# G Protein-coupled receptors as targets for drug design

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REVIEW

**ABSTRACT**

G protein-coupled receptors (GPCRs) are the target for more than 50% of the drugs currently on the market, including about 25% of the 100 top-selling drugs. They are considered the most important molecules in the field of drug discovery and design today, mostly due to their role as receptors in many of the basic processes in the body, and because they are present in all tissues. Unfortunately, a structure-based rational design is very difficult for GPCRs; the structures available for modeling purposes are only for family A. Despite this fact, research has continued and progressed, using combined structure-based techniques. This review intends to summarize this work.

*Keywords*: GPCR, 7TM, receptor, virtual screening, docking, molecular modeling, drug design

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**RESUMEN**

**Los receptores asociados a proteínas G como blancos en el diseño de fármacos.** Los receptores asociados a proteínas G (GPCRs), son el blanco de más del 50% de los medicamentos que se encuentran actualmente en el mercado, e incluyen cerca del 25% de la lista de las 100 medicinas más vendidas en el mundo, estas moléculas son consideradas hoy en día como las más importantes para el diseño de fármacos, fundamentalmente por su papel como receptores de la mayoría de los procesos básicos del organismo, además de estar presentes en todos los tejidos. Desafortunadamente el diseño racional basado en la estructura se hace muy difícil para las GPCRs, las estructuras que existen son solo de la familia. A pesar de esto, las investigaciones han continuado y progresado utilizando técnicas combinadas. Esta revisión trata de resumir este trabajo.

*Palabras clave*: GPCR, 7TM, evaluación virtual, acoplamiento molecular, modelación molecular, diseño de fármacos

## Introduction

G protein-coupled receptors (GPCRs) form the largest family of membrane proteins responsible for communication between the cell and the environment. These proteins recognize extracellular messengers and transduce the signal to the cytosol. GPCRs bind to a wide variety of molecules, including ions, amino acids, peptides, lipids, and nucleotides. They control the activity of enzymes, ion channels and vesicular transport, principally through the catalysis of GDP-GTP exchange on heterotrimeric G proteins. They are involved in diverse biological functions including the senses of smell, taste and sight, and the regulation of appetite, digestion, blood pressure, reproduction and inflammation [1] the reason why they are involved in a wide variety of pathologies.

Each cell expresses a few dozen different GPCRs, which implies that its homeostasis can be influenced by numerous transmitters. A particular GPCR is often expressed in several tissues. It can be found in the periphery and in the central nervous system. Its roles in these tissues may be different although the second messengers that result from the initial activation are probably the same. The organ that is possibly most dependent on GPCR activity is the brain, where practically all the GPCRs are expressed. They are involved in synaptic transmission mechanisms and most of our senses depend directly on the activation of specific GPCRs.

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GPCRs have proven to be particularly amenable to modulation by small molecule drugs and are the targets of approximately half of the current prescription drugs, as well as the targets of a large number of therapeutics and GPCRs provide opportunities for the development of new drugs with applications in all clinical fields.

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## Characteristic features of GPCRs

GPCRs are integral membrane proteins with seven transmembrane helices. The N-terminal segment is extracellular and the C-terminal segment is located in the cytosol. The transmembrane (TM) domains are more conserved among GPCRs than the extracellular or intracellular domains. There are several signature amino acid motifs which provide us with their identity as GPCRs; for example, the LxxxD motif in the TM II, the DRY motif at the end of the TM III and the NPxxY motif on the TM VII (Figure 1). Usually, the intracellular domain III (between TM V and TM VI) and the carboxy terminal are considered to play certain roles in G-protein coupling [2].

GPCRs are divided into families according to their sequence homology. Family A represents the largest subgroup of receptors and includes catecholamines, neuropeptide, chemokine, glycoproteins, lipid and nucleotide receptors. Family A is characterized by several highly conserved amino acids and a disulphide bridge. Most of these receptors also have a palmitoylated cysteine in the carboxy-terminal tail. Ligand binding within the transmembrane region of the receptor seems to occur mainly in a cavity flanked by TMs III, V, VI and VII. The crystal structure of rhodopsin [3, 4] has indicated that the transmembrane domains of this family are "tilted" and "kinked" (Figure 2a). Family B contains receptors for a large number of peptides

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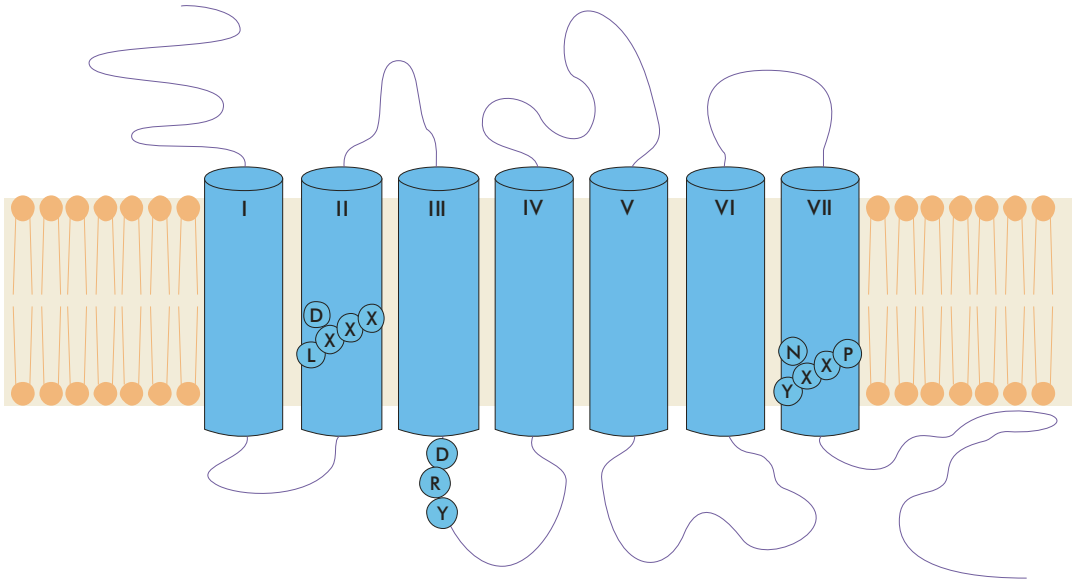


