Strategies to Discover Unexpected Targets for Drugs Active at G Protein–Coupled Receptors

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Annu. Rev. Pharmacol. Toxicol. 2011. 51:117–44

First published online as a Review in Advance on September 20, 2010

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi:
10.1146/annurev-pharmtox-010510-100553

0362-1642/11/0210-0117$20.00

Keywords

high-throughput screening, polypharmacology, receptorome, valvulopathy, drug side effects, similarity ensemble approach

Abstract

G protein–coupled receptors (GPCRs) are an evolutionarily conserved family of signaling molecules comprising approximately 2% of the human genome; this receptor family remains a central focus in basic pharmacology studies and drug discovery efforts. Detailed studies of drug action at GPCRs over the past decade have revealed existing and novel ligands that exhibit polypharmacology—that is, drugs with activity at more than one receptor target for which they were designed. These "off-target" drug actions can be a liability that causes adverse side effects; however, in several cases, drugs with less selectivity demonstrate better clinical efficacy. Here we review physical screening and cheminformatic approaches that define drug activity at the GPCR receptorome. In many cases, such profiling has revealed unexpected targets that explain therapeutic actions as well as off-targets underlying drug side effects. Such drug-receptor profiling has also provided new insights into mechanisms of action of existing drugs and has suggested directions for future drug development.

INTRODUCTION

GPCR: G protein–coupled receptor

GPCR receptorome: the entire complement of G protein–coupled receptors in the genome

Polypharmacology: the specific binding and activity of a compound at two or more molecular targets

G protein–coupled receptors (GPCRs) are a large superfamily of receptors that regulate a vast number of physiological processes. Because of their important physiological roles, GPCRs have emerged as essential therapeutic targets and a major focus of drug discovery efforts. The development and establishment of medications targeted at GPCRs have proven successful for treating an array of disease indications, including immune, oncologic, cardiovascular, neurological, and metabolic disorders. Although GPCRs are common, even classic, therapeutic targets, a large portion of the GPCR receptorome remains unexploited from both basic science and drug discovery perspectives. Indeed, roughly 150 GPCRs are orphan receptors whose endogenous ligand(s) and physiological function(s) are unknown; however, these orphan receptors will likely include valuable targets for future drug development. GPCR pharmacology thus represents a rich area for continued research in basic science as well as drug discovery.

The dominant paradigm throughout drug discovery has been the magic-bullet concept (i.e., designing maximally selective ligands to act on individual drug targets). This paradigm has also been the standard approach for GPCR drug discovery, whereby the focus has been on identifying and developing high-affinity small molecules that target a single receptor. This approach has proven successful, particularly for diseases that have clearly defined mechanisms, etiology, and pathophysiology and that can be treated by targeting a particular receptor. A major reason that discovery efforts favor this selectivity-based concept is because drugs that exhibit polypharmacology (i.e., nonselectivity) frequently display deleterious side effects when advanced into clinical trials. Thus lack of GPCR selectivity can increase "off-target" actions that result in toxicity and potentially lethal side effects. Surprisingly, however, many of the most clinically effective drugs appear to exert their superior clinical actions via the modulation of many GPCRs (i.e., they are "magic shotguns"). These seemingly contrasting outcomes of drug polypharmacology, i.e., side effect liability versus enhanced efficacy, suggest the need for more efficient approaches to define drug action(s) at GPCRs.

Here we review physical screening and cheminformatic approaches that illuminate drug activity at GPCRs and other selected targets. We also relate several informative case studies in which small-molecule profiling at GPCRs has revealed unexpected targets for drugs and natural products. These examples also provide a rich data set that reveals new targets and new opportunities for existing medications and also clarifies some GPCRs that can cause serious side effects.

G PROTEIN–COUPLED RECEPTORS AND THEIR SIGNALING

GPCRs represent an evolutionarily conserved family of signaling molecules comprising approximately 2% of the human genome (1, 2, 3). GPCRs transduce signals elicited by both endogenous and exogenous ligands to regulate and modulate nearly every cellular and physiological process (4, 5). GPCR ligands are remarkably diverse and include environmental stimulants (e.g., photons, odorants, tastants, and viruses) and endogenous ligands (e.g., small-molecule neurotransmitters, amino acids, polypeptides, hormones, nucleotides, ions, and lipids). Not surprisingly, GPCR dysregulation is also associated with many human diseases (6), thus explaining why GPCRs remain both successful therapeutic targets and the focus of intense research in both academia and industry (7).

All GPCRs are predicted to contain an arrangement of seven-transmembrane-spanning helices, an extracellular N terminus, and an intracellular C terminus. This basic structure gives rise to other common names for GPCRs: seven-transmembrane (7TM) receptors or heptahelical receptors. A canonical example of this structural arrangement is exemplified by bovine rhodopsin,

whose crystal structure reveals 7TM helices oriented such that the receptor N terminus is extracellular, the C terminus is intracellular, and the helices in a bundle are linked by three intracellular and three extracellular loops (8). Other GPCRs for which high-resolution structures are available show a similar overall arrangement (9, 10). Mutagenesis studies, molecular modeling, and screening of large numbers of ligands have been used to define ligand-binding sites of GPCRs. Common binding sites for endogenous ligands include the N terminus and/or extracellular loops for endogenous peptide ligands, or, in the case of small molecules such as biogenic amines, a hydrophilic pocket formed between the transmembranes near the extracellular face (4, 11). Most small-molecule drugs are directed toward this hydrophilic pocket. This region is suitable for controlling the activity of receptors in which native ligands bind within or even outside this pocket (4). In general, the third intracellular loop, as well as the intracellular C-terminal regions of GPCRs, is a site for receptor/G protein interactions.

The basic paradigm of GPCR signaling typically involves three basic components: GPCRs, heterotrimeric G proteins, and various G protein–dependent and G protein–independent effectors (12). GPCRs couple to heterotrimeric G proteins, and in their ligand-activated conformation, GPCRs function as guanine nucleotide exchange factors (GEFs) for Gα subunits. Upon binding agonist, GPCRs undergo conformational shifts that promote the Gα subunits to exchange GDP for GTP and to become activated. Binding of GTP uncouples the Gα and Gβγ proteins from the receptor, enabling them to independently interact with, and modulate, various intracellular effectors such as second messenger generating enzymes and ion channels. This basic GPCR signaling scheme provides the biochemical mechanism of transduction, whereby the extracellular signal of the hormone or drug is converted into an intracellular response (13–15).

More recently, studies indicate some GPCRs can signal independently of canonical G protein pathways. G protein–independent signaling by GPCRs is typically mediated by β-arrestin proteins, which can function as scaffolds for downstream effector molecules (16). β-arrestins have historically been defined as mediators of desensitization and signaling attenuation for GPCRs. Upon binding agonist, most GPCRs (the β-adrenergic receptor is the classic example) are phosphorylated by G protein–coupled receptor kinases (GRKs); this process promotes the recruitment and binding of β-arrestin proteins to the receptor. β-arrestin facilitates the endocytosis of GPCRs and prevents G protein coupling, which, in turn, leads to signaling desensitization (17, 18). In addition to their well-established role in GPCR desensitization, β-arrestins can also enable GPCR-mediated "arrestinergic" signaling by functioning as scaffolds for downstream effector molecules such as the extracellular regulated kinases (ERKs) (16, 19).

