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This article discusses in silico methods that employ receptor-based modeling, highlighting some new directions and ideas in the field of GPCRs.

I want a new drug: G-protein-coupled receptors in drug development

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Huey Lewis and the News summed it up nicely in their 1980s hit record: 'I want a new drug, one that won't make me sick, one that won't make me crash my car, or make me feel three feet thick'.
The song could be an anthem for drug discovery in the pharmaceutical industry. We all want new and better drugs with fewer side effects, which are effective for combating the major diseases of our time: cancer, heart disease, obesity and autoimmune diseases. How do we get these new drugs? There are currently some new ideas in drug discovery, centered on that staple diet of the pharmaceutical industry, the G-protein-coupled receptor (GPCR) superfamily. *In silico* methods, employing receptor-based modeling, offer a more rational approach in the design of drugs targeting GPCRs. These approaches can be used to understand receptor selectivity and species specificity of drugs that interact with GPCRs. In addition, there are various novel approaches, such as the design and potential utility of drugs that target more than one GPCR ('dual specificity' drugs).

G-protein-coupled receptors (GPCRs) are ubiquitous throughout the plant and animal kingdoms [1–4]. They have a major role in regulating the overall homeostasis of complex organisms, such as mammals, but are also found in primitive species such as *Dictyostelium* (slime mold) [5] and yeast [6]. The GPCR superfamily is diverse [7,8], and sequencing of the human genome has revealed >850 genes that encode them [9,10]. The diversity of the GPCRs is matched equally by the variety of ligands that activate them, including odorants [11], taste ligands [12], light [13], metals [14], biogenic amines [15], fatty acids [16], amino acids [17], peptides [18], proteins [19], nucleotides [20], lipids [21–23], Krebs-cycle intermediates [24] and steroids [25].

GPCRs are one of the most important drug targets for the pharmaceutical industry, and >30% of all marketed therapeutics act on them [26] (Figure 1). However, these drugs target only ~30 members of the family, mainly biogenic amine receptors, so there is enormous potential within the pharmaceutical industry to exploit the remaining family members, including the >100 orphan receptors [27] for which no existing ligands have so far been identified.

Traditional drug development follows a standard path to uncover new drugs. The first step in this process generally encompasses a HTS approach against the drug target of choice using

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Sabine Schlyer was born in Nuremberg, Germany, and studied German literature at the University of Regensburg. She received her PhD in Chemistry from the University of California, Davis, USA, where she studied nonequilibrium reaction dynamics under Professor Susan Tucker. As a postdoctoral researcher at Berlex Biosciences and Sandia National Laboratories, she worked on the development and application of scoring functions. She joined Berlex Biosciences as a scientist in 2002, where her work has mainly been focused on modeling of G-protein-coupled receptors (GPCRs). She currently works in close collaboration with the group of Dr Vaidehi Nagarajan at the City of Hope on GPCR structure prediction, as well as virtual screening of GPCRs.

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Richard Horuk was born in Lebenstedt, Germany and studied Biology at Trent Polytechnic in the UK. In 1980, he obtained a PhD in Biochemistry from Birkbeck College, University of London, UK, studying insulin structure–function under Professor Sir Thomas Blundell. In 1981, he worked as a postdoctoral fellow in the laboratory of Dr Martin Rodbell at NIH. In 1983, he joined Dr Jerrold Olefsky at the University of California, San Diego, USA, to pursue further studies on insulin action. In 1986, Horuk joined the Dupont Company, where he undertook research on interleukin-1 (IL-1) receptors, and was the first to discover both the type II IL-1 receptor and the soluble IL-1 receptor. In 1991, Horuk joined Genentech and started a research program on chemokines and their receptors. He was the first to discover that the Duffy antigen, a portal of entry for the malarial parasite *Plasmodium vivax*, was also a chemokine receptor. These findings were the first demonstration that GPCRs could act as vehicles of entry for pathogens, and set the stage for findings that showed that chemokine receptors were HIV-1 co-receptors. Horuk joined Berlex BioSciences in 1994 and is presently a Principal Scientist in the Department of Immunology, where his research interests are centered on GPCRs. Horuk is a member of the American Society for Biochemistry and Molecular Biology and the American Association of Immunologists, and he is an Editor of the *Journal of Biological Chemistry*.

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Top 20 drugs sales (2003) in US$ billions

Drug	Target	Sales
Lipitor	enzyme	5.8
Zocor	enzyme	4.4
Prevacid	enzyme	4.0
Procrit	agonist	3.3
Zyprexa	GPCR	3.2
Epogen	agonist	3.1
Nexium	enzyme	3.1
Zoloft	GPCR	2.9
Celebrex	enzyme	2.6
Neurontin	analgesic	2.4
Advair diskus	GPCR	2.3
Plavix	GPCR	2.2
Norvasc	ion channel	2.2
Effexor XR	SSRI	2.1
Pravachol	enzyme	2.0
Risperdal	GPCR	2.0
Oxycontin	GPCR	1.9
Fosamax	osteoporosis	1.8
Protonix	GPCR	1.8
Vioxx	enzyme	1.8

FIGURE 1

The top 20 prescription drugs based on total sales in 2003. Here, drugs that target G-protein-coupled receptors (GPCRs) are highlighted in red (source: San Francisco Chronicle, 1st October 2005, page A9).

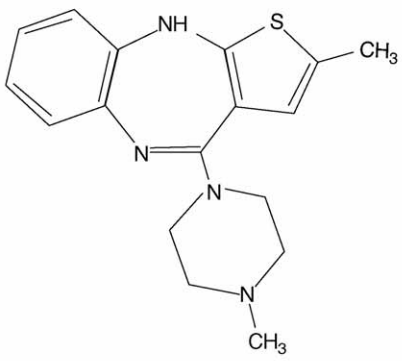
inhouse small-molecule compound libraries. Although HTS forms the central core of drug-finding programs in the pharmaceutical industry and has been extremely successful, it is now often supplemented by *in silico* methods to maximize the probability of finding attractive novel lead compounds. Structure-based *in silico* approaches have been extremely challenging for GPCRs, given the lack of structural information; in fact, only one tertiary structure for a GPCR, bovine rhodopsin, has so far been reported [28]. However, the use of *in silico* methods employing receptor-based design strategies offers a promising parallel approach (to HTS) to overcome the current limitations in the rational design of drugs targeting GPCRs.

Drug selectivity for GPCRs

Drug selectivity whereby a small molecule developed against one GPCR displays off-target effects by crossreacting with other members of the family is a well-recognized problem in the development of drugs targeting GPCRs. For example, β-blockers such as atenolol were mainly developed as cardiovascular agents based on their ability to regulate high blood pressure and angina by antagonizing β_1-adrenoceptors [29]. Unfortunately, β-blockers also crossreact with β_2-adrenoceptors found in smooth muscle and in the lungs [30]. Thus, they can give rise to side effects that can be life threatening in severe asthmatics. Further examples of drug selectivity issues are provided by α_1-adrenoceptor and α_2- adrenoceptors. These receptors are highly related in sequence, making it difficult to design small-molecule antagonists to specifically block one and not the other. Most of the antagonists designed to target α_1-adrenoceptors still have residual activity on α_2-adrenoceptors [31]. Nevertheless, even when a compound has only weak activity for a related receptor, it can give rise to unwanted side-effects when used therapeutically in humans.