Figure 1. Schematic drawing depicting the 7 helices, the conecting loops and some conserved amino acid motifs in GPCRs. The LxxxD motif in the TM II, the DRY motif at the end of the TM III and the NPxxY motif on the TM VII.

such as calcitonin, glucagon, gonadotropin-releasing hormone and parathyroid hormone. These receptors are characterized by a relatively long amino terminus that contains six conserved cysteine residues, which presumably form a network of disulphide bridges (Figure 2b). This amino terminus seems to play a key role for most ligands, but it is not sufficient and additional interactions are found in the extracellular loops. Family C is the metabotropic containing the metabotropic glutamate receptors, GABA receptors and the calcium sensor receptor. These receptors are characterized by a long amino terminus and carboxyl tail. The amino terminus is folded as a separate ligand binding domain which is often described as being like a "Venus fly trap" (Figure 2c) [1, 5, 6].

Ligand binding to GPCRs promote conformational changes leading to G-protein coupling, the initiation of signal transduction pathways and ultimately cellular response. Studies based on electron paramagnetic resonance and fluorescence spectroscopy [7] suggested the need of an outward movement of the cytoplasmic end of TMs III and VI [8, 9], as well as an anti-clockwise rotation of TM VI around its helical axis, when viewed from the extracellular side, for its activation. Other helices probably adjust their positions upon activation as well.

Each GPCR has its own selectivity to G proteins (Figure 3), however, the specific sequences activating each G protein (Gs, Gi, Gq, G12, etc.) are as yet unknown, although there is a proposed theory that basic amino acids are important for G protein coupling [10].

Even though it is known that for many classes of receptors constitutive or ligand-induced oligomerization is essential for signaling [11], only a monomeric model for GPCRs is generally accepted. Since the mid-1990s, many reports have successively shown oligomerization of the GPCRs, examples of this are the H2 histamine receptor [12] and the β2-adrenergic receptor [13, 14]. Now, oligomerization is widely accepted as a universal aspect of GPCR biology.

After the first reports of GPCR homo-oligomers, it was shown that some receptor subtypes formed hetero-oligomers, for example AT1-AT2 angiotensin receptors [15] and A1 adenosine-D1 dopamine receptors [16], and that these "heteromeric" receptors had functional characteristics that differed from homogeneous populations of their constituent receptors. The generation of new properties through hetero-oligomerization indicated a possible mechanism for a generating diversity of functions among GPCRs that had not previously been anticipated [5].

## GPCRs in drug discovery

GPCRs have been shown to be excellent targets for pharmaceutical treatments; along with kinases, GPCRs constitute the most widely screened classes of signal transduction targets [17]. Many major diseases involve the malfunction of these receptors making them the most important drug target for pharmacological intervention. In particular, the subfamily of biogenic amine-binding GPCRs has provided excellent targets for the treatment of several central nervous system diseases, such as schizophrenia (mixed D2/D1/5-HT2 receptors), psychosis (mixed D2/5-HT2A receptors), depression (5-HT1 receptor), or migraine (5-HT1 receptor). This GPCR subfamily has also provided drug targets for other disease areas such as allergies (H1 receptor), asthma (β2 receptor), ulcers (H2 receptor), or hypertension (α1 antagonist, β1 antagonist) [18] (Table 1).

GPCR agonist or antagonist drugs have been therapeutically successful because of their direct activity on the cell surface [19]. GPCRs comprise 50-60% of the drugs now on the market, including about 25% of the 100 top-selling drugs [20].

In commercial terms, GPCRs will continue to predominate as drug targets. The total human genome

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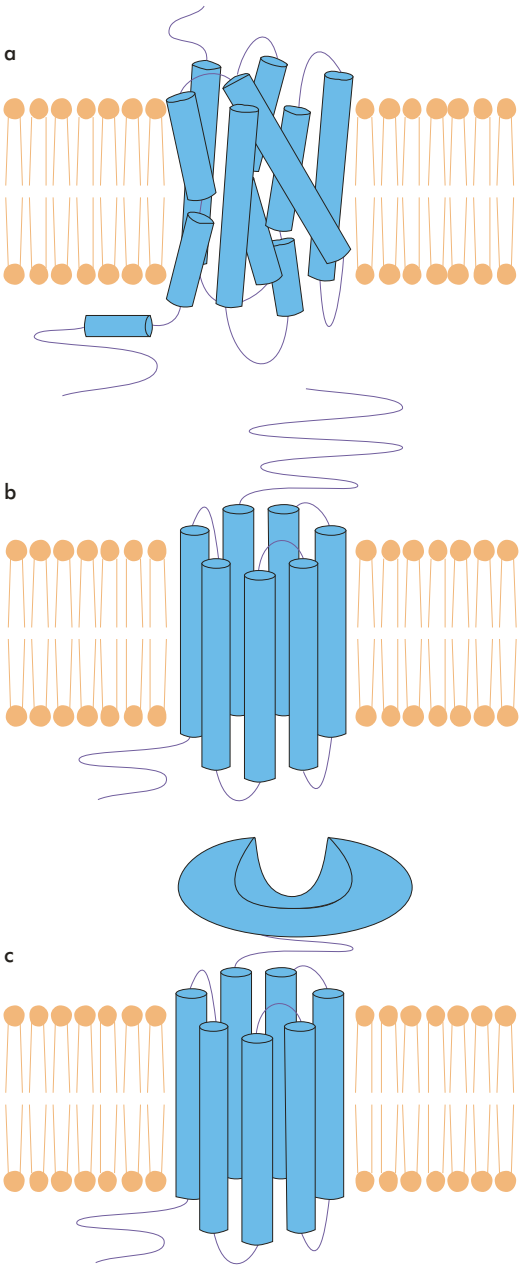


Figure 2. Schematic drawing of the GPCR families. (a) Family A has a short amino terminus and the transmembrane domains are "tilted" and "kinked" (b) Family B has a relatively long amino terminus that contains cysteine residues (c) Family C has a long amino terminus folded as a "Venus fly trap" domain.