Analysis of the human genome has revealed 735–802 GPCR open reading frames. Approximately half of these putative genes are olfactory or taste receptors, and the remaining (~375) are nonolfactory (5, 11, 20). Based on sequence homology and pharmacological similarities, human GPCRs are grouped into one of five families: A (rhodopsin), B (secretin), C (glutamate), adhesion, and Frizzled/Smoothened/Taste2 (20). GPCR family A is the largest family, and its members recognize a diverse array of ligands including odorants, biogenic amines, neuropeptides, peptidergic hormones, lipids, nucleotides, proteases, and even photons for the prototypical family A receptor rhodopsin (4). The family B receptors are responsive to hormones and peptides. The family C receptors are activated by amino acids, ions, and tastants. Adhesion receptors are hypothesized to interact with extracellular matrix or membrane-bound proteins, whereas Frizzled and Taste2 receptors are activated by Wnt proteins and tastants, respectively.

In addition, there are many orphan GPCRs, i.e., receptors for which neither endogenous native ligand nor function is known. A large number, approximately 150, of putative or validated GPCRs are orphan receptors. Orphan GPCRs represent a rich area for continued research, and deorphanization efforts have revealed several important GPCR-ligand relationships (21). For

example, deorphanization of several orphan GPCR neuropeptide receptors (ghrelin, orexin, and neuropeptide B/neuropeptide W) has demonstrated that they play essential roles in the control of metabolism. Some of these GPCRs are now targets for treating disorders such as obesity (22). Therefore, the GPCR receptorome remains not fully tapped, especially when considering that the native ligands and pharmacology of many orphan receptors remain elusive.

Cheminformatics: computational approaches using computer software and hardware to predict chemical and pharmacological properties of chemicals; they can be used in drug discovery and development programs for in silico chemical screening and analysis

G PROTEIN–COUPLED RECEPTORS AS DRUG TARGETS

Because GPCRs regulate a vast number of physiological processes, they are ideal targets for small-molecule drugs used as "magic bullets." A large number of drugs targeted to GPCRs have been developed for indications that include immune, cardiovascular, metabolic, neurodegenerative, psychiatric, and oncologic diseases. Although an estimated 40–50% of all marketed drugs act directly to modulate GPCR signaling pathways, less than 20% of GPCRs (nonodorant receptors) are currently targeted (5, 23). This number of drugs is expected to increase as new functions for GPCRs are discovered, and accordingly, GPCR targets remain an ongoing focus in drug discovery efforts.

Drug Activity at G Protein–Coupled Receptors: Selective and Nonselective Actions

Development of drugs that target a single GPCR has been the major approach for GPCR drug discovery over the past few decades. A highly successful example was the early development of selective β_2-adrenergic agonists (e.g., salbutamol/albuterol) commonly used to treat asthma and pulmonary diseases (24). These selective β_2-adrenergic agonists, which relax bronchial airway smooth muscle, have fewer off-target actions at β_1-adrenergic receptors (which can cause serious cardiac side effects, such as tachycardia and other arrhythmias). However, selective action of a drug at a single GPCR is not always predictive of favorable outcomes, especially for diseases whose etiology and pathophysiology are elusive or complex.

Defining selective or nonselective drug activity at GPCRs, although straightforward in concept, remains a challenging undertaking. This arises largely from the sheer number of GPCRs (~375 nonolfactory receptors) in the human genome that can serve as off-targets and from the fact that GPCR signaling varies depending on the cellular milieu (25, 26). Additionally, because no universal readout of drug activity for all GPCRs is available, multiple functional activities must be assessed in drug screening studies. A rapidly evolving method to predict drug activities at GPCRs and other potential targets relies on cheminformatics, which can predict both on- and off-target activities of GPCR-targeted compounds (27, 28). This approach can speed the process of defining selective and nonselective actions of GPCR drugs. Currently, no public or private entity has the scientific resources to screen the entire GPCR receptorome, although some such entities are close, including our own. Frequently, however, screening small molecules for activity at a large number of GPCRs (e.g., 10–100) has been used. Much easier to define, and to test, is the activity of many small molecules or drugs at a single GPCR, which uses at most a few robust readouts of receptor activity.

Although we have seen the development of many successful drugs that selectively target receptor isoforms and subtypes, it has been difficult to achieve such targeting of GPCRs that contain homologous residues within their orthosteric binding sites (i.e., site of endogenous ligand binding). For example, attempts to develop agonists that are highly selective for individual muscarinic acetylcholine receptor (mAChR) subtypes have failed because of the high conservation of the orthosteric ACh-binding site (29), although subtype-selective muscarinic agonists would likely

be useful in a number of disease settings, such as neuropsychiatric diseases (30). For such highly homologous GPCRs, development efforts aimed at the allosteric modulation of the binding of endogenous ligands may prove a better strategy. Indeed, positive allosteric modulators of M1 and M4 mAChRs that have been developed show superior selectivity at these receptors compared with previously developed orthosteric agonists; these modulators show promise for the treatment of neurodegenerative and neuropsychiatric diseases (31, 32).

hERG: human ether-a-go-go K+ channel

Polypharmacology at G Protein–Coupled Receptors: Liability or Opportunity?

As noted above, the dominant paradigm in drug discovery continues to be the design of maximally selective ligands that target individual GPCRs. This approach has proven successful, particularly for diseases that have clearly defined mechanisms, etiology, and pathophysiology. Because drugs that exhibit polypharmacology may have increased potential for side effects, undesirable off-targets are commonly screened for during drug discovery and development. For example, cardiac side effects resulting from inhibition of the hERG (human ether-a-go-go K+ channel) can cause prolongation of the cardiac Q-T interval and, in rare instances, fatal arrhythmia. Interaction with the hERG is thus routinely assessed through screening studies early in preclinical development (33). The underlying assumption of this discovery approach is that safer, more effective drugs will result from designing highly selective ligands whose undesirable, potentially toxic side activities have been removed (34). Although rational approaches to drug design may remove off-target toxicities and side effects, highly selective drugs are not superior in clinical efficacy for many diseases. For many central nervous system (CNS) disorders, in particular psychiatric diseases such as schizophrenia and depression, GPCR drugs that are nonselective commonly demonstrate better clinical efficacy (see sidebar, Magic Shotguns versus Magic Bullets for CNS Disorders?) (35).

Is polypharmacology at GPCRs a liability or an opportunity? The answer depends on which receptor(s) and which compound(s) exhibit activity. Ultimately, defining both selective and nonselective actions for drugs targeted at GPCRs will elucidate drug action and receptor pharmacology. In many cases, such profiling has revealed unexpected targets that can explain therapeutic actions as well as potential side effects. Such drug-receptor profiling has also provided new insights into mechanisms of action of current drugs.

MAGIC SHOTGUNS VERSUS MAGIC BULLETS FOR CNS DISORDERS?

The assumption that highly selective drugs are more effective than nonselective drugs is not always supported, particularly for central nervous system (CNS) disorders in which pathology is complex. In terms of etiology, it is now widely accepted that the major mental illnesses are polygenic (134, 135), with substantial environmental and, perhaps, epigenetic components (136). Because the precise molecular etiology of diseases such as depression and schizophrenia remains unknown, choosing the appropriate molecular target(s) for drug discovery is especially risky. A fundamental difficulty with developing novel CNS therapeutics is the appreciation that the most widely prescribed CNS medications, especially those for mood disorders—lithium, anticonvulsants, and antidepressants (137)—and schizophrenia (138) have complex and ill-defined mechanisms of action. The discovery that the most clinically effective CNS drugs are pharmacologically complex and exhibit pleiotropic actions (that is, they act as "magic shotguns") has made the development of "magic bullets" (that is, drugs selective for a single molecular target) less likely. Given that selectively nonselective drugs are likely to be more beneficial than single-action agents in many CNS disorders, defining the polypharmacology of drug action is an important step in the development and validation of relevant and important targets for CNS disorders. (For reviews, see References 35 and 139.)

