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REVIEW

Rational Design of Drugs Targeting G-Protein-Coupled Receptors: Ligand Search and Screening

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Abstract—G protein-coupled receptors (GPCRs) are transmembrane proteins that participate in many physiological processes and represent major pharmacological targets. Recent advances in structural biology of GPCRs have enabled the development of drugs based on the receptor structure (structure-based drug design, SBDD). SBDD utilizes information about the receptor–ligand complex to search for suitable compounds, thus expanding the chemical space of possible receptor ligands without the need for experimental screening. The review describes the use of structure-based virtual screening (SBVS) for GPCR ligands and approaches for the functional testing of potential drug compounds, as well as discusses recent advances and successful examples in the application of SBDD for the identification of GPCR ligands.

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INTRODUCTION

G protein-coupled receptors (GPCRs) have a special place in structural biology. These heptahelical transmembrane proteins are widely represented in the human body and constitute one of the largest classes of membrane proteins [1]. They participate in the transmission of extracellular signals initiated by the binding of endogenous agonists or receptor photoactivation and regulate cellular function by modulating the activity of receptor partner proteins. Natural GPCR ligands are chemically diverse compounds that include amino acids, ions, proteins, peptides, nucleotides, biogenic amines, and lipid bioeffectors. GPCRs play a primary role in such important physiological functions of the human body as vision, perception of taste and smell, regulation of the nervous, immune and cardiovascular systems, and maintenance of homeostasis and cell density in tissues. Dysfunctions of GPCRs lead to severe diseases, which makes these receptors important biological targets in basic and applied medico-pharmaceutical research. About 700 drugs, i.e., 35% of all drugs approved by the US Food and Drug Administration (FDA), target 108 unique GPCRs. Approximately 321 of them are currently in clinical trials, of which ~20% target 66 novel GPCRs that do not have approved drugs [2]. The malfunctions targeted by GPCR-specific drugs are diabetes, obesity,

Abbreviations: BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IP_1, IP_2, and IP_3, inosito mono-, di-, and triphosphate; SBDD, structure-based drug design; SBVS, structure-based virtual screening.

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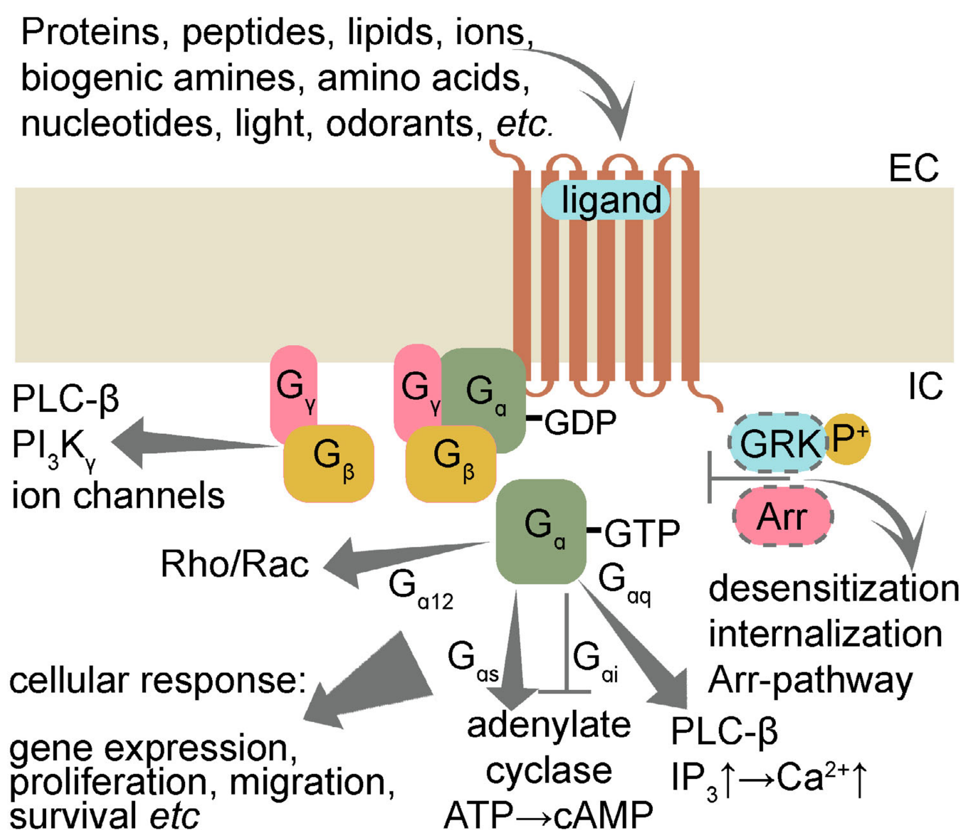