Antagonists of peptidergic receptors, such as the chemokine receptor family, that are highly related to other rhodopsin family members also show crossreactivity with other GPCRs. This appears to be especially true for crossreactivity of chemokine receptor antagonists with biogenic amine receptors. The piperazine-based CCR5 inhibitors produced by Schering–Plough are reported to crossreact with muscarinic receptors [32]. The CCR2 spiropiperidine inhibitor series developed by Roche crossreacts with the 5-hydroxytryptamine (5-HT) receptor 5-HT_{1A} and the α_1-adrenoceptor [33], and the CCR2 inhibitor produced by SmithKline Beecham crossreacts with 5-HT receptors [34]. The 4-hydroxypiperidine CCR1 inhibitor series produced by Berlex shows crossreactivity with several biogenic amine receptors, including dopamine and muscarinic receptors [35]. The CCR5 inhibitors developed by Merck are derived from templates identified in their tachykinin receptor antagonist program [36,37]. Surprisingly, as shown by these examples, the selectivity of chemokine receptor antagonists with members of the biogenic amine subclass of receptors (adrenoceptors and muscarinic, 5-HT and dopamine receptors) appears to present more of a potential problem than with other peptide ligand GPCRs. Because selectivity issues can limit the potential therapeutic use of these receptors, a more detailed understanding of receptor–ligand interactions is necessary to eliminate undesired activities while maintaining potency for the chemokine receptor target.

However, as we will see later (see section on dual receptor antagonists), drugs that exhibit GPCR promiscuity can, in some circumstances, be desirable and there are examples of drugs that target more than one GPCR, which is therapeutically advantageous. A good example of this is the antipsychotic drug olanzapine (Zyprexa®), which was developed by Lilly to compete in the central nervous system market with the rival drug clozapine (Clozaril®), marketed by Novartis. Although both drugs were initially developed to target dopamine D_2 receptors, olanzapine binds with high affinity to >12 GPCRs [38] (Figure 2). At first glance, this broad range of activities might seem to limit the



Receptor	Binding affinity (nM)
5-HT_{2A}	4
5-HT_{2B}	12
5-HT_{2C}	11
5-HT_3	57
dop D_1	31
dop D_2	11
dop D_4	27
musc M_1	1.9
musc M_2	18
musc M_3	25
musc M_4	13
adr a_1	19
adr a_2	230
hist H_1	7

FIGURE 2

Specificity of drugs that target G-protein-coupled receptors (GPCRs). The structure of olanzapine is shown. It is a promiscuous GPCR ligand that binds to several biogenic amine receptors with high affinity. Modified, with permission, from Ref. [38].

therapeutic usefulness of olanzapine but that has not been the case and it is, in fact, one of the most successfully marketed drugs of the past few years. In this case, the ability of the drug to block 5-HT and dopamine receptors is probably an advantage because it is thought that both receptors have a role in schizophrenia.

Species specificity for drugs targeting GPCRs

One of the more challenging problems in drug development stems from the fact that potent small-molecule inhibitors for a specific human GPCR can very often turn out to have poor affinity for the equivalent non-human receptors. There are numerous examples in the literature. For example, the triarylpyrrole glucagon receptor antagonist L-168,049, which has significant activity for the human glucagon receptor (IC_{50} = 1.3 nM), has very weak affinity for the rat, guinea pig and rabbit glucagon receptors ($IC_{50} > 1$ μM) [39]. Other examples are the neurokinin-1 receptor antagonist CP-96,345, which has a 90-fold higher binding affinity for the human versus the rat receptor [40] and the non-peptide benzodiazepine-based antagonist L365260, which has a 20-fold higher binding affinity for the human versus the canine cholecystokinin CCK_2 receptors [41].

Two further examples of species specificity are exemplified by recent work with the adenosine A_3 and the gonadotropin-releasing hormone receptor (GnRH). In the first example, several pyrazole pyrimidine compounds were recently identified as potent antagonists of the human A_3 receptor (K_i = 40 pM) but were almost inactive on rat A_3 receptors ($K_i > 1$ μM) [42]. In the second example, several quinolone antagonists of the GnRH receptor showed strict species specificity for human versus rhesus and rat, with K_is of 3.4 nM, 87 nM and >10 μM, respectively [43]. The human and rhesus receptors differ by only eight residues, and mutation of two of these in rhesus GnRH – P203S, and V300L – converts it into a high-affinity receptor for the compound. Mutation of these two residues in rat, together with T24M and E208Q, gives the mutated receptor an affinity of 240 nM, which is greater than a 40-fold increase over the wild-type (WT) receptor.

Drug substances that are limited in specificity to human target proteins can be problematic during drug development because they are difficult to test in surrogate animal efficacy models. Without efficacy data, it can become very difficult to justify further development of the drug, given the considerable risks and costs involved. Sometimes the species specificity of a drug can be exploited to understand better how it interacts with the receptor. For example, the antihypertensive agent losartan (Cozaar®) is a potent angiotensin II (AT_1) receptor antagonist that was originally developed to replace angiotensin-converting enzyme (ACE) inhibitors to treat hypertension. It binds to rat AT_1 receptors with a K_i of 2.2 nM but binds poorly to frog AT_1 receptors, with an affinity almost 100,000 times lower ($K_i \geq 50$ μM) [44] (Figure 3a). Mutation of 13 specific residues in the frog receptor imbued it with high affinity for losartan (K_i = 2 nM), thus identifying the specific receptor-binding site for the drug. Similarly, the human and rat 5-HT_{1B} receptors share 93% sequence identity but differ significantly in binding to many drugs (Figure 3b) [45]. Replacement of a single amino acid in the human receptor – namely threonine 355 – with the corresponding asparagine found in rodent 5-HT_{1B} receptors, renders the pharmacology of the receptors essentially identical. For example, (−)-propranolol binds to the rat 5-HT_{1B} receptor with an affinity of 0.06 nM, to the human 5-HT_{1B} WT receptor with an affinity of 10.9 nM and to the human 5-HT_{1B} Thr355Asn mutant receptor with an affinity of 0.013 nM [45]. As we shall see later, identification of the ligand-binding pocket in GPCRs is a prerequisite for rational drug design, and *in silico* approaches often need to be combined with known experimental data to arrive at an accurate description of the binding site.

