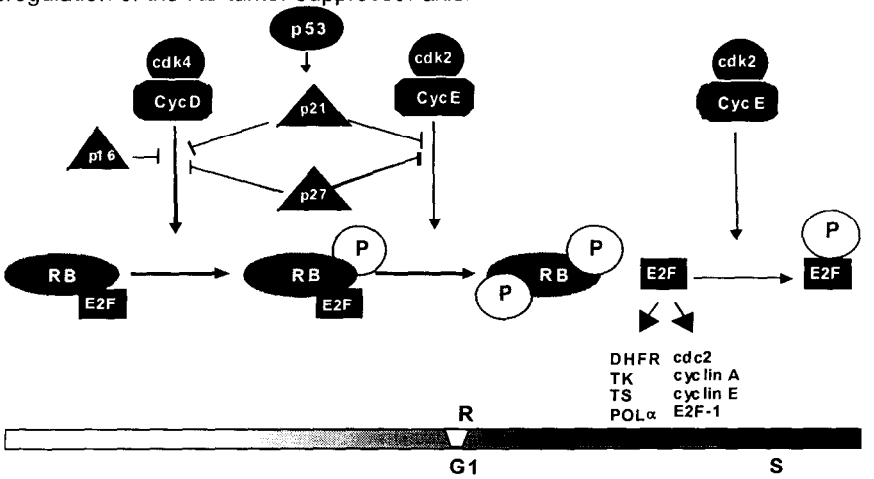
dependent kinase function has made the cdks and their regulatory pathways compelling targets for the development of novel chemotherapeutic agents. This chapter will summarize recent advances in the development of small molecule inhibitors of the cyclin-dependent kinases as well as recent advances in the understanding of checkpoint controls. More detailed reviews are available discussing the biology of the cell cycle, molecular prerequisites of cancer, the clinical potential of cyclin dependent kinase inhibitors, inhibitors of cyclin dependent kinases, and more generally, inhibitors of protein kinases (6-15).

INHIBITORS OF CYCLIN DEPENDENT KINASES

Mechanistic rationale – In addition to epidemiological and genetic evidence that restriction point control is aberrant in essentially all human cancer, there is growing mechanistic support for the potential of cdk2 inhibitors in the treatment of proliferative disease. Phosphorylation of RB by cdk4/cylin D and cdk2/cyclin E complexes frees

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transcription factors of the E2F family and initiates the transcription of a host of genes that are critical for the cell to pass the G1/S boundary. E2F-dependent gene transcription is required only for a brief period, and E2F itself is a substrate for cdk2/cyclin A complex, which negatively regulates the transcriptional activity of E2F at G1/S through phosphorylation (16). Treatment of cells with membrane-permeable peptides that inhibit the activity of cdk2/cyclin A allows E2F-dependent gene transcription to continue into S phase, and this unregulated transcriptional activity causes the cell to initiate its apoptotic machinery (17). Therefore, inhibitors of cdk2 would be expected to cause apoptosis in cancer cells, which are dependent upon the misregulation of the RB tumor suppressor axis.



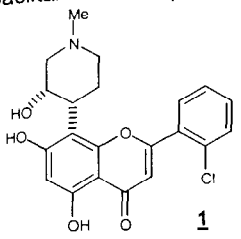
An additional advantage to the development of inhibitors of cdk2 is the fact there is a wealth of structural information describing the enzyme in complex with cyclin A as well as the endogenous inhibitor p27 (18,19). Modeling and crystallographic information have been used by numerous groups in the design of inhibitors of the cyclin dependent kinases(*vida infra*).

Flavopiridol – Flavopiridol (NSC-649890, 1) is the most widely studied inhibitor of cyclin-dependent kinases, and is the first inhibitor of cyclin-dependent kinases to reach clinical trials. Flavopiridol was originally derived by synthesis of analogues of a natural product isolated from the plant, *Dysoxylum binectariferum* (20), and was characterized by its ability to inhibit tumor cell proliferation and its ability to block cells in the G1 and G2 phases of the cell cycle (21). Further examination of flavopiridol's mechanism of action led to the realization that it is a potent inhibitor of cdk1, cdk2 and cdk4, competitive with ATP binding (22,23).

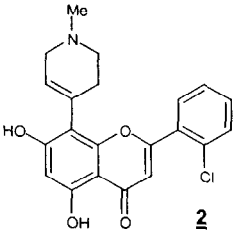
The inhibition of cdk2 and cdk4 activity corresponds temporally to hypophosphorylation of Rb and the development of a G1 arrest in MCF-7 and MDA-MB-468 cells. In a panel of NSCLC cell lines, flavopiridol induces cell cycle arrest and apoptotic cell death (24). Flavopiridol is also a 16 μM inhibitor of glycogen phosphorylase, mimicking caffeine in its binding. This inhibition is synergistic with glucose in stabilizing the T-state of the enzyme, raising the possibility that there may be physiological sequelae at physiological concentrations of both ligands (25). In addition, flavopiridol may have the potential for utility in the treatment of HIV-1 (26).

Flavopiridol is currently undergoing phase I and phase II clinical trials under the supervision of the National Cancer Institute (NCI). Recent reviews have been published covering the clinical trials of this compound (27,28). The initial drug treatment regimen was a 72-hour infusion once every two weeks, and the dose limiting toxicity was found to be diarrhea, consistent with earlier preclinical observations.

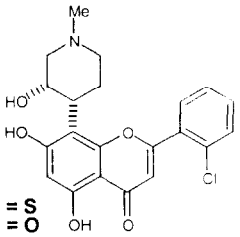
Further evaluation in phase II studies showed that this dose-limiting toxicity is inversely related to hepatic glucuronidation (29). However, cytotoxic activity as a single agent has not been observed on this dosing schedule (30-32). Flavopiridol is currently undergoing Phase II clinical trials and additional Phase I trials in combination with paclitaxel and cisplatinum. As the first cdk inhibitor in man, flavopiridol will pioneer the



1



2



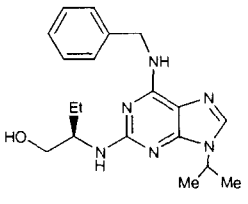
3 X = S
4 X = O

treatment strategy from the standpoints of efficacy and clinical trial design, which should enable a more rapid development of improved second-generation cdk inhibitors.

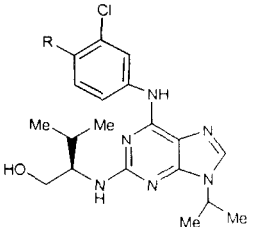
Analogs of Flavopiridol – Studies conducted of piperidinyl ring and/or the chromone-modified flavopiridol analogs identified **2** as a 1.1 and 0.8 μM inhibitor of cdk1/cycB and cdk4/cycD, respectively. In the same assay, the IC_{50} of flavopiridol was 0.2 μM against both cdk/cyclin pairs (33). A recent publication describes the SAR of thio- and oxoflavopiridol analogs (34). The exact thio and oxo congeners of flavopiridol, **3** and **4**, are selective inhibitors of cdk1/cycB, with activities of 0.11 and 0.13 μM. The X-ray structure of **3** bound to cdk2 is compared with that of flavopiridol.

Bicyclic ATP Mimetics: Purines –The C2, N6, N9 substituted purines olomoucine **5** and roscovitine **6** were the first purine inhibitors of cyclin dependent kinases to be evaluated extensively (35). Olomoucine is a 6 μM inhibitor of cdk1, while roscovitine is some 30-fold more potent, and is also highly selective for the cdk family compared with a panel of more distantly related kinases (36). Olomoucine and roscovitine undergo rapid hepatic metabolism in vitro and in vivo; the $t_{1/2}$ for olomoucine in the mouse is reported to be 0.57 hr (37,38).

A number of compounds of this chemical class have been evaluated and found to possess micromolar inhibitory activity against cdk1, cdk2 and cdk5 and to arrest tumor cell growth at the G1/S and G2/M boundaries *in vitro*. These findings are consistent with the inhibition of cyclin-dependent kinases as a mechanism of action. Purvalanol A **7** and purvalanol B **8** are potent inhibitors of cyclin dependent kinases (cdk1/cycB IC_{50} = 4 nM and 6 nM; cdk2/cycE IC_{50} = 35 nM and 6 nM, respectively) (39).

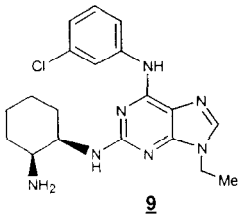


5 R = H
6 R = Me

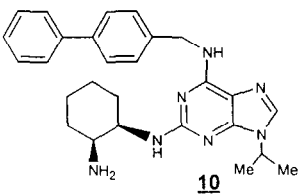


7 R = H
8 R = COOH

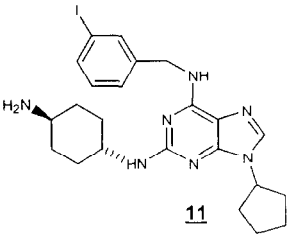
The olomoucine analog CGP-79807 (**9**) is under preclinical evaluation (40). A related series of diaminocyclohexyl purines **10** that are substituted by a biphenyl methyl group at N6 are reported as potent cytotoxic agents in cells(*ca.* 1 nM), despite *ca.* 1 μM activity against the isolated cdk2/cyclin E complex (41). Compound **11** is currently in preclinical development as a 50 nM inhibitor of cdk2 (42).



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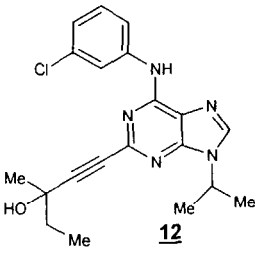


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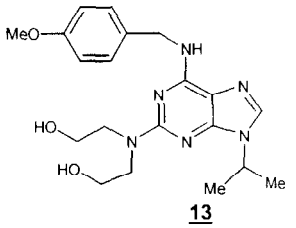


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C2 alkynyl purine **12** has an IC_{50} against cdk1/cyclin B of 60 nM (43). A purine-based inhibitor of cdk2 related to CVT-313 (**13**) is being developed to block cell proliferation in settings of coronary artery bypass and myocardial disease (44). Its structure has not been disclosed.



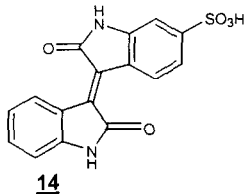
12



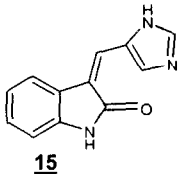
13

Bicyclic ATP Mimetics: Indolinones – The indolinone template is commonly found among inhibitors of both serine/threonine and tyrosine kinases. Indirubin 5-sulfonic acid **14** inhibits cdk1/cycB, cdk2/cycE and cdk4/cycD1with potencies of 55, 150, and 300 nM, respectively, and is *ca.* 100X selective for the cyclin dependent kinase family of enzymes (45). The imidazolyl substituted indolinone **15** inhibits these enzymes with potencies of 40, 22, and 200 nM, respectively (46). X-ray crystallography reveals that the 5-sulfonic acid interacts with Lys33 and Asp145 in the active site of the cdk2 enzyme.

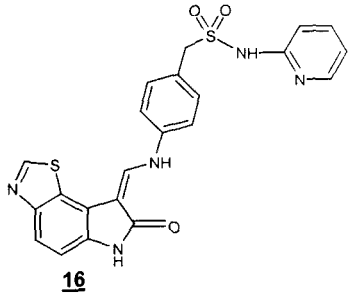
A series of patent applications covering indolinone and 7-aza indolinone inhibitors of serine/threonine and tyrosine kinases have been filed(47,48). Of particular interest is GW-8510 **16**, a 10 nM inhibitor of cdk2/cycE. Administration of **16** prior to treatment



14



15

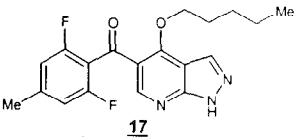


16

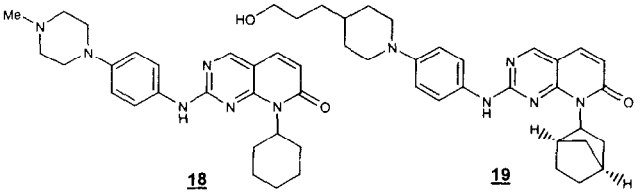
of rat pups with etoposide or cyclophosphamide-doxorubicin prevented the hair loss normally induced by these cytotoxic regimens (49). The poor systemic exposure of **16** may be key to its reversible protective effect on normal proliferating hair follicles (50). While there is no validated animal model of chemotherapy-induced alopecia, this study dramatically illustrates that it may be possible to safely block proliferation of normal cells with cdk2 inhibitors, and thereby protect them against the damage caused by chemo- and radiotherapy.

Bicyclic ATP Mimetics: Miscellaneous – Pyrazolopyridine **17** is a 6 nM and 9 nM inhibitor of cdk1/cycB and cdk2/cycE, respectively (51). Pyridopyrimidinones **18** and **19** have been reported as inhibitors of cdk4/cycD (52). Compound **18** has modest

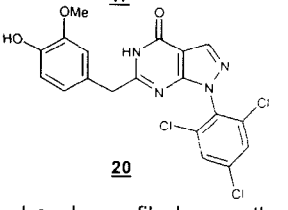
selectivity (cdk1/cycB=79 nM, cdk2/cycE=20 nM, cdk4/cycD=4 nM, FGFr=51 nM). However, the less basic pyridopyrimidinone **19** is highly selective for cdk4/cycD with IC_{50} values against these enzymes of >40000 nM, 165 nM, 8 nM and 8600 nM, respectively.



17



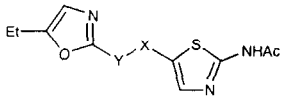
18 **19**



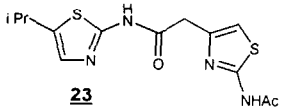
20

A patent describing a series of pyrazolopyrimidin-4-ones has been filed recently (53). Although no data is provided, a number of compounds are specifically claimed, including **20**.

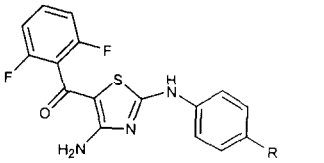
Monocyclic ATP Mimetics: Aminothiazoles – Several series of aminothiazole cdk2/cycE inhibitors have recently been published. Methylthio-oxazolyl aminothiazoles **21** are potent and selective inhibitors of cdk2/cycE (54,55). The ethenyl analog **22** is claimed in a subsequent patent application (56). A related aminothiazole **23** is a 0.5 μM inhibitor of cdk2/cycA (57).



21 X,Y = SCH_2
22 X,Y = CH=CH



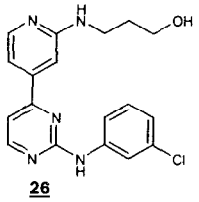
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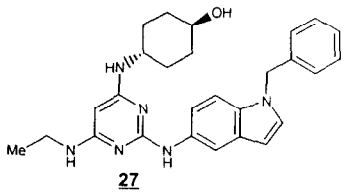
24 R = 4-N-methyl-1-piperazinyl
25 R = SO_2NH_2

A series of 4-amino substituted aminothiazole inhibitors of cyclin-dependent kinases have been published. AG-12275 **24** is a cdk4 selective inhibitor, while AG-12286 **25** is a *pan* cdk inhibitor. These compounds inhibit cdk1/cycB, cdk2/cycE, and cdk4/cycD at 320, 220, 3, and at 2, 6, and 12 nM, respectively. Both compounds show > 1000X selective for the cdk family relative to PKC (58-60).

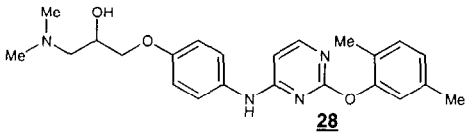
Monocyclic ATP Mimetics: Pyrimidines – The 2-amino substituted pyrimidine is one of the most common templates for both serine/threonine and tyrosine kinase inhibitors. Extensive preclinical research was carried out on **26**, but it has now been discontinued (61). A more recent 2,4,6-triamino substituted pyrimidine **27** is a relatively specific inhibitor of cdk4/cycD (62).



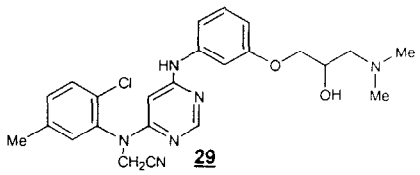
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27



28

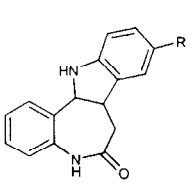


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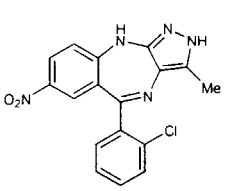
A recently filed series of patent applications covers 2,4- and 4,6-substituted pyrimidines as inhibitors of cdks and focal adhesion kinase. The 2,4-substituted pyrimidine **28** inhibits cdk4/cycD with an IC_{50} of 1.2 μM, while a similarly substituted 4,6 analog **29** is reported to have an IC_{50} of 70 nM (63-66).

Miscellaneous Inhibitors of Cyclin Dependent Kinases – Paullones are fused benzazepin-2-ones discovered at the NCI using the COMPARE algorithm to search for novel chemotypes with patterns of anti-tumor activity related to the cdk inhibitor flavopiridol (67-69). Alsterpaullone **30** inhibits cdk1/cycB, cdk2/cycA, cdk2/cycE, cdk5/p25, and GSK-3β with potencies of 35 nM, 15 nM, 200 nM, 40 nM, and 4 nM, respectively, and is a 70 nM inhibitor of HCT-116 cell growth in vitro (70,71). The 9-CN analog **31** is a 24 nM inhibitor of cdk1/cycB but is 100X less active at inhibiting HCT-116 cell growth; alsterpaullone is in preclinical development at NCI.

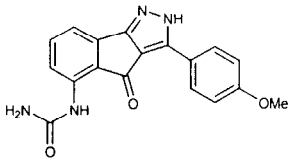
Pyrrazolobenzodiazepine **32** has been claimed as a specific inhibitor of cdk2 in a



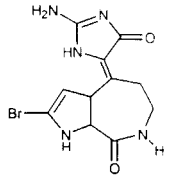
30 R = NO_2
31 R = CN



32



33

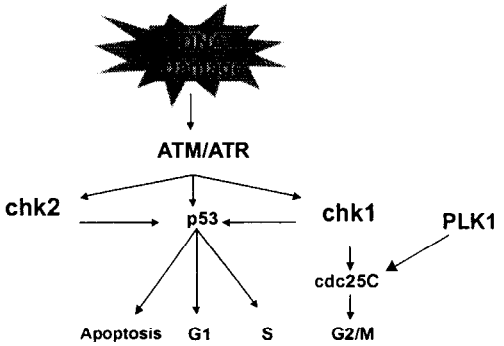


34

recent patent filing (72). This compound has been reported to have activity between 0.01 and 1.0 μM in the cdk2/cycE and three cell proliferation assays. **33** is a recently described 5-aminoindeno pyrazole inhibitor of cdk2/cycE and cdk4/cycD (73). Hymenialdisine **34** is an alkaloid derived from the marine sponge *Hymeniacidon* (74). Like alsterpaullone, hymenialdisine is a potent inhibitor of cdk1/cycB, cdk2/cycE, cdk5/p25, and GSK 3-β, with IC_{50} values of 22 nM, 40 nM, 28 nM, and 10 nM, respectively (75).

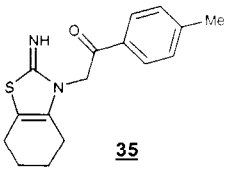
CELL CYCLE CHECKPOINTS IN CANCER

DNA damage checkpoint –DNA damage triggers the stabilization of the p53 tumor



suppressor protein, which mediates both cell cycle arrest and apoptotic responses to genomic damage. The central regulatory role that p53 plays in determining cell cycle progression, and the multiplicity of inputs and outputs, has been likened to a node on the internet (76,77). Tumor cells lacking p53 may bypass these checkpoints and proceed through S and G2 phases of the cell cycle despite the presence of damaged genetic material. For this reason, the loss of p53 is a source of genetic instability for cancer cells, and predisposes them to further mutation.

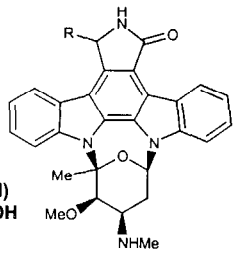
The dose limiting side effects of cytotoxic drugs are due to p53-driven apoptotic cell death of normal epithelial cells. It has been found that the temporary inhibition of p53 function by PFTα (**35**) protects mice from lethal doses of γ-irradiation (78), raising the possibility that the co-administration of p53 inhibitors concomitantly with radiotherapy or cytotoxic drugs might provide an extra margin of safety, allowing for higher dosing. The ability of **35** to temporarily block apoptosis in normal cells may also make it useful in the inhibition of chemotherapy-induced alopecia, which requires functional p53 (79).



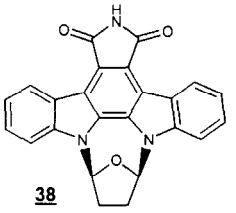
35

G2/M checkpoint – The transition from G2 to mitosis requires the activation of cdk1/cyclin B by cdc25C phosphatase, and the accumulation of the activated kinase complex in the cell nucleus (80,81). The ATM and ATR proteins recognize DNA damage and regulate the downstream Chk1 kinase, which inactivates cdc25C phosphatase by phosphorylation (82). ATM also directly targets p53, and activates Chk2, which stabilizes p53 by phosphorylating it on Ser20 (83). However, in the absence of functional chk1 kinase, this activation is insufficient to maintain a G2/M arrest (84). In many tumor cells with compromised p53 status, inhibition of Chk1 kinase would therefore bypass the G2/M DNA damage checkpoint, and drive the damaged cell into mitosis. Forcing genetically damaged cells into mitosis by abrogation of the G2/M checkpoint would be expected to be highly toxic to cancer cells and synergistic with many chemotherapeutic treatments.

Staurosporine (**36**), UCN-01 (**37**) and the related SB-218078 (**38**) are all indolocarbazole inhibitors of the Chk1 kinase (85). Interestingly, the non-chiral SB-218078 is a more selective inhibitor of Chk1 relative to cdk1 and PKC than either staurosporine or UCN-01 (85). The solid state structure of the Chk1 kinase with an analog of ATP has been described, which should enhance design efforts of inhibitors of this enzyme (86).



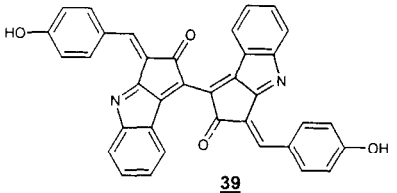
36 (R = H)
37 (R = OH



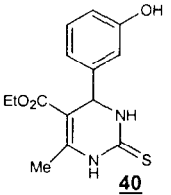
38

	Chk1	cdk1	PKC
36	8 nM	6 nM	5 nM
37	7 nM	100 nM	4 nM
38	15 nM	250 nM	1000 nM

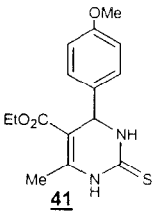
PLK1 is a kinase that functions to activate cdc25C phosphatase and trigger mitosis. Overexpression of PLK1 transforms NIH3T3 cells, and is correlated with poor prognosis in some cancers. A dominant negative PLK1 induces G2/M arrest, followed by apopotosis in SAOS-2 and U-20S tumor cell lines (87). Scytonemin (**39**) is a pigment from blue-green algae that inhibits human PLK1 with an IC_{50} of 2 μM (88).



39



40



41

Mitotic assembly checkpoint - The G1/S and G2/M checkpoints monitor the integrity of the genome prior to DNA replication and segregation of sister chromatids, respectively. In contrast, the mitotic assembly checkpoint assesses the status of the mitotic spindle,

and blocks the onset of anaphase until the kinetochores form stable attachments to microtubules. The initiation of the mitotic spindle checkpoint may also involve the tumor suppressor protein p53 (89). The proper functioning of the mitotic spindle apparatus involves a number of molecular motors (90). The inhibition of the Eg5 mitotic spindle motor protein by monastrol (**40**) causes a distinctive mitotic arrest phenotype in which the centromeres do not separate, and the chromosomes spread out in a starburst pattern (91). The closely related analog **41** is not active in this assay. It is not known whether the inhibition of the Eg5 motor protein has any potential utility in the treatment of cancer.

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SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

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Chapter 15. Targeting the Estrogen Receptor with SERMs

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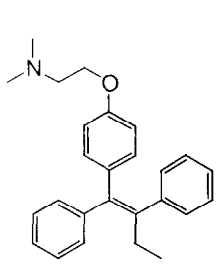
Introduction – Estrogens represent a diverse structural array of compounds that function via binding to two nuclear localized receptors - estrogen receptor α and β (ER) (1,2). Members of this group of ligands includes the classic steroid hormones 17β-estradiol, estrone, and estriol; phytoestrogens such as coumestrol and genistein; synthetic estrogens like diethylstilbestrol; and xenobiotics like bisphenols and several chlorinated hydrocarbon pesticides (3). A relatively diverse group of compounds, which have been classified as selective estrogen receptor modulators or SERMs, are also members of this estrogen family (4). When an estrogen binds to the ER a series of events occurs (e.g. receptor dimerization, receptor conformational changes, biochemical modifications, coactivator interaction, and DNA binding) culminating in gene transcription which affects changes in cellular / tissue physiology. What distinguishes the SERMs from the other estrogens is that they demonstrate both ER agonist and antagonist activity dependent upon the cell type and gene promoter targeted.

The primary clinical application of estrogens is estrogen replacement therapy (ERT) in menopausal and postmenopausal women. Menopause is associated with a reduction in ovarian production of estrogens. Physiologically, this results in vasomotor instability (including an increase in hot flushes), vaginal dryness, incontinence, changes in blood lipid profile (e.g. increased LDL cholesterol), and a reduction in bone mass (osteoporosis). Replacement with estrogens alleviates all of these symptoms to some extent, however, unopposed estrogen replacement also results in unacceptable uterine stimulation and an increased risk of developing breast cancer. In order to alleviate the uterine stimulation, estrogens are combined with progestins, known as hormone replacement therapy (HRT). This completely eliminates the negative impact of estrogens on the uterus. However, the effect of estrogens on the breast appears to remain unaffected by this combination therapy.

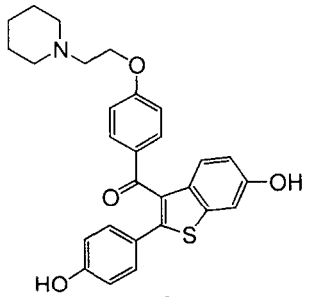
Alternative therapies for HRT are desired because of these associated negative side effects. When the first SERM, tamoxifen (**1**) was initially characterized, it was classified as an antiestrogen in the treatment of breast cancer. Subsequent to its development for the treatment of breast cancer, it was demonstrated that tamoxifen exhibited estrogen agonist effects on the skeleton and liver (5). Unfortunately, like ERT, it also stimulated the uterine endometrium resulting in an increased risk of endometrial cancer. In an effort to maximize the positive characteristics of tamoxifen and eliminate the negative effects, other SERMs have been developed. This second generation of compounds includes raloxifene (**2**), droloxifene (**3**), idoxifene (**4**), and levormeloxifene. Of this group, only raloxifene has been developed and marketed for the treatment and prevention of osteoporosis. Additionally, raloxifene modestly reduces LDL cholesterol, however it has been shown to exacerbate vasomotor instability. It does not stimulate the uterus and reduces the risk of developing breast cancer (6). Third generation SERMs, TSE-424 (**5**) and lasofoxifene (**6**), are in Phase III development and demonstrate an improved profile over raloxifene, however no SERM has been shown to function as an equivalent replacement for HRT. The current candidates lack uterine and breast stimulation, but also lack the vasomotor relief,

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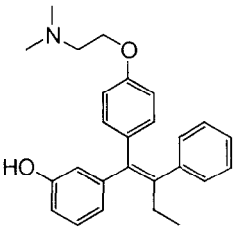
vaginal lubrication, greater efficacy on the skeleton and improved lipid profiles associated with classical HRT. The benefit that newer SERMs or combination therapies will demonstrate to improve upon the current SERMs' effectiveness will be known in the near future.



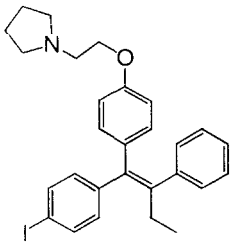
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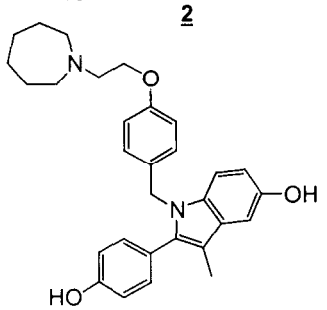
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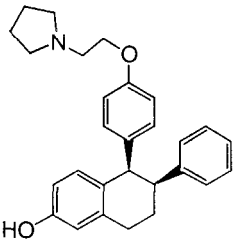
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Structural basis for SERM selectivity - Most non-steroidal selective estrogens reported to date share a common pharmacophore consisting of 2 aryl groups separated by two atoms, often in a stilbene type arrangement. Additionally, SERMs typically bear a third aryl group possessing a 4-aminoethoxy substitution. It had been speculated that when selective estrogens bind to ER, their stilbene like cores mimic the action of 17β-estradiol where at least one of the two aryl groups of the core generally contains a phenol group and overlays with the A-ring phenol of an estratriene nucleus (e.g. 17β-estradiol). The third aryl group which bears the 4-aminoethoxy side chain would then project from a position which corresponds to the 11β-position of the estratriene nucleus (7). Indeed, recent X-ray co-crystallographic studies of 4-OH tamoxifen (a high affinity metabolite of tamoxifen (1)) or raloxifene with the ligand binding domain of human ERα demonstrate this to be the case (8,9). A similar binding orientation was observed for raloxifene in rat ERβ (10). When either of the SERMs bound to the receptor, a large conformational change was induced wherein helix 12 was moved to a groove formed between helices 3 and 5. This is in contrast to the situation with the non-steroidal estrogen agonist diethylstilbestrol (DES) or 17β-estradiol which both lack an amine bearing side chain. When these estrogen agonists bind the receptor, helix 12 is folded over revealing a groove formed by residues from helices 3, 4, 5, and 12 (Figure 1) (8). This groove provides a binding site for the LXXLL motif of various nuclear receptor coregulators, which recognize this portion of the receptor (11). The net result for the SERM occupied receptor is that helix 12, displaced by the bulky SERM side chain, is in a position that blocks the interaction of the receptor with certain nuclear coactivator proteins. Interference of coactivator recruitment can interfere with cellular transcription and thus allow a SERM to behave as an antagonist on target genes within a given cell type. The particular balance of coregulatory proteins and target promoter(s) in a given cellular backdrop likely determines ultimate SERM selectivity (12).

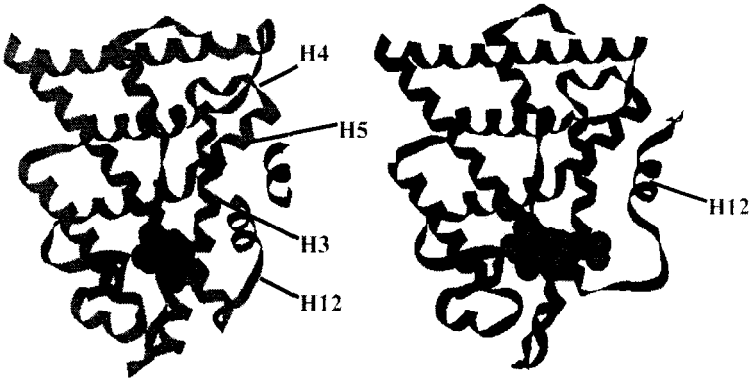


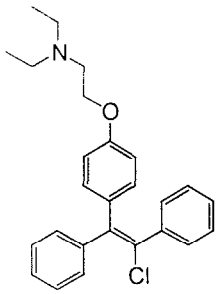
Figure 1. Ribbon representation of X-Ray crystal structures of the ligand binding domains of ERα/DES with a co-crystallized coactivator fragment (left) and ERα/4-OH tamoxifen (right). The sidechain of 4-OH-tamoxifen has displaced helix 12 from the agonist bound conformation (8).

It has been demonstrated that small structural modifications to SERM molecules often results in large differences in both *in vitro* and *in vivo* potency as well as tissue selectivity (13-15). Nevertheless, certain general determinants of tissue selectivity have been made. In particular, it has been observed that the nature and orientation of the side chain is critical to the estrogen's profile. In a series of 2-phenyl benzothiophenes, it was demonstrated that small changes in the amine of the side chain resulted in large changes to the compound's ability to stimulate uterine hypertrophy (15). Another key determinant of selectivity between structural classes is the relative orientation of the side chain. Templates, such as triphenylethylenes (e.g. tamoxifen) where the side chain is in the plane of the stilbene system tend to cause increased uterine hypertrophy compared to templates where the side chain is capable of orienting itself orthogonal to the stilbene plane. This orthogonal orientation may come about through attachment of the side chain to a hinge atom linker, or through placement of the side chain on a non-aromatic, cyclic system which allows for the side chain to occupy an axial position (15). Interestingly, in cases where the side chain is connected in a triphenylethylene, coplanar type arrangement, the nature of the terminal amine seems much less critical to compound selectivity, if necessary at all (16-18).

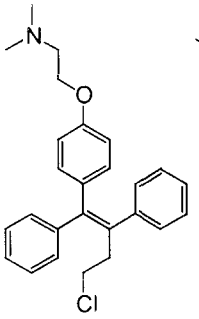
MOLECULAR CLASSES OF SERMS

Triphenylethylenes (TPE's) – Triphenylethylenes were among the first reported structural classes of SERM molecules and tamoxifen (**1**) and clomiphene (**7**, shown as E-isomer) are probably the best known examples. Tamoxifen has been widely used for decades as a treatment for breast cancer and its indication was recently expanded for the prevention of breast cancer in women at risk (19). While long-term use of tamoxifen was associated with a 45%-50% decrease in breast cancer incidences in longterm studies of women at increased risk for the disease, there was a concomitant increased incidence in uterine cancer, deep vein thrombosis and hot flushes (20). The hyperplastic effect of tamoxifen on the uterus is of some concern and considerable efforts have been expended modifying the structure in order to decrease its trophic action on uterine tissue. Toremifene (**8**) is currently marketed for the treatment of breast cancer but apparently also suffers from similar uterine liability (21). Droloxifene (**3**) and idoxifene (**4**) were both recently terminated from late stage clinical trials (22,23).

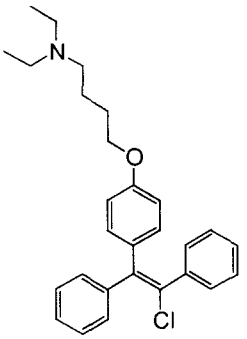
Additional triphenylethylenes under investigation include MDL-101,232 (**9**), a clomiphene analogue with extended sidechain and FC-1271a (**10**) (a human metabolite of toremifene) (24-26). Both compounds displayed estrogen agonist activity on bone as well as in decreasing total cholesterol, however, both compounds also demonstrated significant uterine effects when dosed in ovariectomized animals (27,28). It appears that the triphenylethylene class of SERMs generally continues to suffer from increased uterine liability relative to many of the other SERM classes.



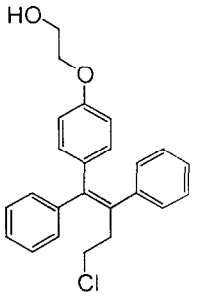
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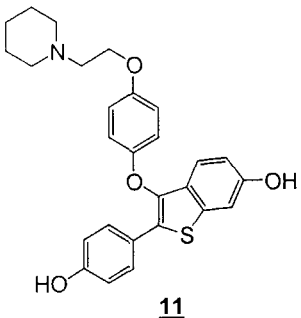


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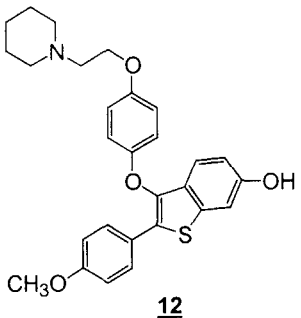


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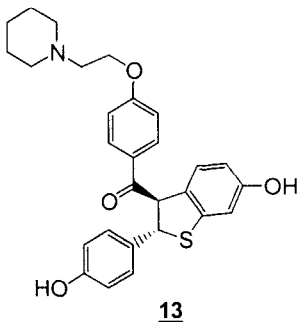
Benzothiophenes – The FDA approved raloxifene (**2**, Evista®) in December 1997 for the treatment and prevention of osteoporosis in postmenopausal women (29). In a double-blind randomized trial of 7705 postmenopausal, osteoporotic women (MORE study), raloxifene at 60 mg/day reduced the risk of vertebral fracture in women by 30-50% (depending on pre-existing fracture status of patient group) over a 36 month period but also increased the risk of deep vein thrombosis (30,31). In addition, a 90% reduction of invasive ER+ breast cancer incidences was reported in raloxifene treated groups (60 mg/day and 120 mg/day groups combined) and there was no increase in endometrial cancer among women in the raloxifene treated groups (32). In a separate study evaluating the effect of raloxifene (60 mg/day) on hot flushes, it was demonstrated that raloxifene, like tamoxifen, increased the incidences of hot flushes in postmenopausal women (33). Currently raloxifene is being evaluated in a large 10 year study (STAR) to compare its profile to tamoxifen in effecting long-term reductions in the incidence of postmenopausal ER+ breast cancer among women with increased risk for the disease (34).



11



12



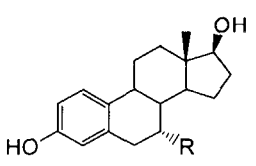
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Several reports in the literature describe modifications to the benzothiophene core with the goal of improving potency and selectivity. Exchange of the carbonyl hinge at the three position of the benzothiophene with an N, O, S atom, or methylene group resulted in compounds with increased antiestrogenic potency in an MCF-7 cell proliferation assay. Particularly noteworthy was the replacement of the carbonyl group

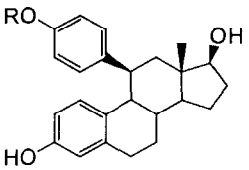
with an oxygen atom resulting in **11** which displayed high antiestrogenic potency in a 4-day immature rat uterine assay (ED_{50} = 0.006 mg/kg s.c. against ethynyl estradiol (0.1 mg/kg p.o.) compared to an ED_{50} = 0.05 mg/kg s.c. for raloxifene).

However, the potency advantage dissipated somewhat when comparing oral doses of the two compounds in the same model (ED_{50} = 0.25 mg/kg p.o. for **11** vs ED_{50} = 0.55 mg/kg p.o. for raloxifene) (35). Methylation of the 4'-phenol of **11** results in arzoxifene (**12**), a SERM with substantially enhanced oral activity relative to raloxifene. Evaluation of **12** in 6 month ovariectomized rats revealed that it was from 30 to 100 times more potent than raloxifene in preventing ovariectomy-induced effects on body weight, serum cholesterol, and bone density, while displaying marginal estrogen agonist activity on the uterus (36,37). The increased oral bioavailabilty is presumably due to decreased intestinal glucuronidation since the 4'-OH phenol is not available for conjugation (38). Additional recent modifications to the benzothiophene core of raloxifene include reduction of the double bond between the 2 and 3 position of the benzothiophene resulting in the racemic dihydroraloxifene analogue (**13**) with 2,3-trans stereochemistry. This compound displayed receptor affinity and MCF-7 cell proliferation inhibition potency similar to raloxifene (39).

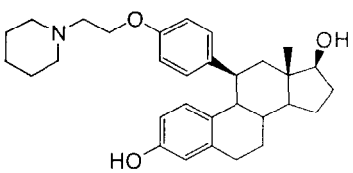
Steroids - Since the principle endogenous ligand for the ER receptor is 17β-estradiol, a steroid hormone containing the estratriene nucleus, it is not surprising that many attempts to modify the structure chemically in order to alter its pharmacological profile have been made. It has been shown that the placement of long aliphatic amide (**14**) and sulfoxide (**15**) containing side chains at the 7α position resulted in compounds possessing what has been described as "pure" antiestrogen activity (40-42). It has also been demonstrated that the placement of similar aliphatic chains (or long aliphatic side chains with phenoxy linkers) into the 11β position of the estratriene nucleus (**16**) can also result in compounds with such activity (43). While such compounds, most notably **15** (ICI-182780 (faslodex)), have advanced in the clinic for the treatment of ER-dependent breast cancer; their selective estrogen activity is more restricted compared to raloxifene or tamoxifen since that they do not display estrogen agonist like activity on the many parameters where ER agonist activity is desired, such as bone, cardiovascular or the CNS (44,45). Nevertheless, placement of side chains that more closely resemble those of raloxifene at the 11β position of the estratriene nucleus resulted in compounds such as **17** that prevented bone loss as well as lowered cholesterol in ovariectomized rats while demonstrating minimal uterine effects when compared to 17β-estradiol (46,47). This result is consistent with the x-ray co-crystal of raloxifene and ERα that places the raloxifene sidechain in a position of the receptor that is coincident with the 11β position of 17β-estradiol (9).



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15 R = $(CH_2)_9SO(CH_2)_3CF_2CF_3$



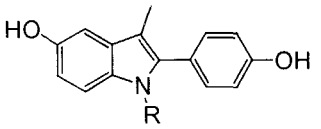
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R = $(CH_2)_5SO_2(CH_2)_3C_2F_5$



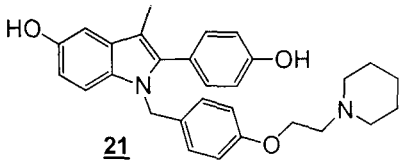
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Indoles - The 2-phenyl substituted indole has been used as a non-steroidal estrogen scaffold (48-50). Conversion of these previously reported indole estrogen agonists to compounds with improved selectivity profiles was accomplished through the introduction of aliphatic side chains originating from the 1-position and often terminating with amines, such as ZK-119010 (**18**) (51,52). While initial reports described **18** as a compound that caused no uterine stimulation in mice, a later report

demonstrated that the compound had some uterotrophic activity in immature female rats (52,53). Longer chain compounds like **19** and **20** were subsequently identified with potent antiestrogenic activity in MCF-7 cell assays and did not display estrogen agonism in a mouse uterine wet weight assay (54,55). More recently, 2-phenyl indoles with rigid side chains have been reported as molecules possessing highly desirable SERM-like profiles. TSE-424 (**5**) demonstrated potent antagonism of 17β-estradiol stimulated proliferation of the MCF-7 breast cancer cell line (IC_{50} = 0.19 nM) and displayed little or no uterine stimulation when dosed in either immature or ovariectomized rats. Additionally, in a 6-week ovariectomized model of rat osteopenia, **5** was able to protect against bone loss and lower total cholesterol with oral doses of 0.3 mg/kg. Interestingly, **5** inhibited activity at hepatic lipase and apo(a) promoters in hepG2 cells similarly to 17β-estradiol while raloxifene and tamoxifen were inactive. The SERM (**5**) has completed phase II clinical trials for the treatment and prevention of osteoporosis in postmenopausal women and is presently advancing through phase III clinical trials (56,57). The piperidine-containing SERM ERA-923 (**21**) competitively and potently inhibited binding of 17β-estradiol on both ERα and ERβ. In its agonist mode, **21** lowered cholesterol and protected against bone loss in an ovariectomized rat model while in MCF-7 cells, **21** inhibited estradiol-stimulated growth with an IC_{50} = 0.7 nM. In nude mice implanted with MCF-7 breast tumor cells, **21** (3-10 mg/kg/day given orally) inhibited estradiol-stimulated growth. Additionally, **21** inhibited proliferation of a human endometrial line, EnCa-101, human ovarian BG-1 cells, and an MCF-7 variant that is inherently resistant to tamoxifen. SERM (**21**) displays no uterotrophic effects when given alone to rats or mice. Two Phase I trials with **21** given to healthy, postmenopausal women have been completed and a Phase II evaluation in women with metastatic breast cancer is currently underway (58,59).

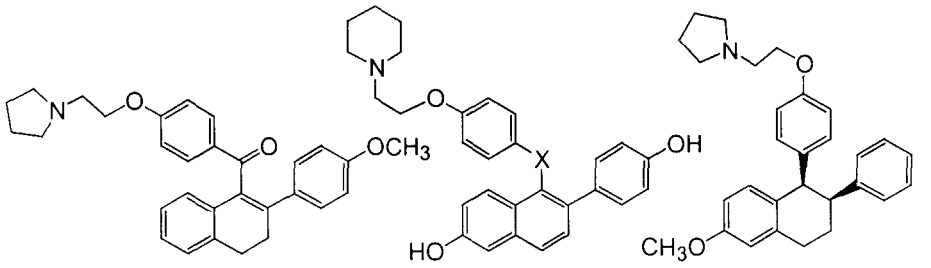


18 R = $(CH_2)_6$-N$(CH_2)_4$
19 R = $(CH_2)_{10}CON(CH_3)$n-Bu
20 R = $(CH_2)_9SO$-$(CH_2)_3CF_2CF_3$



21

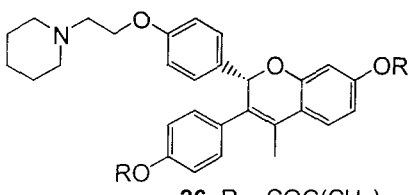
Napthalene Derivatives – Napthalene, dihydronapthalenes, and tetrahydronapthalene scaffolds have served as versatile cores for molecules demonstrating selective estrogen action. Molecules related to the dihydronapthalene trioxifene (**22**) were recently described where the carbonyl hinge was replaced by oxygen (**23**) or CH_2 (**24**) (60-62). SERM (**24**) displayed little or no uterine stimulation in ovariectomized rats while protecting against bone loss and reducing cholesterol with oral doses as low as 0.1 mg/kg per day (61). Lasofoxifene, the tetrahydronapthalene (**6**), is currently in



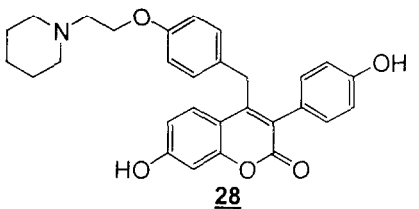
22 **23** X = O **25**
24 X = CH_2

phase III clinical trials as a salt of its (-) isomer (63). SERM (**6**), closely related to the much earlier reported estrogen (**25**), protected against lumbar vertebral bone loss in ovariectomized rats with an ED_{50} of <1 ug/kg/day p.o. (64-66). In human phase II clinical trials, **6** prevented bone loss in postmenopausal women with a dose of just 0.25 mg/day (67). The excellent oral potency of **6** is attributed to reduced intestinal glucuronidation of the phenol.

Benzopyrans and Arylcoumarins – Certain benzopyrans and arylcoumarins have been described as compounds having SERM activity. EM-800 (**26**) is the (S)-(+) bis-pivaloylated prodrug of its progenitor, EM-652 (**27**). In human breast cancer cell lines (ZR-75-1 and T-47D), **27**, was from 27 to 60 times more potent as an antagonist than its (R) enantiomer (68). The ability of **27** to block both the 17β-estradiol-dependent interaction of ERα and ERβ with the coactivator (SRC-1) and the in vitro interaction of SRC-1 with the ligand binding domains of both receptors suggested that the compound fully impedes both AF-1 and AF-2 ER function in these systems. This was regarded as important to the molecule's ultimate potential in treating breast cancer since the AF-1 domain of ER displays ligand-independent activity and mediates growth factors as well as the ras oncogene and MAPK pathways. 4-OH-tamoxifen, in contrast, only inhibits cellular transcription through the ligand dependent AF2 domain (69). In a comparison with raloxifene, **26** was tested for its ability to prevent bone loss and lower serum cholesterol in a 37-week rat ovariectomized model. It was concluded from the study that **26** was from 3-10 times more potent than raloxifene on preventing bone loss in the proximal tibia and femur and in reducing total cholesterol. Both compounds were similarly efficacious, but **26**, unlike raloxifene, produced no discernable stimulation of the rat uterine epithelium (69). Arylcoumarins, exemplified by **28**, have been recently described and reported to exhibit SERM activity. In a 3-day immature rat uterine assay, subcutaneous dosing of compound **28** at 1 mg/kg/day demonstrated no significant increase in uterine wet weight while, in the same assay at the same dose, raloxifene increased the wet uterine weights by 50% which was significantly different from control. Compound (**28**) also inhibited both estradiol induced proliferation of MCF-7 cells and IL-6 expression in human osteoblasts (70).



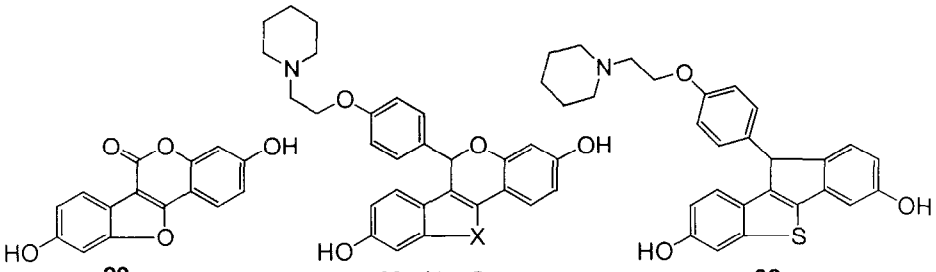
26 R = $COC(CH_3)_3$
27 R = H



28

Constrained SERMs with tetracyclic cores – Recently, several SERMs have been described wherein the core consists of a tetracycle that simultaneously constrains the stilbene phenols into a coplanar arrangement as well as fixes the side chain into an axial conformation. The phytoestrogen coumestrol (**29**) acted as the precursor to the SERM (**30**) where the carbonyl of the lactone served as the attachment point for the basic side chains. The importance of the basic side chain for SERM selectivity is demonstrated by the fact that **30** is a potent antagonist of estrogen dependent stimulation of MCF-7 cells (IC_{50} = 0.7 nM). Additionally, **30** did not stimulate uterine hypertrophy in ovariectomized rats through a range of doses but is able to partially inhibit uterine hypertrophy caused by ethynyl estradiol in immature rats. Coumestrol, in contrast, failed to inhibit estrogen induced MCF-7 cell proliferation and caused uterine hypertrophy in an ovariectomized rat model (71,72). SAR in the series demonstrated that the preferred amine for optimal selectivity was a piperidine ring. Additionally, it was demonstrated that the napthalene fused compound (**31**) displayed

excellent *in vivo* potency on several estrogen sensitive parameters including cholesterol levels, bone mineral density, and inhibition of estrogen stimulated uterine hypertrophy. Since **31** was not remarkably potent on certain *in vitro* measurements such as estrogen receptor affinity (ERα RBA = 0.22 vs 0.34 for raloxifene) or inhibition of estrogen stimulated MCF-7 cell proliferation (IC_{50} = 0.4 nM vs 0.4 nM for raloxifene) it is possible that the superior *in vivo* potency of **31** in preventing bone loss in the ovariectomized model of rat osteopenia (0.01 mg/kg/day vs 1.0 mg/kg/day for raloxifene) is evidence for its having increased bioavailability relative to other SERMs such as raloxifene (72). A molecule from a related series of indenobenzothiophenes (**32**) (which can be viewed as a conformationally locked version of raloxifene) proved itself a potent antagonist in an MCF-7 cell assay (IC_{50} = 0.1 nM) (73).



29 **30** X = O **31** X = CH=CH **32**

Conclusion – The approval of raloxifene for the treatment and prevention of osteoporosis in postmenopausal women illustrates the value of SERMs for the treatment of at least one of the maladies associated with this patient population. Additional evidence from clinical studies indicate that raloxifene, like tamoxifen, lowers the risk of ER+ breast cancer, but unlike tamoxifen, raloxifene does not appear to increase the risk of endometrial cancer or uterine bleeding. Encouraged by raloxifene's early success, much recent research dedicated to improving the pharmacological properties of the earlier generation SERMs has resulted in compounds with improved bioavailability and selectivity. To date, however, SERMs have not been shown to positively affect many other symptoms of the menopause and postmenopause period such as hot flushes, vaginal dryness, or urinary incontinence. Since these "quality of life" issues are what prompt many women to seek hormone replacement therapy in the first place, it is likely that the search for newer and better SERMs or SERM combinations will occupy the attentions of the research community for some time to come (74-76).

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Chapter 16. Vasopressin Receptor Modulators: From Non-peptide Antagonists to Agonists

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Introduction - The cyclic peptide hormone arginine vasopressin (AVP) or antidiuretic hormone plays an important role in water control by regulating a subset of membrane water channels known as aquaporin-2 channels in the kidney resulting in water reabsorption (1). In addition, AVP effects vascular smooth muscle cells as well as the release of corticotrophin from the anterior pituitary gland. The net effect of AVP is to regulate the largest single component of the body, the volume of water. While the biological target has been attractive, the development of a modified peptide derivative containing a disulfide linkage has provided a substantial challenge. The peptide Desmopressin (dDAVP) is currently the only vasopressin mimetic which is commercially available. It is administered intranasally, by injection or orally at high doses (2,3). Peptide vasopressin receptor antagonists were also developed a decade ago and unforeseen agonist-like effects were observed in clinical studies (4). New non-peptide modulators of the vasopressin receptor have been discovered and are being developed in this area of drug discovery.

Vasopressin is secreted from the posterior pituitary in response to either an increase in plasma osmolality detected by brain osmoreceptors or decreased blood volume or blood pressure measured by arterial baroreceptors or low-pressure volume receptors (5-7). Once released vasopressin interacts with three characterized G-protein mediated receptor subtypes V_{1a}, V_{1b} and V_2 each having different tissue distributions and signaling pathways (8). Interaction of AVP with the V_{1a} receptor, which is predominantly located in vascular smooth muscle, hepatocytes and blood platelets, causes contraction and proliferation of vascular smooth muscle, platelet aggregation, coagulation factor (Factor VIII) release, and glycogenolysis. The V_{1b} receptors are found in the anterior pituitary and control ACTH and β-endorphin release (9). The V_2-receptors are localized in the collecting duct of the kidney and regulate the insertion or removal of aquaporin-2 channels from principal cells (1). The effects produced by the modulation of the V_2-receptor subtype provide the readily observed and easily quantifiable clinical observation (antidiuresis). Changes in urine volume and electrolyte concentration (osmolality) provide easily obtained endpoints or markers to measure the pharmacological effects.

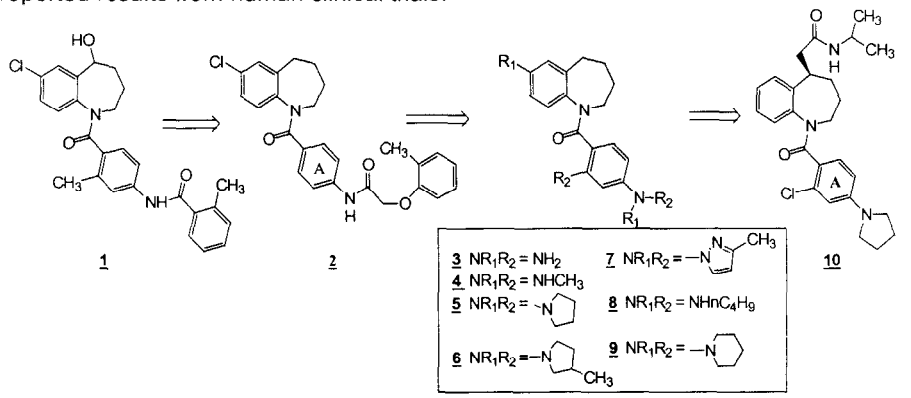
Drug discovery efforts seeking non-peptide vasopressin receptor antagonists have been documented in the literature for the past ten years with the first disclosure of a rat selective V_{1a}-receptor antagonist reported in 1991 (10). In this first example preceding the use of *in vitro* stably cloned and expressed human receptors for improved predictability, clinical studies produced disappointedly weaker effects. The discrepancy between animal and human pharmacology was explained by species differences in the V_{1a}-receptor and provided an important lesson in translating animal pharmacology into clinical events (7). The drug discovery effort evolved into paying closer attention to species differences.Compounds with selective V_{1a}-, V_2- and mixed V_{1a}/V_2-antagonist effects and, most recently, selective V_2-agonists or vasopressin mimetics have been investigated using cloned human receptors.

The most common disease states targeted for vasopressin receptor modulators have been summarized (11-14). V_2- and mixed V_{1a}/V_2-receptor antagonists are targeted for congestive heart failure, liver cirrhosis, SIADH (syndrome of inappropriate secretion of antidiuretic hormone), hyponatremia, and hypertension. A

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selective V_{1a}-receptor antagonist that is currently targeted for vasorelaxation has shown clinical effects in dysmenorrhea and a series of patents suggest that V_{1a}-receptor antagonists may have utility in the reduction in ocular hypertension and glaucoma (15,16). The therapeutic utilities for vasopressin receptor agonists include diabetes insipidous, nocturnal enuresis, and nocturia. While there are claims that urinary incontinence may be included as a therapeutic indication, V_2-receptor agonists exert their effect in the kidney and have little to no reported effects on bladder smooth muscle. Results from clinical studies on non-peptide V_2-receptor agonists have not been disclosed.

Vasopressin V_2-Receptor Agonists - Reports of non-peptide mimics of vasopressin or vasopressin receptor agonists have recently occurred (17,18,21,25,27,29). These reports provide an important benchmark since they represent only the third instance where non-peptides have been discovered which mimic the effects of natural peptide ligands that activate their G-protein coupled receptors (7-GPCR) [cholecystokinin (CCK) and angiotensin (AT-1) being the first two (19,20)]. Thus far, there are no reported results from human clinical trials.

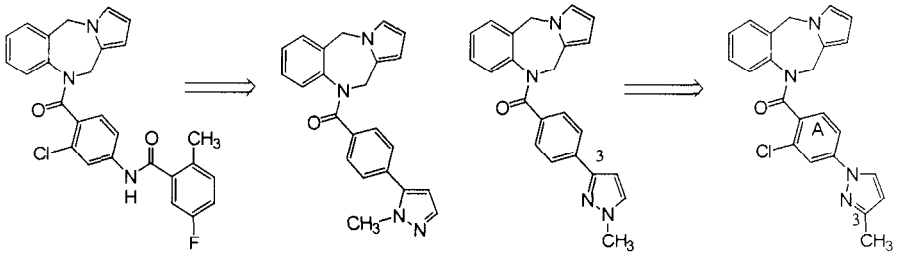


Discovery of these two compounds occurred independently within the same time frame through observations of their antidiuretic effects in *in vivo* models utilized in the search to find non-peptide V2 -receptor antagonists. A derivative of the nonpeptide V_2-antagonist OPC-41061 (**1**), the phenoxyacetyl derivative **2** showed an antidiuretic effect after oral administration using Brattleboro rats which are naturally deficient in AVP (rat V_{1a} IC50 = 5.1 uM; V_2 IC_{50} = 0.038 uM; female rats 2 hr at 1 mpk, urine volume = 0 mL versus 10 mL for control) (21). This observation stimulated a medicinal chemistry effort where the amido-tailpiece was shown to play an important role in agonist activity. As in the case of the angiotensin AT-1 non-peptide agonist ligands, there was a very strict limitation to substituent size. As a result of a pharmacokinetic study, the phenoxyacetyl moiety was found to be labile and the agonist activity was attributed to the parent aniline **3**. The conversion of a V_2-receptor antagonist into an agonist required that the terminal benzamide moiety of the antagonist be replaced by a small anilino group such as N-methyl **4**, pyrrolidinyl **5**, 3-methylpyrrolidinyl **6**, or a 5-membered heteroaromatic ring such as pyrazole **7**. Increasing the size of the amide substituent to n-butylamino **8** or piperidinyl **9** was deleterious to agonist activity. Substitution of a chloro or methyl group at the 2-position of the A-aryl benzamide moiety enhanced V_2-agonist–like activity. A chloro substituent at the 7-position of the benzazepine ring was of limited value.

The pharmacological profile of N-isopropyl derivative OPC-51803 (**10**) has appeared (22). OP-51803 (**10**) competitively displaced [^{3}H]-AVP from cloned human V_2 -and V_{1a}-receptors in a concentration dependent manner, although it minimally

displaced [^{3}H]-AVP from human V_{1b}-receptors at 100 μM. The Ki value for **10** for V_2-receptors was 91.9 nM and for V_{1a}-receptors was 819 nM. The EC_{50} for cAMP production for **10** was 189 nM. The dose response curve of **10** was shifted to the right with a selective V_2-antagonist at 1 μM and 0.1 μM. In male Sprage Dawley rats with normal AVP levels, OPC-51803 (0.03-0.3-mg/kg, p.o.) produced a dose dependent antidiuretic effect without changes in blood pressure; heart rate or inhibition of AVP-induced pressor responses were observed at 30 mg/kg, p.o. (23).

Non-peptide vasopressin mimetics were also discovered where the secondary amide bond on the tailpiece in the selective V_2-receptor antagonist WAY-VPA-985 (lixivaptan, **11**) was replaced with a five-membered heterocyclic ring intended to improve physical properties of the ligand. Initially, a mixture of pyrazoles **12** and **13** was tested (24). In a *in vivo* antagonist model, a decrease in urine volume was observed for the mixture of **12** and **13** rather than the expected increase elicited by antagonists. Separation of the mixture and structure activity studies resulted in the assignment of the majority agonist activity to regioisomer **13**. The resulting discovery program led to the development of WAY-VNA-932 (**14**) where the methyl group at the 3-position enhanced the antidiuretic activity over the unsubstituted pyrazole and that a methyl group at the 5-position greatly diminished the agonist-like effect (25,26).



11 **12** **13** **14**

The investigation of substituent effects in the tailpiece suggested that the free rotation of the pyrazole ring or its ability to adopt a coplanar relationship with the benzoate ring (A-aryl) was important for agonist-like activity. Other isosteric five membered heterocyclic rings such as pyrrole, triazole and imidazole were also effective (18). The *ortho*-chloro group in the A-aryl ring, which was found in WAY-VPA-985, imparts selective V_2- antagonist effects by weakening the interaction with the V_{1a}-receptor and directly enhances the V_2-agonist effects in **14** over the unsubstituted analog. Other effective ortho substituents in the A-aryl region include fluoro, bromo, methyl, trifluoromethyl groups. The pyrrole ring of thetricyclic headpiece may be replaced by pyridine (27). A model for agonist-like activity was proposed based on x-ray analysis of **14** (Figure 1) (26).