Fig. 1. General principle of GPCR signal transmission: a selective ligand approaches from the extracellular space and activates the receptor which undergoes conformational changes and activates bound G protein (heterotrimer consisting of the α-, β-, and γ-subunits). This initiates heterotrimer dissociation and exchange of GDP for GTP in the nucleotide-binding site of the $G_α$ subunit, which initiates a particular reaction cascade. For example, $G_{α12/13}$ triggers the Rac/Rho small GTPase pathway; $G_{αs}$ stimulates and $G_{αi/o}$ inhibits adenylate cyclase, an enzyme that catalyzes conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP); $G_{αq}$ stimulates phospholipase C-β (PLC-β), which cleaves inositol triphosphate (IP_3) from phosphatidylinositol 3,5-bisphosphate (PI-3,5-P_2), resulting in the increase in the IP_3 concentration and triggering of the calcium release from the intracellular depots. The generated second messengers initiate the appropriate cellular response. $G_{βγ}$ subunits also trigger a number of signaling pathways, for example, by interacting with ion channels, lipid kinases (e.g., phosphoinositide 3-kinase-γ, PI3Kγ) and phospholipases (PLC-β). Prolonged activation can result in the receptor phosphorylation, for example, by GPCR kinase (GRK), which leads to the binding of arrestin (Arr) and inhibition of the G protein-mediated signaling [5]. EC, extracellular region; IC, intracellular region.

cardiovascular disorders, Alzheimer's disease, and other disfunctions of the central nervous system [3]. There is no doubt that GPCRs will remain at the forefront of drug development for a long time to come.

Transmission of extracellular signals by GPCRs occurs in response to the receptor activation by a ligand resulting in changes in the receptor conformation (Fig. 1). Orthosteric ligands bind in the main binding pocket which also binds to endogenous receptor ligands, while allosteric ligands bind in additional pockets and affect the functioning of the receptor regardless of the main ligand, thus increasing the possibilities for the pharmacological regulation of the receptor [4]. In the classic case of activation, changes in the receptor conformation activate heterotrimeric G proteins, signaling molecules named for their ability to bind and convert GTP/GDP. G proteins are classified into four main families according to the type of their α-subunits that is determined based on their structural homology, function, and type of triggered signaling pathways: $G_{i/o}$, G_q, G_s, and $G_{12/13}$ [5].

In addition to G proteins, there are a number of transducer proteins that directly interact with GPCRs, such as β-arrestins and G protein-coupled receptor kinases (GRKs) [6]. Modulating proteins JAK (Janus kinase) [7], RAMP (receptor activity-modifying protein) [8], RGS (regulator of G protein signaling) [9], and others, that also take part in signal transmission. Activated GPCRs can be phosphorylated by GRK and other effector kinases. Phosphorylation patterns (often referred to as "barcodes" in the literature) determine the signaling through various arrestins and alter its kinetic parameters [10].

One of the most important phenomena in the functioning of GPCRs is the functional selectivity of ligands (biased signaling): specific ligands (including

allosteric modulators [11]) cause activation/inhibition of particular signaling pathways associated with different G proteins or arrestins, thus directing signaling cascades in a certain direction. Activation of different signaling pathways through the same GPCR can have important pharmacological consequences, since one signaling pathway can facilitate disease therapy and the other can be associated with the therapy side effects. Understanding the mechanisms of biased signaling can help in the development of ligands that would specifically modulate selected signaling pathways without affecting others, thereby minimizing the side effects of treatment [12].

Elucidation of the functioning of GPCRs requires structural information on their different conformational states [2]. Currently, structural and functional studies of members of the GPCR superfamily advance very rapidly; the 3-D structures have been resolved for 165 unique receptors out of 876 GPCRs encoded in the human genome (according to the https://gpcrdb.org/resource; accessed on 06/19/23). However, each GPCR molecule poses new challenges, mainly because of the low stability, amphiphilic nature, and high conformational mobility of these receptors. The methods for the expression and crystallization of GPCRs, collection of crystallographic and cryogenic electron microscopy (cryo-EM) data, application of bioinformatics approaches for identification of GPCR ligands, and functional tests used for selecting potential drugs from predicted compounds are constantly optimized and modernized. Thus, the success in the search for new GPCR ligands can be explained by an increasing number of high-resolution GPCR structures and expansion of virtual libraries of compounds for further "on-demand" synthesis. Addition of new methods for the analysis of receptor–ligand interactions to the already existing "toolkit" used in the development of GPCR-targeting drugs makes this process more "rational."

In this review, we described the principal steps in the structure-based drug design (SBDD) from the point of view of GPCR structural biology with a special emphasis on the structure-based virtual screening (SBVS) of ligands and analysis of GPCR functional activity *in vitro*.

STRUCTURE-BASED VIRTUAL SCREENING (SBVS)

SBVS is a computer-aided search for potentially active molecules in virtual compound libraries. It relies on the analysis of the three-dimensional structure of the target protein and the features of protein interaction with potential ligands, mostly at the early stages of drug development.

SBVS usually starts with the processing of the protein three-dimensional structure, which includes addition of necessary hydrogen atoms, verification of the atom valences and multiplicity of bonds, removal of water molecules (when these molecules are not involved in the interaction with the ligand or the software does not explicitly account for them), restoration of replaced amino acids, removal of ligands present in the original structure (since protein holo forms are usually better suited for molecular docking [13]). and selection of protein region for docking. The information on the known ligands can be useful. When used with the data on specially formulated inactive compounds (decoys) [14, 15], it allows to perform the retrospective analysis in order to validate the structure prepared for docking and to select the optimal docking parameters [16].

