Chapter 4

An Overview on GPCRs and Drug Discovery: Structure-Based Drug Design and Structural Biology on GPCRs

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Summary

G protein-coupled receptors (GPCRs) represent 50–60% of the current drug targets. There is no doubt that this family of membrane proteins plays a crucial role in drug discovery today. Classically, a number of drugs based on GPCRs have been developed for such different indications as cardiovascular, metabolic, neurodegenerative, psychiatric, and oncologic diseases. Owing to the restricted structural information on GPCRs, only limited exploration of structure-based drug design has been possible. Much effort has been dedicated to structural biology on GPCRs and very recently an X-ray structure of the β2-adrenergic receptor was obtained. This breakthrough will certainly increase the efforts in structural biology on GPCRs and furthermore speed up and facilitate the drug discovery process.

Key words: GPCRs, Drug discovery, Overexpression, Functional receptor, X-ray crystallography, Structure-based drug design.

1. Introduction

The drug discovery process has passed through dramatic changes during the past 20 years. The requirements for drug manufacturing and especially the safety aspects related to the final medicine have become immense. This development has made the drug discovery and development processes both time-consuming and labor intensive. Not surprisingly, development of drugs has become extremely expensive. In addition, the success rate of bringing new successful drugs to the market has been worryingly low. One approach to speed up drug discovery and also to reduce the adverse effects of developed drugs has been to apply structure-based drug design. There are a number of examples of success. For
instance, structural information has played an important role in lead optimization in drug screening programs (1). Furthermore, high-resolution structures of HIV proteinase (2) and influenza virus neuraminidase (3) have contributed directly to the development of AIDS (Agenerase® and Viracept®) and flu (Relenza®) drugs, respectively. In total, more than 10 drugs can today be considered as designed based on known high-resolution structures of target molecules.

Despite their prominent position as drug targets, very modest progress in structure-based drug design has been observed for membrane proteins. Although some 70% of the current drug targets are membrane proteins, minimal direct efforts in structure-based drug discovery has been conducted on membrane proteins. The simple reason is the very small number of high-resolution structures available for membrane proteins in general and more specifically for drug targets. Among the more than 35,000 structures deposited today in public databases less than 200 exist on membrane proteins (4).

2. GPCRs as Drug Targets

GPCRs represent a broad spectrum of drug targets as they are the mediators for so many essential biological activities. Their function can be triggered by such different components as neurotransmitters, peptides, hormones, chemokines, amino acids, calcium ions, odorants, and even light, which results in signal transduction events on the cell, tissue, organ, and whole organism level to adjust to environmental requirements. Not surprisingly, GPCRs have been targeted for many types of maladies including cardiovascular, metabolic, neurodegenerative, neurological, virological, and tumorigenic diseases.

It is estimated that the human genome contains approximately 800 GPCRs of which a relatively large number is represented by odorant receptors. The ligands for many odorant receptors are still unknown and therefore they are called orphan receptors. Also several non-odorant receptors belong to the group of receptors for which ligands are not available. In total, some 100 GPCRs are classified as orphan receptors and are considered as potentially interesting novel drug targets as described in more detail below (5).

Although GPCR signaling is mediated through G proteins, it has fairly recently become evident that other signaling pathways are possible. For instance, c-Src tyrosine kinase interaction with the proline-rich SH3 domain in the third intracellular loop of the β3-adrenergic receptor activates the extracellular signal-regulated
kinase (ERK)–mitogen-activated protein kinase (MAPK) cascade (6). Another example includes the interaction between β-arrestin-1 and c-Src, which facilitates the β2-adrenergic receptor-dependent activation of the ERK–MAPK pathway (7). Moreover, the β-arrestin-1–c-Src interaction plays an important role in glucose transport mediated by endothelin receptors (8) and the activation of the STAT (signal transducer and activator of transcription) transcription factor (9). These “alternative” pathways might present interesting opportunities for the development of novel drugs.

Among the 200 top selling drugs today a quarter is based on GPCRs with annual sales exceeding $200 billion worldwide (10). Included in the best-selling GPCR-based drugs are salmeterol, an anti-asthmatic β2-adrenergic agonist; olanzapine, an antipsychotic serotonin 5-HT2/dopamine receptor antagonist; and clopidrogel, an antithrombotic P2Y12 purinergic receptor antagonist (11). Other “blockbuster” drugs targeted to GPCRs are H1-antihistamines (fexofenadine, cetirizine, and desloratadine) and antihypertensive angiotensin II receptor antagonists (losartan, valsartan, cardesartan, and irbesartan).