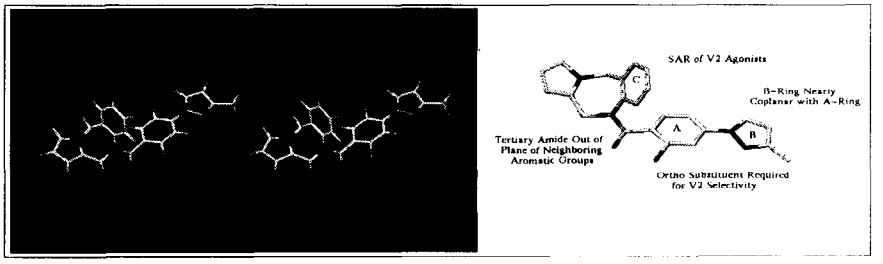
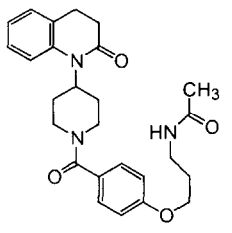


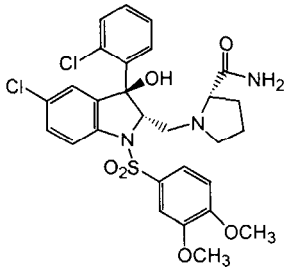
Figure 1.

A preliminary pharmacological profile was reported for **14** (25,26). Binding experiments in stably transfected CHO cells indicated that vasopressin and **14** competitively bind to V_2- and V_{1a}-receptors (**14** V_2 Ki = 39.9 nM, V_{1a} Ki = 465 nM; AVP V_2 Ki = 0.84, V_{1a} Ki = 0.22 nM). The agonist–like effects of **14** were observed *in vitro* by the stimulation of cAMP in LV_2-cells (EC_{50} = 0.72 nM). The agonist-like effect was reversed by the addition of the vasopressin receptor antagonist **15**. Oral efficacy was observed in Sprague-Dawley rats (ED_{50} = 0.14 mpk), conscious dogs (ED_{50} = 0.3 mpk), conscious monkeys (ED_{50} = 0.5 mpk) and in naturally AVP deficient Brattleboro rats (ED_{50} = 0.1 mpk).

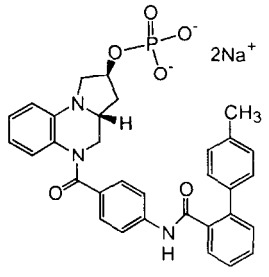
Vasopressin Receptor V_{1a}-Antagonists. The V_{1a}-vasopressin receptor has provided a challenge for discovery. The first non-peptide V_{1a}-receptor antagonist OPC-21268 (**15**) was discovered from random screening and chemical optimization was based upon the rat V_{1a}-receptor. While the human and the rat V_{1a}-receptor show 96% sequence identity the affinity of **15** for the human V_{1a}-receptor (IC_{50} = 880 nM) was significantly weaker for the rat V_{1a}-receptor (IC_{50} = 25 nM). The affinity differences for the rodent versus human receptors translated into poor to no therapeutic effects in human clinical trials. Since these first experiments, the human vasopressin receptors have been cloned and stably expressed in CHO cells (28). While the rodent *in vivo* models remain a convenient paradigm for drug screening, the strong evidence for species differences suggest that both human and rodent binding should be determined and that compounds with similar affinities in different species are more likely to have animal effects which are predictive of the human clinical state (29). The influence of species related amino acid differences was tested using site directed mutagenesis of rat to human for the V_{1a}-receptor and molecular modeling (30,31). A 27-fold increase in affinity for the human V_{1a}-receptor was observed for **15** for a glycine to alanine shift at residue 337. Molecular modeling provides a conceptual model of the binding sites and suggests that peptides and non-peptide agonists and antagonists bind to different regions on the receptor.



15



16



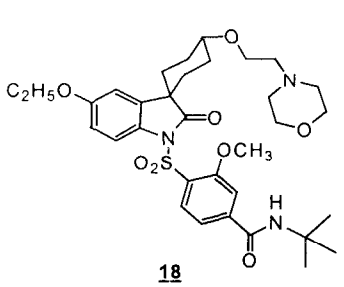
17

The Sanofi V_{1a}-receptor antagonist SR-49059 (**16**, Relcovaptan) remains the agent furthest advanced in development for this vasopressin receptor subtype. SR-49059 (**16**) is an effective antagonist of vasopressin V_{1a}-receptors when dosed orally. The only published clinical studies concerning relovaptan relate to its effects on the cardiovascular system and the uterus (15,32,33). A single 300 mg oral dose of **16** in hypertensive males was able to block AVP induced platelet aggregation and exerted a transient blood pressure reduction (34). SR-49059 (**16**) antagonizes vasopressin-induced contractility of myometrium and uterine arteries of non-pregnant womenin a dose-dependent manner. On the basis of these findings, orally active substances that inhibit vasopressin effects on uterine contractions by blocking V_{1a}-receptors, have demonstrated a therapeutic effect in dysmenorrhea (35,36). A literature description of the structure activity relationship has not appeared, however, a patent disclosure is available (37). In an alternate therapeutic indication,

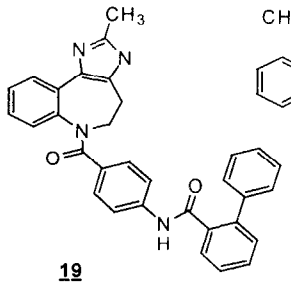
compound **17** was tested in rabbits by eye drop administration and showed a hypotensive effect in a dose dependent manner (16). The patent abstract suggests V_{1a}-receptor mediated effects, however, the corresponding alcohol has been reported as a selective V_2-receptor antagonist.

Vasopressin Receptor V_2-Antagonists. The largest compound equity lies in the V_2-receptor antagonist area. There are five compounds reported at some stage of clinical development ranging from either a selective V_2-receptor profile (WAY-VPA-985 (**1**, lixivaptan), OPC-41061A (**11**, tolvaptan), and SR-121463A (**18**)) to a mixed V_{1a}/V_2-receptor profile (YM-087 (**19**, conivaptan) and OPC-31260 (**20**)) (38-42).

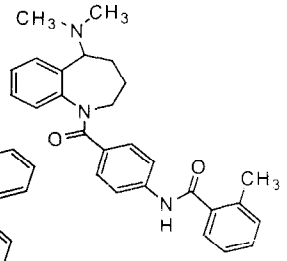
The animal models that measure urine volume and osmolality have been predictive of activity in man. The most recent collective synopsis of the clinical studies suggests that all of the compounds act as aquaretics by significantly increasing the excretion of free water, but their impact on the sustained control of hyponatremia, maintenance of blood volume, effects on endogenous AVP levels and improved morbidity and mortality is still unknown (43). In fact, the risk benefit ratio remains to be established (which is the charter of later stage clinical trials). While the clinical assessment of the individual compounds is underway combination therapies such as diuretics and selective V_{1a} - and V_2 –inhibitors are being considered (44).



18



19



20

There are two scaffolds for the vasopressin V_2-receptor ligands. The N-sulfonyl-indoline moiety has been used extensively and requires significant modification to achieve selective V_{1a} (**16**, SR-49059) versus V_2-receptor selectivity (**18**, SR-121463A) (45). The scaffold benzoylated benzazepine (or benzodiazepine) ring has been adopted by numerous groups and has been the predominant template for new derivatives. In this second scaffold, either selective V_2- or mixed V_{1a}/V_2- but not selective V_{1a}-receptor antagonists have been reported.

Based upon the single crystal X-ray structure of WAY-VNA-932 (**14**) and computer modeling, three-dimensional models have been generated which summarize the SAR for this series (Figure 2) (45). The benzazepine ring is puckered and the tertiary amide carbonyl is essential for activity. It connects the benzazepine headpiece to the tailpiece, thus providing a three-dimensional architecture to the molecule. The benzoylamido tailpiece controls the agonist-antagonist properties and V_{1a}-V_2-selectivity for the target compounds. The ortho substituent in the A-aryl group provides V_2-receptor selectivity by weakening the affinity of the ligand for the V_{1a}-receptor. Chloro, methyl and methoxy have been used with either the chloro or methyl groups being most efficient. Meta-substituents to the carboxyl group have little effect on selectivity but in the case of the methoxy analog improved physical characteristics were observed (54). The secondary amide moiety connecting the two aryl groups is coplanar to the A-aryl moiety. The ortho-substituent on the B-aryl group forces the phenyl group out of co-planarity with the carbonyl. Phenyl groups are accommodated at the ortho-position on the B-aryl moiety while the smaller methyl group is adequate. *Meta*- and *para*-substituents to

the carboxyl on the B-aryl group are accommodated and have limited impact on the target activities.

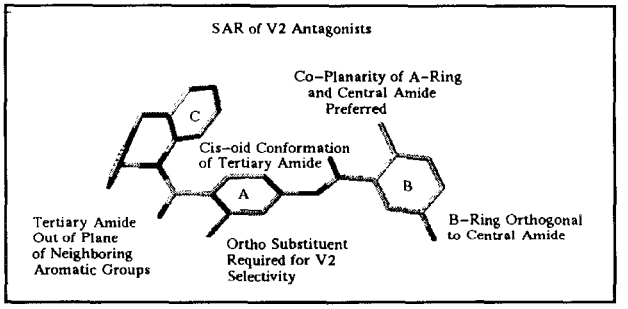
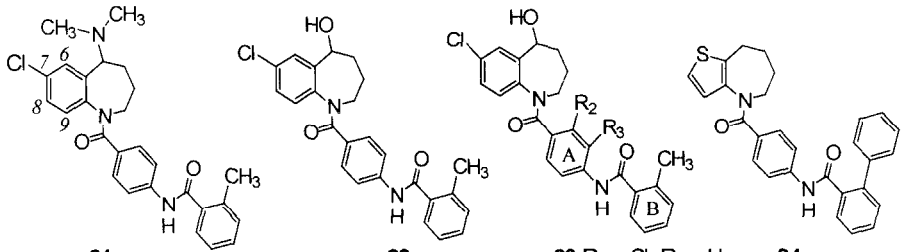


Figure 2.

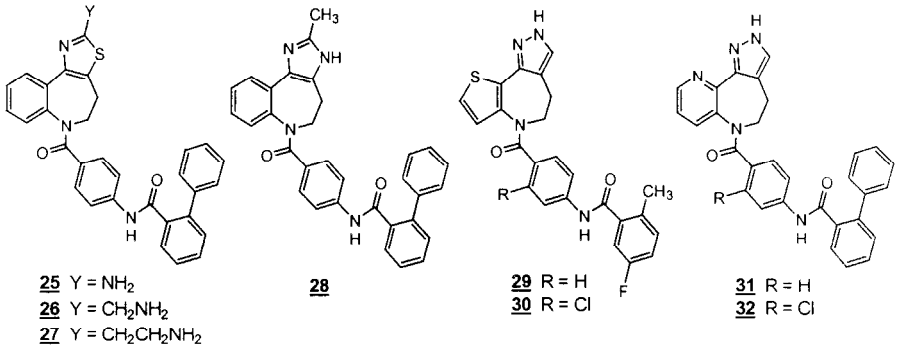
The sytematic transformation of OPC-31260 (**20**) into a mixed V_{1a}/V_2-receptor antagonist into OPC 41061 (**1**) provides exemplification into the structure activity relationships of this template (46,47). The introduction of a chloro substituent into the 7-position of OPC-31260 (**20**) to give **21**, increased the affinity at V_{1a}-receptor and had little effect on V_2-receptor affinity and urine volume. Substitution of a chloro group at the 6-, 8-, or 9-position had little effect on V_{1a}-receptor affinity and a deleterious effect on V_2-receptor affinity and urine volume. Replacement of the dimethylamino moiety in **21** with a hydroxyl group to afford **22** showed a marked improvement in the V_2-receptor affinity but not on the oral activity and selectivity compared to OPC-31260 (**20**). The addition of a 2-chloro **23** or 2-methyl group to the A-aryl group weakened the affinity for the V_{1a}-receptor and enhanced the affinity for the V_2-receptors. The selection of OPC-41061 (**1**) was based on the better selectivity for V_2-receptors and its potent oral activity (rat, ED_{50} = 0.54 mg/kg, p.o.).



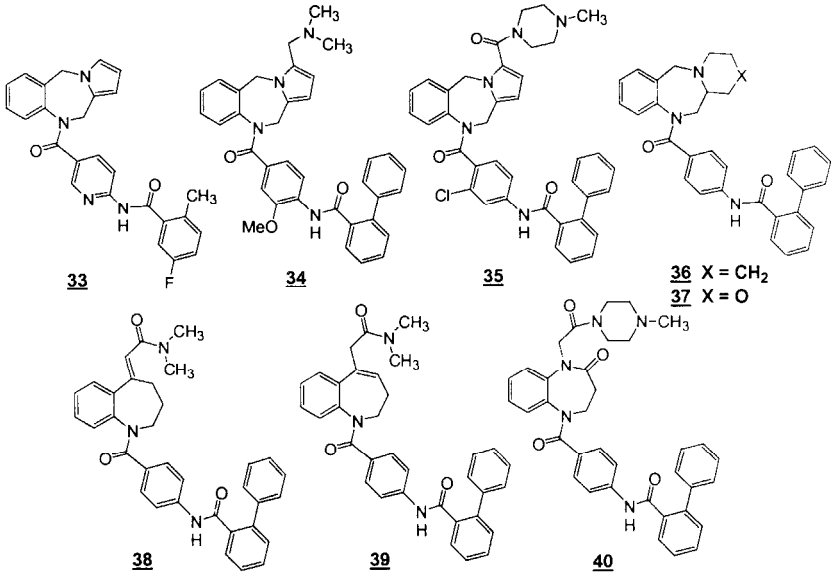
21 **22** **23** R_2 = Cl, R_3 = H **24**

The need for an azepine ring with a single fused aromatic moiety (benzazepine) can be expanded to heteroaromatic groups such as thiophene (thienylazepine) and is exemplified in **24** which has potent *in vitro* vasopressin receptor affinity (rat V_{1a} IC_{50} = 50 nM, V_2 IC_{50} = 300 nM) (48). The program that discovered YM-087 targeted compounds with strong and equal affinity for both the V_{1a}- and V_2–receptor subtypes. The attachment of a basic moiety either as part of or attached to the fused azole ring provided optimal potency (49). Thiazoles **25-27** with an amino or amino alkyl group attached to the 2-position of the azole ring and imidazole **28** were the most potent derivatives while maintaining an equal balance between V_{1a}- and V_2-receptor affinity. The effect of the chloro substituent in the tailpiece for V_2-receptor binding is exemplified in two related series the pyrazolo-pyridinoazepines **29** and **30** and the pyrazolo-thienylazepines **31** and **32** (50,51). While these compounds are less potent than the imidazobenzodiazepines, the addition of chloro substituents to **29** and **31** provided significant increases in selectivity, 8- and 20-fold,

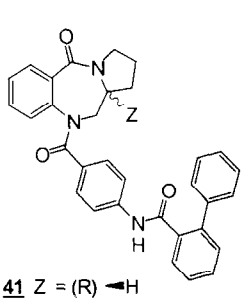
respectively for **30** and **32**, for the V_2-receptor versus the mixed V_{1a}/V_2-receptor activity.



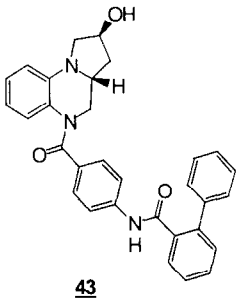
The search for an improved physical chemical profile was extended to WAY-VPA-985 (**11**). The substitution of a pyridine ring for the 3-chlorophenyl ring inWAY-VPA-985, did not improve aqueous solubility and resulted in compound **33** which has been characterized as a mixed V_{1a}/V_2-receptor antagonist (52,53). In a further attempt to improve aqueous solubility compounds **34** and **35** were reported (54). Compound **34** is the result of replacing the 3-chlorophenyl with a 2-methoxyphenyl and the addition of a dimethylaminomethylene group to the pyrrole. The two changes to the structure increased aqueous solubility (0.72 mg/mL, 30% PEG) but also produced a compound with only a five-fold selectivity for the V_2-receptor (vs. 200-fold for WAY-VPA-985) or effectively a mixed V_{1a}/V_2-receptor antagonist. Compound **35** attached a N-methylpiperizinylcarbonyl fragment to the pyrrolo-benzodiazepine ring and substituted the 2-phenyl for the 2-methyl group in the benzoyl tailpiece. Compound **35** retained the selective V_2-receptor profile, however, with four-fold weaker oral activity than WAY-VPA-985 (50). Substitution of the heteroaromatic ring in the pyrrolobenzodiazepines with a saturated heterocyclic ring such as piperidine **36** or morpholine **37**, produced significantly active compounds and provided a basic nitrogen atom to improve aqueous solubility (55).



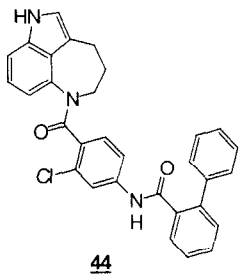
The attachment of a carboxamide moiety to the head piece is found in V_2-receptor agonist **10**, in the improved aqueous soluble pyrrolobenzo-diazepine **35** and also in the substituted benzazepines **38**-**40** (56,57). Restricting the aminocarbonyl group to the entgegen site by the introduction of the alkylidene group at the 5-position of the azepine ring was effective in increasing the binding affinities for **38** (V_{1a} pKi = 9.89; V_2 pKi = 10.32) (57). The lability of compound **38** to acid or base was seen in the conversion to the endocyclic olefin **39**. Compound **39** had significantly diminished affinity for the vasopressin receptor (V_{1a} pKi = 6.55; V_2 pKi = 9.09). One solution to the isomerization issue was provided with the preparation of 1,5-benzodiazepine rather than 1-benzazepine derivatives. The second nitrogen of the benzodiazepine ring removes the asymmetric center and the issue of the endocyclic double bond. Compound **40**, an N-methyl-piperizine derivative, was relatively selective for the V_2-receptor (rat V_2 IC_{50} = 2.9 nM; rat V_{1a} IC_{50} = 200 nM) and, in normal hydrated conscious rats, induced both an increase in urine volume and a decrease of urine osmolality in a dose-dependent manner. (1 to 10 mg/kg, p.o.). The net result of the latter studies suggests that while the chloro substituent on the A-aryl ring of the tailpiece improves V_2-receptor selectivity, the piperizinyl amide moiety present in **36** and **40** may also influence V_2-selectivity. In each of the later references, substitution of the A-aryl ring was not reported.



41 Z = (R) ◄H
42 Z = (S) ····H



43



44

An alternate modification of the pyrrolobenzodiazepine results in the pyrrolobenzodiazepinone **41** and **42**. The S-enantiomer **42** has five-fold weaker affinity for the rat V_2-receptor (IC_{50} = 1.18 nM) versus the R-isomer (IC_{50} = 0.216 nM) but was more efficaceous in rats upon administration at 30 mg/kg, p.o. (urine volume ED_{300} = 0.31 mg/kg, p.o. vs. 0.78 mg/kg, p.o. for **42**) in the urine volume assay (58). In the same report, ring contraction of the benzodiazepine ring in **41** produced the pyrroloquinoxaline **43** which has potent and selective rat V_2-receptor antagonism with potent oral activity (ED_{300} = 0.22 mg/kg, p.o.). Further pharmacological characterization of **43** suggest potent aquaretic effects via selective V_2-receptor antagonism (59,60). The selectivity is achieved and not tested without substitution in the A-aryl ring. Chemically reorganizing the pyrroloenzodiazepine ring results in indoloazepine **44** which results in vasopressin receptor antagonism, albeit with weaker affinity (61).

Results of the later stage clinical trials will provide evidence for the utility of vasopressin receptor modulators in the treatment of cardiovascular and related diseases.

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Chapter 17. Selective Androgen Receptor Modulators (SARMs)

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Introduction – Testosterone (T) is an endogenous ligand for the androgen receptor (AR) and is essential for the development and maintenance of the male reproductive system and secondary male sex characteristics (1). T also plays diverse physiological and pathophysiological roles in both men and women. T can be considered as a non-tissue-selective androgen since it has indiscriminate biological activities in both anabolic (bone and muscular) and androgenic (reproductive) target tissues. The separation of the anabolic from the androgenic activities of steroidal androgens was a major effort several decades ago (2). Indeed, anabolic steroids known to partially separate anabolic and androgenic activities have been available to physicians for many years but have undesirable safety profiles, especially the potential for hepatotoxicity (3,4). Androgen therapy (still mainly T preparations) has been used to treat a number of male disorders such as hypogonadism; however, unlike female hormone replacement therapy (HRT), it is not widely used as a male HRT largely due to its trophic activity for the prostate.

There is a growing interest in finding selective male hormones or antihormones for aging related disorders given the increase in life expectancy. Aging men experience a gradual decrease in circulating male hormones, which results in osteopenia, loss of lean body mass and reduced sexual function (5). Concomitantly, they experience an increase in the risk of benign prostatic hyperplasia (BPH) and prostate cancer which are androgen dependent (6,7). Tissue-selective androgen receptor modulators (SARMs) with agonist activity in most target tissues but the prostate and other SARMs with antagonist activity only in prostate are likely to fulfil important unmet medical needs for a growing aging population (8). Relative to the successful development and commercialization of selective estrogen receptor modulators (SERMs), the development of SARMs is in a very early stage. An understanding of the molecular basis of the profound physiological and pharmacological effects of androgens on their target tissues has grown rapidly during the last decade and has been helpful in the design of drug discovery efforts. This review will focus on the physiology and pathophysiology of androgens in major target tissues, the therapeutic potentials of SARMs, and the AR pharmacophores that have been reported in recent years. An earlier review of a related topic was previously written for this series (9).

Mechanism of AR Action – Androgens work primarily through the AR that is expressed in various tissues of the body (10). The AR is a member of the steroid/thyroid hormone nuclear receptor superfamily, which in humans has more than 30 members including the other steroid hormone receptors (estrogen (ER), progesterone (PR), glucocorticoid (GR), and mineralocorticoid (MR) receptors) as well as the receptors for many other non-peptide hormones such as retinoids, thyroid hormone, and vitamin D (11). The receptor protein consists of three main functional domains: the ligand-binding domain (LBD), the DNA-binding domain and the amino-terminal domain (12). The structure of the LBD, determined by X-ray crystallography of a number of different nuclear hormone receptors, is composed of 12 alpha helices and a small beta sheet. The helix H12 functions as a "lid" covering the ligand binding pocket and it is in the "closed" configuration when an agonist occupies the binding pocket and is "open" in the absence of a ligand or in the presence of an antagonist (13,14). To investigate ligand-binding specificity, a three-dimensional homology model of the human AR LBD

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was constructed based on the crystal structures of other closely related steroid receptors (15). Recently, the crystal structure of the human AR LBD complexed to steroidal androgen R1881 was determined and it reveals insights into the binding mode of androgens to AR (16).

The molecular pathway of AR action is characterized by several major events (17). After high affinity binding to the androgen in the target cell, the AR releases the heat shock proteins that were bound to the AR in the absence of a ligand, is phosphorylated, and translocates into the cell nucleus (18-21). The conformationally more compact ligand-receptor complex homodimerizes in an antiparallel fashion and binds to a regulatory DNA sequence within the promoter of target genes known as a hormone response element (22,23). This complex then recruits other proteins termed cofactors that bind at the amino-terminal domain or the LBD of the receptor to induce gene transcription (24). Regulation of transcription determines the levels of new proteins produced, and thus, regulations of numerous events such as cell function, growth, and differentiation.

The AR acts in a tissue- and gene-selective fashion and the mechanisms of the selectivity are just beginning to be understood. It is believed that specificity is dependent upon a combination of the regulation of AR expression, differential DNA binding at the promoter of regulated genes, and tissue specific protein-protein interactions (12,25). The level of AR in the cells of any particular tissue is regulated in response to multiple signals and the resulting AR expression profile can determine the response to various compounds. The amount of AR can also be regulated, changing in response to environmental stimuli. For example, it is known that AR expression in kidneys of rodents is upregulated after castration and downregulated by androgens. Two androgen regulated transcription start sites have been identified within the promoter of the AR gene and a number of hormones and growth factors are believed to modulate androgen effects on different target tissues through modulating AR expression (26). The identification of specific target genes and cofactors in different tissues or cells is another area through which a greater understanding of tissue-specificity is being pursued. A significant effort using these approaches concentrates on selectivity in prostatic tissue (27-29). Other pathways may also contribute to the androgen tissue specificity, such as cross-talk with other tissue-specific signaling components, non-genomic effects and heterodimerization with other receptors (30-32).

Therapeutic Potential – Currently, the prime uses of androgen are in the treatment of reproductive disorders and primary or secondary male hypogonadism (33,34). The clinical exploration of anabolic effects of androgens on non-gonadal disorders has also been documented, including bone disease, hematological disease, neuromuscular disease, rheumatological disease, wasting disease, and female androgen deficiency (35). The discovery and development of SARMs can provide tissue-specific molecules for a specific indication. For example, an ideal SARM to treat osteopenia or osteoporosis in elderly men would be a compound that is stimulatory in bone and muscle but has reduced activity in the prostate and sex accessory tissues (8). The therapeutic potential of SARMs should be expanded beyond the current androgen applications with better or total separation of the anabolic and androgenic activities of androgens.

Androgens and Bone – In contrast to estrogens, much less work has been done on the effects of androgens on bone, however, several pieces of evidence seem to implicate androgens in bone formation. T is responsible for the pubertal rise in bone mineral density (BMD) in males (36). In addition, cortical BMD is approximately 15% higher in men than in women and males with genetic androgen insensitivity have lower cortical BMD than normal men (37). Recently, two separate studies on healthy older men with low T levels indicated that bioavailable T, body mass and physical activity positively

correlated with femoral neck BMD (38,39). Accelerated bone loss (femoral neck BMD decline) occurs in prostate cancer patients with androgen deprivation therapy (chemical or surgical) (40,41). Androgen also plays a role in female bone metabolism. A two-year double-blind study in surgically menopausal women showed that estrogen/androgen combination therapy increased spine/hip BMD more than estrogen alone ($P<0.002$) (42). The addition of a weak androgen to estrogen treatment also enhanced bone protection in postmenopausal women (43). Women with complete androgen insensitivity syndrome (CAIS) still showed moderate deficits in spine BMD even with excellent compliance to exogenous estrogen (44).

It is unclear how much of the anabolic effect on bone is contributed directly by T or through its metabolites. Estradiol, converted from T by aromatase, is involved in maintenance of bone in men but the role of DHT (5α-dihydrotestosterone), reduced from T by 5α-reductase, is not fully established (45). One study concluded that skeletal muscle may be more important than estrogens/androgens in preserving bone mass in elderly men, which suggests an indirect effect of androgens through muscle (46). Bone is a dynamic organ and the adult skeleton regenerates completely every ten years through constant bone remodeling that is regulated by numerous factors and hormones (47-49). Loss of sex steroids increases the rate of bone remodeling by up-regulation of the production of both osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells). This effect is mediated by a variety cytokines, such as interleukin-6 and results in a transient acceleration of bone mineral loss by faster bone resorption than bone formation (50),. In addition, it leads to deeper erosion than normal by osteoclasts through the prolongation of the lifespan of osteoclast cells and the shorter lifespan of osteoblast cells (51,52). Androgens, including nonaromatizable ones, have identical effects to those of estrogen on the biosynthetic activity and the birth/death of bone cells *in vitro* and *in vivo*, at least in rodents (53). However, unlike the "antiresorptive agents" such as estrogens and SERMs (as well as bisphosphonates and calcitonin), androgens not only inhibit osteoclast formation but also increase cortical bone formation primarily through stimulation of perioateal bone formation (54,55). Along with parathyroid hormone, oxytocin analogues and statins, SARMs are viewed as novel pharmacotherapeutic approaches to stimulate anabolic effects in bone (56).

Androgens and Muscle – Androgens are responsible for the increased muscle size and strength in men relative to women. It has been reported that T replacement augments fat-free mass and muscle size and strength in hypogonadal men (57). Very high doses of T produce further increase in fat-free mass and strength in eugonadal men but the functional "quality" of the extra muscle was less than that of muscle gained through exercise (58). Women with abnormally high serum androgen levels due to polycystic ovary syndrome (PCOS) also show significantly higher lean body mass (59). Loss of muscle mass results in a syndrome termed "frailty", which refers to weakness, immobility and balance instability. Mechanism(s) and pharmacological interventions of age-related skeletal muscle impairment have gained considerable interest in recent years (60). It is generally believed that the T-induced increase in muscle size represents muscle hypertrophy, not hyperplasia (61). The AR is expressed in myocytes and androgens affect skeletal muscle size by controlling protein synthesis (10,62). The AR also mediates androgen-induced hypertrophy in cardiac myocytes (63). The best example of indirect effects through the neuromuscular junction is spinal and bulbar muscular atrophy associated with Kennedy's disease, which is apparently caused by AR mutations in the first exon, although the detailed mechanism has not been established (26). One study suggests that T upregulates insulin-like growth factor-1 (IGF-1) in muscle with or without stimulating growth hormone (GH) secretion (64). A mechanism mediated by antiglucocorticoid effects of androgens was also proposed (65). The androgen effects on muscular function may also be related to its effects on neuromuscular transmission (66).

Androgens and behavior – It is widely accepted that androgens play an essential role in male sex behavior. However, androgens have a much less influence on human behavior than animal behavior due to the significant impact of intrapsychic, social and cultural factors. A number of clinical studies of androgen replacement in castrated or hypogonadal men demonstrated effects on various aspects of sexual behavior (67). Only a fraction of the physiological level of T is needed for healthy men to maintain sexual function; supraphysiological doses of T did increase psychosexual stimulation but not sexual activity or spontaneous erection (68). The serum DHT concentration was also significantly correlated with orgasmic frequency in young healthy men (69). Physiological T levels in women are only 10% of those in the normal men and plasma T levels peak around the time of ovulation. Clinical studies have also shown that androgens enhance sexual behavior in women (44,70).

While androgens have a clear impact on behavior, certain behaviors can also affect androgen levels in humans. It was observed that sexual behavior caused T levels to rise in both men and women (71,72). Long-term extreme psychosomatic stress can result in a drop in T to hypogonadal levels. Physical exercise (5-30 min) normally results in increased T levels during exercise, independent of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion and decreased T levels to below baseline after exercising. Male athletes have lower androgen levels than untrained men in a resting state (73).

The published data demonstrates the positive correlation between androgen levels and cognitive ability in humans (67). Men often score better on tests of visual-spatial skills than women and enhancement of spatial skills was observed after T treatment in elderly men as well as in female-to-male transsexuals (74-76). The serum concentration of T, non sex hormone binding globulin (SHBG)-bound saliva T and DHT all positively correlated to superior skills on spatial tests (77). It was reported that T reduced neuronal secretion of beta-amyloid peptides in murine N2a cells and rat primary cerebrocortical neurons, suggesting a protective effect of T against the development of Alzheimer's disease (78).

T and human aggression has been extensively studied, however, the data are not consistent. In some studies, endogenous T levels positively correlated with aggressiveness in both men and women but no significant correlation was reported in other studies (79,80). Some reports show increased aggressiveness in anabolic steroids abusers, but no significant change of aggression was observed in men who received high-doses of T for potential hormonal male contraception (68,81). Assertive or aggressive behavior in turn can alter T levels. The relation of mood with T is not fully established. It is believed that sex hormone levels have an influence on mood, however, contradictory historical data were reported on the relationship between T and depression and elation (67). A recent randomized, double blind study showed that the andrenal precursors of steroid biosynthesis dehydroepiandrosterone (DHEA) and its sulfate, significantly enhanced self-esteem with a tendency for improved overall well-being in patients with Addison's disease (82).

The mechanisms of AR regulation in neuronal tissue remain unclear. Animal studies showed that the hypothalamus, hippocampus, the preoptic-septal region, and the limbic system are important target areas for sex steroid action (83). The AR is expressed in rat neurons and DHT upregulates neural AR in mice and rats (84-86). In the hamster, androgen was found to affect neural development in the hypothalamus and amygdala (87).

Androgens and the prostate – The prostate is a male sex accessory gland that secretes high levels of zinc, citric acid, cholesterol and a number of proteins and

enzymes that contribute to the human ejaculate. T plays an essential role in prostate growth, development, differentiation and function directly through the interaction of the more potent androgen DHT with the AR (88). Only the glandular epithelial cells in the prostate are androgen-dependent and they undergo apoptosis following androgen blockade which leads to a decrease in size of the prostate gland (89,90). Physiological T replacement in hypogonadal men dramatically increases prostate volume (34). BPH and prostate cancer are prostate conditions that occur with age in the presence of androgens. The decrease in total T, free T, or bioavailable T with aging might be the result of an age-dependent increase in the binding-capacity of SHBG (91). BPH and prostate cancer do not occur in men castrated early in life or men with 5α-reductase deficiency but there is no direct evidence that androgens cause BPH or prostate cancer. Young men rarely have BPH or prostate cancer even though T level peaks around age twenty. The promotion of prostate cancer risk by dietary fat is believed to occur through its effect on sex hormones (92). PSA is an androgen-dependent enzyme secreted by the prostate and is widely used as a marker in diagnosis, staging and post-treatment follow-up of prostate cancer (93). Unfortunately, it also rises with endocrine-independent progression of prostate cancer (94). Serum PSA is also detectable in women and has been proposed as a biochemical marker of androgen action in women (95).

Androgens and the hair and skin – Androgens are the major regulators of human hair growth and are responsible for the development of axillary and pubic hair in both sexes, and facial and chest hair in men. Elevated androgen levels inhibit hair growth in the scalp of both men and women. This occurs more frequently in men and is known as androgenetic alopecia or male pattern baldness. Studies attempting to link baldness with prostate cancer risk have been reported (96). Hirsutism is a female condition of development to male pattern hair growths and is associated with higher levels of androgens and/or greater sensitivity to normal levels of androgens (97). The paradoxical tissue-specific effects of androgens on the hair follicles of different body sites are poorly understood. Genetic factors play a role in hair growth as evidenced by the different hair growth patterns among families and races (98). Androgen effects on hair growth are mainly through DHT. Two isoforms of 5α-reductase have been identified and type 2 is linked with post-pubertal hair growth in males (9). Men with 5α-reductase deficiency have axillary and female pattern pubic hair but little beard growth, suggesting that T is responsible for the terminal pubic and axillary hair development. The androgen effects are mediated through the AR and patients with XY CAIS lack any body hair or baldness even with high circulating androgen levels (99). It was proposed that androgens act in the terminal hair follicle via dermal papilla cells by altering the production of regulatory paracrine factors, which then affect the activity of other follicle components (100). Androgen target genes were identified in the dermal papilla cells from the bald frontal scalp of stumptailed macaques and may reveal the mechanism of androgen inhibition of the follicular cell growth in androgenetic alopecia (101).

Sebum is the secretion of sebaceous glands located in hair follicles. Sebaceous gland development and sebum production are androgen-dependent processes that are also regulated by numerous other hormones (102). The type 1 enzyme is the dominant form of 5α-reductase in sebaceous glands and DHT is the active androgen (103). Sebum production is normal in the patients with 5α-reductase deficiency since they usually have a type 2 isozyme deficiency (104). Hypogonadal men may suffer from dry skin due to their androgen deficiency and teenagers may develop acne related to the elevation of circulating androgen levels at puberty (103).

Androgens and spermatogenesis – T is produced in the Leydig cells of testis and is capable of initiating, maintaining and re-initiating the formation of spermatozoa. The production of T is directly controlled by LH that in turn is controlled by the hypothalamic

gonadotropin-releasing hormone (GnRH). Another gonadotropic hormone FSH, which is also controlled by GnRH, is required for the full development of normal number of germ cells (105). The AR is found in different kinds of cells of the testis and T upregulates AR expression in these cells (106). It is assumed that T action in testis is mediated through DHT and it was observed that DHT and T had similar effects in stimulating spermatogenesis at comparable doses (107). However, 5α-reductase inhibition does not suppress spermatogenesis in young men (108). Estrogen also plays a role in spermatogenesis as aromatase inhibitors affect the development of spermatids in the monkey (109,110). T inhibits GnRH release as a feedback, which in turn interrupts LH and FSH secretion in the pituitary gland and thus reduces further T production. Exogenous androgens in healthy men can reduce GnRH release, which results in the suppression of spermatogenesis through both reduced FSH and testicular T. Many studies have used androgens (alone or in combination with a progestin or estrogen) as male hormone contraceptives (111). A monkey study reveals that T-induced inhibition of spermatogenesis is more likely the result of FSH reduction rather than the suppression of testicular androgen levels (112).

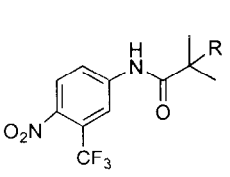
Androgens and Kidney – It is known that androgens stimulate renal hypertrophy and erythropoietin (EPO) production, which then influences the production of hemoglobin in bone marrow. Clinical data showed that prostate cancer patients developed anemia after receiving combined hormonal blockade (113). T replacement dramatically increased hematocrit in hypogonadal men from mildly anemic (with values in the female range) to mid-normal range in three months (34). Androgens were used to treat anemia caused by chronic renal failure before the introduction of recombinant human EPO and there is still an interest in the exploration of the potential use of androgens in dialysis patients (114). A study reported that androgens at pharmacological doses increased serum EPO levels in anemic patients with non-severe aplastic anemia and myelodysplastic syndromes but not in non-anemic patients (115). Animal studies suggest that T must be converted to DHT in renal cells to have biological activity (116).

Androgens and cardiovascular risk factors – Androgens can affect the cardiovascular system but not to the extent of the other target tissues discussed above. Clinical and animal data on androgen effects on cardiovascular risk factors are contradictory, perhaps since the results are often complicated by other factors, such as obesity and insulin sensitivity (117). Clinical studies suggest that normal levels of endogenous androgens have a neutral or even anti-atherogenic effect in men and in postmenopausal women (118-120). Exogenous T also has neutral effects on lower limb atherosclerosis (121). Age-related androgen declines in healthy men have no effect on lipids and a three-year T replacement in hypogonadal men resulted in no lipid changes (122,34). A recent six-week study in which hypogonadal men were treated with T gel resulted in a 15% decrease in all cholesterol fractions (123). AR was detected in human megakaryocytes and platelets suggesting that androgens may play a role in thrombotic disease (124).

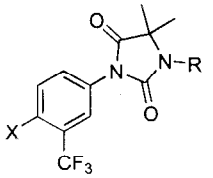
Steroidal Modulators – T is the safest steroidal androgen and has been widely used in treating androgen deficiencies for decades; however, there is currently no oral formulation because of its poor bioavailability. Intramuscular injections are available but suffer from T levels that fluctuate between supraphysiological and subphysiological doses (125). A number of preparations (T implants, scrotal and non-scrotal transdermal T) improve the efficiency of T delivery and maintain T levels closer to physiological concentrations (126). Recently, a T gel preparation has been developed and used in the treatment of hypogonadal men (123). In addition to treating androgen deficiencies in males, transdermal T has also been reported to improve sex function and psychological well-being in women, particularly those who have undergone ovariectomy and hysterectomy (70).

There are no new steroidal antiandrogens that have been reported as effective pharmacological agents since the last review of the subject in this series (9). In general, steroidal anti-androgens are non-selective for the AR, and decrease serum T levels by suppressing the formation of gonadotropins (127). The primary biological activity of cyproterone acetate (CPA), the most potent oral antiandrogen, is as a progestin that is formulated with ethinylestradiol as a contraceptive (Diane 35™). Diane 35™ has been used to treat female acne and hirsutism and the recent addition of finasteride improved the treatment of hirsute women in clinical studies (128).

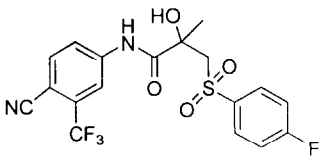
Non-steroidal AR modulators – Anilide analogues are the first scaffold of non-steroidal antiandrogens discovered, and three analogues are used therapeutically in prostate cancer patients (9,129). Antiandrogens are playing an evolving role in prostate cancer treatment and new approaches such as monotherapy and intermittent therapy are being developed (127,130-132). Flutamide (Eulexin, **1a**) was the first approved non-steroidal antiandrogen as a prodrug, and is given in combination with LHRH agonists. The active metabolite, 2-hydroxyflutamide (**1b**) is rapidly cleared so that it requires frequent dosing (3 times per day). Nilutamide (Anandron, **2a**) is administered once a day, but its side effect profile includes hot flashes, transient night blindness, and alcohol intolerance. Bicalutamide (Casodex, **3**) is an improvement over both flutamide and nilutamide in terms of its pharmacokinetics and side effect profile (133). The newer analogue RU 58841 (**2b**) was reported to show potent topical antiandrogenic activity in a macaque model of androgenetic alopecia without significant systemic effects, which suggests a potential topical application for androgen related skin disorders (134). Casodex is marketed as a racemic mixture where the (*R*)-enantiomer is the eutomer (135). A series of anilides derived from Casodex was recently disclosed and the achiral analogue **4** has excellent antiandrogenic activity *in vitro* (IC_{50} = 0.87 nM) comparable to Casodex (IC_{50} = 0.88 nM) (136).



1a R = H
1b R = OH

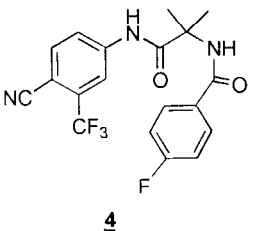


2a X = NO_2; R = H
2b X = CN; R = $(CH_2)_4OH$

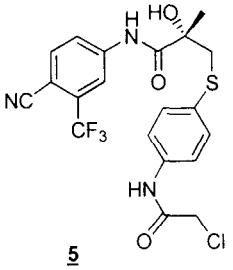


3

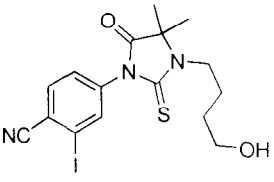
A series of AR agonists was recently discovered by modifying the anilide antagonist pharmacophore, which include in bicalutamide analogue **5** and nilutamide analogue **6** (137,138). Analogue **5**, with the (R)-configuration, demonstrated a 10-fold higher AR binding affinity than the (S)-enantiomer and showed moderate AR transcriptional ability (EC_{50} = 100 nM) as compared to DHT (EC_{50} = 1 nM) in a cotransfection assay. The agonist **6**, identified in the development of radiolabeled ligands to aid non-invasive imaging of prostate tumor cells, displayed high binding affinity to the rat AR and potent agonist activity, comparable to DHT, in a cotransfection assay.



4

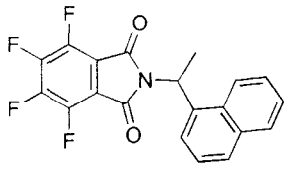


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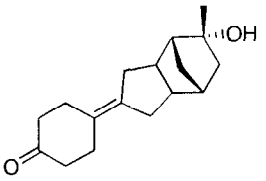


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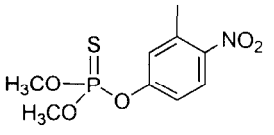
The phthalimide antagonist **7** evolved from a class of tumor necrosis factor-α (TNFα) inhibitors. At a 1 μM concentration, **7** antagonized T-induced chloramphenicol acetyl-transferase (CAT) reporter activity (T at 10 nM) to baseline (139). Interestingly, the stereochemical configuration determines whether these analogs have AR antagonist activity alone or AR antagonist along with TNFα inhibitory property; both possibilities could be beneficial for cancer treatment (139). Another scaffold **8** was designed to mimic DHT; however, it behaves as a weak antagonist (IC_{50} = 1 μM) *in vitro* (140). The organophosphate insecticide fenitrothion **9** demonstrated potent antiandrogenic activity similar to flutamide in a 7-week-old rat model (141).



7

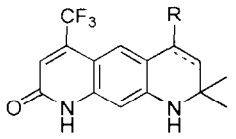


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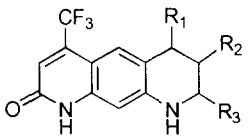


9

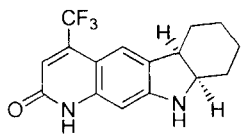
The linear tricyclic quinolinones were discovered as an entirely new AR modulator scaffold and their ability to modulate the transcriptional activity of the AR in a cotransfection assays depends on the substitution pattern of the third ring. Analogues **10a** and **10b**, with geminal dimethyl groups at C-8, antagonize half-maximal DHT-induced agonist response at ~30 nM, comparable to 2-hydroxyflutamide (IC_{50} = 43 nM) (142). Analogue **11a** (LG121071) with a 6-ethyl substituent demonstrates agonist activity (EC_{50} = 4 nM) and 100% efficacy relative to DHT (143). Analogues **11b** (LG121104) and **11c** have EC_{50}'s of 3 nM with 114% and 107% relative efficacy, respectively (144,145). Other modifications also generated a potent series of modulators, such as the indoline agonist analogue **12** (LG121100) (EC_{50} = 5 nM, 91% efficacy) (146), a coumarin agonist analogue **13** (EC_{50} = 1 nM, 138%) (147), and a quinoline antagonist analogue **14** IC_{50} = 9 nM, 83%) (148).



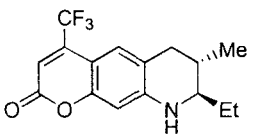
10a R =H
10b R =Me; $\Delta^{6,7}$



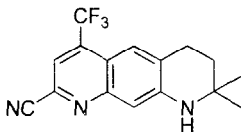
11a R_1 =Et; R_2 = R_3 = H
11b R_1 =H; R_2 =Et, R_3 = -trans-Et
11c R_1, R_2 = -cis-$(CH_2)_4$-; R_3 = H



12



13



14

The cross-reactivities of these novel AR modulators with other steroid hormone receptors were examined by using the cotransfection and competitive binding assays. Most of the potent analogues exhibit selectivity for AR relative to ER, MR, GR, and PR. The antiandrogens **10a** and **10b** were studied in a rodent model. At doses of 20 and 40 mg/kg po, both antagonists demonstrated the ability to inhibit the growth of the ventral prostate and seminal vesicles (sex accessory organs) in both immature and mature male rats with potencies and efficacies comparable to flutamide. Unlike Casodex and flutamide, there was no concomitant increase in serum LH or T levels with drug administration (142). An important *in vivo* proof of principle was achieved with the agonist analogue **11a** (143). In a 2-week LH suppression assay, both DHT at

1 mg/kg (s.c.) and LG121071 at 20 mg/kg (po) were able to restore LH to normal physiological levels.

Conclusion – Androgens contribute to the physiological differences between male and female and are also implicated in many pathophysiological occurences in both sexes. Advances in our understanding the mechanisms of androgen action and in the development of new drug discovery tools should make it possible to develop tissue-selective pharmaceutical agents to treat a variety of diseases or conditions that involve the AR for increased lifespan and improved quality of life.

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Chapter 18 . Antagonists of β_2 Integrin-Mediated Cell Adhesion

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Introduction - The β_2 integrins comprise a family of dynamically regulated cell adhesion molecules involved in the trafficking and activation of leukocytes. The critical role of β_2 integrins in health and disease has long been appreciated. Widely distributed within the immune system, the β_2 integrins participate in a broad spectrum of cell-cell and cell-matrix interactions that facilitate immune surveillance, trafficking to sites of inflammation, and resolution of infection. Work published through the year 2000 on the biology and chemistry of these proteins, including the discovery of novel inhibitors of their binding and function, is reviewed in this chapter.

BIOLOGY OF THE β_2 INTEGRINS

The β_2 integrins are large heterodimeric receptors that are defined by a common β_2 subunit (CD18) associated with unique, but homologous α chains (CD11a, CD11b, CD11c, CD11d). The members of this family include: (1) lymphocyte function-associated antigen-1 (LFA-1, $\alpha L\beta_2$, CD11a/CD18), (2) macrophage-1 antigen (Mac-1, $\alpha M\beta_2$, CD11b/CD18, CR3), (3) p150,95 ($\alpha X\beta_2$, CD11c/CD18, CR4) and (4) $\alpha D\beta_2$ (CD11d/CD18) (1).

LFA-1 is the most widely expressed of the β_2 integrins and is found on the surface of most leukocyte subsets (2). Originally appreciated for its role in cytolytic T cell killing, LFA-1 is now recognized as a key player in several homeostatic pathways including lymphocyte recirculation, trafficking to sites of inflammation, antigen presentation and T cell activation. The major ligands for LFA-1 include members of the intercellular adhesion molecule family: ICAM-1, ICAM-2, and ICAM-3 (3-5). The expression pattern of each ligand is distinct. In normal tissues, ICAM-1 is expressed at low levels on endothelial cells, epithelial cells, lymphocytes and monocytes. In the presence of various inflammatory stimuli, however, ICAM-1 expression is substantially upregulated (3,6). This, coupled with the finding that elevated levels of ICAM-1 are associated with numerous chronic human diseases (including multiple sclerosis, rheumatoid arthritis and psoriasis), supports a central role for this ligand at sites of inflammation (7-9). The expression of the other ligands for LFA-1 is more restricted. ICAM-2 is expressed constitutively on endothelial cells, platelets and mononuclear cells (10-12). Expression of ICAM-3 is restricted to leukocytes, with the highest levels on resting lymphocytes. Two additional members of the ICAM family, ICAM-4 and ICAM-5, support LFA-1 binding *in vitro*. ICAM-4 (LW blood group) is expressed on red blood cells (13). ICAM-5 is expressed specifically on neurons within the telencephalon (14). The physiological roles for these two ligands are currently unclear.

Mac-1 and p150,95 are predominantly expressed on cells of the myeloid lineage, although both have also been detected on subsets of activated T cells (2). Together these integrins play an important role in the innate immune response promoting trafficking, phagocytosis, and activation of monocytes, neutrophils, and mast cells. One of the hallmarks of Mac-1 biology is the rapid release of intracellular stores of Mac-1 following exposure to various inflammatory stimuli (15,16). In contrast to LFA-1, Mac-1 binds a diverse array of ligands including members of the immunoglobulin superfamily (ICAM-1)(17, matrix components (fibrinogen and factor X), microbial products, and proteins of the complement pathway (C3bi) ((17-23).

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Antigen p150,95 has been studied less extensively than Mac-1. This integrin binds components of the extracellular matrix (fibrinogen, collagen) and the complement system (C3bi) (24-26). The sharing of some ligands and their similar cellular expression pattern suggests that Mac-1 and p150,95 may have overlapping functions.

$\alpha D\beta_2$ is the most recently identified member of the β_2 integrin family. This receptor is expressed at low levels on subsets of neutrophils, monocytes, eosinophils, and lymphocytes, but is dramatically upregulated on certain specialized cell types such as macrophage foam cells in atherosclerotic plaques (27). ICAM-3 and VCAM-1 have been identified as ligands for $\alpha D\beta_2$ (27-29).

Patients with leukocyte adhesion deficiency type I (LAD I) have provided unique insights into the biology of human β_2 integrins (30-33). As a result of discrete mutations in the CD18 gene, the leukocytes in these patients fail to express normal levels of β_2 integrins. These patients suffer from recurrent, potentially life-threatening microbial infections, delayed wound healing, and persistent granulocytosis. At sites of infection, LAD neutrophils are notably absent despite elevated levels of neutrophils in the blood. This apparent paradox is explained by *in vitro* analyses demonstrating severe defects in adhesion, chemotaxis, and superoxide production in these cells. Although evident, defects in the lymphocyte compartment are much less severe and include a decrease in proliferation and cytotoxicity *in vitro* and a decrease in antibody production *in vivo*. Responses to viral infections are adequate in these patients and no loss of delayed-type hypersensitivity responses is observed, suggesting that other adhesion molecules are capable of compensating *in vivo* for the absence of LFA-1. The recapitulation of the LAD I phenotype in CD18 null mice further underscores the importance of the β_2 integrin family in homeostasis (34).

While important for defining the role of the β_2 integrins in development and disease, LAD I patients and CD18 deficient animals do not allow an assessment of the individual contributions of β_2 integrin family members. Towards this end, mice deficient in either LFA-1 ($CD11a^{-/-}$) or Mac-1 ($CD11b^{-/-}$) have been generated (33,35-41). In marked contrast to the CD18 null mice, neither the LFA-1 nor the Mac-1 deficient mice develop persistent bacterial infections. This suggests that the LAD I phenotype likely results from the simultaneous loss of more than one β_2 integrin or that compensatory changes occur in the single knockout animals. Despite this difference, LFA-1 and Mac-1 deficient mice exhibit distinct functional defects. Studies of numerous, independently derived, LFA-1 deficient mouse lines confirmed the role of LFA-1 in mediating homotypic aggregation, cytotoxicity, adhesion, and activation *in vitro*. The central role of LFA-1 in lymphocyte recirculation was also confirmed in two independently derived $LFA\text{-}1^{-/-}$ lines. LFA-1 deficient neutrophils also showed defects in adherence to ICAM-1 and endothelial monolayers, similar in magnitude to those observed with CD18 deficient neutrophils. The correlation of this *in vitro* effect with diminished neutrophil trafficking in an air pouch model of inflammation provides evidence for a broader role of LFA-1 in leukocyte trafficking and is supported by studies revealing a decrease in the number of infiltrating neutrophils in a thioglycolate-induced model of peritonitis. In a model of LPS-induced skin inflammation, however, LFA-1 played a minimal role in trafficking suggesting that the involvement of LFA-1 in neutrophil extravasation may be stimulus/site dependent or alternatively, may reflect differences in independently derived LFA-1 deficient mouse lines. Delayed-type hypersensitivity interactions were diminished in two LFA-1 null lines and unaffected in a third further illustrating the potential role of stimulus and mouse strain-dependent effects. One of the most striking features of LFA-1 deficient animals was their diminished capacity to reject tumors and control their metastatic spread. Similar to LAD patients, LFA-1 deficient animals showed normal cytotoxic/anti-viral responses *in vivo*.

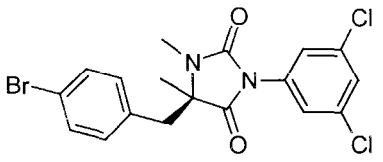
Mac-1 ($CD11b^{-/-}$) deficient mice have also provided insights into the particular role of this integrin in immunity. *In vitro*, defects in neutrophil adhesion, phagocytosis, and oxidative burst are evident. *In vivo*, Mac-1 deficient animals display a striking phenotype. In thioglycolate and air pouch models of inflammation, increased numbers of neutrophils accumulate as a result of diminished apoptosis. Though increased in number, these neutrophils are functionally defective in phagocytosis and elicitation of oxidative burst and, one study links these deficits to the changes observed in the apoptotic index. In an independent study, neutrophil trafficking to implanted fibrinogen-coated discs was unaffected by the absence of Mac-1. Adhesion and degranulation, however, were markedly diminished. Thus, Mac-1 deficient neutrophils were capable of entering sites of inflammation, but were severely compromised in adhesive and respiratory functions. In an animal model of nephritis, Mac-1 depletion protected against the development of proteinuria (42). Protection was also seen in a mast-cell dependent model of acute septic peritonitis supporting a role for Mac-1 in mast cell function (43).

Given their respective roles in leukocyte trafficking and activation, members of the β_2 integrin family have long been considered attractive targets for therapeutic intervention. At present we are beginning to see these concepts tested in humans. A phase II clinical trial with the humanized anti-CD11a monoclonal antibody hu1124 has provided some validation of LFA-1 as a target for psoriasis (44). Ongoing clinical studies with monoclonal antibodies against CD11a, CD11b and CD18 will soon provide additional insights into the role of these integrins in other human diseases including ischemic stroke, hemorrhagic shock, and kidney transplant.

INHIBITORS OF β_2 INTEGRIN-MEDIATED CELL ADHESION

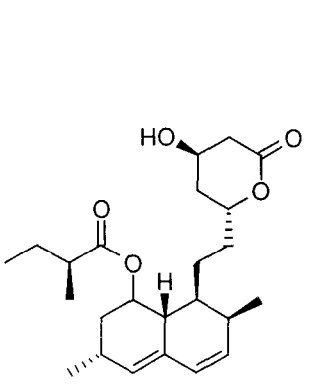
The biological activity of β_2 integrins is tightly regulated. Affinity modulation by divalent cations, activating antibodies, and inside-out signaling dramatically affects ligand binding as do increases in receptor clustering/avidity (45-54). Several domains within the $\alpha\beta$ heterodimer participate directly or indirectly in ligand binding. Most notable among these is the inserted or I-domain of the α subunit. Crystal structures for LFA-1 and Mac-1 I-domains reveal a dinucleotide binding fold with a metal ion-dependent adhesion site or MIDAS (55,56). Amino acids within and adjacent to the MIDAS coordinate Mg^{+2} and provide key contacts for ligand engagement (56-61). At the cell surface, the I-domain extends from a β-propeller domain that is essential for the biosynthesis of intact heterodimer (62-65). Recently, several lines of evidence have led to a model of LFA-1 affinity modulation in which the relative position of the β-propeller and the I-domain are controlled by conformational changes emanating from the C-terminus of the I-domain (66-69). Several of the antagonists discussed below block LFA-1 function by binding to this region of the protein.

There have been numerous reports of novel β_2 integrin inhibitors over the past few years. This review will cover three broad classes of inhibitors, including (1) direct antagonists of β_2 integrin binding to ligand, (2) small molecules that act indirectly by modulating the cell surface expression or activation state of these integrins, and (3) inhibitors that suppress expression of β_2 integrin ligands.

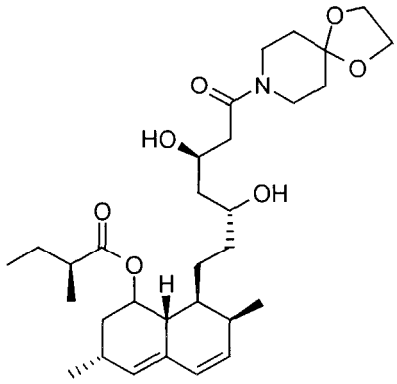


1

Selective Antagonists of LFA-1 Binding - Three structurally distinct classes of molecules have been reported as antagonists of the LFA-1/ICAM protein-protein interaction. These molecules all bind to an allosteric site on the LFA-1 I-domain opposite from the residues known to support ICAM-1 binding. In a molecular binding assay, compound 1 (BIRT0377) antagonizes ICAM-1 binding to LFA-1 with a Kd of 26 nM, but does not block ICAM-1 binding to the closely related Mac-1 (70-71). This compound inhibits the adhesion of activated T-cells (SKW3 cells) to immobilized ICAM-1 (IC_{50} = 2.6 μM) and also blocks superantigen-induced IL-2 production *in vitro* (IC_{50} = 0.85 μM) and *in vivo* (64% inhibition at 50 mg/kg, p.o.). The binding of 1 to LFA-1 affects the conformational equilibrium of LFA-1 such that the protein is locked into a low-affinity state, strongly supporting the hypothesis that it is acting via an allosteric mechanism (72). The hydantoin core serves to orient the two aromatic rings into lipophilic pockets at the C-terminus of the LFA-1 I-domain. Binding data indicates that both rings are essential for binding and little variability in the dichlorophenyl ring is tolerated. The bromobenzyl group occupies a more promiscuous site but there is still the need for the aromatic component and little tolerance for polar functionality.



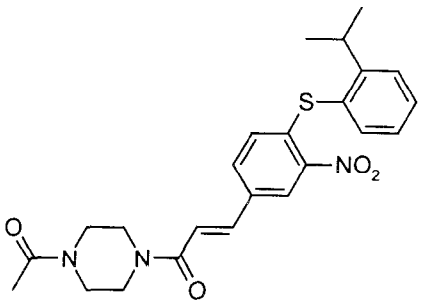
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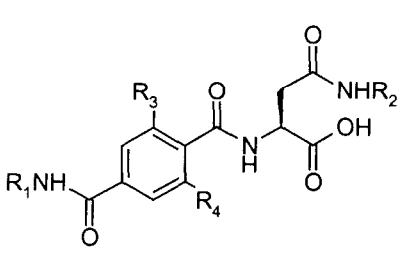
Compound 2 (lovastatin) also binds to the same region on LFA-1 and inhibits an LFA-1/ICAM-1 binding assay with an IC_{50} of 2.4 μM (73-75). Originally, it was thought that this HMG-CoA reductase inhibitor might be hydrolyzed to the seco-acid form to provide a ligand for the Mg^{+2} of the MIDAS containing region. However, the hydroxy acid had an IC_{50} of 14.1 μM in the binding assay, thus negating this hypothesis. Subsequent analyses by both NMR and X-ray crystallographic techniques localized the binding of lovastatin to the C-terminal regulatory site on the I-domain. SAR studies have not been published on this series but patent applications focus largely on derivatives in which the lactone has been replaced. An example is compound 3 that is reported to inhibit a mouse model of thioglycolate-induced peritonitis at 0.1 mg/kg (s.c.). Binding data are not available for many of these compounds, but presumably these changes have been designed

to increase binding to LFA-1 while minimizing the HMG-CoA reductase activity of the parent compound.

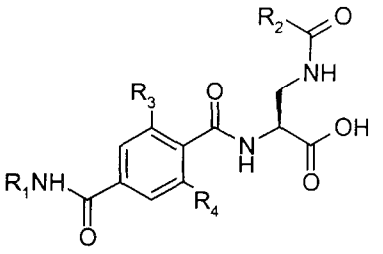


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Compound **4** (A-286982) exemplifies a third class of compound that binds at the I-domain C-terminus. In an LFA-1/ICAM-1 binding assay, this compound has an IC_{50} of 44 nM, and was optimized from a 1.7 μM screening hit (76-77). The compound has a potency of 35 nM in a B cell (JY) homotypic aggregation assay. Compound **4** has poor oral bioavailability, a feature reportedly corrected in later series derivatives. SAR studies on this series demonstrated the common requirement of electron-deficient aromatic rings and a high degree of lipophilicity for optimal affinity to this binding site.

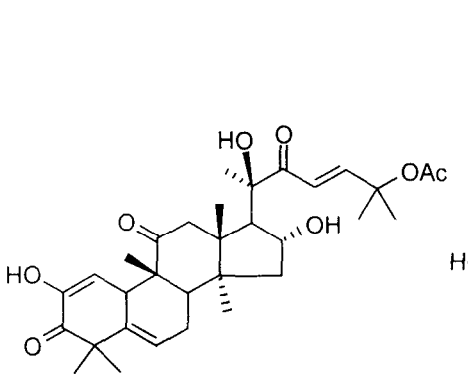


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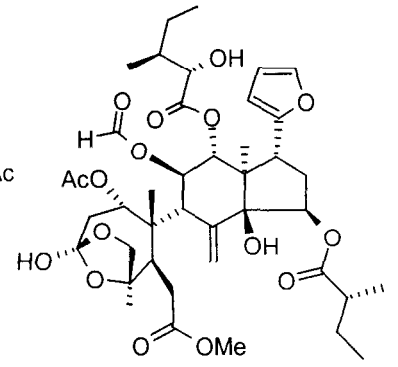


6

Dual Antagonists of LFA-1 and Mac-1 Binding - In the β_1 integrin field, several laboratories have had success in designing small molecules by exploiting peptide epitopes (e.g., R-G-D and L-D-V) known to mimic a portion of the binding interface between β_1 integrins and their ligands. A similar approach has been taken for β_2 integrins, taking advantage of the critical interaction between Glu-34 on ICAM-1 and the Mg^{+2} of the LFA-1 MIDAS domain. This approach has yielded compounds such as **5** and **6** which block both LFA-1 and Mac-1 binding to ICAM-1 (78). Several hundred derivatives are reported in this patent application. Compound **5** (R_1 = $CH(CH_3)$-Ph(3-OH); R_2 = H; R_3 = Cl; R_4 = H) had an IC_{50} of 10 nM in the Mac-1/ICAM-1 binding assay and an IC_{50} of 14 nM in the LFA-1 binding assay Compound **6** (R_1 = CH_2-Ph(3-OH); R_2 = 2-thienyl; R_3 = Cl; R_4 = H) is reported to have an IC_{50} of 1 nM in a Mac-1/ICAM-1 binding assay. No data is available on the effect of these compounds on Mac-1 binding to its other ligands such as C3bi.