Next, a virtual library containing potential ligands is selected. If necessary, the library is converted into a format suitable for a chosen software package and used for molecular docking. In molecular docking, ligands from the virtual library are positioned in the binding pocket of the target in various conformations. The docking algorithms account for the target structure, interactions between the atoms, and energy parameters of the system in order to estimate the binding strength of the ligand and to predict its affinity. The ligands are ranked based on the scoring function values, and the most promising candidates are selected for further experimental studies, which include their synthesis and assessment of biological activity [16].

Virtual compound libraries. The chemical space of drug-like organic compounds is enormous and, according to various estimates, ranges from 10^{20} to more than 10^{60} molecules [17]. Analysis and even storage of such a number of virtual compounds are currently impossible. For example, only recently the computing power and methods for the optimization of calculations have allowed to exceed 1 billion of studied molecules [18].

One of the approaches to creating databases with a large number of compounds is generation of theoretically synthesized molecules based on the rules of chemical stability. Such databases contain molecules with a limited number of non-hydrogen atoms typical for organic compounds (C, N, O, S, halogens) [19, 20]. Despite a great chemical diversity of the resulting compounds, no methods for their synthesis have been developed and, moreover, some molecules are too complex to be used in further research. Because of this, the work with virtual libraries involves the use of various filtering algorithms and additional restrictions [21].

An alternative approach is using the building blocks to generate virtual libraries of compounds that can be then synthesized from structural units using already proven reactions [22, 23]. This approach has certain advantages, including rapid "on-demand"

Publicly available commercial databases and chemical spaces used for SBVS

Database	Number of small molecules*
ZINC (https://zinc20.docking.org)	>2.3×10^8
ChEMBL (https://www.ebi.ac.uk/chembl/)	2.6×10^6
PubChem (https://pubchem.ncbi.nlm.nih.gov/)	1.2×10^8
REAL Space (Enamine) (http://enamine.net/compound-collections/real-compounds/real-space-navigator)	3.6×10^{10}
GalaXi (WuXi LabNetwork) (https://www.biosolveit.de/infiniSee)	1.2×10^{10}
eXplore (eMolecules) (https://www.emolecules.com/explore)	7×10^{12}
Freedom Space (Chemspace) (https://chem-space.com/compounds/freedom-space)	1.8×10^8
CHEMriya (OTAVA's Chemicals) (https://otavachemicals.com/products/chemriya)	1.2×10^{10}

Note. * Data for 2023 [36].

chemical synthesis of molecules (make-on-demand compounds) and identification of ligands with chemical novelty (since the method does not use the information on already known active molecules) [24]. However, the main disadvantage of combinatorial virtual libraries is a limited variety due to the use of a specific set of reactions and a finite number of building blocks [21].

There is also a great interest in the application of generative deep learning models to increase the diversity of virtual libraries. The model is trained on a small sample of known molecules represented by their molecular descriptors, including molecular graphs, SMILES [25], and others [26]. Based on these data, the neural network creates a new chemical space that contains compounds that combine and vary the features of the original sample. This approach requires careful control both at the stage of model training and during validation of the resulting molecules and assessment of their diversity [27-29]. However, a model trained using additional input data about known ligands allows to work with a rationally compiled virtual library of compounds aimed at the target protein (focused library) [30, 31].

Due to the computational difficulties with storing and processing information, large virtual libraries (chemical spaces) sometimes do not explicitly list all molecules they contain, but are instead specified by the rules necessary to virtually construct compounds on-the-fly [16, 32, 33]. Of particular value are the algorithms for searching, comparing, and selecting molecules in such spaces. These include comparative pharmacophore analysis (e.g., FTrees method; https://www.biosolveit.de/infiniSee), molecular fingerprint similarity (e.g., Tanimoto coefficient [34]), comparison of 3D structures of compounds (e.g. ROCS algorithm; https://www.eyesopen.com/rocs), and many others. Among other things, such methods allow to create collections of various chemical compounds (diversity libraries) that are small virtual libraries of organic molecules representing the diversity of an entire chemical space or database [35]. Collections of chemical compounds are widely used for primary virtual screening as they allow efficient selection of potential hit compounds for further optimization with significant savings of computing resources.

In addition to the listed approaches used for creating chemical spaces, there are numerous databases containing information about molecules that had already been studied and characterized. Some of the databases and chemical spaces that may be useful in SBVS [36] are shown in the table.

Molecular docking, which was first described in the 1980s [37, 38], is currently one of the most common SBDD approaches. It is used to model at the atomic level the interactions between a small molecule and a target protein and allows to predict the most stable position and energetically favorable ligand conformation in the binding site. It should be noted that the predicted high affinity of a ligand does not guarantee its effectiveness and does not determine the nature of its effect on the receptor. To predict the biological activity of potential ligands, some auxiliary approaches are employed, for example, molecular docking using a certain conformation of the receptor (e.g., associated with an agonist, antagonist, or inverse agonist), analysis of molecular descriptors of the ligands, analysis of the molecular dynamics of the receptor–ligand complex,

and some others [39]. Nevertheless, the problem of determining the type of biological activity of a ligand based on its structure remains relevant for computer modeling and does not have a universal solution.