Table 1 Selected public-sector small-molecule screening resources

Name of screening resource	Web site of resource	User and comments
NIMH Psychoactive Drug Screening Program	**http://pdsp.med.unc.edu/**	A; free and confidential screening to qualified users
NIH Chemical Genomics Center	**http://ncgc.nih.gov/**	G; advertises screening capacity of up to 1×10^6 compounds per day
Scripps Research Institute Molecular Screening Center	**http://mlpcn.florida.scripps.edu/**	NP; an NIH-supported HTS and large chemical repository
Molecular Libraries Screening Centers Network	**http://mli.nih.gov/mli/secondary-menu/mlscn/**	G; a 10-center network offering diverse services such as FLIPR and HCS
Rockefeller University HTS Resource Center	**http://www.rockefeller.edu/highthroughput/highthroughput.php**	A/NP; one of the oldest NP HTS facilities
Kansas University HTS Laboratory	**http://www.hts.ku.edu**	A; funded by NIH COBRE mechanism

A, academic; C, commercial; COBRE, Centers of Biomedical Research Excellence; FLIPR, fluorescent imaging plate reader; G, government; HCS, high-content screening; HTS, high-throughput screening; NIH, National Institutes of Health; NIMH, National Institute of Mental Health; NP, nonprofit.

METHODS TO DEFINE DRUG ACTIVITY AT THE G PROTEIN–COUPLED RECEPTOROME

Drug Screening Approaches for G Protein–Coupled Receptors

Various approaches are available for screening the GPCR receptorome. Several public-sector small-molecule screening centers available to researchers are listed in **Table 1**. Ideally, GPCR profiling can be used both to identify the molecular targets for endogenous or synthetic ligands and to elucidate drug actions at many receptors. These various approaches for defining drug activity at GPCRs are most informative when combined, and they can reveal unexpected and potentially therapeutic targets for drugs active at GPCRs. Several of these approaches, some of which are amenable to high-throughput screening, are described in the following sections.

Radioligand-Binding Screens

Historically, radioligand-binding assays have been used to identify compounds that target and bind to GPCRs (see, for instance, References 36 and 37). Radioligand-binding experiments in which a test ligand competes with a receptor-specific, high-affinity radiolabeled ligand for binding to receptor-containing whole-cell or membrane preparations have been used to identify ligand-receptor interactions. The development of assays to investigate these ligand-GPCR interactions using multiwell (96+) formats have accelerated the throughput. For multiwell ligand-binding screens, the scintillation proximity assays (SPAs) (38) or the less frequently employed fluorescence polarization (FP) assays (39) can also be used to increase throughput. Radioligand-binding assays enable equilibrium binding or kinetic displacement measurements so that both equilibrium and kinetic dissociation constants (e.g., K_D values) can be obtained. Because biochemical binding data are less prone to errors by off-target effects than are cellular data, radioligand-binding data typically provide a robust first screen for both on- and off-target effects of drugs at GPCRs. This basic drug-binding information indicates a test compound's affinity, and when combined to assess drug binding across multiple receptors, it also provides information regarding the selectivity of binding. An advantage of binding assays is that they can be performed with frozen

tissue or cellular membranes as opposed to live cells (which are required for most functional assays).

However, there are several major drawbacks to radioligand-binding screens: (*a*) they rely primarily on radiolabeled ligands; (*b*) they do not readily distinguish between the functional properties of ligands (e.g., agonist, partial agonist, inverse agonist, and antagonist); (*c*) they may not be suitable for identifying allosteric ligands that bind at sites distinct from the primary (orthosteric) binding site (40); and (*d*) they are not well suited for deorphanizing receptors because, by definition, no ligand is known to bind to an orphan GPCR. To date, no commercial or public entities have the resources to screen the entire GPCR receptorome by using binding assays, although with the advent of the Molecular Libraries Program (**http://nihroadmap.nih.gov/molecularlibraries/index.asp**), such a capacity may be available in the future. At present, in the public domain, the NIH/NIMH-Psychoactive Drug Screening Program (NIH/NIMH-PDSP) has the single largest collection of receptors composed of receptorome superfamilies; the collection includes GPCRs, transporters, and ligand-gated ion channels for which compounds can be screened. A unique property of the NIH/NIMH-PDSP is that many receptors are probed simultaneously against a given ligand. Although radioligand binding is not easily amenable to high-throughput screening (HTS), it provides essential information about drug affinity. Drug-binding studies performed in parallel across many GPCRs and other targets can reveal key information about drug polypharmacology as well as unexpected or undesired off-target actions.

High-throughput screening (HTS): the process of using automated assays to search through large numbers of compounds for desired activity, resulting in larger output, lower cost, and faster results than other screening techniques

FLIPR: fluorescent imaging plate reader

Functional Screens

Although ligand-binding assays have historically been a standard approach to identify compounds that target GPCRs, current profiling efforts have moved toward the utilization of cell-based functional assays for defining drug activities at GPCRs. Functional screens based on receptor activation/signaling represent a complementary approach for identifying receptor-interacting compounds by ligand binding. In general, functional screening of GPCRs relies on receptor activation of G proteins to modulate effector activity and generate second messengers [e.g., calcium or cyclic adenosine monophosphate (cAMP)], which, in turn, activate signaling cascades that typically involve kinases to ultimately elicit a cellular response. Fluorescent measurements of Ca^{2+} release induced by inositol trisphosphate (IP_3) that was produced following activation of $G\alpha_{q/11}$-coupled receptors have become a staple of HTS (41). The most universal platform for assessing GPCR-induced calcium release and dynamics is the fluorescent imaging plate reader (FLIPR) (42) and related platforms. FLIPR-type instruments enable low-level optical detection of fluorescence generated by calcium indicators (among others) in conditions with precise temperature control and fluid handling in 96-, 384-, 1536-, and higher-well formats (**Figure 1**). There have also been many successful efforts to adapt non-$G\alpha_{q/11}$-coupled receptors (e.g., $G\alpha_s$- or $G\alpha_i$-coupled) to signal through the phospholipase C β (PLCβ)/Ca^{2+} pathway (41, 43). To achieve this, investigators have taken advantage of "promiscuous" $G\alpha_{15}$ and $G\alpha_{16}$ proteins, which can nonspecifically couple GPCRs to activate PLCβ (44). In addition, chimeric $G\alpha_{q/11}$ proteins, which have their C-terminal amino acids exchanged with those of $G\alpha_s$ or $G\alpha_{i/o}$, have been engineered to allow the activation of PLCβ by $G\alpha_s$- or $G\alpha_{i/o}$-coupled receptors (41, 43, 44). However, to date no truly "universal coupling" system exists, and typically one has to sequentially test a variety of chimeric and promiscuous G proteins. Furthermore, these artificial G protein–coupling systems may identify ligand potencies and efficacies that are nonphysiological and pharmacologically irrelevant (45, 46). Ultimately, the artificial systems must be validated using native receptors and their functional activity readouts (47, 48).