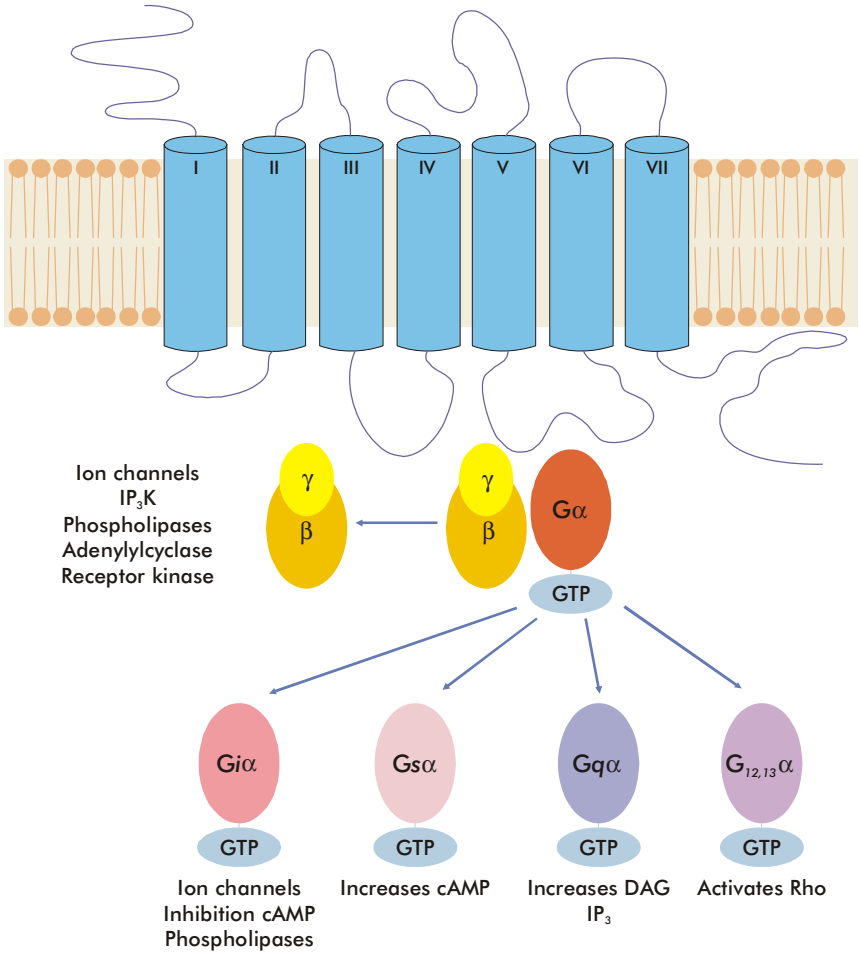


Figure 3. Schematic drawing depicting the 7 helices and the different pathways upon G protein coupling. Each GPCR has its own selectivity to a particular G protein and each G protein has its own signal pathway.

consists of approximately 35 000 genes. Further analysis suggests that approximately 10% of these genes could be targets for drug intervention in the treatment of diseases. These approximately 3000 genes include those encoding for receptors, ion channels, enzyme inhibitors and GPCRs that might be encoded by 750 of these genes. Almost half of these sequences are likely to encode sensory receptors, leaving around 400 receptors that could be considered as potential drug targets [21]. In addition, current GPCR-based drugs only target ~30% of the approximately 200 known GPCRs already identified in the genome, so there are still enormous opportunities for further drug discovery in this field.

## Structure-based drug design

Structure-based drug design is widely used in the development of novel drugs. Using structural-based methods it is possible to select compounds with biological activity for synthesis and biological assay. Ligands or target structural information is needed for this approach, which are then divided in ligand- or target based methods.

Ligand-based methods are traditionally used when no protein structure is available. The 2D ligand information can be used to develop new ligands with a structural similarity or the 3D pharmacophore search is used to identify ligands with similar steric and electrostatic features that are recognized at the target binding site and are considered responsible for the biological activity.

Target-based methods require 3D structure of the target and effective scoring procedures. The knowledge of the three-dimensional structure of the protein target

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is used to identify compounds that can bind to the target with a high specificity, resulting in the inhibition or activation of the target and its effector system.

## Ligand-based drug design

The limited availability of structural data makes ligand-based drug design a very important technique in GPCRs studies. The natural ligand can provide a good starting point in the lead finding process. The aminergic GPCR ligands are extensively used for this purpose [22], because they have the physicochemical requirements for oral absorption (small, moderately lipophilic molecules that tend to exhibit minimal hydrogen bonding potential) [23]. Structure-activity relationships (SAR) can be directly derived from natural ligands and their analogues. The resulting pharmacophore models can then be employed for virtual screening to identify lead structures with novel scaffolds. The molecules generated in this manner will share characteristics of the ligand and might have the potential to displace the ligand from the receptor. This is exemplified for the adrenegic β-receptors (Figure 4). Both the β-agonists (isoprenaline and salbutamol) and the β-blockers (propanolol and atenolol) share significant chemical features with adrenaline [22].