The process of molecular docking can be divided into two main stages. The first one is finding the best position of the ligand in the binding site (algorithm Search). In the simplest case, the ligand and the target protein can be considered as two rigid bodies that form a system with six degrees of freedom (translational and rotational). This method is used as the fastest approach for initial screening of small molecule databases. However, the resulting estimate is often unacceptably approximate. More accurate docking takes into account the conformational degrees of freedom of the ligand, protein (usually only a small fragment directly involved in the complex formation), or both of them [40, 41]. Calculating all possible conformations for a large number of molecules in this case is time-consuming, and numerous algorithms have been developed and tested for optimizing the search for the system energy minimum. The algorithms that take into account the flexibility of the ligand, can be divided into 3 types: systematic, stochastic, and deterministic (there are also hybrid approaches). The features of these algorithms and their use in various software packages were comprehensively described in reviews [42, 43].

Once promising ligand conformations are selected, a scoring function must be applied to rank and select the best compounds, which is achieved by assessing changes in the system energy caused by formation of the ligand–receptor complex. To do this, the scoring functions make a number of simplifications and evaluate approximate contribution of physical phenomena that determine molecular interactions in the complex. The scoring functions are usually divided into 4 main types: physics-based, empirical, knowledge-based, and machine learning-based (there are also some combined approaches). More information on the scoring functions can be found in [44, 45].

Despite a large number of SBVS software packages (the most popular of which are listed in the review by Maia et al. [46]), it is impossible to select a program that would always ensure the best results. A software providing identification of a larger number of hit compounds and their better elimination has to be chosen on a case-by-case basis. Therefore, an approach called consensus docking is sometimes employed, which uses several different programs for molecular docking. The results of consensus docking can help to identify ligands with the highest affinity and to increase the accuracy in the final selection of compounds [47, 48].

After virtual screening, the resulting compounds can further be selected, if necessary, based on molecular properties determining their bioavailability (one of the most well-known sets of criteria is the Lipinski's rule of five [49]), similarity of molecular fingerprints and other molecular descriptors, and indicators predicting false positives and anomalous activity of ligands (PAINS criteria [50]) in order to search for unique chemotypes. The selected molecules (from several tens to several hundreds) are analyzed, and compounds with the highest performance indices are tested in *in vitro* functional tests to select hit compounds and to eliminate inactive substances.

There are at least several examples when the use of SBVS methods for GPCRs has made it possible to obtain compounds with a submicromolar activity [24, 51, 52].

ANALYSIS OF LIGAND FUNCTIONAL ACTIVITY *in vitro*

Selected hits and leading compounds obtained by virtual screening and theoretical optimization should be tested experimentally. The main approaches to testing involve determination or comparison of kinetic and thermodynamic binding constants (first of all, dissociation constant K_d) and quantitative characterization of cellular response by detecting formation/dissociation of protein–protein complexes and changes in the concentration of second messengers and expression profiles of reporter proteins. The measured values include the ligand molar activity (potency), the half-maximal effective concentration (EC50, agonist concentration that causes a biological response equal to half the maximum possible for a given ligand after reaching the curve saturation), as well as the half-maximal inhibitory concentration (IC50) for antagonists and inverse agonists. When comparing the effectiveness of ligands, relative competitive substitution constants are used that reflect the efficiency of ligand replacement in the binding site with another ligand.

***In vitro* functional assays characterizing receptor–ligand interactions.** Methods using fluorescent polarization of ligands [53] and affinity selection mass spectrometry (AS-MS) [54, 55] are among the most common *in vitro* procedures for the large-scale screening of GPCRs. Radioligand analysis based on the binding of a radiolabeled ligand to the receptor [56], scintillation proximity assay (SPA), and biosensor technologies based on the surface plasmon resonance [57, 58] are used for the screening at the laboratory scale (dozens of ligands). Other promising methods include microscale thermophoresis (MST) [59, 60], nano differential scanning fluorimetry (NanoDSF), NMR spectroscopy-based screening [61], isothermal titration calorimetry (ITC) [62], and bio-layer interferometry (BLI), which is used to study interactions with macromolecular ligands [63]. The binding constants determined