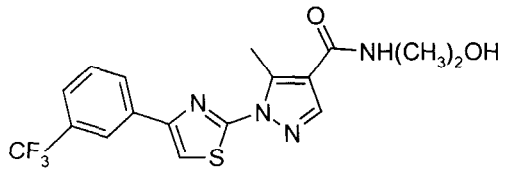
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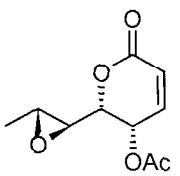
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Compounds Detected Through Cell-Based Adhesion Assays - There have been several reports of compounds that block leukocyte cell adhesion by undisclosed mechanisms. These compounds were discovered using cell-cell or cell-isolated protein adhesion assays and little data is available on them. These assays all require external activation of the cells. While it is possible that some of these compounds are directly antagonizing β2 integrin/ligand binding, the more likely scenario is that they interfere with signaling events or cytoskeletal rearrangements that are required for integrin activation or expression of integrin ligands.

Compounds **7** and **8** were identified through a natural products screening program (79,80). These compounds inhibit the binding of LFA-1 expressing JY cells to ICAM-1 expressing HeLa cells with IC_{50} values of 180 nM and ~25 nM, respectively. A follow-up study on compound **7** indicated that that this compound was mediating its effect through LFA-1 in this cell-cell assay. No further information is available on compound **8**.

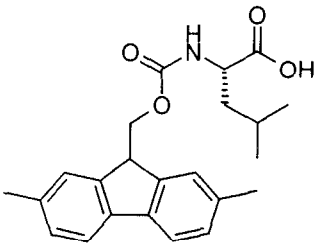


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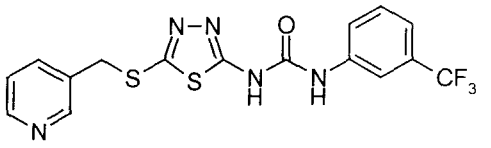


10

The thiazole **9** (RWJ-50271) is a very selective inhibitor of LFA-1 interactions (81). The compound has an IC_{50} of 5μM in assays measuring the binding of HL60 cells to ICAM-1. Interestingly, the compound does not block Mac-1 expressing cells from binding to rICAM-1. Since **9** does not affect LFA-1 expression, these data indicates that it may acting as an inhibitor of LFA-1 activation. This compound is active in a mouse DTH model (>50% inhibition at 50 mg/kg, p.o.). Compound **10** (asperilin) also blocks the β_2 integrin-mediated binding of HL60 cells to rICAM-1 (82) with an approximate IC_{50} of 12.5 μg/mL. No effect is seen on VLA-4- or E-selectin-mediated binding.

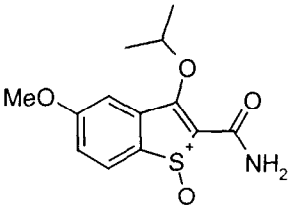


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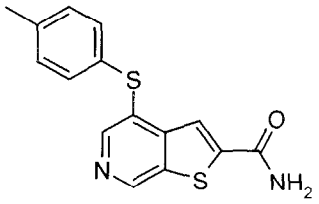


12

Compound **11** (NPC-15669) is a member of the leumedin class of antiinflammatory agents (83). These compounds block the upregulation of Mac-1 on neutrophils and inhibit the stimulation-dependent adherence of neutrophils to HUVECs. Compound **11** has been shown to have anti-ischemic properties in animal models. The thiadiazolyl urea **12** blocks both Mac-1- and LFA-1-transfected CHO cells from binding to rICAM-1 in SPA assays with IC_{50} values of 1.3 μM and 0.2 μM, respectively (84-85). The compounds are also active in cell aggregation assays. It is unclear whether the series interferes with the intracellular activation processes or with the extracellular binding events.



13



14

Antagonists of β2 Integrin Ligand Expression - In addition to blocking β_2 integrin binding or activation, it is also possible to modulate β_2 integrin-mediated cell adhesion by preventing the expression of inducible ligands. For example, an antisense agent directed against ICAM-1 is currently undergoing clinical trials for Crohn's disease (86). Compounds **13** (PD 144795) and **14** (A-205804) also inhibit the expression of several integrin ligands presumably through interference with the NF-κB pathway (87-89). Compound **13** inhibits the TNF-α induced expression of ICAM-1, E-selectin and VCAM-1 on endothelial cells with IC_{50} values in the 4 to 10 μM range. Northern blot analysis confirmed a reduction in mRNA for these endothelial adhesion molecules. The *S*-enantiomer possesses most of the biological activity. Compound **13** was orally active in several rodent models of inflammation.

Compound **14** is a member of a large class of compounds that also inhibits ICAM-1 and E-selectin expression (IC_{50} = 140 and 45 nM, respectively). VCAM-1 expression is inhibited to a much lesser degree (>4 μM for **14**) providing an interesting level of selectivity for this series. SAR has been published on this series indicating the need for proper thienylpyridine isomer as well as the amide in the 2-position of the ring for optimal potency. The 4-methylphenyl substituent is amenable to replacement and substitution, however both the aromatic and lipophilic

components appear to be desired. Initial PK data show that compound **14** has poor exposure following oral dosing in rats and that the amide and sulfide are primary sites of metabolism.

Summary - The biology of the β_2 integrins suggests that they could be attractive targets for the intervention in autoimmune and inflammatory diseases. In the short term, results from the clinical trials using biological reagents should provide critical insights into the validity of this hypothesis. The recent progress in the discovery of small molecules that interfere with β_2 integrin activation and function provides new reagents for probing the role of these proteins *in vitro* and *in vivo*. Furthermore, the discovery of an allosteric binding site on LFA-1 provides unique insights for understanding how small molecule antagonists might effectively disrupt the interaction of a large cell surface receptor and its ligand.

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Chapter 19. DPP-IV Inhibition and Therapeutic Potential

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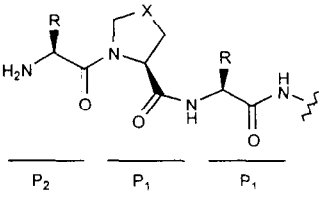
Introduction - Dipeptidyl peptidase IV (EC 3.4.14.5; DPP-IV) is a widely distributed serine protease initially employed as a cell surface marker enzyme for studies of membrane protein turnover, glycosylation events, membrane polarization, and organ-specific differences in the regulation of protein expression (1-7). Interest in DPP-IV, especially its serine protease function, has significantly increased as a consequence of the evolving role for DPP-IV as a modulator of bioactive peptides involved in endocrine, neuroendocrine and immune functions (8-14). DPP-IV's structural characteristics, proteolytic substrate requirements, and the profile of key and new inhibitors of its catalytic function will be discussed. In addition, the current understanding of the biological profile and potential therapeutic value of DPP-IV inhibition will be reviewed.

Structural characteristics - The structure and remarkable stability of DPP-IV have been extensively studied (10,15-18). With a half-life of more than 96 hr, DPP-IV is constitutively expressed by a variety of cell types, particularly on differentiated epithelial cells of the intestine, liver, prostate tissue, corpus luteum, and kidney proximal tubules as well as leukocyte subsets, such as T-helper lymphocytes and subsets of macrophages (19). The DPP-IV gene and its associated cDNAs from human, mouse, and rat tissue have been cloned (20-22). A single gene encoding DPP-IV has been localized to human chromosome 2 (2q24.3) and encodes two mRNA species sized at approximately 2.8 and 4.2 kb which are present in most tissues producing DPP-IV (23,24). The DPP-IV mRNA steady-state levels parallel protein expression. The protein sequence of the human, mouse, and rat DPP-IV enzymes are highly conserved, with >85% sequence identity between the three species (25). As a highly glycosylated type 2 membrane glycoprotein, DPP-IV is composed of two identical subunits of approximately 110 kDa molecular mass. The deduced structure of human DPP-IV spans 766 amino acids and produces a protein with a molecular weight of 88.3 kDa. The predicted DPP-IV structure provides six highly conserved cytoplasmic amino acids, a 22 amino acid hydrophobic transmembrane domain, and a 738 amino acid extracellular domain (26). The extracellular region can be divided into 3 regions the N-terminal glycosylated region starting with a 20-amino acid flexible stalk region, a cysteine-rich region and a 260 amino acid C-terminal region containing the catalytic triad. A soluble form of DPP-IV from human serum has recently been purified and characterized and is identical in sequence and function to the membrane-bound human kidney form except that it lacks the transmembrane domain and starts at the N-terminal Ser^{39} (27). The C-terminal regions (residues 625-752) of the human and rat sequence are completely conserved (25). The consensus sequence for the active site serine residue in humans is Gly-Trp-Ser^{630}-Tyr-Gly and correlates with the consensus sequence GXSXG proposed for serine proteases (28). As a member of the prolyl oligopeptidase (POP) and α/β hydrolase-fold serine protease family, DPP-IV possesses a novel orientation of its catalytic triad residues (Ser-Asp-His) (23); inverse to that found in classical serine proteases (His-Asp-Ser) (29,30). In the absence of an x-ray structure, a homology model using the recently published x-ray structure of propyl oligopeptidase (POP) has been proposed (31).

Proteolytic Substrate Requirements - DPP-IV is a highly specific aminopeptidase that cleaves Xaa-Pro, and to a lesser extent, Xaa-Ala dipeptides from the amino terminus

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of natural and synthetic peptides (9,32). To a much lesser extent, the P_1 site can also accommodate unnatural amino acids, such as hydroxyproline, dehydroproline, or pipicolic acid (33-36). DPP-IV substrates require a protonatable 1^o or 2^o amine at the P_2 site, a *trans* configuration for the amide bond connecting the P_1 and P_2 site, L-chirality at the P_2 site when P_1 is proline, and a $P_{1'}$ group which is not proline or hydroxyproline (37). Almost any amino acid can occupy the P_2 position. However, those containing a hydrophobic group are preferred. In the case of alanine at P_1, (R)-amino acids at P_2 are allowed. The proline ring can tolerate an oxygen or sulfur (X = O, S) without loss of activity (36). In addition to the classical serine protease mechanism, a pathway which invokes a stabilized tetrahedral intermediate through the formation of an oxazolidine ring with the P_2-P_1 peptide bond has been proposed (38,39). Conventional synthetic substrates like Gly-Pro-pNA, as well as other Xaa-Pro-pNA or Xaa-Pro-AMC dipeptides have been used as tools to determine DPP-IV activity (39-41). The simple tripeptides Ile-Pro-Ile (diprotin A) and Val-Pro-Leu (diprotin B) are competitive substrates with a low turnover rate (42).



THERAPEUTIC POTENTIAL OF DPP-IV INHIBITION

With its unique substrate specificity and localization as an ectoenzyme at the plasma membrane, DPP-IV plays an important role in initiating N-terminal degradation of a number of neuropeptides, peptide hormones and chemokines with N-terminal Xaa-Pro and Xaa-Ala. However, most studies reporting cleavage of synthetic peptide substrates are under *in vitro* conditions, making predictions of a physiological role for DPP-IV in the control of biological activity of most substrates speculative. A number of recent reviews support divergent roles for DPP-IV in the modulation of biological activity of a variety of substrates (9-11,43). Indeed, DPP-IV-mediated cleavage of peptide hormones has been shown to lead to total inactivation, partial inactivation, altered receptor affinity, or no alteration of biological function, depending on the substrate being investigated. This potential for synergistic and antagonistic interplay between the processed substrates, along with the difficulties associated with detection and quantification of small DPP-IV-processed substrates, has hampered efforts to establish a precise role for DPP-IV in any given process.

Attempts to determine the therapeutic potential of inhibiting DPP-IV activity *in vivo* have been further complicated by a multiplicity of enzymes reported to exhibit DPP-IV-like activity; including quiescent cell proline dipeptidase (QPP), dipeptidyl peptidase-IVβ (DPP-IVβ), dipeptidyl peptidase-8 (DPP-8), fibroblast-activation protein (FAP or CD8) and dipeptidyl peptidase II (DPP-II) (44-51). DPP-IV deficient or knockout animal models have been valuable in establishing the role for DPP-IV in individual pathways. Both a DPP-IV-deficient rat sub-strain (DPP-IV negative Fischer 344 rats) and a DPP-IV knockout mouse are fertile and healthy (52,53). Biological function and turnover of four Xaa-Ala peptide substrates have been investigated in these models, including two of the most potent insulinotropic agents presently known, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), an intestinal growth factor, glucagon-like peptide-2 (GLP-2), and growth-releasing hormone (GRH). Recent literature on the potential therapeutic value of DPP-IV inhibitors has focused mainly on GLP-1 and GIP as the rationale for use of DPP-IV inhibitors in type 2 diabetes.

Type 2 diabetes - GLP-1 stimulates glucose-dependent insulin secretion, inhibits glucagon release, slows gastric emptying, promotes growth and differentiation of β-cells, and stimulates insulin gene expression and biosynthesis (54-58). Numerous studies support a rate-limiting function for DPP-IV in inactivating GLP-1 *in vivo*

(59-63). More importantly, the contribution of DPP-IV in the regulation of glucose levels through GLP-1 inactivation has recently been confirmed by the examination of GLP-1 effects in both the DPP-IV knockout mouse and the hyperglycemic, GLP-1 receptor knockout mouse (64). Due to these multiple benefits of GLP-1 augmentation, DPP-IV inhibition has been recognized as a mechanistic approach of potential value to the treatment of type 2 diabetes (8). Studies with exogenous GLP-1 and DPP-IV resistant GLP-1 analogs have shown that normalized glucose levels are achieved in type 2 diabetics by increasing circulating levels of active GLP-1 by 3- to 4-fold (65-68). Val-pyrrolidide, a structurally related DPP-IV inhibitor (K_i = 0.4 μM), completely inhibited the NH_2-terminal degradation of GLP-1 and significantly augmented the insulin response to glucose in GLP-1 infused pigs and mice (69,70). A competitive, reversible DPP-IV inhibitor, Ile-thiazolidide (K_i = 0.13 μM), prevented the degradation of infused GLP-1 which resulted in augmented insulin responses to glucose and enhanced glucose clearance in both normal and obese rat models (71,72). A potent and selective DPP-IV inhibitor (K_i = 11 nM), **7** normalized glucose excursion in an obese fa/fa rat model and augmented active GLP-1 levels 3-fold in humans (73-75). Studies with Val-pyrrolidide, DPP-IV-knockout mice and glucose intolerant GIP-knockout mice confirmed DPP-IV's role in GIP inactivation and provide another mechanism for the control of glucose-dependent insulin secretion via DPP-IV inhibition (64,76).

Growth factors - *In vitro* and *in vivo* studies in mice, rats, and humans have shown that DPP-IV is the primary inactivation pathway for the intestinal growth factor, GLP-2 (77-79). Approaches that prevent GLP-2 degradation, such as the use of DPP-IV resistant GLP-2 analogs , DPP-IV deficient rats, or DPP-IV inhibitors have been more effective at promoting small intestinal mucosa growth than natural GLP-2 in normal rats alone (8,78). A DPP-IV inhibitor may therefore be considered therapeutic in conjunction with GLP-2 administration to encourage mucosal regeneration in patients with intestinal disease (66,80). However, the importance of DPP-IV inhibition to preserve intact GLP-2, at least in man, is questionable due to the recently reported low inactivation rate ($t_{1/2}$ > 1h) (79).

In vitro and *in vivo* studies in rat, pig, and cattle have established DPP-IV mediated proteolysis as a major route of GRH degradation and inactivation (81-84). Increases in hormone activity have been shown *in vivo* in both DPP-IV resistant GRH analogs in cattle and DPP-IV inhibitors co-administered with GRH in rats (82,84). In situations of sub-normal development or dwarfism, DPP-IV inhibition may potentially reduce the amount or frequency of GRH dosing.

Other functions of DPP-IV - Known as CD26 in the immunology field, DPP-IV is a well-established marker of T-lymphocyte activation (14,17,85). DPP-IV is present on the surface of activated T-cells where it participates in T-cell modulation through direct physical interactions with CD45 and possibly other T-cell surface molecules. With DPP-IV mutants which are devoid of the catalytic site, it has been demonstrated that DPP-IV enzymatic activity is not required for normal T-cell function (14).

Human DPP-IV also serves as a membrane-anchoring protein for ecto-adenosine deaminase (ADA), which has been postulated to detoxify extracellular adenosine or 2'-deoxyadenosine and to modulate the co-stimulatory function of CD26 on T-lymphocytes (86,87). The binding of ADA to DPP-IV does not require DPP-IV enzymatic activity and is mediated at least in part by residues in the non-catalytic cysteine-rich domain of DPP-IV (88,89).

Other observations - Alterations of DPP-IV expression or serum activity occur in several clinical and experimental cases of altered immune function (90). Serum DPP-IV activities are significantly increased in cases of allograft rejection, anorexia

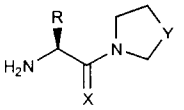
nervosa and periodontal disease, but are decreased in systemic lupus, rheumatoid arthritis, pregnancy, depression, and schizophrenia (91-98). HIV-infected patients have normal serum DPP-IV activity but with a decreased number of DPP-IV-positive lymphocytes (99). DPP-IV has been employed as a cell surface marker in the histological evaluation of a wide range of tumor types. Tumors have been described with either increased or decreased expression of CD26/DPP-IV, and this divergent expression has been associated with both an increased and decreased aggressiveness of growth of the tumors in question. Tumors with high cell-surface DPP-IV activity/expression include B chronic lymphocytic leukemia, basal cell carcinoma, T cell lymphoma, thyroid carcinoma, breast cancer, hepatocellular carcinoma, and lung tumors, while tumors with reduced or absent DPP-IV activity/CD26 expression include squamous cell carcinoma and melanoma (88,100-108). Presently, it is unclear whether changes in DPP-IV expression contribute to, or are a reflection of, the transformed status of the cells. Causal relationships between DPP-IV serum levels and disease states have yet to be determined.

Under controlled situations where proline is a major dietary amino acid, DPP-IV has been shown to play an important role in intestinal proline absorption and recovery of proline through re-absorption from the renal proximal tubuli (109). With a normal, non-proline rich reference diet administered over four weeks to both normal and DPP-IV deficient (DDR) Fischer 344 rats, no difference in the growth rate of either group was observed (109). However, significant weight loss was observed for the DDR rats compared to control when fed a diet that contained 20% of proline-rich gliadin as the sole source of protein over the same time period. Since proline is not a major component of dietary protein and is readily synthesized from L-glutamate, the potential clinical relevance of these differences is unclear.

KEY DPP-IV INHIBITORS

A variety of design strategies have been applied to the inhibition of DPP-IV, and have recently been reviewed (38). For this discussion, only the most viable inhibitor classes will be reviewed along with recent additions to the field.

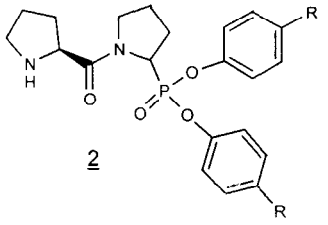
Peptide-like Inhibitors - As seen with substrates, most inhibitors require a protonatable 1° or 2° amine. The simplest inhibitors of DPP-IV are aminoacyl pyrrolidides and thiazolidides **1** (X = O, Y = CH_2, S). These are competitive, reversible inhibitors with IC_{50} values in the range of 2-6 μM for R = i-Pr (110). Substituting the pyrrolidine ring with 6- or 7-membered rings or acyclic amines results in a loss of potency (111). Replacing the P_2-site valine with cyclohexylglycine increases potency by a factor of 7 (112). Converting the amide to a thioamide (X = S) reduces potency by about half (R = i-Pr) (113). Furthermore, compounds where X = Y = S are more potent than those where X = S, Y = CH_2 (41,114,115).



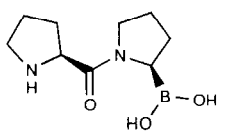
1

Incorporation of an electrophilic group on the proline mimic at P_1 provides a reactive center to accept DPP-IV's catalytic serine residue, thus producing more potent inhibitors. Paradoxically, the desirable properties of the electrophilic group cause these molecules to either have limited stability in biological media or cyclize with the P_2-site amino group. Dipeptide phosphonates (**2**) are irreversible inhibitors forming a very stable phosphonate ester with the active site serine (116). This stability is shown in an *in vitro* study with **2** (R = H, IC_{50} = 100 μM) which regained only 10% of DPP-IV activity after 4 weeks (117). A single injection of the R,S racemate (1, 5 or 10 mg i.v.) in rabbits reduced DPP-IV activity in plasma, circulating mononuclear cells and various tissue beds to less than 20% which took 20 days for complete recovery. Selectivity for DPP-IV inhibition was seen over aminopeptidase P and post-proline cleaving enzyme and no pathological alterations were observed in the histologic

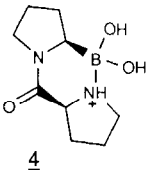
examination of a number of tissues (34,118). Electron withdrawing groups are the preferred R-groups with an inverse relationship between stability to hydrolysis and *in vitro* potency. For example, one of the most potent inhibitors (R = COOMe with an IC_{50} = 0.016 μM) has a half-life of 19 min in plasma. The acetamide (R = 4-NHAc) provides the best balance (IC_{50} = 0.4 μM, $t_{1/2}$ = 320 min) (34). Replacing the proline phosphonate at P_1 with piperidine (homoproline phosphonate) increases the inhibitory potency by certain members of this class by 2-10 fold (137).



2

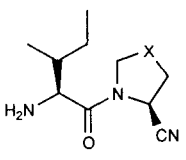


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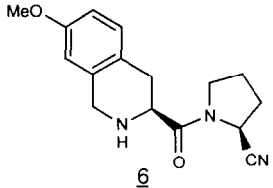


4

Prolineboronic acid (boroPro) dipeptides **3** are transition-state analogs that are reversible, slow-binding inhibitors of DPP-IV with IC_{50} values in the low nanomolar range (35,119). Active inhibitors require the (S) configuration of the boroPro at P_1 and an (S)-amino acid at P_2. Pro-boroPro **3** has an IC_{50} of 20 nM (120). However, it loses activity in a time-dependent manner in aqueous buffer due to cyclization to inactive **4** ($t_{1/2}$ = 160 min) (121). *In vivo* studies in rats show that oral dosing of **3** at 0.3 mg/kg almost completely inhibits serum DPP-IV after 1 hr (35). At low pH (~ 2) **3** retains the *trans* peptide bond and remains in its acyclic form (122). Cyclic analogs such as **4** can also be administered orally. The acidic conditions found in the stomach are sufficient to reconstitute the linear form **3** and regain its inhibitory activity (123). It is interesting to note that **3** is the only member of the Xaa-boroPro family to demonstrate selectivity, although modest, against DPP-II (38- fold) (119).

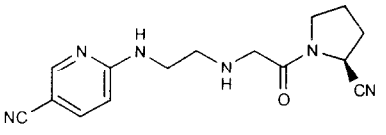


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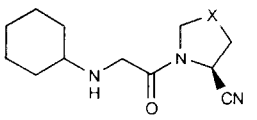


6

A nitrile at the catalytic site, more commonly used in cysteine protease inhibition, provides potent DPP-IV inhibition as it had previously for another member of the oligopeptidase family, post-proline cleaving enzyme (124). As slow-binding, reversible inhibitors of DPP-IV, aminoacylpyrrolidine-2-nitriles show good potency (low nM K_i), good stability, and > 500-fold selectivity against DPP-II (41,125). For example, the P_2 site Ile derivative **5** (X = CH_2) has a K_i for DPP-IV of 2.2 nM and a half-life in pH 7.4 buffered solution of 48 hr (126,127). The most potent member of the series contains cyclohexylglycine at P_2 site (K_i = 1.1 nM) (112). The analogous thiazolidide **5** (X = S) exhibits increased potency (K_i = 0.41 nM, $t_{1/2}$ = 27 hr (pH = 7.4)) (126). Tetrahydroisoquinoline analog **6**, a conformationally restrained tyrosine derivative, inhibits DPP-IV with an IC_{50} of 4 nM (128). It has recently been shown that the alkyl group of the P_2 amino acid need not reside on carbon. Moving the group from carbon to nitrogen produces potent peptoid-like molecules, e.g. **7** (IC_{50} = 7 nM) (73,129). As seen with the carbon and sulfur analogs of **5**, the thiazolidide **8** (X = S, IC_{50} = 7 nM) is more potent than the pyrrolidide analog **8** (X = CH_2, IC_{50} = 90 nM) (129,130).

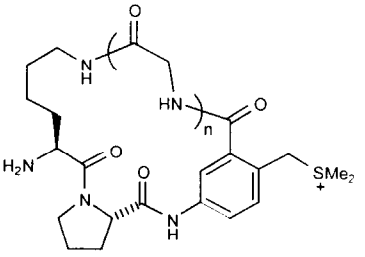


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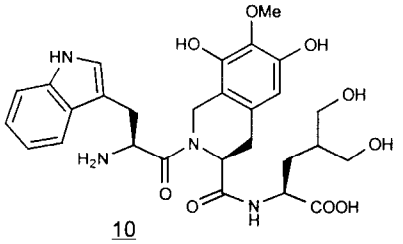
8

The novel cyclopeptide **9** irreversibly inhibits DPP-IV by a "double-hit" mechanism. The catalytic serine reacts at the proline carbonyl forming an ester bond. This unmasks a quinoniminium methide electrophile that is attacked by a nucleophile in the vicinity of the catalytic serine to form a second irreversible bond thus blocking the catalytic site (131). The cyclopeptide with two glycine residues (n = 2) has an IC_{50} of 12 nM. Increasing the ring size (n = 4) improves inhibitory activity (IC_{50} = 3 nM). These compounds are selective against the DPP-IVβ isoform (IC_{50} = 1 μM and 0.61 μ M, respectively).

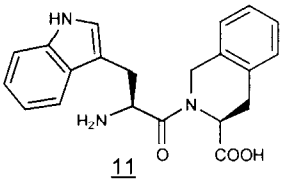


9

The natural product TMC-2A (**10**), isolated from the fermentation broth of *Aspergillus oryzae* A374, is a modest inhibitor of DPP-IV (IC_{50} = 8.1 μM) (132,133). The critical core structure responsible for the inhibitory activity of **10** is TSL-225 (**11**), an uncompetitive inhibitor with a K_i value of 3.6 μM (134) and an IC_{50} of 5.7 μ M (135).

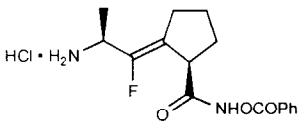


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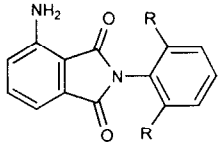


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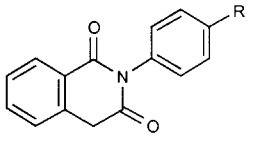
Nonpeptide Inhibitors - Replacing the amide bond of a peptide with a fluoroolefin simulates its bond length, bond angle, hydrogen-binding and steric requirements. The hydroxamic acid-based inhibitor **12** (K_i = 188 nM) demonstrates that the *trans*-double bond conformer efficiently mimics the *trans* P_2-Pro amide bond of its amide counterpart, which is a poorer inhibitor (K_i = 30 μM) (136). As with other O-benzoylhydroxylamine inhibitors, DPP-IV is irreversibly inactivated (137). Unable to internally cyclize, **12** is stable in buffer (pH 7.6) with a half-life of 103 hr. As expected, the *cis*-double bond conformer is 70-fold less potent than the *trans* conformer (136).



12

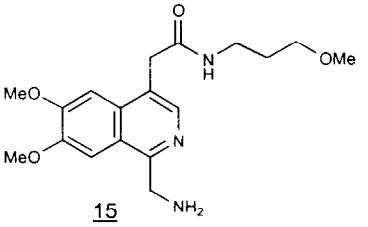


13 R = Me, Et, i-Pr

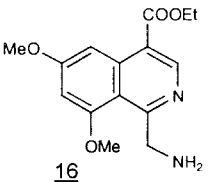


14 R = OMe, SMe

A library of 230 isoquinoline-based 4-acetic acid amides was synthesized in response to a high-throughput screening lead. The most potent member of the series is **15** (IC_{50} = 5.43 μM) (139). In a similar series, it was found that the truncated 4-carbethoxy analog **16** is even more potent (IC_{50} = 0.32 μM) (140).



15



16

Conclusion - Significant progress has been made in the identification of highly selective DPP-IV inhibitors with pharmaceutical potential. Due to the emerging evidence that therapeutic efficacy may be achieved in the treatment of type 2 diabetes, through inhibition of incretin hormone degradation, considerable additional efforts are anticipated in the coming years. Potent and selective orally active inhibitors have been identified that will allow careful evaluation of the safety and tolerability profile of this new therapeutic class. The availability of compounds with proven mechanism of inhibition and established selectivity for DPP-IV relative to other related proteases will be of further value in the clarification of the role played by this enzyme in a diverse array of physiological processes.

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SECTION V. TOPICS IN BIOLOGY

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Chapter 20. Bioinformatics in the Drug Discovery Process

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Introduction — Bioinformatics is the part of molecular biology that involves working with biological data, typically using computers, with the goal of enabling and accelerating biological research. It is ubiquitous in drug discovery; few if any projects are computer-free. Bioinformatics spans a wide range of activities: data capture, automated recording of experimental results; data storage and access, using a multitude of databases and query tools; data analysis; and visualization of raw data and analytical results. Although the first two categories rely on computer skills, the others also require mathematics, molecular biology, and biochemistry. Until recently, most people working in bioinformatics started in one discipline and picked up the others on the job. New interdisciplinary programs of study, though, integrate these subjects.

Bioinformatics is web-centric. Most academic groups and many companies make computing tools and data available on the web. An online supplement to this article (http://www.cgen.com/science/armc-2001.htm) contains links to relevant websites. Key databases are also reviewed in each year's first issue of Nucleic Acids Research (1).

This article briefly reviews recent bioinformatics publications, with a focus on tools and databases used to speed drug target discovery and selection. Subjects not covered include genetic, physical, and radiation hybrid mapping; genetics; genotyping and high-throughput screening; molecular and cellular simulation; and automation.

WHOLE-GENOME SEQUENCE ANALYSIS

The newly available human genome sequence includes the sequence of almost every gene. Initial versions of the transcriptome, the expression level of transcribed sequences in particular tissues under specific conditions, and of the proteome, the proteins in a cell under given conditions and their interactions, are also available. These data are transforming biology and the pharmaceutical industry. Manual analysis of such vast amounts of data is impractical; computerized tools are needed to reap the full benefit of these resources. Genomics has become a major source of drug targets, and bioinformatics is crucial for finding and validating novel targets so as to minimize investment in laboratory resources.

Sequencing the Human Genome — The most important recent genomics papers are about the draft sequencing of the human genome (2, 3). Two versions were published: one by the Human Genome Project (HGP), which includes 20 sequencing centers in six countries, and one by the company Celera. The primary sequencing articles are accompanied by articles describing bioinformatics analyses of the data.

Current technology can sequence DNA segments of up to about 1,000 base pairs (bp; kb for thousand bp and Mb for million bp). A longer stretch is sequenced by breaking it into small fragments, sequencing one or both ends of each, and joining the pieces, so-called shotgun sequencing. The process of joining short sequences is called fragment assembly; it relies on the assertion that two sequences with virtually identical regions are likely from the same location, in the absence of complications from repeats and sequencing errors. Assembly tools include Phrap, FAK, the TIGR assembler, and CAP (4-7). The HGP divided the genome into pieces of 200-350 kb that were

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sequenced individually and then joined. Celera, though, fragmented the entire genome. Previous assemblers reconstructed entire microbial genomes, but the Celera Assembler was the first to apply whole-genome shotgun sequencing to a target this complex (8).

The HGP sequence and automatically-generated annotation are freely available from the websites of the National Center for Biotechnology Information (NCBI), Ensembl, and UCSC. The Celera sequence is available from the Celera website under conditions described there. Comparisons of the sequences reveal that they are globally similar but have local discrepancies, probably mostly the result of mis-assembly (9, 10)

This review can mention only a few of the many interesting conclusions. The most striking finding may be the number of protein-coding genes, which both groups estimated as 30,000-40,000. This is lower than the previously estimated 70,000-120,00 genes (11), though recent analyses presaged this result (12, 13). These numbers are surprising because they are not much larger than the 20,000 genes in the nematode or the 12,000 in the fruit fly. This shows that genome complexity does not depend only on the number of genes. Alternative splicing seems to be key to the greater intricacy of humans (14). Analysis also revealed evidence of horizontal transfer of bacterial genes into vertebrates as well as extremely long duplicated regions.

Sequencing Other Genomes — More than 600 organisms are being sequenced and several dozen genomes are complete. One criterion in choosing an organism to sequence is its medical importance. Microbial and viral genomes help identify drug targets in pathogens; eukaryotic genomes advance understanding of human genetics and physiology. Plant and animal sequencing have also led to significant advances in sequencing and bioinformatics technologies.

A gene's genomic context, including nearby genes and transcription control regions, is needed to understand the gene's function. Comparative genomics, i.e., comparing conserved sequence regions in multiple organisms, uses conservation of sequence and higher-order genomic structure to gain insight into gene function. PipMaker compares long DNA sequences to identify conserved regions without prior knowledge of gene structures (15). The Artemis Comparison Tool (ACT), a DNA sequence viewer, displays genomic structure as well as similarities and differences between two genomes (16).

NCBI provides tools for comparative genomics in addition to comprehensive cross-links between various databases (17). HomoloGene includes curated and calculated orthologues and homologues in six eukaryotes; LocusLink is an interface for queries about their genetic loci. The Human/Mouse Homology Maps display syntenic regions, i.e., regions that contain similar genes, usually in the same order.

Clusters of Orthologous Groups (COGs) are phylogenetic classifications of proteins from 34 complete genomes in 26 major phylogenetic lineages (18). BLAST (see below) is used to find the best match for each protein in each genome. Proteins that are best hits for each other are clustered. Clustered proteins are likely to have similar functions; a COG is a ciuster with proteins from three or more lineages. COGs yield functional predictions for poorly characterized genomes. Results do not depend on BLAST cutoff scores and so are unaffected by different evolutionary times between pairs of genomes.

Sequence Alignment Tools — Database searches to find similar sequences that are putative homologues, i.e., that appear to have a common evolutionary ancestor, are at the core of sequence analysis. Proteins with similar sequences or domains typically have similar structure and often function, though the converse is not always true. Proteins that are at least 25% similar are generally homologous (19).

Almost synonymous with database searching, Basic Local Alignment Search Tool (BLAST) may be the most widely used bioinformatics tool (20). BLAST is optimized for speed but sacrifices only minimal sensitivity in searching databases. More sensitive algorithms like that of Smith and Waterman find optimal alignments but are slower (21).

Newer tools address features of nucleotide sequences (22, 23), modeling phenomena such as frame shifts, codon insertions and deletions, and introns. Improved sensitivity comes at the price of computation time, though hardware accelerators make some such algorithms reasonable for high-throughput environments.

Distinguishing signal from background noise is difficult when looking for homologues that are extremely distant on the evolutionary time scale. Sensitivity is improved by focusing on a protein's functional domains, which may have distinct origins and functions. Multiple alignment of sequences from a domain in several organisms reveals patterns of conservation, including highly conserved residues, and the patterns can be used to recognize distant homologues. Databases that use patterns are based on multiple alignments that have been either manually optimized or created automatically (24-27). InterPro centralizes protein information from many databases (28).

Some new tools use existing sequence databases. BLAST now accommodates sequence gaps. Another version combines alignments into a position-specific scoring matrix and searches with that matrix. This iterative process yields a Position-Specific Iterated version (PSI-BLAST) (29) that provides improved annotation (30). Sequence queries can also be compared to collections of position-specific scoring matrices (31).

GenBank contains more than 11 billion bp in more than 10 million sequences and continues to grow exponentially. A BLAST search against all of GenBank takes three times as long as it did a year ago! MEGABLAST compares large sets of long nucleotide sequences up to 10 times as fast as do previous programs, but is best for comparing sequences that differ because of sequencing errors (32). The race between database growth and search speed continues; algorithmic and hardware improvements will be needed to continue the pace of biological discovery.

Recognizing and Predicting Genes and Other Sequence Features — One post-sequencing challenge is comprehensive genome annotation, including at least the putative start, stop, and structure of each gene, and preferably information about transcription regulatory regions. Similarity searches can identify some novel genes, but many human genes are not very similar to known sequences from other organisms.

Ab initio gene prediction finds genes in a (typically eukaryotic) genomic sequence using sequence only, without knowing expressed or translated regions, though possibly using known genes as examples. This is challenging, as eukaryotic genes are complex and less than 2% of the human genome codes for proteins. RefSeq, a non-redundant, curated database of mRNA and protein sequences, contains examples that illustrate this complexity (17): genes have many exons (median 7 / mean 8.8), long introns (1,023 bp / 3,365 bp, with large variance, and many longer than 50 kb), short internal exons (122 bp / 145 bp), and short coding sequences (1,100 bp / 1,340 bp). They also

Methods such as GrailZ, GenScan, Genie, and HMMGene (33-36) use probabilistic pattern recognition methods to identify protein coding regions based on gene-related patterns of DNA composition. The latter two programs also integrate sequence similarity to known coding regions and expressed sequence tags (see below).

EST analysis — An expressed sequence tag (EST) is a partial expressed sequence from a (generally anonymous) cDNA. Large collections of ESTs promised a shortcut to finding most genes before the genome sequence was available and were to obviate difficulties of computational gene prediction (37). Although their early promise was not completely fulfilled, ESTs are quite useful. Examination of EST assemblies provided the first hints of the prevalence of alternative splicing in the human genome (38, 39) and led to the detection of many putative SNPs (see below) (40, 41). ESTs are useful molecular landmarks and those from multiple tissues can be used to answer questions about tissue-specific genes and to identify novel proteins (42, 43).

GenBank contains more than three million human and almost two million mouse EST sequences, and proprietary collections include millions more. ESTs are typically short, 3'-skewed, and of relatively low quality; many represent only the 3' UTR; and the databases are highly redundant and poorly annotated. As a result, searching EST databases directly is not terribly efficient. More useful information is found by clustering ESTs and mRNAs based on sequence overlaps, yielding sequences that are longer, of higher accuracy, and that better represent the underlying genes. Databases of EST clusters include UniGene (44), the TIGR Human Gene Index (45), and STACK (46); they are compared in (47). GeneNest adds to UniGene by providing consensus sequences and visualization (48). A current challenge is integrating EST clusters and genomic data to improve gene and transcript predictions by eliminating EST artifacts.

Polymorphism analysis — Most common sequence variation, or polymorphism, consists of single nucleotide polymorphisms (SNPs), mutations that replace one base pair with another. SNPs serve as genetic markers to track familial inheritance; some are functionally important, affecting disease susceptibility and response to medication (49). The dbSNP database (50) holds more than 1.5 million unique SNPs, more than half from The SNP Consortium (51). About 1.42 million have been mapped to the human genome using MEGABLAST (52). To find SNPs, several copies of DNA from a region, preferably from ethnically diverse individuals, are compared for sequence differences. SNP detection technology and the use of SNPs in genotyping are reviewed in (53).

Phred (54), a basecalling program for sequencer traces, estimates an error probability for each base, and this quality score can help differentiate polymorphism from sequencing errors. Other errors can be found by aligning sequences to a high-quality reference sequence. The neighborhood quality standard (NQS) requires high Phred scores and good alignment to the reference sequence around a putative polymorphic site (51). POLYBAYES uses the NQS information and the multiple alignment of ESTs in a cluster to model the probability that a site is polymorphic (55); this allows it to detect paralogous genes as well as sequencing errors.

METABOLIC AND REGULATORY PATHWAYS AND NETWORKS

The previous section focuses on individual genes and proteins. Understanding how proteins interact, though, is crucial to drug development, as modifying one protein's behavior affects others as well. Gene expression is often used as a surrogate for protein expression, as the former is easier to measure with current technologies, but they do not correlate perfectly (56). Both are used to model cellular dynamics (57, 58).

Gene Expression Profiling — Gene expression is measured to find specific genes that are differentially expressed and groups of genes that are co-regulated (59). At present, the most popular technology for measuring gene expression is DNA "chips" or "microarrays," which perform massively parallel Northern blots (60). Where scientists previously studied a few genes at a time, chips allow efficient genome-wide analysis of expression variation. Expression can also be measured with non-microarray methods, including Serial Analysis of Gene Expression (SAGE) and variants (61). Chip and SAGE results agree well on both absolute and relative expression levels (62), though proper analysis is needed to account for SAGE experimental biases (63-65).

A differentially expressed gene may be implicated in the phenotypic differences between samples (66). A typical experiment compares expression at different developmental stages (67, 68), between normal and disease states (69, 70), or under differing environmental conditions (71). The EST cluster databases described above can also be used for expression profiling across multiple libraries (72).

Co-regulation is recognized from similar expression levels in multiple states. Many methods have been used to measure profile similarity and to cluster genes with similar expression patterns (73-76). Some works cluster tissues as well, as co-regulation of

genes associated with multiple regulatory mechanisms may only be recognized by examining a subset of the tissues (77).

A common difficulty in prescribing medical treatment is differentiating between diseases with similar phenotypes. Microarray analyses can distinguish between normal and tumor tissue or between cancers (78-81). The latter paper includes an algorithm that identifies multiple cancers in tissues without foreknowledge of different diseases. Microarrays are also used to compare cell lines. Cell line clusters based on expression levels differ from those based on drug responses (82); changes in expression can have striking effects on drug sensitivity and resistance. An elegant use of microarrays is to verify and supplement computational predictions of gene structure (83).

Promoters, portions of genes that contain information to turn genes on and off, can be identified using clusters from microarray analysis (84). A motif that appears upstream of co-regulated genes may be a promoter (85-88). Promoters and expression clusters can be uncovered simultaneously (89, 90). Upstream regions of similar genes from multiple organisms can also be compared, with the required level of motif conservation depending on the phylogenetic distance between the organisms (91).

Proteomics — Proteomics studies networks of proteins by measuring, among other things, protein expression. Protein activity is regulated by post-translational modification and degradation; these cannot yet be predicted from DNA sequence. Proteomics measures protein expression directly, not via gene expression, thus achieving better accuracy. Current work uses 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry. New separation and characterization technologies, such as protein microarrays and high-throughput chromatography, are being developed.

In 2D-PAGE, samples to be compared are run through separate gels; corresponding spots that look different may indicate differential expression. Comparing spots has been time-consuming and error-prone, but new tools vastly improve both speed and accuracy (92). Spot intensity is measured to estimate expression level and mass spectrometry is used to identify proteins from spots of interest (93). Tools for analyzing gel images and mass spectra are described in (94). Proteins from organisms that are not well represented in protein databases can sometimes be identified based on proteins from other species using mutation-tolerant methods (95). Differential expression can be measured by labeling samples with different tags (96).

Control sequences (standards) are typically used to calibrate mass spectrometry measurements. Internal standards added to samples can confound identification; external standards in different spots may not yield sufficient precision. Both suffer because calibration varies even over the time needed to process a sample plate. A recent approach corrects for systematic deviations by first using a low-precision database search. The proteins found are used to estimate calibration parameters and a second search uses greater precision, taking account of the recalibration (97).

Metabolic and Regulatory Networks — Cellular processes result in large part from proteins interacting with each other and with other cellular components. Some research tries to identify interactions; other efforts use interactions to reverse-engineer metabolic and regulatory networks. Appropriate intervention points for therapeutic agents can be gleaned from an understanding of these networks. Comprehensive protein-protein interaction maps have been published for *Saccharomyces cerevisiae* (98) and *Helicobacter pylori* (99). Groups are working on maps for other organisms and for specific pathways in humans.

Cross-species comparisons can be used to infer interactions. A phylogenetic profile is a list of species in which a protein is expressed. Proteins with the same profile are likely to be in a pathway that occurs only in certain organisms (100). Conservation of relative gene position identifies sets of genes that occur together, in the same order, in multiple species (101); this approach has been more informative in prokaryotes than in

eukaryotes (102). Domain fusion looks for domains from two proteins in one organism that are contained in a single protein in a second organism (103, 104). Fusions may have an evolutionary advantage from more efficient interactions if the domains are in a common pathway. The protein with fused domains is called a "Rosetta stone" because it helps explain the relationship between the separated proteins in the other organism.

Studying all tissues under all conditions to elucidate all interactions is a tall order. Recent work draws on existing research reports. Text mining tools extract interactions reported in document collections such as Medline. One approach is to compile a list of proteins and look for entries separated by appropriate verbs, such as two protein names with "phosphorylate" in between (105-107). A second approach parses text completely and looks for interactions (108, 109). The latter is more comprehensive but existing tools cannot fully evaluate all text. Other tools skip parsing altogether, using co-occurrence of protein names to deduce functional relationships (110, 111).

Network complexity is illustrated by the Boehringer Mannheim "Biochemical Pathways" wall charts. Most reconstruction focuses on pathways, non-branching portions of networks. Databases of reconstructed pathways include KEGG (112), EcoCyc and MetaCyc (113), and DIP (114). Reconstructing pathways from interaction data is quite difficult. Some tools simultaneously recognize interactions and reconstruct pathways (115-117), often using statistical techniques (118-121).

Mathematical models incorporate gene and protein expression to deduce pathway structure. Stoichiometric, thermodynamic, biochemical, and other constraints are used to model expression over time (122-127). Comparing pathways can yield functional insights; comparisons based on sequence (128) and on Enzyme Commission numbers of pathway components (129) have been developed. Reconstructed networks can predict output compounds based on input nutrients and required precursors (130).

PROTEIN SECONDARY AND TERTIARY STRUCTURE

Predicting protein three-dimensional structure is an important open problem not only in bioinformatics, but in general high performance computing. Structure being the key to function, determining a protein's structure is a key step toward elucidating its role. The subfield of protein-ligand docking is useful in rational drug design. Laboratory prediction is time consuming and expensive, so researchers have been working on computerized prediction for several decades. Exact computational prediction is difficult (131, 132), but sophisticated algorithms to find approximate solutions continue to be developed.

The Critical Assessment of Methods of Protein Structure Prediction (CASP) tracks progress in this field bi-annually. Computational groups predict structures of proteins whose structures have been found in the laboratory before the latter results are released. The predictions are assessed by experts. Tools are classified as using one of three approaches: comparative modeling looks for amino acid similarity to proteins of known structure; fold recognition predicts folds in regions that do not share amino acid similarity with known structures; and *ab initio* tools work directly from physical principles. Results of CASP 3 defined the technology as of 1998 (133-135). CASP 4 took place in 2000, and the upcoming publication of its results will define the current state of the art.

WORKING WITH COMPLEX DATASETS

The difficulties of genomic analysis are compounded by data complexities. Datasets are large, ill-structured, and noisy. Combining data from multiple sources is challenging, as even basic terms have multiple meanings. A gene, for example, may refer to the stretch of genomic DNA that encodes a protein (sometimes including the promoter), the exons in that region, the coding sequence only, the resulting mRNA transcript, etc.

Data Management and Integration — Most genomic data are stored in flat files or commercial relational databases, but object-oriented databases are finding increasing use. AceDB, a non-commercial database created as part of the *Caenorhabditis elegans*

sequencing project, has a flexible data model that is easily extended to handle new kinds of data (136). It includes displays and other tools for use with genomic data.

Gene expression, as a new research area, has benefited from integrating databases that are geographically dispersed, with different formats and terminology. Standards for storing, reporting, and exchanging data have been proposed (137-139); an alternative is to combine data sources in a centralized, curated database (140, 141). Data storage standards have also been proposed for protein interactions and pathways (142).

Groups working in established areas often cannot retrofit standards to their existing databases. One way to enhance interoperability is to add standard, object-oriented interfaces (143-146). Another is to create tools that access distributed databases, but present a user interface that makes the databases appear integrated (147). A standard for exchanging many kinds of biological data, based on XML, is described in (148).

Several databases contain different kinds of data integrated by locus. Often these contain similar data from different sources, such as multiple radiation hybrid maps (149). Sometimes they include different kinds of data (51, 150-153) or more complex representations, such as the genes contained in a metabolic pathway (154) or data on protein interaction and function (155). InterPro is perhaps the most comprehensive of such databases, and includes web links to many data sources (28).

Gene Ontologies — Database integration is crucial for comparative genomics, large-scale inference of characteristics of novel genes in one organism from known properties of genes in other organisms. This requires automatic combination of different data sources. Ontologies are structured, controlled vocabularies. An ontology for biological function in *Escherichia coli* is described in (156). More recently, the Gene Ontology Consortium has built ontologies describing a gene's molecular function, the biological processes that use it, and its cellular location (157). Tools for sharing ontologies exist (158), but results of using ontologies for database integration have yet to be published.

Visualization — Computational methods cannot yet rival the ability of the human eye to pick out patterns. Many visualization tools show annotated genomic sequences. An early example is Genotator (159), which shows color-coded annotations such as predicted genes, promoters, splice sites, and putative homologies in parallel to the sequence. Different levels of detail can be viewed by zooming in and out. Artemis is a newer tool that allows calculations on the sequence or its CDS features, such as G+C content and amino acid properties such as hydrophobicity (16). This area continues to develop rapidly, with new tools constantly emerging. A recent focus is comparing both sequence and rearrangements, as discussed above (160-162).

Most recent visualization tools build on the availability of web browsers. Re-usable interface components, typically in Java, for displaying common genomic objects are available (163, 164). Displays of 3D molecular structures may be the most striking visualization tools; experience with these and other display tools helps researchers understand how to take advantage of visual perception in the design of new tools (165).

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Chapter 21. The Role of Protein Structure Prediction in Drug Discovery

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Introduction - As we move into the post-sequencing phase of many genome projects, attention is becoming increasingly focussed on the correct identification of gene products. Assigning a possible function to a gene is an important first step to characterising its role in the various cellular processes, and without this information, it is impossible to realise the true value of genome sequencing. Of course, straightforward sequence comparison algorithms are by far the most widely used techniques for making an initial identification of a particular gene product. The identification of common ancestry between a new gene product and a gene of known function allows some inferences to be made regarding the function of the new gene. How reliably the function can be extrapolated to the new gene depends on a number of factors, but the principle factor is of course the degree of sequence similarity observed.

New developments in sensitive sequence comparison, particularly recent extensions of the BLAST algorithm to sequence profiles or techniques based on Hidden Markov Models have resulted in the routine detection of ever more remote homologous relationships (1,2). Of course, as more and more remote relationships are being considered, it becomes less clear as to how reliably one can map the function of one gene to another (3,4). Nevertheless, sensitive sequence comparison algorithms are still the most vital technology that we have for rapidly characterising new gene products. The importance of this development cannot be underestimated. Until its release, the ability to generate results comparable with those now routinely produced by PSI-Blast was restricted to specialist researchers, who combined *ad hoc* combinations of database search and profile generation software and judicious hand editing (to remove over-dominant sequence clusters and to know when to stop iterating in the absence of easily interpreted statistics).

Because PSI-BLAST is so easy to use and so universally popular, it is important here to also provide a word of caution (5). The algorithms underpinning PSI-Blast are complicated and use a variety of assumptions. One of these assumptions is that the sequences being compared are both globular proteins, which is often not the case. This assumption becomes an issue when two proteins only share a low level of sequence similarity, as unrelated non-globular proteins could achieve scores similar to those between related globular proteins. This is because globular proteins tend to make full use of the available 20 amino acids throughout their sequences whereas transmembrane regions (with their bias towards hydrophobic residues), signal peptides and coiled coils do not. It is therefore critical that all low complexity regions, as they are known, are masked out, to ensure that the only regions being compared have high complexity and that the statistics are equally applicable for any sequence comparison in a database search.

Despite these improvements in sequence comparison techniques, there are still many gene sequences which cannot be functionally characterised by sequence comparison. These sequences are found either to match no other proteins at all, in which case they are labelled sequence "orphans", or they only match other proteins which are also functionally uncharacterised. Apart from direct experimental characterisation, using gene knockout experiments or expression array techniques for example, what else can be done? One possible avenue for obtaining functional insights into novel genes is to look at the

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possible structure of the protein encoded by the gene in question. In this way, 3-D structural information can also be used as part of the process for identifying distant homologues (i.e. those which are not readily identified by PSI-BLAST).

METHODS FOR STRUCTURE PREDICTION

Obtaining a protein structure experimentally can be a time consuming process whether it is done by X-ray crystallography or by NMR spectroscopy. Given the evident importance of 3-D structure in providing insights into the function and mechanism of proteins, it is reasonable to consider the applicability and reliability of available structure prediction techniques. Is there a role for protein structure prediction in structurally characterising a protein. Clearly, a satisfactory theoretical approach to accurately modelling the structure of many proteins would have a great impact on genomics as a whole. However, if the use of prediction algorithms is going to be generally accepted by the biology community at large, then it is essential that the reliability of these methods be assessed in such a way as to convince this rather sceptical audience. Although individual authors of automatic prediction methods do attempt to properly benchmark their methods and attempt to provide useful measures of confidence alongside their predictions, there still remains the possibility that the published results are somewhat better than might be expected in cases where the true structure is not known. The recent Fourth Critical Assessment in Structure Prediction (CASP4) Experiment was carried out in 2000, along similar lines to the previous 3 similar experiments, and this continues to allow some indication to be gained as to the reliability of truly blind predictions using different approaches. Detailed results from the experiment will be published in a special issue of the journal "Proteins", along the same lines as for CASP3 (7). The raw data from the CASP4 evaluation is also available across the Internet (URL http://predictioncenter.llnl.gov).

COMPARATIVE MODELLING

At present, the most accurate method for predicting protein structure is to make use of comparative modelling techniques to infer the structure of a target protein based on the structure of a related template protein. The reliability and simplicity of this class of method stems from the fact that it is limited to predicting the structure of proteins which are closely related to the template protein of known structure. The comparative modelling process can be divided into five basic steps: alignment of the target sequence with the sequence of a protein of known 3-D structure; building of a framework structure based on the alignment; loop building; addition and optimization of side chains; and finally model refinement.

In recent years there has been a definite advance in the accuracy of sequence alignments for target-template pairs which are only distantly related. Indeed, some of these pairs would previously have been considered to be so distantly related as to be only suitable for fold recognition. This has come from the common usage of sensitive sequence profile alignment methods such as PSI-BLAST or one of the several methods based on Hidden Markov Models as discussed earlier (1).

For comparative modelling to be used routinely for genome annotation, it should be possible to build good quality 3-D models without requiring human intervention. Given the fact that progress does seem to have been made in terms of full automation of comparative modelling, and producing accurate sequence-structure alignments, it is not surprising that comparative modelling techniques form a central part of structural genomics initiatives. It has been demonstrated that a large fraction of the yeast genome can be automatically modelled by homology to known 3-D structures using the program MODELLER, but so far progress has been limited to ORFs with relatively high sequence similarity to the template protein structures (8).

PROTEIN FOLD RECOGNITION AND THREADING

In the absence of suitable homologous template structures with which to build a model for a given sequence, and the slow progress that is evident in the ab initio prediction field, fold recognition algorithms provide another option for constructing useful tertiary structural models. 3D structure information can be used as part of the process for identifying distant homologues (i.e. those below about 25% sequence identity) as one can assess the fit of a sequence to a protein structure using empirically derived probabilities or propensities (also known as energy potentials). Many of these methods are referred to as threading, of which the first was THREADER (9). This algorithm has been continuously developed and refined (e.g. 10), along with related approaches developed in other laboratories (11), but these methods still have the following advantages and disadvantages. Threading methods try to predict the fold of a protein in the absence of any sequence similarity (i.e. down to 0%) using a large library of folds as its database (fold means the approximate main chain trace of a protein structure). Its problem is that there exist no robust statistical assessments of significance and therefore there will always be a top prediction even if the fold of the query sequence is not in the fold library. This situation is very similar to the problems with profile based methods before the advent of PSI-BLAST. Threading is also CPU intensive and may take many hours on a relatively powerful machine to complete a database search.

In addition to the problem of identifying true from false positives, there is also the problem of alignment accuracy. From the results of the CASP experiments, the conclusion we reach is that in order to produce reasonably accurate 3-D models with fold recognition methods there should be an evolutionary relationship between the target protein and at least one template structure of known 3-D structure. Despite the fact that the sample sizes in the CASP experiments are small (30-40 target domains), it would appear that where there is at least some detectable sequence similarity, fold recognition methods based on sequence-profiles are presently sufficient to build useful models. Beyond these cases, however, fold recognition methods not reliant on sequence alignment (i.e. true threading methods which ignore the sequence of the template proteins) are much more limited in their ability to recognize folds, and to the accuracy of the models they can produce. Nevertheless, even these relatively poor models may be enough to gain some insight into the function of a new gene sequence. Even fold recognition algorithms which are able to correctly recognize folds but are entirely incapable of producing sensible alignments may offer some advantage in the narrowing-down of potential gene functions.

It has become clear that these algorithms are now beginning to converge, with many different groups all heavily relying on sensitive sequence comparison in addition to more traditional fold recognition methods. To develop the principle of threading further Jones has recently developed a new method called GenTHREADER which is faster and has a more robust scoring scheme (that makes it accessible to non-specialists) (12, 13). GenTHREADER is considerably different in concept from THREADER, being based on the observation that for real relationships the alignments resulting from comparison of a sequence/profile are often mostly correct, even though the score too low to suggest statistical significance. It was proposed that when 3D structure information is available for either the sequence or the profile, and additional assessment of structural compatibility may be able to 'rescue' these 'lost' relationships. For the *M.Genitalium* genome, Jones was able to confidently assign as 3D structure to at least one domain of 46% of the open reading frames (ORFs) (12). Figure 1 shows the distribution of protein archiectures found in *M.Genitalium.* Figure 2 shows a simple example where a crude functional assignment can be obtained by the use of GenTHREADER on an uncharacterised ORF from M. genitalium.

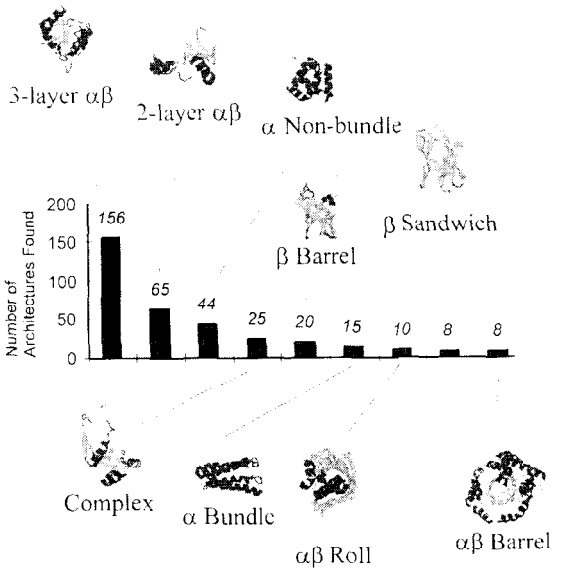
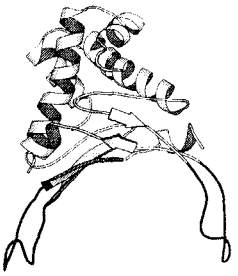


Figure1: The distribution of protein fold architectures (64) in the *M. genitalium* genome, assigned by GenTHREADER (12).



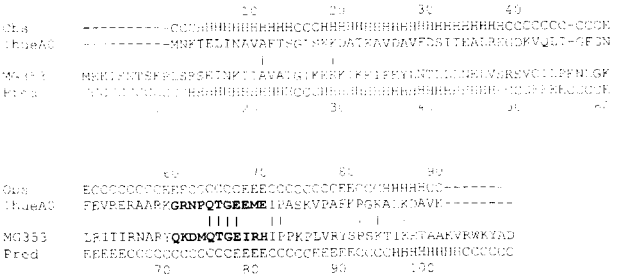


Figure 2: A typical example of a fold assignment which provides some functional insight just from the fold alone. In this case the gene can be assigned broadly to a class of DNA binding proteins by virtue of the fact that a confident match has been found to another DNA binding protein (1HUE) and that some sequence conservation is apparent in the arm of the protein which interacts with DNA (the proteins binds as a dimer).

FOLD RECOGNITION METHODS FOR GENOME ANALYSIS

When mining large amounts of sequence data, fold recognition methods can contribute significantly to target prioritization. Depending on one's strategy, one might for instance wish to concentrate on protein folds that are likely to be drugable or those for which one already has an assay available. This will immediately remove most of the sequences from consideration and leave a more compact list for subsequent validation.

Given the potential benefits of assigning a correct structure to a newly discovered gene product, it is unsurprising than several groups have applied existing fold recognition algorithms to genome analysis. These techniques can be classified into roughly 3 classes: sequence profile methods (1-2,14), structural (3-D-1-D) profile methods (15-16) and threading algorithms (17-18).

The first attempt at assigning folds to genome sequences made use of a structural profile method. Fischer & Eisenberg (19) used a development of the original 3-D-1-D profile method (15) to assign folds to the ORFs found in *M. genitalium*, the smallest known bacterial genome. They found that approximately 16% of the ORFs could be assigned to a known fold by means of straightforward sequence comparison, and that an additional 6% could be assigned to a known fold at high confidence using their fold recognition method. Of course, as the structure databases are now much larger, it is very likely that these fractions would now be somewhat higher.

Although many different threading (purely pair potential based fold recognition) methods have been developed, only a single attempt at applying these methods to genome analysis has been described (20). The ProFit method has been applied to analyse the ORFs in *M. pneumoniae*, a slightly larger genome than *M. genitalium* (18). In this work, to save time, proteins which could be matched to known structures by straightforward sequence comparison were excluded from the analysis along with proteins longer than 200 residues (which were assumed to be multidomain proteins). Of the 124 ORFs remaining, Grandori was able to recognize folds for 12, giving a recognition rate of 10%. Interestingly, a number of disagreements were reported when the results were compared with the results from Fischer & Eisenberg's work (by identifying *M. pneumoniae* homologues in *M. genitalium*). This is not surprising given the relatively low overall reliability of pure fold recognition algorithms, but more surprising because in some cases both predictions were apparently very significant.