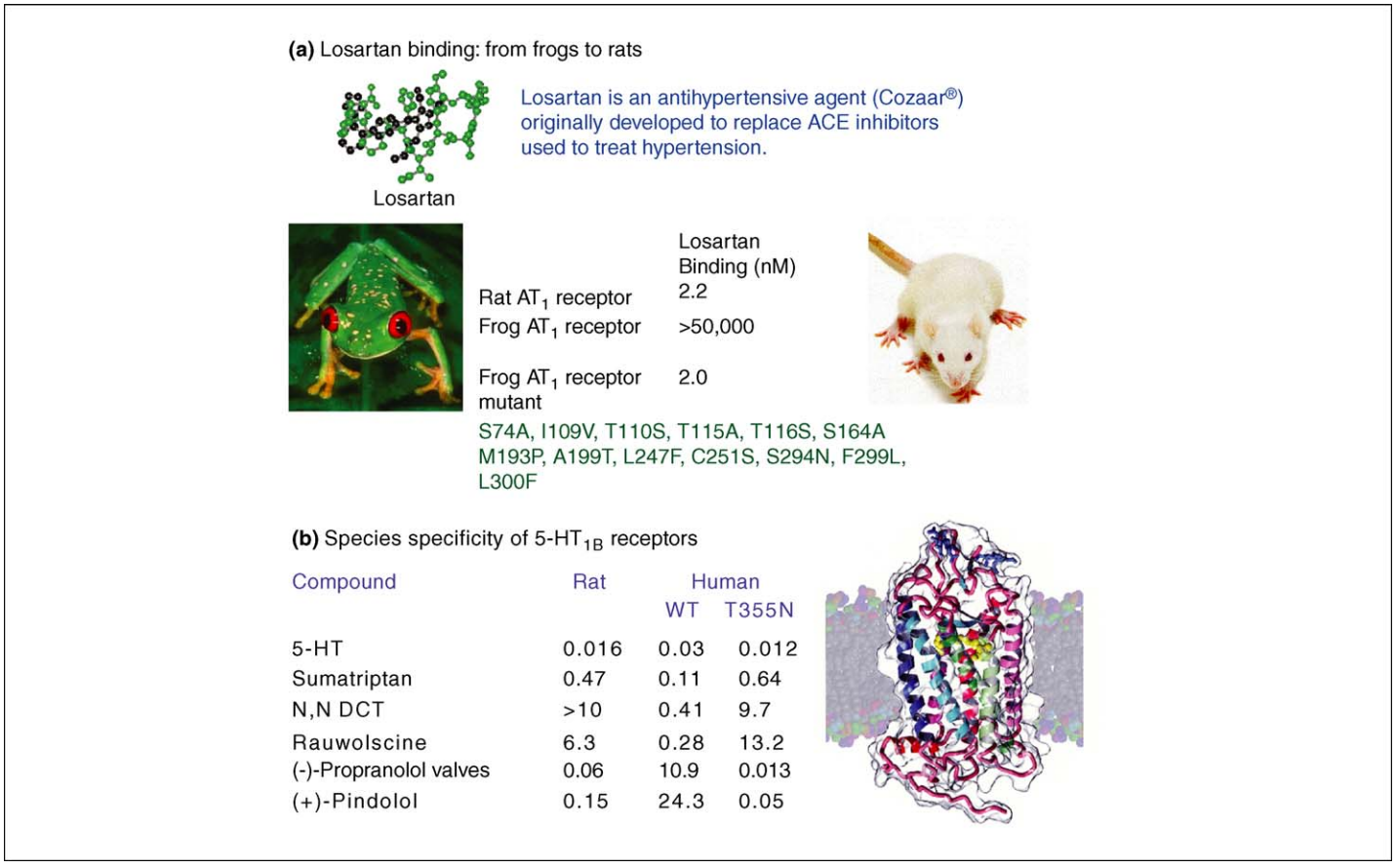


FIGURE 3

Species specificity of drugs that target G-protein-coupled receptors (GPCRs). (a) Losartan binds with high affinity to the rat AT_1 receptor but with low affinity to the frog AT_1 receptor. **(b)** A single amino acid change, Thr355Asn, explains the pharmacological distinctions between the rat and human 5-HT_{1B} receptors. Modified, with permission, from Ref. [45].

An interesting approach to circumvent the problem of species specificity is to generate transgenic animals in which the rodent receptor is replaced by the human protein. One of the CCR3 antagonists produced by Merck binds with high affinity to the human CCR3 receptor but does not bind to the mouse receptor. Merck created transgenic mice in which the mouse receptor was replaced with the human CCR3 gene (Figure 4). The humanized mice were then shown to respond to the CCR3 antagonist, which inhibited the migration of mouse eosinophils in a chemotaxis assay [46]. These studies, of course, assume that the human receptor expressed in these transgenic animals functions biologically in an identical manner to the endogenous mouse receptor. A simpler approach would be to use primate models, if they exist; however, the major drawback here is the expense.

In silico methods of drug discovery for GPCRs

Molecular modeling offers a useful adjunct to the more traditional methods of drug discovery discussed above, and if good models can be generated, they might be helpful in understanding the problems of selectivity and species specificity. Theoretical prediction of the 3D structure of a molecule using state-of-the-art modeling techniques can, in some cases, rival that of the experimental approach, X-ray crystallography. This is especially true for GPCRs, for which only one X-ray structure, that of bovine rhodopsin, exists. Two distinct computational methods have been used in predicting the 3D structure of GPCRs: homology modeling and *de novo* structure prediction. These approaches have found increasing vogue in the drug discovery process for GPCRs, and we will illustrate their utility here by reference to a variety of biogenic amine and peptidergic receptors.

Homology modeling

All homology models are based on the crystal structure of bovine rhodopsin [28] and are constructed in several stages (Box 1). Numerous reports of GPCR homology models are described in the literature, and if we believe all of the success stories, it is now just a trivial exercise to rapidly build accurate GPCR homology models – or is it? For the purposes of our discussion, we will take the best-case scenario, in which a GPCR has high sequence homology to bovine rhodopsin. For such a GPCR, the overall structure of the protein, and possibly the location of the small-molecule binding site within the transmembrane domain, might be predicted fairly accurately but small differences in the exact helical arrangement might greatly affect the accuracy of the small-molecule binding site. Even small errors in crucial receptor regions can render these models almost worthless for structure-based drug design. For many class A GPCRs, which have low sequence homology with bovine rhodopsin, homology models therefore cannot accurately predict the helical topology, and even less so the

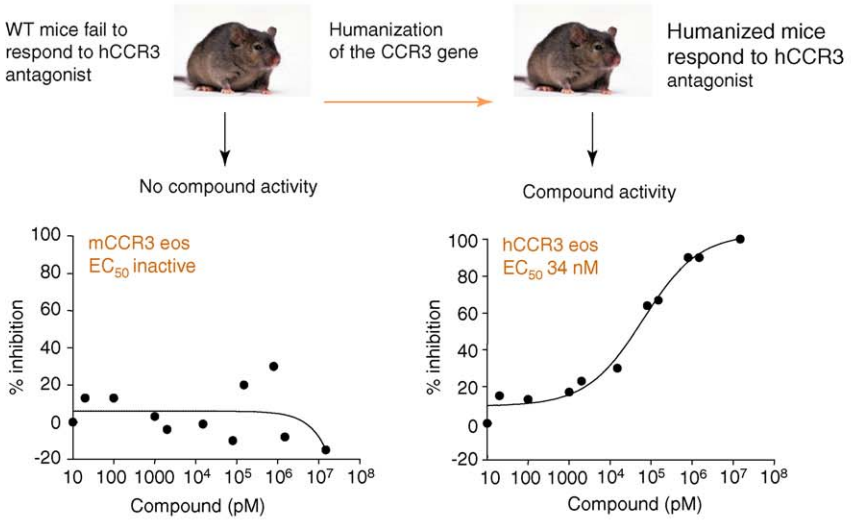


FIGURE 4

Species crossreactivity of CCR3. A human CCR3 antagonist does not elicit chemotactic responses in wild-type (wt) mice but crossreacts with transgenic mice expressing human CCR3. Modified, with permission, from Ref. [46].

topology of the small-molecule binding site. The pros and cons of homology modeling for several GPCRs are discussed below.

How accurate are homology models?

This is an important question because it will define the usefulness of the model to explain small-molecule structure–activity relationships, as well as its utility in the virtual screening of compound libraries to identify new leads. Homology modeling has been successful for some classes of receptors, such as the biogenic amine receptors. For these receptors, most homology models correctly identify the agonist and antagonist binding site within the seven-transmembrane domain and are able to predict important hydrogen-bond interactions between the small molecule and key residues in the receptor, such as the conserved Asp in transmembrane (TM) domain 3. However, it is less clear what impact homology models will have in structure-based design for peptidergic receptor targets or other classes of rhodpsin-like GPCRs. Two recent studies have addressed this question in distinctly different ways, and it is worthwhile to examine each approach and the conclusions drawn.

In the first study, the authors examined the impact of the crystal structure of bovine rhodopsin on attempts to build homology models of peptidergic GPCRs such as the CCK_1 receptor [47]. Comparison of the CCK_1 receptor model with the structure of rhodopsin revealed that, although these proteins shared several common features, such as the conserved (E)DRY box and NPXXY motifs, differences in primary sequences resulted in marked changes between the two 3D structures [47]. For example, TM3 of the CCK_1 receptor exhibits two kinks, owing to proline and glycine residues in the CCK_1 receptor sequence, instead of a regular α-helix, as seen in rhodopsin. By contrast, TM1 of the CCK_1 receptor is a regular α-helix, owing to the absence of proline residues, whereas Pro53 in rhodopsin bends this helix more inwards. Lastly, TM7 adopts a more regular α-helical structure in the CCK_1 receptor than it does in rhodopsin. These differences in helical packing result in a different orientation of the residues involved in protein–ligand interactions in the binding site.