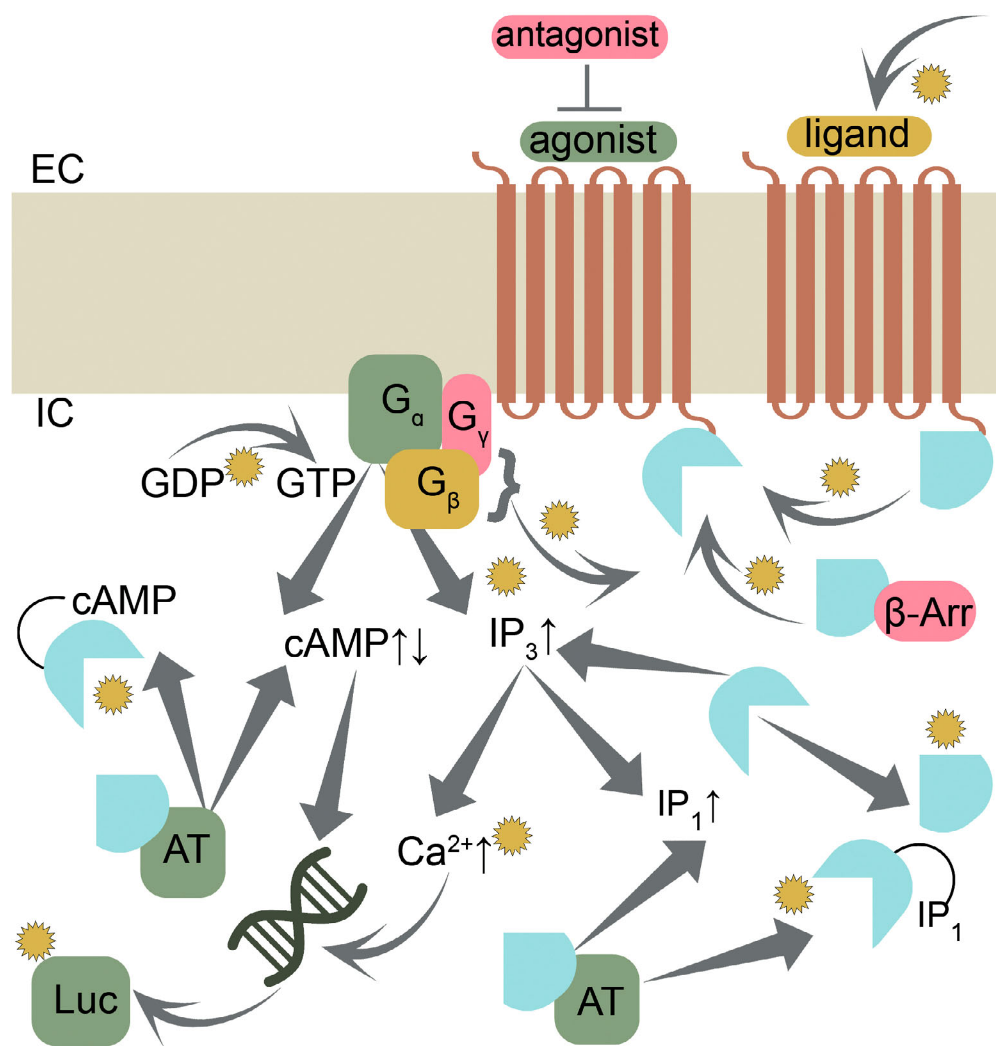

Fig. 2. Methods for analysis of intracellular GPCR signaling used in the screening of compound libraries; the "sun" sign denotes detectable chemiluminescence, fluorescence signal caused by the interaction of molecules modified with the sensors, assembly of fluorescent proteins, or resonance energy transfer that occurs when macromolecules approach each other. EC, extracellular region; IC, intracellular region.

with these approaches generally do not allow to reliably identify the pharmacological type of a ligand (orthosteric agonist/antagonist/inverse agonist/allosteric modulator) or to assess the actual effectiveness of ligand action on the intracellular response after its interaction with the receptor. It should be also mentioned that the low stability of purified GPCRs makes their screening outside cells or native membrane systems challenging, so *in vitro* assays use stabilized receptor constructs.

Functional tests in cultured cells are used in cases when the *in vitro* binding data are insufficient and the levels of receptor activation and its pharmacological profile have to be assessed. Ligand binding to GPCRs induces intracellular signaling pathways, resulting in biochemical reactions, protein–protein interactions, and changes in the intracellular concentration of second messengers, i.e., events that can be assessed quantitatively. Some of these events occur immediately after receptor activation (transformation of heterotrimeric G-proteins and arrestins); the other range from the cytoplasmic reactions to the activation of transcription factors controlling gene expression. Figure 2 shows the classical scheme of GPCR activation and processes that can be assessed quantitatively using luminescent or fluorescent sensors in order to evaluate the corresponding cellular response.

Agonist binding leads to the conformational changes in the receptor, which results in the G protein activation, including replacement of GDP with GTP in the α-subunit of the heterotrimeric G protein. By detecting accumulation of GTPγS (hydrolysis-resistant radioactive analogue of GTP), it is possible to detect GDP replacement with GTP upon G protein activation. Further improvement of this technology has led to the development of non-radioactive DELFIA (dissociation-

enhanced lanthanide fluorescence immunoassay) based on the TRF (time-resolved fluorescence) method that uses fluorescent chelated lanthanide elements [64].

Structural changes in the heterotrimeric G protein that occur upon GPCR activation can be detected using the split-luciferase approach. The most popular method is the NanoBiT technology from the Promega company, in which the interaction between the G_{α} and $G_{\beta\gamma}$ subunits of the G protein is monitored using the NanoLuc luciferase-based enzyme complementation system. The SmBiT and LgBiT fragments are fused to different subunits, so that the interacting subunits produce luminescence in the presence of a substrate, while their dissociation results in the luminescence disappearance [65, 66]. This approach can be extended to other protein–protein interaction as well.