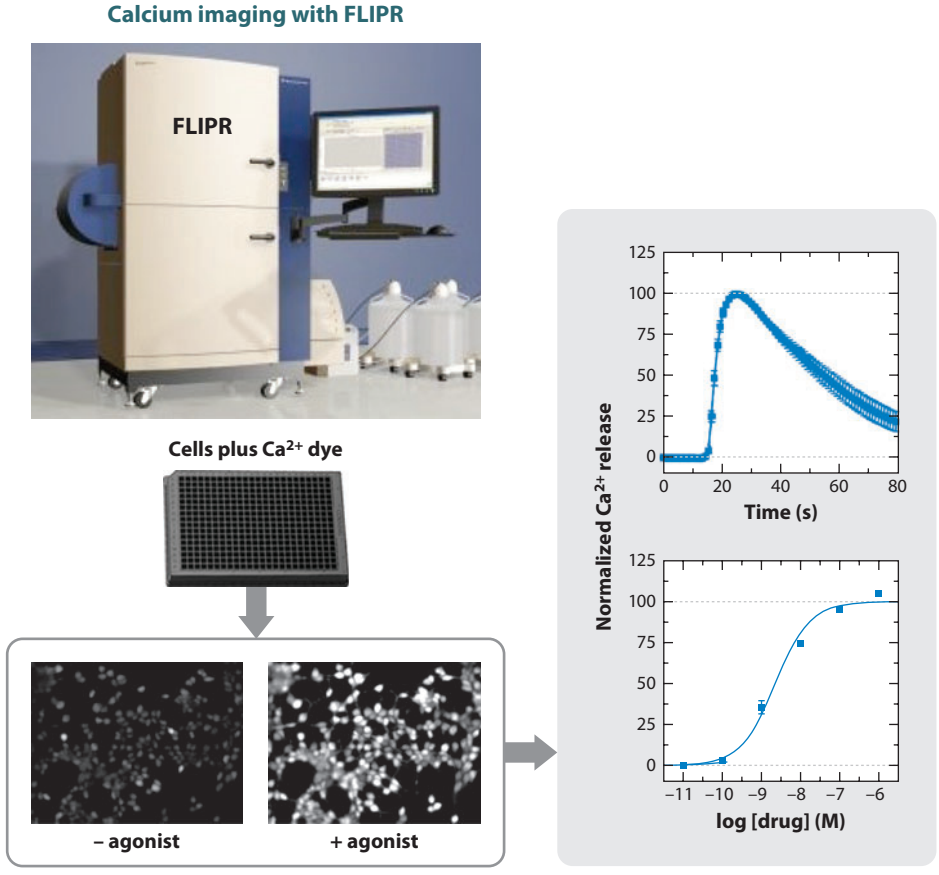


Figure 1

Assessing drug activity at G protein–coupled receptors (GPCRs) using the fluorescent imaging plate reader (FLIPR). For FLIPR-based assays, cells expressing GPCRs are plated in 96-, 384-, 1536-, or higher-well formats, after which compounds are added robotically. FLIPR-like machines quantify Ca^{2+} transients automatically and simultaneously in every well, allowing for high-throughput screening (HTS) and ultra-HTS of 10^4 to 10^6 compounds per day. The graphs in the gray box illustrate that precise quantification of agonist potency and efficacy can be obtained. Modification of these assays can allow screening for antagonists and allosteric modulators.

In addition to assessing calcium as second messenger, several approaches amenable to HTS are available to assess $G\alpha_s$- and $G\alpha_{i/o}$-coupled modulation of adenylyl cyclase and cAMP levels. Recent advances in fluorescence- and bioluminescence-based sensor development have provided sensitive indicators for cAMP levels in living cells (49), although these approaches are not typically amenable to HTS. The recent development of a split-luciferase-based sensor provides a novel bioluminescence-based approach to measure cAMP levels in living cells that is amenable to HTS (50). Using such assessments of cAMP can provide key functional information about drug actions at both $G\alpha_s$-coupled and $G\alpha_{i/o}$-coupled receptors (51).

GPCR functional assays have also been developed to assess the phenomenon of β-arrestin translocation (recruitment) to a GPCR. As mentioned previously, during agonist activation of most GPCRs, cytoplasmic β-arrestin proteins translocate to the plasma membrane and form complexes with the activated and phosphorylated receptors. This β-arrestin translocation can be

used as a functional readout for GPCR agonism or antagonism via several approaches. For example, bioluminescence resonance energy transfer (BRET) between a *Renilla* luciferase-β-arrestin fusion protein and a GPCR engineered to contain a yellow or green fluorescent protein (GFP) can report real-time translocation of β-arrestin to an activated GPCR (52, 53); such approaches have also been used for HTS screening of GPCRs (54). Additional recently developed technologies to assess β-arrestin translocation couple GPCR-mediated β-arrestin recruitment to a gene-reporter readout; these approaches are also amenable to HTS (55, 56).

One general concern in developing or using any functional screen, especially when using heterologous expression systems, is whether a GPCR is correctly targeted to the cell surface in a particular cellular setting without appropriate chaperone and trafficking proteins (43, 57). These concerns have prompted the use of constitutively active GPCRs (i.e., mutated receptors that are active in the absence of ligand) to establish which G proteins, cell system, or cellular milieu is acceptable for screening a given receptor (43, 58).

A number of other heterologous expression systems have been used to monitor ligand-dependent GPCR activation. GPCR-mediated pheromone signaling in the yeast *Saccharomyces cerevisiae* has been genetically altered to enable activated mammalian GPCRs (59), which are heterologously expressed in these yeast, to stimulate a mitogen-activated protein kinase (MAPK) pathway. This pathway leads to transcriptional activation of pheromone-responsive promoters that drive reporter gene expression (60). Yeast-based GPCR expression systems have been used to deorphanize GPCRs (61), serve as small-molecule screening platforms (60, 62), and define patterns of GPCR functional selectivity (63). Yeast-based systems have also been used to evolve GPCRs to create novel pharmacological profiles (64, 65). As most mammalian cellular systems express a variety of endogenous GPCRs, yeast offer the unique advantage of a "clean" expression system because the lone GPCR (Ste2) can be deleted without adversely affecting yeast physiology (60). *Xenopus laevis* melanophores offer another heterologous expression system to screen for mammalian GPCR activation (43, 58, 66). Melanosomes, organelles containing dark melanin pigment, found within melanophores will aggregate on inhibition of adenylyl cyclase (AC) activity or disperse upon stimulation of PLCβ and AC, resulting in cell lightening or darkening, respectively (58).