On the other hand, non-aminergic ligands do not exhibit the most desired physicochemical requirements for oral absorption. Especially for peptide-binding GPCRs the identification of a non-peptidic ligand is crucial for drug discovery to avoid the inherent pharmacokinetic problems frequently associated with peptide lead structures like poor oral bioavailability or metabolic instability [22].

However, especially for peptide-binding GPCRs, the screening of diverse or focused compound sets still remains a successful lead finding approach, which has led to the discovery of several potent, non-peptidic GPCRs ligands [24-26]. Such compounds have been classified as functional mimetics as they elicit agonist or antagonist activity, but do not necessarily mimic the structure of the native ligand. Examples for the successful use of peptide-derived structure-activity relationship to design non-peptidic GPCR ligands are described for the SST receptor [27], the opiate receptor [28], the thrombin receptor [29], the growth hormone secretagogues receptor [30], and the urotensin II receptor [31].

Ligand-based three-dimensional quantitative structure-activity relationship (or 3D-QSAR) methods and comparative molecular field analyses (CoMFA)[32-33], have supported the chemical optimization of numerous GPCR lead compounds. Thus, a CoMFA study enables chemical modifications that are beneficial or detrimental for biological activity. There are studies of CoMFA in optimizing GPCR-directed ligands as described, for example, for the dopamine receptors [34-36], the serotonin receptors [37-39], the endothelin receptor [40], and the adenosine receptors [41, 42].

CoMFA models can also be used to recognize molecular features that are responsible for the selectivity of the ligands. A series of aryl piperazines that were active against the 5-HT1A receptor have collateral affinities for the α1-adrenergic receptor [43]. A separate CoMFA model was derived for each receptor and the comparison of the models indicated that bulky substitutes at the meta position of the aryl moiety would increase selectivity for the 5-HT1A receptor; the a1 receptor, in contrast, does not tolerate large residues at this position. Furthermore, increasing the length of the alkyl chain linking the arylpiperazine with a hydantoin moiety was very beneficial for the desired selectivity.

Table 1. Some drugs acting through GPCRs

| Trademark | Generic name | Company | Disease | Target receptor |
|---|---|---|---|---|
| Claritin | loratadine | Schering-Plough | allergies | H1 antagonist |
| Zyprexa | olanzapine | Eli LillyTatemoto | schizophrenia | mixed D2/D1/5-HT2 |
| Cozaar | losartan | Merk & Co | hypertension | AT1 antagonist |
| Risperdal | risperidone | Johnson & Johnson | psychosis | mixed D2/5-HT2A |
| Leuplin/Lupron | leuprolide | Takeda | cancer | LH-RH agonist |
| Neurontin | gabapentin | Pfizer | neurogenic pain | GABA B agonist |
| Allegra/Telfast | fexofenadine | Aventis | allergies | H1 antagonist |
| Imigran/Imitex | sumatriptan | GlaxoSmithKline | migrane | 5HT1 agonist |
| Serevent | salmaterol | GlaxoSmithKline | asthma | β2 agonist |
| Zantac | ranitidine | GlaxoSmithKline | ulcers | H2 antagonist |
| Pepcidine | famotidine | Merk & Co | ulcers | H2 antagonist |
| Zofran | ondansetron | GlaxoSmithKline | antiemetic | 5-HT3 antagonist |
| Dovan | valsartan | Novartis | hypertension | AT1 antagonist |
| Duragesic | fentanyl | Johnson & Johnson | pain | opioid agonist |

## Privileged structures

The term "privileged structure" [44] is accepted as "a single molecular framework that is able to provide ligands for diverse receptors" and it is considered that the modification of such structures could be an alternative in the search for new receptor agonists and antagonists.

This term refers to scaffolds or molecular fragments that seem to reappear in hits, with a relatively high frequency, within a particular group of receptors. The use of privileged structures as a basis for library design has been used to find compounds with good affinity.

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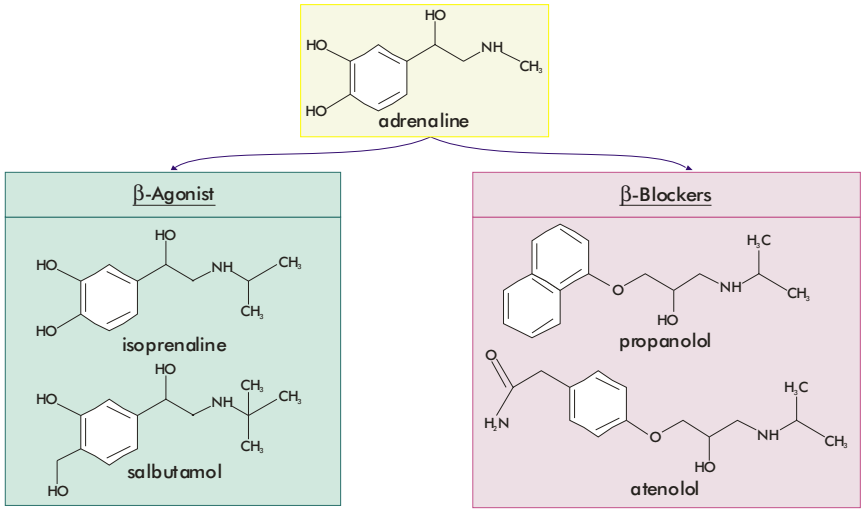


Figure 4. The molecules generated by ligand-based drug design will share chemical features of the natural ligand. Compounds drawn with ACD/ChemSketch.Freeware v10.

Examples of GPCR privileged substructures are biphenyl, 1,1-diphenylmethane, xanthines, 4-arylpiperidines, 4-arylpiperazines, and spiropiperidines (Figure 5). Some of the privileged substructures are not restricted to one GPCR subfamily. Spiropiperidine moiety can be found within ligands of biogenic amine receptors as well as within compounds acting on chemokine and peptide-binding GPCRs. These structures have been notably used for peptide receptors such as somatostatin agonists (Figure 5, structure 1) as well as growth hormone secretagogues (Figure 5, structure 2) [45, 46].