Some tests used in ligand screening are aimed at detecting events occurring downstream of the receptors, e.g., at changes in the levels of second messengers (cAMP, calcium, IP_1, IP_3, etc.) that change with the activation of various G_{α} subunits ($G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha q}$). Depending on the α-subunit in the G protein, ligand binding induces activation of different signaling pathways and formation and release of different second messengers. Changes in the intracellular cAMP concentration occur as a result of activation or inhibition of adenylate cyclase by G proteins with the $G_{\alpha s}$ or $G_{\alpha i}$ subunits, respectively. $G_{\alpha s}$ activates adenylate cyclase, leading to the increase in the concentration of intracellular cAMP, while activation of $G_{\alpha i}$ inhibits adenylate cyclase and decreases the level of cAMP in the cell. Many screening methods are based on changes in the concentration of intracellular cAMP upon modulation of the activity of a group of receptors. At the same time, the G protein with the $G_{\alpha q}$ subunit activates PLC, which catalyzes formation of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 stimulates the opening of endoplasmic reticulum calcium channels and calcium release to the cytoplasm, IP_3 is then enzymatically converted into IP_1 and IP_2.

Another important pathway in GPCR activation is the β-arrestin signaling cascade. β-Arrestins 1 and 2 are ubiquitously expressed cytosolic adapter proteins that have been originally discovered as inhibitors of GPCR signaling. In some cases, β-arrestins regulate only receptor desensitization and internalization, similar to the visual arrestin in rhodopsin desensitization [67]. However, β-arrestins often do not simply block activated GPCRs, but trigger endocytosis and activation of kinases, leading to the initiation of specific signaling pathways in the endosomes. It was also found that the signaling pathways initiated by β-arrestins are independent of the G protein activation. Identification of ligands that block the activation of G proteins but promote the binding of β-arrestins, or *vice versa*, has resulted in the discovery of biased signaling, i.e., selective triggering of intracellular signaling pathways. It was found that β-arrestin signaling activates MAPK (mitogen-activated protein kinase) in a G protein-independent manner and ultimately initiates expression of controlled genes [68].

Methods for assessing cAMP in $G_{\alpha s}$- and $G_{\alpha i}$-associated pathways. There are several methods to measure changes in the cAMP concentration upon receptor activation. The HitHunter technology from DiscoverX is based on the complementation of fragments of the cleaved β-galactosidase protein [69]. It uses a small fragment of β-galactosidase conjugated with cAMP and a complementary large fragment of the same protein. After addition of antibodies and enzyme substrate, the conjugated cAMP binds to the antibodies, thus preventing β-galactosidase complementation and substrate conversion, and, therefore, causing the disappearance of the signal. If GPCR activation results in the activation of adenylate cyclase and cAMP accumulation of in the cytoplasm, this endogenous cAMP competes with the conjugated cAMP for binding with the antibodies, leading to the assembly of active β-galactosidase. In the presence of the substrate, the reaction catalyzed by the enzyme causes emittance of the chemiluminescence signal which can detected by a luminometer.

Another example of chemiluminescent analysis is the AlphaScreen technology from PerkinElmer [70]. It uses donor and acceptor microparticles, which, when brought together, transfer singlet oxygen generated during donor photoactivation to the acceptor. The donors are photoactivatable phthalocyanine-based microparticles coated with streptavidin. Biotinylated synthetic cAMP binds to the donor and to the anti-cAMP antibodies conjugated with the acceptor particles containing the dye Thioxene that reacts with the singlet oxygen to generate light. When the donor and acceptor are close to each other, the chemiluminescent signal increases. Endogenous cAMP competes with synthetic cAMP for binding with the antibodies and disrupts the donor (biotinylated cAMP)–acceptor complex, resulting in the decrease in the chemiluminescent signal.

The concentration of cAMP in a cell can be determined by the fluorescent polarization method [71]. Synthetic cAMP conjugated to a fluorescent tag emitting polarized light binds to the anti-cAMP antibodies and produces polarized emission in polarized light due to the reduced degree of rotational freedom in the bound form. As the concentration of endogenous cAMP increases, the labeled cAMP is competitively displaced from the complex with the antibodies, which increases in its chaotic mobility and, as a consequence, reduces detectable fluorescence polarization.

Another approach to measuring the cAMP concentration employs cAMP-dependent enzymes. For example, the GloSensor technology [72] uses a modified

cAMP-dependent luciferase. The binding of cAMP causes conformational changes in luciferase and results in the concentration-dependent activation of luminescence. A similar principle is used in the case of cAMP-dependent EPAC sensors, which are chimeric proteins that consist of a protein domain whose structural rearrangements change the distances between the BRET (bioluminescence resonance energy transfer) pair of luciferases and/or FRET (fluorescence resonance energy transfer) pair of fluorescent proteins attached to this domain [73]. The use of these approaches for studying GPCRs can be found in recent publications [74, 75].

A common approach is the application of reporter systems in which the luciferase gene is placed under the control of the multimerized cAMP response element (CRE), which regulates the activity of the gene promoter, thereby allowing to detect changes in the cAMP from changes in the luminescent signal [76, 77].