High-Content Screening Approaches for Profiling G Protein–Coupled Receptors

High-content imaging systems have been increasingly employed for screening and defining drug actions at GPCRs (26, 67). High-content screening (HCS) combines high-resolution fluorescence microscopy of cells in 96- or 384-well format with automated image analysis (**Figure 2**). There are several advantages to using HCS-based approaches. For example, cellular HTS (e.g., calcium flux using FLIPR) conventionally monitors the mean response of the whole cell population in the well of a microtiter plate. By contrast, HCS can distinguish the response of many individual cells in microplate wells (68). Because cellular responses can differ with respect to cellular differentiation, the stage of the cell cycle, the level of transgene expression, or natural variability, HCS can detect this information in individual cells and can include or exclude cells of interest. The flexible and varied information that can be profiled using HCS to perform microscopy-based assessments of cells provides numerous potential readouts for activation or antagonism of GPCRs. For example, agonist-induced GPCR internalization/endocytosis occurs for most GPCRs (18, 69), and monitoring the cell surface versus the intracellular distribution of GPCRs using HCS can define agonist or antagonist actions at receptors (70). Similarly, activation of most GPCRs results in recruitment and translocation of β-arrestins from the cytoplasm to the plasma membrane, and

GFP: green fluorescent protein

High-content screening (HCS): a screening platform and technique that combines high-resolution microscopy of cells with automated image analysis; this cellular-based screening technique can distinguish drug responses from individual cells in microplate wells

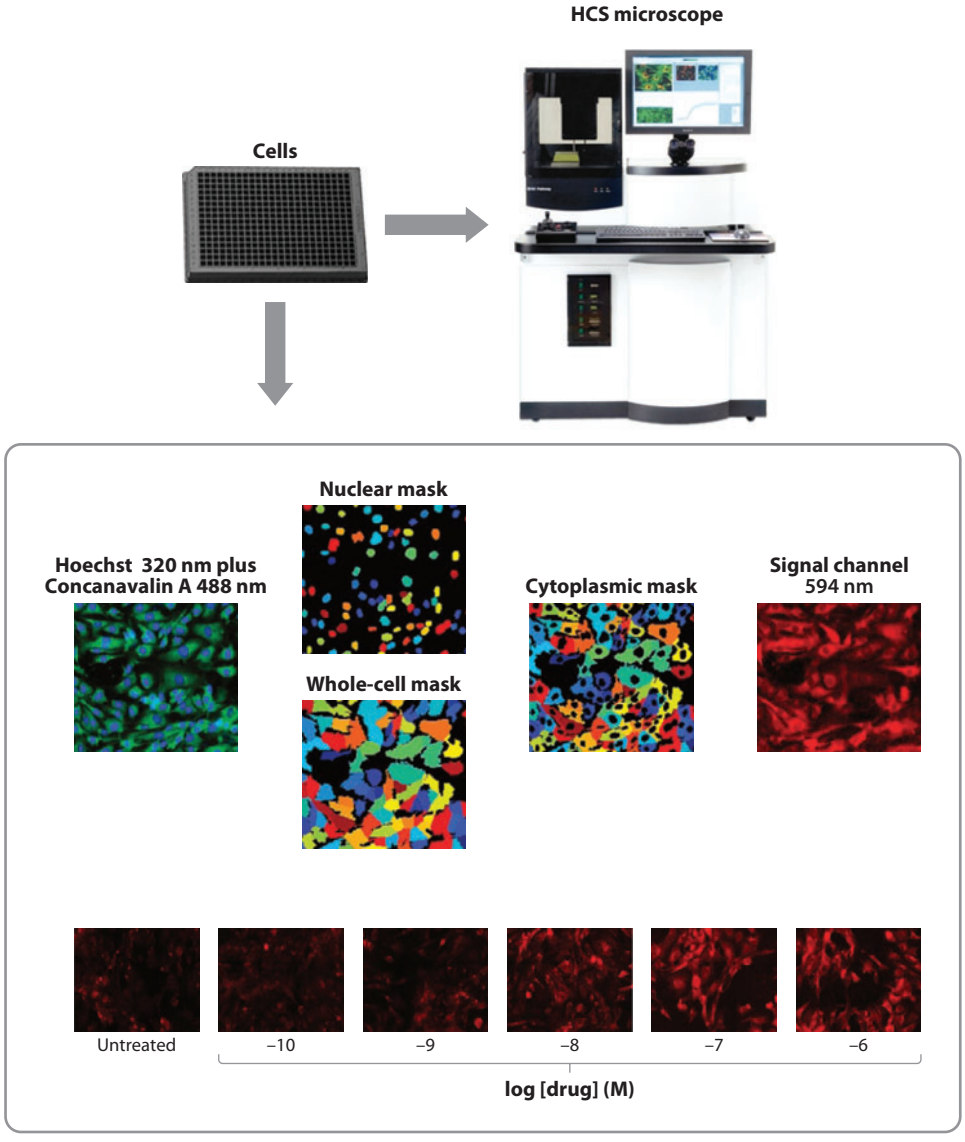


Figure 2

High-content screening (HCS) of G protein–coupled receptor (GPCR) activation: focus on extracellular regulated kinase (ERK) phosphorylation. For HCS-based screens, GPCR-expressing cells are plated in 96-, 384-, or 1536-well format and then exposed to drugs. Typically after fixation and visualization of signaling pathway components via fluorescence, individual wells are analyzed via automated microscopy-based imaging systems. As with fluorescent imaging plate reader–based screening, reliable estimations of agonist and antagonist potencies and efficacies can be obtained, as shown in the lower inset. Cellular masks of nuclear and cytoplasmic regions of cells were determined based on Hoechst and Concanavalin A fluorescence. Using imaging processing software, cytoplasmic masks for individual cells are overlaid in the ERK channel (594 nm here), thus allowing determination of cytoplasmic ERK signal intensity (for further details, see Reference 26). Modified from Reference 26 with permission.

this can be profiled in living or fixed cells through the use of HCS as a functional readout of test ligand activity (52).

In addition, using fluorescence detection of downstream GPCR signaling responses by HCS provides numerous possibilities. The monitoring of the ERK pathway is a broadly applicable readout because activation of numerous GPCRs—in some cases, independent of their G protein coupling (16, 19)—induces ERK phosphorylation and signaling. In HCS applications, a phosphoERK-specific antibody is employed through the use of immunofluorescence staining (26) (**Figure 2**). ERK phosphorylation is detectable via HCS within a few minutes of GPCR stimulation, so that the relatively long incubation time required to evaluate test compounds in gene reporter assays can be avoided (71). The $G_{12/13}$-coupled GPCRs signal, in part, by the activation of the small monomeric GTPase Rho. In an HCS application of this $G_{12/13}$ signaling, the cytosol-to-plasma membrane translocation of a GFP-tagged RhoGEF is employed to monitor and quantify GPCR/$G_{12/13}$ activation (72). These and many more G protein signaling readouts (e.g., protein translocations, morphological changes, or various kinase phosphorylation cascades) can be monitored in the HCS format (26, 73, 74, 75).

Screening Approaches for Deorphanizing G Protein–Coupled Receptors

Because, by definition, no known endogenous ligand can bind to an orphan GPCR, ligand-binding assays are typically useless for deorphanizing GPCRs. Instead, functional activity screening approaches are the best option to identify ligands that are active at orphan receptors. The advantage of using screening to deorphanize GPCRs is that very large numbers of test ligands (natural, endogenous, or synthetic) can be efficiently tested for activity at a single orphan receptor. Deorphanization by screening relies on expression of a cloned orphan GPCR in a heterologous expression system that, ideally, will report the general functional activity of the orphan receptor. Because little is typically known about either the signaling or the G protein–coupling mechanisms for the orphan GPCRs, using a heterologous or engineered system that reports general receptor activity is ideal and useful. For example, coexpressing an orphan GPCR with "promiscuous" $G\alpha_{15}$ and $G\alpha_{16}$ proteins can enable an orphan GPCR to nonspecifically couple to activation of PLCβ, resulting in calcium flux (44). Alternatively, expression of chimeric $G\alpha_{q/11}$ proteins, which have their C-terminal amino acids exchanged with those of $G\alpha_s$ or $G\alpha_{i/o}$, could enable an orphan receptor that normally couples to $G\alpha_s$ or $G\alpha_{i/o}$ to instead couple to the activation of PLCβ (41, 43, 44). These sorts of approaches that enable an orphan receptor to activate PLCβ to induce calcium flux are amenable to HTS via FLIPR-type platforms.