Benzodiazepine based medications are currently in use for several types of central nervous system receptors and in ligands of ion channels and GPCRs throughout the body, and this family is still successfully exploited. Very potent oxytocin antagonists were found by further decorating the two nitrogens of a 1,4-benzodiazepine (Figure 5, structure 3) [47]. Similarly, a 1,4-benzodiazepine library (Figure 5, structure 4) was used to identify ligands for cholecystokinin-B from a set of 168 compounds [48].

Arylpiperazines are another very versatile template, in particular for dopaminergic, serotoninergic and adrenergic receptors. A library of 300 discrete arylpiperazines was examined to identify a nanomolar ligand (Figure 5, structure 5), for the D3 receptor showing good selectivity over the corresponding D2 receptor [49]. However the use of a promiscuous template can result in hits with binding at other receptors, for example other arylpiperazine molecules (Figure 5, structure 6) showed a good affinity at the D3, 5HT2a and a1A-adrenergic receptors [50]. In this case, the lack of selectivity was not a problem because it curiously solved the adverse effects of classical antipsychotics.

Even though the usage of privileged substructures for lead finding offers the chance to quickly identify new lead compounds against novel GPCR targets, receptor selectivity has to be addressed within the lead optimization process, because compounds sharing a privileged substructure often reveal activity against many GPCRs, and could even elicit activities on other yet unknown GPCR mediated mechanism, with adverse side effects.

## Target-based drug design

Target-based drug design is another approach for discovering compounds exhibiting biological activity, but it needs the tridimensional structure of the target. In the case of GPCR, that poses a great problem. With the exception of bovine rhodopsin [3, 4], the atomic level structures of other GPCRs, in particular for potential GPCRs drugs, are yet unknown. Rhodopsin is unique among GPCRs; it consists of two building blocks, an opsin protein and a reversibly covalently bound ligand, retinal (Figure 6). The other available structure of a 7TM (7 transmembrane helices) protein is that of bacteriorhodopsin [51], but bacteriorhodopsin is not a GPCR and in contrast to the beliefs of many authors for a large number of years, its 3D structure is significantly different from that of rhodopsin [6].

In the absence of experimentally determined structures for GPCRs, computational protein modeling becomes an important approach for structure-based drug discovery for GPCRs.

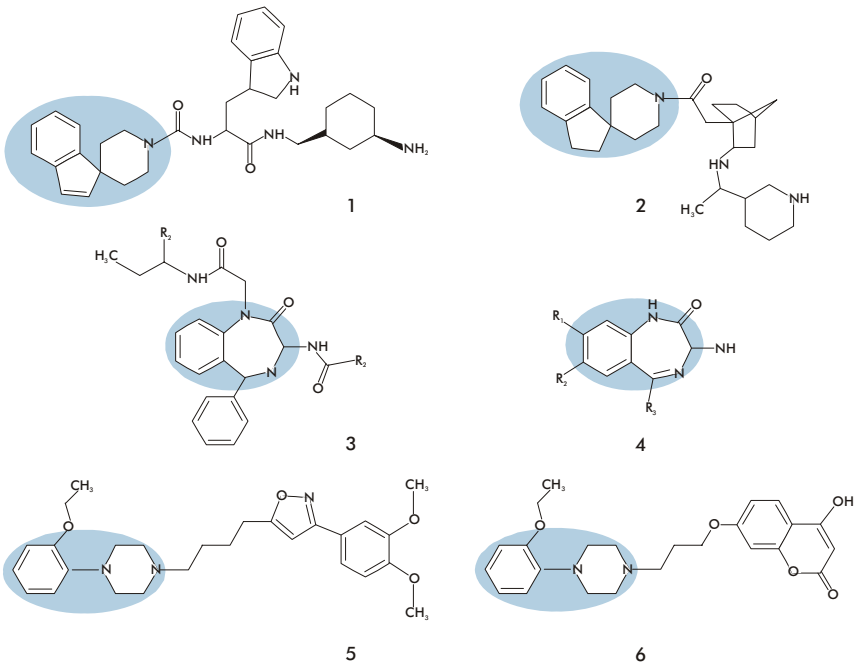


Figure 5. Examples of GPCR privileged substructures (1, 2) Spiropiperidines (3, 4) Benzodiazepines (5, 6) Arylpiperazine. Compounds drawn with ACD/ChemSketch.Freeware v10.

## Modeling GPCRs

The most straightforward approach to determine the 3D structure of GPCRs is homology modeling. Although bovine rhodopsin reveals a low sequence similarity to other GPCRs, the specific arrangement of the 7TM helices stabilized by a series of intramolecular interactions mediated by several backbone and side-chain atoms seems to be conserved among the Family A receptors. Rhodopsin thus represents an improved structural template for the understanding of experimental data available for related 7TM receptors and for generating improved molecular models of other Family A receptors [6].

Homology modeling methods for GPCRs with sufficient accuracy for structure-based drug design would have an enormous impact on drug discovery. This goal faces several serious challenges. Many targets of interest for drug development share rather low sequence identity (30%) with rhodopsin. The transmembrane helices can frequently be aligned with reasonable certainty aided by certain highly conserved residues (*e.g.*, DRY on TMIII, NPxxY on TMVI) and GPCR models based on a template with an identity of 20-30% can thus be expected to be of greater accuracy than when modeling other types of proteins based on a template with a low-sequence identity.

Rhodopsin-based homology models have been developed for the dopamine D2 receptor [52], opioid receptors [53] and the $\alpha$2 adrenergic receptor [54].