3. Conventional Drug Discovery Approaches

Before initiation of any drug screening program it is highly recommended to invest resources in target validation (12). In this context, bioinformatics approaches including database mining, comparative homology and species analysis, and in silico expression studies are essential. Moreover, localization studies by in situ hybridization, RT-PCRs, and microarrays are excellent means for expression comparison in tissues originating from healthy and diseased individuals. Furthermore, it has been demonstrated that mutations in GPCRs can induce disease as is the case for constitutive activity of certain GPCRs (13) and it is important to validate the effect of various mutations. Once targets have been defined drug screening can commence.

Typically, classic drug screening programs have relied on pharmacological evaluation of GPCRs by radioligand binding assays (14). In this context, large chemical libraries are screened in binding assays for hits. The advent of recombinant protein expression methods has substantially facilitated the drug screening process as is described in more detail below. Assay development has focused strongly on automation and miniaturization and, for instance, fluorescence intensity, fluorescence polarization, time-resolved fluorescence resonance energy transfer (TR-FRET), and fluorescence macroconfocal technology (FMAT) methods have been utilized for high-throughput screening in 96-, 384-, and 1536-well
The conventional process involves further chemical modifications of discovered hit molecules on so-called lead compounds. These compounds will be further tested for potency by saturation binding assays, but also for specificity by performing pharmacological evaluation on related receptor families and as well as on receptor subtypes.

Much attention is today paid to the composition of the chemical libraries used for the screening process. The design of chemical libraries has become more and more important and approaches have been taken to generate ligand-based libraries and relying on physicochemical and substructural properties. Structure-based library design is described in more detail below.

The majority of drug screening efforts have recently shifted from binding assays to functional determination of receptor coupling to G proteins. The advantage of change in strategy is the possibility to evaluate agonists, antagonists, partial agonists, and inverse agonists. A number of cell-based assays measuring cAMP stimulation, inositol phosphate accumulation, and intracellular Ca$^{2+}$-release have been established. Application of Ca$^{2+}$-sensitive dyes has allowed fluorescence imaging in automated 384-well format. Other screening approaches include the establishment of stable cell lines for second messenger and reporter gene detection ($\beta$-lactamase and luciferase) for detection of transcriptional regulation of promoter elements activated by GPCRs. Moreover, transient expression of GPCRs in melanophores from the neural crest of *Xenopus laevis* has allowed monitoring functional activity by measurement of light absorption based on pigment dispersion. Today agonist activation and antagonist inhibition has been evaluated for more than 100 GPCRs in the melanophore system. Although application of functional screening assays for GPCRs has broadened the drug discovery process and facilitated finding new drug molecules, a number of other approaches have been taken as presented below.

### 4. Chemical Libraries and Structure-based Drug Design

Although the first high-resolution structure of a GPCR became available only recently, approaches have previously been made to use structural information in drug design. One approach has been two- and three-dimensional mapping of the ligand–GPCR interaction sites applying homology models of rhodopsin and site-directed mutagenesis to determine structure–activity relationships (SARs) for ligands. Moreover, structural information on GPCR ligands has presented the basis for design of chemical
libraries for screening purposes (21). Pharmacophore-based design of combinatorial libraries was applied to design a novel series of indolyl sulfonamides as selective high-affinity serotonin 5-HT₆ receptor ligands and resulted in the identification of some novel compounds (22). Pharmacophore models have also been used for virtual screening approaches to identify nonpeptidic ligands for peptide-binding GPCRs such as the somatostatin receptor known for its poor bioavailability and low metabolic stability (23). Nonpeptidic antagonists for the urotensin II receptor could be identified based on truncated peptide derivatives of the cyclic 11 amino acid peptide urotensin II (24). Alanine scanning and NMR spectroscopy resulted in the identification of the Trp-Lys-Trp motif in the cyclic part of the human urotensin II. Likewise, when two different pharmacore models were established 172 virtual antagonist hits were identified for the muscarinic M3 receptor leading to three compounds with a novel scaffold (25). Other nonpeptidic GPCR ligands have been designed for opioid (26), thrombin (27), and somatostatin (28) receptors.