Another general functional response of most activated GPCRs is β-arrestin recruitment and translocation to the activated receptor (as explained above). Therefore, expressing an orphan GPCR in a cell system that reports β-arrestin recruitment (e.g., using BRET; see previous section) can indicate test ligand activity at the orphan receptor; β-arrestin recruitment using BRET or other approaches is also amenable to multiwell HTS (76). Another useful heterologous system for screening-based deorphanization studies is the yeast *Saccharomyces cerevisiae*. GPCR-mediated pheromone signaling in yeast that are genetically engineered to enable orphan GPCRs to stimulate a MAPK pathway leads to transcriptional activation of pheromone-responsive promoters that drive reporter gene expression; these approaches can also enable selection and growth only of yeast whose orphan GPCR has been activated by test ligands (60). Indeed, yeast-based GPCR expression systems can be used to deorphanize GPCRs (61), and such approaches are ongoing in our lab as well as elsewhere (77). These and many other approaches can be used to bring the efficient power of functional activity screening to the major challenge of deorphanizing the GPCR receptorome (21, 76, 78).

COMPUTATIONALLY SCREENING THE G PROTEIN–COUPLED RECEPTOROME

Cheminformatics and Bioinformatics Approaches for G Protein–Coupled Receptor Drug Discovery

The human genome project accelerated the identification of potential members of various receptor superfamilies, including the GPCR superfamily. Given the large amount of information available regarding GPCR pharmacology, many investigators have attempted to use this information to predict GPCR molecular targets for known drugs and drug-like small molecules. Several large databases serve as repositories of GPCR-ligand interactions. These include the following.

- **K_i Database** (**http://pdsp.med.unc.edu/kidb.php**) is a curated database of, mainly, GPCR-ligand affinity values. K_i Database (KiDB) currently has ~50,000 annotated affinity values (**Figure 3**) and incorporates links to PubMed, PubChem, UniGene, and a nonolfactory GPCR receptorome anatomical database (79). To our knowledge, KiDB represents the largest publicly accessible database of GPCR-ligand pharmacological data.
- **IUPHAR 7TM Database** (**http://www.iuphar-db.org/DATABASE/ReceptorFamiliesForward?type=GPCR**) has an annotated and searchable list of GPCRs and their ligands, along with affinity values. Unique to this database, an expert consensus-approach is used to provide a "consensus" K_i value for a particular GPCR-ligand pair.
- **GPCRDB** (**http://www.gpcr.org/7tm/**) is an annotated database of molecular biological and pharmacological information on GPCRs. Some ligand-binding data are provided for selected GPCRs, although the information is not readily searchable.
- **PubChem** (**http://pubchem.ncbi.nlm.nih.gov/**) provides both raw screening data as well as certified efficacy data at a large number of molecular targets, including GPCRs. The information is readily searchable and cross-annotated with PubMed.
- **WOMBAT**, which stands for World of Molecular Bioactivity (**http://www.sunsetmolecular.com/**), is representative of several commercial (i.e., non-public-domain) databases that contain information culled from the world's medicinal chemistry literature. WOMBAT contains >76,000 GPCR-ligand pairs.
- **DrugBank** (**http://www.drugbank.ca/**), a database of drugs approved mainly by the U.S. Food and Drug Administration (FDA), lists molecular targets but does not provide information regarding drug affinities.
- **GLIDA** (**http://pharminfo.pharm.kyoto-u.ac.jp/services/glida/**) is a GPCR-ligand database that, like DrugBank, provides a list of various molecular targets but does not provide detailed information regarding drug affinities.
- **ChEMBL** (**http://www.ebi.ac.uk/chembldb/**) is a small-molecule-centric database of drugs and drug-like molecules that provides a wealth of pharmacological and chemical information culled mainly from the medicinal chemistry literature.

Predicting Drug Activity at G Protein–Coupled Receptors and Non-GPCR Targets

Using information derived from the databases described above, we (27, 28) and others (80, 81) have attempted to predict the interactions of known drugs as well as drug-like small molecules at GPCRs and non-GPCR targets. Such use of computational methods to predict chemical and pharmacological properties of chemicals is named cheminformatics. Recently, for instance, we were able to predict and experimentally validate many off-target GPCR interactions for a number of FDA-approved medications (e.g., methadone, fluoxetine, and domperidone) using an approach

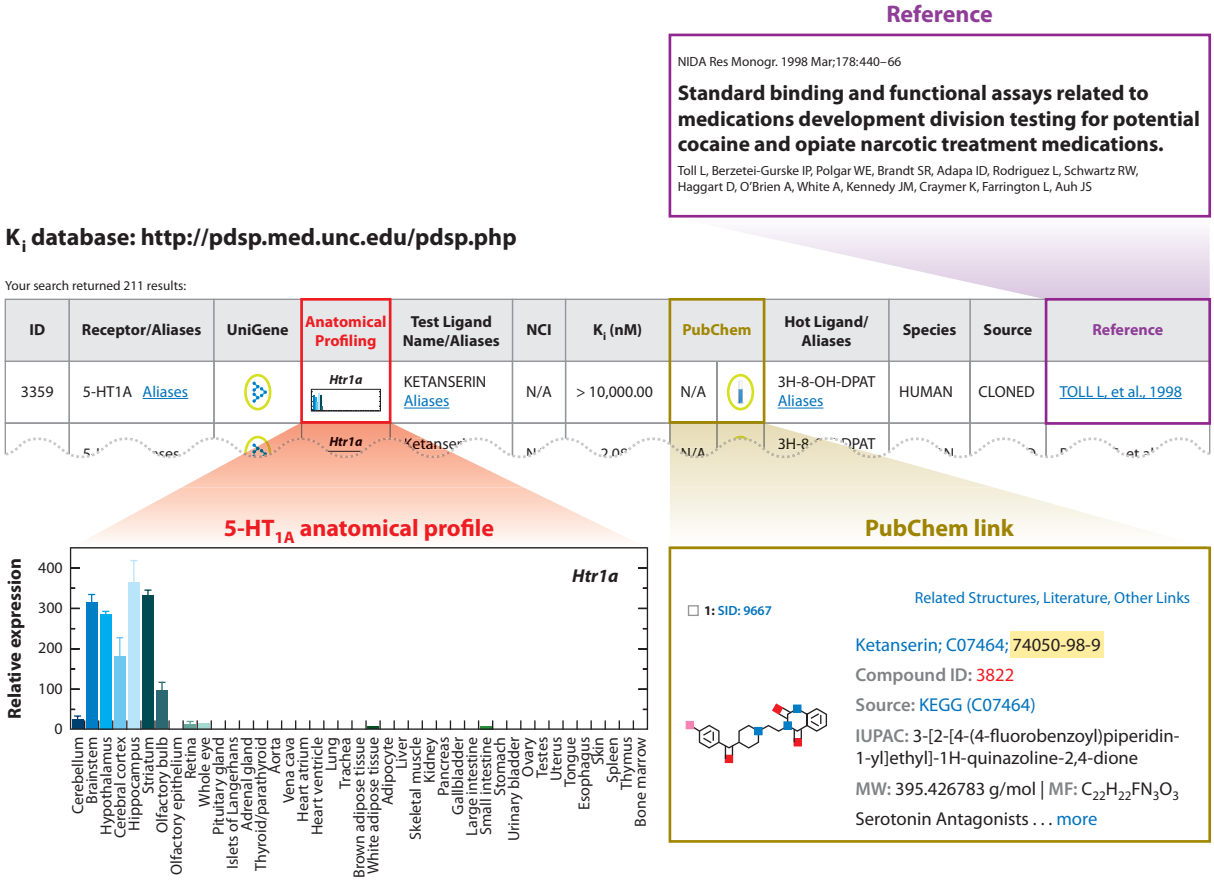


Figure 3

The NIH/NIMH-Psychoactive Drug Screening Program (NIMH-PDSP) K_i Database (KiDB), a curated database of, mainly, G protein–coupled receptor (GPCR)-ligand affinity values. KiDB, which features ~50,000 annotated affinity values, incorporates links to PubMed, PubChem, UniGene, and a nonolfactory GPCR receptorome anatomical database. Shown are results from a query for the serotonin receptor antagonist ketanserin. The main results window lists all receptors in the database that return ketanserin K_i values (*main table*; only the first one listed is shown in its entirety). Links to other resources associated with the K_i value are also available, including the anatomical profiling for the receptor (*red box*), the PubChem listing for the compound (*dark yellow box*), and the original reference in PubMed (*purple box*).