Not only homology modeling can be used to obtain GPCRs models. The PREDICT algorithm [55, 56] is a *de novo* approach for modeling the 3D structure of any GPCR that is not based on a homology to a known structure of rhodopsin or bacteriorhodopsin. The PREDICT method relies on the primary sequence of the receptor itself and on structural constraints imposed

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by the membrane environment. PREDICT is based on the physicochemical properties of a single sequence and is therefore substantially different from the existing modeling approaches that rely on known structures or multiple sequence alignment. It was demonstrated that PREDICT was able to reproduce the known experimental structure of rhodopsin [6]. The quality of the PREDICT models for drug discovery purposes was validated by their successful utilization in virtual screening [57]. Examples of this are Dopamine D2, neurokinin NK1, and neuropeptide Y Y1 receptors [58].

Threading assembly refinement (TASSER) is another method that was recently developed [59]. This methodology combines threading and ab-initio algorithms to span the homologous to non-homologous regimens. Only the sequence of the given GPCR is needed and no other extrinsic knowledge (*e.g.*, active sites and binding regions, experimental restraints, etc.) is incorporated into the structure prediction approach. Also, distinct from many other GPCR modeling methods that only attempt to model the TM helical regions [58, 60, 61], TASSER generates reasonable predictions for the loop regions. This is especially important in GPCR modeling as the extracellular loops are often critical in determining ligand specificity [62-64]. Therefore, full-length TASSER models might offer substantial advantages over traditional comparative modeling methods and are likely to be of greater aid in understanding the ligand and signaling interactions of GPCRs.

On our experience the modeling of a GPCR is not, and will never be, an easy task as long as no new crystal structures are elucidated, having only one template to choose from and this being from a single family, all the prediction methods can only be validated using the structure of rhodopsin and this could be strongly biased when used for the prediction of GPCRs from the rest of the families.

## Docking and Virtual screening with GPCRs models

A three-dimensional model of the human melanocortin 4 receptor (hMC4R) was constructed [65], using the transmembrane helices and the C terminal domain of bovine rhodopsin, and simulating both intracellular and extracellular loop domains on homologous loop regions in other proteins of known 3D structure and further refining the structure by minimization and dynamic calculations. The model was tested by docking with a triplet peptide (RFF) ligand. The ligand-receptor interactions found, were consistent with mutational and biochemical data.

Another study was done [66] using only the helical centers of the rhodopsin structure for predicting the 3D structures of rhodopsin and of the b1 adrenergic receptor. The binding mode of adrenaline docked into this b1 adrenergic receptor model was in good agreement with the experimental data.

In another approach [67], it was suggested that GPCRs models based on the rhodopsin X-ray structure are therefore expected to be closer to their inactive form than to their activated, agonist-bound state. Therefore, they have optimized rhodopsin-based models for the agonist and antagonist by energy minimization with one known agonist or antagonist docked to the active site, respectively, even though in the case of an agonist it might not be enough to generate correct models of an activated state of a GPCR. The ligand-bound models of several receptors generated this way, including dopamine D3, vasopressin V1a, β2 adrenergic and opioid receptors, proved successful in computational screening tests. They also did "cross-docking" experiments and showing that virtual screening against the new agonist-bound states of related GPCRs is selective enough to distinguish not only true ligands from randomly chosen drug-like molecules but also true hits from chemically related inactive compounds [67]. Applied to three human GPCRs, such receptor models are accurate enough for discriminating known agonists from randomly chosen "drug-like" molecules. Most importantly, they were able to retrieve in the virtual hit lists true agonists whose chemical structures had not previously been used for generating the pharmacophore and reûning the receptor model [67].

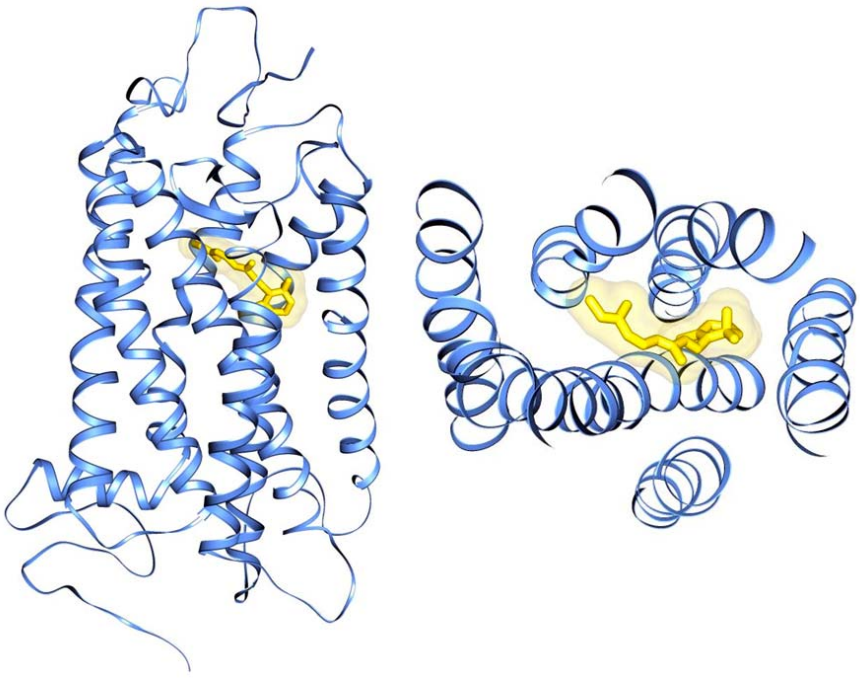

Figure 6. Rhodopsin consist of two blocks, a 7TM protein called opsin (gray) and retinal as a ligand (black). Retinal is attached to Lys296 through a protonated Schiff base bond. Activation of rhodopsin is caused by the photo-isomeric change from 11-cis to -trans retinal. Drawings use rhodopsin structure (PDB: 1L9H) and Chimera [93].