Despite the fact that both approaches made use of basic pairwise sequence comparison methods to detect obvious homologues to known structures, it is clear that better sequence comparison algorithms could have been applied, and these techniques might well have assigned a greater number of folds to ORFs. In answer to this, a number of groups (21-24) have used PSI-BLAST (1), which is an iterative sequence profile method based on the standard gapped-BLAST method (1). PSI-BLAST has proven to be not only a very sensitive sequence comparison method, but also to be very reliable. To get the best results from PSI-BLAST, however, it should be used in both "directions" (21,24). Normally, each ORF is scanned against a set of PSI-BLAST profiles, each of which corresponds to a single protein structure or structural domain. Despite the fact that these profiles are slow to calculate, this process only has to be done once for each sequence of known structure. Assigning folds to ORFs using this procedure is thus fairly efficient. To achieve the second search direction, a PSI-BLAST profile must be calculated for each ORF, and this profile can be scanned against a library of sequences relating to known structures. Given that the calculation of a single PSI-BLAST profile takes 10 minutes on average using a modern workstation, for large genomes, this second approach is very computationally expensive, and relatively impractical. Despite this disadvantage, extra matches can be found when both searches are carried out.

In another study, attempts were made to exploit this asymmetry in profile comparisons by means of a comparison algorithm based on the alignment of one profile with another (25, 26). This technique, BASIC, requires profiles to be computed for each sequence in the 3-D structure library and also for each ORF. These two sets of profiles are then compared by means of a local dynamic programming method.

As already mentioned, Jones has developed a hybrid method for assigning folds to genome sequences, called GenTHREADER (12), which was used successfully to assign folds to the genome of *Mycoplasma genitalium*, where analysis of the results showed that as many as 46% of the proteins derived from the predicted protein coding regions had a significant relationship to a protein of known structure.

Teichmann et al. have compared the results from several attempts at assigning folds to the *M. genitalium* (MG) genome (27). Being the smallest bacterial genome, MG provides a useful benchmark for different approaches to fold assignment as most groups have made predictions for this genome. Despite the fact that it was found that a high degree of agreement was apparent between the different algorithms, some results were not found by all techniques. This suggests that to maximise success in assigning folds to genomes, some kind of consensus of algorithms might be useful. At present, this is difficult as there are no agreed standards for how structural annotations should be represented.

A number of Web resources are available which provide access to precompiled fold assignments for different subsets of genomes. These resources are predominantly based on PSI-BLAST comparisons e.g. the GTOP database at the National Institute of Genetics in Japan. The database contains fold assignments for 26 completed genomes based on PSI-BLAST similarity searches, and can be accessed from the following URL:

http://spock.genes.nig.ac.jp/~genome/summary.html

To date, the only available set of comprehensive fold assignments using fold recognition techniques are those derived by GenTHREADER, which have been compiled for 25 complete genomes (plus the currently confirmed gene products from the draft human genome) and have been stored in a Web-accessible database at the following URL:

http://insulin.brunel.ac.uk/genomes

A preliminary analysis of these data shows that whilst certain folds are prevalent in all genomes, certain folds are more common in some genomes than others. The Rossmann-like fold is the most commonly occurring fold in almost every organism studied, mainly by virtue of the high recurrence of the P-loop hydrolase's superfamily. One interesting exception to this is the human genome, in which the immunoglobulin fold currently appears to be the most common fold.

FULLY AUTOMATIC VS COMPUTER AIDED PREDICTION METHODS

One feature of the CASP process that continues to be a concern is the difficulty in separating out wholly automatic predictions from those which have been made using various degrees of human intervention. It is not at all clear how much of the success shown in CASP comes from the algorithms which are being used and how much comes from the expert biological knowledge of the humans using the algorithms. It is very clear that humans do add some value, and all of the most successful groups at CASP4 made use of the scientific literature to identify functionally related proteins from a shortlist of possibilities. It therefore seems fair to say that human intervention is required to make the best predictions, but what can a non-expert hope to achieve using just automated methods alone? Also, is it possible to achieve the same high levels of success shown in CASP4 on many targets. If, instead of 30 or so targets, which could easily be analysed individually by human predictors, there had been, say, 1000 targets, how good would the results have been? Clearly, if fold recognition, or protein structure prediction more generally, is to play an important part in structural genomics then it is essential that we characterise the success of fully automated methods for structure prediction.

Fischer et al. have attempted to address this issue by creating a sub-section of the CASP process, called CAFASP (Critical Assessment of Fully Automated Structure Prediction) (28). The basic idea of CAFASP is to evaluate Web-based prediction tools in a fully automated fashion, thus eliminating the possibility of human assistance in the prediction process. CAFASP1 was carried out shortly after the CASP3 meeting, and must therefore be taken into account a pilot study as the predictions were not blind. Nonetheless, the results were interesting and the process allowed some technical issues to be resolved in good time for CAFASP2, which was an official part of CASP4.

Although the detailed results for CAFASP2 are available for viewing from the associated Web site (http://www.cs.bgu.ac.il/~dfischer/CAFASP2), it is possible to broadly conclude that although skilled human intervention is clearly beneficial, entirely automated methods still performed fairly well. The bad news is that, as with the human predictors in CASP4, the success of the automatic methods mainly came from relatively easy targets in the superfamily category. Targets in the analogous category were predicted very poorly by the automatic servers.

Ab initio Methods. In cases where it is not possible to build a useful model by comparative modelling, it might be hoped that methods might be available to calculate a 3-D structure directly from the amino acid sequence by means of pure physics or a knowledge of the rules of protein folding. This has proven to be, and remains, a very difficult challenge. It is not difficult to understand the practical applications of an entirely general method for predicting the tertiary structure of novel gene products, but is there any hope that such approaches will provide useful levels of success in the short to medium term?

Of course, not all ab initio methods are aimed at the prediction of tertiary structure. A great deal of progress has been made in recent years in methods which attempt to predict protein secondary structure. The reason there remains great interest in secondary structure prediction is because it is often used as a component of a wide-range of 3-D prediction methods. Indeed, although it is rarely used in isolation, accurate secondary structure prediction is exploited by the vast majority of prediction groups taking part in CASP. It has also been suggested that careful analysis of accurate secondary structure predictions can also provide functional information on a new gene sequence (29).

Up until around two years ago, the best and by far the most widely used method for predicting secondary structure was the PHD method developed by Burkhard Rost (30). At CASP3, however, the PSIPRED method showed a

marked improvement in prediction accuracy over previous methods (31). Although PSIPRED was very similar to PHD in concept (using two levels of neural networks to analyse sequence profiles) it used PSI-BLAST to provide more sensitive and more accurate sequence profiles. Added to this was a highly redundant training set including nearly 2000 separate profiles. At CASP4, PSIPRED was still ranked at the top of 20 or so methods evaluated, achieving an overall 3-state prediction accuracy (Q3 score) of 80.6% for all 40 target domains with no obvious sequence similarity to existing structures.

Predicting new folds - Somewhat more interesting than secondary structure prediction is of course the *ab initio* prediction of protein tertiary structure. The concept behind most ab initio approaches to protein structure is quite simple. Firstly, a large number of different chain conformations are generated for the target protein. At the very simplest these conformations can be enumerated exhaustively - i.e. virtually every distinct 3-D structure is generated for the protein. Clearly, this is only practical for very small proteins as for larger proteins the number of possible conformations grows exponentially with the number of amino acid residues. One way of slightly reducing the number of possible structures is to build the structure on a fixed lattice, which restricts the positions of the atoms in the structure to a fixed number of coordinates. For larger proteins, more intelligent search strategies are needed, which include molecular dynamics, simulated annealing, genetic algorithms and a number of other efficient search strategies.

Traditionally, very little success has been demonstrated in the *ab initio* prediction of protein tertiary structure in the various CASP experiments. However, in the 2nd CASP experiment, the best *ab initio* prediction was close enough (α-carbon RMSD of 6.2 Å) for it to be confidently claimed that at least the fold was correctly reproduced in the model (32). This prediction was generated by a Monte Carlo approach where fragments of protein structures are spliced together, and the resulting chain conformations evaluated using a simple energy function.

At CASP3 the group of David Baker took these ideas further with some success (33). As an aside, this kind of approach to folding proteins has become nicknamed "mini-threading" by some predictors. This terminology is perhaps useful to distinguish such knowledge-based prediction methods from methods which attempt to simulate protein folding using physical principles, but is otherwise quite misleading.

At CASP4, however, the Baker group showed an even higher degree of success across a much wider range of target folds than previously seen at earlier CASP experiments, where earlier successes had been limited to mainly alpha-helical folds.

WHAT CAN STRUCTURE TELL US ABOUT PROTEIN FUNCTION ?

Supposing that we have calculated a reasonable model for a protein of unknown function using one of the classes of methods described, the question still remains as to how useful protein structure is for elucidating the function of a protein.

At the most basic level there are apparent trends between the broad functional class of a protein and its structural classification. In a recent survey of the structures in PDB (35), for example, Thornton and colleagues show that the majority of enzyme structures are found to be in the $\alpha\beta$ fold class, as are nucleotide-binding domains. Hegyi & Gerstein (36) have also looked more at the relationship between fold classification and enzyme classification (E.C. number), where they used BLAST (1) to cross-reference between the SCOP database and SWISSPROT. In terms of fold class biases, their data is in broad agreement with the observations made earlier (35). However, by extending the data set by counting not just entries in PDB, but also the homologues of these

structures in SWISSPROT, Hegyi & Gerstein were also able to make some statements about the statistical relationship between functional class and protein topology. They found that the average number of functions found to be associated with a particular fold is 1.2 for both enzymes and non-enzymes, and 1.8 for enzyme-related folds alone. Furthermore, they found the average number of folds for a given function to be 3.6 (2.5 for enzymes alone). One interpretation of this is that, on average at least, the correct prediction of a protein's fold might be a very good indicator as to its function. Unfortunately, this evident good news is somewhat marred by the observed biases in fold distributions. The superfolds (Figure 3) such as the $(\alpha\beta)_8$ (TIM) barrel have been long known to be associated with a very large number of functions (37). Hegyi & Gerstein found the top five "multifunctional folds" to be the TIM barrel, the hydrolase fold, the Rossmann-like fold, the P-loop containing NTP hydrolase fold, and the ferredoxin-like fold.

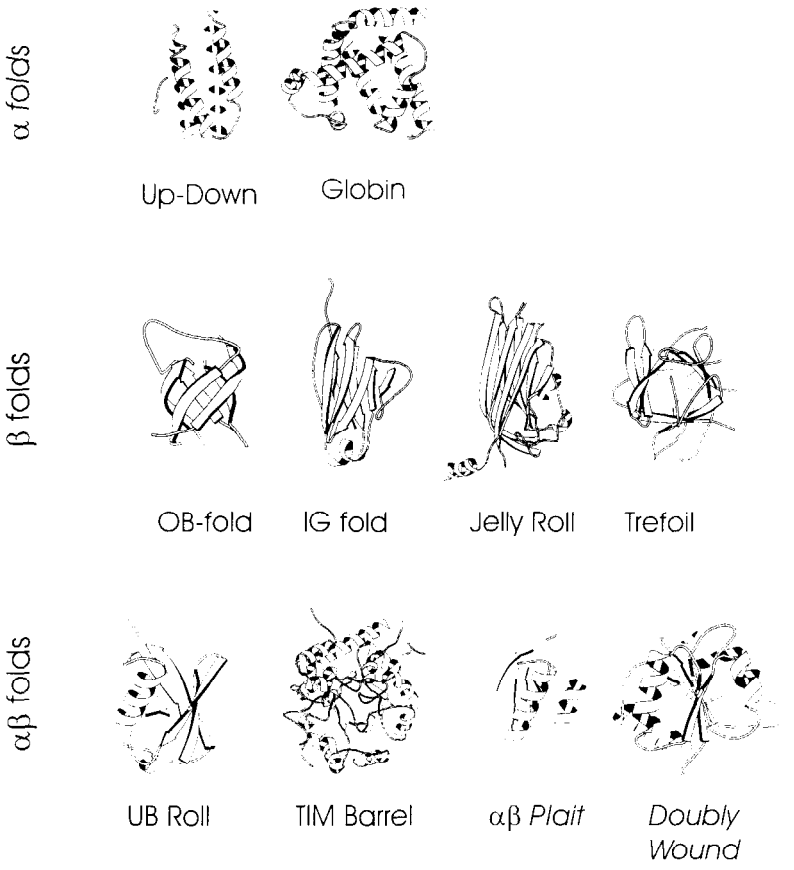


Figure 3: The 10 so-called "superfolds" currently found in the CATH protein structure classification scheme. These folds account for more than 50% of the observed structural similarities between protein domains. Despite the recurrence of these folds, there appears to be no indication of common ancestry between many of the proteins which exhibit these folds.

From this we can see that assigning the TIM barrel fold or a Rossmann-like to a particular gene product will give very little information as to its function. In

all probability, in the case of the TIM barrel fold, the gene would encode an enzyme (as almost all proteins with the TIM barrel fold are enzymes) but apart from this, very little functional information would be gained. On the other hand, one positive point to make about these structural similarities is that whenever a non-superfold structure is assigned to a new gene, based on current observations it would appear that the functions of the template protein and the target protein would be expected to be broadly similar.

TARGET DISCOVERY IN THE POST-GENOMIC ERA

We have seen that correct fold assignment can offer some insights into the potential function of a new gene sequence. So how do these and other methods perform in practice? As we have already noted, the first draft of the human genome has recently been released, though complete coverage of the genome and publication of a "gold standard" copy of the entire sequence is projected to take until 2003. Nevertheless, the race is now on in the pharmaceutical and biotechnology industry to identify genomics based targets which can be fed into their drug discovery pipelines. These targets are selected not just from the genomic DNA data, but also from the wealth of EST (Expressed Sequence Tag) data, both public and proprietary, that currently exists.

An essential requirement of a suitable target is an understanding of the function of the gene product. By combining annotation transferred from the proteins identified through sequence comparison, or profile and threading based searches such as Psi-BLAST (1), Hidden Markov models (3) and GenTHREADER (12) with gene expression data we can greatly enhance our ability to identify proteins which may only be distantly related to well annotated proteins. Indeed, while a sequence that is annotated as having similarity to a sequence of unknown function is of rather limited use, a sequence identified as having a fold similar to a serine protease, with a conserved catalytic triad, is very useful. If mRNA expression data were also available, it might even be possible to make an educated guess at its biological function. For example a serine proteinase that is highly expressed in the blood may suggest a role in blood coagulation even if one had not heard of Factor X (38). In the same manner, a protein which has been identified as functioning in a disease relevant pathway is of very high interest in the drug discovery industry. If the protein's biochemical function is unidentifiable it remains unassayable and does not present itself as a viable target. A recent relevant example of this is the nicastrin protein. Nicastrin was recently identified as apart of a multiprotein complex involved in the cleavage of the beta-amyloid precursor protein (39). One of the cleavage products of the beta-amyloid precursor protein, the neurotoxic beta-amyloid peptide, is found in the plaques of brains of Alzheimers patients. As nicastrin was shown to be directly involved in the production of the peptide, it presents itself as a target for therapeutic intervention. The authors used sequence based bioinformatic approaches to identify the biochemical function of nicastrin and were only able to identify unannotated homologues in model organisms (39). Using fold recognition techniques, we were able to identify nicastrin as having a central domain that belongs to the aminopeptidase/transferrin receptor superfamily (40).

Estimates of the number of genes in the human genome have ranged from 40,000-145,000 genes, but the current consensus now seems to be settling on an even lower value of around 30,000 genes (41,42). Current molecular drug targets are based on a narrow set of, what the pharmaceutical industry knows as, "drug friendly" protein families. These include proteases, kinases, nuclear hormone receptors, GPCR-like 7 tm proteins, chemokines, cytokines, and adhesion molecules (43). These are proteins that have been successful in screens for obtaining small molecule agonists/antagonists, hence the term "drug friendly". In order to maximise the probability of success and minimise the attrition rate in the pharmaceutical industry, which is currently running at

95%, many companies are focussing their efforts on identifying and characterising novel members of drug friendly protein families through genome wide searches. This has proved successful to date. For example, the indentification of novel GPCR's and cytokine receptors (44,45) using sequence based homology searches, coupled with lab based characterisation has produced interesting new targets. However, these homology based searches can not identify low homology members of protein families. The ability to carry out sensitive genome wide searches, annotating on the basis of structure, maximises target identification efforts. Identification of all homologues of a serine protease, both close and distant, helps enable selectivity issues to be considered prior to entrance of a molecular target into a small molecule-screening program, decreasing the possibility of toxicology issues further downstream.

Once a genome wide view of these protein families is known, prioritisation of the sequences for movement into the drug discovery pipeline begins. In order for this to happen, the biological significance of the protein must be identified and a disease relevance associated with it.

TARGET VALIDATION

Target validation can have different meanings, but broadly relates to identification of the biological role of a protein, and assessment if alteration of the activity/level of the protein will impinge on a disease phenotype. Such methodologies enable researchers to narrow down the number of potential drug targets to those that may truly enable disease modulation through small molecule intervention. Measurement of the mRNA level of identified gene sequences in a normal vs. diseased tissue can give an initial indication if a gene is involved in the disease process. This can be achieved in a high throughput manner using gene expression profiling (microarrays). This involves quantitative hybridisation of a large panel of cloned genes or synthetic oligonucleotides with the total cDNA derived from a particular cell or tissue type (46,47). If the mRNA levels of a particular gene are differentially regulated in the diseased vs. normal tissue it is an indication that perturbation of that gene product may play a role in disease pathogenesis (48,49). Further validation is then required that specific knockdown or over-expression of the gene leads to alteration of the desired phenotype. This can be done using a number of technologies, such as antisense [50], retroviral and adenoviral based methods (51,52). This is just one method used currently to validate a gene for drug discovery. It is beyond the scope of this current review to explain each of the different methodologies that may be used for target validation, but there have been several good reviews on the subject (53). A good example of this technology in use was to validate the fact that overexpression of the EGR1 transactivatior gene could potentially regulate a number of steps involved in initiation and progression of prostate cancer [54]. It was known that the EGR gene was overexpressed in prostate cancer. The EGR gene was overexpressed in a prostate cancer cell line using adenoviral based approaches. Using oligonucleotide arrays, a number of EGR1-regulated genes were identified. A number of the regulated genes were growth factors that had previously been implicated in enhancing tumour progression as well as some cell cycle related genes. Using this global transcriptome (genome wide mRNA expression) technology, the role of EGR1 in prostate cancer was validated. These sort of "gain of function" experiments are useful in identification of signal transduction pathways. A gene such as EGR1 itself may not be a well suited molecule for small molecule intervention, but one of the proteins that is under its regulation may well be and hence present itself as an alternative target. In another example, superoxide dismutase (SOD) was identified as the molecular target of certain oestrogen derivatives and that this is the basis for selective killing of human leukaemia cells, but not normal lymphocytes (55). Selectivity relies on the fact that SOD levels are low in cancer cells, dependence on active SOD is high, thus inhibition of SOD is highly effective. In this example microarrays were able to

identify the molecular target of oestrogen in malignant cells. If transcriptome data is to be utilised to its fullest, high quality annotation of the maximum amount of genes is necessary.

One of the interesting methods employed today in order to place a molecule in a pathway, hence biological process, is proteomics. Proteomics refers to the ability to monitor genome wide protein expression. Proteins rarely exist alone in cells, they interact with other proteins, and exist in complexes that make up signal transduction pathways. A serine protease of unknown function is of little use to pharmaceutical/biotechnology companies. By identifying what other proteins the serine protease interacts with you may place it in a pathway that may give you disease relevance. Physical/experimental methods such as yeast two-hybrid (56,57) and functional proteomics (58,59) enable researches to identify protein-protein interactions.

Microarrays are now being used in order to analyse both protein-protein interactions and protein amounts. Although the approach is not nearly as robust as transcriptome analysis, the microarray approach to investigation of proteins is increasingly sensitive. A microarray approach has been used to study the role of cholesterol in the biosynthesis of beta-amyloid peptides (60). Antibodies to the target, here the beta-amyloid peptide are covalently linked to a chip in order to make a microarray. The profile of amyloid beta (A beta) peptide variants secreted into the media of human cultured cells that express the amyloid precursor protein was examined using Surface Enhanced Laser Desorption/Ionization (SELDI) ProteinChip technology from Ciphergen Biosystems. An anti-A beta polyclonal antibody (anti-NTA4) was used to capture and purify multiple immunoreactive A beta fragments from a single microliter of media onto the array. Fragments retained on the surface of the array were detected directly by mass system to provide information on the identity of different A beta-variants secreted from the cultured cells. Using this information the investigators were able to directly measure how alteration of cholesterol levels alters the levels of the different amyloid beta peptides.

The next step in microarrays would be the introduction of a more global analysis of the proteome, where at least 5000-10000 proteins could be analysed using the same principle. Although still relatively time consuming, advances in the production of single chain antibodies coupled with selection of potent antibodies for each protein of interest through the use of phage display technologies, will certainly increase the likelihood of this.

In a similar manner genome wide prediction of protein-protein interactions would be of unquestionable importance. The approach developed by two groups independently is remarkably simple, and therefore elegant, in its concept (61-64). There are now many examples of protein that interact with one another within a particular organism that exist as domains of a single protein in another. This suggests that by finding two distinct hits to a query protein using, what is essentially, a BLAST search with a bit of post processing one is able to build a database of predicted protein-protein interactions. Of course the method will not work for all cases and it is clear that EGF and other promiscuous domains will give poor quality results. Notwithstanding this, it is a very exciting development and Marcotte *et al.*, have attempted to provide a comprehensive view of protein interactions by considering not only these predictions but also the data available from databases such as the Yeast Protein Database.

CONCLUSIONS

This review has hopefully demonstrated that protein structure prediction does have a role to play in helping researchers in the pharmaceutical and biotechnology industries to identify, annotate, and functionally characterise new genes. What we have described here are some of the current cutting edge technologies that are in currently being deployed in the pharmaceutical industry.

The number of methodologies used to identify and validate genes of potential therapeutic interest will undoubtedly grow in both scope and throughput in the future to the point where a researcher has not only a transcriptiome wide view of the genome, but a reliable proteome view entailing all expressed proteins and potential interaction partners.

It seems that *ab initio* methods for protein structure prediction, whilst sometimes achieving interesting results on fragments of proteins, are unlikely to be used for genome analysis. The success of *ab initio* algorithms has never been tested rigorously on a large number of test cases, and so the chance of finding a reasonable model for a target protein is unknown. However, results in the four CASP experiments suggest that the chances of any single algorithm producing a reasonable model for a given sequence is very low.

Fold recognition algorithms, on the other hand, have now reached a point where fair models for target proteins can be found on a routine basis, especially where a homologous template structure can be found. Not surprisingly, therefore, different fold recognition methods have already been applied to the problem of assigning folds to genome sequences. The simplest and most reliable predictions are based purely on sequence similarity, and in particular PSI-BLAST (1) is proving to be a valuable tool for detecting remote homologous relationships between protein sequences. At the other extreme, fold recognition methods which typically ignore sequence similarity and make use of structural information have also been applied, but with somewhat less success. Hybrid methods, which combine sequence comparison and fold recognition methods are expected to be an important development in this area. Such methods can detect homologous relationships just beyond the detection threshold of methods such as PSI-BLAST, as evidenced by the results for the first such algorithm (37).

So how is the relationship between computational approaches to protein folding and the ongoing structural genomics projects likely to develop? It is clear that protein structure prediction is never likely to challenge experimental methods in the determination of accurate structural models for proteins. The role of protein structure prediction and modelling lies in the rapid analysis and annotation of proteins, the analysis of proteins for which experimental structure determination has proven to be difficult, and the extrapolation from existing experimental structures to other members of the protein's family and superfamily.

Eventually, of course, the library of protein folds will be complete and protein structure prediction will become a more or less academic problem. Eventually there will almost always be available a closely related protein of known structure with which to build an accurate model for any given target protein. How long until this occurs? It is hard to give an accurate answer to this question. Going by current rates of experimental structure determination and the consequent growth of the structure databases, we may not have a complete set of protein folds for another 15 years or perhaps longer. On the other hand, the structural genomics initiatives may well stimulate the development of novel methods for rapid structure determination and so reduce this time. In either case, will theoreticians suddenly become obsolete? Unlikely. There will remain a rich selection of unsolved problems to keep theoreticians in structural biology busy for as long as they wish: protein misfolding, protein-protein interactions and biomolecular recognition, drug design, membrane proteins, *de novo* protein design and even nanotechnology are just a few of the many challenging future possibilities for continuing theoretical studies of protein structure.

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Chapter 22. Proteomics: Defining Protein Function in the Post Genomics Era

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Introduction - The flow of research from gene to protein to function is currently under a revolution. Never before have there been as many actual or predicted gene sequences within the public domain. Coupled with the enormous amount of data provided from transcriptional profiling efforts, many researchers are at a loss as to how to gain relevant knowledge from all of this information. The number of genes within the human genome has been estimated to be somewhere between 30,000 to 40,000. Taking into account alternative splicing and post-translational modification, the number of corresponding protein products will be much higher. In order to begin to address the daunting task of describing function to these proteins, a number of approaches have been implemented. This article will attempt to review the various technologies currently employed towards this goal and how these efforts will impact the drug discovery process.

A blueprint of the human genome has recently been completed (1,2). This impressive scientific achievement has posed many more questions than it has answered. It is now apparent that the scientific community has a tremendous amount of genetic information with only limited tools on how to attribute function to this information. Even if the entire genome could be properly annotated to identify all of the open reading frames (ORFs) such that we were armed with a validated sequence for every human gene, the ability to attribute function to the proteins encoded by all of these genes is our next big challenge. Proteomics refers to the effort to determine the biological function of multiple gene products. In its broadest sense, proteomics describes the multi-parallel examination of proteins or protein function. Although this process has been performed for decades on single or limited gene sequences, never before have we been faced with the daunting number of genes currently available. Although exceptional tools are available for the analysis of genetic information, the output from these studies still is tied to the nucleic acid, not the protein moiety. Since proteins are the mediators of function within the host and are the targets for the drug discovery process, data derived from the genome sequencing projects must be converted to functional information about proteins on an unprecedented scale.

Proteomics can be loosely broken down into methodologies which are: 1) aimed at novel target identification, 2) designed to elucidate function of a chosen set of gene products, or 3) designed to identify relationships between proteins or protein families (such as protein: protein interactions). This review will serve to provide a general overview of the experimental procedures employed in this field but is not an exhaustive analysis of this exploding new effort. Where possible, the detail of various aspects is referenced to recent publications that cover more detail.

EXPERIMENTAL APPROACHES TOWARDS NOVEL TARGET IDENTIFICATION

Novel targets have two common definitions. Targets which have never been seen before in the literature and thus their novelty derives from their discovery, or targets which are known but have never been associated with a given cellular process or disease state and thus their novelty derives from their association with the specific process. Both categories of protein targets are areas of aggressive investigation.

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By far the workhorse of the early target discovery efforts has been the two dimensional gel separation of complex protein mixtures followed by mass spectrometry identification (2D - MS). In this technique, proteins from a given biological sample are separated by isoelectric focusing (IEF) to provide the first dimension. Following this separation, the proteins are then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to provide the second dimension. The protein gel is then visualized by a variety of methods to produce an image of hundreds to thousands of proteins. Spot identification of a

Table I Common Methodologies used in Proteomics

Target Identification
- 2D gel MS
- LC-LC MS
- ICAT MS
- Protein Profiling Chips

Functional Screening
- High Throughput Screening
  - biochemical and biological
- Knock-out models
  - *in vitro* and *in vivo*
- Computer modeling

Protein:Protein interaction mapping
- Immunoprecipitation coupled to MS
- Yeast two-hybrid
- Bacterial two-hybrid
- Phage display
- mRNA display

given feature within this gel is performed by excision of the spot from the gel, proteolytic digestion of the protein (usually by trypsin) and then analysis of the resulting peptide fragments by mass spectrometry (MS) or tandem MS. This approach has identified hundreds of protein targets and provided excellent leads for protein discovery. However, this technology has many disadvantages that have limited its use in a large-scale effort. These disadvantages include the use time consuming labor-intensive techniques, the fact that it is non-quantitative and the finding that the majority of the proteins identified by this technique represent the most abundant proteins limiting the discovery process to only a subset of cellular proteins (see 3 and references therein for details). Advances such as isotope-coded affinity tag (ICAT) peptide labeling (4) and multidimensional liquid chromatography coupled to tandem MS (LC-LC MS) (5) should expand this core competence and add additional value to the use of MS as a readout (3,6).

Transcriptional profiling of mRNAs has been highly valuable for the identification and prioritization of gene products based on their expression profiles in a given disease state, functional setting, drug response cascade, etc (7-10). In this setting, two or more mRNA populations are labeled with separate fluorescent signals, combined and then used to probe microarrays containing cDNA products or oligonucleotides corresponding to a given gene sequence. Post hybridization, the filters are analyzed for the relative intensities of each test population at a given feature to determine the expression level within the original sample. Although this technology has been able to define expression profiles of genes that are either up- or down-regulated under various conditions, there have been difficulties in relating these mRNA changes to protein expression changes. In particular, a lack of correlation has been observed between changes in mRNA and protein expression levels (11, 12). For this reason, relying on the information gained solely from transcriptional profiling may be misleading when attempting to make prioritization decisions. There has been considerable effort to develop tools to ease analysis of protein expression levels and, thereby, to decipher protein involvement in specific states. Not surprisingly, this has become a very active field in both academia and industry.

With protein profiling, a microarray of specific protein "binders" must first be created. This effort will carry with it all of the hurdles that previously challenged transcriptional profiling (i.e. surface chemistries, content issues, detection methodologies and data interpretation) together with the additional burden of working with a protein molecule rather than a nucleic acid. Nevertheless, there are many efforts towards the development of protein affinity arrays using a variety of different approaches. The most obvious application is to use existing monoclonal antibodies (mAbs) attached them to a solid surface to develop an assay within the context of a micro-ELISA type setting. Early work from Stanford has shown that this approach can yield encouraging results. However, only about 20% of the tested antibodies were able to provide specific interactions with their corresponding ligand in this setting (13). Another approach is to create a set of affinity reagents to be leveraged for their use on solid phase. In one example of this, phage display selection of single chain antibody fragments (scFv) has been employed to identify synthetic affinity reagents from large antibody libraries. These reagents can be used for a variety of approaches, including the creation of antibody arrays (see 14 for review). Since whole antibodies, as well as their fragments, can yield sub-optimal or non-active binding agents for this application, other groups have approached this area by designing and creating novel affinity mimics which can be used in the place of conventional antibodies or antibody fragments. One such mimic is the tenth fibronectin type three repeat (^{10}FnIII). This is a very stable molecule that displays three surface loops analogous to one half of an antibody binding pocket. By creating very large libraries of ^{10}FnIII molecules where these three loops are randomized, high affinity binding molecules, which are active on a chip surface, have been successfully created (15). The combination of all of these efforts will hopefully lead to the creation of a microarray that can profile the protein content of any given biological sample.

FUNCTIONAL SCREENING

Once identified by using some of the methods discussed above, a protein target often has no associated function or has been put into a new setting where a previously defined function may not be its mode of action. When there are only a handful of protein targets of interest, straight forward functional characterization using low throughput, labor-intensive screens can be applied. However, with the increasing number of newly-discovered protein products, this type of analysis needs

to be adapted for a higher throughput. High-throughput biological screening has been used for many years in an attempt to quickly identify the function of a set of proteins. For this, a wide net of simple phenotypic screens are employed to obtain an "hit" which can then be screened with more labor-intensive and directed assays. It is analogous to small molecule screening but at the protein level. Often this is done with cellular assays but also can involve direct biochemical screens for binding or enzymatic activities. One of the huge bottlenecks in this application is the production of the proteins to be tested. There is also a limitation on the actual number of screens that can be performed in an accurate manner. Coupled together, these limitations significantly decrease the overall throughput and the value of this type of screening will be dependent on the ability to adapt these assays to a more minaturized/microscale platform.

Other approaches leverage the genetic information for the proteins of interest as the reagent rather than the protein itself. That is, the gene that encodes the new protein is used as a tool to remove the protein in cells by use of anti-sense or homologous recombination mediated genetic knock out. For antisense applications, oligonucleotides corresponding to the complement of the genetic material to be targeted are delivered to a cell in an attempt to shut off the transcription of the mRNA (or directly degrade it) and thus turn off protein production. Once shut off, the effect of the loss of that protein is used to help decipher its potential function. These experiments have been successfully applied in a variety of different areas (16,17) and have recently moved from *in vitro* cell based assays to whole animal models (18). Another application that leverages genetic information is gene targeting by homologous recombination in mouse embryonic stem cells. This is a powerful technique to determine the physiological function of any gene product in a living host. Here, the same concept applies as for the antisense knockout but this application is directed at removing a protein function in context of a mouse embryo. Although classically used for the "final" validation of a given protein target, advances in the speed of development and increased numbers of knock-out mice has slowly seen this technology used more as a screening application. Unfortunately, the biological characterization of the resulting knockout mouse still presents a high experimental burden.

In an attempt to gain information on a given protein without the burden of massive screening, some investigators have turned to structural genomics. Structural genomics uses a program of high-throughput X-ray crystallography and NMR spectroscopy to determine protein structures (see 19 for review). This information is then coupled with computer software to perform a number of different "assays." Since many protein functions are domain mediated, sophisticated searches of the structural databases can quickly identify potential functions for an unknown protein based on homologous domains found in proteins with known function. In addition, virtual screening can be performed using a number of different computer simulation programs. In this application a given crystal structure is utilized to perform computer based docking experiments with known or virtual synthetic libraries. Molecules predicted to interact with the active site of the protein target are then synthesized and checked for functional activity in a lower throughput manner.

Unfortunately, the ability to carry out many of the assays mentioned above in a highly parallel manner has been a limiting step. As the number of potential proteins of interest escalates, these types of screens will be limited in their applications. In addition, all of these assays are designed to elucidate the function of a given protein by some predetermined assay or with some predetermined data set. Last, most of these assays are used to investigate receptors, surface antigens or soluble ligands. As the investigation of protein function moves forward, many investigators have turned to the huge number of intracellular protein pathways as potential targets for

therapeutic intervention. Here, a new set of screens must be designed to begin to attribute function to this different set of proteins.

PROTEIN:PROTEIN INTERACTION MAPPING

Protein:protein interaction mapping refers to the study of a given protein target in context of the other cellular proteins with which it interacts. Signal transduction pathways are an often complicated series of protein:protein interactions that translate an extracellular signal into a specific cellular response. Since these pathways are responsible for the integration of cellular events, understanding the different components of a pathway can lead to a better understanding of cellular responses such as proliferation, apoptosis, or drug susceptibility. For these studies, the target protein is used as "bait" to capture the interacting proteins known as the "prey". However, the target protein can be used as bait in the absence of knowing much about it in biochemical or biological terms.

A further application of the 2D-MS technology discussed previously has been employed in this area. In this application a target protein is immunoprecipitated under conditions where protein complexes will remain intact. Following this, any interacting partners which were complexed with the protein are identified using 2D-MS (20,21). Although this approach has been successful, it carries with it the limitations of 2D-MS already discussed as well as the requirement of an affinity reagent for, or the incorporation of a tag on, the target protein. This decreases the utility of this application for genome-wide analysis.

The yeast two-hybrid technology has been employed for over a decade and has identified many protein:protein interactions (22). This assay takes advantage of the GAL4 transcriptional activator which has a domain required for DNA binding which can be physically removed form another domain that activates transcription. A "bait" protein is produced as a fusion molecule with the DNA binding domain and interacted with a library consisting of fusions with the transcriptional activator domain. If the bait forms a protein:protein interaction with a member of the library, the functional reconstitution of the two domains results in transcriptional activation within the nucleus of the yeast host. Using this technique, massive protein:protein interaction maps have been constructed across Caenorhabditis elegans (23), Helicobacter pylori (24), as well as the yeast Saccharomyces cerevisiae (25-27). Once created, this large amount of information needs to be collected, analyzed and the interactions annotated in such a way as to be useful. As highlighted recently, the creation of networks of interactions (by any method), coupled with information from other biological experiments, will provide an invaluable tool in the proteomics arena (28,29).

However, the yeast two-hybrid technology brings with it many limitations (30). First, poor reproducibility from experiment to experiment has been observed and could arise from a range of different experimental steps. Second, false positives have continually plagued this technology and remain a burden for the validation of the interactions identified. Third, the library construction is host associated and therefore the size of the library is limited, as is the representation of rare genes. Last, this technology is performed within the nucleus of a host and brings with it the limitations of performing selections within this host environment. That is, during the selection step it is impossible to vary conditions (such as salt concentrations, pH, etc.) or control the selection stringency by varying the wash conditions. In an attempt to remove some of these limitations and create new selection capacities, alternatives to this technology have emerged.

Recently, a bacterial two-hybrid selection system has been described (31). Although this is still host associated, library size has increased as compared to yeast two-hybrid and the interactions take place in the cytoplasm rather than the nucleus. However, such host mediated methodologies still carry with them limitations at the level of the selection step and even further improvement has come from the application of *in vitro* display technologies in the protein:protein interaction mapping efforts.

Phage display was first described over 15 years ago and represents the first library technology to link genotype to phenotype (32). In this technology, a library of genetic sequences is transformed into bacteriophage and the resulting proteins are displayed on the surface of the phage. Selections for protein function can be carried out by panning the library across the target and selecting for phage particles displaying the peptide or protein that interacts with the bait protein. Upon isolation of the phage, the genetic material that encodes for the selected protein can be identified. Although successfully used for a great number of synthetic based peptide or protein libraries (33), phage display has not been widely used with libraries generated from cDNA sources. Because of the success of using phage display in peptide based selections, some have approached this area by first selecting random peptides that interact with a protein target. Following this, bioinformatics is used to analyze the available databases to determine whether a protein contains the peptide sequence or similar domain to the sequence selected. One must then determine if that peptide sequence confers a binding capacity to the protein identified, or if it is normally buried within the protein and is not available for interaction. Since this type of approach is one step removed from the cellular target, recent studies have focussed on using cDNA libraries and have shown the successful phage display selection of cellular proteins interacting with the cytoplasmic tail of surface receptors or with transcription factors (34-37). Once again, this technology is still limited by a host component in terms of the bacteriophage displayed library as construction of the library within this host limits the size of the library that can be created and also biases the proteins represented to those that can be processed through the prokaryotic machinery.

The technology of mRNA display has recently emerged as a further improvement for the *in vitro* display field. First described in 1997, this technology creates libraries of proteins that are covalently attached to the mRNA which encode them (38). Similar to phage display, this links phenotype with genotype but does so in an entirely *in vitro* environment. Because of this, the size of the libraries created are very large ($>10^{13}$), the selection conditions are completely controllable, and the steps involved readily automated (39,40). This selection technique has been successfully applied to peptide based selections (41,42), the selection of antibody mimics (15) as well as proteomic based selections (43). In the last example, a mixture of different tissue specific mRNA display libraries were used to select protein:protein interactions mediated by the apoptotic protein Bcl-XL. Known and novel partners of this important regulatory pathway were identified. Since this technology is completely host independent, the library construction is not subject to the inefficiencies of other systems and all of the steps in the procedure are easily automated. This provides significant advantages as we look forward to a genome-wide analysis of protein:protein interactions from human tissues.

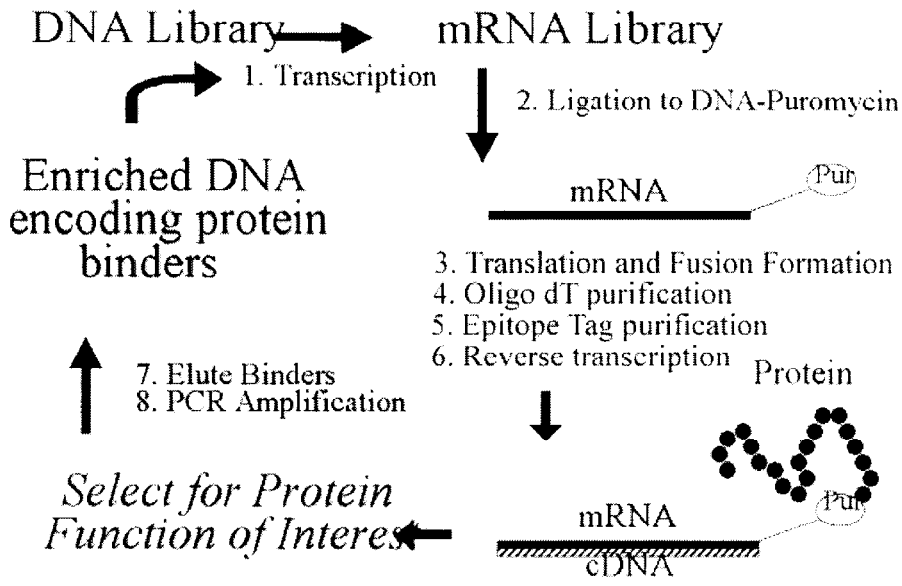


Figure 1: ***In vitro*** **selection using mRNA-display.** A DNA library is constructed as a PCR product to engineer appropriate flanking sequences at both termini. This library is then *in vitro* transcribed to create an mRNA library. A poly-dA linker containing a puromycin (Pur) moiety is then ligated onto the 3' end of the mRNA. Upon *in vitro* translation of this library, puromycin becomes covalently attached to the C-terminus of the protein product. Following this, the mRNA-Protein fusion molecules are dissociated from the ribosome, purified, and a cDNA strand synthesized on the mRNA template using a reverse transcription reaction. This library of fusion molecules is then subjected to a selection step by incubation with a target protein, washing and elution. The eluted cDNA products are amplified by PCR to produce an enriched library of binders and the process is repeated.

CONCLUSION

The publication of the human genome this year has opened up a new age of discovery. In response to this, a wide variety of techniques are now available for both the discovery of new targets and the subsequent assignment of function to these targets. The methodologies discussed in this article yield results with different levels of validation and must be interpreted in context of information coming from other sources. Relying on one approach to give the final answer will not be prudent as we move forward on the quest to assign function to the human proteome. The combination of novel target discovery with biological and biochemical screening will result in an enormous amount of relational data sets. These need to be properly annotated with computer software to become a knowledge base rather than a series of data points (44). Contributing to this knowledge base will be methodologies not discussed in this review but still just as important to the overall goal. One such example worth noting here is the need to create genome wide affinity reagents for the final analysis of these protein targets. Although not directly discussed for this purpose, the agents created for the protein profiling chips (mAbs, scFv's and antibody mimics) will also need to be leveraged for conventional antibody type procedures such as western blot analysis, FACs analysis, immunoprecipitation, immunohistochemistry, cellular localization, and potentially, functional knock out reagents. Since the production of thousands of mAbs by conventional techniques is not a practical solution, leveraging the various *in vitro* display technologies towards this goal will be important for future success. Combining all of these techniques, reagents, and tools towards the goal of attributing function to the human proteome will hopefully result in the identification, prioritization and validation of hundreds to thousands of clinically relevant targets.

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Chapter 23 . Therapeutic Monoclonal Antibodies : History, Facts and Trends

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Introduction - During the last three years it has become evident that monoclonal antibody (mAb) therapies are effective in a wide range of diseases. As a result, the development of mAb technology and antibody therapeutics has attracted the attention of biotechnology and pharmaceutical companies alike. The current estimated market for these agents is about $25 billion each year. In this review, we will summarize the critical molecular biology, immunology and clinical research discoveries and the recent industry trends which 111 years after the first use of a therapeutic anti-serum, finally ensure the development of safe and useful therapeutic antibodies.

HISTORY AND PERSPECTIVES

From serum therapy to genetically manipulated human antibodies - The importance of *natural immunity* has been widely recognized and defined by the generation or presence of protective, therapeutic antibodies to infectious or foreign agents (1). Original reports of survivors from notable epidemics were frequently interpreted as signs of special powers that identified these individuals for posterity. This belief is linked to the concept, dated from ancient Greek and Egyptian cultures, that these lethal diseases were punishments to serious faults, and thus survivors must have been spared because they were chosen and their faults forgiven, -hence holding a special standing in society. Such is the case of a medical doctor who lived in France in the 16th century, Michel de Nôtredame, also known as Nostradamus. His unexpected survival to the plague that affected Aix and Lyons in 1546 and 1547 made him famous and people believed that he held special powers due to his resistance to lethal infectious diseases (1,2). As we know today, he survived because of a rapidly responding immune system. The acquired immune response is composed of two arms, cellular and humoral. Antibody responses make up the humoral response and as was demonstrated through originally crude experiments, immune protection from disease can be passively transferred through serum containing antibodies.

Von Behring and Kitasato in 1890 not only contributed significantly to the discovery of antibodies but they demonstrated for the first time that anti-toxins (antibodies) are effective in vivo (1). In his "Nobel winning" experiments, von Behring injected patients with anti-toxin containing sera which had been prepared by immunization of animals with diphtheria or tetanus toxin. The anti-toxin neutralized the lethal toxin in-vivo and saved the lives of many children and adults far before the discovery of antibiotics. The development of antiserum for the treatment of acute diseases caused by soluble toxins (e.g. snake bites) find their original foundation in von Behring's work. However, the risk of anaphylactic shock, which was frequently induced by the injection of heterologous serum (from horse, goat, rabbit) severely limited the use to life threatening and otherwise untreatable rare conditions. It took more than a half century to understand the precise mechanism of anti-toxic serum therapy and the lethal side effects. Nevertheless, the early clinical studies opened the door for antibody therapy.

With the advent of mAb development and advanced genetic manipulation of antibodies novel therapeutic approaches were born. Initially, mouse monoclonal antibodies were tested (first FDA approval in 1986) in the clinic. These therapeutic antibodies were directed

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against immunologic targets in acute diseases; TNF alpha and IL1 in endotoxin shock (3,4), CD3, IL2r p55 in acute graft rejection (5,6) and graft versus host disease (7), respectively. Soon, however, it became evident that mouse monoclonal antibodies were not suitable for chronic administration. In order to improve the clinical performance, human/ mouse chimeric antibodies were produced and numerous mouse antibodies were humanized by genetically splicing extensive human frameworks into the mouse protein. Later, by the insertion of human immunoglobulin genes into immunoglobulin gene deficient knock out mice, human antibodies were produced in mice (8). Human mAbs and genetically modified small antibody-like mono (Fv) or bi-specific fragments are produced today by expressing and selecting antibody like proteins on the surface of lambda phages and by producing large quantities via current biotechnological methods, *in-vitro*, and *in-vivo*, (e.g. in the milk of transgenic goats). Antibodies have been used as research tools, diagnostics and as therapeutic agents. One segment of therapeutic Ab application is the delivery of irradiation or toxins to targets cells with the goal of killing the contacted cell. For this purpose the Abs are conjugated to either beta ray emitting isotopes or potent metabolic toxins (immunotoxins). The advantage of using Abs to deliver the toxins is found in the exquisite specificity of the antigen recognition portion of the molecule. The more recent chimeric, humanized and fully human antibodies and immunotoxins being profiled in the clinic demonstrate dramatically improved clinical efficacy which expectedly correlates with the technology development of recent years (Table 1). By now, basic target selection, mechanism of action, pharmacological and clinical paradigms have been postulated, tested and allow the investigation of therapeutic Abs for the treatment of a variety of diseases, including autoimmune, inflammatory, infectious diseases and cancer. Expression of antibodies is possible in plants.

	86-88	89-90	92-94	95-97	98-00
Type of antibodies					
Mouse	1			1	
Chimeric			1	1	3
Humanized				1	4
Therapeutic areas	Immune		Cardio	Inflammation Cancer	Infectious Cancer Inflammation Immune

Table I: FDA approval of therapeutic antibodies

INTRODUCTION TO ANTIBODY STRUCTURE AND FUNCTION

Antibodies and Fc receptors - Antibodies represent a dominant class of serum proteins; reaching 10 mg/ml in the serum following immunization or during the course of severe chronic infections, or as a result of proliferative diseases of antibody producing cells. The major classes of immunoglobulins (Ig) include IgG, IgA, IgM, IgD and IgE. IgG represents the protective, high affinity antibodies circulating in the blood (with a half life of about three weeks) with neutralizing activity for infectious agents, bacteria, viruses or soluble toxins. IgG molecules are composed of two heavy chains and two light chains which are linked to each other by disulfide bonds. An antibody is composed of multiple Ig domains. A heavy chain contains four Ig domains, while the light chain has only two. The N terminal Ig domain of a heavy chain and a light chain aligns in the Ig molecule and forms the antigen-binding pocket. One IgG molecule has two antigen binding sites, this portion of the protein

can be separated from the constant region by enzymatic digestion which cleaves the molecule to the antigen binding F(ab)2 and the constant, Fc fragments. Peptide sequence of the N terminal Ig domain is variable, a property achieved by recombination and somatic mutation mechanisms. The two variable segments that together make the antigen binding pocket contain outstandingly important peptide sequences in the complementarity determining regions (CDRs) responsible for interaction with the antigen. CDRs represent the most variable peptide sequences in the Ig molecule and are coded by variable (V), diversity (D) and joining (J) genomic segments. The total possible recombinations of various VDJ segments and somatic mutation are responsible for the diversity of antigen binding sites that exceeds10^6 possible permutations. Invariant parts of Ig chains are termed constant. The five different heavy chain constant region genes determine the Ig class (IgD, IgM, IgE, IgA and IgG subclasses). Ig classes differ in their effector activities which are associated with the distal, Fc portion of the molecules. Specific Ig binding receptor mediated activation or inhibition of macrophages, granulocytes and mast cells, as well as intestinal, endothelial and placental transfer of Ig molecules are dependent on various forms of Ig class specific Fc receptors.

Monoclonal antibodies - IgG is produced by B cells and plasma cells. Each B cell makes only one type of Ig molecule, all molecules carry the same variable sequences because VDJ recombination is irreversible, and it takes place at an early stage of B cell clonal development and because of allelic exclusion that ensures protein expression from only one IgG allele in a cell. During immunization, rising serum titers of antibodies represent a mixture of antibodies produced by a large population of B cell clones, thus serum derived antibodies are polyclonal.

Milstein and Kohler discovered monoclonal antibody production in 1975 (9). In their method, mice were injected with a protein antigen to initiate an immune response, the spleen of the animal was harvested and the antibody producing-B cells of the spleen were immortalized by cell fusion with a myeloma cell line. This fusion allowed the cells to live indefinitely expressing and secreting large amounts of antibody that could be harvested and screened for functional activity. Since this time antibodies have held tremendous promise and have been equated to a "magic bullet" because of their specificity and disease modifying potential. The application of antibodies is broad ranging including: research tools, diagnostics, and therapeutics. The first therapeutic antibodies were mouse monoclonal antibodies that were selected against cytokines and cell surface proteins of proinflammatory, immunologic or cancer cells.

THERAPEUTIC ANTIBODIES

Mouse monoclonal antibodies -The first reported successful antibody therapy targeted a malignant B-cell lymphoma, in a patient who received a lymphoma specific mAb. (10). In this and numerous other anti-cancer therapy regimens the mechanism of action was the targeted binding of the mAb to the cancer cell, which would then be lysed by complement activation through the cell surface-bound antibodies. Although theoretically possible, clear in-vivo evidences for this mechanism of action are often weak, and often suggest Fc receptor mediated activation of phagocytotic and NK cells. The targeting concept was advanced by the discovery of selective tumor-cell surface antigens, which are not expressed on non-malignant cells and do not exist in soluble forms. If the tumor is readily accessible to the antibody, the therapy is generally more effective. If the Ab is targeted at a tumor-specific antigen, tumor lysis may be enhanced by conjugation of beta and alpha emitting isotopes (131I, 90Y, 211 astatin, 212 bismuth,) to the Ab. An additional application for the labeled Abs is diagnostic imaging, however this often requires the addition of hazardous gamma emitting isotope (e.g. 111Indium). Two important examples of anti

cancer mouse mAbs are Ovarex (CA125) for ovarian cancer (11) and Panorex (17-IA) for colorectal cancer (12). The best use of these reagents is as adjuvant to surgical and/or tumor suppressive therapy to eliminate residual tumor mass and as a palliative agent in therapy resistant cases. Chronic use of murine mAbs is limited because of their immunogenicity and short half life. However, cancer therapy remains in the focus, mostly due to the successful anti breast cancer drug, the humanized anti HER2 mAB, Herceptin which not only targets but inhibits tumor cell growth via down modulating an active signaling molecule HER2 (13).

The development of mAB technology boosted the functional characterization, immunologic definition and CD clustering of surface antigens of lymphocytes and antigen presenting cells. This process coincided with the early phase of cytokine discovery. Both of these fields generated new concepts in immunology by the recognition of critical surface antigens (CD3, CD4, CD20, CD25, CD28, CD40, CD52 integrins etc) and the first interleukins (IL1-IL9, chemokines and TNF family of cytokines) via their blockade with mABs in-vitro and in mice. The straight forward approach was to test the mABs in human diseases. The best known early mABs were directed against selected inhibitory epitopes of CD25, the alpha chain of the IL2 receptor (anti-Tac), CD3 (OKT3/Orthoclone), depleting CD4 specific sites (OKT4A, B-F5, 16H5, VIT-4 etc.) and ICAM-1 (BIRR-1) (4-7) which have been shown to be effective in the treatment of acute graft rejection, graft versus host disease, and showed limited improvement in some cases of severe inflammatory diseases like rheumatoid arthritis and inflammatory bowel disease (14). The most successful product of this era, the CDw52 specific Campath-1h was humanized later. This reagent is a potent inducer of complement mediated lysis and its best application is the purging of T cells from bone marrow samples used for transplantation, however it was shown to be a successful T cell depleting agent in chronic inflammatory and autoimmune diseases as well (15). The first anti cytokine blocking agents were also mouse mABs directed against TNF alpha, IL1, IL2, IL6. These antibodies were tried in acute and chronic inflammatory diseases with more failures than success. The failures were due to two important deficiencies of mouse mABs (i) the induction of immune response and potentially life threatening anaphylactic reactions, (ii) short half life of 20 hours, as opposed to 21 days of circulating self antibodies. In order to block soluble factors that are produced at distant inflammatory sites higher initial concentration of antibodies with prolonged high concentration in plasma would be required, but has been unachievable to date. The failures stimulated the first attempts to eliminate the limitations of mouse mABs by removing immuno adjuvant glycosylation, chimerisation, humanization and finally the production of fully human antibodies and antibody like Fv reagents. The benefit of human and humanized antibodies is clear and these are expected to completely suppress the use of mouse mABs in human therapy. The human and humanized therapeutic antibodies will allow the development of novel concepts (e;g immunostimulation, cross linking and stimulation of hematopoetic receptors etc.) for long term therapy. At the same time mouse mABs will remain the reagents to test new hypotheses in vitro and in experimental models.

Advances in development of therapeutic antibodies - While murine-derived monoclonal antibodies are well tolerated in the acute therapeutic setting, use, as chronic therapy, is limited due to the severe and sometimes fatal human anti-mouse antibody responses that are induced when murine antibodies are used in the clinical setting. This response is also referred to as the Human Anti-Mouse Antibody response (HAMA) (16) .

The advent of molecular engineering and isolation of the genes that encode antibody molecules created an opportunity for genetic modification of antibody genes with the goal of eliminating the HAMA response. The HAMA response is elicited due to the "foreign" nature of the antibody itself. The concept was to replace the foreign components of the murine antibody with human antibody sequences thereby reducing or eliminating their

immunogenicity. Despite the variability in amino acid sequence within the variabke heavy and light chain domains, the overall structure of an antibody is well conserved. This conservation turned out to be critical for the genetic engineering of immunoglobulin (Ig) molecules. A second feature of Ig molecules, that was advantageous for genetic manipulation, was the genetic organization of their genes. Each domain of the protein molecule is encoded within a separate exon allowing domain swapping and genetic modification of specific domains to be completed with relative ease (17).

In the early 1980's with the availability of molecular genetics and the antibody-encoding genes, the first human-mouse chimeric molecules were developed which replaced the murine constant (murine CH) domains with human constant (CH) domains (Fig 1 and ref. 18,19). These chimeric molecules retained the sensitivity and specificity (determined by the variable domains) of the murine antibody, acquired the effector functions associated with human constant domains and were designed to reduce the immunogenicity of the molecule. While a significant portion of the murine antibody was retained, these modifications yielded Ig proteins that were overall ~75% human sequence. The ability to exchange different constant domains varied the isotype and effector functions, allowing potential therapeutic antibodies to be "taylored" for specific functions. In addition, inclusion of the human constant regions generated, not only less immunogenic antibodies for chronic therapy, but also increased the *in vivo* stability and half-life of the antibodies (20).

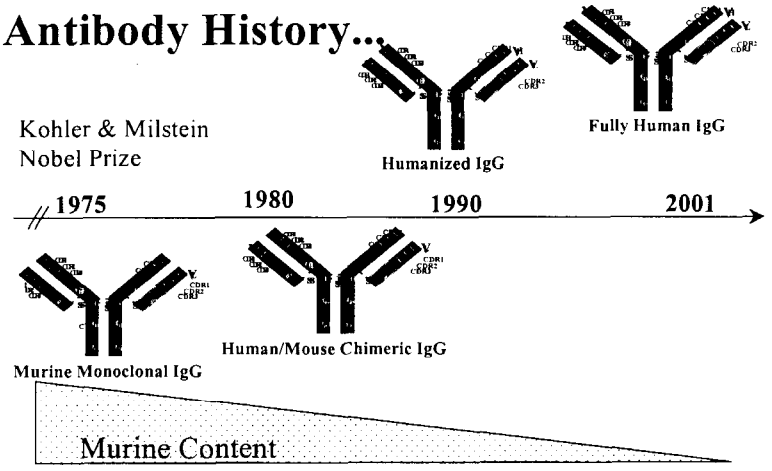


Figure 1: antibody history

Several chimeric antibodies have received FDA approval and have been commercially successful in both the acute and chronic therapeutic setting. Two of the best known examples include Abciximab (ReoPro;Centocor/Eli Lill/Fujisawa) and Infliximab (Remicade; Centocor/ScheringPlough/Tanabe). Abciximab is an Fab fragment of a chimeric human-mouse monoclonal Ab that binds the glycoprotein IIb/IIIa and vitronectin receptors on human platelets and is indicated for acute blockade of platelet aggregation and subsequent thrombosis following percutaneous transluminal coronary angioplasty (14). Abciximab was approved in 1994. Infliximab (Remicade), approved in 1998, is a murine-human chimeric antibody that targets TNF-alpha (14). This antibody is indicated for chronic use in Crohn's disease and rheumatoid arthritis. Despite the reduction in murine content in these chimerized antibodies, HAMA responses have been reported to

occur. The response is rare when chimeric antibodies are used in the acute setting but is more frequently observed when utilized in the chronic setting.

A refinement of the genetic engineering of chimeric antibodies came with the generation of humanized, CDR-grafted and human-resurfaced-humanized antibodies. To test the possibility of transplanting only the CDR fragments of a rodent antibody on to a human antibody framework while retaining the binding specificity, Jones and Morrison grafted the CDR from a rodent anti-hapten antibody onto a human antibody framework (18-19). These studies demonstrated that binding specificity and affinity could be transferred onto a human antibody with only the CDR segments and opened the possibility for genetic manipulation to further reduce the immunogenicity of therapeutic antibodies while retaining the specificity and function. In addition to further reducing immunogenicity, these studies also lead to a greater understanding of antibody structure and clarification of the nature of the critical contacts between the CDRs and the donor framework residues. The findings suggested that there must be very careful consideration of the human acceptor framework if the binding specificity and affinity of the humanized antibody is to match that of the parent rodent antibody. This was exemplified in the studies demonstrating that a single amino acid change in the framework region of the humanized Campath-1 antibody changed it from being nearly inactive in antigen binding to high affinity binding activity of the parent molecule (21). Antibodies humanized by CDR-grafting are overall about 95% human in sequence (See Fig 1).

A growing number of humanized antibodies have been approved since the first in 1997 and the number of humanized antibodies entering clinical development continues to rise. One of the first humanized antibodies approved was Daclizumab (Zenapax; Protein Design Labs/Hoffman-La Roche). This humanized IgG1 monoclonal antibody targets the alpha subunit of the human high-affinity interleukin-2 receptor and is currently utilized in acute kidney transplant rejection (14). Soon thereafter, Trastuzumab (Herceptin; Genentech/Hoffman-La Roche) was approved in 1998. This humanized IgG1 kappa monoclonal antibody has had enormous commercial success for the treatment of metastatic breast cancer in tumors that over express the human epidermal growth factor receptor 2 protein (14).

Alternative methods to further reduced murine content in humanized antibodies utilize a resurfacing or veneering technique. With this approach the remaining murine amino acids, contained in the CDR regions and adjacent FR segments, are replaced one by one with amino acids that correspond to human variable regions (17). The difficult engineering challenge with both humanizing and resurfacing of antibodies is the retention of affinity and specificity. Determining which modifications will retain high affinity for antigen while minimizing the immune response is a significant undertaking and further selecting which murine amino acids to replace (resurfacing) adds an additional complexity. For these reasons, alternative approaches were sought which were more easily adaptable to higher throughput antibody development.

While the risk of HAMA is certainly minimized in chimeric and humanized antibodies, fully human monoclonal antibodies are preferable as they are not expected to elicit an undesirable immune response. To date, greater than twelve fully human antibodies have been or are currently being profiled in clinical studies and there have been no reports of neutralizing Human Anti-human antibody (HAHA) development. Until recently however, generating fully human antibodies was not possible because a key step in the process – generation of the antigen-specific B cell hybridomas– could only be done in rodent systems. Today, three methods are now available to produce fully human antibodies: 1) phage display 2) transgenic animals which contain the human antibody gene repertoire and 3) direct isolation and cloning of human antibody-producing B cells.

Phage display libraries were a giant step forward for the in vitro expression and identification recombinant human antibodies. With this technique, genes encoding the surface proteins of a phage are genetically altered to allow the insertion of the fragment of an immunoglobulin genes necessary for antigen binding (22). These fragments are called single chain Fv and consist of the antigen binding domains of the VH and VL segments connected by a short peptide linker. The immunoglobulin gene is expressed as a fusion protein on the surface of the phage. Phage display libraries containing greater than 10^9 recombinant scFv fusion proteins can be generated and easily screened. This method allows for the enrichment and isolation of antibodies by virtue of their binding to antigen. In addition, it can be used to mimick *in vivo* affinity maturation (23). Affinity maturation is a natural process of somatic mutation and antigen-driven selection which yields antibodies of higher affinity. Phage libraries can be generated containing the genes encoding the CDR fragments that have been randomly mutated. This process allows for re-screening and identification of phage with altered binding specificity or increased avidity. A number of fully human antibodies, developed by phage display are currently in clinical trials. These include: D2E7 (Cambridge Antibody Technology/BASF Pharma), J695 (Cambridge Antibody Technology/BASF Pharma/Genetics Institute), and CAT 152 (Cambridge Antibody Technology/BASF Pharma/Genetics Institute).