devised by Michael Keiser and Brian Shoichet (UCSF School of Pharmacy) named the similarity ensemble approach (SEA) (**http://sea.bkslab.org/**) (28).

SEA relates receptors and proteins based on the set-wise chemical similarity among their ligands. This chemocentric approach considers ligand structure and chemistry where the motivating hypothesis is that two similar molecules have similar properties and bind to the same group of proteins (27, 82). SEA can be used to rapidly search compound databases and to build cross-target similarity maps. Unlike bioinformatic methods, which might use the sequence or structural similarity among GPCRs, SEA adapts the Basic Local Alignment Search Tool (BLAST) algorithms to compare targets by the similarity of the ligands that bind to them, expressed as expectation values (for a recent example, see Reference 83). For instance, using 1,216 drugs in the U.S. Environmental Protection Agency DSSTox database and 344 GPCRs, Drs. Keiser and Shoichet have annotated

Similarity ensemble approach (SEA): a bioinformatic approach that relates proteins based on the set-wise chemical similarity among their ligands and that can be used to rapidly search compound databases to predict compound-target interactions and build cross-target similarity maps

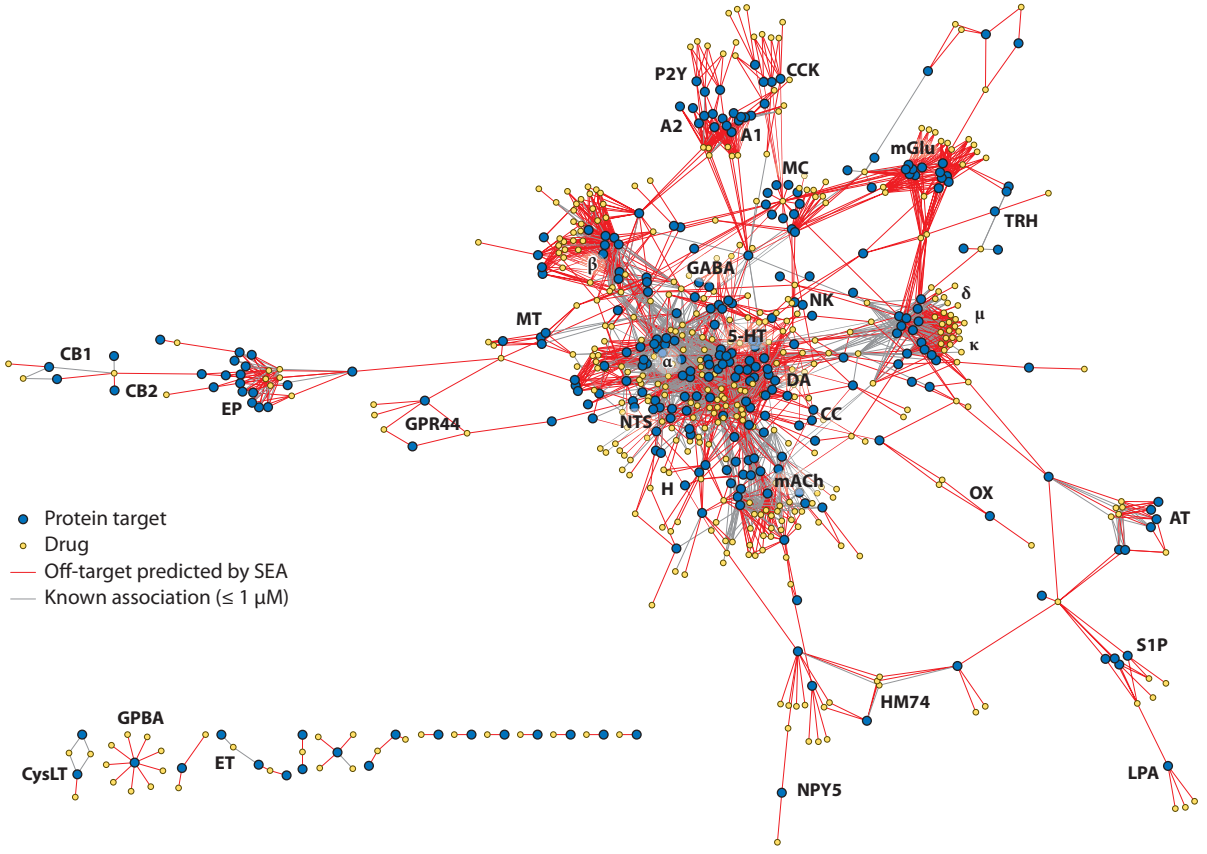


Figure 4

Predicting drug-GPCR networks using cheminformatics and the similarity ensemble approach (SEA). Using 1,216 drugs from the U.S. Environmental Protection Agency DSSTox database and 344 G protein–coupled receptors (GPCRs), Michael Keiser and Brian Shoichet (UCSF School of Pharmacy; **http://sea.bkslab.org**) have annotated known and predicted drug-GPCR networks using SEA. The results indicate that 285 GPCRs (of which 122 are human) and 403 drugs have interactions and are included in this network. Drugs and receptors are linked as per the known drug-target network (*lines in gray*); the red edges represent SEA off-target predictions with *E* values $\leq 10^{-10}$. Numerous off-target drug-GPCR interactions are predicted by SEA and can be compared with known drug interactions. Most predicted interactions arise from drug interactions among similar GPCRs; however, several off-target actions are also predicted for drugs interacting with different GPCRs across different groups. Receptor key: α, α-adrenergic; β, β-adrenergic; δ, δ opioid; κ, κ opioid; μ, μ opioid; 5-HT, 5-hydroxytryptamine (serotonin); AT, angiotensin II; A*x*, adenosine A*x*; CB, cannabinoid; CC, C-C chemokine; CCK, cholecystokinin; CysLT, cysteinyl leukotriene; DA, dopamine; EP, prostanoid receptors EP, DP, IP; ET, endothelin ET-A and ET-B; GABA, gamma-aminobutyric acid B; GPBA, G protein–coupled bile acid; GPR44, G protein–coupled receptor 44; H, histamine; HM74, HM74 nicotinic acid; LPA, lysophosphatidic acid (Edg-7); mACh, muscarinic acetylcholine; MC, melanocortin; mGlu, metabotropic glutamate; MT, melatonin; NK, neurokinin; NPY5, neuropeptide Y5; NTS, neurotensin; OX, orexin; P2Y, purinergic P2Y and pyrimidinergic P2Y; S1P, sphingosine 1-phosphate; TRH, thyrotropin-releasing hormone.

known and predicted drug-GPCR interactions using SEA (**Figure 4**). The results indicate that 285 GPCRs (of which 122 are human) and 403 drugs have interactions and are included in the network. Numerous drug-GPCR interactions are predicted, most arising from drug interactions among similar receptor families. Our recent studies have utilized the predictive power of SEA for identifying unexpected targets for existing drugs. We subsequently validated many new off-target activities predicted by SEA through the use of physical screening methods at GPCRs and other

targets. For example, antagonism of the β_1-adrenergic receptor by the transporter inhibitor fluoxetine (Prozac®), inhibition of the 5-hydroxytryptamine (5-HT) transporter by the ion channel drug ifenprodil (Vadilex), and antagonism of the histamine H_4 receptor by the enzyme inhibitor delavirdine (Rescriptor®) were predicted by SEA and then validated (28). Such approaches hold promise for continued prediction and identification of off-target activities of drugs among GPCRs and other putative targets.