Moreover, ligand-based three-dimensional quantitative SAR (3D-QSAR) methods have been applied for lead optimization. Using comparative molecular field analysis (CoMFA) for the correlation of the steric and electronic field environment a number of GPCR lead compounds were optimized (29, 30). For instance, successful ligand optimization for dopamine (31, 32), serotonin (33, 34), endothelin (35), and adenosine (36, 37) receptors has been reported for CoMFA. Moreover, ligand selectivity has also been addressed by demonstrating side affinities for a series of aryl piperazines active against the serotonin 5-HT1A receptor for the α1-adrenergic receptor (38). When the ligand-based CoMFA method was combined with 3D receptor modeling of serotonin receptor subtypes, the serotonin 5-HT2C/2B indoline urea lead series showed only minor side affinity against the serotonin 5-HT2A receptor (39, 40).

The progress in chemogenomics has also had a big impact on the design of targeted libraries. The PREDICT technology was developed for 3D structure modeling of GPCRs (41). PREDICT does not require a structural template and can be used for any GPCR amino acid sequence. Consequently, PREDICT has been applied for dopamine D2, neurokinin 1, neuropeptide Y1, and chemokine CCR3 receptors and demonstrated good agreement with data from a large number of experiments.

Virtual screening has become important in drug discovery because of potential time and cost reductions. For instance, applying the 2.8 Å-resolution X-ray structure of bovine rhodopsin as a homology model for antagonist screening of three human GPCRs (the dopamine D3 receptor, the muscarinic M1 receptor, and the vasopressin V1a receptor) (42) showed that it was possible to distinguish known antagonists from randomly chosen molecules.
Three different docking programs (Dock, FlexX, Gold) were used in combination with seven scoring functions (ChemScore, Dock, FlexX, Fresno, Gold, Pmf, Score). In another approach, PRE-DICT was applied for virtual screening of several GPCRs resulting in enrichment factors of 9- to 44-fold better than what was obtained from random screening (43). Moreover, a practical scoring function was applied to assess the druggability of compounds, which consisted of 12 metrics taking into account physical, chemical, and structural properties and undesirable functional groups (44). Evaluation of the 12-metric scoring function for 44 different databases including more than 3.8 million commercially available compounds indicated that the majority of compounds that did not show satisfactory druggability had a high molecular weight and high logP values and also indicated the presence of reactive functional groups.

5. Novel Approaches Including Dimerization and Orphan GPCRs

In addition to conventional screening of molecules reacting with GPCRs more adventurous approaches have been to target pathways other than those involving G proteins (20). Typically, a number of GPCRs can signal through interaction with arrestins and other cellular proteins. For instance, receptor–protein interaction occurs between the angiotensin 1A receptor and the C terminus of the Janus 2 kinase (JAK2) through activation of the STAT transcription factor (9). Investment in targets for alternative pathways might bring substantial rewards as the signaling through G proteins has been so well documented and fairly few novel discoveries are anticipated. On the other hand, employing combinatorial chemistry and advanced chemical libraries for the screening procedure might be productive (16).

There are two other interesting relatively novel approaches for drug discovery on GPCRs. Approximately a decade ago the existence of GPCR dimers and higher multimers was documented (45). Interestingly, it was demonstrated that GABA_B receptors required both GABA_B-r1 and GABA_B-r2 subunits in a dimer composition to obtain functional receptors on the plasma membrane (46). Other GPCRs such as taste receptors can also form dimers. Most interestingly, the heterodimeric T1R1+T1R3 combination generates the umami receptor, whereas the T1R2+T1R3 heterodimer defines the sensor for sweet taste (47, 48). Moreover, when the leukotriene BLT1 receptor was expressed in Escherichia coli inclusion bodies low-affinity binding homodimers were obtained after refolding, which could be reverted to high-affinity
binding after addition of a heterotrimeric Gα12β1γ2 complex to the refolded GPCRs (49). This suggested the requirement of GPCR dimerization.