Detection of inositol phosphates and calcium upon activation of the $G_{\alpha q}$ pathway. Several platforms are currently available for determining the concentrations of IP_1, IP_3, and calcium. The above-described AlphaScreen and polarized fluorescent tags are also applicable for the detection of the second messenger IP_3 [78].

Beside measuring IP_3, similar approaches are used to measure the concentration of another second messenger, IP_1 (an intermediate in the IP_3 degradation). It has been shown that the addition of lithium chloride to cells leads to the inhibition of dephosphorylation and accumulation of IP_1 in the cell, thus allowing to measure its concentration as a consequence of GPCR activation [78]. This is a competitive assay based on the homogeneous time-resolved FRET (HTRF) [79], in which d2-labeled IP_1 acts as a fluorescence acceptor, while terbium cryptate-labeled anti-IP_1 monoclonal antibody (mAb) acts as a fluorescence donor. With the increase in the concentration of endogenous IP_1, the labeled analogue is replaced in the antibody binding site and, as a result, the FRET signal decreases. This approach was implemented in the IP-One assay from Cisbio and was previously used by us in the study of cysteinyl leukotriene receptors [52, 80, 81].

Another popular test involves measuring the concentration of intracellular calcium using cell-penetrating calcium-sensitive fluorophores (Fluo-3, Fluo-4 and their optimized analogues) and fluorescent readers based on fluorescent imaging plate reader (FLIPR) technology. Such readers can provide relatively fast (3-5 s) real-time fluorescence measurements [82].

Additionally, changes in the intracellular calcium can be detected from the activation of expression of luciferase reporter genes under control of the calcium-dependent promoters (NFAT system from Promega) [83].

β-Arrestin pathway. The key event in the activation of this signaling pathway in most cases is receptor phosphorylation by GRK. The early studies of GPCR phosphorylation relied on radioactive assays in whole cells, which required high radioactivity levels and did not allow to identify individual phosphorylated serine and threonine residues. More recently, such studies have been focused on the analysis of phosphoproteome, which has provided limited quantitative information. Currently, the prevailing approach is the use of antibodies that specifically recognize phosphorylated GPCRs [84].

Other approaches to studying the activation of β-arrestin signaling pathways [85] are based on the analysis of multiple changes in the cells using fluorescent and luminescent assays. One of these methods involves monitoring receptor migration in the cell after its internalization using labeled ligands, antibodies against various receptor epitopes, or conjugated fluorescent labels (as implemented in the Transfluor Assay technology by Molecular Devices) [86].

Another approach is direct detection of β-arrestin binding to the receptor, in particular, using the BRET-based methods [87]. BRET uses a fluorescent dye-conjugated receptor and a luciferase-conjugated β-arrestin, or *vice versa*. When the receptor binds to β-arrestin, the two labels come together and the bioluminescence energy is transferred to the fluorophore.

The Tango assay [88] is a reporter system activated by the TEV protease conjugated to β-arrestin. When β-arrestin approaches the receptor fused to a transcription factor through the TEV protease recognition site, the factor is cleaved from the receptor by the protease, and the transcription factor is translocated to the nucleus, where it activates transcription of β-lactamase. β-Lactamase catalyzes the cleavage of the substrate with two fluorescent tags, thus disrupting the FRET between the donor and acceptor. The fluorescent signal from the intact substrate disappears and the signal from the cleaved substrate appears.

An alternative method is the PathHunter technology from DiscoverX [89], which is based on the complementation of protein fragments, in this case, fragments of β-galactosidase. If β-arrestin is conjugated with a large inactive fragment of β-galactosidase, and the receptor is conjugated with a small one, then the binding of the receptor with β-arrestin brings the fragments together, and β-galactosidase cleaves the substrate with the generation of a chemiluminescent signal.

Detection of GPCR dimerization. Many GPCRs form homodimers, heterodimers, or oligomers in the plasma membrane [90]. Dimerization can significantly change the array of activated signaling pathways [91]. There are several common approaches to the detection of receptor dimerization. Some of them are based on FRET or BRET, with donor and acceptor tags

conjugated to the C-termini of the receptors [92, 93]; others are based on the complementation of β-galactosidase fragments (PathHunter) [94]. When receptors, one of which is conjugated to the large and the other to the small fragment of β-galactosidase are co-expressed, addition of an agonist leads to the receptor dimerization and enzyme assembly with the generation of chemiluminescence in the presence of the substrate.

Practical aspects in selection of functional tests in cells. Currently, approaches providing simultaneous detection of activation of several GPCR-mediated signaling pathways and activation of different G protein subunits using BRET (e.g., Truepath assay [90]) are becoming increasingly popular, as they allow to study the pharmacological profile of receptor activation and to directly investigate biased signaling.

In practice, the choice of the functional test is based on the scientific problem itself, methods for detection of results of receptor activation and selected biochemical pathway occurring immediately after receptor activation and downstream (changes in the concentration of second messengers and expression of reporter genes), and possibility of false data arising due to the crosstalk with independent signaling pathways [95, 96].

Technical and economic considerations, such as availability of reagents and signal detection systems, dynamic range of the detection method, possibility of scaling up for screening purposes, and possibility of validation for meeting preclinical testing criteria, should be taken into account as well.