The accuracy of the rhodopsin-derived CCK_1 receptor model was tested by asking whether the natural ligand CCK could be docked into the modeled structure in a configuration that agreed with the experimentally established interaction sites between CCK and the CCK_1 receptor. Docking of the ligand CCK into the model of CCK_1 revealed some major problems with ligand binding that required adjustments to be made to the rhodopsin-derived CCK_1 receptor model. One problem was that extracellular loop (ECL) 2 of

BOX 1

G-protein-coupled receptor modeling approaches

Homology models

The first step in building a homology model involves aligning the protein of interest to one or more nonredundant homologous sequences and evaluating the alignment using a scoring matrix. Subsequently, the transmembrane domains (TMs) are determined by identifying conserved residues between the query protein and bovine rhodopsin. The individual helices are oriented with respect to each other and to the membrane, using rhodopsin as a template for the helical backbone. Side chains in common with the rhodopsin template are copied directly from rhodopsin; differing side chains are added from a side chain library according to known side chain preferences, followed by extensive energy minimization to eliminate steric clashes. The extracellular and intracellular domains generally have very low homology to the corresponding domains in rhodopsin, and therefore alternative techniques have been developed to model the loop regions and termini of G-protein-coupled receptors (GPCRs). The resulting structures are subsequently refined in a stepwise process using minimization techniques, initially keeping the helix backbone atoms fixed and only removing side chain close contacts. Frequently, molecular dynamics simulations are carried out, again fixing the α-carbons of the TM, to reach a thermally equilibrated state; this is generally followed by another minimization procedure, in which all atoms are allowed to move. The quality of the final structure is validated with various tools that analyze protein structural motifs.

the modeled receptor was buried within the receptor helical bundle (based on the closed loop structure of rhodopsin) and was thus covered by the amino-terminal domain of the receptor, which prevented the ligand from binding to ECL2. To circumvent this problem, the first 29 amino-terminal residues of CCK_1 had to be removed, so that the ECL2 could present its binding domain to the ligand. When the ligand was docked into this modified CCK_1 receptor, its carboxy-terminal amide came close to Asn333 of the CCK_1 receptor but remained at a noninteracting distance. The side chains of crucial amino acids in the CCK_1 ligand-binding site were not positioned to interact with their counterparts on CCK, and none of the residues of CCK known to have a major role in the affinity and activity of CCK could pair directly with amino acids of the rhodopsin-derived CCK_1 model. The model had to be extensively refined to enable crucial residues of the receptor to interact with their counterparts on CCK.

However, even with these changes, many crucial residues in the ligand-binding site of the CCK_1 receptor remained at noninteracting distances from their respective partners in CCK. A modification of this TM arrangement to reflect particular structural features of CCK_1 not present in rhodopsin yielded a CCK_1 receptor model, which was highly divergent from the rhodopsin structure. The failure to generate a good working model of the CCK_1 receptor is perhaps not too surprising, given that several key residues are located in ECL2, and it is extremely challenging to model loop conformations correctly owing to a lack of structural information. This exercise should serve as a cautionary note that not all GPCRs are created equal, and thus lend themselves well to homology modeling.

A different tack was taken by Bissantz *et al.* [48]. The authors generated rhodopsin-based homology models of six GPCRs: three virtual protein–antagonist complexes (the D_3 receptor, the muscarinic receptor and the vasopressin V_{1A} receptor) and three virtual protein–agonist complexes (the D_3 receptor, β-adrenoceptor and δ-opioid receptor). The initial apoprotein models were minimized, and agonists and antagonists were manually docked into the binding site. However, the binding cavity in the TM domain was too small to accommodate the ligands, and the initial models had to be refined in the presence of the small-molecule ligand.

The accuracy of the models was tested by screening them against a virtual library of 1000 compounds, including ten known antagonists that were different from those used to build the models. For the three protein antagonist complexes, the virtual screening results were encouraging. For example, for the D_3 receptor, high hit rates of up to 30% were observed, and the configuration of the bound ligand in the binding site agreed well with data from experimental mutagenesis studies. In addition, ligands that were structurally unrelated to the antagonist used in the refinement of the D_3 receptor model were found as hits from the virtual screen. This shows that even though the active site of the receptor was refined with only one antagonist, the resulting model was still broad enough to accommodate a wide variety of ligands.

By contrast, the agonist-bound models did not produce good hit rates, and agonists belonging to different structural classes from those used in the refinement were not identified. Furthermore, structurally different agonists could not be docked reliably into the binding site. Thus, in contrast to the antagonist-bound models, the agonist-bound models were highly biased toward the agonist used in the refinement procedure. To remedy this problem, the authors generated a multiligand-based model which, in addition to the chemical features of the ligand, also takes into account the structural features of the receptor. The new receptor model derived from this approach was better able to accommodate agonists belonging to different structural classes and avoided biasing the receptor structure toward one particular agonist. These models were much better suited for virtual screening than single-ligand-based models. For the D_3 receptor, the multiligand-based receptor model not only gave high hit rates in a virtual screen, but the hit lists also included agonists from structurally diverse templates. Furthermore, even agonists that were not represented in the initial multiligand pharmacophore were identified in the consensus hit lists. In summary, the receptor models generated by these approaches seemed to be suitable for the identification of new GPCR agonists and antagonists by virtual screening, and should thus be useful for drug design.

CCR5 homology models

The finding, several years ago, that the chemokine receptor CCR5 is one of the major co-receptors for HIV-1 invasion [49] has resulted in the rapid development of a large variety of small-molecule CCR5 antagonists [50]. Correspondingly, there have been numerous attempts to explain the mechanism of CCR5 binding for some of these compounds, and several homology models of CCR5 in complex with a variety of CCR5 antagonists have been reported in the literature [51–57]. We will consider two approaches here that exemplify some of the problems encountered using bovine rhodopsin as a template for a CCR5 homology model.

Some of the structural motifs in CCR5 are distinctly different from those of bovine rhodopsin and this has important implications for rhodopsin-based homology modeling of CCR5. These differences have been considered by some, but not all, of the CCR5 homology models [58,59]. For example, Govaerts *et al.* have described an approach that combines mutation studies with receptor modeling to elucidate the mechanism of chemokine-induced activation of CCR5 [58,59]. In their initial study, Govaerts *et al.* [58] focused on the role of the TXP (where X represents a variable hydrophobic residue) motif in TM2. Pro2.58 of the motif (residue numbers are given according to the notation of Ballesteros and Weinstein [60]) is completely conserved across all mammalian chemokine receptors, whereas Thr2.56 is present in 47 out of 55 mammalian chemokine receptors.

By contrast, although TM2 of bovine rhodopsin has high sequence identity to the TM2 of chemokine receptors from the cytosolic border up to the TXP motif, it strongly diverges between the TXP motif and the start of ECL1. In bovine rhodopsin, the TXP motif is replaced with a GGF motif, which causes TM2 to be distorted so that its extracellular part is close to TM1. By contrast, the presence of the TXP motif orientates the extracellular part of TM2 away from TM1 and toward TM3 and the center of the helix bundle. This is a striking structural difference between the chemokine receptor family and rhodopsin. This would not have been captured by a homology model in which nonconserved side chains are replaced using a rotamer library, and in which the backbone conformation of the helix is kept identical to that of rhodopsin, without correcting for structural changes such as helical bends

that are induced by certain amino acids. To accommodate the structural difference induced by the TXP motif in TM2, Govaerts *et al.* [58] replaced the extracellular part of TM2 of their homology model with a model peptide that includes the TXP motif and assessed its conformational properties as well as its structural consequences in the context of the seven-helix assembly. Their approach produced a more realistic structure of CCR5 (Figure 5), which is distinctly different from that of rhodopsin on the extracellular side of the TM bundle.