An alternative to phage display for the generation of fully human antibodies is the use of transgenic mice expressing the human antibody gene repertoire. In this approach, mice carrying the human immunoglobulin gene segments in their germline configuration, rearrange and express fully human antibodies in the murine B cells (24). In this system, as in the conventional hybridoma technology, mice are immunized with antigen to elicit a specific immune response. B cells are then isolated and somatically fused with an immortalized myeloma cell line. One possible advantage of the transgenic approach over phage display is that this method does not involve genetic manipulation or *in vitro* selection of mutated V regions to identify antibodies of higher affinity. Rather, it taps into the natural *in vivo* affinity maturation process for expansion of higher affinity antibodies (Table 3). Because only the murine immunoglobulin genes in these mice have been deleted (and replaced with a very large portion of the human immunoglobulin loci) these mice respond to antigenic stimulation comparable to their wild type counterparts but their antibody response is of human origin not mouse. Agents currently in clinical trials which were developed with this technology include: ABX-EGF (Abgenix), ABX-IL8 (Abgenix), and MDX-CD4 (Medarex).

Finally, the third method for generating fully human antibodies is by direct immortalization of B cells isolated from individuals who have been previously exposed to a specific antigen, pathogen or virus, or immunization of SCID mice reconstituted with human immune response cells. One example application of this technology is the identification of anti-cancer therapeutics. In this approach B cells are isolated from a cancer patient and fused with another non-antibody secreting immortalized cell. The fused cells are then screened for antibodies reactive with specific tumor antigens.

Over the past few years, there have been vast improvements in genetic engineering for expression of chimeric and humanized antibodies. Also novel technologies are now available through phage display and transgenic approaches for producing fully human antibodies. What advances lies ahead? The newest interests lie with the development of bispecific antibodies and radiotherapy and toxin-fusion antibodies. Bispecific antibodies combine the VH and VL of two different antibodies into one molecule (25). This approach has broad therapeutic application and beckons back to the original description of antibodies as the "magic bullet," with antigen or cellular targeting in one arm and effector function in the other.

Table 3. Advantages of phage display versus transgenic fully human antibodies

Phage Display	Transgenic Approach
High throughput; rapid identification	Longer Ab generation time
In vitro affinity maturation	Native affinity maturation
Bacterial expression system	Mammalian expression system
Yields single chain antibodies	Yields complete antibody
Fully human	Fully human

Radiotherapy and toxin-fusion is a technology that has been used extensively in area of diagnostics (26). More recent application of this technology has pushed the coupling of specific toxins and radioisotopes to target-specific antibodies. The most promising application of this technology is the coupling of antibodies specific for tumor cells to a radioisotope. Delivery of toxins or radioisotope to specific diseased-tissue or cells would provide a significant advancement to the use of antibodies as therapeutics.

CONCEPT OF THERAPEUTIC ANTIBODIES AND THEIR USE IN CLINICAL PRACTICE

Passive immunization works where active immunization may fail or be inappropriate, either because the immune response to the agent may take too long or require a specific combination of factors to be mounted appropriately or because lack of an intact immune system. Slow growth viruses, such as rabies, are best treated using antibodies already prepared against the virus rather than waiting for the patient to develop them, which may take decades and no longer protect them from the pathogenic agent. Subjects who are immunosuppressed may also benefit from receiving passive antibodies when exposed to infections or foreign agents (27). This practice is not unusual: it is standard procedure to inject gammaglobulin to children or elderly exposed to Hepatitis A, Parvovirus, pneumonia and other infectious diseases; as well as to mothers who have a blood type lacking her husband's red blood cells' surface antigens (28, 29). In contrast to active immunization, using passive immunization provides immediate concentrations of antibodies that will bind the foreign agent, while at the same time inhibiting the natural immune response to produce these antibodies. This latter feature is the principle for using gammaglobulin (IgG) in mothers with high risk of developing antibodies against their offspring. In the former examples, passive immunity will provide immediate protection where it would take too long or where it would be unlikely that the patient ever makes active antibodies against the foreign agent. Hence, passive immunity will not provide long-term immunity against the same agent . Passive immunity using specific or non-specific antibodies may use antibodies raised in other animals, such as horse serum or pooled gammaglobulins from humans. These are available for intramuscular injections or as lyophilized intravenous gammaglobulin or IVIg. IVIg has been available since the early 20^{th} century. The indications for using IVIg broadened in the late 20^{th} century to include autoimmune diseases such as polymyositis, dermatomyositis, and others including the treatment of inmmunodeficient patients like hemophiliacs and patients with AIDS (30-32). The reason for using IVIg in patients with immunodeficiency is to replace the lack or limited amount or quality of the therapeutic antibody response in these subjects and protect them from lethal infections. The use in autoimmune diseases is less clear and has mainly been based on isolated observations and anecdotal reports. Although, it's consistent benefits have been reported (27,28,30-34).

A collection of the specific pathways that are involved in chronic intractable diseases like some types of cancer, degenerative and autoimmune diseases are better known as

more basic research has furthered the understanding of the role of each of these pathways in these diseases. As a more consistent picture evolves, some mediators of disease have been identified as main players in the pathogenesis of life-threatening diseases. The role of surface molecules, soluble receptors and specific cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) in autoimmune inflammatory diseases provided the rationale for generating specific neutralizing antibodies to them or to their receptors. Animal models using antibodies to cytokines have shown beneficial effects in terms of the disease activity and progression. However, in practice, the limitations of using specific antibodies in humans has met the challenge of commonly found adverse events that include the identification of the specific therapeutic antibodies as foreign antigens that trigger an immune reaction against them. These adverse events include fever, malaise, muscle aches and pains, headaches, skin rashes, nausea and vomiting (31). At the center of this reaction is the generation of antibodies to the foreign therapeutic agent used. With the generation of "humanized" or human monoclonal antibodies generated against specific pathogenic agents, the representation of the variable and hypervariable regions that provide the specificity is dramatically increased. Hence, the recipient of these monoclonal antibodies is more likely to recognize the variable and hypervariable regions as foreign and mount an undesired adverse immune response to the treatment, creating anti-idiotypic antibodies.

In summary, one of the main advantages of therapeutic antibodies is that they can be made to target the pathogenic mechanism and even target the specific affected organ. This decreases the prevalence of adverse events and organ toxicities commonly seen with non-specific therapies. However, the therapeutic antibodies used may trigger an immune response when identified as "foreign" by the recipient. The "foreignness" may be within the constant regions, when the source of these antibodies is non-human or within a small part of the constant regions when they are of human origin but contain uncommon allotypic sequences. To avoid these two conditions, specific humanized monoclonal antibodies can be engineered with non-antigenic constant regions. When using human or humanized non-antigenic constant regions, the variable and hypervariable regions may still be identified as foreign and trigger an immune response with creation of antibodies to these regions. However, this immune response is infrequent and treatable, and thus the possibility of using monoclonal humanized antibodies broadens more and more in clinical practice, without the major risk of triggering immune-related adverse events.

CONCLUSION

It is clear that the application and utility of therapeutic antibodies will continue to grow. An updated summary of the monoclonal antibodies that have received a product license approval by the FDA as therapeutic antibodies since 1996 can be found on internet (35). In addition to their therapeutic application novel techniques are on the horizon for diagnostics and imaging. One area of recent focus is identifying improvements in Ab production and purification. While significant advances have been made to improving the stability, half life and immunogenicity of current therapeutic antibodies, the cost of manufacturing and purifying Abs still limits their application to diseases which will tolerate the high expense and parenteral administration. Recent advances in protein expression have already yielded improvements in animal and plant transgenic expression systems.

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SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

Editor: George L. Trainor, DuPont Pharmaceuticals Company,
Wilmington, Delaware

Chapter 24. Pharmacokinetics and Design of Aspartyl Protease Inhibitors

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Introduction: Aspartyl proteases are involved in a large number of peptide and protein cleavage events of medicinal importance, and drugs which inhibit the action of aspartyl proteases have been proposed as therapy in a number of diseases. The successful development of HIV-1-protease inhibitors for the treatment of HIV disease and AIDS has provided clinical support for this approach, and a variety of programs in the pharmaceutical industry currently target aspartyl proteases. This article will summarize recent work to develop superior second-generation HIV protease inhibitors, particularly focusing on improvements in the pharmacokinetics and dosing schedules of current clinical candidates. Additionally, this article will highlight recent progress toward the clinical evaluation of nonpeptidic inhibitors of renin. Recent efforts to improve computational tools and methods useful for inhibitor design will also be discussed.

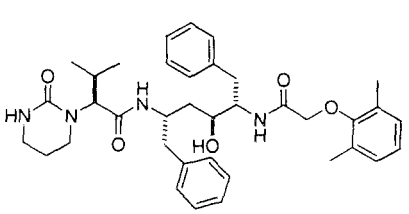
HIV CLINICAL CANDIDATES

The HIV-1 protease (HIV PR) is necessary in the viral replication cycle to cleave the *gag* and *gag-pol* gene products (1-3). After an enormous effort to identify and develop inhibitors of the HIV protease, the succesful compounds have become an important component of the succesful HAART (highly active antiretroviral therapy) program in the fight against HIV disease. There are currently six marketed HIV-PR inhibitors in the U.S. The first five agents to be approved were saquinavir (Invirase®), ritonavir (Norvir®), indinavir (Crixivan®), nelfinavir (Viracept®), and amprenavir (Agenerase®). The properties of these drugs have recently been reviewed (4-11). The current marketed drugs are all peptidomimetics containing transition state isosteres (11), and all have pharmacokinetic (PK) profiles which necessitate large doses of drug to be administered several times daily in order to achieve good antiviral efficacy. Although these drugs are highly successful in many patients, therapy failure due to mutated virus or patient noncompliance in complicated dosing regimens is still a problem, as are side effects (12-16, see also chapter on infectious diseases in this volume). Current clinical candidates have focused on providing increased antiviral efficacy against mutant strains of HIV by improving both potency and PK. The benchmarks for new HIV inhibitors will likely include antiviral efficacy (EC_{90}) in the presence of human serum in the low nanomolar range, and sufficient PK to cover EC_{90} at trough, by several-fold, with a reasonable once daily dose. The best agents will also show the broadest activity against mutant strains of HIV.

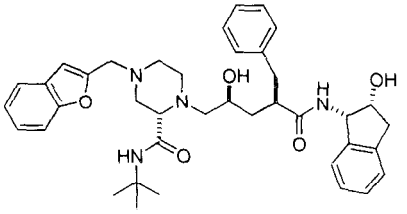
Kaletra® - A combination of lopinavir (ABT-378 (**1**), 400 mg) and ritonavir (100 mg) was approved for U.S. sale in October, 2000. Lopinavir contains the same 2-hydroxy-1,4-diaminobutane transition-state isostere as ritonavir (RTV) and was designed using the X-ray crystal structure of RTV bound to HIV-PR. RTV is sensitive to mutant virus that arise with mutations in Val 82 in the protease, losing 12-52 fold potency against these viral strains. Lopinavir was designed by eliminating a crucial interaction between Val 82 and the P3 group of RTV, and reoptimizing the structure with this interaction missing. The PK of **1** as a single agent is poor: the bioavailibility in rat is 25% with a short half-life, and no plasma

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levels are seen after oral dosing in either monkeys (10 mg/kg) or in dogs (5 mg/kg) (17). In contrast, the inclusion of RTV in the formulation at low concentrations results in inhibition of cytochrome 450 3A (cyp 3A)-mediated clearance of **1** and raises the total drug exposure >100 fold in humans (18-20). This general principle for increasing exposure by inhibition of cyp 3A has been expanded to many other HIV PR drug combinations (*vida infra*), but this method of improving drug exposure is not optimal, as the PK of other drugs administered to the patient may also change. At the clinical dose in humans (400 mg **1** and 100 mg of RTV twice daily (b.i.d.)), Kaletra® achieves a C_{max} of 15.2 μM at a t_{max} of 4.2 h. The C_{trough} = 8.75 μM, Clearance = 6.4 L/h, AUC over a 12 hour dosing interval = 132 μM h, and $t_{1/2}$ = 5.5 h (18,21,22). In *in vitro* studies, **1** shows a K_i for WT HIV of 1.3 pM and inhibits 93% of WT HIV activity in cells at 0.5 nM (17). The compound shows an EC_{50} of 102 nM in the presence of 50% human serum, and is 98-99% protein bound in humans (19). Antiviral activity of **1** against a variety of Val 82 mutant HIV strains is excellent, with binding Ki's of less than 4 pM against several strains, and only a 6-13-fold loss in potency against patient isolates containing multiple mutations. The trough concentration of **1** generated by Kaletra® is >75 fold the protein-binding adjusted EC_{50} for WT HIV, which is significantly higher than the 13-fold drop in potency observed against the panel of mutant HIV strains above. In a phase II clinical trial of Kaletra® + stavudine and lamivudine with 100 patients, no patient discontinued by 48 weeks because of virologic rebound (21). At 96 weeks, 97% of patients by on-treatment analysis had a viral load of <400 copies of HIV mRNA/mL, and an intent-to-treat analysis (missing value = failure) showed 83% of patients with a viral load of <400 copies/mL (23). Some adverse events were noted, with 2 of 100 patients failing therapy because of drug-related events.



1

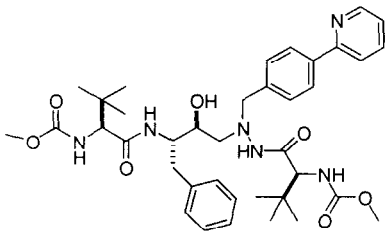


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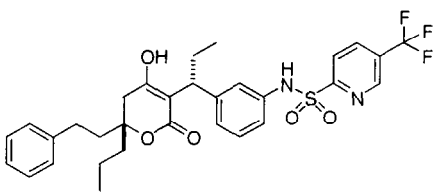
MK-944A (**2**, L-756,423) - Compound **2** incorporates the P2-P2' binding elements and 4-hydroxy-5-aminopentanamide isostere from indinavir (IDV). The compound seeks to improve on the PK properties of IDV by modifying a site of metabolic lability identified in human dosing of a radiolabeled form of the drug (24). The benzofuranyl methyl group in P3 replaces a 3-pyridylmethyl group in IDV that is the site of extensive metabolic degradation. Compound **2** a is a potent inhibitor of WT HIV PR (Ki = 0.049 nM) and has an EC_{95} of 25-50 nM in MT4 cells against the IIIb isolate (25). The compound is 70% bioavailable in dogs (10 mg/kg at 0.5 M in citric acid), with a C_{max} of 28 μM, a T_{max} of 42 min, and 800 nM drug levels in plasma after 8 h. An i.v. dose of 2 mg/kg provides a volume of distribution of 2.6 L/kg, and a relatively short $T_{1/2}$ of 54 min. Interestingly, no drug (<30 nM) was detectable in the plasma of monkeys after a 10 mg/kg dose. The compound is 2.7% free in human serum and maintains a 20-50 nM EC_{95} in the presence of α1 acid glycoprotein (AAG) or 50% human serum. The results of a phase I clinical study with **2** were recently reported (26). The C_{max} and AUC of **2** were reportedly lower than anticipated, presumably due to first-pass metabolism, although $t_{1/2}$ was a relatively long 8.5-9 h. Co-dosing of **2** and 800 mg of IDV, however, produced a robust 20-30 fold increase in AUC, presumably due to IDV saturation of cyp 3A-mediated first pass metabolism. Co-

dosing of 1600 mg **2** with 800 mg IDV once daily (q.d.) with food resulted in an AUC_{0-24} of 123 μM , a 13 .3 μM C_{max}, and 825 nM drug levels at 24 h post dose, well above the protein-binding adjusted IC_{95}. This agent will be examined in both q.d. and b.i.d. regimens. Compound **2** shows a similar resistance profile to IDV, with 163-510 fold loss in activity (Ki of 8-25 nM) against three highly resistant clinical isolates (27,28).

BMS 232,632 (**3**, CPG 73,547) - The hydroxyethyl hydrazine isostere was designed as a mimetic of the related 2-hydroxy-1,3-diaminopropane isostere, but has several advantages, including the elimination of one stereogenic center and ease of synthesis (29). Compound **3** has good potency against WT HIV PR (K_i = 10 pM), potent antiviral activity in HIV-infected PBMCs (EC_{90} = 8-12 nM) and showed very promising PK in mice. The presence of 40% human serum or 2 mg/mL AAG raises the EC_{50} by 5-8 fold. In a phase II trial, **3** dosed 400 mg q.d. for 14 days produced steady-state drug levels with a C_{Max} = 7.64 μM, t_{max} = 2.2 h, AUC = 41.7 μM h, C_{trough} = 225 nM, and $t_{1/2}$ = 5.3 h (30). Higher doses may be necessary to achieve sufficient coverage of the protein-binding adjusted IC_{90} for mutant strains with this agent, but a reversible elevation in unconjugated bilirubin levels was observed at a dose of 600 mg q.d. Co-dosing of 400 mg of **3** with 100 mg of RTV q.d. increased the AUC by 2.4 fold, the $t_{1/2}$ to 7 h, and increased the C_{trough} to 1.45 μM, well above the EC_{90} for mutant strains. In *in vitro* testing, **3** retains activity against a broad panel of PR-resistant clinical isolates with a <6-fold loss in potency, suggesting it will be necessary for multiple mutations to arise for significant loss in efficacy (31).



3

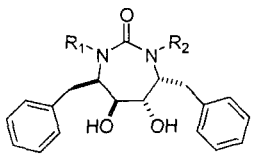


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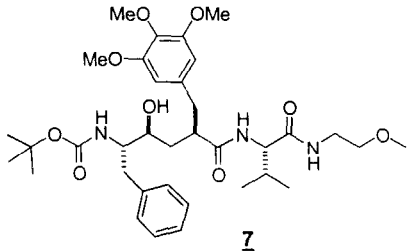
Tipranavir (**4**, PNU-140,690) - The identification of phenprocoumon as a weak inhibitor of HIV PR lead to the synthesis of several related series of substituted dihydropyrones (32-34). Compound **4** is a sulfonamide-containing inhibitor with high *in vitro* potency (K_i = <10 pM) and good antiviral efficacy (EC_{90} = 100 nM). The compound is highly protein bound in 75% human serum containing 10% fetal bovine serum (>99%) and shows a 1.4 μM IC_{90} under these conditions (34). In phase I studies, compound **4** dosed at 900 mg or higher t.i.d produced steady state trough concentrations above 1 μM (35). In Phase II studies **4** was examined alone at 1200 mg b.i.d. and was also co-dosed with RTV at two different concentrations: 300 mg **4** + 200 mg RTV and 1200 mg **4** + 200 mg RTV b.i.d. Concentrations of **4** at trough measured 0.76, 21, and 67 μM respectively (36). Early efficacy measurements show that regimens containing **4** in treatment-naïve patients demonstrated a 1.4 to 1.6 log HIV RNA reduction after 2 weeks of treatment. Due to its unique structure, **4** has a very flat resistance profile, inhibiting 98% of a panel of 105 multidrug resistant clinical HIV isolates with a less than fivefold decrease in IC_{50} (37,38).

DMP-450 (**5**) - The cyclic ureas were designed based on the concept of cyclizing an inhibitor containing a 2,3-dihydroxy-1,4-diaminobutane isostere and incorporating a mimetic of a conserved structural water in the crystal structure of HIV PR (39,40). Compound **5** has a 0.28 nM Ki for WT HIV PR and an IC_{90} of 130 nM. The compound is between 90-93% protein bound, and the apparent EC_{90} increases 4.5-

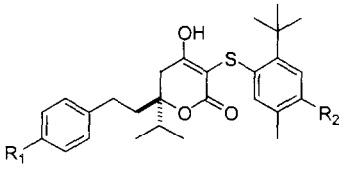
8.4 fold in the presence of human serum and AAG. In phase 1 studies at 750 mg, **5** showed a C_{max} = 6.5 μM, a 5.7 h $t_{1/2}$, and trough levels of >1 μM at 6 h (41). In phase 2 studies **5** at 1250 mg b.i.d. generated a C_{max} of 11.5 μM, a somewhat shorter $t_{1/2}$ of 2.7 h, an AUC of 33.6 μM, and a C_{min} of 875 nM (42). Good antiviral activity and tolerability was observed at this dose with a 2.4 log decrease in viral



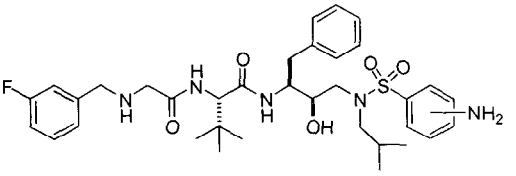
5, $R_1 = R_2 =$ (3-NH_2)$PhCH_2$-
6, R_1 = Bu, R_2 = 3-(NH_2)indazoleCH_2-



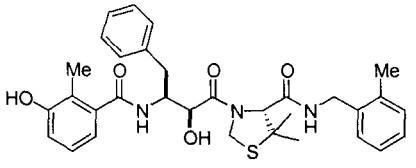
7



8, $R_1 = NH_2$, $R_2 = CH_2OH$
9, R_1 = OH, $R_2 = NH_2$



10, *p*-NH_2
11, *m*-NH_2



12

load at 12 weeks and efficacy similar to IDV out to 48 weeks, both in % of patients with <400 copies of HIV RNA and ITT analysis of patients with <50 copies of viral RNA. Compound **5** is affected *in vitro* by a single mutant virus (I84V) with a 10 fold loss in potency and multiple mutations can cause losses in potency of >100 fold. Additional candidates from this class including compound **6** have advanced to clinical trials based on favorable *in vitro* profiles (43). Compound **6** is active against wild type HIV with an EC_{90} = 56 nM but the further development of this compound has not been described.

Lasinavir (**7**, BMS-234,475, CGP 61,755) - Compound **7** was originally synthesized using SAR from a series of dual renin-HIV PR inhibitors incorporating the 4-hydroxy-5-aminopentanamide isostere (44). The compound has an IC_{50} for WT HIV-PR activity of 1 nM and an EC_{90} of 30 nM. It also shows *in vitro* inhibition of cathepsin D with an IC_{50} of 100 nM. The compound binds to AAG producing a 4-fold higher EC_{90} (122 nM) in MT-2 cells and an 8-fold lower EC_{90} (364 nM) in primary lymphocytes (45). No clinical data is available for **7**, and it is possible that the compound may not be sufficiently potent or selective.

PD 178,390 (**8**) - A second series of dihydropyrone inhibitors of HIV PR with structural similarity to the tipranavir series has been reported (46). Compound **8** has a Ki of 0.11 nM and an EC_{50} of 200 nM, which increases 2-fold in the presence of 40% human serum albumin (EC_{90} without HSA is 1 μM.). The activity drops up to 14 fold against mutant HIV PR *in vitro* with up to 2 mutant residues, and the compound has promising PK in mice and dogs. A related compound PD 178,392 (**9**) with

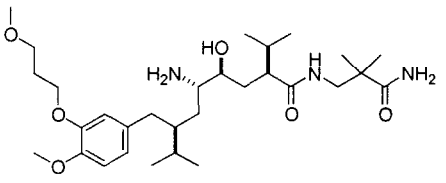
similar antiviral activity (EC_{90} = 1 μM) and good PK in animals has also been reported (47), however this agent loses up to 49-fold activity against mutant HIV-PR *in vitro*.

DPC 681 and DPC 684 (**10**, **11**) - Substituted aminoacyl sulfonamides using the 2-hydroxy-1,3-diaminopropane scaffold are potent HIV-PR inhibitors (48,49). Compounds **10** and **11** inhibit WT HIV with a Ki of 10-20 pM and show extremely potent viral suppression with EC_{90}s of 1-4 nM (50). In human serum the free unbound fractions are 1.6% and 1.9%, respectively. The compounds have an excellent resistance profile, losing less than 5 fold in potency versus clinically resistant HIV PR with multiple mutations (51). Blood levels of 1 μM at trough are predicted to yield 90% suppression of highly resistant HIV clinical isolates.

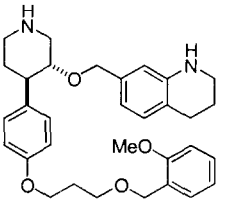
AG-1776 (**12**, JE-2147) - A number of inhibitors of HIV PR containing the allophenylnorstatine isostere have shown good potency and bioavailability in animal models (52-54). Compound **12** was advanced into clinical trials in 1998 (55). The compound is a potent inhibitor with a K_i of 0.33 nM and an EC_{50} of 27 nM against WT HIV and shows robust antiviral activity against a panel of resistant clinical HIV mutant virus (56). Only a two-fold loss in potency against 7 strains with appreciable resistance to other PI's was demonstrated, and multiple mutations were necessary to select resistant strains *in vitro* (57). The compound shows good PK in both rats and dogs (56), but human PK data is not yet available.

RENIN CLINICAL CANDIDATES

The development of renin inhibitors for the treatment of hypertension not yet met with the clinical success seen with HIV PR inhibitors. The reasons include difficulty in obtaining compounds with sufficient PK properties and the clinical success of the angiotensin II receptor blockers, which were cheaper to synthesize (10,58). Recent publications show that work in this area continues, with several clinical candidates emerging that have nonpeptidic structures and promising PK.



14



15

SPP-100 (**14**) - A successful strategy to synthesize nonpeptidic inhibitors of renin evolved from the observation that the S1 and S3 subsites of the enzyme are contiguous and can be accessed with one extended binding element (59-61). Compound **14** was designed using this approach (62). The compound shows a 0.6 nM Ki against recombinant human renin and gives the same IC_{50} in the presence of human plasma. The compound shows a robust and long-lasting (up to 24 h) lowering of mean arterial pressure (MAP) in sodium-depleted marmosets, with a maximum of Δ -25-30 mm Hg observed during the first 4 h of dosing. Additional animal or human PK data is not yet available for this compound.

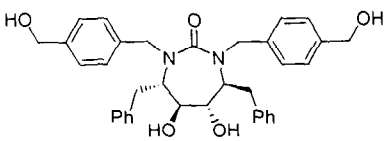
Piperidines - Compound **15** is a member of a totally novel class of renin inhibitors recently reported (63-65). The piperidine inhibitors bind the catalytic aspartates through the piperidine nitrogen atom and require an induced-fit reorganization of the

enzyme during binding (64). Compound **15** as a racemate shows a K_i for recombinant renin of 0.7 nM, and an IC_{50} of 37 nM in the presence of human plasma. In sodium-depleted marmosets a 3 mg/kg dose produces a Δ MAP_{max} = -19 mm Hg and maintains a Δ MAP of -10 mm Hg after 20 h.

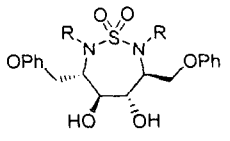
COMPUTATIONAL DEVELOPMENTS IN ASPARTYL PROTEASE INHIBITOR DESIGN

The large body of structural and SAR data generated in the course of developing of HIV protease inhibitors has provided validation data for many new computational methodologies. Due to space constraints, this section will focus on the application of computational methodologies to the development of HIV PR inhibitors.

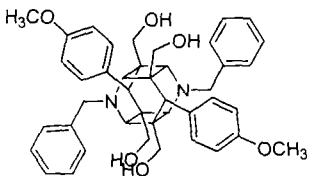
Comparative molecular field analysis (CoMFA) models have been generated for several series of HIV protease inhibitors. The inihibitory data for 118 cyclic ureas has been used to generate a one such model (66). Three individual training sets were generated, each containing 93 compounds with the remaining 25 used as test sets. A single conformation for each molecule was taken from the crystal structure, if available, or from minimization in the active site. The alignment was derived from superimposing the proteins and extracting the inhibitor position from the active site. The resulting CoMFA models had approximately equal contributions from steric and electrostatic effects and yielded predictive correlation coefficients from 0.699 to 0.727. DMP 323 (**16**) has served as the basis for the design of a series of cyclic sulfamides, **17**, which have been evaluated by a CoMFA model (67). These compounds were hypothesized to have a symmetric binding mode, as does **16**. However, subsequent crystallographic studies demonstrated that the P1'/P2' groups were reversed relative to the cyclic ureas (68). To further explore the SAR, a series of both symmetric and asymmetric analogs were made and the resulting SAR was rationalized using CoMFA (69). Because of the unknown orientation of the asymmetric inhibitors, models were generated for all possible alignments. A model was found with a q^2 of 0.68 and seemed to be consistent with the available structural data.



16



17

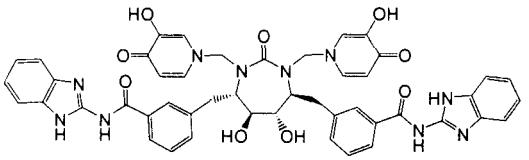


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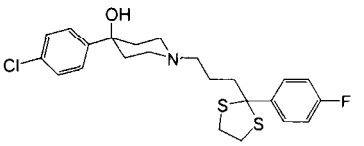
The inclusion of calculated binding energies has been found to enhance the predictive ability of a CoMFA model generated using 38 compounds similar to indinavir (70). Binding energies were derived from the mimized complex allowing all atoms within 3.0 Å of the inhibitor to move. The best model reported used the minimized conformations of the inhibitors aligned using the Atom Fit method and AM1-ESP partial charges. The predictive ability of this model was increased from a

q^2 of 0.42 to 0.65 when the interaction energies were included as an additional descriptor. CoMFA field alignment was also used in the design of a C2 symmetric cage structure, **18**, based on the apparent C2 symmetry observed in the cyclic ureas and azacyclic ureas (71). Alignment of the two inhibitors using CoMFA field fitting confirmed steric and electrostatic similarity. GRID analysis indicated that the electrostatic properties of this molecule were similar to the azacyclic urea A-98881 as observed in the crystal structure in complex with HIV.

The linear relationship between the calculated interaction energy and the experimental Ki for linear HIV protease inhibitors has been well established (72). This relationship has now been explored with the cyclic urea inhibitor DMP 450 (**5**) and 10 related analogs using the cvff force field (73). Regression analysis of the $\Delta\log K_i$ and ΔE_{compl}, both relative to **5**, was carried out and a correlation coefficient of 0.97 was found. Using this model, a DMP 450 derivative with P1/P1' N-(3-hydroxy-4-pyridone)yl-methyl and P2/P2' 3-(N-benzimidazol-2-yl) carboxamido-benzyl, **20**, was predicted to have K_i's of 0.002 nM, 0.019 nM, 0.001 nM, and 0.018 nM against the wild type, I84V mutant, V82F mutant, and I84V-V82F double mutant enzymes respectively.



20



21

Detailed free energy studies have been carried out for several systems. The relative binding affinity of Boc-PheΨAla-Val-NH_2 and Boc-PheΨGly-Val-NH_2 was calculated using stochastic boundary molecular dynamics which dramatically reduced the size of the simulation and the computational expense (74). This simulation predicted a binding free energy of –1.89 kcal/mol which is within 2% of the experimental value. Free energy calculations were also used to examine the 5-phenyl derivative of haloperidol thioketal (**21**), a 15μM HIV PR inhibitor (75). The 5-phenyl compound was predicted to have a $\Delta\Delta G_{bind}$ of –4.5 ± 2.0 relative to **21**. However, this large structural change led to poor convergence and the authors caution that the results are more qualitative than quantitative. The contribution of the hydroxyl groups on **16** has been studied using free energy perturbation and continuum electrostatic calculations (76). The calculated $\Delta\Delta G_{bind}$ of the alcohol relative to the uncyclized diol was found to be +2.2 kcal/mol, consistent with the experimental finding that the alcohol is 2.4 fold more active than the corresponding diol. The $\Delta\Delta G_{solv}$ of the alcohol to diol was found to increase by 1.6 kcal/mol, suggesting that the alcohol would be more soluble.

A 542 ps molecular dynamics trajectory of HIV protease in complex with SDZ283-910 and an 805 ps trajectory of unliganded enzyme were generated (77). From the trajectories of Trp6A/Trp6B and Trp42A/Trp42B transition dipole moments the theoretical fluorescence anistropy decay was calculated and found to be in good agreement with experimental data. This agreement confirmed that long simulations starting from a high resolution crystal structures can accurately capture the atomic detail of conformational change on inhibitor binding.

A series of cyclic cyanoguanidines has been studied using QSAR (78). The descriptors used in this study were the Hansch and Leo hydrophobicity parameter (79) and several indicator parameters. The indicator parameters denote the

presence of either an OH/NH2, an aromatic, an etherial moiety, or an ortho moiety in the P2/P2' site. From the equations derived, the general conclusion was that inhibition is correlated with the hydrophobic parameter and could be enhanced by the inclusion of OH/NH2 groups in P2/P2' and the absence of aromatic groups.

Conclusion - Advances continue in the development of nonpeptidic aspartyl protease inhibitors. With the help of numerous advances in computational tools for design, clinical candidates show improvements in key pharmacokinetec parameters that allow improved efficacy. Next-generation HIV PR inhibitors will need further improvements in PK to allow a q.d. dose that provides clinical suppression of highly resistant strains of HIV without the need for concurrent inhibition of Cyp-mediated metabolism. Clinical testing of novel renin inhibitors will provide information about some new nonpepdide inhibitor motifs. These new structures also provide the groundwork for programs designed at inhibiting new, therapeutically relevant aspartyl proteases, such as the BACE protein involved in Alzheimer's disease (80).

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Chapter 25. ADME by Computer

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Introduction – The Absorption, Distribution, Metabolism and Elimination/Excretion (ADME) characteristics of pharmaceuticals are important properties to be considered in the development of novel therapeutic agents. Many of the compounds entering phase I-III clinical trials must be discontinued, often times due to issues directly related to ADME. Toxicity is also an important factor to consider for attrition and is related to ADME. Toxicity, and computational methods relating to it (such as the MULTICASE program (1), used routinely by the FDA), is itself worthy of an entire review, but is not in the scope of this review. In recent years, the number of predictive approaches to ADME using computational techniques has increased dramatically. Hybrid approaches, those that combine computational techniques with easily obtainable experimental data, are also becoming more prevalent. While there has been no significant breakthrough in many of the areas associated with computational approaches to ADME (in particular, distribution and elimination/excretion), there have been modest but noteworthy improvements in the predictive methods. There are also several review articles that have been recently published on the subject of predicting ADME properties (2-5). With the foundation of the 1990's as a background, the field of ADME by computer has taken off, and shows no signs of slowing down.

ABSORPTION & DISTRIBUTION

Absorption is of paramount importance in drug design. Currently, there is a heavy emphasis on producing drug candidates that have good oral absorption which hopefully extends to good oral bioavailability. The obstacle to good, general, and robust models of absorption has always been finding a large and consistent data set. Several attempts have been made to model absorption, and most are attempted with small datasets. Many of these attempts have been highlighted at meetings and conferences, such as the recently held ACS meetings in Anaheim (spring of 1999), San Francisco (spring of 2000) and Washington D.C. (fall of 2000).

Human Intestinal Absorption - Human intestinal absorption is a direct measure of the ability of a drug to enter the human biological system. Using the logit transform [logit(value) = ln{(value - A)/(B - value)} where A = -10.0, B = 110.0] of the absorption values, a model using the MolSurf parameters gave a reasonable correlation for 20 drug compounds. The MolSurf descriptors utilized quantum chemical calculations and an analysis of the "molecular valence" region of molecules. Hydrogen bonding terms were the dominant contributor in the equation, which had an $r^2 = 0.9$ and a $q^2 = 0.685$. (6). The Molsurf and VolSurf parameters were also used with partial least squares (PLS) to model the absorption values of 19 tetrapeptides (7). A comparison of the rule of five to dynamic polar surface area (PSA_d) for 10 endothelin receptor antagonists revealed an improvement over the simple rule of five (8,9). PSA_d is obtained by using a Boltzmann weighting scheme to calculate the average PSA for compounds that can adopt different conformations. Dynamic non-polar surface area ($NPSA_d$) was calculated but not found to be a better predictor of absorption when combined with PSA_d. Not surprisingly, $NPSA_d$ correlates well with cLogP. A recent and interesting finding is that single conformer PSA performs as well as PSA_d in models for absorption (10). For a data set of 20 drug compounds with known fraction absorbed values, a classification model performed well and generated a

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good classification on a larger data set as an external test. The model classified compounds into one of three classes, good, OK and poor absorption (10).

A simpler approach that employs single conformer PSA and AlogP98 with scatterplots was capable of classifying compounds into either well or poorly absorbed classes. The well-absorbed data set contained 199 compounds and the poorly-absorbed data set had 35 compounds. By using two descriptors, PSA and AlogP98, bi-plots were constructed to show the region of well absorbed compounds. Ellipses within the scatter plot, reprocessing 95% and 99% confidence regions, were calculated for the well absorbed dataset. Additional data sets from the Comprehensive Medicinal Chemistry database and the Physicians Desk Reference were used to validate the model (11). The HYBOT/HYBOT-PLUS program was also employed to build more traditional QSAR models for two data set of passively absorbed drugs (12). A non-linear equation (FA = 1/(1+10)^-Z, Z = linear combination of HYBOT descriptors) was used to predict logit(FA) values. The HYBOT H-bond factor values for acceptors and donors performed best, giving an r^2 = 0.973, q2 = 0.971. Non-linear models were much better than linear regression models, indicating the complex nature of absorption (12). Given the effective permeation of a drug compound (based on cell permeation studies) it is also possible to simulate more traditional mechanisms of absorption based on diffusion properties and physical intestinal characteristics (13), however the requirement of measured permeation values does introduce a time and resource allotment. Conventional wisdom holds that the charged species of an ionizable drug compound is not transported. However, for the two drugs alfentanil and cimetidine, pK_a calculations and MolSurf parameters show that if the unionized form of the drug is less than 10% at a given pH, then the charged form of the drug is transported and plays a major role in affecting the transport mechanism (14).

Caco-2 and MDCK – A substitute for actual intestinal absorption values is the use of Caco-2 and MDCK cell lines for measuring permeability. Since these cell lines are routinely used in the pharmaceutical industry, there is more data available (at least in-house). Measures of apparent permeation (P_{app}) are accepted as meaningful substitutes for actual human intestinal absorption values. Consequently, modeling of these permeability measures is an important substitute for modeling intestinal absorption.

The VolSurf program takes 3-Dimensional molecular fields and converts them into meaningful descriptors. These parameters can be used to build models for Caco-2 and MDCK permeability (15,16). The use of PSA_d was again effective for modeling Caco-2 P_{app} values for 19 oligopeptides. Unlike the human absorption studies, combining PSA_d and $NPSA_d$ was effective (17). General library optimization using the rule of five and PSA values can be effective. If PSA values less than 140 $Å^2$ are used, a high probability of good Caco-2 permeation values could be expected for a small library of TNF-alpha inhibitors (18).

MolSurf parameters have been used to model Caco-2 P_{app} values effectively in conjunction with ACDlogD predictions for some small (17-20 compounds) data sets. The ACDlogD values (which are rapidly calculated) were actually the most important for modeling of Caco-2 data and also lends credence to the aforementioned charge/uncharged transport mechanisms (19). Along the lines of rapid descriptor calculations, an useful approach for calculating PSA rapidly uses fragments and topological 2-dimensional information to determine the 3-dimensional PSA quite well. The PSA values can then be used to model Caco-2 P_{app} values and other properties. The speed advantages of this approach compensates for the slight falloff in the quality of the models produced (20).

Solubility and logP – Solubility plays a critical role in the absorption characteristics of a drug compound. A compound with poor solubility may not achieve high enough levels in the stomach and intestine to be absorbed adequately. It is conceivable that overly soluble compounds will not be absorbed either, but this is typically not the case in drug design. A major contributor to solubility is the octanol/water partition coefficient, or logP and its related distribution coefficient, logD. The logP parameter has been found to be quite important in many models that estimate solubility, but logP alone does not completely explain all observed solubilities. The pK_a of a drug compound also plays a role in solubility and absorption , and there have been some advances in the area of predicting pK_a values (14,21).

There have been many attempts at estimating the solubility of organic compounds over the years, and more recently, drug and drug-like compounds. Most notably, a Monte Carlo simulation algorithm has been used to determine the energetic properties (Coulombic and Lennard-Jones interaction enrages in water) and accurate counts of hydrogen bond donors and acceptors of 150 drug compounds for predicting thermodynamic aqueous solubility (22). The accurate H-bond counts derive from a radial distribution function of water molecules in close proximity to the drug compounds. A four term equation consisting of the Lennard-Jones interaction energy, solute acceptor H-bond count, number of non-conjugated amines and number of nitro groups in the compound, gives an r^2 =0 .88, q^2 = 0.87.

One of the problems with predicting solubility has always been finding a way to estimate the lattice energy of a compound. The experimental melting point can be used as an estimate of lattice energy, and this term in conjunction with logP and other terms affords excellent correlations with solubility. However, there is currently no reliable method for predicting melting point. An attempt to calculate the entropy of melting based on the calculated flexibility number (determined from chain atoms and ring systems), and the symmetry number was successful (23). These two parameters correlated well with the entropy of melting. This may be a useful avenue for accessing the troublesome lattice energy term.

The logP of a compound is a often used parameter in the drug design paradigm. In fact, the well known ClogP method is widely used. There are some new methods for calculating logP and the associated logD. Using the AHYBOT program, the logP for a set of 15 triazole and thiadiazole compounds. The actual logP was determined from an HPLC procedure (24). Another program, PrologD, was used to calculate the logD values of 47 compounds each with different reported logD values at different pH values. An 80% acceptance rate was observed for the PrologD program (25). A data set of 200 drug and drug-like compounds with reported logP values were used to build a model based on Monte Carlo simulations in water. The final equation consisted the solvent accessible surface area (SASA), acceptor solute H-bonds (from the aforementioned radial distribution function), the number of non-conjugated amines, and the number of nitro + acid groups. This produced an r^2 = .90 for the 200 compounds (26). With the use of a neural network, a model with error estimation for individual compounds has been developed that predicts the logP of over 1000 organic compounds. The descriptors in the neural network include electrostatic potentials from quantum calculations as well as atom types (27).

A unique molecular hashkey method was recently proposed. In this context, a molecular hashkey is a real valued vector that tries to describe the surface properties of a small molecule. Using K nearest neighbor methodology, a model for predicting logP performs well for 862 compounds (28). The same method was successfully applied to absorption using a neural network (28). A method termed MS-WHIM (molecular surface-weighted holistic invariant molecular descriptors) has been developed which offers an alternative strategy to 3-dimensional model building.

The advantage of using this method is that it is not dependent upon the molecular superposition (29). The MS-WHIM descriptors were used to calculate logP for 268 small organic compounds. A 3-component PLS model using the MS-WHIM descriptors gave an r^2 = 0.771and a q^2 = .709 (21). The traditional atom count fragment count methods are also being improved, and some new correction factors for certain atom types have been employed in logP calculations (30).

Estimates of water/chloroform partition coefficients for lipophilicity, known as $logP_{chl}$ are more often being seen as a better alternative to traditional octanol/water partition coefficients. In lieu of this, a predictive method for $logP_{chl}$ has appeared. For a set of 335 compounds, a final equation for $logP_{chl}$, gave an r^2 = 0.971. Some of the descriptors are still experimentally determined, but it is hoped that the ABSOLVE program will be expanded to able to predict $logP_{chl}$ from structure directly (31). There are also some methods for predicting the logP and logD values of peptides, which may offer further insight into the complexities of the logP characteristics of drug compounds (32,33).

Distribution & Blood-Brain Barrier - Of fundamental importance to any CNS drug is its ability to cross the blood-brain barrier or BBB. This barrier is formed by the highly selective capillaries of the central nervous system, and can reduce or restrict the penetration of drugs from the circulatory system. Passage of drugs through the BBB may occur via passive diffusion or from various specific uptake mechanisms. Although many of these transport mechanisms supply nutrients to the brain, a number of mechanisms exist which can transport substances out of the brain. P-glycoprotein (Pgp) is a efflux pump commonly implicated in the removal drug compounds from the brain. Recent computational chemistry efforts have examined both passive diffusion using primarily QSAR methods, as well as Pgp issues using more traditional molecular modeling. While BBB is an important feature of distribution, other areas of distribution have not been heavily explored (34).

Researchers interested in passive diffusion of drugs through the blood-brain barrier have typically focused on the log(BBB) as the biological endpoint to be modeled. This quantity is defined as the partition coefficient between drug located in the brain and that in the systemic system. Many predictive models have been published in the past (35-37). Most of the research reviewed here has built upon these earlier findings, and in many cases uses essentially the same set of data to build QSAR models.

The MolSurf descriptors have been applied to the previously used log(BBB) data sets of 63 compounds. This method resulted in predictive cross-validated model, q^2 = 0.782, and the authors concluded that hydrogen bonding descriptors as well as descriptors relating to the polarizability of electrons (i.e.: groups containing conjugated and aromatic substructures as well as large halogens) are important (38). These same authors also developed a simpler system for the calculation of partitioning behavior using simple "rule-of-five" type descriptors available via ACD-labs commercial software (39). The aforementioned VolSurf parameters have also been used to build a discriminant PLS classification model that classified BBB+ and BBB- with 75% accuracy (15).

Topological descriptors of the sort developed by Keir and Hall have also been used to construct a PLS-QSAR model (40-42). Using essentially the same data set as above to train the model, these authors were able to generate very predictive (q^2 = 0.867, r^2 = 0.922) models, although the topological descriptors make interpretation of the model less straight forward.

There seems to be some consensus that the polar surface area of the molecule appears to be the predominant descriptor for the prediction of passive diffusion into the brain (43,44). The first article to make this claim established a predictive and simple QSAR relation between the dynamic polar surface area, obtained from time-averaged molecular dynamics simulations, and brain penetration (r^2 = 0.84) (44). Here, brain penetration was found to decrease with increased polar surface area. Analysis of 776 CNS and 1590 non-CNS drugs showed an apparent cutoff at approximately 120 $Å^2$, above which drugs stand little chance of getting passively diffused into the brain .

Subsequent studies have shown that further improvement may be obtained by adding a logP term to polar surface area QSAR equations (43). Using the ClogP octanol-water partition calculation yields a simple equation with log(BBB) (r^2 = .876) and the second uses MlogP (r^2 = 0.841). These equations are straightforward to interpret and may be calculated quickly.

Not all researchers find the correlation with polar surface area as satisfying. It has been argued that a more rigorous approach is to correlate the blood-brain partition coefficient to the free energy of solvation as has been done in the past (37). In this spirit, the generalized Born/surface area (GB/SA) continuum solvation model was used as a high-throughput solution to the calculation of solvation free energies (45). The results look statistically similar when compared to other methods, and the calculation of the free energy of solvation appears simple. The final analysis looks at 8700 compounds with reported CNS activity, 96% of which have free energies of solvation higher than -50 kJ/mol. The authors suggest that this value may be used as a cutoff point for compound evaluation.

Two separate research groups have attacked the problem by asking a more general question: can CNS drugs be pharmacologically profiled based on current data sets of CNS active drugs? One group trained a neural network to classify CNS actives and inactives using simple chemical descriptors such as molecular weight, number of donors/acceptors as well as 166 2-dimensional fragment-based descriptors (46). Here, the MDDR database, which consists of 15000 CNS active drugs and over 50000 inactives, was used as a training set for the neural networks. The neural network was able to identify 92 % of a validation set of 275 compounds with known CNS activity.

In a separate work, the VolSurf program is presented as another means of classifying drugs as being CNS active or inactive (47). This is an automated program that takes 3-dimensional molecular structures and calculates GRID fields, converts these to VolSurf descriptors that are simplified 1-dimensional representations of the 3-dimensional GRID fields. Some simple, rule-of-five-like descriptors are also included. The program then calculates a principle-components analysis (PCA) and a partial least squares model (PLS). For the first training set, the 1^{st} principle component is shown to separate CNS actives and in-actives, although a high false-positive rate was found (only 65 % of the CNS in-actives were identified as such) when applied to an external validation. This lack of accuracy is rationalized as being due to the multiple mechanisms that may stop a drug from entering the brain, such as metabolism and efflux processes. The partial-least squares analysis performs equally as well. Analysis of the PLS coefficients appear to be consistent with previous interpretations of blood-brain partitioning. Hydrophilic interactions result in poor brain penetration and hydrophobic interactions tend to help brain penetration. Two new descriptors, critical packing and the hydrophilic-lipophilic balance, also appear important. The latter describes the balance between the 2 types of molecular regions to determine if one effect dominates or if they are roughly equal. The critical packing defines a ratio between the hydrophilic and lipophilic part

of the molecule. These descriptors appear to be similar to those used by previous researchers to relate various partitioning data to polar surface area and hydrogen-bonding features of compounds (43,44,48).

In almost all of the above studies a single general theme may be seen: CNS drugs must be of modest hydrophilicity in order to passively cross the BBB. This attribute is reflected in a number of the model's primary descriptors: polar surface area, free energy of solvation, as well as various other hydrogen-bonding descriptors. These descriptors are presumably well correlated with each other and reflect the general differences between the environment of the central nervous system and that of the circulatory system. In none of these models is transport out of the brain by Pgp considered.

Recent QSAR modeling studies have been reported that attempt to correlate Pgp inhibition with simple molecular properties (49). They found that hydrogen bond acceptor "units" correlate to Pgp substrate binding, a result that runs counter to the general belief that a basic-nitrogen is required to inhibit Pgp. Certainly a structural model of the sort described in the following section for the various metabolizing agents would be of great use for Pgp studies, and enable classical computational techniques (docking, dynamics and CoMFA models) to be applied to problems associated with Pgp interactions. One recent computationally derived structural model has been published which builds upon extensive experimental data (50).

METABOLISM & ELIMINATION/EXCRETION

Metabolism - With the exception of very polar substances most drugs are lipid-soluble and are reabsorbed from the kidney back into the bloodstream. These compounds undergo metabolism, generating more polar species that may avoid renal reabsorption and be excreted into the urine. Metabolites may either be pharmacologically similar to the parent, harmless but not pharmacologically active, or posses life-threatening toxicity. Clearly, researchers must know which of these categories their drug falls into and be able to modify a drug such that the metabolites are not toxic and have pharmacologically relevant lifetimes prior to being metabolized. A variety of methods have been published over the last two years with the goal of predicting the metabolic fate of a drug from its physicochemical structure. Perhaps reflective of the extreme variety of chemical pathways which a drug may be metabolized, the breath of computational techniques having been applied to such problems is fairly large. These can be classified into four primary categories: 1. models based on protein structure, 2. statistical structure-metabolism relationships, 3. pharmacophore modeling and 4. predictions based on expert systems.

Perhaps the most prevalent computational technique applied to metabolism in the recent literature has been the construction of protein structure homology models of various cytochrome P450s. These include models of CYP2B1, CYP2B4, CYP2B6 CYP4A1, CYP4A4, CYP4A11, CYP1A1, CYP1A2, CYP1A6, CYP2C9 and CYP2E1 (51-55). All of these models were built using the CYP102 crystal structure (56). A majority of these models were used to examine binding interactions of specific substrates and compare with experimentally determined regioselectivity, or to examine and rationalize active site interactions. Another strategy is to use the homology model to assist in the functional studies of CYP mutants (54).

Homology modeling techniques have also been employed to study the possible structures of various CYP isoforms and to investigate binding modes of key residues for particular substrates (57,58). In addition to building the homology models and docking substrates, molecular dynamics simulations were run in order to achieve time-averaged interaction energies between the enzyme-substrate complexes. This

method allowed for the calculation of the relative frequency that each abstractable hydrogen approached to within 4.0 Å of the ferryl oxygen, allowing for the prediction of product species and direct validation of this technique to experimentally determined products (58).

In combination with pharmacophore modeling and molecular orbital calculations, homology modeling techniques have been used to investigate the interactions of 40 different substrates with CYP2D6 (59). Here, a pharmacophore model was built using 2 compounds as templates: debrisoquine and dextromethorphan. Subsequent compounds were overlapped to these 2 templates using the SYBYL molecular operating environment. Homology models were constructed from x-ray structure of CYPs 101, 102 and 108. Twenty models were built, and a consensus structure was chosen using the previously developed pharmacophore model and the PROCHECK program as guides. An aspartic acid residue (ASP301) was determined to be the primary binding site for the basic nitrogen for most substrates. The authors also proposed a glutamine residue (GLU216) as a possible alternative site for larger compounds. The compounds overlapped with the pharmacophore were then oriented into the active site of the 2D6 homology model and minimized. Combining these 2 methods gives compound orientations and general pharmacophore distances which can then be used in conjunction with the molecular orbital calculations to help predict possible sites of metabolism.

CYP2C9 is the major isoform found in the human liver, and has been studied using both homology modeling and CoMFA, a generally used statistical method that incorporates the three-dimensional structure of a set of compounds (60). The idea underlying CoMFA (Comparative Molecular Field Analysis) is that differences in a target property are often related to differences in the shapes of the non-covalent fields surrounding the tested molecules. To put the shape of a molecular field into a more general statistical analysis, the magnitudes of its steric (Lennard-Jones) and electrostatic (Coulombic) fields are sampled at regular intervals throughout a defined three-dimensional region. As one may expect, the most important aspect of such a study is the relative alignment of the individual molecules when their fields are computed. Using a homology model as a guide for molecular alignments has allowed researchers to predict the 3-dimensional features that correlate with CYP2C9 inhibition for 14 compounds (60). The QSAR generated by a PLS analysis of the 3D field-data resulted in q^2 values ranging from 0.6 to 0.8. In a similar work, a small set of heterocyclic amines was docked into a homology model of CYP1A2 using the AUTODOCK program. Molecular fields were calculated using the GRID program and a QSAR was generated with a $q^2 = 0.79$. A similar method used the CoMFA model and homology model in an iterative sense to "mutually validate" both models. Here, only regions with a high interaction potential were included in the next generation CoMFA model, leading to a model with a reduced number of input variables (11 from 325 initial points) and, presumably, a more accurate model (61).

The MS-WHIM program produces 36 descriptors that contain information about the molecular structure in terms of size, shape and electrostatic contribution. The method was tested on a set of 16 rigid coumarin-like compounds and 78 similarly rigid polyhalogenated aromatics that are inhibitors of CYP2A5. The q^2 values were found to be around 0.7 (29). Subsequent studies had significantly lower q^2 values (62,63). Other strategies at building a 3-dimensional pharmacophore model have been reported for CYP3A4, CYP2B6 and human *in vitro* intrinsic clearance with varying degrees of success (62-64).

More traditional QSAR approaches to metabolism have also been published within the last two years. In one study, an attempt was made to predict the metabolism of benzoic acids (n = 22) as measured by rat urine excretion of parent

compounds (65). Quantum chemical descriptors such as HOMO, dipole moment components and heat of formation were used, as well as some of the more common descriptors such as CMR, ClogP, pK_a and surface area. In general 4-7 parameters were needed, and correlations (r^2 values) ranged from about 0.53 to 0.83. In a separate work, an attempt was made to obtain a more general equation for human blood *in vitro* metabolism data of more than 80 compounds belonging to seven different classes: beta-blockers, angiotensin converting enzyme inhibitors, opioid analgetics, soft corticosteroids, short-acting anti-arrhythmic agents and buprenorphine prodrugs (66). They used the novel parameter "inaccessible steric angle," a measure of the solid angle of a particular atomic site that is essentially blocked by surrounding atoms. A good correlation was reached using only the inaccessible steric angle for the carbonyl oxygen and carbon atoms (r^2 = 0.71). Adding qlogP increases this number to 0.80. In a study involving aliphatic alcohols and acids a QSAR equation was constructed which correlated the Frontier orbital populations (HOMO and/or LUMO population) on the alpha-carbon as well as the overall length of the molecule. Good correlations were achieved (r^2 values ranged from 0.77-0.94 for small data sets) (55).

Others have taken an approach similar to that of Lipinski (8), where attempts are made to identify the particular logP ranges common to various CYP isoforms (67). Here, it is reasoned that the site of metabolism can be generally correlated to the disposition of hydrogen bond acceptors and donors, as well as the molecular size and shape (67).

"Expert systems" represent the final class of computational techniques applied to the prediction of metabolically related biological endpoints. Instead of making correlations to biological data as traditional QSAR does, these programs depend upon empirical assessment of the mechanism in question to define a set of rules. With respect to metabolic issues, these programs generally involve the prediction of possible metabolites from a given parent structure in hopes of identifying possible toxic products. These programs reflect the inter-relation between ADME and TOX predictions. A review of toxicologically related computational modeling is prohibited by the length restrictions of this manuscript. Interested readers may inquire into some of the more historical works in this area, or some of the more recently published offerings (1,68-74).

Conclusions - Several recent advances in the field of predicting ADME properties have been made over the last two years. Efforts at predicting logP seem to be numerous, while distribution (aside from BBB) and elimination/excretion are being largely ignored. Interestingly, not many advances in the area of predicting solubility have been made. Absorption is an important area and a great opportunity exists for computational methods to make inroads. However, the lack of quality data (including internal data) precludes any real advances. Absorption is a multiple mechanism phenomenon, which immediately lowers the chances that any regular QSAR method will succeed. The same case can be made for both metabolism and Pgp related biological endpoints, where structure-based models seem to appear more often than the traditional QSAR methods. Given the nature of the problems involved in predicting ADME properties, the fact that more methods and publications are appearing is encouraging. It is hoped that in the future, numerous and cleaner data will allow for an expanded attack of ADME by computer.

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Chapter 26. PET Ligands for Assessing Receptor Occupancy In Vivo

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Introduction: Positron emission tomography (PET) is emerging as a powerful research tool that may help reduce the significant cost of bringing new drugs from discovery to the marketplace by improving the efficiency and bridging of both preclinical and clinical research on new drug candidates. PET is a relatively non-invasive clinical and research imaging technique that is available at a limited number of medical centers world-wide (~40 in the US, a comparable number in Europe, as well as in Japan and other parts of the world)(1). This powerful imaging methodology is gaining acceptance as a means of designing dosage levels and regimens for CNS drugs. This emerging interest in PET as a tool in drug discovery, research and development is illustrated by the fact that there are now regular scientific meetings specifically devoted to the subject (for a list, see www.snidd.org). A common theme of these meetings is that PET technology is most useful when: 1) there is no easily measurable clinical pharmacodynamic marker or 2) substantial time is required before an indication of efficacy can be observed (as is the case for many CNS drugs and cancer treatments). Several review articles and books on the use of PET in drug development have appeared (2-10). This review will focus on reports of new tracers for CNS PET receptor, enzyme and transporter imaging published since the beginning of 2000.

Neuroreceptor Specific PET Tracers - The primary use of PET imaging in clinical CNS drug development has been to determine the relationship between drug dose (or plasma level) and occupancy of the target binding site in the brain (receptor, active site of an enzyme or a transporter binding site, hereafter referred to collectively as receptors). These studies involve the use of specifically designed radiotracers with high affinity and selectivity for these target binding sites to obtain tomographic images of radiotracer bound to receptors of interest. *In vivo* competition studies can thus be conducted to determine the level of occupancy provided by unlabeled drug candidates. This is the only methodology that can provide a direct measure of receptor occupancy in living humans.

Since the first reported clinical neuroreceptor imaging study in 1983 a wide variety of tracers have been developed in academic and government labs that are suitable for use in receptor occupancy studies (11, 12, 13). However, when new drug targets are involved, new tracers must be developed. Sometimes, the drug candidate can be labeled and used as a PET radioligand (e.g. [^{11}C]raclopride (14, 15) and [^{11}C]MDL-100,907 (16)), but this is rare. When the drug candidate itself does not have suitable properties to be used as the radioligand or if it cannot be labeled rapidly with an appropriate radionuclide, alternative target-specific novel radiotracers must be developed for use in *in vivo* competition studies. It is noteworthy however that it is not always possible to develop radiotracers for PET receptor occupancy studies especially when the target receptors are present in the brain at low concentrations. Most CNS receptors for which PET tracers have been developed are found in the brain at concentrations on the order of 5 nM or higher. Receptors with concentrations as low as ~ 0.5 nM (extrastriatal D_2 receptors) have been successfully labeled but this requires a tracer ([^{11}C]FLB457) with very high affinity (20 pM for D_2 receptors) and very low non-specific binding.

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PET studies have typically been carried out using radiotracers labeled with the short-lived positron emitting radionuclides, primarily C-11 or F-18 ($t_{1/2}$ = 20 and 110 min. respectively). For example, PET imaging using either N-[^{11}C]-methylspiperone or [^{11}C]raclopride has been used to demonstrate that most of the current drugs used for the treatment of schizophrenia block post synaptic dopamine D_2 receptors with occupancies > 70% at therapeutic doses and that extrapyramidal side effects such as Parkinsonism, dystonia and restlessness are most prevalent with D_2 occupancies > 80% (17, 18). The characteristics of the most commonly used PET radionuclides are listed in Table 1.

TABLE 1. POSITRON EMITTING RADIONUCLIDES.

Radionuclide	Half-Life Minutes	Photons (keV)	Nuclear Reactions
Oxygen-15	2.01	511	$^{14}N(d,n)^{15}O$ $^{14}N(p,n)^{15}O$
Nitrogen-13	9.98	511	$^{16}O(p,\alpha)^{13}N$ $^{13}C(p,n)^{13}N$
Carbon-11	20.4	511	$^{14}N(p, \alpha)^{11}C$ $^{11}B(p,n)^{11}C$ $^{10}B(d,n)^{11}C$
Fluorine-18	109.8	511	$^{18}O(p,n)^{18}F$ $^{20}Ne(d, \alpha)^{18}F$
*Bromine-76	16.3 hours	511	$^{76}Se(p,n)^{76}Br$
*Iodine-124	4.2 days	511	$^{124}Te(p,n)^{124}I$

The most widely used of these radionuclides (^{11}C, ^{18}F, ^{13}N and ^{15}O) are cyclotron produced, and, given their very short half-lives, must be used at the site of production. Recently, as a result of the rapidly growing application of [^{18}F]FDG (fluorodeoxyglucose) studies in oncology, several regional [^{18}F]FDG production centers have been established which can supply the tracer to hospitals within a 2 hour shipping radius of the production site (given the 110 min half life of F-18). This is not practical with the shorter-lived isotopes. *Bromine-76 and *Iodine-124 are available at only a few PET centers, however, given their half-lives, production and distribution to PET centers remote from the cyclotron is viable. Similar studies to those using PET can be done via Single Photon Emission Computed Tomography (SPECT or SPET) using radioiodinated tracers labeled with ^{123}I ($t_{1/2}$ = 13 hr) (19), however, PET imaging is now generally preferred.

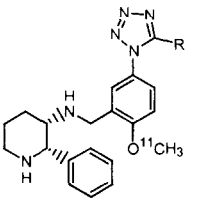
Neuroreceptor Quantitation and Occupancy Studies - Neuroreceptor tracers have been used to establish the level of occupancy achieved by therapeutic doses of several classes of drugs, as well as to investigate potential treatment related changes in receptor concentrations. The recent development of high resolution, dedicated, small animal PET cameras has spurred investigation of a more extensive use of this technology at the preclinical stage by conducting imaging experiments in smaller animals, including rat and mouse (20, 21, 22, 23). Important bridging validation studies can also be conducted in non-human primates to give a proof of concept for a novel radiotracer in non-terminal studies that are

very similar to those that will be used in clinical programs. Also, application of PET imaging technology to both preclinical and clinical drug development, has the advantages of 1) producing accurate estimates of occupancy *in vivo,* 2) being relatively non-invasive, 3) permitting repeated measurements (in humans and animals which also allows for the use of the subject or animal as its own control prior to drug treatment), and 4) allowing replication and corroboration of animal studies in humans. The latter feature is significant in light of potential species differences and in ethical terms as the use of animals can be reduced and the quality of data improved by these non-terminal minimally invasive procedures.