The biggest impact dimerization can have on drug discovery is most likely on the activation of different signaling pathways, receptor desensitization and sensitization, and modulation of GPCRs (45, 50). For this reason, the number of potential drugs could increase significantly and programs on oligomeric GPCRs could be included in drug screening strategies. The action of drug molecules on additional sites in comparison to monomeric GPCR binding sites would also have an impact on drug design. In this context, novel designs of dimeric ligands with two covalently linked monovalent ligands could possibly more efficiently induce or stabilize dimeric GPCR conformations (51). In order to prevent the potential protein–protein interaction in dimers, enhancement or disruption of oligomerization could be promoted in drug design.

Another approach has been to target orphan GPCRs. By definition, these are receptors for which no ligand has been defined yet. Despite extensive deorphanization programs some 100 orphan GPCRs still exist (52). Orphan receptors are considered as potentially interesting targets as the initiation of several drug discovery programs indicate. In this context, nociceptin/orphanin FQ has been evaluated for pain and anxiety (53), orexin/hypocretin for narcolepsy (54) and food intake (55), ghrelin for obesity (56), and metastin for potency in oncology (57). The impact of orphan GPCRs is difficult to evaluate as only few receptors have been deorphanized, so far. One concern has been the relatively low endogenous levels of their ligands and their potential signaling through G protein-independent pathways. Drug development programs on orphan receptors can therefore be considered risky, but the rewards through finding novel treatment for disease, when successful, will also be substantial.

6. Overexpression of GPCRs

To support drug screening activities and structural biology initiatives it is essential to obtain high-level expression of GPCRs. As the seven-transmembrane topology of GPCRs makes the expression more difficult and demanding compared to soluble protein, it is not surprising that more or less every available expression system has been tested (58). Both prokaryotic and eukaryotic expression systems have been used frequently and recently also cell-free E. coli- and wheat germ-based systems have been applied. Although
cell-free translation has mainly been used for soluble proteins, novel development has also allowed reasonable expression of membrane proteins (59, 60). E. coli vectors are the most frequently used prokaryotic vectors. GPCRs have been expressed both in inclusion bodies (61) and the plasma membrane (62). The approach of production in inclusion bodies generates relatively high expression level, but the drawback is that extensive refolding exercise is required to restore the GPCR (63). On the other hand, targeting GPCRs to the plasma membrane can generate functional GPCRs. However, the membrane insertion often results in growth regression of bacterial host cells and reduced recombinant protein yields due to the GPCR toxicity. Improved expression has been achieved by introduction of mutations and deletions in the target GPCR and engineering fusions to, for instance, maltose binding protein (MBP) (64). This approach has resulted in production of milligram levels of rat neurotensin receptor in E. coli cultured in fermentors (65). In addition to E. coli, Halobacterium salinarum (66) and Lactococcus lactis (67) have been applied for GPCR expression. The yields obtained have so far, however, been relatively modest.

Yeast-based expression of GPCRs has received plenty of attention. The most frequently used yeast hosts are Saccharomyces cerevisiae (68, 69) and Pichia pastoris (70). Particularly, the application of P. pastoris has resulted in high binding activity (up to 100 pmol/mg) and impressive yields (5 mg/L) of a large number of GPCRs (71). Likewise, Baculovirus vectors carrying GPCRs have been introduced into insect cells resulting in robust expression of functionally active GPCRs (72). Up to 16 GPCRs were expressed from Baculovirus vectors in parallel resulting in 250 pmol/mg receptor (73). The production of GPCRs in insect cells cultured in bioreactors has provided sufficient material for structural studies on GPCRs as described below. Application of mammalian expression systems for the overexpression of GPCRs has been to some extent hampered by time-consuming and expensive procedures and low expression levels. Despite that a mutant HEK293 cell line allowed production of up to 6 mg/L of rhodopsin (74). A number of viral vectors such as adenoviruses, vaccinia viruses, lentiviruses, and alphaviruses have also been used for GPCR expression. Particularly, Semliki Forest virus (SFV) vectors, an alphavirus, have been applied for the expression of more than 100 GPCRs in different cell lines (75, 76). The SFV system has shown high expression levels measured by saturation binding (>100 pmol/mg) and functional coupling to G proteins (intracellular Ca\textsuperscript{2+} release, inositol phosphate accumulation, cAMP stimulation, and GTP\gammaS binding). Furthermore, the system has been scaled up to allow large-scale production in multiple liter volumes in spinner and roller flasks as well as in bioreactors.
Although no high-resolution structure of therapeutically interesting GPCRs had been solved until recently, reasonable information has been available for GPCRs from other structures. Tertiary models of various GPCRs have been built based on the high-resolution structure of bacteriorhodopsin obtained from *Halobacterium salinarium* (77). A further improvement occurred when the three-dimensional structure of bovine rhodopsin became available (78) as modeling could switch to this mammalian receptor. However, the real breakthrough was seen very recently when the high-resolution structure of the human β2-adrenergic receptor–lysozyme complex was solved by receptor overexpression in Baculovirus-infected insect cells (79, 80). The fusion protein was bound to the partial inverse agonist carazolol at a 2.4 Å resolution. Despite a similar location of carazolol in the β2-adrenergic receptor and retinal in rhodopsin, structural differences in the ligand-binding site and other regions were observed. This clearly signifies the shortcomings of using rhodopsin as a template for GPCR modeling.