In a further study, Govaerts *et al.* [59] showed the importance of an aromatic cluster, in the extracellular region of TM2 and TM3, in the activation of CCR5. The experimental and structural data together strongly indicate the importance of the TM2–TM3 interface for receptor activation and suggest that the aromatic cluster has a crucial role in the conformational changes of the receptor required for activation. In the accompanying modeling studies, the authors used the same approach as before, building model peptides of TM2 and TM3 and using molecular dynamics (MD) trajectories to generate geometries, the other TM domains were based on rhodopsin. The TM2–TM3 interface was then optimized by MD simulations of the seven-helix bundle. Along with experimental mutational analysis, Govaerts introduced the same mutations into the model and studied the TM2–TM3 interface using MD simulations. The original model and the model with the mutations agreed very well with the experimental data on the activation mechanism of CCR5. These studies clearly showed that a CCR5 structure based solely on the rhodopsin crystal structure would not be able to explain the activation mechanism of CCR5 following chemokine binding. This work serves as a further example of how meaningful structural information from rhodopsin homology models can only be obtained by thoroughly analyzing differences between rhodopsin and other GPCRs and then adjusting the model accordingly.

De novo structure prediction

In contrast to homology modeling, bovine rhodopsin is not used as a template for the *de novo* modeling of GPCRs. Instead, a model of the protein is built from first principles, based solely on its amino acid sequence. Two groups have described this approach in detail and we discuss them both below [61,62].

A series of papers from the Goddard group describes their computational strategies and techniques for predicting the tertiary structure of GPCRs (MembStruk) and for predicting a putative binding site for small-molecule ligands in the TM, which might or might not overlap with the retinal binding site in rhodopsin (HierDock) [62–64]. The MembStruk protocol consists of five steps, and we will only refer briefly to it here because of space constraints (Box 2). We direct those readers who are interested in a fuller account of the method to consult the original work [62]. Using this approach, the Goddard group was able to predict accurately a rhodopsin structure covalently bound to retinal, close to that observed for the experimental structure. The docked configuration of retinal in the crystal structure of rhodopsin differs by 0.62 Å from the crystal structure configuration of retinal. In the predicted structure of rhodopsin, the difference between the predicted configuration of retinal in the model of rhodopsin and its configuration in the crystal structure is 2.92 Å. Given these encouraging results, the Goddard group applied their structure-prediction algorithms to several other receptors including the β_2-adrenoceptor, which is discussed in more detail below.

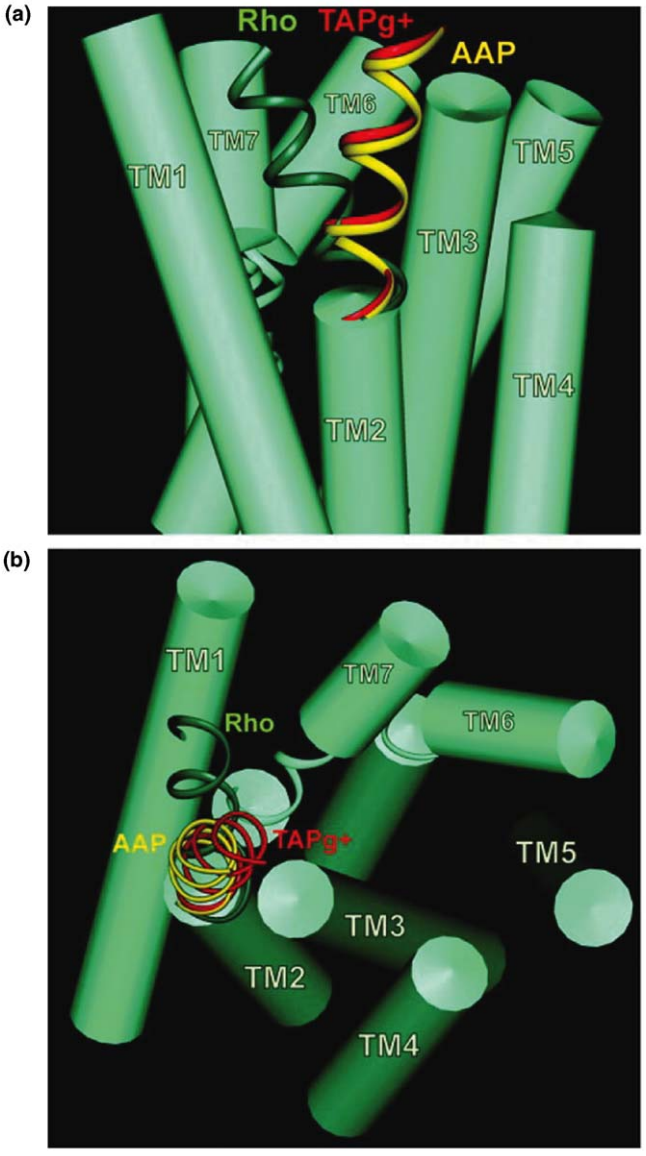


FIGURE 5

The TXP motif in the second transmembrane helix of CCR5 is a structural determinant of chemokine-induced activation. (a) The representative structure of the AAP (yellow ribbon) and TAP *g*+ (red ribbon) motifs are positioned in the rhodopsin template, respecting the homology between CCR5 and rhodopsin. The two representative helices were superimposed on the cytoplasmic end of TM2 in the rhodopsin structure, using backbone atoms up to position 2.54. Rhodopsin (turquoise) helices are shown as cylinders, except for the extracellular part of TM2, which is shown as a ribbon. In bovine rhodopsin, the TXP motif is replaced with a GGF motif, which results in striking differences in the orientation of TM2 between CCR5 and rhodopsin, as observed in this figure. The influence of the threonine on the proline kink is clearly visible, because it bends the helix inside the bundle. This panel is viewed from the side of the protein. **(b)** The same representation that is shown in (a) viewed from the extracellular side. Modified, with permission, from Ref. [58].

A 3D structure of the human β_2-adrenoceptor was first generated using the MembStruk method [63]. Subsequently, the agonist- and antagonist-binding pockets were identified with

BOX 2

The *de novo* model of Goddard

In the approach developed by Goddard *et al.* at Caltech, the first step in the model-building process is the prediction of the transmembrane (TM) regions based on the primary amino acid sequence, using the hydrophobicity profile of a multiple sequence alignment of the query sequence with an ensemble of protein sequences [62]. The predicted TM predictions for bovine rhodopsin are in good agreement with the crystal structure.

The assembly and initial optimization of the seven-helix bundle is carried out by optimizing the relative translation and rotation of the helices. The translational and rotational orientation of the helices is crucial because it determines the shape and chemical properties of the small-molecule binding site in the TM domain. Because the sequence homology between bovine rhodopsin and the protein of interest can be low, even in the TM domain, rhodopsin might not necessarily be a good template to model the helix packing of any G-protein-coupled receptor (GPCR). To overcome these limitations, a Monte Carlo-like systematic conformational search algorithm that overcomes energy barriers and finds a low energy conformation of the helix bundle is used. To assemble the initial helix bundle, canonical α-helices are built for each TM using extended side chain conformations, and the helical axes are then oriented along the helical axes of the 7.5 Å frog rhodopsin crystal structure, without using information of atomic positions or helical translations and rotations. Each helix from the optimized helix bundle is subsequently optimized individually to take into account helix bends, due to, for example, the presence of proline and glycine residues. Next, a lipid bilayer of 52 molecules of dilauroylphosphatidylcholine is added and the combined protein-lipid system undergoes a rigid body MD simulation, where the lipid and protein are treated as rigid bodies. During this process, the helices are also allowed to adjust their translational position within the helix bundle. In the final steps, intracellular and extracellular loops, including side chains, are added to the helices, and disulfide bonds are formed where necessary. The wholly assembled protein then undergoes a full atom minimization in vacuum to yield the final predicted protein structure. Using this procedure, the predicted apo structure of bovine rhodopsin differs from the crystal structure of rhodopsin by less than 3 Å.