P-Glycoprotein - An important characteristic of PET tracers for use in CNS receptor imaging is rapid penetration of the blood-brain barrier and this generally requires modest lipophilicity (Log P ~ 1 – 3). However, this is not always sufficient to assure adequate brain penetrability. P-glycoprotein is a membrane bound pump present in the capillary endothelium of the CNS and it functions by pumping some foreign compounds from the brain very rapidly. The importance of this to the discovery of novel PET tracers for labeling CNS receptors was clearly demonstrated (24) in a rat study of [^{18}F]MPPF, a radioligand for imaging of 5-HT$_{1A}$ receptors. A 5 – 10 fold increase in brain uptake of [^{18}F]MPPF (a Pgp substrate) was observed in rats pretreated with the Pgp inhibitor, cyclosporin A.

NEUROKININ-1 RECEPTOR

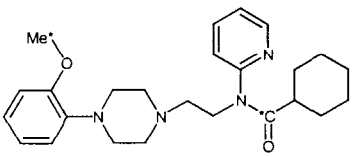
Substance P, NK1 Selective Radiotracers - Neurokinin-1 (NK$_1$) antagonists are currently receiving substantial attention due to their potential use in a number of indications including treatment of emesis, pain, inflammation and several psychiatric disorders (depression and anxiety). Human and monkey PET studies have been reported using a new NK$_1$ selective PET tracer [^{18}F]L-829,165 but the details of these studies have not yet been disclosed (25). Results of monkey PET studies with 2 labeled NK$_1$ antagonists [^{11}C]GR203040 (**1**) and [^{11}C]GR205171 (**2**) were recently reported (26). Although the structure and NK$_1$ affinities of these two tracers are very similar, results suggest that only [^{11}C]GR205171 is useful for imaging NK$_1$ receptors in monkey.



1 R=H, [^{11}C]GR203040
2 R=CF$_3$, [^{11}C]GR205171

SEROTONIN RECEPTORS AND TRANSPORTER

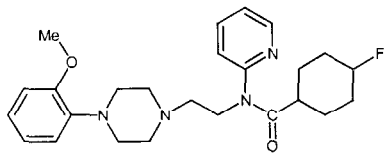
Serotonin 5-HT$_{1A}$ Receptors - The first successful tracer for imaging 5-HT$_{1A}$ receptors, [O-Methyl-^{11}C]WAY100635 (**3**), was developed through an academic / industry collaboration (27). Although this tracer has been used to successfully image 5-HT$_{1A}$ receptors in humans, it suffers from the liability of forming a radiolabeled metabolite that not only crosses the blood-brain barrier, but also labels 5-HT$_{1A}$ receptors, complicating the use of this tracer in quantitative PET studies. This has led to a substantial effort to develop improved 5-HT$_{1A}$ selective PET tracers. WAY100635 has also been labeled in the carboxyl position ([*Carbonyl*-^{11}C]WAY100635 (**3**)), reducing concern for labeled metabolites, and this tracer is now widely used in clinical research and drug development studies (28). PET occupancy studies of several novel 5-HT$_{1A}$ antagonists in monkeys using [*Carbonyl*-^{11}C]WAY100635 indicated that this tracer



3

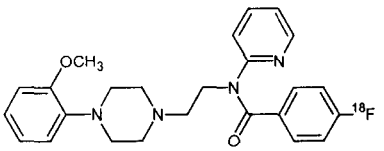
is useful for dose finding studies (29). Similar studies were conducted in healthy volunteers using this tracer to measure 5-HT_{1A} receptor occupancy achieved by treatment with a controlled release formulation of pindolol (30). However, like [O-methyl-^{11}C]WAY100635, it is rapidly metabolized and cleared from plasma and modeling studies are hampered by the short half-life of C-11. Consequently, the search continues for a comparable radioligand, with a higher and more sustained delivery to brain in hope of developing a tracer that is more useful for PET modeling studies, as well as for other types of studies such as measurement of disease or drug related changes in endogenous serotonin levels (31). The proceedings of a symposium on 5-HT_{1A} receptor selective PET tracer held at the Karolinska Institute in 1999 have been published (32). At this meeting, an overview was provided of the developments that led to the identification of [O-methyl-^{11}C]WAY100635 as well as recent attempts to develop superior radioligands (33).

F-18 Labeled 5-HT_{1A} Tracers - F-18 labeled tracers provide two advantages over C-11 tracers, both related to the significantly longer half-life of F-18 which makes if feasible to use F-18 labeled tracers at sites remote from the accelerator required to make the radionuclides. In addition, imaging experiments can be carried out for much longer times than with C-11 and this is sometimes an important consideration for conducting quantitative kinetic modeling studies. Toward this end, several new F-18 labeled 5-HT_{1A} ligands have been reported, including [^{18}F]FCWAY (**4**) an F-18 labeled analog of [carbonyl-^{11}C]WAY-100635 in which a trans-fluorine is incorporated at the 4-position of the cyclohexyl group) (34). Recent studies in monkeys suggest that this tracer is promising for imaging human 5-HT_{1A} receptors (35). Plasma metabolite analysis identified [^{18}F]fluorohexane-carboxylic acid and [^{18}F]fluoride, neither of which cross the blood-brain barrier. Analysis of kinetic curves demonstrated that [^{18}F]FCWAY has similar kinetic characteristics to [*carbonyl*-^{11}C]WAY100635.



4

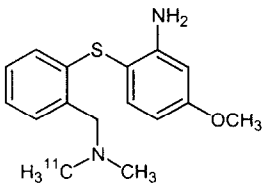
Another 5-HT_{1A} tracer, [^{18}F]MPPF (**5**), was first reported in 1997 and continues to show promise (36, 37). Quantitative imaging studies in healthy volunteers were recently reported (38, 39). After injection of [^{18}F]MPPF, radioactivity was rapidly taken up in the brain, with the highest accumulation in the medial temporal cortex. Low levels of radioactivity were found in cerebellum and basal ganglia. A good correlation was found between the calculated binding potentials and literature values for 5-HT_{1A} receptor densities. Blocking experiments with pindolol showed a decrease of 40% in the region/cerebellum ratios of the target areas. Binding potentials were 4 to 6 times lower than for [*carbonyl*-^{11}C]WAY100635, indicating that [^{18}F]MPPF has a lower *in vivo* affinity. These results suggest that [^{18}F]MPPF can be used for the quantitative study of 5-HT_{1A} receptor in the living human brain and may be sensitive to endogenous serotonin concentrations within the brain.



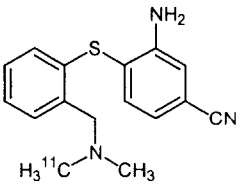
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Serotonin Transporters - Interest in imaging the serotonin transporter (SERT) stems primarily from its role in psychiatric diseases, especially depression. A series

of phenylthiobenzylamines were prepared and evaluated in rats (40). Two of these, [^{11}C]DAPP (**6**) and [^{11}C]DASB (**7**), were subsequently taken on to human studies (41). Preliminary studies in healthy volunteers showed that both had similar brain uptake and kinetics with highest uptake in regions rich in SERT (midbrain, thalamus, striatum) and lowest in the cerebellum giving target-to-cerebellum ratios of about 2.5. Specificity was demonstrated by pretreatment with citalopram which reduced binding in target regions by about 80% (down to the level of the cerebellum). These results suggest that both tracers show promise for PET studies of SERT.



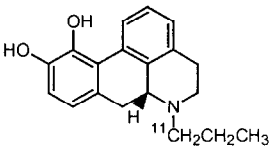
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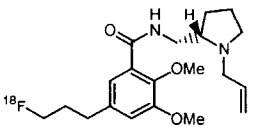
DOPAMINE RECEPTORS AND TRANSPORTER

Dopamine D_2 Receptors - A wide variety of labeled D_2 antagonists have been developed for imaging D_2 receptors. Imaging neuroreceptors with radiolabeled agonists might provide valuable information on the *in vivo* agonist affinity states of receptors. The radiosynthesis of [^{11}C](-)-NPA (**8**), as well as biodistribution in rodents, and imaging studies in baboons have recently been reported (42). Rodent studies showed high uptake of [^{11}C](-)-NPA in D_2 receptor-rich areas with striatum /cerebellum ratios of 1.7, 3.4, and 4.4 at 5 min, 30 min, and 60 min post injection, respectively. Pretreating the animals with haloperidol reduced the striatum/cerebellum ratio at 30 min postinjection to 1.3. [^{11}C](-)-NPA was also evaluated in baboon PET studies. Specific binding to D_2 receptors was confirmed by selective blockade of tracer binding by pretreatment with haloperidol suggesting that this tracer may be useful for probing D2 receptors in humans.



8

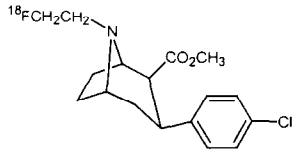
[^{18}F]Fallypride (**9**) is a highly selective, high-affinity (K_D = 30 pM), dopamine D_2 receptor ligand (43). The high affinity makes it a suitable candidate for visualizing both striatal and extrastriatal binding in the brain. Dynamic PET studies of two macaque monkeys provided specific signals in the striatum, thalamus, frontal, and temporal cortices. Modeling studies using the cerebellum as a reference tissue provided distribution volumes of 26, 29, 3.8 and 1.7 for the caudate, putamen, thalamus and frontal = temporal cortex, respectively. These results suggest that this tracer may be suitable for visualizing striatal and extrastriatal dopamine D_2 receptors in the human brain.



9

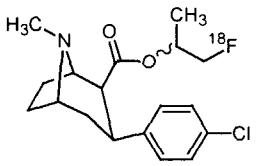
Dopamine Transporters - The dopamine transporter (DAT) is a membrane bound protein that terminates the neurochemical action of dopamine via reuptake of synaptic dopamine. Interest in DAT stems from its role in the psychostimulant effects of cocaine, as well as the loss of dopaminergic terminals in Parkinson's disease. The importance of DAT has lead to substantial efforts to develop PET and SPECT tracers for DAT imaging. Although a wide variety of C-11, F-18 and I-123 labeled tracers have been prepared and tested, this remains an active area of research, and four new, promising [^{18}F]-labeled tracers were recently reported.

FECNT was labeled with F-18 and evaluated it in rats (44). Results indicated that the brain uptake of [^{18}F]FECNT (**10**) is selective and specific for DAT-rich regions. Imaging studies in monkeys demonstrated high [^{18}F]FECNT uptake in the caudate and putamen with caudate-to-cerebellum and putamen-to-cerebellum ratios of ~ 10 at 60 min. [^{18}F]FECNT uptake in the caudate/putamen peaked in less than 75 min and exhibited higher caudate- and putamen-to-cerebellum ratios at transient equilibrium than reported for other DAT selective PET tracers. Displacement experiments in Rhesus monkeys showed that radioactivity in the putamen was displaced with an average half-time of 10.2 min. suggesting that [^{18}F]FECNT may be useful imaging DAT in humans using PET.



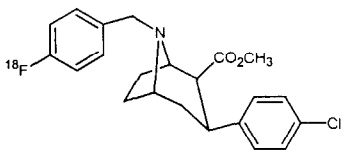
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The same group also reported on [^{18}F](R)-FIPCT (**11**) and [^{18}F](R)-FIPCT (**12**), either of which can be made at high specific activity in a single radiochemical step(45). Both diastereomers have high affinity and selectivity for the DAT over the serotonin transporter. PET imaging studies in monkeys demonstrated high uptake in the caudate and putamen and provided caudate-to-cerebellum ratios of 2-5 – 3.5. [^{18}F](R)-FIPCT reaches transient equilibrium in the striatum in < 90 minutes and should be useful for quantitative studies of striatal DAT sites in humans. [^{18}F](R)-FIPCT showed prolonged retention in the striatum and may be useful for mapping extrastriatal DAT sites.



11 (R), 12 (S)

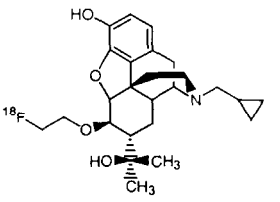
Another promising [^{18}F]-labeled tropane analog has been reported. [^{18}F](+)-FCT (**13**) which has high affinity and selectivity for DAT and had a higher accumulation in the caudate and putamen than the cerebellum (46). Modeling studies indicated that [^{18}F](+)-FCT displayed reversible binding kinetics *in vivo*. Displacement of [^{18}F](+)-FCT radioactivity in the basal ganglia by intravenous injection of cocaine confirmed the reversibility of binding. These data suggest that [^{18}F](+)-FCT may be a suitable radiotracer for studying DAT function with PET.



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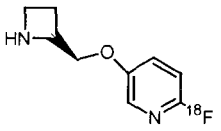
OTHER RECEPTORS

Opiate Receptors - Results of preliminary imaging studies of a new, F-18 labeled analog of diprenorphine ([^{18}F]DPN (**14**)), in healthy men showed a binding pattern comparable to that of a control group imaged with [^{11}]diprenorphine (47). Rat brain autoradiography studies showed that [^{18}F]DPN binds to mu, kappa, and delta opioid receptors. The longer half-life of F-18 provides the advantage of extended scanning periods, allowing for more flexible interventions (e.g., displacement studies), as well as use of the tracer at PET centers without an on-site cyclotron.



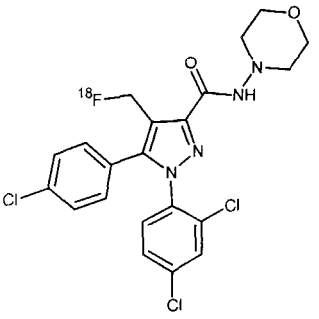
14

Nicotinic Receptor - Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of ligand gated ion channels which are widely distributed in the human brain. Multiple subtypes exist which mediate the effects of nicotine and are involved in a number of disorders such as Alzheimer's disease, Parkinson's disease and schizophrenia (48). Efforts to develop PET tracers for imaging nAChRs have focused on three general classes of compounds, including, nicotine and its analogs, epibatidine and related compounds, and 3-pyridyl ether compounds, including A-85380 (49, 50). The novel radioligand, [^{18}F]fluoro-A-85380 (**15**) was synthesized via nucleophilic displacement of an iodine from the corresponding, N-protected iodopyridine followed by deprotection (51). The regional distribution in mouse brain was consistent with binding to nAChRs and was blockable by pretreatment with unlabeled A-85380 and other ligands that bind selectively to nAChRs. PET studies in monkey, showed highest binding of radioactivity in brain regions enriched with the alpha$_4$beta$_2$ subtype (52). The thalamus/cerebellum ratio was similar to that previously determined for an analog of epibatidine, however this tracer is expected to be significantly toxic and appears to be a suitable candidate for imaging nAChRs in human brain.

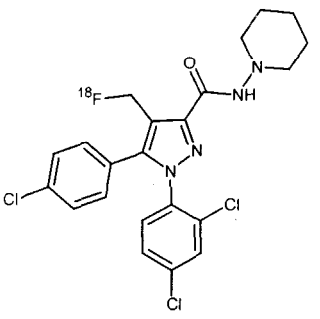


15

Cannabinoid Receptors - Products derived from *cannabis sativa* are of interest, not only because of their potential as drugs of abuse, but also because of a variety of potential therapeutic indications, including analgesia, antiemesis and appetite



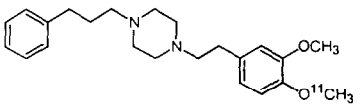
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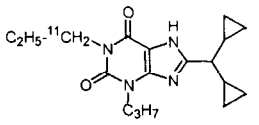
modulation. Two types of cannabinoid receptors have been identified, CB_1 and CB_2, with CB_1 being found predominantly in the brain. SR141716A is the first potent and selective antagonist of CB_1 receptors, and has been labeled with tritium and used for labeling CB_1 receptors in rat brain (53). An I-123 labeled analog of SR141716A has also been reported and shown to be useful for SPECT imaging of brain cannabinoid receptors (54). Recently, the syntheses of two [^{18}F]-labeled analogs of SR141716A have been reported (55). [^{18}F]SR147963 (**16**) and [^{18}F]SR144385 (**17**) both were made by incorporation of the label into a fluoromethyl group via nucleophilic displacement of a bromine followed by a multistep synthesis to provide the final labeled product. Although both radioligands had appropriate regional brain distribution for cannabinoid receptors and binding was blocked by SR141716A, the target to non-target ratios were modest (1.7 and 2.5 for [^{18}F]SR147963 and [^{18}F]SR144385, respectively) suggesting that alternative tracers with higher affinity and lower lipophilicity should be sought.

Sigma-1 Receptors - SA4503 is a selective sigma-1 antagonist (IC_{50} = 17.4 nM, sigma1/sigma2 = 103) that has been labeled with C-11 and evaluated in rat PET imaging experiments (56). In blocking studies, SA4503 and haloperidol significantly reduced brain uptake of [^{11}C]SA4503 (to approximately 30% of the control). Although approximately 20% of the radioactivity was found as C-11-labeled metabolites in plasma at 30 min post injection, no C-11-labeled metabolites were detected in the brain. These results suggest that [^{11}C]SA4503 (**18**) has potential for mapping sigma1 receptors in the central nervous system.



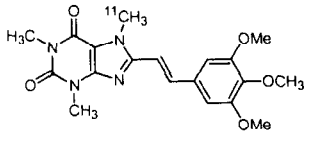
18

Adenosine Receptors - Adenosine is an endogenous neuromodulator that functions through interactions with a variety of adenosine receptor subtypes (A_1, A_{2a}, A_{2b}, and A_3) and may play a role in a variety of neurological disorders, including Huntington's chorea, Parkinson's Disease, and Alzheimer's Disease. KF15372 (**19**) is a high affinity, selective ligand for A_1 receptors and it has been labeled with C-11, with the label being incorporated into a propyl group (57). PET studies in anesthetized monkey showed that the regional distribution of radioactivity in the brain was consistent with binding to the A_1 receptor and this binding was significantly reduced by a blocking dose of cold KF15372 suggesting that this tracer may be suitable for imaging A_1 receptors in humans.

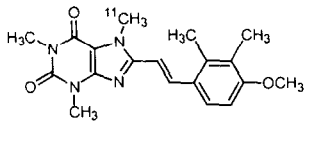


19

KF18446 is a potent A_{2a} antagonist that has been labeled with C-11 by methylation of the corresponding des-methyl compound (58). *In vitro* receptor autoradiography studies with rat brain sections showed a high striatum/cortex binding ratio (5.0) and low nonspecific binding (<10%). Subsequent PET imaging studies in rats with [^{11}C]KF18446 (**20**) and a structurally similar analog, [^{11}C]KF21213 (**21**) clearly visualized the striatum, a region with high adenosine A_{2a} receptor concentration (59). [^{11}C]KF21213 was found to be the more promising of the two tracers for mapping adenosine A_{2a} in the central nervous system by PET.



20



21

Summary - Interest in using PET for assessing receptor occupancy *in vivo* continues to increase and development new PET radioligands remains an active area of research. This methodology has gained acceptance as an important tool for the drug industry in clinical trials of CNS drug candidates. The recent availability of high resolution, small animal PET cameras provides an opportunity for extension of the application of this technology to preclinical drug discovery programs.

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Chapter 27. Existing and Emerging Strategies for the Analytical Characterization and Profiling of Compound Libraries

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Introduction - Combinatorial chemistry, parallel solution phase and solid phase organic synthesis (SPOS) are a new wave of chemistry technologies that have unquestionably revolutionized the drug discovery process. First introduced and pioneered in the late 1980's and early 1990's, combinatorial chemistry enables exquisitely large numbers of compounds to be generated in a fraction of time otherwise required by classical medicinal chemistry methods (1-3). Based on solid phase synthesis principles, the technique involves what is commonly referred to as split-couple-recombine (or split and mix) methodology to produce in a single step one or several mixtures (i.e., libraries) of products, typically through a randomization process (4). In the true combinatorial chemistry split and mix approach, functionalized polymeric resin (e.g., Wang, Rink resins) containing thousands to millions of individual beads functionalized with accessible "reactive" sites on the bead surface necessary to facilitate solid phase synthesis are treated with a single monomer (or synthon). This monomer is added in large excess relative to the amount of functionalized bead material, permitted to react, and excess reagent is removed, typically by filtration. Following this initial coupling event, the beads are distributed evenly into a set of "pots" (the number of "pots" being equivalent to the number of monomers to be used in the second coupling reaction). Following addition of the second monomer set, the beads are then recombined and sorted again. The process of monomer addition, sorting and recombination continues until the last set of monomers is added. Typically, this approach generates bead mixtures containing hundreds, thousands and even millions of compounds, each bead containing between 0.5–5 nanomoles of material (depending on the size of the individual bead), sufficient for high throughput biological screening. Combinatorial chemistry has been the focus of numerous reviews; a particularly comprehensive one published in 1999 (5).

Combinatorial chemistry clearly laid the foundation for revolutionary advances in medicinal chemistry over the last decade. Though the technique of split and mix synthesis still remains popular for some bench chemists, it has been replaced largely by the technique of directed parallel solution phase and parallel solid phase organic synthesis (6-8). The shift from combinatorial libraries to parallel synthesis libraries has been driven by the desire to screen discrete compounds that are available in larger quantities and are more rigorously characterized. Coffen, in a recent report, thoroughly investigated the merits of both solid and solution phase parallel synthesis (9). Parallel (or array) synthesis allows for the generation of a large collection of individual compounds (unlike combinatorial chemistry, which produces mixtures containing a large collection of molecules). Another advantage of parallel solution phase and solid phase parallel synthesis is that it provides a means for generating much larger quantities of material whilst capitalizing on its amenability to automation. Automated parallel synthesis has captured the imagination of the pharmaceutical industry and today is a standard laboratory tool for the medicinal chemist.

Combinatorial chemistry and automated parallel synthesis, now mainstays in the pharmaceutical, biotechnology, agrochemical and material science industries, have forced the analytical community to develop technologies to keep pace. The

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conundrum facing the analytical community is that existing analytical techniques have been inherently serial-based and parallel synthesis techniques are inherently parallel. Over the past few years, the analytical community has addressed this conundrum by introducing a new wave of cutting edge technologies to support combinatorial library characterization, purification and screening. This review will cover state-of-the-art methods for the high throughput mass spectrometric and NMR analysis, quantification and purification of combinatorial and parallel synthesis libraries. In addition, hardware and data acquisition and data reduction software improvements made in both the mass spectrometry and NMR fields will be highlighted. Additionally, methods where MS and NMR have been applied to the study of receptor:ligand and enzyme:substrate interactions will be presented. Emerging technologies, including high throughput absorption and metabolism screening and high throughput parallel analysis will be presented.

ANALYSIS AND DECONVOLUTION OF COMBINATORIAL LIBRARIES

True combinatorial libraries are synthesized as mixtures on polymeric resins (beads). Although each library may contain a mixture of thousands to millions of molecules, each individual bead is synthesized containing a unique synthetic product. The techniques of NMR, FT-IR and mass spectrometry have been used routinely for the characterization of combinatorial libraries

NMR of Materials on Resins - Quite often it is necessary to monitor solid phase synthesis with out cleaving the material from the resin. While the spectra obtained from a dry resin sample exhibit extremely large line widths, it is possible to obtain "high resolution" spectra of materials on resins. The poor quality of the spectra of a dry resin arises from several factors. The extremely slow molecular motions in the solid state cause a significant broadening of the NMR lines. By swelling the resin into a gel state with a suitable solvent much narrower lines can be obtained. While the line widths are still too large for proton NMR to be of much use, fair results have been obtained for ^{13}C and results have also been reported for ^{31}P and ^{19}F (10-12). The other major factor contributing to the broad lines is the varying magnetic susceptibilities present in the heterogeneous sample. Borrowing from the technique of magic angle spinning (MAS) that has been used for decades in solid state NMR, the line broadening contribution from magnetic susceptibilities can be greatly reduced if the sample is spun at 2-3 kHz about the magic angle (13-15). Combining these two techniques provides a method of obtaining high resolution NMR of gel state resins. Much work has been done on exploring which resin and solvent combination provides the highest quality spectrum (16). Resins with long tethers, such as Tentagel®, provide the highest quality spectra, while resins with short tethers, such as Wang®, provide low quality data. This result is not surprising, as Tentagel's® long polyethylene glycol tether provides for a great deal of intra-molecular motion at the end of the tether. The use of MAS gel NMR has been widely applied to monitoring the progress of SPS reactions using various nuclei (13,17,18). This technique is so sensitive that data has been obtained from one single macro resin bead (17,19).

The application of 2-dimensional NMR experiments in MAS gel NMR of resins has been extensively explored (20-23). Improvements in resolution have been achieved by using the 2D J resolved spectrum and projecting on to the chemical shift axis the un-tilted spectrum. Not only does this method provide improved resolution, but the intensities of the broad resonance of the resin polymer are greatly reduced or eliminated. This work has been extended to demonstrate the application of other 2D NMR experiments such as total correlated spectroscopy (TOCSY), heteronuclear multiple quantum spectroscopy (HMQC), and spin-echo correlated

spectroscopy (SECSY) as methods for monitoring reactions in solid phase synthesis.

FTIR of Materials on Resin - Single bead Fourier Transform Infrared (FTIR) spectroscopy has been used for both qualitative and quantitative reaction monitoring of bead-bound materials and has been found to be specifically advantageous for deriving on-resin conversion yields and assessing reaction kinetics (24-27). The technique is ostensibly non-destructive (i.e., does not require liberation of the material from the resin for analysis). Reaction monitoring by FTIR involves the detection of the organic functional group interconversions via chemical reaction or by appearance or disappearance of functional groups carried by building blocks or protecting groups introduced or removed during a reaction. A major limitation of the conventional FTIR methods are that they are not amenable to full automation for high throughput applications. Recent work utilizing FTIR imaging has demonstrated that the FTIR can be used in a high through-put parallel fashion to identify the chemical enities on individual beads (28). A second limitation is that presence of multiple components causes severe spectral overlaps, making quantitative determinations problematic.

Mass Spectrometry of Materials on Resin - There have been few reports of direct mass spectrometric analysis of bead bound materials for reaction monitoring. Unlike MAS-NMR and FT-IR, monitoring solid-phase reactions by mass spectrometry typically involves partial liberation of the compound from resin prior to mass spectrometric analysis. Secondary ion imaging mass spectrometry (SIMS), a technique similar in concept to matrix assisted laser desorption ionization (MALDI), differing principally in that no matrix is used to aid in desorption and ionization of the bead-bound compound, has been used for reaction monitoring (29). The utility of MALDI-TOF for reaction monitoring of combinatorial libraries has also been demonstrated (30). Two common techniques for liberating material from beads are to use acid- or photo-labile linkers to the resin and to use electrospray ionization or MALDI-MS to analyze the liberated product (31,32). It has been shown that it is possible to get a direct MALDI-TOF mass spectrometric analysis of a peptide attached via a photocleavable linker to a resin (33). In this experiment, the sample was irradiated and released directly from the resin bead with multiple pulses of a 337nm N_2 laser.

More typically, mass spectrometry has been used for analysis of bead-bound materials following biological screening. In these experiments, active beads are isolated and the compound is cleaved from the resin and analyzed by mass spectrometry. Molecular weight information alone is insufficient to deduce unequivocally the identity of the compound. This is because in a typical combinatorial library, the molecular weight redundancy is extremely high. Tandem mass spectrometry can be a useful tool for structural assignment of bead-bound materials (34). Tandem mass spectrometry (MS/MS) information significantly reduces the ambiguities due to molecular redundancy because in addition to molecular weight information, the technique provides a wealth of structural information. However, interpretation of MS/MS spectra is not possible in a fully automated way, and therefore limits its practical value for the characterization of large combinatorial libraries. In a recent paper compound libraries designed a priori taking into account the potential for molecular weight redundancy, facilitate direct analysis of library components by mass spectrometry, offering an alternative to labor-intensive structural elucidation by NMR or tandem mass spectrometry (35).

Mass Spectrometry Based Encoding Strategies - Attaching a synthetic, molecular tag to the bead during the course of the combinatorial library synthesis is an alternative strategy for deducing the identity (albeit indirectly) of bead-bound

compounds. Numerous elegant encoding and decoding strategies have been developed over the past several years (36-39). The polyamide "hard tag" was shown to be a particularly effective encoding technique because these tags are found to be compatible with a wide range of synthetic chemistries. Originally, these tags were released from the bead surface and analyzed by gas chromatography. A significant speed advantage in the analysis of these secondary amine tags was achieved when a fast LC/MS method was adopted (40). A clever bead encoding strategy using stable isotopes of carbon and hydrogen (^{13}C and ^{2}H) and a decoding strategy incorporating electrospray ionization mass spectrometric detection has been developed (41).

Each of the encoding strategies has its advantages and limitations. The advantage of coding strategies is that each bead has a unique identifier (the tag) which greatly facilitates compound identification. The principal limitation is that the decoding strategy is an indirect determination of the compound on the bead. The determination of the code implies the structure of the material on the bead but does not confirm it unequivocally. Typically, partial release of the target compound is required followed by secondary analysis by mass spectrometry analysis. In order for this approach to be practically useful, automated and reliable bead picking apparatus is required.

HIGH THROUGHPUT ANALYSIS OF PARALLEL SYNTHESIS LIBRARIES

Analysis by NMR - For decades NMR has been the method of choice for the confirmation of molecular structure in medicinal chemistry. The NMR spectrometer's sample handling hardware and software has been developed over the years to deal with standard sample formats. The use of automation in the NMR laboratory has been developed to the point were running a hundred or more proton spectra a day on a single system is considered routine. Samples are generally prepared in 5 mm tube in a suitable deuterated solvent. The experiment information is placed in a queue and the samples in rack. A robot arm that is controlled by the NMR software removes and inserts samples from the magnet when required. While this method of handling NMR samples works very well for traditional chemistry methods in which a chemist produces only a few intermediates and products daily, it does not scale well to the hundreds to thousands of samples that can be produced on a daily basis using combinatorial synthetic methods. Modern spectrometers are quite capable of running several hundred of these types of samples a day, but in addition to the time required to run the experiments there is also the time required to prepare two hundred NMR samples, the time required to clean the tubes after use and the costs for the deuterated solvents and the NMR tubes. In addition this method requires milligram quantities of compound in order to produce satisfactory results. The demand on sample requirements and solvent costs can be diminished by using smaller diameter NMR tubes, however, these tubes are also more difficult to handle.

To overcome these inherent problems with traditional NMR methods, manufacturers have borrowed upon their experiences with LC/NMR to develop "tubeless NMR" accessories. The basic layout of a "tubeless" NMR consists of a standard NMR equipped with a flow cell, also called a direct inject, NMR probe. Sample handling is done typically by a liquid handler, such as the Gilson 215 (Gilson, Inc). Neat or prepared samples are contained in 96 well microtiter plates on the liquid handle and are transferred to the NMR with one of a variety of "plumbing" configurations (42). The spectrometer's computer controls not only the NMR but the liquid handler as well. Samples are aspirated from the plates and pushed into the probe using the syringe on the liquid handler. While the sample is being injected the outlet of the probe is opened to avoid back pressure. Typical sample volumes are 100-120 microliters, with additional solvent and/or air gaps used to push the

sample into the probe cell. Once the NMR data has been collected the sample is aspirated from the probe and either returned to the well or discarded. Prior to injecting the next sample the probe is rinsed with an appropriate solvent. Because of the versatility of the hardware and software, many variations of the process are possible, for example just prior to running each sample the sample could be prepared by dissolving it in an appropriate solvent. Throughput of 12-20 samples per hour can be achieved with these systems. At first that glance, this may not appear to provide a significant of an advantage over traditional robotic sample changers which can run around between 10-15 samples per hour, but once the elimination of sample preparation and clean up are taken into account, there is a significant advantage to "tubeless NMR". The cost of running such a large number of samples can be further reduced by the use of protonated solvent instead of their deuterated counterparts, however the resulting requirement for solvent suppression and the increased complexity of the spectra generally out ways the savings benefit. One of the distinct advantages the high throughput NMR is that the data can provide useful quantitative information. By the simple addition of a known quantity of a stable internal standard it is possible to obtain both purity and yield estimates on every compound in the library.

One limitation to high throughput NMR is that the bottleneck in the process shifts from data acquisition to data interpretation. The NMR vendors are starting to supply some simple tools to assist in this process, but the greatest advances are coming from the companies that have been providing spectral databases and prediction programs. Predicted spectra, either from chemical shift databases or chemical shift rules, are compared to the observed spectra and a confidence value is determined. This data can then be presented to the user in the familiar red and green dot format that allows a chemist to see at a glance the NMR results of a whole plate. There is a distinct advantage to using the chemical shift database method, as the end user can generally augment or even replace the commercial database with a proprietary one, thus providing increased accuracy in the predictions.

High Throughput Fast HPLC/MS Analysis - By far the most common analytical technique in place today for the characterization of parallel synthesis libraries is liquid chromatography/mass spectrometry (LC/MS). In the early 1990's, flow injection analysis mass spectrometry (FIA-MS) was the technique of choice for the medicinal and synthetic chemist to monitor their solution phase synthesis reactions. FIA-MS gained such enormous popularity because of the advent of walk-up or "open access" mass spectrometry (43-45). LC/MS is in essence an extension of FIA-MS. LC/MS has replaced FIA-MS as the standard tool in just about all pharmaceutical laboratories, allowing the chemist to monitor reactions or characterize final products in a fully automated manner. LC/MS offers the important advantage over FIA-MS of more complete characterization of the library product. LC/MS systems are typically outfitted with orthogonal detection methods, such as UV and evaporative light scattering detection (ELSD). LC/UV/ELSD/MS, as a hyphenated technique, enables the chemist to not only rapidly determine the identity of the synthetic product but determine its purity as well. Fast LC/UV and LC/UV/MS methods suitable for combinatorial library analysis have been developed (46,47). Each of these methods incorporated short analytical columns (4.6 mm i.d. x 5 cm) operated at ultra-high flow rates (3-5 mL/min). Both groups showed that chromatographic resolution operating these columns at ultra-high flow rates was not compromised, and provided a rapid quality control assessment tool for the medicinal chemist. More recently introduced are ultra-fast LC/UV/ELSD/MS and LC/UV/MS/MS methods, respectively for applications in high throughput discovery (48,49). It has been demonstrated that with both of these methods relatively high peak capacity is maintained in the chromatographic separation as a function of gradient duration time, as shown in Figure 1. Whereas only a handful of years ago a typical LC/MS run was 30-40

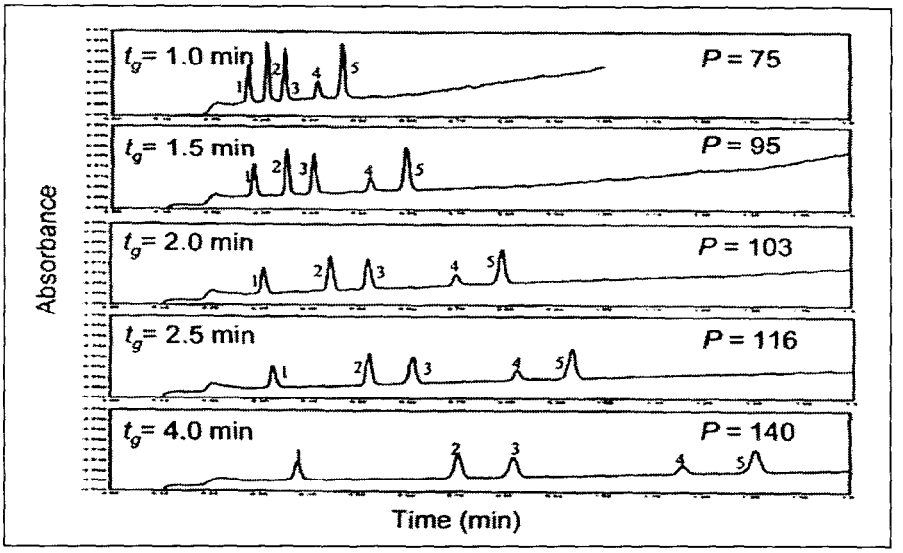


Figure 1

minutes, today, LC/MS run times have been reduced by an order of magnitude, to 2-4 minutes in duration, including re-equilibration.

Parallel Flow Injection and Parallel LC/MS Analysis of Compound Libraries - Serial based LC/MS methods have a practical upper limit on how fast a separation can be carried out in order to accurately characterize compound libraries. In some instances, higher speed analysis tools are required. Two such applications where higher speed analytical tools are required are in the area of characterization of corporate collections (central compound management repositories) and general screening (e.g., gene family) libraries. Corporate collections are typically the first "libraries" in the pharmaceutical arsenal to be screened against a new biological target to identify potential "hits." In the era of mergers and acquisitions, corporate collections are now known to exceed 1,000,000 discrete compounds! The quality of corporate collections and general screening libraries are under continued scrutiny by research teams. Corporate collections are historical in nature and may contain compounds that have been stored as dry powders or thin films for decades. General screening libraries are often purchased from outside sources and their quality is often suspect. One objective taken on by organizations is to eliminate compounds from the corporate collections and general screening libraries known to be impure or known to have degraded upon prolonged storage. However, to screen a 1,000,000 member library by even the ultra-fast serial-based LC/MS technique would take several months to years (and that is assuming 24/7 unattended operation). The only practical way to analyze these compound collections is to purchase numerous systems or to incorporate parallel sampling methodologies. Parallel LC/MS FIA-MS and parallel LC/MS technologies have been introduced in recently and are gaining significant attention (50-54). In a recent presentation an entire 96-well microtiter plate was assessed by parallel FIA-MS in as little as 5 minutes with the potential of generating molecular weight profiles on up to 20,000 compounds in a single day (51).

Parallel LC/MS is now being utilized for routine sample characterization, offering the same advantages over parallel FIA-MS that serial based LC/MS offers over serial based FIA-MS analysis. Figure 2 shows an example of the experimental

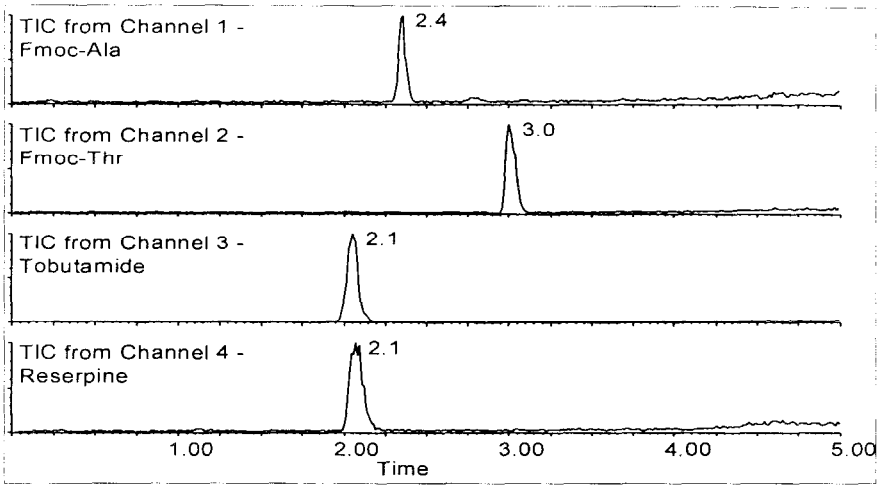


Figure 2

results for a parallel LC/MS analysis of a combinatorial library. In this set-up, a binary HPLC system delivers flow equally to an array of HPLC columns by aid of a simple 4-way tee (splitter). Samples are introduced simultaneously onto the of columns using a multiple probe autosampler (e.g., Gilson eight channel multiple probe autosampler or Leap multiple probe autosampler). Samples are analyzed by gradient elution chromatography and detected using an indexed electrospray ionization source, enabling the effluents from the array of columns to be readily deconvoluted. Innovations in parallel sampling devices, such as the MUX ion source and the rapid switching valve sampling device are enabling much higher throughput analyses to be achieved (53, 55). Recently, members of this laboratory have successfully performed parallel LC/UV/ELSD/MS analysis on a fast scanning quadrupole mass spectrometer in a high throughput drug discovery setting (56). An example of a parallel 8-column LC/UV/MUX/MS quadrupole analysis is shown in Figure 3.

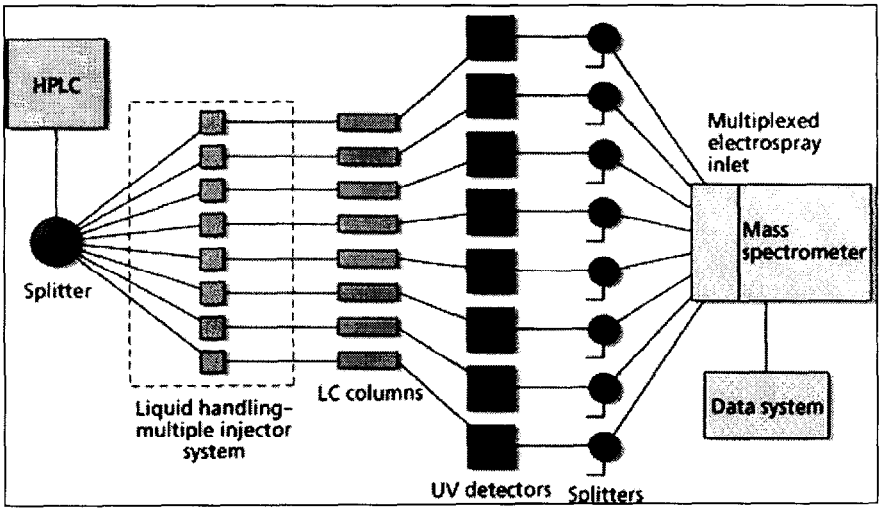


Figure 3

Underpinning these high throughput techniques is automated sample acquisition and automated data processing. It is of paramount importance to have automated measurements, otherwise these faster acquisition methods are rendered useless. Although most mass spectrometry vendor software packages provide excellent plate viewing tools, customized applications (using VB and applescripts) greatly facilitate automated data interpretation. In particular, a combination of applescripts and visual basic applications have been developed to facilitate compound identification and determination of purity based on UV and/or ELSD response.

HIGH THROUGHPUT PURIFICATION OF PARALLEL SYNTHESIS LIBRARIES

Parallel Flash Chromatography for Intermediate Purification - Flash chromatography typically involves the use of bare silica resin dry or slurry packed into large diameter cartridges (typically teflon or glass). Flash chromatography is not new to the medicinal chemist. In fact, the technique has been in wide spread use for large-scale intermediate purification for decades. Alas, flash chromatography is not immune to innovations. Similar to advances in parallel HPLC and parallel LC/MS, flash chromatography is being impacted by the parallel synthesis revolution. As increasing numbers chemists are adopting high throughput organic synthesis as part of their repertoire the reliance on new tools to facilitate their reaction work-up has increased. Larger numbers and larger quantities of scaffolds (templates) are required to support the demands of parallel synthesis. In the past few years, a number of semi-automated and fully automated flash chromatography workstations have been introduced. For a list of manufacturers involved in flash product development, one need go no further than the 2001 list of vendors at the Pittsburgh Conference. The most recently introduced flash chromatography workstations enable compounds to be purified in parallel using time-based fractionation. Alternatively, automated flash chromatographic workup of reaction intermediates is analogous to automated HPLC final product purification, where samples are robotically loaded onto the silica cartridges and fraction collection is initiated based on UV response.

UV and Mass Directed Fraction Collection of Final Products - Purification is becoming increasingly important in the high throughput discovery laboratory. What is absolutely clear is that neither the medicinal chemist nor the biologist is interested in compromising the integrity of the biological assay results by screening impure compounds. In addition, what both the chemist and biologist desire are qualified hits that may be further interrogated as potential lead candidates. The dogma of SPOS literature is that solid phase synthesis leads to pure products because the reaction is carried out on solid support and excess reagents can be used to drive the reaction towards completion and can be removed readily. Experiences by all groups incorporating solid phase organic synthesis have realized that the purity of the final product is not guaranteed, even when significant development effort has gone into optimizing the solid phase reaction chemistry. Another reason final product purification has become so important is that researches have found that the scope of reactions amenable to solid phase synthesis are limited relative to solution phase synthesis (9). And clearly, in solution phase parallel synthesis, purification is a must.

Solid-phase extraction, ion exchange chromatography, crystallization, common techniques in the medicinal chemistry laboratory have been used effectively for compound purification. However, by far the two most popular and powerful techniques for purifying parallel synthesis-derived compound libraries are via automated RP-HPLC and RP-HPLC/MS. Fast semi-preparative and preparative chromatography methods have become the norm for high throughput purification (46). Analogous to fast LC and LC/MS, short columns, typically 20 mm i.d. x 5 cm or

30 mm x 7.5 cm columns, operated at 35-50 mL/min are used. Crude samples (50-100 mg) are loaded directly on column and separated by running fast generic gradients, typically 10-100% acetonitrile (containing 0.05% - 0.1% TFA) in as little as 5-10 minutes. Fraction collection is achieved based on UV or other analog signal input (e.g., ELSD) or based on a target mass reaching a specific threshold value (57, 47). Mass-directed fraction collection offers the advantage of permitting only the target compound (whose mass is known prior to purification) to be isolated. UV-directed fraction collection typically involves the collection of multiple fractions per chromatographic run. A recent review discusses some of the advantages and limitations of the two techniques for purifying compound libraries (58).

DRUG SCREENING AND LEAD GENERATON

Screening by NMR - As a result of the application of combinatorial chemistry in drug discovery a much larger number of compounds must be screened for biological activity. High throughput biological screening has become essential in the discovery process. In order to achieve the maximum throughput, compounds have been screened as mixtures instead of as discrete compounds, and when a "hit" is identified, the mixture must be "deconvoluted" in order to identify the active component. Frequently when the individual compounds are re-tested none of them show any significant activity and the original activity of the mixture is assumed to arise from the combination of several weak interactions. NMR has been explored as a complementary method of screening for activity of mixtures and discrete compounds. Many of the quantifiable properties that are observed in NMR spectra are directly related to the size and shape of the molecule in solution. The relaxation times (T_1 and T_2), the nuclear Overhauser effect (nOe), and the diffusion rate (D) are strongly dependant on the apparent size of the molecule, and each of these can be used in drug screening by NMR. When a small molecule (ligand) binds to a much larger molecule (receptor) then the ligand behaves as if it where as large as the receptor. This greatly affects the properties mentioned above: the NMR line width of the ligand gets broader, the intramolecular nOe's go from positive to negative, and the diffusion rate is much slower. The advantage these NMR techniques have over more traditional screening methods is that NMR can distinguish which species in a mixture is binding and obtain an estimate of the dissociation constant (K_d). The nOe method as been demonstrated to be able to identify the single oligosaccharide, in a mixture of 15, that was binding to *A. aurantia agglutinin* (59).

By far, the majority of the work has focused on the use of diffusion editing of NMR signals (60-63). This method relies upon the difference in the diffusion rates of small molecules, ligands, versus those of larger molecules, receptors. The small ligands diffuse through the solution much faster than the larger receptors. A pulsed field gradient (PFG) experiment is constructed that filters out the signals of the faster moving ligand molecules and shows only those for the slower moving molecules. When a ligand binds to the receptor it spends part of it's time behaving as a large molecule and it's signals reappear in the NMR spectrum. By running the experiment at various gradient strengths it is also possible to calculate the disassociation constant.

Two-dimensional NMR has been demonstrated as a useful method of drug screening (64). This method relies upon detecting the changes in the $^{15}N/^{1}H$ amide chemical shifts of the ^{15}N enriched receptor in a heteronuclear single quantum correlated (HSQC) NMR spectra when a ligand binds to it. To achieve the throughput levels desired, mixtures of 100 compounds must be screened in a single experiment. As a consequence of screening this many compounds in one experiment the concentration of each compound as well as the receptor must be reduced. Lowering the receptor concentration results in a significant increase in the

amount of time required to acquire data that has adequate signal-to-noise (S/N) levels. To overcome this problem cryogenically cooled probes and preamplifiers were used that have superior S/N characteristics. Recently this NMR screening method was used in a fully automated process to screen over 300 compounds (65).

Each of these methods have their own advantages and disadvantages. With the diffusion method it is not required to deconvolute a mixture to determine the active compound and an estimate of the dissociation constant for each compound in the mixture can be obtained . In the 2D HSQC method described by Fesik, the mixture must be deconvoluted if any binding is detected. This makes the Fesik method inappropriate for the use on focused libraries in which some level of binding is expected for most compounds, but ideal for leads discovery screening. In both methods the presence of a very strong binding compound will mask the presence of moderate to weak binders that may be of interest. While both methods require the use of significant quantities of receptor, the diffusion method uses unlabeled receptor while the 2D HSQC method requires ^{15}N labeled receptor.

Lead Generation by NMR - While leads generation and drug screening are closely related and use similar methodology the goals are different enough to warrant separate treatment. The goals of leads generation and optimization are to first identify an active pharmacophore and to optimize this activity using structure-activity-relationships (SAR). The initial lead discovery has generally been accomplished by screening large diverse libraries of compounds, then the lead is optimized by synthesizing a large number of related compounds. Recently two NMR methods of leads generation and optimization have been described that attempt to do this using smaller initial libraries (66,67).

The "SAR by NMR"™ method uses the 2D HSQC experiment describe above to discover and optimize leads (67). The essence of this method is to identify two small molecules that bind weakly to different, but proximal, sites of the receptor. After identifying the first weak binder, a second binder is screened for in the presence of a saturating amount of the first binder. Then by connecting these two components together with a suitable linker a potent binder may be generated. This technique has been demonstrated with a variety of target molecules (67-72). The location of binding to the receptor can be determined either from analysis of the chemical shift perturbations in the NMR data to determine exactly which amides in the receptor are perturbed, this requires that the NMR assignments of the receptor be known, or by competitive binding studies with known binders. Recently this work has been extended to determining the exact binding site and the orientation of the binding ligand (73). After first discovering a suitable ligand, minor structural changes are made to it and the effect this has on the amide chemical shifts are determined. Structural changes in the ligand that have little or no affect on the observed chemical shifts are assumed to have taken place on regions of the ligand that are not part of the binding interaction. Once the binding and non-binding regions of the ligand are determined it is possible to optimize the binding characteristics by modifications in the binding regions of the ligand, while optimizing the physical properties (solubility, protein binding, etc.) by modifications in the non-binding regions of the ligand.

An alternate method of leads generation is the SHAPES strategy described by Moore (66,74) The key objective in this method is to reduce the initial screening library down to a relatively small library of compounds that consists of simple molecules that have the frameworks mostly commonly found in successful drugs. This library of compounds is then screened against a receptor using transfer nuclear Overhauser enhancement (tnOe). The dissociation constants, K_d, for the hits from the SHAPES screening are then determined by diffusion NMR experiments as describe above. Follow up focused libraries based on the best SHAPES hits can

then be screened using traditional or NMR based screening methods. Using this method the authors achieved a ten fold increase in hit rates over traditional high throughput methods.

These two leads generation methods are very complementary to each other. The "SAR by NMR"™ method is based on observing the NMR signals of the receptor, which requires that the receptor be amenable to NMR studies (it must not be too large and it must be available enriched in ^{15}N). The SHAPES method which relies on observing affects in the ligand works best on larger receptors, MW > 60 kDa. A SHAPES library of compounds would make an good starting library for the "SAR by NMR"™ method of leads generation.

Drug Screening by Mass Spectrometry - Analogous to NMR, mass spectrometry has been shown to a very powerful technique for the screening of both synthetic and natural products combinatorial libraries for biological activity. One caveat to the mass spectrometric-based screening strategy is that in the screening of synthetic combinatorial libraries, it is important that molecular weight redundancy be kept to a minimum so that molecular weight information alone can be used to identify the active component(s).

The most common affinity screening method involves pre-incubation of a combinatorial library with soluble receptor under optimized binding conditions (75). Following incubation, the mixture is applied to a size exclusion chromatographic column or to a size exclusion spin column. On column, bound ligands pass freely through the sizing column unretained while the unbound ligand are retained slightly. Protein-bound ligands are then identified by rapid desalting on a C18 or C18-like RP-HPLC cartridge and analyzed by mass spectrometry. In several experiments a high affinity (sub-micromolar) binder was identified from a 700+ member combinatorial peptide library mixture. Recently several other of the more common screening techniques were reviewed, including affinity chromatography-mass spectrometry, Pulsed Ultrafiltration-mass spectrometry, immunoaffinilty ultrafiltration-mass spectrometry, hydrophilic interaction chromatography-mass spectrometry (81), affinity capillary electrophoresis-mass spectrometry, and frontal affinity chromatography-mass spectrometry to name only a few (76-83).

Substrate optimization offers a second application of mass spectrometry in the area of high throughput screening of combinatorial libraries. Combinatorial library substrate optimization involves the pre-incubation of a target enzyme with a library of potential substrates. Typically, mass spectrometry either by itself or in combination with HPLC is used to assess both the amount of parent remaining and product formed over a time-course incubation and to determine, from their time-course incubations, kinetics of the enzyme:substrate interaction, reported as a semi-quantitative relative k_{cat}/K_m value. The objectives of such an assay are to develop novel, highly active substrates that provide the following potential benefits. The first being a more sensitive substrate, allowing for better screening detection sensitivity and therefore, the requirement for less enzyme in the high throughput in vitro screening assay and the potential for inhibitor design based on the structure-activity relationships that can be derived from the substrate optimization experiments. Early applications of LC/MS to the design and optimization of substrates for gelatinase, HIV protease and $pp60^{c\text{-}src}$ tyrosine kinase were reported (84-86). In these studies, relatively small combinatorial peptide libraries were used, varying the peptides at a single position (e.g., in the case of the enzyme targets, on either side of the cleavage site, denoted P_1 and P_1'). In a recent study a 1000-member combinatorial (non-natural amino acid containing peptide) library, synthesized as 10 discrete pools of 100 compounds each was screened for substrate activity against TNF convertase, probing substrate specificity while simultaneously varying at the P_1' and P_2' cleavage

sites (87). In this method, the cleavage product passes freely through the affinity support where as unmodified peptides are retained irreversibly on the affinity support, greatly facilitating the identification of optimal substrates. This approach led to a 10-20 fold improvement in substrate activity and provided insights into both substrate and inhibitor design.

HIGH THROUGHPUT ADME MEASUREMENTS BY MASS SPECTROMETRY

Advances in high throughput analysis, purification and biological screening are effectively addressing the analytical bottleneck in combinatorial chemistry. In a highly cited publication, the most pressing bottleneck in drug discovery is not high throughput synthesis and biological screening but in the design and synthesis of compounds with favorable Absorption, Distribution, Metabolism and Excretion (ADME) properties. Compound attrition is reported to be attributable principally to poor pharmacokinetics (historically accounting for almost 40% of development attrition) (88). *In Vitro* ADME profiling of compound libraries, therefore, has been investigated as a means for reducing compound attrition as compounds progress towards nomination status.

Traditionally, evaluation of the physicochemical (e.g., log P, solubility, pKa) and biochemical (e.g., cytochrome P450 metabolic stability, inhibition, plasma protein binding, cell permeability, etc.) properties of compounds have been relegated to relatively late stages of discovery programs. Furthermore, these experiments have been performed strictly within the purview of dedicated drug metabolism and pharmacokinetics (DMPK) groups. Automated mass spectrometric data acquisition and post-data acquisition processing tools have become increasingly available and have enabled discovery teams to begin evaluating compounds for their ADME and physicochemical properties at earlier and earlier stages of discovery programs. The hope and promise of this approach has been to use this information to guide synthetic strategy and to increase the probability that the compound(s) nominated for pre-clinical development will be more likely to have suitable "drug-like" properties.

In vitro models have been developed and widely incorporated by pharmaceutical companies to provide the discovery teams within insights into compound metabolism, biological activity, and bioavailability.

Microsomal Stability Screening by Mass Spectrometry - Human liver microsomes, S9 fractions and hepatocytes have all been widely incorporated to model intrinsic clearance mechanisms, that is, as a way to assess a compound's likely metabolic fate after administration. Mass spectrometry has become an increasingly important tool for these biochemical assays because of the inherent sensitivity and selectivity of the technique. In an analogous manner to screening combinatorial libraries for biological activity, mass spectrometry is being used for high throughput metabolism screens. Typically, target compound (1-5 μM) is incubated with microsomes (1 mg/mL protein concentration) in the presence and absence of NADPH, a co-factor required for enzymatic activation, over a defined time-course period. Following quenching of the reaction with acetonitrile or acid, the sample is analyzed by fast chromatography-mass spectrometry to determine the amount of substrate (test compound) remaining over the course of the incubation period. A recently presented method of increasing sample throughput consists of using a multi-column parallel LC/MS for microsomal stability determinations that greatly reduced analysis and post data acquisition processing time, permitting up to 4 plates of compounds to be profiled over 4 time-points in triplicate in a single day (89).

Cassette Dosing - An alternative approach to increasing the throughput of in vitro ADME analysis is to perform the technique of cassette dosing for in vivo

pharmacokinetic studies (90). Although cassette dosing has not been used extensively in early cytochrome P450 metabolism studies, owing to the fact that these metabolizing enzymes are characterized by promiscuous binding sites, the technique has found value in cell permeability screens. The colorectal carcinoma cell line (Caco-2) is an industry-accepted standard for assessing compound permeability and has been found to be reasonably well correlated with human absorption/bioavailability. In the Caco-2 assay, compounds are administered to the apical (top) layer of the Caco-2 chamber and are transported across the membrane into a basolateral compartment by a) passive, b) active or c) paracellular transort mechanisms. Recently presented was a ultra-high throughput analysis of a 300,000+ member combinatorial library, analyzing pools of 2500 compounds each to identify highly permeable compounds (91).

Metabolite Identification by LC/NMR/MS - Both microsomes and Caco-2 cells contain the cellular machinery to carry out metabolizing (enzymatic) reactions. S-9 fractions and hepatocytes are comprised of both phase I and phase II cytochrome P450 metabolizing enzymes, which participate in the conversion of substrate into water soluble conjugates which are readily eliminated in urine. Knowledge of the sites of metabolism are critical to pre-clinical development. A technique that is gaining increasing attention is HPLC/NMR/MS. Numerous reports have surfaced recently showing the power of this hybrid technique for metabolite identification and structural elucidation studies.

CONCLUSIONS

Next Generation NMR - Further developments in hardware, software, and experimental procedures will greatly increase the role that NMR plays in drug discovery. Applications of cryogenically cooled probes in this area are just now starting to be published (64). The high sensitivity of these probes translates into significant decrease in experiment time, sample requirement, or both. Recent work in the area of multi-sample NMR probes shows promise of providing increased capacity in high throughput NMR (92,93). These probes allow data to be collected on several samples simultaneously or on one sample while the another sample is being changed.

By far the greatest changes in the future will be in software tools that automate the analysis of all the data collected in these NMR experiments and integrates this information into computational databases. Screening by NMR will only become practical on a large scale if software for the automated analysis and reporting tools are available.

Next Generation Mass Spectrometry - The landscape of how mass spectrometry is being applied in combinatorial chemistry is clear. Synthetic throughput achievable by the medicinal chemistry has now rendered LC/MS as one of the "rate-limiting" steps in the discovery process. Although advances in sample analysis throughput have been clearly demonstrated, there is a limit as to how fast a separation and analysis can be achieved while maintaining good separation efficiency and quality analysis. The trend recently has been towards implementing parallel analysis methods as a means for keeping pace with the parallel synthesis revolution. Recently, novel interfaces enabling up to 4 to 8 samples to be processed in parallel, increasing the sample analysis throughput dramatically over conventional, serial-based LC/MS analyses. The idea of parallel separation and analysis is no longer a concept but has captured the imagination of the mass spectrometry community. The future of parallel analysis and purification is clear and it is indeed bright.

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SECTION VII. TRENDS AND PERSPECTIVES

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Chapter 28. To Market, To Market - 2000

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Pfizer Global Research & Development
Fresnes, France

With a total of 35 newly launched therapeutic chemical and biological entities, last year (2000) was equally productive as 1999 (1-5). In 2000, the US and Europe were the most frequent choices for the first introduction of new active entities with 12 and 11 respectively. There were 7 NCEs launched in Japan last year. In terms of originators, Europe held the top position with 14 original NCEs generated, followed by the US and Japan with 11 and 6, respectively. The companies Abbott, Pfizer, Pharmacia, Tanabe Seiyaku and UCB all introduced 2 original drugs. Among these companies, Pfizer and Pharmacia not only marketed the corresponding pharmacological agents but also discovered them. It should also be noted that Orion was also responsible for 2 NCEs introduced last year.

Interestingly, the most represented therapeutic area last year was gastroenterology with 5 new agents, mainly antisecretory and antiulcer agents. The (*S*)-enantiomer of omeprazole, Nexium (esomeprazole magnesium), is the first proton pump inhibitor to be developed as a pure optical isomer and provides superior acid control compared to the racemic mixture. Diotul (dosmalfate), an aluminium complex of diosmin, has demonstrated very poor absorption and, consequently, a low incidence of side-effects. Azuloxa (egualen sodium) is a water-soluble analog of guaiazulene, a natural product with anti-ulcer properties. Stogar (lafutidine) acts as a long-lasting H_2 antagonist with additional gastroprotective action. Lotronex (alosetron), a 5-HT_3 antagonist from the " setron " class was the first introduced oral treatment against diarrhea-predominant IBS in women, but it was withdrawn from the US market only nine months after its first launch due to the incidence of serious cases of ischemic colitis.

The cardiovascular field was also well represented with 4 NCEs. Tikosyn (dofetilide), a seemingly unique class III antiarrhythmic agent, has a very specific mechanism of action on a single potassium channel without any effect on the conduction system. The synthetic peptide modeled on hirudin, Angiomax (bivalirudine), a direct inhibitor of thrombin, was more efficacious than heparin in coagulation disorders. Simdax (levosimendan) is a myofilament Ca^{2+} sensitizer that acts *via* a unique mechanism and has a considerable potential in the treatment of heart failure. Zantipres (zofenopril calcium) is a second-generation ACE inhibitor with concomitant cardioprotective effects.

There were 4 NCEs introduced in the CNS field last year. Zeldox (ziprasidone hydrochloride) was the sixth atypical antipsychotic launched for the treatment of schizophrenia, with a complex binding profile for 5-HT and dopaminergic receptors. Precedex (dexmedetomidine hydrochloride), a full α_2-agonist which is much more selective than clonidine, is a novel agent for sedation during intensive surgery and allows patients to rapidly recover. Ceredist (taltirelin) was the first orally-active drug marketed for the improvement of ataxia due to cerebrospinal degeneration. Keppra (levetiracetam), a second-generation analog of piracetam and aniracetam, provides a useful alternative to conventional anticonvulsants for the treatment of epilepsy.