Virtual screening based on GPCR models may be particularly important in cases when either limited or no ligand information is available [18]. This is true for most of the pharmaceutically relevant GPCRs, for which only the endogenous ligand is known. Recent publications report successful applications of GPCR models in virtual screening [67-70], indicating the general relevance of GPCR models and their usefulness for structure-based drug design. For example, a virtual screening for the D3 receptor using a homology model of this receptor was done and eight, out of 20 experimentally tested compounds showed Ki values better than 1 μM [70].

Different virtual screening strategies were compared for the identification of biogenic amine-binding GPCR

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antagonists, starting from virtual libraries, consisting of the antagonist of these target receptors (the a1A, 5HT2A, D2, and M1 receptors) and additional drug-like molecules [18]. And the results were also in agreement with the expectations.

In another attempt to explore the suitability of GPCR homology models for the purpose of virtual screening, a homology model for the a1A receptor was generated [69]. Applying two-dimensional (2D) queries and a 3D-pharmacophore model as a prefilter, they docked 23 000 ligands into the a1A receptor homology model, 37 out of the 80 compounds that were selected for experimental testing, showed a Ki value better than 10 μM, and 24 of these compounds were even binding in the sub-micromolar range. The hit rates achieved with these models were similar to those typically reached when the target protein is given by a crystal structure, suggesting that docking into rhodopsin-based GPCR models, is indeed a feasible approach for the identification of novel ligands.

Even when it is widely accepted that docking to models is more challenging and less successful than docking to crystallographic structures, surprisingly little work has been done to quantify the accuracy of docking to homology models or to improve the existing methods particularly for docking to homology models. The true fact is that any reasonable structure could produce a good hit rate on ligand design or screening if one is careful enough in the selection of the docking experimental parameters.

## Orphan GPCRs

Even though GPCRs have been intensely investigated as potential drug targets, their structural and functional diversity [71, 72], still offer opportunities to develop novel drugs. The analyses of the human genomic sequence suggest that there may be 750 human GPCR-encoding genes, of which approximately 160 cannot be functionally characterized either on the basis of sequence homology or by association with known endogenous ligands [21]. These are referred to as orphan GPCRs (oGPCRs) which bind (as yet) unknown ligands [73, 74].

Reverse pharmacology is an approach that can be used for GPCRs deorphanization. Based on the idea that GPCRs are targets of neurotransmitters, peptides, hormones and other transmitters, it can be expected that orphan GPCRs are also activated by transmitter molecules. Then the orphan GPCR is used as a target to test potential transmitters [73, 75].

The first efforts to identify ligands for orphan GPCRs began in the mid-1980s. At that time, the number of known potential transmitters was large. This became reverse pharmacology in an important approach to this aim. The first successful deorphanization of orphan GPCRs were reported in 1988 (5-HT1A receptors [76] and dopamine D2 receptors [77]). The strategies used were the same, that is, the orphan GPCR was expressed by DNA transfection in eukaryotic cells, membranes of these cells were then used as targets to determine the binding of potential transmitters. During the first part of the 1990s, the application of the reverse pharmacology strategy led to the pharmacological characterization of many GPCRs (Table 2).

Reverse pharmacology has been adapted to allow for the screening of a large battery of potential transmitters on batteries of orphan GPCRs by using high-throughput screening techniques. This has made it possible to match several dozens of orphan GPCRs to their ligands [19, 73]. But all these ligands had been previously discovered and there was a need to identify new transmitters.

While over the years, numerous orphan GPCRs have been matched to specific ligands, there are over one hundred GPCRs that do not bind any known transmitters [78]. In the mid-1990's a parallel approach was devised to use orphan GPCRs as targets to find novel, still non-described transmitters. This has been termed the "orphan receptor strategy" [73, 79]. The method consists of expressing an orphan GPCR by transfection into eucaryotic cell lines, preparing a tissue extract expected to contain the transmitter specific to the orphan receptor and monitoring the activation of the GPCR by applying finely fractioned tissue extract over these engineered cell lines. The activation of the orphan GPCR is monitored by measuring second messenger responses. Positive extracts are fractionated biochemically until the active component is isolated and characterized [78]. This approach has led to the discovery of dozens of bioactive peptides. The orphan receptor strategy was first applied in 1995 to the discovery of a novel neuropeptide called orphanin FQ or nociceptin (or OFQ/N).

Traditionally the existence of a transmitter was postulated on the basis of a particular physiological response and was isolated using that response as an assay. The orphan receptor strategy reverses this approach and allows the isolation of transmitters with unknown physiology and linkage to a disease process. The suc-

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Table 2. Some ligand-orphan GPCR pairings identified using reverse pharmacology strategy

| Receptor | Ligand | Year | Major function | Refs |
|---|---|---|---|---|
| Adenosine $A_1$, $A_{2A}$ (RDC7,RDC8) | Adenosine | 1990-1991 | Platelet function, anxiety | [82,83] |
| ORL-1 | Nociceptin/Orphanin FQ | 1995 | Stress, pain | [94] |
| Orexin-1 and 2 | Orexins/Hypocretins | 1998 | Food intake, sleep-wakefulness | [95, 96] |
| GPR10 | Prolactin-releasing peptide | 1998 | Sleep, absence seizure | [97] |
| APJ | Apelin | 1998 | Unknown | [98] |
| GHS-R | Ghrelin | 1999 | Food intake, GH secretion | [99, 100] |
| SLC-1(MHC1) | MCH | 1999 | Food intake | [101, 102] |
| GPR14 | Urotensin II | 1999 | Vasoconstriction | [103] |
| Histamine H3 (GPCR97) | Histamine | 1999 | Central nervous system-obesity, psychiatry | [88] |
| FM-3/4 | Neuromedin U | 2000 | Unknown | [104, 105] |
| Histamine H4 GPRv53 | Histamine | 2000 | Inflammation, eosinophil chemotaxis | [89] |
| GPR54 | Mestatin | 2001 | Cell proliferation, development | [106, 107] |
| GPR73 a/b | Prokineticin ½ | 2002 | Angiogenesis, circadian rhythm | [108, 109] |
| GPR7 and GPR8 | NPB and NPW | 2002 | Food intake, unknown | [110, 111] |
| GPCR135 and GPCR142 | Relaxin | 2003 | Unknown | [112, 113] |
| GPR91 | Succinate | 2004 | Increases blood pressure | [114] |

cess of this approach, however, is a big leap towards understanding the transmitter system by using the receptor as a vehicle to unravel its physiological function [78].