To predict the ligand-binding site, the entire protein is scanned by mapping the void regions with spheres, which are subsequently clustered into overlapping regions of 10 $Å^3$. To identify the energetically most favorable binding site, one (or a set of) ligand(s) is docked into each region; conformations with <90% of buried surface area are discarded, and the remaining conformations are ranked according to ligand–protein interaction energy. The thus-identified putative ligand-binding region is now subjected to a series of further hierarchical refinements and, finally, the protein–ligand complex with the best binding energy is subjected to protein side-chain optimization followed by minimization, where all protein and ligand atoms are allowed to move.

HierDock. The predicted structure was then validated by docking eight known agonists and antagonists into the binding site and analyzing the binding configuration of the protein–ligand complex. Important residues experimentally implicated in agonist and antagonist binding, such as Asp113 in TM3, Ser-203, -204 and -207 in TM5, and Phe290 and Ans293 in TM6 [65–67], were found to interact with the small-molecule ligand in the modeled structure as well. Furthermore, the docked complexes were ranked according to protein–ligand binding energies and the relative predicted binding energies corresponded well with the experimentally determined values. Specifically, the model was able to distinguish β_2-adrenoceptor agonists from antagonists by differences in their binding modes. All agonists were found to interact with at least two of three conserved serines (Ser203, Ser204 and Ser207) in TM5 through a hydrogen bond network. By contrast, Ser207 was not involved in hydrogen bonding to the antagonists.

This model enabled the correct classification of seven known and three unknown compounds. Additionally, two α_1-adrenoceptor-specific compounds, atenolol and xamoterol, were docked into the β_2-adrenoceptor model and found not to bind. A third α_1-adrenoceptor-specific compound, metoprolol, was classified as a β_2-adrenoceptor antagonist. Furthermore, the model provided a rational explanation for the difference in binding affinity between epinephrine and norepinephrine. Comparison of the binding modes of both agonists in the β_2-adrenoceptor-binding pocket revealed that epinephrine tilts toward Ser204 and makes strong hydrogen bond interaction with the protein, whereas norepinephrine tilts away and thus forms a much weaker hydrogen bond, accounting for the different affinities toward the receptor (Figure 6).

Lastly, all β_2-adrenoceptor agonists are known to interact with Asn293 in TM6; this was also reflected in the predicted structure. This interaction has not previously been shown to be important experimentally for antagonists. Consistent with this, none of the antagonists docked into the model was predicted to interact with Asn293. Without going into further detail, it is important to note that several experimentally observed interactions, lipophilic and hydrophilic, are reproduced in the predicted complexes from the Goddard group. These studies indicate that the *de novo* method and binding site identification are reliable and can be successfully applied to other GPCRs.

As we have discussed above, even with carefully built rhodopsin-based homology models of class A GPCRs, it can be challenging

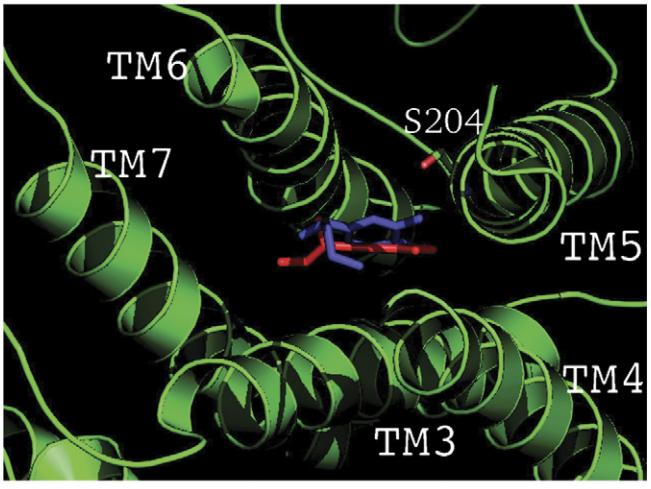


FIGURE 6

Comparison of the binding sites of epinephrine and norepinephrine to the β_2-adrenoceptor. Epinephrine (blue) tilts toward S204 and makes strong contact, whereas norepinephrine (red) tilts away and makes weaker contact. This interaction accounts, in part, for the higher affinity of epinephrine for the β_2-adrenoceptor. Reproduced, with permission, from Ref. [63].

to explain agonist and antagonist binding [48]. A recent study by Furse *et al.* [68] serves to illustrate this point further. These authors constructed homology and *de novo* models of the β_2-adrenoceptor. The homology model was built using the standard procedures already described. The *de novo* model was constructed starting with ideal α-helices corresponding to each TM segment, which were subsequently arranged into a seven-helix bundle using manual and semiautomated model-building procedures to generate a large number of different topologies. For further details of the model construction and the addition of the loops, we refer the reader to the original work [68].

The authors then docked several known agonists and antagonists into both models and compared the results. They found that their *de novo* model provided a better explanation for agonist–receptor interactions than did the homology model, which could only modestly accommodate very small agonists such as epinephrine or isoproterenol. Furthermore, the *de novo* model accommodated a much wider variety of ligands, and reflected the experimental fact that Asn293 in TM6 has an important role in ligand–receptor interactions. In the homology model, this residue points away from the binding pocket. The most notable difference between the two models (other than the loop regions) is the exact rotation and tilt of TM6. It is known experimentally for both the bovine rhodopsin and the β_2 adrenoceptor that the rotation and tilt of TM6 are involved in the activation mechanism. Thus, it is reasonable to assume that TM6 of bovine rhodopsin does not constitute a good template for agonist binding because the rhodopsin crystal structure complex reflects the inactive form of bovine rhodopsin, in which the protein is in complex with the potent inverse agonist 11-*cis*-retinal.

Another interesting *de novo* structure prediction method called PREDICT has been described by Shacham *et al.* [69]. Their approach (Box 3) does not rely on known structures as templates; instead, the 3D protein structure is predicted based solely on its own sequence, using knowledge-based structural constraints, and structural constraints imposed by the presence of a phospholipid membrane. We now discuss one example in which the PREDICT procedure was used to generate a model of the D_2 receptor. The quality of this model was then assessed by testing it in a receptor-based virtual screen.

The 2D structure-building procedure for the D_2 receptor followed the general PREDICT method [61,69]. Over 2000 2D conformations

BOX 3

The PREDICT method of *de novo* modeling

The PREDICT method uses the concept of 'structural decoys' to predict G-protein-coupled receptor (GPCR) structures. It generates many alternative structures, which are simultaneously optimized. This technique avoids a structure becoming trapped in a local minimum without sufficiently exploring its conformational space. The PREDICT procedure consists of four main steps: first, the 'coarse' application of the PREDICT algorithm; second, the 'fine' application of the PREDICT algorithm; third, a molecular dynamics refinement; and fourth, the generation of a virtual protein–ligand complex. As in the Goddard approach, the first step is to determine the transmembrane domain (TM). Initially, the TM is only approximately determined. For further optimization, a longer stretch of amino acids is used, from which, in the later stages of the refinement, the correct TM length is determined using 3D optimization and energy scoring criteria.