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In the area of antiallergic respiratory drugs, 2 NCEs were marketed against allergic rhinitis : Baynas (ramatroban), a potent antagonist of prostaglandin PGD_2/PGH_2 and thromboxane TxA_2 receptors and Talion (betotastine besilate), the seventh marketed non-sedating histamine H_1 antagonist exhibiting diverse antiinflammatory effects.

The class of antiinfectives was modestly represented last year. The only antiviral agent was Kaletra (lopinavir), a new HIV protease inhibitor launched in combination with small doses of ritonavir, a marketed HIV protease inhibitor. This original formulation uses the complementarity of pharmacokinetic profiles to accentuate the antiviral properties of lopinavir. Zyvox (linezolid), the first of a new class of antibacterial oxazolidin-2-ones, was introduced for the treatment of infections caused by serious Gram-positive pathogens, complementing the available arsenal against community-acquired infections. Zefnaft (liranaftate), a new topical antifungal for the treatment of dermatophycoses, was found to be more active than other thiocarbamates of this class. In addition, 2 antimalarial drugs, arteether and bulaquine, were launched last year.

In the anticancer area, Targretin (bexarotene) was the first launched retinoid X receptor agonist selective versus retinoid A receptors, that has demonstrated less toxicity than the broad spectrum or RAR-selective retinoids. Aromasin (exemestane) is a new synthetic steroid belonging to the third-generation of orally active aromatase inhibitors which have displayed potent activity against estrogen-dependent tumors and postmenopausal breast cancer. Mylotarg (gemtuzumab ozogamicin) was the only new biological entity launched last year and was also the first antibody-targeted antineoplastic agent for the treatment of patients with acute myeloid leukemia (AML).

In the area of metabolism regulators, 2 NCEs were introduced. Welchol (colesevelam hydrochloride), a non-absorbable hydrogel launched for the treatment of LDL hypercholesterolemia, exhibited significantly less side-effects than other established bile acid sequestrants. Zometa (zoledronate disodium), a new third-generation bisphosphonate, was shown to be superior to pamidronate in the treatment of tumor-induced hypercalcemia.

Prezios/Oxarol (maxacalcitol) was the third synthetic vitamin D analog marketed for the management of secondary hyperparathyroidism in patients with chronic renal failure, with only minor effects on calcium and phosphate metabolism.

Almogran (almotriptan) was the fifth triptan to be introduced for the treatment of acute attacks of migraine, exhibiting the best oral bioavailability in this class.

In 2000, tzo imaging agents, OptiMark (gadoversetamide) and Datscan (ioflupane), were launched for magnetic resonance imaging and for the diagnosis of Parkinson's disease respectively.

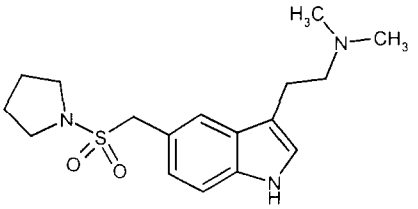
Finally, nine other biological entities were launched in 2000, although they are not considered as NBEs:

- Natural products or vaccines such as Hexavac® from Aventis (a combined hexavalent vaccine against diphteria, tetanus, pertussis, polyomyelitis, hepatitis B and haemophilus influenzae type b), M-Vax™ from Avax (an autologous cancer vaccine for melanoma, custom-made from the patient's own tumor cells) and Group C meningococcal conjugate vaccines (Neis Vac-C® from Baxter and Menjugate® from Chiron);
- new formulation or new modification of already existing products such as Insulin glargine from Aventis (Lantus®, a long-acting, once-daily insulin analog), peginterferon alfa-2b from Schering Plough (PegIntron®, the first pegylated

interferon with sustained delivery used for therapy of chronic viral hepatitis C) and Tenecteplase from Genentech (TNKase®, a modified form of human tissue plasminogen activator with improved half-life used as thrombolytic agent for treatment of acute myocardial infarction);

- existing products launched for new indications such as pneumococcal 7-valent conjugate vaccine from Wyeth-Ayerst (Prevnar® launched for active immunization of infants and toddlers against Streptococcus pneumoniae - serotypes : 4, 6B, 9V, 14, 18C, 19F and 23F-) and interferon gamma-1b from Genentech (Actimmune® indicated for delaying the time to disease progression in patients with severe malignant osteopetrosis).

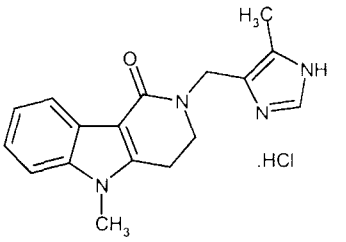
Almotriptan (antimigraine) (6-9)



Country of Origin : Spain
Originator : Almirall Prodesfarma
First Introduction : Spain
Introduced by : Almirall Prodesfarma
Trade Name : Almogran
CAS Registry No : 154323-57-6
Molecular Weight : 335.47

Almotriptan was first marketed in Spain as a new medicine against acute attacks of migraine. It is the fifth agent belonging to the "triptan" class to be launched after sumatriptan, naratriptan, zolmitriptan and rizatriptan. This close structural analog of sumatriptan can be prepared in six steps from 4-nitrobenzylsulfonyl chloride with a Fischer indole synthesis as the key step. Almotriptan acts as a dual 5-HT_{1D}/$_{1B}$ agonist with a 35 to 51-fold selectivity versus 5-HT_{1A} and 5-HT_7 receptors respectively as well as having insignificant affinity for the most relevant nonserotonergic receptors (K_i > 1μM). Its agonistic effect on 5-HT_{1D} receptors of trigeminal sensory neurons turns off neurogenic inflammation by inhibiting the release of neuropeptides such as calcitonin gene-related peptide, neurokinin A and substance P. Concomitantly, its action on the 5-HT_{1B} receptors in meningeal arteries relieves the vasodilatation of these vessels associated with migraine attacks. Almotriptan causes selective concentration-dependent vasoconstriction of human meningeal and temporal arteries (with EC_{50} of 0.03 and 0.7 μM) compared to basilar (EC_{50} = 3.5 μM) and pulmonary arteries (EC_{50} > 10 μM) or rabbit mesenteric and renal arteries (EC_{50} > 100 μM). Although it is predominantly cleared by the kidneys as unchanged drug (45%) or transformed into inactive metabolites by monoamine oxidase A (MAO-A) and CYP3A4 enzymes in the liver, almotriptan has the highest oral bioavailability (70%) of the triptans and has a half-life of 3.5 h. The therapeutic dose of 12.5 mg is well tolerated, shows a rapid onset of action (30 min) and low recurrence rate compared to sumatriptan.

Alosetron hydrochloride (irritable bowel syndrome) (10-15)

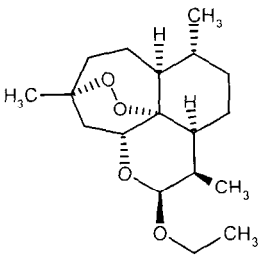


Country of Origin : UK
Originator : Glaxo-Wellcome
First Introduction : US
Introduced by : Glaxo-Wellcome
Trade Name : Lotronex
CAS Registry No : 132414-02-9
Molecular Weight : 330.86

Alosetron was launched in March 2000 in the US as a new oral treatment for diarrhea-predominant irritable bowel syndrome in women. This structurally-related analog of ondansetron is prepared by alkylation of the pyrido[4,3-b]indol-1-one skeleton with the appropriate trityl-protected chloromethylimidazole. Although its mechanism of action is not fully understood, alosetron is known to be a potent and selective 5-HT_3 antagonist with a superior pharmacodynamic profile than its predecessor ondansetron. Besides being present in the central nervous system, 5-HT_3 receptors are located on neurons of both enteric and sensory nervous systems. Alosetron may act on several of these sites leading to the regulation of intestinal secretion, gastrointestinal contractility and gastric emptying. Clinically, it has been found that a twice-daily dose of 1mg of alosetron not only improves bowel function, stool frequency and stool consistency but also affects the pain severity and sense of urgency in IBS. Despite its short half-life (1.5h) and extensive metabolism (at least 12 metabolites have been identified in urine), alosetron shows a long duration (10h) of inhibition of the serotonin-induced skin flare response in man. Due to reported cases of constipation and ischemic colitis as well as rare fatalities of patients under treatment with Lotronex, Glaxo-Wellcome withdrew the drug from the US market in November and is currently running further clinical trials.

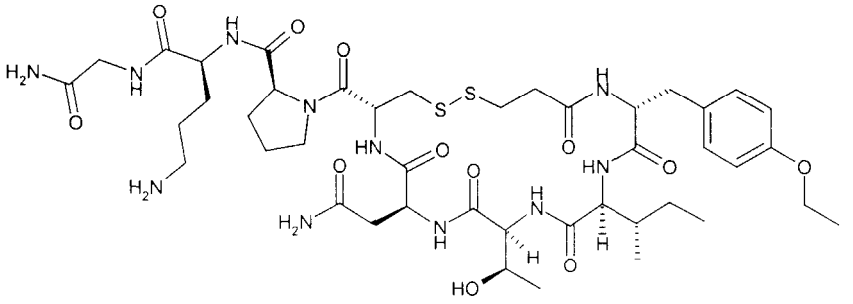
Arteether (antimalarial) (16-18)

Country of Origin : India
Originator : Central Drug Research Institute
First Introduction : Netherlands
Introduced by : Artecef BV
Trade Name : Artemotil
CAS Registry No : 075887-54-6
Molecular Weight : 312.19



Arteether reached the market last year in the Netherlands as a solution in sesame oil, administered by i.m. injection, for the treatment of severe malaria infections in children and adolescents. Artheether, a sesquiterpene acetal with an endoperoxide bridge, is an ether derivative of the naturally occuring compound artemisinin, the active component of Chinese herbal remedies. As the other structural analogs of artemisinin, such as artesunate or artemether, it acts rapidly against *Plasmodium*, the parasite responsible for the disease, during the early blood stage of its development. It also exhibits a gametocytocidal activity against *Plasmodium falciparum*, reducing its potential for transmission. Because the only controlled clinical studies had been performed in children and adolescents, this new antimalarial drug was only approved for young patients. Further studies in adults treated with 150 mg i.m. artemotil, once daily for three consecutive days, indicated that the drug was efficient, rapidly acting (parasite clearance time meanly 38 h) and well tolerated. A new promising achievement in the regression of malaria is the combination of artemisinin derivatives with other long-lived antimalarials as mefloquine or pyrimethamine/sulfa.

Atosiban (preterm labor) (19-22)



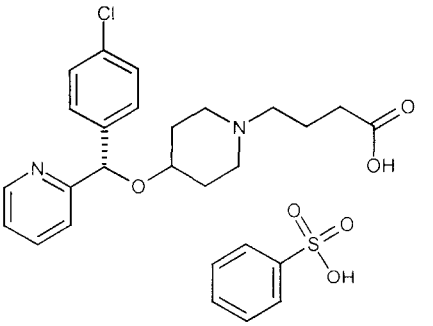
Country of Origin : Sweden
Originator : Ferring AB
First Introduction : UK
Introduced by : Ferring AB

Trade Name : Tractocile, Antocin
CAS Registry No : 090779-69-4
Molecular Weight : 994.19

Atosiban was introduced in the UK as an injectable inhibitor of preterm labor, a major cause of infant morbidity and mortality. This peptidic oxytocin analog is an antagonist of the vasopressin V_{1a} receptor and of the oxytocin receptor which is found in dramatically increased concentration in the uterine myometrium of pregnant women near term. It competitively inhibited contractions in the pregnant guinea pig uterus induced by oxytocin and vasopressin. In a multicenter, double-blind, placebo-controlled trial, treatment with atosiban caused pregnancy prolongation for up to 7 days in women with more than 28 weeks of gestation. In a comparative clinical trial, atosiban showed a comparable tocolytic action (uterine relaxant) to ritodrine but the former was significantly better tolerated, especially with regards to maternal cardiovascular side effects. In healthy volunteers, plasma levels of atosiban decreased bi-exponentially with an initial and a terminal half-life of 21 min and 1.7 h respectively.

Betotastine besilate (antiallergic) (23-25)

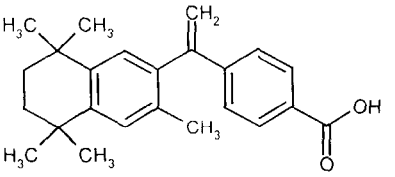
Country of Origin : Japan
Originator : UBE
First Introduction : Japan
Introduced by : UBE and Tanabe Seiyaku
Trade Name : Talion
CAS Registry No : 190786-44-8
Molecular Weight : 547.06



Betotastine was introduced in Japan for the treatment of allergic rhinitis. This structurally-related derivative of chlorpheniramine and ebastine is prepared by condensation of optically-resolved 4-[1-(4-chlorophenyl)-1-(2-pyridyl)-methoxy]piperidine with ethyl 4-bromobutyrate followed by ester hydrolysis. Betotastine is the seventh marketed non-sedating histamine H_1 antagonist. Its very low sedative side effect is due to very poor penetration in the central nervous system. Besides its potent and long-acting activity in models of allergic rhinitis, betotastine was also shown to act as a PAF antagonist

and inhibit LTD_4 in tracheal smooth muscle and ileum, IL-5 production by human peripheral blood mononuclear cells as well as eosinophil infiltration in the airway and peripheral blood. As a consequence, it is currently being developed against other allergic and respiratory disorders.

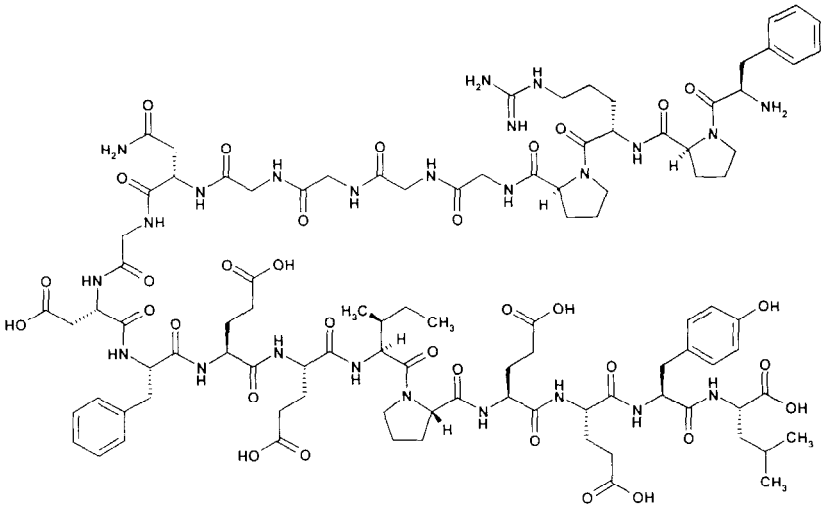
Bexarotene (anticancer) (26-29)



Country of Origin : US
Originator : Ligand
First Introduction : US
Introduced by : Ligand
Trade Name : Targretin
CAS Registry No : 153559-49-0
Molecular Weight : 348.49

Bexarotene was launched in the US for the treatment of manifestations of cutaneous T-cell lymphoma in patients who are refractory to at least one prior systemic therapy. The four step synthesis of bexarotene involves a double Friedel-Craft alkylation of toluene with 2,5-dichloro-2,5-dimethylhexane followed by acylation with monomethylterephthalic acid chloride, then Wittig methylidenation. Bexarotene is the first retinoid X receptor (RXR) agonist to be selective versus retinoid A receptors (RAR). Its activation of the three RXRα, β, γ isoforms induces cell differentiation and apoptosis and inhibits cell proliferation in several models of cancer. In phase II/III clinical trials, 48% of patients with refractory or persistent early-stage cutaneous T-cell lymphoma achieved a complete or partial response when treated with 300 mg/m^2/day of bexarotene. It was shown in phase I trials that this second-generation retinoid was substantially less toxic than the broad-spectrum or RAR-selective retinoids.

Bivalirudin (antithrombotic) (30-37)



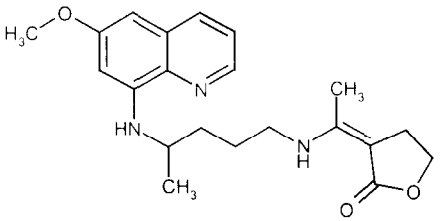
Country of Origin : US
Originator : Biogen
First Introduction : New Zealand
Introduced by : The Medicines Company

Trade Name : Angiomax
CAS Registry No : 128270-60-0
Molecular Weight : 2179.3

Bivalirudin was launched in New Zealand as an anticoagulant for i.v. treatment of patients with unstable angina undergoing percutaneous transluminal coronary angioplasty. Bivalirubin is a synthetic 20 amino acid peptide rationally modeled on hirudin (residues 53-64), the most potent and specific naturally-occuring known inhibitor of thrombin, the enzyme that plays a key role in hemostasis and blood clot formation. This peptide is a direct thrombin inhibitor that maintains the unique bivalent binding properties of hirudin to the catalytic site and to the fibrin-recognition exosite of the enzyme, so acting directly on thrombin with high affinity and specificity. *In vitro* studies demonstrated that alpha- and zeta-thrombins, both with the higher fibrinogen-procoagulant activities, were inhibited. In rats receiving high doses of bivalirudin, the platelet deposition in carotide was reduced by 63% compared to controls. The results of clinical studies, conducted only in patients receiving concomitant aspirin, suggested that the use of bivalirudin was more efficacious and more predictable than unfractionated heparin, with fewer bleeding complications. Despite some unresolved developmental issues, the attractive properties of this novel agent could make it a useful alternative to heparin in a variety of coagulation disorders.

Bulaquine (antimalarial) (38-40)

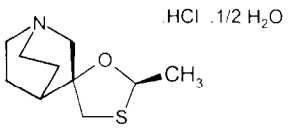
Country of Origin : India
Originator : CDRI
First Introduction : India
Introduced by : CDRI
Trade Name : Aablaquin
CAS Registry No : 079781-00-3
Molecular Weight : 369.47



Bulaquine is a derivative of primaquine that was introduced in India in combination with chloroquine as a treatment against malaria. The compound is prepared by condensation of primaquine with 3-acetyl-γ-butyrolactone in presence of a catalytic amount of base. Bulaquine kills the latent tissue stage (hypnozoites) of the parasite *Plasmodium vivax* accumulated in the liver and responsible for relapses. Its efficient anti-relapse activity was demonstrated against sporozoite induced *Plasmodium cynomolgi* infection in rhesus monkey. Bulaquine is safer than primaquine, the preceding candidate against malaria relapse. Although it is stable at pH 7-8, bulaquine is hydrolyzed to primaquine under acidic conditions.

Cevimeline hydrochloride (anti-xerostomia) (41-44)

Country of Origin : Israel
Originator : Israel Institute for Biological Research/ Snow Brand
First Introduction : US
Introduced by : Daiichi Pharmaceutical
Trade Name : Evoxac
CAS Registry No : 153504-70-2
Molecular Weight : 244.78

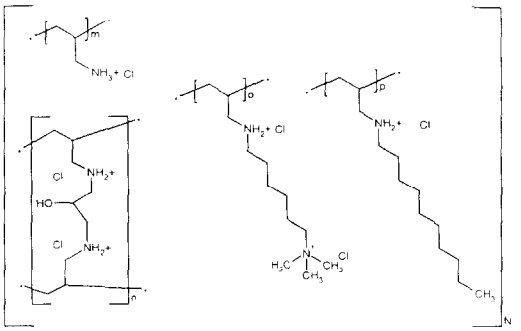


Although it was initially developed as a cognition enhancer, cevimeline was launched in the US for the treatment of dry mouth symptoms (xerostomia) in patients with Sjögren's syndrome. Cevimeline is a racemic mixture of *cis*-oxathiolanes that can be obtained with a three step synthesis starting from quinuclidin-3-one followed by separation from its 9-12-fold less potent *trans*-diastereomer. This conformationally rigid analog of acetylcholine is a

dual muscarinic M_1/M_3 agonist, selective versus M_2, M_4 and M_5 receptors. It is the fifth M_1 agonist that has failed in clinical trials against Alzheimer's disease. On the contrary, the sialagogic effects of cevimeline caused by its stimulation of M_3 receptors in salivary and lacrimal glands were demonstrated in randomized double-blind placebo-controlled clinical trials (30 mg t.i.d. oral dose). Cevimeline (1-3 mg/kg i.v.) was as potent in dogs as pilocarpine (0.1-0.3 mg/kg i.v.), the only prior drug efficacious against xerostomia associated with Sjögren's syndrome, but the effects of cevimeline lasted around 2-fold longer. No cardiovascular side effects were reported with cevimeline, unlike pilocarpine which has a 40-fold higher affinity for the M_2 receptor. Cevimeline seems to bind extensively to tissues (volume of distribution 6 L/kg in man) since it was found to be less than 20% bound to human plasma proteins. It is metabolized into the *cis* and *trans*-sulfoxide, a glucuronide conjugate and the *N*-oxide.

Colesevelam hydrochloride (hypolipidemic) (45-49)

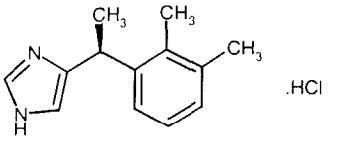
Country of Origin : US
Originator : GelTex
First Introduction : US
Introduced by : Sankyo and Pfizer
Trade Name : Welchol
CAS Registry No : 182815-44-7



Colesevelam hydrochloride was launched as Welchol (formerly known as Cholestagel) in the US for the reduction of elevated levels of serum LDL cholesterol and accordingly, the decrease of the risk of vascular disease in patients with primary hypercholesterolemia. This orally administered cationic hydrogel is a non-absorbable, water-insoluble polymer of an hexanaminium chloride with N-(2-propenyl) decanamine, 2-propen-1-amine hydrochloride and chloromethyloxirane. It acts as a powerful bile acid sequestering agent, this binding and blockage of bile acids having the end result of compelling the removal of LDL cholesterol from the blood stream into the liver. In animals fed with a cholesterol-rich diet for several weeks, colesevelam demonstrated a good maintenance of low serum cholesterol levels, this activity being significantly greater when compared with cholestyramine. In several placebo-controlled studies, this agent decreased total cholesterol levels by 6 to 10% and LDL cholesterol levels by 9 to 20%. Combination therapy with the co-administration of a HMG-CoA reductase inhibitor (or statin) and colesevelam produced an additional reduction of 8-16% in LDL-cholesterol levels above that obtained with the statin alone. Due to its unique water-absorbing hydrogel formulation, this polymer is not absorbed at all from the GI tract, and thus, it is said to have a lower rate of side-effects (as the constipating effect) than the previously marketed bile acid sequestrants. Colesevelam hydrochloride may be used as a monotherapy or as a dual therapy with statins.

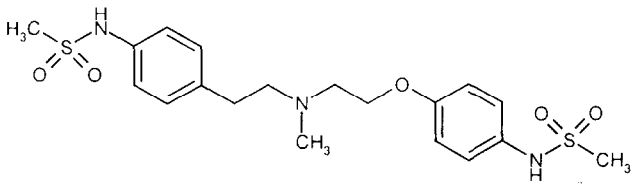
Dexmedetomidine hydrochloride (sedative) (50-53)

Country of Origin : Finland
Originator : Orion
First Introduction : US
Introduced by : Abbott
Trade Name : Precedex
CAS Registry No : 145108-58-3
Molecular Weight : 236.74



Dexmedetomidine was launched in the US as an i.v. infusion for sedation of initially intubated and mechanically ventilated patients during treatment in an intensive care unit. This imidazole derivative is the (*S*)-enantiomer of medetomidine that can be obtained *via* fractional crystallization of the tartrate salt of the racemic mixture. Dexmedetomidine is a full agonist of α_2 adrenoceptors with 1300-fold selectivity versus α_1 compared to the less selective partial α_2 agonist clonidine, a veterinary hypnotic. Dexmedetomidine is unique compared with currently available sedatives in that it provides sedation, analgesia and anxiolysis with the ability of patients to be easily awakened. Besides, it causes minimal respiratory depression unlike other available drugs such as benzodiazepines or opioids. Pharmacological studies in transgenic mice showed that the sedative, anesthetic and analgesic effects of dexmedetomidine are specifically due to the stimulation of the α_{2A} subtype receptor. Like other α_2 adrenoceptor agonists, dexmedetomidine can provoke hypotension and bradycardia probably because of its non-selective action on peripheral α_{2B} subtype receptors in vascular smooth muscle. Dexmedetomidine is extensively metabolized into methyl and glucuronide conjugates which are mainly eliminated by renal excretion. It was found to be a CYP2D6 inhibitor but less potent than the clinically relevant standard quinidine.

Dofetilide (antiarrhythmic) (54-60)

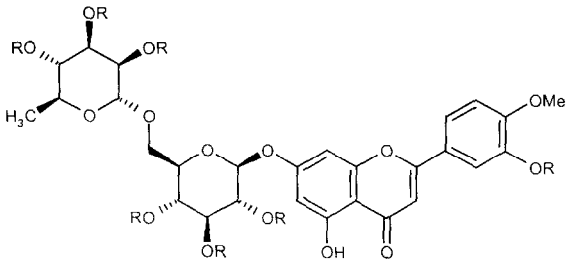


Country of Origin : US
Originator : Pfizer
First Introduction : US
Introduced by : Pfizer
Trade Name : Tikosyn
CAS Registry No : 115256-11-6
Molecular Weight : 441.14

Dofetilide was launched in the US as a novel class III antiarrhythmic for treatment of cardiac patients with highly symptomatic atrial fibrillation . This bisarylsulfonamide can be obtained by a three step synthesis starting from 4-nitro-N-methylphenethylamine and involving simultaneous nitro reduction and mesylation on both aromatic amine functions. In contrast to other class III antiarrhythmic agents such as amiodarone, dofetilide potently and selectively inhibits a single potassium channel, Ikr, the rapidly acting component of the delayed rectifier potassium current. Accordingly, by blocking the open state of Ikr, dofetilide is able to prolong the effective refractory period (ERP) in both atrial and ventricular myocardium and the monophasic action potential duration. Moreover, as it

targets only one cardiac ion channel, it does not produce any effects on the sinus node, cardiac conduction system and other extracardiac organs, making it unique among established class III agents. Several pharmacological studies with models using different animal species indicated that dofetilide was a potent and highly selective class III antiarrhythmic agent devoid of cardiodepressive effects. During clinical trials in patients with paroxysmal atrial or supraventricular fibrillation, dofetilide was found to increase atrial and ventricular refractory periods without affecting conduction or sinus node function. If increases in the QT/QTc interval after oral or intravenous dofetilide are expected, as for other class III antiarrhythmic agents, other electrocardiographic intervals are unaffected.

Dosmalfate (antiulcer) (61-63)



R = $SO_3[Al_2(OH)_5]$

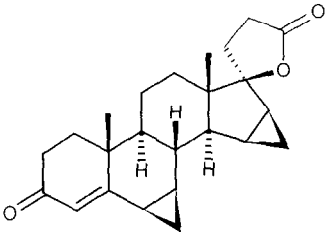
Country of Origin : Spain
Originator : Faes
First Introduction : Spain
Introduced by : Faes

Trade Name : Diotul
CAS Registry No : 122312-55-4
Molecular Weight : 2103.55

Dosmalfate, a heptakis(hydrogensulfate) aluminium complex of diosmin, was launched in Spain for the prevention and treatment of gastroduodenal lesions induced by NSAID therapy. Dosmalfate can be prepared from diosmin by initial treatment with a pyridine sulfur trioxide complex followed by sodium hydroxide and finally aluminium hydroxychloride. In several pharmacological models of acute and chronic ulcers, this new cytoprotective agent has demonstrated significant protection against damage to the gastric mucosa or esophagus erosion, hemorrhage or perforation. Several mechanisms of action could be involved, partly mediated by the endogenous prostaglandins. In several clinical studies with patients receiving long-term NSAID therapy for chronic inflammatory disease, the efficacy of dosmalfate in preventing gastric ulcer was high and comparable to that of misoprostol, but with significantly less adverse events. In rats and humans, dosmalfate, administered orally at very high doses, displayed a very poor absorption and consequently, a very low incidence of side effects, particularly on CNS, cardiovascular or respiratory systems.

Drospirenone (contraceptive) (64-67)

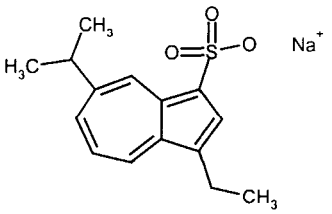
Country of Origin : Germany
Originator : Schering AG
First Introduction : Germany
Introduced by : Schering AG
Trade Name : Yasmin
CAS Registry No : 067392-87-4
Molecular Weight : 366.50



The steroid drospirenone in combination with the estrogen agonist ethinylestradiol was introduced in Germany as a new oral contraceptive for women. This analog of the aldosterone antagonist spironolactone can be synthesized in five steps from 3β-hydroxy-15β,16β-methylene-5-androsten-17-one. Since its binding profile for steroid receptors is very similar to progesterone, drospirenone mimics the progestogen agonistic activity as well as the anti-androgenic and anti-mineralocorticoid properties of the endogenous hormone. In rats, drospirenone inhibited ovulation at 6.3 mg/kg/day p.o. Compared with currently available progestins which lack anti-mineralocorticoid activity, drospirenone did not cause weight gain that could result from fluid retention in clinical studies. Its combination with ethinylestradiol was well tolerated and did not engender adverse effects on blood pressure or plasma lipid levels. Drospirenone is rapidly absorbed in man with an oral bioavailability of 76%. It is extensively metabolized since over 20 different metabolites were observed in the urine and in the feces, resulting for instance from hydrolysis of the lactone in the plasma or reductive conjugation of the enone to the 3-sulfate ester of 4,5-dihydrodrospirenone. Its elimination is bi-exponential with an initial and a terminal half-life of 2 and 25-33h respectively.

Egualen sodium (antiulcer) (68-71)

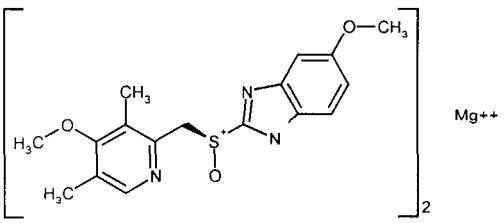
Country of Origin : Japan
Originator : Kotobuki Seiyaku
First Introduction : Japan
Introduced by : Kotobuki Seiyaku
Trade Name : Azuloxa
CAS Registry No : 97683-31-3
Molecular Weight : 300.35



Egualen, formulated as a sodium salt, was marketed last year in Japan for the treatment of gastric ulcers and gastritis. It is a water-soluble sodium sulfonate analog of guaiazulene, a natural azulene with anti-ulcer activity. Egualen can be prepared from an 7-isopropyl azulen-2-yl carboxylate by successive 3-acetylation, reduction, decarboxylation and finally, sulfonylation in 1-position. The mechanism of action is not well defined ; egualen is a weak inhibitor of the enzyme system H^+/K^+ ATPase and a weak antagonist of prostanoid receptors ; concomitantly, it was shown to possess some anti-inflammatory properties. In animal models of gastric ulcers induced with cortisone or acetic acid, oral treatment with egualen sodium promoted healing of ulcers and, at higher doses, mucosal regeneration. The data available from clinical studies for egualen are limited ; in one trial, the combination of egualen sodium and cimetidine administered to patients with unhealing gastric ulcers produced an appreciable rate of healing without any adverse events.

Esomeprazole magnesium (gastric antisecretory) (72-77)

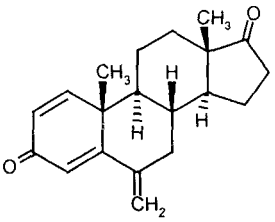
Country of Origin : UK
Originator : AstraZeneca
First Introduction : Sweden
Introduced by : AstraZeneca
Trade Name : Nexium
CAS Registry No : 119141-88-7
Molecular Weight : 711.15



Esomeprazole, formulated as a magnesium salt, reached the market as a treatment for acid-related diseases such as gastro-esophageal reflux (GERD) disease including peptic ulcer disease and reflux esophagitis. Esomeprazole (formerly perprazole) is the active (*S*)-enantiomer of omeprazole (1988) and the first proton pump inhibitor developed as an optical isomer. It can be obtained by several routes such as asymmetric oxidation of the pro-chiral pyridylmethyl benzimidazole sulfide, separation from the racemic sulfoxide by chiral chromatography or separation of a diastereomeric mixture obtained from the racemic compound and a chiral acid, followed by hydrolysis. Biochemical studies have shown that esomeprazole irreversibly inhibits the gastric H^+/K^+-adenosine triphosphatase (ATPase), an enzyme system involved at the secretory surface of the stomach's parietal cells responsible for the secretion of gastric acid. Compared with racemic omeprazole in healthy subjects, esomeprazole has higher bioavailability, is absorbed more rapidly and exhibits a more uniform and predictable dose-response with higher plasma levels, leading to less inter-individual variability between slow and rapid metabolizers. In extensive clinical trials in patients suffering from GERD symptoms, esomeprazole provided superior acid control and significantly reduced the healing time compared to omeprazole.

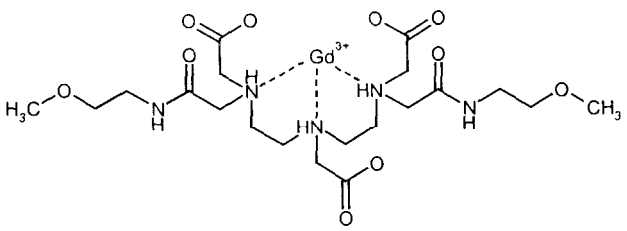
Exemestane (anticancer) (78-85)

Country of Origin : Italy
Originator : Farmitalia Carlo Erba
First Introduction : US; Canada; western Europe
Introduced by : Pharmacia
Trade Name : Aromasin
CAS Registry No : 107868-30-4
Molecular Weight : 296.41



Exemestane was launched in US and other countries for the treatment of estrogen-dependent tumors and postmenopausal breast cancer. It is a novel steroidal compound structurally related to the natural substrate for the biosynthesis of estrogen, androstanedione, and can be synthesized by methylidenation of androsta-1, 4-dien-17beta-ol-3-one in 6 position then oxidation of the alcohol function. Exemestane is an irreversible inactivator of the aromatase enzyme system, so inducing *in vivo* a dose-related sustained suppression of serum estrogen and minimal endocrine activity. It is the first steroidal representative of the third-generation of orally active aromatase inhibitors with a highly potent and selective mechanism of action, displaying good tolerability and safety profile. In rats with DMBA-induced mammary tumors, 10 to 100 mg/kg of exemestan administered po twice-daily for 4 weeks resulted in 76 to 88% regression. In women failing anti-estrogen therapy with tamoxifen, this agent has demonstrated high activity in locally advanced or metastatic disease. In addition, it may also have potential for breast cancer prevention.

Gadoversetamide (MRI contrast agent) (86-89)

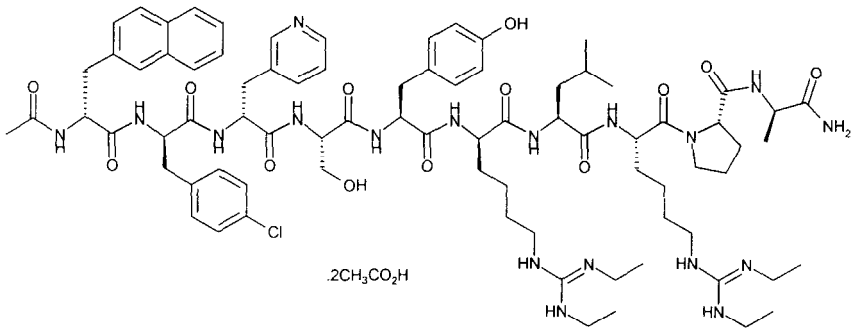


Country of Origin : US
Originator : Mallinckrodt
First Introduction : US
Introduced by : Mallinckrodt

Trade Name : OptiMARK
CAS Registry No : 131069-91-5
Molecular Weight : 661.77

The contrast agent gadoversetamide was launched in the US as a solution for i.v. injection to be used prior to magnetic resonance imaging (MRI) in patients with anomalous blood-brain barrier or anomalous vascularity in the central nervous system or in the liver. This chelate obtained by complexation of paramagnetic gadolinium(III) ion and DTPA-bis-(methoxyethylamide) ligand enhances visualization of lesions including tumors in the brain, spine and liver due to the effect of Gd(III)-water interaction on the proton magnetic relaxation of water. The small molecular size gadoversetamide belongs to the class of extracellular contrast agents. A phase III, multicenter, double-blind, parallel group trial showed that gadoversetamide was well tolerated and as efficient as gadopentetate dimeglumine (Schering) in hepatic magnetic resonance imaging of patients with suspected liver pathology.

Ganirelix acetate (female infertility) (90-93)



Country of Origin : US
Originator : Roche Bioscience
First Introduction : Germany
Introduced by : Organon

Trade Name : Orgalutran
CAS Registry No : 129311-55-3
Molecular Weight : 1690.44

Ganirelix acetate was introduced in Germany as prefilled syringes for subcutaneous injections that inhibit premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation. This decapeptide analog of luteinizing hormone-releasing hormone (LH-RH) is the second third-generation LH-RH antagonist to be launched after citrorelix (Asta Medica). This highly bioavailable compound immediately blocks the endogenous release by the pituitary gland of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), the hormone that induces ovulation. After discontinuation of the treatment, the pituitary-gonadal function is rapidly recovered due to its short-half life. As a consequence, ganirelix at daily doses of 0.25 mg s.c. efficiently prevented LH surges during clinical trials in infertile women under controlled ovarian hyperstimulation with recombinant FSH before *in vitro* fertilization or similar reproductive techniques. Unlike first and second-generation gonadotropin-releasing hormone antagonists, ganirelix has minimal histamine-releasing effects thus avoiding the formation

of edema of the face and extremities. Ganirelix is very resistant to hydrolysis and, in contrast to the already established cetrorelix, has good water solubility.

Gemtuzumab ozogamicin (Anticancer) (94-98)

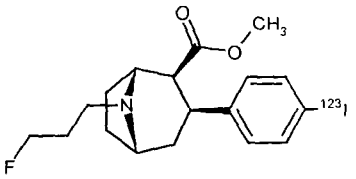
Country of Origin : US / UK
Originator : Celltech Group / Wyeth-Ayerst Research (AHP)
First Introduction : US
Introduced by : Celltech Group / Wyeth-Ayerst Research (AHP)
Trade Name : Mylotarg
CAS Registry No : 220578-59-6

Class : Recombinant protein
Type : Antibody-targeted antineoplatic agent
Molecular Weight : 152 kDa
Expression system : NS0 human myeloma cells
Manufacturer : Wyeth-Ayerst Laboratories

Gemtuzumab ozogamicin was launched last year as the first antibody-targeted antineoplastic agent for treatment of patients with acute myeloid leukemia (AML). This immunoconjugate consists of an anti-CD33 humanized mouse monoclonal antibody (IgG4) linked via a bifunctional linker to the cytotoxic antibiotic calicheamicin. Once, the anti-CD33 binds to its antigen expressed on AML blast cells, a complex forms that is internalized, eventually releasing the calicheamicin derivative inside the cell. The released antibiotic binds to the minor groove of DNA and after Bergman cyclization induces double-strand breaks and cell-death. Results from both phase I and II clinical trials suggested that this drug, when administered (9 mg/m^2 as 2 i.v. infusions separated by 14 days) as a single agent to patients in first relapse, demonstrates an acceptable safety profile, while achieving meaningful clinical remission in 32 to 43% of individuals. Gemtuzumab ozogamicin is considered at least as effective as conventional therapy but presents a more favorable tolerability profile as compared to standard chemo-therapeutic drugs commonly used to treat AML. This agent received accelerated approval from the FDA for the treatment of patients in first relapse who are 60 years of age or older and who are not considered candidates for cytotoxic chemotherapy.

Ioflupane (diagnosis CNS) (99-103)

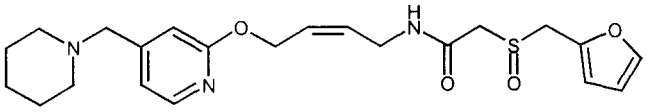
Country of Origin : UK
Originator : Research Biochemicals Int
First Introduction : UK
Introduced by : Nycomed Amersham
Trade Name : DatSCAN
CAS Registry No : 155798-07-5
Molecular Weight : 427.39



Ioflupane was launched last year as a new imaging agent for investigation of the dopaminergic neurons and the early diagnosis of Parkinson's disease and related syndromes. This (^{123}I) iodine-labeled agent was developed for imaging with single-photon emission computed tomography (SPECT) ; it binds to dopamine transporters on cerebral neurons, enabling the detection of an eventual loss of functional neurons, particularly in the striatal region. The use in clinical routine of SPECT technique with this new agent generates reliable and reproducible results in healthy control subjects as well as in patients with Parkinson's disease. So, it displays a quantification of striatal dopaminergic function and can help in the identification of the parkinsonian syndromes and their differentiation from syndromes due to other diseases. The major metabolic reactions in healthy humans

are hydrolysis of the ester bond to the free acid and N-dealkylation to the ^{123}I-labeled nor-analog.

Lafutidine (gastric antisecretory) (104-107)

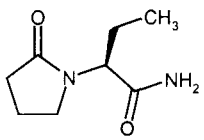


Country of Origin : Japan
Originator : Fujirebio
First Introduction : Japan
Introduced by : UCB and Taiho
Trade Name : Stogar, Protecadin
CAS Registry No : 118288-08-7
Molecular Weight : 431.19

Lafutidine was launched in Japan for the treatment of gastritis, reflux oesophagitis and peptic ulcers. It can be prepared in eight steps from 4-(2-tetrahydropyranyloxy)-2(*Z*)-buten-1-ol. Lafutidine is a potent and longer-acting H_2 antagonist compared to other marketed compounds of its class such as cimetidine and famotidine. In contrast to other commercially available H_2 antagonists, lafutidine also exerts a gastroprotective action probably *via* capsaicin-sensitive afferent nerves. It was clinically effective in the treatment of nonsteroidal antiinflammatory drug-induced ulcer in patients refractory to existing antiulcer agents.

Levetiracetam (antiepileptic) (108-112)

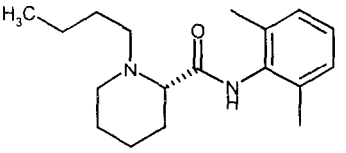
Country of Origin : Belgium
Originator : UCB
First Introduction : US
Introduced by : UCB
Trade Name : Keppra
CAS Registry No : 102767-28-2
Molecular Weight : 170.21



Levetiracetam was first introduced in the US as an adjunctive therapy in the treatment of partial-onset seizures in adults with epilepsy. This second-generation analog of piracetam can be prepared by condensation of (*S*)-2-aminobutyramide with 4-chlorobutyryl chloride. Although its mechanism of action is not well established, it was shown that [^{3}H]-levetiracetam reversibly binds to a specific site predominantly present in the membranes of the brain. Unlike conventional anticonvulsants such as phenytoin, carbamazepine, valproic acid, phenobarbital, diazepam and clonazepam, compounds structurally-related to levetiracetam, such as piracetam and aniracetam, also have affinity for this site. Levetiracetam reveals a broad and unique profile in animal seizure models, including promising antiepileptogenic properties. Besides being rapidly and almost completely absorbed in man (oral bioavailability>95%), it possesses a favorable pharmacokinetic profile since it is not hepatically metabolized but only partly hydrolized into the inactive carboxylic acid by enzymes in a number of tissues including blood cells, it is minimally bound to plasma proteins (<10%) and does not inhibit or induce hepatic enzymes. Therefore levetiracetam has a low potential for drug interaction, providing a useful alternative as adjunctive therapy to treat seizures refractory to conventional anticonvulsants.

Levobupivacaine hydrochloride (local anesthetic) (113-115)

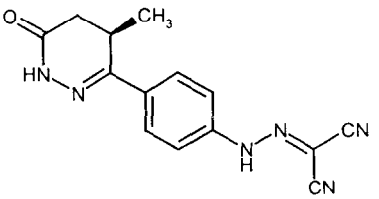
Country of Origin : UK
Originator : Chiroscience
First Introduction : US
Introduced by : Purdue Pharma
Trade Name : Chirocaine
CAS Registry No : 27262-48-2
Molecular Weight : 288.22



Levobupivacaine was first launched last year in the US for the production of local anesthesia for surgery and obstetrics and for post-operative pain management. It is the (*S*)-enantiomer of the long acting, highly potent local anesthetic bupivacaine (Marcaine) that can be prepared by a three step sequence from (*S*)-pipecolic acid or from (*S*)-lysine by oxidative deamination and stereospecific ring closure to (*S*)-pipecolamide core structure. Levobupivacaine exhibits its long-acting local anesthetic effect by blocking neuronal sodium channel ion flow in nerve axons. Clinical studies demonstrated an efficacy and a general profile closely resembling those of the racemic bupivacaine currently in use ; however, it produced an enhanced safety profile, in particular substantially reduced (about one-third) cardiotoxicity (less effect on myocardial contractility and QT_c prolongation) and CNS depressive side effects. Onset and duration of blockade were also equivalent or even better.

Levosimendan (heart failure) (116-120)

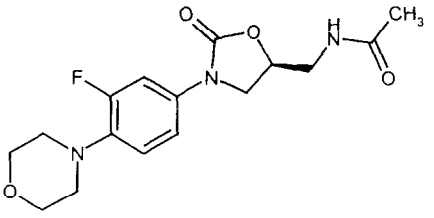
Country of Origin : Finland
Originator : Orion
First Introduction : Sweden
Introduced by : Orion
Trade Name : Simdax
CAS Registry No : 141505-33-1
Molecular Weight : 280.29



Levosimendan was introduced in Sweden as an i.v. infusion for the treatment of acute heart failure or refractory symptoms of chronic heart failure in cases where conventional treatment (e.g., diuretic or ACE inhibitor) is not sufficient. Levosimendan is the (*R*)-enantiomer of simendan that belongs to the same class as pimobendan (Boehringer Ingelheim). Levosimendan is an innovative myofilament calcium sensitizer that increases myocardial contractility by selectively binding to the N-terminus of troponin C and by stabilizing the Ca^{2+}-bound conformation of this contractile protein. It also activates ventricular and arterial adenosine triphosphate-regulated potassium channels which causes vasodilatation in vascular smooth muscle and protects myocardium against infarction. Its low phosphodiesterase III inhibiting activity is probably not responsible for its positive inotropic, lusitropic and dilating effects. Unlike other cardiotonic drugs, levosimendan is able to produce positive inotropic effects without prolonging myocardial relaxation or increasing the incidence of malignant arrythmias. It was clinically shown to have a lower risk of mortality in patients with heart failure when compared to placebo and dobutamine. Since it has a larger potential, levosimendan is currently under further clinical evaluation as a chronic treatment for congestive heart failure.

Linezolid (antibiotic) (121-128)

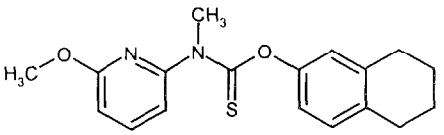
Country of Origin : US
Originator : Pharmacia Corp.
First Introduction : US
Introduced by : Pharmacia Corp.
Trade Name : Zyvox
CAS Registry No : 165800-03-3
Molecular Weight : 337.14



Linezolid reached the US market for the treatment of patients with infections caused by serious Gram-positive pathogens, particularly skin and soft tissue infections, community-acquired pneumonia and vancomycin-resistant enterococcal infections. Linezolid is the (*S*)-enantiomer of an oxazolidin-2-one synthesized in a multistep process from 3,4-difluoronitrobenzene, the key step being the cyclization of a carbamate, using a chiral epoxyester, into an enantiomerically pure oxazolidin-2-one. Linezolid can be considered as the first of a new class of antibacterial agents known as oxazolidinones, its mechanism of action being related to the inhibition of early ribosomal protein synthesis without directly inhibiting DNA or RNA synthesis. *In vitro* studies demonstrated that linezolid was effective, at potency levels similar to vancomycin, against staphylococcal, streptococcal and pneumococcal infections (MIC values in the range of 0.5 to 2 µg/ml), enterococcal species including VRE and VSE (MIC values about 4 µg/ml), but also other vancomycin-resistant bacteria. Linezolid is rapidly absorbed orally, its bioavailability is nearly complete at 250 mg dose giving a Cmax to MIC ratio sufficient to have pathogenic strain eradication in the clinical setting. It is considered that this new promising agent may offer new options for therapy of multi-drug infections.

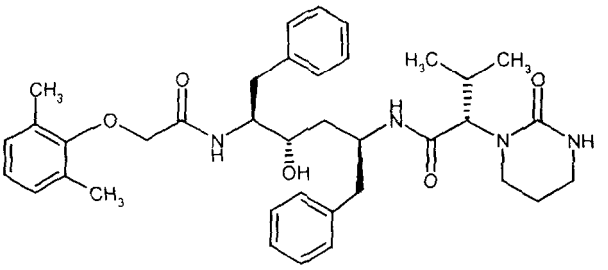
Liranaftate (topical antifungal) (129-130)

Country of Origin : Japan
Originator : Tosoh
First Introduction : Japan
Introduced by : Tosoh
Trade Name : Zefnart
CAS Registry No : 088678-31-3
Molecular Weight : 328.43



Liranaftate was launched in Japan as a new topical antifungal for the treatment of dermatophycoses. This compound belonging to the thiocarbamate class of antifungals can be prepared by condensation of 5,6,7,8-tetrahydro-2-naphthol with the corresponding N-pyridylthiocarbamoyl chloride. It is a potent and specific inhibitor of squalene epoxidase and consequently a blocker of ergosterol biosynthesis in fungi, without any detectable effect on mammalian cholesterol biosynthesis in rat liver at therapeutic dose levels. Liranaftate was found to be significantly more active than the other thiocarbamate tolnaftate against several dermatophytes, including *Trichophyton mentagrophytes*, and against certain yeasts, such as *Cryptococcus neoformans*. On the other hand, it was inactive against a variety of Gram-positive and negative bacteria. When applicated as a 1 or 2% cream during clinical trials, it was well tolerated and no systemic absorption was observed.

Lopinavir (antiviral) (131-135)

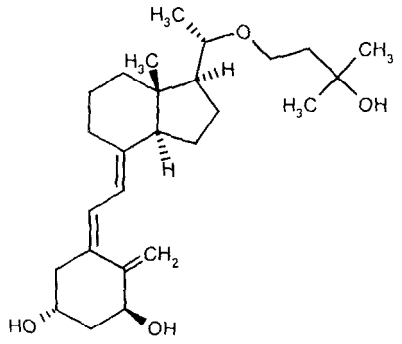


Country of Origin : US
Originator : Abbott
First Introduction : US
Introduced by : Abbott; Triangle Pharm. Inc.

Trade Name : Kaletra
CAS Registry No : 192725-17-0
Molecular Weight : 628.82

Lopinavir, the sixth HIV protease inhibitor in the "navir" class, was launched in co-formulation with ritonavir, another HIV protease inhibitor already marketed (Abbott, 1996) ; this original formulation was introduced as Kaletra for use in combination with either nucleoside or non-nucleoside reverse transcriptase inhibitors for the treatment of AIDS in adults and children. Lopinavir is a peptidomimetic compound with a structural core identical to that of ritonavir, on which terminal groups, particularly a modified valine, were introduced by peptide coupling procedures. Lopinavir is a potent competitive inhibitor of HIV-1 protease exhibiting high potential against ritonavir-resistant mutations. In several animal species, pharmacokinetic studies with the lopinavir/ritonavir association showed that the modest properties of lopinavir were significantly improved in presence of ritonavir, in terms of Cmax and duration of action. Ritonavir inhibits the P450 isoenzyme CYP3A4 and the human liver microsomal metabolism of lopinavir, so strongly amplifying plasma levels of this latter component. In AIDS patients, the plasma HIV RNA level was considerably reduced and the CD4+ T-cell counts increased after administration of lopinavir combined with relatively small doses of ritonavir. Kaletra is intended to be used jointly with other antiretroviral agents.

Maxacalcitol (vitamin D) (136)

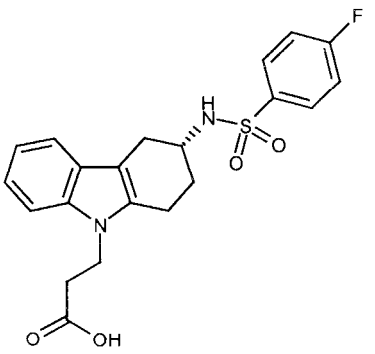


Country of Origin : Japan
Originator : Chugai
First Introduction : Japan
Introduced by : Chugai
Trade Name : Prezios, Oxarol
CAS Registry No : 103909-75-7
Molecular Weight : 418.31

Maxacalcitol is the third synthetic vitamin D analog, after paricalcitol and doxercalciferol, to be marketed for the treatment of secondary hyperparathyroidism

(SHPT) associated with chronic renal failure. It is a new vitamin D3 derivative with an oxygen atom in 22-position as main structural feature (22-oxacalcitriol, OCT). This vitamin D receptor agonist has a strong inhibitory effect on synthesis and secretion of parathyroid hormone in the setting of severe parathyroid hyperplasia. Maxacalcitol induced only minor effects on calcium and phosphate metabolism unlike an agent such as 1, 25-(OH)2D3 that produced hypercalcemia and hyperphosphatemia. Maxacalcitol is currently in clinical development for psoriasis (ointment formulation for topical treatment).

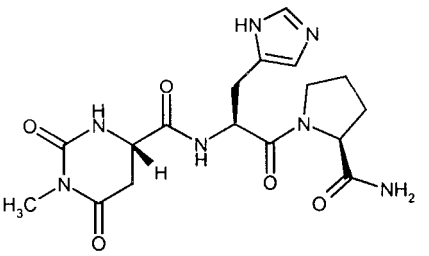
Ramatroban (antiallergic) (137-141)



Country of Origin : Germany
Originator : Bayer
First Introduction : Japan
Introduced by : Bayer
Trade Name : Baynas
CAS Registry No : 116649-85-5
Molecular Weight : 416.12

Ramatroban, originally developed for the treatment of cardiovascular pathologies as thromboembolism, was finally introduced in Japan for the treatment of allergic rhinitis. It is a (3*R*)-enantiomer that can be synthesized in 5 steps from 3-oxo-1,2,3,4-tetrahydrocarbazole by stereoselective reductive amination, using *S*-phenethylamine as the chiral source, followed by hydrogenolysis, sulfonylation and finally 2-step N-carboxyethylation. Ramatroban is a potent antagonist of prostaglandin receptors (PGD_2, PGH_2) and thromboxane receptors (TxA_2) ; accordingly, it blocks the contractions induced by thromboxane or TxA_2-mimetics in animal and human airway smooth muscle. It also prevents, when administered i.v., p.o. or by aerosol, bronchoconstriction induced by PGD_2 or antigen. In animal models of nasal allergy, ramatroban inhibits antigen-induced neutrophil infiltration into nasal mucosa and also inhibits nasal symptoms. In several species including humans, it is well-absorbed, extensively protein-bound (>95%) and eliminated mainly as a glucuro-conjugate ; in man, its terminal half-life is 2 to 3 hours.

Taltirelin (CNS Stimulant) (142-147)

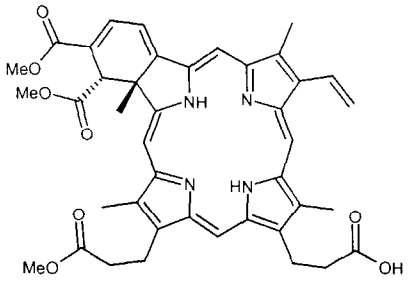


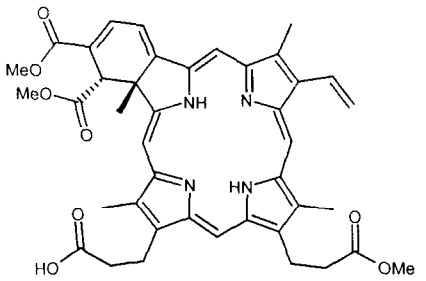
Country of Origin : Japan
Originator : Tanabe Seiyaku
First Introduction : Japan
Introduced by : Tanabe Seiyaku
Trade Name : Ceredist
CAS Registry No : 103300-74-9
Molecular Weight : 405.42

Taltirelin was marketed in Japan for the treatment of neurodegenerative diseases, in particular the improvement of ataxia due to spino-cerebellar degeneration (SCD); it is the first orally-active drug in this indication. This synthetic thyrotropin-releasing hormone (TRH) analog is prepared by condensation of (*S*)-1-methyl-4,5-dihydroorotic acid with L-histidyl-L-prolinamide. It was shown that the *S*-configuration for all 3 chiral centers is crucial for CNS

activity. Taltirelin is a potent agonist of the TRH receptors that shows significant effects on the cerebral monoamine systems when administered i.p. in rats. As TRH at 10-fold higher doses, taltirelin has been found to increase the extracellular levels of dopamine and its metabolites (DOPAC and HVA) as well as precursors and/or metabolites of noradrenaline and serotonin in different areas of the rat brain. Taltirelin produced an anti-ataxic activity in the Rolling mouse Nagoya, an ataxic mutant mouse. In several clinical studies performed in patients suffering from various forms of SCD, taltirelin demonstrated a statistically significant increase in the global improvement of ataxic symptoms and other neurologic abnormalities.

Verteporfin (photosensitizer) (148-151)





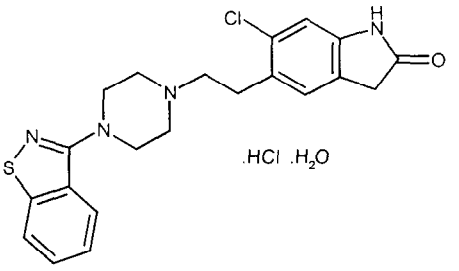
Country of Origin : Canada
Originator : QLT Inc.
First Introduction : Switzerland
Introduced by : QLT and Ciba Vision

Trade Name : Visudyne
CAS Registry No : 129497-78-5
Molecular Weight : 730.83

Verteporfin was introduced in Switzerland as a second-generation photosensitizer for photodynamic therapy of wet age-related macular degeneration in patients with subfoveal choroidal neovascularisation. It is constituted of a mixture of two regioisomers resulting from nonselective monohydrolysis of the methyl tetraester. Upon irradiation with a low-energy nonheat-generating laser light of 689nm wavelength, both regioisomeric benzoporphyrins are responsible for the local generation of singlet oxygen leading to free radical damage of neovascular endothelial cells in subfoveal lesions of patients with age-related macular degeneration. The lipophilic drug is injected i.v. as a liposomal formulation in order to favor its uptake by plasma lipoproteins and distribution to cells with a high expression of low density lipoprotein receptors such as tumor and neovascular endothelial cells. Verteporfin is also being developed for the treatment of other eye diseases, non-melanoma skin cancer and psoriasis.

Ziprasidone hydrochloride (neuroleptic) (152-156)

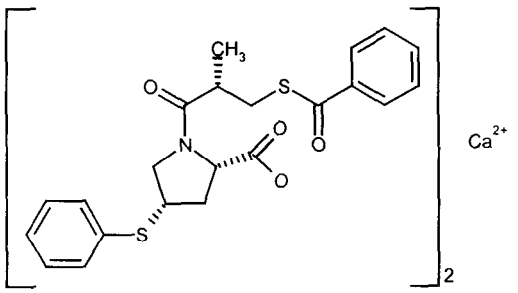
Country of Origin : US
Originator : Pfizer
First Introduction : Sweden
Introduced by : Pfizer
Trade Name : Zeldox
CAS Registry No : 122883-93-6
Molecular Weight : 412.11



Both p.o. and i.m. formulations of ziprasidone were launched in Sweden for the treatment of schizophrenia and agitated psychoses. It is the sixth marketed atypical antipsychotic after clozapine, risperidone, olanzapine, sertindole and quetiapine. The synthesis of ziprasidone involves a novel one-step process for the preparation of 3-(1-piperazinyl)-1,2-benzisothiazole followed by coupling with a chlorooxindole fragment. Ziprasidone is a very potent 5-HT_{2A}/D_2 antagonist with a ratio of about 11 in favor of the serotonin receptor. It also shows very high 5-HT_{2C} antagonistic activity, high 5-HT_{1A} agonistic and 5-HT_{1D} antagonistic activity, as well as moderate antagonism of α_1 and H_1 receptors and moderate norepinephrine and serotonin reuptake inhibition. Its complex binding profile for serotonin and dopamine receptors resulted during clinical trials in high antipsychotic efficacy with low extrapyramidal side effects and also in antidepressive action with low propensity for weight gain in opposition to other atypical and typical neuroleptics. An intramuscular formulation of ziprasidone was demonstrated to be superior to haloperidol, a conventional neuroleptic, for the short-term treatment of agitation in acutely psychotic patients. When administered orally in the fed state, this well-tolerated agent which strongly binds to plasma proteins shows a bioavailability of about 60% which is almost 2 fold greater than in the fasted state. It is transformed into 4 circulating major metabolites by different enzyme systems. The small QTc prolongation observed with ziprasidone was found to be comparable to other antipsychotic drugs and it is considered to be without significant risk.

Zofenopril calcium (antihypertensive) (157-161)

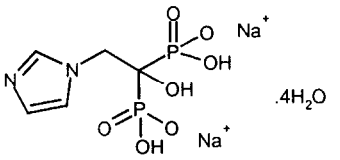
Country of Origin : US
Originator : Bristol-Myers Squibb
First Introduction : Italy
Introduced by : Menarini
Trade Name : Zantipres
CAS Registry No : 81872-10-8; 81938-43-4
Molecular Weight : 429.11



Zofenopril calcium was introduced last year as a second-generation angiotensin-converting enzyme (ACE) inhibitor for the treatment of acute myocardial infarction. Zofenopril, rapidly hydrolyzed by cardiac esterase, is actually a *S*-benzoyl prodrug of the active component zofenopril-sulfhydryl (zofenoprilat), the latter being the ACE inhibitor responsible for the improvement of postischemic contractile function and for the reduction of cardiac cell death. It was suggested that ACE inhibition alone was not sufficient to explain the cardioprotective effects ; the antioxidant properties demonstrated *in vitro* and *in vivo* could partly explain the strong anti-ischemic effects. In rats with CHF after myocardial infarction, zofenopril attenuated ventricular enlargement and cardiac hypertrophy. It was also shown in isolated globally ischemic rat hearts that the cardioprotective effect of zofenopril was stereoselective. Clinical studies in healthy volunteers comparing zofenopril with enalapril demonstrated that the rate of hydrolysis of the former was faster ; at 30 or 60 mg, ACE was completely inhibited in most volunteers until 12h after administration. In patients with anterior acute myocardial infarction, a 6-week treatment with zofenopril significantly reduced the incidence of death or severe CHF and also improved the chance of surviving the next year.