After *in vitro* functional tests, successful candidates are assessed in more complex *in vivo* systems, for example, functionally differentiated cells and tissues [97, 98] and organoids [99, 100], tested for ADME and toxicity [101] and then move to preclinical testing in animals.

CONCLUSION

Development of functional tests for GPCRs and bioinformatics methods, complemented by advances in structural biology, have provided researchers with a full toolset for design and verification of new ligands using the SBDD method.

With less than 10 years passing since the resolution of most GPCR structures, it is too early to judge the success (or failure) of SBDD method in the studies of GPCRs. Besides, pharmaceutical companies usually do not publish early research results in order to reduce competition. However, below we present a few examples of drugs developed using SBDD that are currently in clinical trials.

In 2012, the crystal structure of A_{2A} adenosine receptor ($A_{2A}AR$) published in 2011 [102] was used in the search of receptor antagonists for their use in immuno-oncology. *In silico* screening identified a number of potentially suitable chemical compounds. Analysis of the orthosteric ligand-binding site in the crystal structures of several receptor–ligand complexes revealed a previously unknown region in the orthosteric pocket, which was involved in the binding of agonists, but stayed unoccupied in the case of previously known antagonists. Artificial antagonists with the chemical groups binding in this newly discovered pocket region were highly specific. The use of SBDD has led to the development of AZD4635, an antagonist with a higher selectivity for $A_{2A}AR$ compared to the homologous A_1AR. In the preclinical trials, AZD4635 showed an efficacy as an antitumor immunomodulator both as a monotherapy and in combination with an anti-PD1 antibody [103, 104]. It is currently tested in phase II clinical trials in patients with prostate cancer (https://clinicaltrials.gov/study/NCT04089553).

Another example of successful application of SBDD is the search for selective agonists of the muscarinic receptors M_1R and M_4R. Activation of these receptors is expected to help in the treatment of cognitive and psychiatric disorders, Alzheimer's disease, and schizophrenia. At the same time, an agonist of M_1R and M_4R should not activate homologous M_2R and M_3R receptors in order to avoid the side effects from the digestive and cardiovascular systems. Traditional approaches to the selection of agonists have failed to provide a required selectivity, since the ligand-binding sites in phylogenetically related M_1R, M_2R, M_3R, and M_4R are very similar. Only the use of receptor structures in combination with various ligands has made it possible to develop highly specific agonists. In 2016, the data on three of these agonists were published: HTL9936 (M_1R agonist), HTL18318 (M_1R agonist), and HTL0016878 (M_4R agonist). Currently, these compounds are at various stages of clinical trials (https://soseiheptares.com/news) [103, 105].

In 2017, the structure of the glucagon-like peptide 1 receptor (GLP1R), a member of class B GPCRs, was obtained using cryo-EM. The binding of GLP1 peptide to the receptor promotes proliferation of pancreatic β-cells and increases insulin levels. GLP1R is one of the most effective targets in the treatment of type II diabetes. The crystal structure of the receptor with an allosteric antagonist provided initial structural information for the rational drug design. In 2020, high-throughput *in silico* screening of small-molecule GLP1R agonists and a series of optimization procedures have led to the discovery of the small-molecule agonist PF-06882961, which, unlike its analogues (the best-known of which is semaglutide), has a high oral bioavailability. Next, the cryo-EM structure of the GLP1R complex with PF-06883365 (PF-06882961

analogue) was analyzed, resulting in the identification of a mechanism underlying the interaction of low-molecular agonists with the receptor and key factors of receptor activation. These data have formed the basis for further rational drug design. At the time of writing this review, PF-06882961 (Danuglipron) has already completed phase I and II clinical trials [106, 107].

The authors of study published in 2023 [108] developed a prototype of the new antidepressant BMK-C205, an antagonist of the corticotropin-releasing factor 1 receptor (CRF1R). This receptor belongs to class B GPCRs; it is expressed in the central and peripheral nervous systems and regulates behavioral, endocrine, immune, and autonomic responses to stress, which makes it an appropriate target for the treatment of stress-related disorders. First, the structure of CRF1R in a complex with the allosteric antagonist BMK-I-152 was determined by serial protein crystallography, thus allowing to identify a number of structural features, in particular, two important hydrogen bonds. The disadvantage of BMK-I-152 as a drug is that it is metabolized too quickly in the liver and, as a result, exhibits low efficiency even when administered intravenously. Based on the structure of the CRF1R complex with BMK-I-152, the authors developed two new antagonists, BMK-C203 and BMK-C205, which showed significantly better results in pharmacokinetic tests. The structures of CRF1R complexes with these new antagonists were determined by serial crystallography. Ultimately, BMK-C205 showed good results as an antidepressant in mice and is very likely to be used in the future to develop human antidepressants using the SBDD method.

These examples illustrate only a small part of SBDD works currently underway in the field of GPCR studies. Given the pharmacological significance of these receptors, all obtained structures are undoubtedly already being used or will soon be used in the search for new drugs. It is likely that some of these drugs will enter the pharmaceutical market in the next decade.

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