In the next step, a large number of structural decoys are constructed, from which the most plausible structure(s) are identified by structural and energetic criteria. To generate the initially large number of decoys, the predicted helices are assumed to be 2D dials, which are formed by the projection of perpendicular ideal helices onto the membrane plane. Geometrical rules are used to reduce the large number of possible packing topologies to only include reasonable close-packing structures (see the original study [69] for details). The still large number of 2D decoys is further reduced by introducing constraints based on experimentally known interaction between helices. The rotational optimization of the helices is then carried out by assigning a hydrophobicity vector to each helix and orientating the helix so that the largest hydrophobic moment points toward the membrane.

After the rotational optimization of the helices, the 2D representation is converted into a 3D reduced structure. This reduced representation preserves the shape of the side chains to a certain degree and enables side chain rotamer motion. The different side chain rotamers are generated from a backbone-independent rotamer library. The initial 3D decoys are continuously optimized by the PREDICT algorithm to minimize the PREDICT energy function, which distinguishes correct from incorrect packing topologies. Furthermore, terms for aromatic–aromatic interactions, cation–p interactions, polar interactions and interactions with the membrane are added. This complex energy function is used in the optimization of the 3D structure.

The optimization of the helix centers was also determined initially in the 2D step, assuming fixed distances between the centers of neighboring helices. However, aromatic residues might disrupt the tight helix packing, and thus the x-y position of each helix is optimized by moving it stepwise in a circle of 1 Å around its initial helix center. The optimization of the helical tilt is performed by tilting three or four adjacent helices together to minimize steric hindrance due to tilting individual helices. The tilt optimization is computationally intensive and thus only performed on the lowest energy conformations. The model selection after each pass of PREDICT is done manually by subjecting each low-energy conformation to a thorough structural analysis and a comparison of the putative binding cavity of the model with experimental data.

After the final structure selection, the reduced side-chain representation of the model is expanded into an all-atom model and is minimized with a distance dielectric constant. The minimized structure is then subjected to an MD simulation to remove unfavorable steric interactions and to enable the helices to adopt kinks as a result of the presence of proline and glycine. The putative small-molecule binding cavity is filled with TIP3P water molecules, which are relaxed during Langevin dynamics. Harmonic constraints are added so that the helices do not unwind during the simulation.

The final step in the whole structure generation procedure is to create a virtual protein–ligand complex, which can then be used for virtual screening. To this end, a known ligand is placed manually into the putative binding pocket according to known experimental data. The virtual protein–ligand complex is then subjected to a free-energy-perturbation-like simulation (to remove the bias introduced with the initial manual placement of the ligand), where the ligand can reorientate itself in the binding pocket. The final structure for virtual screening is obtained by removing the ligand from the binding pocket.

were generated; however, only one had a large enough binding site to accommodate known antagonists, and this was used to generate a virtual protein–ligand complex with the known antagonist fluphenazine (K_i = 0.324 nM). Analysis of the final D_2 receptor structure revealed that the binding site for small-molecule antagonists was located between TM 2, -3, -5 and –6, and included the highly conserved residues Asp114 (TM3), Ser197 (TM5) and Phe389 (TM6), all of which are known to contribute to small-molecule binding within the TM domain. Specifically, TM 3, -5 and -6 were implicated in receptor–agonist interactions. Overall, the virtual ligand-binding sites and the D_2 receptor structure agreed very well with experimental data.

The virtual D_2–fluphenazine complex was then used in a virtual screening validation after removing fluphenazine from the structure. The screening library used in the validation included 10,000 drug-like compounds similar to those for known D_2 receptor ligands. The library was seeded with 33 known D_2 antagonists and ten agonists, representing more than six agonist and ten antagonist scaffolds. The virtual screening for D_2 antagonists yielded a 17-fold enrichment for identifying 50% of the known antagonists in the seeded database, and 85% of known antagonists were identified in the top 10% of the seeded library. For the D_2 agonists, the enrichment factor for identifying 50% of the known agonists is ninefold higher than would be seen by chance, and 70% of the known agonists were found in the top 10% of the screened library. These results are encouraging for the further use of predicted 3D GPCR structures in virtual screening and structure-based lead optimization.

It is interesting to note that Goddard's group [64] also predicted a *de novo* D_2 receptor structure, into which they docked a series of agonists and antagonists. The binding site identified by their automated method includes the same residues identified by Becker *et al.* [61], and that have been validated experimentally. Specifically, Goddard's group was able to distinguish agonists from antagonists based on their interaction with either one or two serines in TM5 [64]. Agonists were found to bind to Ser193 and Ser197 in TM5 and to Asp113 in TM3, thus tightly coupling TM3 and -5, whereas antagonists tightly coupled TM3 and -6.

The three *de novo* structure-prediction methods discussed above provide encouraging results for other receptors not mentioned in this review, and should have a significant impact on the identification and optimization of new drugs that target GPCRs. Although space constraints have limited our discussion of the wealth of interesting GPCR modeling studies that are described in the literature, we would like briefly to mention one other method that is finding vogue in the drug discovery process. This involves combining receptor homology modeling techniques with ligand-based pharmacophore modeling to generate accurate models that should increase the likelihood of finding novel agonists and antagonists. The work of Evers and Klabunde [70], with the α_{1A}-adrenoceptor, serves as a prime example of this approach. The model of the α_{1A}-adrenoceptor that was built was used in a virtual screen of a filtered inhouse compound library (target-unspecific filters, such as molecular weight and number of rotatable bonds, and target-specific filters, such as a pharmacophore model) of 22,950 compounds. Using this approach, 37 compounds with binding affinities below 10 μM were discovered, three of which were found to bind in the single digit nanomolar range. In addition, some of the virtual hits belonged to structural classes not previously known as α_{1A}-adrenoceptor antagonists.

Dual receptor specificity

The traditional single-drug, single-target paradigm in drug discovery is not always necessarily the most successful, or even the most desirable, approach, and there are numerous examples of drugs that mediate their effects through multiple targets. Aspirin is a classic example of such a drug. It has long been in clinical use and is effective therapeutically in reducing inflammation and in a variety of other situations, such as in cardiovascular disease [71]. It is well known that these beneficial effects of aspirin probably derive, at least in part, to its inhibition of specific cyclooxygenase pathways (cyclooxygenase 1 and -2), which lead to the inhibition of prostaglandin and thromboxane synthesis [72]. However, it is apparent that the anti-inflammatory effects of aspirin are also due to its ability to generate novel anti-inflammatory mediators, known as aspirin-triggered 15-epi-lipoxins [73].

Combination therapies (concomitantly administered cocktails of several drugs that target different proteins) are already used to treat cancer and AIDS because no single drug alone would be effective to treat these complex diseases. For the treatment of AIDS, for example, cocktails of several reverse transcriptase inhibitors, protease inhibitors and fusion inhibitors are given. We would argue that if some of these combination therapies could be given in the form of one or two drugs that hit multiple targets, it would be advantageous, not only for the purpose of drug development, but also for the patient. It is clear from the discussion earlier in this review on GPCR specificity that drugs targeting GPCRs can also be promiscuous in their mode of action. Although this is not usually desirable because of off-target effects, it can, in some cases, be beneficial therapeutically, such as in the case of the antipsychotic drug olanzapine, which binds with high affinity to >12 GPCRs [38].