In absence of high-resolution structures, site-directed mutagenesis has been applied to investigate the site(s) of interaction between GPCRs and their ligands. In combination with bioinformatics and modeling, site-directed mutagenesis supported by *in vitro* expression in cells can provide important information on change in binding affinity and functional activity. For instance, mutation of an asparagine residue in the TM2 of the GnRH receptor resulted in complete loss of binding activity (81). However, the presence of a second mutation in the TM7 restored the binding activity, which indicated a close proximity of TM2 and TM7. Studies on the neurokinin-1 receptor (NK1R) showed that three adjacent N-terminal residues (Asn23, Glu24, and Phe25) affected substance P binding (82). Moreover, His108 located on the top of TM3 and Tyr287 on the top of TM7 were shown to interact with substance P (83). Other studies revealed that the binding mode for the nonpeptide antagonist CP 96,345 was different from substance P (84) for NK1R and their binding pockets were also different (85). Furthermore, mutant His197 showed a significantly reduced affinity to CP 96,345 (86). For other GPCRs, it was demonstrated by site-directed mutagenesis that ligand binding at the β2-adrenergic receptor occurred mainly within the membrane-spanning regions flanking TMs 3, 5, 6, and 7 (87) and that three binding sites for 5-hydroxytryptamine (5-HT), propranolol, and 8-hydroxy-N,N-diproprylaminotetralin (8-OH-DPAT) within the highly conserved 7TM domain existed.
for the serotonin 5-HT1A receptor (88). Overall, based on results obtained from molecular modeling and mutagenesis studies it was suggested that family A receptors might share a common binding pocket (89, 90). However, because the same effect can be obtained by allosteric modulations (91) and constitutive signaling (92) this view might be oversimplified.

Other approaches to obtain structural information have been to define the proximity and orientation of TM regions by the introduction of histidine zinc (II) binding sites into neurokinin (93) and opioid receptors (94). Furthermore, distance constraints and flexibility of extracellular loops could be determined for the muscarinic M3 receptor by engineering of cysteine mutants (95). In another approach, fluorescent unnatural amino acids were introduced at defined sites for determination of distances and tertiary structures by FRET technology (96). Finally, electron paramagnetic resonance (EPR) spectroscopy and cysteine cross-linking has provided information on helix orientation, flexibility of receptor loops, and the conformational changes induced by light (97).

8. Conclusions and Future Prospects

GPCRs will continue to serve as the most important drug targets in modern medicine. Although drug screening on GPCRs is anticipated to constitute a large part of these activities other approaches will be applied. Much attention will be given to the design of larger and more specialized (GPCR-oriented) libraries. The development of sophisticated software programs and the advancement in bioinformatics will further improve the possibilities of increasing virtual screening methods. This will certainly significantly reduce both costs and time in drug development. As most of the conventional approaches for GPCR screening have already been explored and the chances of discovery of novel drug molecules diminish, it will be essential to look for new opportunities. Orphan GPCRs might be interesting targets for further exploration as ligand molecules with novel therapeutic properties might be discovered. Moreover, investigation of non-G protein signaling pathways for GPCRs might reveal novel mechanisms of actions and in that context discovery of novel therapeutic targets. Finally, structure-based drug design has indeed seen a major breakthrough through the determination of the first human GPCR structure. It is anticipated that other GPCR structures will follow shortly, which will certainly open up extensive new possibilities for rational drug design approaches.
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