Nowadays finding the natural ligand of an orphan GPCR, is equal to that of finding a novel transmitter. However, finding the natural ligands of an orphan GPCR is a challenge, since neither the biochemical properties of the ligand nor the response that receptor activation will induce are known.

The G-protein signaling pathway is often unknown, and to maximize the chance of success the assay system must be as generic as possible to allow for the detection of a wide range of signaling mechanisms, but also to be amenable to high throughput screening so that the activity of a large number of ligands can be readily measured. Such assay systems rely mainly on measuring changes in intracellular cAMP or calcium levels, either directly or through the use of reporter gene assays [21] and the presence of endogenous receptors, which result in "background" responses to ligands, can be avoided by engineered cell strains that have had the endogenous GPCR genetically deleted [80, 81].

Other approaches can be used in GPCR deorphanization, *e.g.* determining the relationship between receptor-expression patterns and the expression pattern of a putative ligand, thus matching a candidate gene with a pharmacologically identified receptor. This led to the identification of orphan receptors RDC7 and RDC8 as adenosine A1 and A2A receptors [82, 83].

Sometimes sequence homology can be helpful in GPCR deorphanization. The DNA sequence of the orphan receptor is compared with the sequences of liganded receptors, and where they are closely related it is sometimes possible to predict the likely cognate ligand of the orphan receptor. This approach has been successful in some cases. The initial demonstration that OGR-1 could act as a high affinity receptor for the lipid sphingosylphosphorylcholine [84] probably facilitated the more recent identification of two related orphan GPCRs, TDAG-8 and G2A, as receptors for the lipids psychosine [85] and lysophosphatidylcholine [86], respectively. The ligands for the fourth member of this receptor subfamily, GPR 4, have recently been identified. As would be predicted from sequence homology, the li-gands for GPR4 are lysophosphatidylcholine and sphingosylphosphorylcholine [87]. However, using amino acid sequence identity as the basis for such experiments can be misleading as recently demonstrated following the cloning of histamine H3 [88] and H4 [89] receptors. Both these receptors have the lowest recorded identity to other members of their receptor family (~20% overall to H1 and H2), which highlights the fact that it is not always possible to make accurate predictions. Another example is a receptor originally known as P2Y7 (BLT1) that was thought to be a nucleotide receptor based on its similarity to P2Y receptors, but it was shown to be activated by an unrelated ligand, leukotriene B4 [90]. Sequence homology gives only an indication as to the nature of the likely ligand, but it is not yet possible to accurately predict which ligand is likely to bind to a novel receptor simply from an analysis of the sequence of that receptor.

Homology modeling and ligand docking might even be helpful for the deorphanization of GPCRs. Docking into GPCR homology models can be a useful approach for lead finding by virtual screening when either little or no information on the active ligands is available [18]. Once the generation of reliable GPCR structure models of the activated receptor state becomes possible [91], molecular docking might even provide an opportunity for the identification of novel agonists.

Some work has been done to virtually screen for ligands of orphan GPCRs [92]. This approach may be used to contribute to the functional characterization of orphan GPCRs by identifying potential cognate ligands, thereby providing clues to guide the therapeutic regulation of important signaling pathways in the cell. The advantage of this approach is the simplicity of the required input data: proteins are described using only physicochemical properties of primary amino acid sequences, and ligand features are based on the two-dimensional connectivity between constituent atoms and atomic properties. In its application, large numbers of chemical compounds may be screened against a particular orphan GPCR sequence, with a ranked list of putative high-affinity ligands generated automatically on output.

The deorphanization of GPCRs has revolutionized the discovery of novel transmitters and in turn these have revolutionized many fields of biomedical research in which they have been involved. For example, the novel neuropeptides found as ligands of orphan GPCRs have changed our understanding of the mechanisms that regulate sleep or food intake. Many of the deorphanized GPCRs are targets of drug development programs.

## New GPCR structures

From October 2007 to October 2008, during the editing process of this review, new GPCR structures were released to the public by the RCSB, and they were considered a major breakthrough for the Structural Biology. Three proteins from family A: the human β2 adrenergic receptor [115-117], the human A2A adenosine receptor [118] and the β1 adrenergic receptor [119] from the common turkey were engineered and crystallyzed using novel methods yielding high resolution structures (2rh1, 2r4r, 2r4s, 3d4s, 3eml, 2vt4).

Although there are not yet models reported to being built using any of them as a template, the solely existance of the structures is a very important factor to improve the knowledge about the structural regularities and differences amongst the GPCRs, and indeed will help us to biuld better models.

We certainly hope that in the near future we could expect more structures from other GPCR families.

## Conclusions

GPCRs are regarded as the most important molecules in the field of drug discovery and design, their role as receptors in many of the basic processes on the organism and their presence on the surface of cells on all tissues make them excellent targets. Much effort is needed, however, in the deorphanization of GPCRs, matching all currently known molecules with a ligand. There are several initiatives in this field, but their

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difficulty makes the progress very slow. Despite of the fact that the structure of a single family is known, research has progressed, using combined structure-based techniques. There are several groups attempting to purify, fold and crystallize GPCRs with important breakthroughs, and structures could be expected in the near future. This would undoubtedly change the course of structure-based design for GPCR targets.


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