Based on the examples above and the wealth of literature discussed earlier, it is obvious that small-molecule drugs can bind to multiple GPCRs. If this receptor promiscuity could be suitably harnessed in the design of dual inhibitors of GPCRs, it could be a very useful approach in several situations; for example, joint CXCR4 and CCR5 inhibitors would be very useful therapeutically as fusion inhibitors to treat AIDS patients. Although the idea is attractive, the question remains whether the design of dual GPCR inhibitors is possible. Clearly, this will depend on the similarity of the receptor-binding pockets of the GPCRs in question. A classic example is provided by the AT_1 receptor [74] and the endothelin ET_A receptor [75]; the ligands for these receptors, angiotensin II and endothelin, are potent vasoconstrictors, and AT_1 antagonists have already taken their place alongside ACE inhibitors as successful treatments for hypertension [74]. Thus, it is expected that dual antagonists of these two receptors would be of greater benefit in the treatment of pulmonary hypertension, congestive heart failure and arteriosclerosis than either treatment alone. Although the overall sequence identity of AT_1 and ET_A is low, at only 19%, it has proven to be possible to design dual antagonists that bind to both receptors with high affinity, in the low nanomolar range [76,77] (Figure 7). Receptor mutagenesis and modeling studies have revealed that the binding site for the dual AT_1 and ET_A antagonist (L-746,072) on the AT_1 receptor overlaps with the binding site of the subtype-specific AT_1 antagonist (KR-31,016)

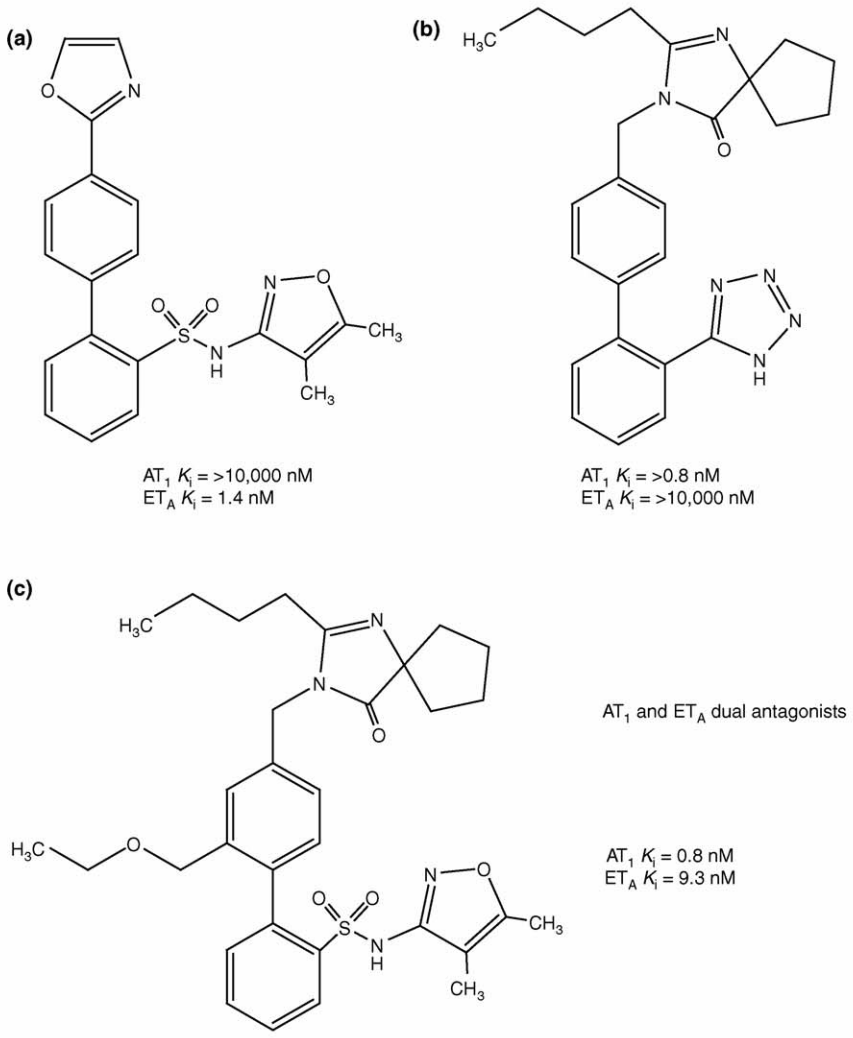


FIGURE 7

The design of the AT_1 and ET_A receptor dual antagonists. This approach is based on the idea that a phenylsulfonamide core is a common component of a class of potent and selective ET_A receptor antagonist **(a)** [90,91]. The biphenyl core of these compounds has a structural similarity to the biphenyltetrazole core of AT_1 receptor antagonists, including irbesartan **(b)**. These observations enabled the design of the potent AT_1 and ET_A dual receptor antagonists exemplified by **(c)** [76,77].

[78] but is not identical with it. This observation is supported by the mutation data for the balanced-affinity AT_1 and AT_2 receptor ligand L-163,017. The same residues affecting L-163,017 binding to AT_1 were also found to be important for L-742,042 (the dual antagonist) binding to AT_1 but not for binding of the subtype-specific and species-selective antagonist KR-31,016 [78].

Recently, a series of tetracyclic indoline butyrophenone analogs, highly potent antagonists of D2 and 5-HT_{2A} receptors, were described [79]. These receptors appear to have a role in schizophrenia, and the promiscuous GPCR antagonist olanzapine, which inhibits these and other GPCRs, has been very successful in treating this disorder (see earlier discussion under section on receptor specificity). Interestingly, the D2 and 5-HT_{2A} receptors share several residues in common that are thought to be involved in ligand binding. These include the Asp3.32, Val5.39, Ser5.43, Ser5.46, Phe5.47, Trp6.48 Phe6.51 and Phe6.52 [80]. Some or all of these residues might also be involved in binding the tetracyclic indoline butyrophenone analogs.

Given the multiple examples discussed above, it is clear that the design of dual receptor antagonists is possible if the binding pockets of the receptors share some common determinants. Several homology models of chemokine receptors have been described. Of particular interest are the highly related receptors CCR1 and CCR3, which share ~59% sequence identity [81,82]. A 2-(benzothiazolylthio) acetamide compound from Takeda binds to both receptors with high affinity, displacing CCL3 with an IC_{50} of 450 nM for CCR1 and displacing CCL11 with an IC_{50} of 33 nM for CCR3 [83]. Similarly, UCB 35625 is a potent antagonist for both receptors, inhibiting CCL3-induced chemotaxis mediated by CCR1 with an IC_{50} of 9.6 nM and CCL11-induced chemotaxis mediated by CCR3 with an IC_{50} of 93.7 nM [84].

The challenge to medicinal chemists will be to design dual-receptor antagonists that are effective in targeting completely unrelated receptors, such as CCR5 and CXCR4. Indeed, some drug companies are already embarking on such approaches; for example, ANORMED recently disclosed compounds that they claimed

were dual CCR5 and CXCR4 inhibitors [85,86]. The specified compound is a benzimidazole tetrahydroquinoline, stated to inhibit HIV-1 NL4.3 or IIIB replication in MT-4 cells with an EC_{50} of 20 μg/ml. It will be interesting to see how such dual antagonists are able to accommodate the receptor-binding cavities of such different binding pockets, and whether they are able to inhibit the binding of chemokines or are purely HIV-1 inhibitory. This field of drug discovery has been discussed in a series of interesting articles, and the reader is directed to these for a fuller discussion than we can provide here [87–89].

Conclusions

In this review, we have attempted to highlight the advances that have been made in applying the principles of computer-based methods to the rational design of drugs that target GPCRs. The fast-paced growth in the computer industry, in terms of both capacity and speed, has led to tremendous advances in the application of computerized modeling approaches in drug design for proteins such as GPCRs, for which little structural information is available. Although none of the approaches described are perfect (we have tried to point out their advantages and disadvantages here), this is a rapidly expanding area of pharmaceutical research that holds great promise for delivering new and improved therapeutics in the near future. In conclusion, if the approaches discussed in this review live up to their expectations, they will bring about a revolution in drug discovery that will lead to novel drugs which are potent and should be of ultimate benefit to the patients of the future.

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