Zoledronate disodium (hypercalcemia) (162-165)

Country of Origin : Switzerland
Originator : Novartis
First Introduction : Canada
Introduced by : Novartis
Trade Name : Zometa
CAS Registry No : 165800-07-7
Molecular Weight : 388.11



Zoledronate was introduced in Canada as a lyophilized powder in vials for infusion as solution in the treatment of tumor-induced hypercalcemia. This third-generation bisphosphonate can be obtained by reaction of 2-(1-imidazolyl)acetic acid hydrochloride with phosphorous trichloride followed by hydrolysis in concentrated hydrochloric acid. Zoledronate originated from a medicinal chemistry program started at Novartis, Switzerland in 1986 and aimed at discovering a follow-up to pamidronate for the treatment of osteoporosis and metastatic bone disease. It has highly potent inhibitory effect on hypercalcemia induced by vitamin D_3 both *in vitro* in mouse calvarial cultures and *in vivo* in the thyroparathyroidectomized rat. It is respectively 100 and 850-fold more potent than pamidronate in the former and latter tests. Clinically, it was demonstrated that zoledronate achieved normalized serum calcium concentrations in a significantly higher percentage of cancer patients compared to pamidronate. The 5 minute infusion with zoledronate was more convenient than the 2 hour infusion necessary with pamidronate.

References

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Chapter 29. New Developments in Animal Healthcare

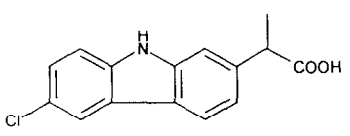
Ashley E. Fenwick
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Introduction - There has been a pronounced shift from the traditional focus on medication for food animals towards companion animals, generally considered to be dogs, cats and horses. Indeed, 2/3 of the pharmaceutical products launched over the last five years have been into this market, which has been the fastest growing sector of the animal health care market. This change has been partly driven by increased demand brought about by advances in veterinary science, an aging pet population and increased willingness of owners to pay for effective treatments. Global economic pressures have also rendered the market for livestock products increasingly difficult. In addition to pharmaceutical products, there is a robust vaccine sector which is more focused on livestock. A significant proportion of pharmaceutical products launched into the animal health market were originally developed for other markets, primarily human health but also crop protection. Indeed, many of the major animal health companies are themselves multinationals with significant human health interests. The shift of focus to companion animals offers an increasing opportunity to leverage compounds originally in development for human use. Many such compounds have been developed using animals and have a profile which is suitable for use in animals if not in humans. In fact, many human medications are used "off label" by veterinarians identifying the potential for cross over products. Without the relevant pharmacokinetics/dynamics in the target species however, it is difficult to make optimal use of human drugs. The markets for animal health products are however smaller than the equivalent human markets and are more price sensitive. The extent of insurance cover is low and owners have to bear the cost of therapy. Cost of goods is therefore a more critical issue and rules out a number of potential cross over products from human health. On the other hand, development is generally cheaper and quicker with a much higher success rate. This results from the ability to take compounds into the target species at a far earlier stage, removing the uncertainties of species variation and identifying adverse effect at a very early stage. Clinical trials also tend to involve lower patient numbers. There are a number of annual reviews of the animal health sector which provide useful information on the market performance and new entrants into it (1,2). This review is intended to survey recent developments in the veterinary medicine field. It will deal mainly with new products for the companion animal market launched over that period and seek to indicate additional compounds which are potentially of interest.

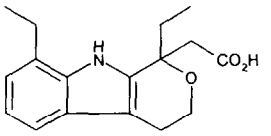
Pain and Inflammation – Nonsteroidal anti-inflammatory drugs (NSAID's) have long been a mainstay therapy for pain and inflammation in humans and are readily available OTC. Simply using the human products is complicated by the different profiles of drugs in animals and humans. Ibuprofen, for instance is not recommended for dogs as higher absorption rates and a longer half life result in higher blood levels and a higher rate of GI complications than in humans (3). NSAID's have been available in the veterinary field for the last 10 years for use with companion animals for both the treatment of osteoarthritis and pain associated with surgery. The treatment of inflammatory disorders in dogs and horses has been reviewed (4-8). Recently several additional compounds have reached the market, including some with an improved safety profile. The move in the veterinary field mirrors the human market with a shift towards COX2 selective compounds to reduce GI liabilities. As in the human field determining the COX1/COX2 selectivity is highly dependent on the assay system used, though comparatively little work has been done using relevant animal enzymes.

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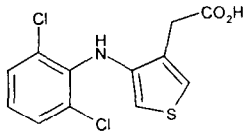
Carprofen **1** the market leader is a reversible COX2 selective inhibitor with a good safety profile in dogs (3,9,10). It is reported as showing selectivity for COX2 of either 129 fold (COX1- canine platelets, COX2-LPS stimulated canine macrophage cell line) (11), or 1.75 fold (COX1-canine macrophage cell line, COX2-LPS stimulated canine macrophage cell line) (12). The relatively low incidence of GI problems supports the more selective figure (3). **1** is sold as the racemate (COX2 IC_{50} 0.1μM) though it has been shown that the S isomer is the more potent enantiomer (COX2 IC_{50} 0.04 μM) by a factor of 200 fold (11). It is also reported as a weak inhibitor of PLA_2 (13). There are also suggestions that it has additional, as yet undefined, anti inflammatory properties; It is certainly reported to modify cell immune responses(3). **1** is well absorbed in dogs (>90% oral absorption) with a half life of about 8 hr (3). Etodolac **2** is reported to be effective in improving the rear limb function of dogs with chronic osteoarthritis (OA) and



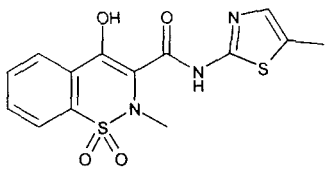
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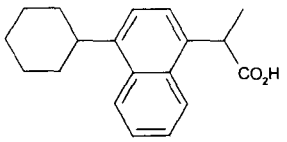
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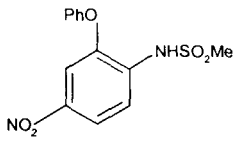
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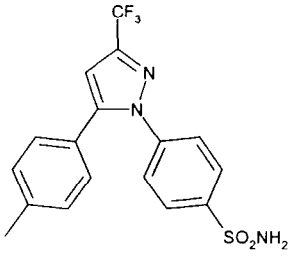


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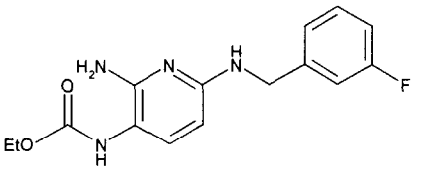
hip displasia (14,15). Toxicity studies in dogs have also shown it to have a low incidence of gastric side effects at therapeutic doses (16). Though it is reported as a selective COX2 inhibitor in humans (COX2 IC_{50} 0.68 μM , COX1 / 2 ratio 179) in dogs it was reported to be slightly COX1 selective (COX2 IC_{50} 2.5 μM , COX1 / 2 ratio 0.5) (11,17). Etodolac has a reported half life of 10 – 14 hr. Extensive enterohepatic circulation in the dog results in high serum concentrations for an extended period (18). Eltenac **3** has been introduced as an injectable for the treatment of horses. It is reported to show beneficial effects for up to 24hr following a single injection and to be safe in horses (19,20). Little other data is available on this compound. Meloxicam **4** is a potent COX2 selective inhibitor (IC_{50} 0.31 μM) with a reported selectivity of 3-12 fold in canine cell lines (11,12). This is consistent with the selectivity reported from human cell lines (21). There is only limited clinical information available for this compound in animals but reports of its use in models suggest it demonstrates potent anti-inflammatory activity with few GI problems (22-24). Vedaprofen **5** was recently launched for dogs and horses. Little data is available on this compound, however it has been shown to be as efficacious in treating dogs as meloxicam with a similar safety profile and has been evaluated in an equine model of acute inflammation (25,26). Nimesulide **6** has also recently been introduced as a selective COX2 inhibitor (IC_{50} 0.06 μM , COX1 / 2 ratio 38 fold in dogs) (11). Though there is little data with regard to its efficacy in companion animals, in humans it is reported to have good gastric safety. **6** is also reported to have a range of additional activities including inhibition of inflammatory mediators (27).

In addition to these marketed agents, there are a number of highly selective COX2 inhibitors being developed for human use which could make their way onto the veterinary market in the future. A patent has recently appeared for celecoxib **7**

specifically for treating non human animals for inflammation related conditions (28). This compound is a highly selective and potent COX2 inhibitor, IC_{50} 40 nM , COX1 / 2 ratio 375 fold for human enzymes (29). However, carprofen does not appear to fit into the published model for human COX2 selectivity, but is selective for the canine COX2 enzyme. It has been suggested therefore that the canine COX enzymes may differ from the human enzymes (11). This could obviously effect the potential utility of the current human enzyme selective compounds.



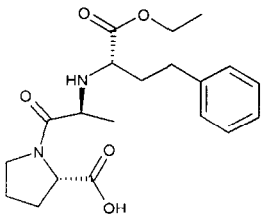
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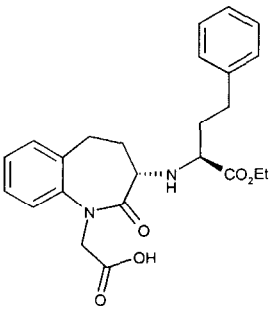
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A patent has also appeared recently claiming the use of flupirtine **8** for alleviating pain caused by degenerative joint disease in dogs and cats (30). This compound appears to be a centrally acting non-opioid analgesic with only weak inhibition of prostaglandin synthesis (31).

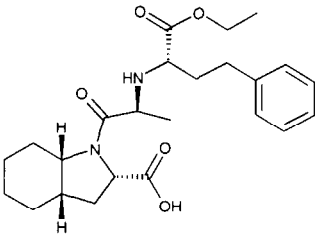
Cardiovascular – The nature of cardiovascular disease in animals differs slightly from humans. Hypertension is not as common in cats and dogs as in humans, though mitral valve disease is more common in dogs (1). In most cases animals are not diagnosed until the onset of heart failure. Angiotensin converting enzyme (ACE) inhibitors are well established as antihypertensives in the human field and have more recently become available for use in dogs.



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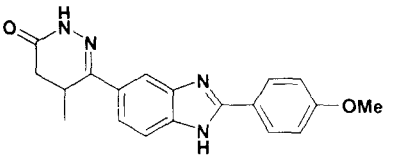


11

Enalapril **9**, a well established potent ACE inhibitor with an IC_{50} of 2.5nM, was introduced to the veterinary market in 1994 (32). Two additional ACE inhibitors have also reached the market more recently, benazepril **10**, which has an IC_{50} 2.8 of nM and ramipril **11**, with a similar level of potency, IC_{50} =2.2nM (33). These are all prodrugs, undergoing cleavage to the diacids *in vivo,* with a duration of action at inhibiting ACE in the plasma of normal beagles which is not significantly different (34). **10**, which has a long half life, (21hr), in humans, has a significantly shorter half life in dogs, (3.5hr) (35,36). They have all been shown to have a beneficial effect in the dogs, decreasing mortality and morbidity, and possibly preventing a deterioration in the condition by preventing left ventricular remodeling (37-41). In line with findings in humans, ACE

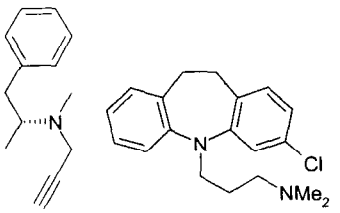
inhibitors have been reported to produce a cough in dogs in approximately 11% of cases (42).

An alternative to ACE inhibitors has been approved for veterinary use. Pimobendan **12** is a positive inotropic agent which has been shown to be effective in treating dilated cardio myopathy in dogs (43). Hitherto digoxin, a weak positive inotrope, has been used off label in dogs, but suffers from a narrow therapeutic index. Pimobendan is used in conjunction with other drugs, such as diuretics and ACE inhibitors, though it is also a weak vasodilator by itself. It is also reported that unlike earlier inotropes, it does not increase myocardial oxygen consumption (44). The activity of the compound, which is a PDE 3 inhibitor amongst other actions, is mainly associated with the (-) isomer (45).

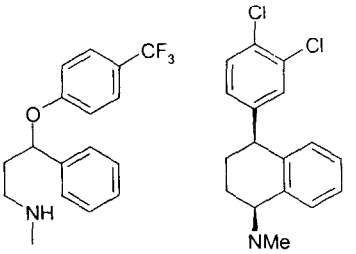


12

CNS and Behavioral – The most common behavioral problems affecting cats and dogs result from aggression, fear/anxiety or age related cognitive dysfunction. 20-40% of veterinary consultations over behavior relates to separation anxiety. The increase in this results from more pets being left on their own for longer as a consequence of socio-economic changes in the pet owning community (1). Three compounds are now licensed for use in this area. Selegiline **13**, also known as L-deprenyl, is a monoamine oxidase (MAO) inhibitor. It is effective in canine Cushings disease and cognitive dysfunction syndrome (CDS) with a good safety profile (46,47). The compound acts on MAO-B which degrades dopamine in the CNS. **13** is also available in Europe for a range of other canine behavioral disorders related to anxiety and aggression. Clomipramine **14**, is a tricyclic antidepressant which inhibits neuronal uptake of both serotonin and noradrenaline, though at therapeutic doses it is likely to mainly inhibit serotonin uptake (48).



13 **14**

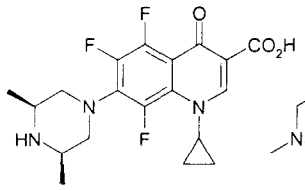


15 **16**

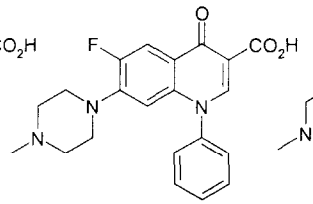
14 has been shown to be effective in treating the adverse behaviors resulting from separation anxiety; destruction, urination and barking (49,50). Ideally drug treatment is coupled to behavioral therapy, under which conditions the problems associated with separation anxiety do not return. No adverse effects were noted as a result of drug withdrawal (50). It has also shown some efficacy in canine compulsive disorder (51). Though beneficial effects were observed whilst undergoing treatment, this did not last beyond the treatment period. Selective serotonin reuptake inhibitors (SSRI's) have been evaluated for canine behavioral disorders but none are as yet approved for use in animals. Fluoxetine **15** was shown to be effective in treating dominance aggression in dogs, whilst **15** and Sertraline **16** were both effective in acral lick dermatitis, which is related to obsessive-compulsive disorder in humans (52,53).The third drug launched

into this area is a veterinary only product aimed at behavior control in cats. It is a synthetic pheromone comprising a mixture of fatty acids; palmitic, pimelic, oleic and azelaic acids (54). The synthetic pheromone has been shown to have beneficial effects on urine spraying and separation anxiety in cats (55,56).

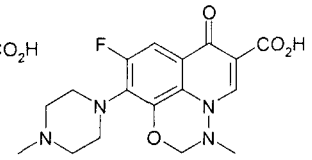
Antiinfectives – This area represents the second largest single area of the veterinary pharmaceutical market. The use of antibiotics in livestock is currently a contentious issue because of concerns over the potential generation of resistance, particularly through their use in feed as growth promoters. Companion animal antibiotic use for treating infections is much less contentious as these species do not enter the human food chain. Drugs from a broad range of pharmacological groups are used with companion animals though those with a broad spectrum are particularly useful (1). There have been a number of new compounds which have reached the market over the last few years, most notably three additional fluoroquinolones for the companion animal sector. Fluoroquinolones were first introduced into the veterinary market in the late 80's. The established market leader, enrofloxacin is now approaching patent expiry. This class of antibiotics exert their effects through inhibition of DNA gyrase and are selective for bacterial enzymes over mammalian. The minimum concentration of



17



18



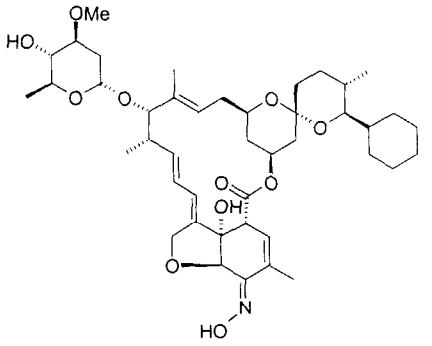
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orbifloxacin **17** which will stimulate bacterial DNA cleavage is 0.48μM, while the concentration needed to produce 50% maximal cleavage via bovine Topoisomerase II is over 2000μM (57). Orbifloxacin is a potent, broad spectrum orally available antibacterial for dogs and cats with typical MIC_{90}'s for relevant strains of around 0.02-0.2 μg/ml (58). In cattle and swine, it has a half-life of 2-3.5hr achieving a maximal concentration of 2-3μg/ml (58). Difloxacin **18** shows lower potency against a range of bacteria having MIC_{90}'s in the range of 0.1–4 μg/ml, but with a superior pharmacokinetic profile (59). It has a much longer half life, 9.3hr, in the dog with a C_{max} of 1.8μg/ml and plasma protein binding of about 50% (60). Marbofloxacin **19** has similar potency to orbifloxacin and has been demonstrated to have good efficacy against a wide range of clinical isolates from cats and dogs, with MIC_{90}'s in the range 0.02-0.4 μg/ml (61). The pharmacokinetic profile of marbofloxacin is superior to orbifloxacin with a half-life of about 10hr, C_{max} of 1.3μg/ml and oral availability of over 90% giving this the best overall profile (62).

Antiparasitics – This is the largest component of the animal health market making up 44% of sales. A large proportion of this sector comprises well established products for the livestock sector which will not be covered in this review but which have been reviewed in the past (63). Though some new compounds have recently been introduced for livestock, the majority of newer products have focused on companion animals, particularly ectoparasiticides for flea infestation. Parasites fall into two main categories; endoparasites such as heartworm, gastrointestinal nematodes and tapeworms, and ectoparasites such as fleas and ticks. Heartworm is a potentially lethal condition in which the worms invade and live in the heart. It is prevalent in regions where mosquitoes, which are an intermediary host, are found. The worms have to be controlled before they reach the adult stage as killing the adult worms could be fatal to the animal. Flea and tick infestations cause a number of problems for companion animals. They are responsible for the transmission of a number of diseases and

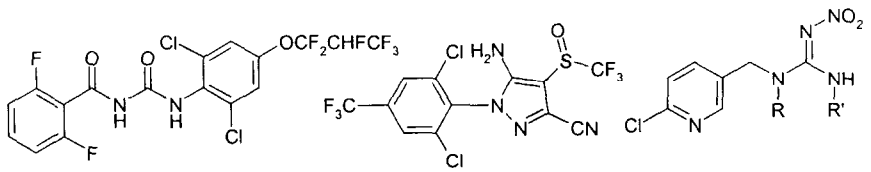
fleabite hypersensitivity can result in flea allergic dermatitis (FAD), one of the most common dermatological conditions in cats and dogs (1).

The treatment of endoparasites was transformed by the discovery in 1976 of the avermectins, a class of macrolide anthelmintics (64). These compounds work by modulating a glutamate gated chloride channel specific to invertebrates (65). Though these are highly potent and efficacious against heartworm, generally they can not be used to control GI nematodes in dogs due to dose limiting toxicity seen in some breeds, particularly collies. This potentially results from deficiencies in the transport of these drugs by P glycoprotein efflux mechanisms in susceptible dogs, allowing increased CNS exposure (66). Older members of this class of compounds have also lacked efficacy against the main ectoparasites, fleas and ticks at safe doses. A new member of the class, Selamectin **20** has for the first time introduced efficacy against fleas and ticks as well as heartworm prophylaxis (67). The drug is applied topically at the base of the neck from where it spreads to the rest of the animal. Efficacy against adult fleas is seen for a prolonged period, with >99% knock down at 30 days after treatment (68). Some tick efficacy is also seen and good control of heartworm in dogs has been demonstrated (69-70). **20** has also been shown to have reduced toxicity in collies compared to other members of this class (71). **20** is the first of the macrolide antiparasitics to show efficacy against fleas at safe doses. Other non-macrolide products have been introduced in the last few years as ectoparasiticides for companion animals.



20

Lufenuron **21** is an insect growth regulator which has been developed for use in both animal heath and crop protection. It works by inhibiting the synthesis of chitin, which is essential for the development of the flea exoskeleton (72). It is not toxic to the adult, working at the egg and larval stages. It is also not effective against ticks. **21** has a long duration of action, being effective for a month following a single dose (73). The compound partitions into the adipose tissue from where it slowly leeches out into the blood resulting in a half life in cats and dogs of about 60 days (74). Adult fleas then take up the drug when feeding and incorporate it into their eggs. A blood concentration of 0.125ppm has been shown to reduce the larval hatch rate to 4% (72). It is also excreted by the fleas where it is ingested by the larvae who feed upon the adult feces. Because it does not kill the adults, **21** takes some time to remove an infestation and is therefore frequently used in conjunction with other agents (75).



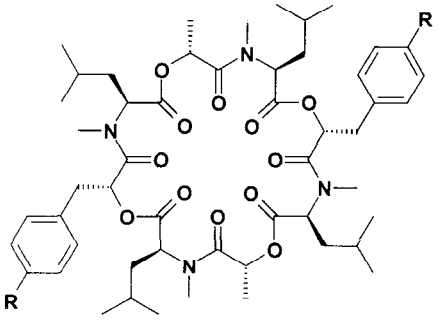
21 **22**

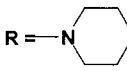
R,R' = CH_2CH_2 **23**
R = Et, R'= Me **24**

Fipronil **22** is a very potent compound, able to kill both adult fleas and ticks. A single topical application reduced the flea populations on dogs and cats by 97% after

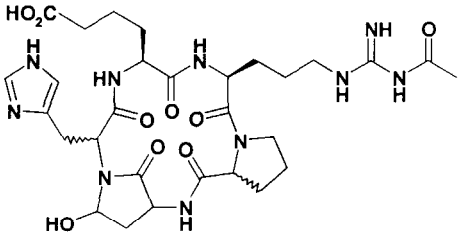
28 days (76). It is claimed to be one of the most potent acaricides available. It works by selectively blocking an insect ligand (GABA) gated chloride channel with little activity against the equivalent mammalian channel , IC_{50} insect 3nM, IC_{50} vertebrates 1100nM, (77). Imidacloprid **23** and nitenpyram **24** both have the same mechanism of action, acting as insect specific nicotinic acetylcholine receptor agonists (78). **23** binds to insect nicotinic acetylcholine receptors with a Kd of between 1–10 nM (79), whilst **24** has an IC_{50} of 57nM against [3H]imidacloprid binding (80). Binding of both of these to various sub types of mammalian nicotinic acetylcholine receptors is in the micromolar range giving around a thousand fold selectivity for the insect receptor (81). A single topical application of **23** removes an existing flea infestation and provides cover for a month (82). In common with fipronil, these compounds do not require the insects to be feeding, working on contact. **23** applied topically is rapidly acting, killing 20 minutes after contact (83). **24** is given orally and results in the death of over 99% of fleas 4hr after a flea challenge (84). It has a short half-life however and has to be used in conjunction with **21** for prolonged flea control (76).

Activity in the antiparasitic area continues to be strong with a number of patents appearing for structures related to the marketed compounds. There are also a number of new and interesting natural products which have been identified as antiparasitic and which appear to be the subject of continuing research. BAY44-4400 **25** was discussed



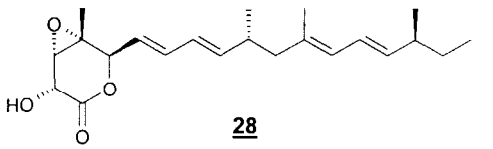
R =  **25**

R = H **26**



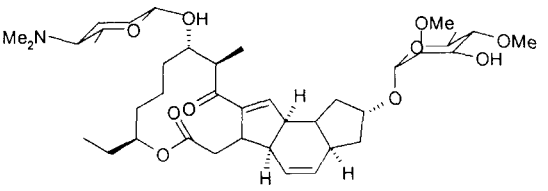
27

at a recent veterinary medicine conference (85). It is derived from a fungal metabolite, PF1022A **26**, which although demonstrating good anthelmintic activity, was limited in its potential utility (86). The introduction of two morpholino groups improves the *in vivo* potency of this structural class, ED_{50} of **25** against *N brasiliensis* 0.63mg/kg vs 10mg/kg for **26** (87). This class of compounds are neurotoxins in nematodes but have no effects against arthropods. They modulate GABA receptors but by a different mechanism to the avermectins (88,89). Another macrocycle isolated from fungi, this time a cyclic peptide, was also recently reported to possess good activity against flies. The peptide, Argadin **27** was isolated from *Clonostachys. sp* and has been shown to be a chitinase inhibitor, with an IC_{50} against blowfly chitinase of 150nm at 37°C (90). Nafuredin **28** has just been reported as a potent anthelmintic isolated from *Aspergillus niger.* compound selectively inhibits NADH-fumarate reductase activity in the helminth mitochondria, 8-200nM against various helminths with no effect on rat liver. **28** has also been demonstrated to have *in vivo* activity against *Haemonchus contortus* in sheep at 2 mg/kg (91). Finally,



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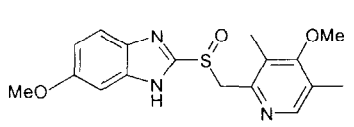
Spinosyn A **29** has recently been reported as showing good efficacy in dogs and cats against fleas and ticks (92,93). This compound was reported in 1991 as an insectacidal natural product isolated from *Saccharopolyspora spinosa* (94). It has now been shown to be effective either orally or topically against fleas and ticks (92,93). A relatively high oral dose of 50 mg/kg rapidly knocks out infestations of both fleas and ticks. Reduction in flea numbers persists at >90% for over 30 days, though persistence against ticks is not seen at this dose (92). A number of other interesting structures which have been reported over the last few years can be found in a review which recently appeared (95).



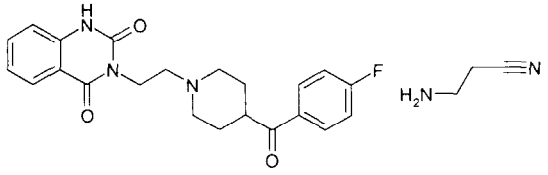
29

Equine Products – Whilst the majority of recent launches into the companion animal market have been aimed at dogs and cats, there have been some specifically aimed at the smaller equine market. Peptic ulcers are an important problem with horses. Up to 50% of young animals and a large proportion of performance animals suffer from this condition (1). The proton pump inhibitor, omeprazole **30**, widely used for humans, has now been approved for use in horses. This compound works in the gastric parietal cells where, in the acid environment of these cells, it is converted to its sulfenamide form which then binds to the hydrogen-potassium-ATP (H^+K^+ATPase) pump inhibiting acid release (96). **30**, dosed at 4mg/kg SID, has been shown to reduce gastric acid secretion by over 90% and to reduce the severity of gastric ulcers in horses (97,98). No similar approved drugs are available for dogs or cats though it is known that the use of NSAIDs does lead to gastric ulceration.

Two products have also been approved for wound healing in horses. Ketanserin **31** has been approved in Europe as a topical agent. It is a selective serotonin S2 receptor antagonist (99). Antagonism of this receptor is reported to reduce the vasoconstriction caused by serotonin and so improve the microcirculation in the wound. It is also claimed to reduce hypergranulation by preventing the activation and aggregation of platelets (100). This is a problem in particular on the legs of horses where the skin is tight resulting in the formation of weakened scare tissue. A second product, beta-aminopropionitrile **32** has also been approved for treating injuries to tendons. The compound is administered by an intratendinous injection and works by inhibition of Lysyl oxidase, IC_{50} 25μM (101,102). There is some evidence that the enzyme turns over the beta-aminopropionitrile to produce an aldehyde, which can then irreversibly inhibit the enzyme, though it could also chelate the copper which is essential for enzyme activity (101). This enzyme is involved with the polymerization and cross-linking of collagen fibrils. Inhibition of it slows down the rate of cross linking allowing for better alignment of the fibrils and a stronger repair (102).



30



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The past few years has seen a clear shift in the focus of the animal health industry towards companion animals. Despite this, there are still a large number of diseases where drugs specifically aimed at companion animals are not available. This will undoubtedly provide the drive for more compounds to be launched for companion animals in the near future. The smaller size of companion animal markets limits the areas which can support a drug discovery program resulting in a continuing emphasis on leveraging compounds, primarily from the human health sector. Antiparasitics will continue to be an area of active research led by the animal health industry, particularly as the emergence of resistance to some important products has already been observed.

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Chapter 30. Intellectual Property Issues Impacting the Pharmaceutical Industry

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Introduction – It is well documented that the discovery and development of drugs is an expensive and risky process. Investment in R&D was expected to be approximately $26.4 billion in the year 2000 (1). The average cost of developing a drug is well over $500 million (1). This cost is recovered by only 30% of drugs sold (1). Thus, it is critical that scientists and patent attorneys work hand in hand to create valid patent portfolios around drugs that allow for the maximal return on this huge investment. It is also important that the COPS (cost of product sold) of each drug be minimized so as to maximize the return on investment. Patent royalties can be a large component of COPS. Finally, the advent of genomics patents means that biotechnology patent law is increasingly important for all types of drug discovery, including the patenting of small molecule drug targets. The many uncertainties facing patent attorneys in biotech patent law will be described.

MANAGING AN OFFENSIVE PATENT PORTFOLIO

The patent attorney who maximizes the value of intellectual property related to his company's marketed drugs will be familiar with the laws of all the major markets, particularly the United States, western Europe and Japan. The web sites of the individual patent offices are good sources of information for individuals beginning their study of patent law (2-4). Furthermore, this familiarity must extend beyond patent law into the laws of trade secrets and regulatory exclusivity attached to the data submitted in support of the drug's approval (5). In the United States, trade secrets are largely the subject of state law but are also regulated by federal law such as the Economic Espionage Act (6). Patent law in the United States has changed dramatically in recent years, due to the American Inventors Protection Act (AIPA) of 1999 (7). Now patent strategists must consider the impact of the law which calls for publication of patent applications and associated file histories, changes in the handling and impact of provisional applications, increases in what constitutes prior art under 35 USC §102(e), and creates rights from the date of publication of the application, among many other changes. New rules were also created to implement the AIPA (8). The web site of the Center for Advanced Research and Study on Intellectual Property of the University of Washington School of Law provides many articles discussing various aspects of patent practice in the United States, Europe and Japan (9).

MINIMIZATION OF COPS BY AVOIDANCE OF ROYALTY STACKING

While small molecule drugs are not immune to the perils of royalty stacking, recombinantly manufactured drugs can be particularly burdened due to patents on a wide variety of elements involved in their manufacture such as cell lines and genetic elements (*e.g.,* promoters). For both small molecule and protein drugs, patented subject matter encountered in the course of discovery and development must be considered if its use is not exempted under an experimental use exemption or statutorily (10). The scope of such exemptions is widely recognized as one of the most difficult issues in patent law (11, 12). Most commentators agree that the purpose of the United States patent system as described in the Constitution, "[t]o promote the Progress of Science and the useful Arts," (13) is threatened if such exemptions are not recognized (11).

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DEVELOPMENT OF LAW RELATING TO GENOMICS PATENTS

Given the potential importance of genomics patents to drug development, it is helpful to have an understanding of the evolution of the law in this area. Due to the unique nature of biotechnology inventions, the way in which traditional legal principles should be applied to these types of inventions has been the subject of much debate. The Court of Appeals for the Federal Circuit has provided some guidance in biotechnology cases by focusing on obviousness law and the disclosure requirements of 35 U.S.C. § 112, ¶ 1. The court has fashioned patentability standards that demand that an inventor's contribution to the art closely match the scope of his claims. Taken as a whole, the court has crafted a jurisprudence that helps ensure the survival of the biotechnology industry as well as justify the enormous investments that must be made to bring products to the market. If the court had formed a more stringent obviousness standard, then many biotechnology companies would have great difficulty acquiring patents, which provide the basis for much of their funding. If the court enforced more relaxed enablement and written description standards, then a relatively smaller number of patentees would gain the ability to control much of the research in the biomedical field, including the discovery of small molecules that interact with protein targets. This would stifle both competition and innovation. Thus, the court has done an admirable job in furthering the goals of the patent system.

The Written Description Requirement in the United States – Throughout the evolution of the written description requirement the courts consistently emphasized the fact-sensitive nature of the issue (14). Courts have even suggested that broadly articulated rules setting forth a standard for fulfillment of the written description requirement are inappropriate (15). The CCPA stated in *In re Driscoll* that the precedential value of written description cases is extremely limited (16). Thus, the unique nature of biotechnology inventions which stems from the redundancy of the genetic code and the ability to easily manipulate DNA and protein sequences has created uncertainty with respect to what will be and what should be sufficient to satisfy the written description requirement.

Despite the fact-sensitive nature of the requirement, the Federal Circuit has applied the standard set forth in *Vas-Cath* to all types of cases including biotechnology cases (17). Regardless of the type of invention, the policy considerations are the same. In order to prevent overreaching by the patentee and yet encourage innovation, it is important that the written description requirement be closely linked with what is necessary to demonstrate conception of an invention. Demonstrating "possession" of an invention is evidencing the "completed conception." Thus, the trilogy of biotechnology cases beginning with *Amgen v. Chugai,* where the Federal Circuit discussed what is necessary to show a completed conception, and concluding with *Fiers v. Revel* and the *Regents of the University of California v. Eli Lilly & Company*, where the court specifically addressed the written description requirement, represents a logical progression of the law as it should be applied to inventions involving DNA and protein (18).

In *Amgen*, Amgen sued Genetics Institute (GI) for patent infringement. The Amgen patent issued on October 27, 1987, and contains claims to the DNA sequence encoding human erythropoietin (EPO), a protein that stimulates the production of red blood cells. GI sought to invalidate the patent under 35 U.S.C. § 102g by showing a prior conception coupled with reasonable diligence to a reduction to practice. Prior to Amgen's cloning of the EPO gene, GI had isolated and purified the EPO protein as well as conceived of a probing strategy using two sets of degenerate oligonucleotide probes from two different regions of the EPO gene to screen a DNA library. The Federal Circuit held that the Amgen patent was not invalidated based on the development of a probing strategy to screen a DNA library even though this strategy

eventually resulted in the actual cloning of the gene by GI (19). GI's earlier work was insufficient to constitute a conception of the DNA encoding EPO.

The court noted that "[c]onception is the 'formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is hereafter to be applied in practice (19)." Applying chemical case law precedent, the court stated, "Conception requires both the idea of the invention's structure and possession of an operative method of making it (19)." Further, the court held:

> Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principle biological property, e.g., encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property (19).

In this particular case, the court noted that an inventor would be unable to envision the detailed constitution of a gene until after the gene had been cloned and characterized.

The Written Description Requirement Is Closely Linked to Conception - In *Fiers v. Revel*, the Federal Circuit clearly set forth the relationship between conception and the written description requirement (20). The court in *Fiers* considered the priority claim of three different parties to the DNA encoding human beta interferon (β-IFN). Revel sought to use the benefit of an Israeli application date as a constructive reduction to practice to prove priority of invention for β-IFN DNA. The court held that the Israeli application did not contain an adequate written description of a DNA encoding β-IFN. The *Fiers* court reasoned that a statement merely referring to DNA encoding β-IFN in conjunction with a method of isolating it by reverse transcription did not indicate Revel was in possession of the DNA. "An adequate written description of a DNA requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it; what is required is a description of the DNA itself (20)."

The court went further noting that the reasoning applied in *Amgen* with respect to what is necessary to show conception also applies to the adequacy of descriptions of DNA. The court stated that "[i]f a conception of a DNA requires a precise definition, such as by structure, formula, chemical name, or physical properties, as we have held, then a description also requires that degree of specificity. To paraphrase the Board, one cannot describe what one has not conceived (20)." The court held that Sugano, another party in the action, was entitled to priority because the disclosure in his application satisfied the written description requirement by disclosing the complete and correct sequence of the DNA which codes for β-IFN. Fiers argued that *Amgen* was distinguishable because in *Amgen* the court was dealing with a situation where the isolation of the EPO DNA was "attended by serious difficulties (20)." Fiers suggested that the standard for proving conception of a DNA is essentially the same as that for proving enablement. The court disagreed and stated that "irrespective of the complexity or simplicity of the method of isolation employed, conception of a DNA, like conception of any chemical substance, requires a definition of that substance other than by its functional utility (20)."

The Description of a Species Does Not Describe a Genus or Other Species - In July 1997, the Federal Circuit again considered the written description requirement for DNA

inventions in an infringement case brought by the University of California (UC) against Eli Lilly & Company (Lilly) (21). Although some have referred to the *Lilly* case as a dramatic departure from previous rulings, (22) the court's decision flows logically from the *Amgen* and *Fiers* decisions and was necessary to promote the progress of biotechnology-related discovery. The *Lilly* case centered on a seven year patent battle over the DNA encoding human insulin. In 1977, researchers at UC cloned the rat insulin gene. After cloning the rat gene, UC filed a patent application claiming plasmids engineered with genes encoding a large genus of vertebrate insulins including human insulin. That patent issued to UC on March 24, 1987, for an invention entitled "Recombinant Bacterial Plasmids Containing Coding Sequences of Insulin Genes" (hereinafter the '525 patent) (23). In 1990, UC sued Lilly for infringing claims of the '525 patent as well as claims in an additional patent (24).

The Federal Circuit relied on the reasoning in *Fiers* to invalidate UC's claims that encompassed the gene encoding human insulin. The '525 patent specification contained a description of the isolation of the rat insulin mRNA, the synthesis and characterization of the rat insulin cDNA, a method of obtaining the human cDNA for insulin using a constructive example incorporating the same method used to obtain the rat cDNA, and the amino acid sequences of the human insulin A and B chains already known in the art. The court held, however, that this was not enough to adequately describe the cDNA encoding human insulin and other vertebrate and mammalian insulins (21). The most fundamental difference between *Lilly* and the *Amgen* and *Fiers* cases is that unlike the inventors in those cases, the UC inventors had actually isolated, cloned, and characterized a cDNA encoding rat insulin. Thus, the *Lilly* case presented the court with an opportunity to provide further guidance and address both whether the description of the rat insulin cDNA adequately described another species (the human insulin cDNA) and whether the description of the rat species adequately described a genus of vertebrate insulins.

In determining the validity of the species claim directed to DNA encoding human insulin, the court noted that, in addition to providing a description of the rat insulin cDNA, the specification provided a general method for obtaining the human cDNA along with the amino acid sequence of human insulin which was already known in the art. However, the court stated, "Whether or not it provides an enabling disclosure, it does not provide a written description of the cDNA encoding human insulin . . . . While the example provides a process for obtaining human insulin-encoding cDNA, there is no further information in the patent pertaining to that cDNA's relevant structural or physical characteristics; in other words, it thus does not describe human insulin cDNA (21)."

Requirements for Description of a Gene - The court provided further that mere knowledge of the human amino acid sequence coupled with a method of isolating the cDNA encoding that sequence does not adequately describe the specific cDNA claimed. The court invoked an obviousness analysis to support this holding. The court cited *In re Deuel* (25) and *In re Bell* (26) for the proposition that an amino acid sequence of a protein does not necessarily render particular DNA molecules encoding that protein obvious nor does disclosure of a method of isolating such DNA render the molecules themselves obvious. "Thus, a fortiori, a description that does not render a claimed invention obvious does not sufficiently describe that invention for purposes of § 112, ¶ 1 (21)."

In addressing whether the '525 patent disclosure contained an adequate written description to support genus claims encompassing cDNAs encoding vertebrate and mammalian insulins, the court noted that every species encompassed by a genus need not be specifically described; however, a description of a single species does not always constitute a description of a genus encompassing it. The court stated that "a

description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus (21)."

Describing A Gene By Function Describes a Wish, Not a Gene - Furthermore, the generic claim itself could not provide its own written description because it claimed the genus by function (cDNA encoding vertebrate or mammalian insulin).

> In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus. In claims to genetic material, however, a generic statement such as 'vertebrate insulin cDNA' or 'mammalian insulin cDNA,' without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function. It does not define any structural features commonly possessed by members of the genus that distinguish them from others. . . . A definition by function, as we have previously indicated, does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is (21).

The Impact of These Rulings - The *Lilly* case has been controversial. Many commentators believe the court overstated the written description requirement. Some suggest that the Federal Circuit's written description requirement for DNA inventions is at odds with the goal of encouraging innovation and discovery because it actually harms the inventor who has made the initial important discovery. However, allowing an inventor to claim more than he or she has actually invented has a tremendous detrimental effect on discovery efforts. Broadly asserted claims based on the discovery of a single gene or piece of a gene could potentially block off entire areas of research and development.

In addition, the *Lilly* case is consistent with the policy of promoting discovery efforts by providing exclusive rights to inventors. This point is clearly illustrated by considering how a decision to uphold the validity of UC's patent in *Lilly* would affect obviousness law. The court stated in *Lilly* that "a description that does not render a claimed invention obvious does not sufficiently describe that invention for purposes of § 112, ¶ 1 (21)." Thus, if there is no basis to conclude obviousness, even less so is there a basis to find a description sufficient to meet the written description requirement. This principle is highly significant in the present context because a determination that a description is sufficient to meet § 112 requirements for a set of claims would surely mean that this description, if published as a prior art reference, would make the invention encompassed by this same set of claims obvious. Thus, the court was in effect saying that a disclosure of the rat insulin gene sequence along with a sequence of the human insulin protein does not make the human insulin gene obvious. Had the court determined alternatively that UC's disclosure adequately described the human gene, thus making it obvious as well, all other researchers who might subsequently clone a gene and corresponding protein from any other species or variant thereof would be effectively blocked from obtaining patents on these molecules. This would

have a profound effect on the industry, especially those biotech companies that depend on the ability to obtain patents as a justification for funding. The enormous amount of time and money companies spend to study DNA and protein variants and their corresponding biological functions would potentially no longer be justified.

Some critics of the decision have suggested that it allows well-funded entities to take advantage of contributions by entities such as academic institutions. This would be accomplished by the commercial entity cloning the human gene using the animal gene disclosed by the academic institution as a probe. This concern is not well-founded given the current environment. Human libraries are readily available so it is not unduly burdensome for smaller entities to clone the human gene. Secondly, because virtually all subsequently cloned genes would be obvious over the first disclosed gene, any advocate of this position must be certain that their institution would be the first to clone any species of a particular gene.

The USPTO's Response to These Cases - The *Lilly* case has generated a large amount of interest among patent practitioners because of its potential impact on the ability to obtain and enforce patents claiming DNA and proteins. In June 1998, the USPTO responded to the *Lilly* decision by issuing a formal request for comments regarding an interim set of guidelines for examination of patent applications under the written description requirement (27). After considering written comments and conducting a public hearing, the Office issued another set of interim guidelines that apply to all technologies and again requested comments (28). Final guidelines were published in the Federal Register on January 5, 2001 (hereinafter "the Guidelines") (29).

While the Guidelines are an admirable attempt to promote consistent examination of applications by applying the law laid down by the Federal Circuit, they do not go far enough. Examiners should be encouraged to be aggressive with respect to making written description rejections in every circumstance where the decisions of the Federal Circuit justify such rejections (30). This affords applicants the opportunity to develop the law on written description through the *ex parte* appeal process. This route is far less expensive and far more satisfactory for both inventors and accused infringers than the painful process of *inter partes* litigation before a federal district court. Furthermore, this will enable the Federal Circuit to provide its guidance much more rapidly and at far less expense to those in the biotechnology industry.

The primary issue that the USPTO and patent practitioners have been struggling with relates to the acceptable scope of DNA or protein claims when the disclosure is a single species. Written description of a claimed genus may be satisfied through sufficient description of a representative number of species. It has never been the case in the chemical arts that every species need be described in a claimed genus. Nor has it been the case that a species falling within a genus but not specifically disclosed necessarily would be considered obvious over the genus.
In determining how to evaluate whether a genus is adequately described, the Guidelines state:

> A 'representative number of species' means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. On the other hand, there may be situations where one species adequately supports a genus. What constitutes a 'representative number' is an inverse function of the skill and

> knowledge in the art. Satisfactory disclosure of a 'representative number' depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed (29).

Thus, the USPTO has determined that what constitutes a "representative number" rests on a determination of variability within the genus. This is problematic especially when claiming a genus such as "vertebrate insulin cDNAs" because it is likely difficult to know whether there is "substantial variation" within a genus until most of the species included in the genus are actually cloned. Further, it is unclear what would amount to "substantial variation." There are numerous examples of proteins that differ by a single amino acid and yet have dramatically different properties. Thus, how to adequately describe a large genus will continue to be the subject of much debate. While the degeneracy of the genetic code and other factors often dictate that fairness to a patentee would not be achieved by limitation of the claim scope to a single species, the line must somehow be drawn to promote the policy of disclosing inventions and not research plans or goals. The following provides a discussion of the breadth of different types of claims involving DNA and proteins that have been allowed by the USPTO and demonstrates the need to make examiners aware of the public policy objectives that are served by aggressively making reasonable and fairly based rejections for lack of written description.

Hybridization and Homology Claims - Particularly troublesome are the types of claims wherein protein and/or nucleic acids are claimed based on homology or the ability to hybridize to a specific sequence under certain conditions coupled with a functional limitation. Numerous patents have been issued with homology-based or hybridization-based claims (30). The USPTO has suggested that these types of claims may be acceptable through the disclosure of a single species because all members of the genus have a specific structural identity with the reference compound and because of the limitation requiring that the stated compounds have a specific biological activity. This approach, however, merely substitutes one linguistic formulation ("percent identity," "homology," or "hybridizes under X conditions" coupled with a required biochemical property) for another linguistic formulation (encoding a particular class of proteins) found insufficient to satisfy the requirement for an adequate written description under the *Lilly* decision (31). While different sets of words have been used, the fact remains that no generic invention has been described.

In *Lilly*, the Federal Circuit held invalid claims directed to "vertebrate insulin cDNA" and "mammalian insulin cDNA" because neither genus was adequately described by the disclosure of a single species, rat insulin cDNA. This claim to DNA encoding a genus of homologous insulin proteins could not have been adequately supported by the disclosure of a single species coupled with a change of wording in the claim from "mammalian insulin" to "proteins at least X % identical to insulin." Further, the potential breadth of these types of claims is enormous which, of course, provides patentees with an incentive to obtain these claims. Lilly's comments to the interim written description guidelines provide an example wherein variants that are at least 95% identical to a protein having 300 amino acids are claimed (30). This genus would encompass any species having between one and fifteen amino acid changes to any of the 20 naturally occurring amino acids, at any location in the protein chain. This genus would potentially encompass thousands of trillions of chemical compounds. Even if billions of these proteins within this mass of thousands of trillions have the required biological activity, the structures of the active compounds are "needles in a haystack."

In *Lilly*, the court held that a genus of cDNAs could be described by recitation of structural features common to a substantial portion of the genus (21). Mere recitation of a biological property or function, without a disclosure of common structural elements was not enough. Homology or hybridization-based claims with functional limitations are generally not adequately described because among the enormous number of compounds falling with the scope of the claim, the typical specification provides no guidance as to what proportion of proteins within this threshold level of homology actually meet the functional limitations or what portion of the protein is important for activity and what is not. Thus, one is actually claiming an unknown number of compounds of unknown structures. As discussed previously, there are countless examples of a single amino acid change negatively impacting biological activity. If there is no structure-activity relationship disclosed and, thus, nothing to identify the claimed biologically active compounds by the structure of the active compounds or equivalent physical characteristics, then there is no written description for the claimed genus. At best, the typical specification demonstrates only that applicants have conceived that functional homologous compounds may exist. However, the specification does not demonstrate a completed conception of which homologous compounds are, in fact, the biologically active ones claimed. Absent evidence of such a completed conception of the invention, an applicant cannot be deemed to have had possession of the generic invention claimed at the time of filing and thus, the written description requirement is not satisfied.

Mechanism of Action Claims - These same defects which should lead the USPTO to reject homology or hybridization-based claims on written description grounds also apply to mechanism of action claims. These types of claims have been the focus of recent discussions due to the lawsuit filed by the University of Rochester in April 2000 against Searle. Rochester claims that Searle's blockbuster drug Celebrex® infringes its patent which covers methods of using compounds that inhibit the enzyme COX-2 as a way to alleviate pain (32). The USPTO may view these types of claims as similar to homology or hybridization-based claims in that the applicant is attempting to claim a genus wherein all members share the property of interacting with a target. However, these types of claims are even more problematic than homology-based claims because the characteristics causing the compound to bind a target are even less well-defined. This type of genus could potentially encompass both small molecules as well as large proteins. Thus, the disclosure of a single target, whether it be a receptor or an enzyme involved in a biological process such as pain transmission, does not adequately describe anything with respect to what binds to that receptor or enzyme.

CONCLUSION

This chapter offers a brief description of some of the more important issues facing patent attorneys working in the corporate pharmaceutical area. However, no article of this scope could begin to embrace all the issues practitioners face on an everyday basis. Biotechnology patent issues have been emphasized in this article due to the increasing importance of genomics in drug discovery.

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15. *In re Wertheim*, 541 F.2d 257, 263 (C.C.P.A. 1976).
16. 562 F.2d 1245, 1250 (C.C.P.A. 1977).
17. *See Lampi Corp. v. American Power Products, Inc.*, 228 F.3d 1365 (Fed. Cir. 2000) (description was sufficient to convey possession of a genus that included both identical and non-identical half-shells as part of a fluorescent light); *Gentry Gallery, Inc. v. Berkline Corp.*, 134 F.3d 1473 (Fed. Cir. 1998) noting that description of a species does not always constitute a description of a genus of which it is a part, the court held that claims directed to sectional sofas where the location of the recliner controls was not limited to the console were invalid because such claims were inadequately described); *Tronzo v. Biomet, Inc.*, 156 F.3d 1154 (Fed. Cir. 1998) (court considered the conical shape to be an essential element and thus, those claims omitting that limitation were invalid as not supported by the specification); *Fiers*, 984 F.2d 1164; *Lilly*, 119 F.3d 1559.
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abacavir sulfate	antiviral	1999	35, 333
acarbose	antidiabetic	1990	26, 297
aceclofenac	antiinflammatory	1992	28, 325
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
adamantanium bromide	antiseptic	1984	20, 315
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical antiinflammatory	1985	21, 323
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
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
amprenavir	antiviral	1999	35, 334
amrinone	cardiotonic	1983	19, 314
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
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
APSAC	thrombolytic	1987	23, 326

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aranidipine	antihypertensive	1996	32, 306
arbekacin	antibiotic	1990	26, 298
argatroban	antithromobotic	1990	26, 299
arglabin	anticancer	1999	35, 335
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
atorvastatin calcium	dyslipidemia	1997	33, 328
atosiban	preterm labor	2000	36, 297
atovaquone	antiparasitic	1992	28, 326
auranofin	chrysotherapeutic	1983	19, 314
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
balsalazide disodium	ulcerative colitis	1997	33, 329
bambuterol	bronchodilator	1990	26, 299
barnidipine HCl	antihypertensive	1992	28, 326
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
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
bicalutamide	antineoplastic	1995	31, 338
bifemelane HCl	nootropic	1987	23, 329
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
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

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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
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
cinitapride	gastroprokinetic	1990	26, 301
cinolazepam	hypnotic	1993	29, 334
ciprofibrate	hypolipidemic	1985	21, 326
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
cyclosporine	immunosuppressant	1983	19, 317
cytarabine ocfosfate	antineoplastic	1993	29, 335
dalfopristin	antibiotic	1999	35, 338
dapiprazole HCl	antiglaucoma	1987	23, 332
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

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denileukin diftitox	anticancer	1999	35, 338
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
dexfenfluramine	antiobesity	1997	33, 332
dexibuprofen	antiinflammatory	1994	30, 298
dexmedetomidine hydrochloride	sedative	2000	36, 301
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
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
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
duteplase	anticougulant	1995	31, 342
ebastine	antihistamine	1990	26 302
ebrotidine	antiulcer	1997	33, 333
ecabet sodium	antiulcerative	1993	29, 336
efavirenz	antiviral	1998	34, 321
efonidipine	antihypertensive	1994	30, 299
egualen sodium	antiulcer	2000	36, 303
emedastine difumarate	antiallergic/antiasthmatic	1993	29, 336
emorfazone	analgesic	1984	20, 317
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
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
exemestane	anticancer	2000	36, 304
exifone	nootropic	1988	24, 302
factor VIIa	haemophilia	1996	32, 307
factor VIII	hemostatic	1992	28, 330
fadrozole HCl	antineoplastic	1995	31, 342
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
flurithromycin ethylsuccinate	antibiotic	1997	33, 333
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
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
formestane	antineoplastic	1993	29, 337
formoterol fumarate	bronchodilator	1986	22, 321
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
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
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
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
ibandronic acid	osteoporosis	1996	32, 309
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
ibutilide fumarate	antiarrhythmic	1996	32, 309
idarubicin HCl	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
iloprost	platelet aggreg. inhibitor	1992	28, 332
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
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
lamotrigine	anticonvulsant	1990	26, 304
lanoconazole	antifungal	1994	30, 302
lanreotide acetate	acromegaly	1995	31, 345
lansoprazole	antiulcer	1992	28, 332
latanoprost	antiglaucoma	1996	32, 311

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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
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
masoprocol	topical antineoplastic	1992	28, 333
maxacalcitol	vitamin D	2000	36, 310
mebefradil hydrochoride	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

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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
mifepristone	abortifacient	1988	24, 306
miglitol	antidiabetic	1998	34, 325
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, 326
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
nabumetone	antiinflammatory	1985	21, 330
nadifloxacin	topical antibiotic	1993	29, 340
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306
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

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nefazodone	antidepressant	1994	30, 305
neflinavir mesylate	antiviral	1997	33, 340
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
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
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
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
olopatadine HCl	antiallergic	1997	33, 340
omeprazole	antiulcer	1988	24, 308
ondansetron HCl	antiemetic	1990	26, 306
orlistat	antiobesity	1998	34, 327
ornoprostil	antiulcer	1987	23, 339
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
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
pantoprazole sodium	antiulcer	1995	30, 306
paricalcitol	vitamin D	1998	34 327
parnaparin sodium	anticoagulant	1993	29, 342
paroxetine	antidepressant	1991	27, 331
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pegaspargase	antineoplastic	1994	30, 306

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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
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
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
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
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
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

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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
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
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin HCl	antibacterial	1992	28, 335
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin HCl	hyperphenylalaninemia	1992	28, 336
saquinavir mesylate	antiviral	1995	31, 349
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
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
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
teicoplanin	antibacterial	1988	24, 311
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
temozolomide	anticancer	1999	35, 349
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
tiopronin	urolithiasis	1989	25, 318
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
tosufloxacin tosylate	antibacterial	1990	26, 310
trandolapril	antihypertensive	1993	29, 348
tretinoin tocoferil	antiulcer	1993	29, 348
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
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
valrubicin	anticancer	1999	35, 350
valsartan	antihypertensive	1996	32, 320
venlafaxine	antidepressant	1994	30, 312
verteporfin	photosensitizer	2000	36, 312
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
vinorelbine	antineoplastic	1989	25, 320
voglibose	antidiabetic	1994	30, 313
xamoterol fumarate	cardiotonic	1988	24, 312
zafirlukast	antiasthma	1996	32, 321
zalcitabine	antiviral	1992	28, 338
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19, 319
mifepristone		1988	24, 306
lanreotide acetate	ACROMEGALY	1995	31, 345
nitrefazole	ALCOHOL DETERRENT	1983	19, 322
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
flupirtine maleate		1985	21, 328
fosfosal		1984	20, 319
ketorolac tromethamine		1990	26, 304
meptazinol HCl		1983	19, 321
mofezolac		1994	30, 304
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
suplatast tosilate		1995	31, 350
tazanolast		1990	26, 309
lodoxamide tromethamine	ANTIALLERGIC	1992	28, 333
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
moricizine hydrochloride		1990	26, 305
nifekalant HCl		1999	35, 344
pilsicainide hydrochloride		1991	27, 332
pirmenol		1994	30, 307
tiracizine hydrochloride		1990	26, 310
celecoxib	ANTIARTHRITIC	1999	35, 335
meloxicam		1996	32, 312
leflunomide		1998	34, 324
rofecoxib		1999	35, 347
amlexanox	ANTIASTHMATIC	1987	23, 327
emedastine difumarate		1993	29, 336
ibudilast		1989	25, 313
levalbuterol HCl		1999	35, 341
montelukast sodium		1998	34, 326
pemirolast potassium		1991	27, 331
seratrodast		1995	31, 349
zafirlukast		1996	32, 321
zileuton		1997	33, 344
ciprofloxacin	ANTIBACTERIAL	1986	22, 318
enoxacin		1986	22, 320
fleroxacin		1992	28, 331
norfloxacin		1983	19, 322
ofloxacin		1985	21, 331
pefloxacin mesylate		1985	21, 331
pranlukast		1995	31, 347
rifabutin		1992	28, 335
rifapentine		1988	24, 310
rufloxacin hydrochloride		1992	28, 335
teicoplanin		1988	24, 311

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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		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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
temocillin disodium		1984	20, 323
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
alitretinoin	ANTICANCER	1999	35, 333
arglabin		1999	35, 335
bexarotene		2000	36, 298
denileukin diftitox		1999	35, 338
exemestane		2000	36, 304
gemtuzumab ozogamicin		2000	36, 306
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
vigabatrin		1989	25, 319
zonisamide		1989	25, 320
bupropion HCl	ANTIDEPRESSANT	1989	25, 310
citalopram		1989	25, 311
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
fomepizole	ANTIDOTE	1998	34, 323
dolasetron mesylate	ANTIEMETIC	1998	34, 321
granisetron hydrochloride		1991	27, 329
ondansetron hydrochloride		1990	26, 306
nazasetron		1994	30, 305
ramosetron		1996	32, 315
tropisetron		1992	28, 337

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
fenticonazole nitrate	ANTIFUNGAL	1987	23, 334
fluconazole		1988	24, 303
itraconazole		1988	24, 305
lanoconazole		1994	30, 302
naftifine HCl		1984	20, 321
oxiconazole nitrate		1983	19, 322
terbinafine hydrochloride		1991	27, 334
terconazole		1983	19, 324
tioconazole		1983	19, 324
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
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
unoprostone isopropyl ester		1994	30, 312
acrivastine	ANTIHISTAMINE	1988	24, 295
astemizole		1983	19, 314
azelastine HCl		1986	22, 316
ebastine		1990	26, 302
cetirizine HCl		1987	23, 331
levocabastine hydrochloride		1991	27, 330
loratadine		1988	24, 306
mizolastine		1998	34, 325
setastine HCl		1987	23, 342

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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
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
budralazine		1983	19, 315
bunazosin HCl		1985	21, 324
candesartan cilexetil		1997	33, 330
carvedilol		1991	27, 323
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
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

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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
tiamenidine HCl		1988	24, 311
tilisolol hydrochloride		1992	28, 337
trandolapril		1993	29, 348
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
tenoxicam		1987	23, 344
zaltoprofen		1993	29, 349
fisalamine	ANTIINFLAMMATORY,	1984	20, 318
osalazine sodium	INTESTINAL	1986	22, 324
alclometasone dipropionate	ANTIINFLAMMATORY,	1985	21, 323
aminoprofen	TOPICAL	1990	26, 298

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
betamethasone butyrate propionate		1994	30, 297
butyl flufenamate		1983	19, 316
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
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
amsacrine	ANTINEOPLASTIC	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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
formestane		1993	29, 337
fotemustine		1989	25, 313
gemcitabine HCl		1995	31, 344
idarubicin hydrochloride		1990	26, 303
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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
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
clopidogrel hydrogensulfate		1998	34, 320
cloricromen		1991	27, 325
enoxaparin		1987	23, 333
eptifibatide		1999	35, 340
ethyl icosapentate		1990	26, 303
ozagrel sodium		1988	24, 308
indobufen		1984	20, 319
picotamide		1987	23, 340
limaprost		1988	24, 306
tirofiban hydrochloride		1998	34, 332
flutropium bromide	ANTITUSSIVE	1988	24, 303
levodropropizine		1988	24, 305
benexate HCl	ANTIULCER	1987	23, 328
dosmalfate		2000	36, 302
ebrotidine		1997	33, 333
ecabet sodium		1993	29, 336

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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, 326
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
abacavir sulfate	ANTIVIRAL	1999	35, 333
amprenavir		1999	35, 334
cidofovir		1996	32, 306
delavirdine mesylate		1997	33, 331
didanosine		1991	27, 326
efavirenz		1998	34, 321
famciclovir		1994	30, 300
fomivirsen sodium		1998	34, 323
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
valaciclovir HCl		1995	31, 352
zalcitabine		1992	28, 338
zanamivir		1999	35, 352
zidovudine		1987	23, 345
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
mexazolam		1984	20, 321
tandospirone		1996	32, 319
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
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
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefozopran HCL	CEPHALOSPORIN, INJECTABLE	1995	31, 339
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
drospirenone	CONTRACEPTIVE	2000	36, 302
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

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
alglucerase	ENZYME	1991	27, 321
erdosteine	EXPECTORANT	1995	31, 342
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
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
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
fluvastatin		1994	30, 300
meglutol		1983	19, 321
ronafibrate		1986	22, 326
modafinil	IDIOPATHIC HYPERSOMNIA	1994	30, 303

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bucillamine	IMMUNOMODULATOR	1987	23, 329
centoxin		1991	27, 325
thymopentin		1985	21, 333
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 mofetil		1995	31, 346
tacrolimus		1993	29, 347
defeiprone	IRON CHELATOR	1995	31, 340
alosetron hydrochloride	IRRITABLE BOWEL SYNDROME	2000	36, 295
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
gadoversetamide	MRI CONTRAST AGENT	2000	36, 304
telmesteine	MUCOLYTIC	1992	28, 337
interferon ß-1a	MULTIPLE SCLEROSIS	1996	32, 311
interferon ß-1b		1993	29, 339
glatiramer acetate		1997	33, 334
afloqualone	MUSCLE RELAXANT	1983	19, 313
cisatracurium besilate		1995	31, 340

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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
clospipramine hydrochloride	NEUROLEPTIC	1991	27, 325
nemonapride		1991	27, 331
olanzapine		1996	32, 313
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
fasudil HCL	NEUROPROTECTIVE	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
bromfenac sodium	NSAID	1997	33, 329
lornoxicam		1997	33, 337
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
verteporfin	PHOTOSENSITIZER	2000	36, 312

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
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
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24, 297
nafamostat mesylate		1986	22, 323
adrafinil	PSYCHOSTIMULANT	1986	22, 315
finasteride	5α-REDUCTASE INHIBITOR	1992	28, 331
surfactant TA	RESPIRATORY SURFACTANT	1987	23, 344
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

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Lyme disease	VACCINE	1999	35, 342
clobenoside	VASOPROTECTIVE	1988	24, 300
maxacalcitol	VITAMIN D	2000	36, 310
paricalcitol		1998	34, 327
doxercalciferol	VITAMIN D PROHORMONE	1999	35, 339
prezatide copper acetate	VULNERARY	1996	32, 314
cadexomer iodine	WOUND HEALING AGENT	1983	19, 316
epidermal growth factor		1987	23, 333