5-HT: 5-hydroxytryptamine (serotonin)

In addition, there is an increasing emphasis on "reverse pharmacology"-based approaches to identify exogenous and endogenous ligands and to assign physiological functions to the ~150 nonolfactory orphan GPCRs (4, 20, 21, 84). Several bioinformatic approaches have been employed to identify ligands for orphan GPCRs. A first approach to narrow possible ligands for orphans is to use sequence homology to match known ligands with orphan receptors that have GPCRs. BLAST, or more sophisticated and accurate programs designed to decipher relationships between family members based on consensus domain profiling and hidden Markov models, has been used to identify closely related receptors and has even been able to provide hints regarding the identity of ligands for some receptors (43, 85). The bioinformatic analysis of receptor sequences has also revealed the potential to predict G protein associations, which, if applied to orphan GPCRs, may help identify the best experimental platform for their deorphanization (86). As well as aiding in such deorphanization, computational genomics approaches may be able to link receptor dysfunction with disease and provide insight into variations in therapeutic responsiveness by identifying genetic mutations and polymorphisms (87). In addition, creating a database of tissue expression profiles for GPCRs, their signaling components, and potential ligand transcripts is one means of determining potential orphan GPCR ligands and functions.

Computational Approaches

Molecular modeling is another in silico method that has classically been used to study receptor structure and function and, more recently, to computationally screen libraries of compounds for both deorphanization and drug discovery efforts. Most GPCR molecular models have been created by homology modeling with the crystal structure of inactive bovine rhodopsin (88, 89) and more recently activated rhodopsin structures. These models have been used to discover novel candidate ligands (90). With the recent availability of high-resolution structures of nonrhodopsin GPCRs (9), computational screening by automated docking to high-resolution structures is now feasible for early-stage drug discovery (91). As more validated molecular structures accumulate, drug candidates may eventually be screened in silico against a "virtual receptorome" to identify possible drug interactions.

Validation of Molecular Targets

A general important point regarding all in vitro experimental systems is that, although extremely useful and efficient, these systems may not reliably predict drugs action(s) in native tissues/cells in situ or in vivo. This constraint can arise from variations between GPCR signaling components in different experimental systems (e.g., level of expression of GPCRs and/or postreceptor signaling molecules in heterologous versus native tissues). In addition, disease-related changes in GPCRs (or their downstream signaling components) may result in drug activity differences in vivo compared with activity identified in screening assays. Therefore, observations of drug action that arise from screening assays in heterologous systems should ultimately be validated in native tissues and with relevant preclinical in vivo studies. Assignment of the physiological roles of GPCRs also lags behind that of receptor profiling and deorphanization. To designate likely physiological roles of

GPCRs in vivo, investigators have determined expression profiles and have generated transgenic animals to knock out the receptor or knock in a constitutively active one at the native gene locus (66, 92). However, as a number of constitutively activated GPCRs are prone to induce cellular hyperplasia, unexpected phenotypes may arise because of cellular transformation in vivo (93, 94). Moreover, although some receptors have similar pharmacology and phenotypes between mice and humans, others frequently differ; these differences may foster false assumptions about the role of specific receptors in human disease (95). Therefore, studies in lower organisms should be interpreted cautiously.

Valvulopathogens: a group of drugs or investigational compounds that induce pathological changes in heart valves and that may result in valvular heart disease (VHD)

DISCOVERING DRUG ACTIVITY AT G PROTEIN–COUPLED RECEPTORS: UNEXPECTED INSIGHTS GAINED FROM CASE STUDIES

When Off-Targets Are Lethal: Valvulopathogens and the 5-HT$_{2B}$ Receptor

The inability of regulatory agencies to efficiently identify serious and potentially life-threatening side effects of many approved drugs has received considerable attention. The withdrawal of rofecoxib (VIOXX® from Merck) and other cyclooxygenase-2 (COX-2) inhibitors because of serious cardiovascular side effects is a pertinent example (96). This inability to identify side effects stems, at least in part, from a lack of information about molecular and cellular mechanisms responsible for drug toxicities and an inability to reliably predict them. Large pharmaceutical companies may not have the scientific resources needed to uncover the cellular and molecular mechanisms of drug toxicities of approved drugs. The inability to identify the target(s) responsible for serious side effects of prescribed medications makes it impossible to avoid these targets via screening in subsequent drug discovery efforts. The case of the fen/phen (fenfluramine/phentermine) appetite suppressant combination is an instructive example of drug profiling at the GPCR receptorome. This profiling identified a toxic-target GPCR that is activated by valvulopathogens. This target identification was accomplished by the screening of available medications and has also allowed the development of a new class of appetite suppressant medications.

In 1997, Connolly and colleagues (97) described valvular heart disease (VHD) as a common and serious side effect of the widely prescribed fen/phen appetite suppressant combination. Later reports indicated that VHD was specifically associated with fenfluramine and its isomer dexfenfluramine (98)—findings that led to the voluntary withdrawal of fenfluramine and dexfenfluramine and >US$20 billion in potential liability against Wyeth Pharmaceuticals. However, a mechanism was not identified until three years later.

Using a systematic screening approach, we analyzed the profile of fenfluramine and its active metabolite norfenfluramine at the GPCR receptorome (99). Additionally, we examined several other off-patent medications known to induce VHD, including methysergide and its active metabolite methylergonovine (99, 100). Because fenfluramine was known to induce 5-HT release, we also screened other drugs that elevate levels of 5-HT but are not known to be associated with VHD, including fluoxetine (Prozac; Eli Lilly) and its main metabolite norfluoxetine. This receptorome screening allowed the rapid identification of the 5-HT$_{2B}$ receptor as the likely target for the cardiac valvulopathic side effects of fenfluramine and related drugs. A similar conclusion was reported by Fitzgerald and colleagues (101), who independently showed an association between 5-HT$_{2B}$ receptor agonist potency and the valvulopathic potential of several approved medications. Subsequently, the 5-HT$_{2B}$ receptor was identified as the likely molecular target responsible for fenfluramine-induced pulmonary hypertension—a fatal side effect of fenfluramine administration (102). We subsequently identified pergolide and lisuride (two dopamine agonist drugs used to

Table 2 Sources of commercially available medications

Medication resource	Web site of resource	Characteristics
MicroSource: The Spectrum Collection	**http://www.msdiscovery.com/spectrum.html**	2,000 active and structurally diverse compounds including known drugs, bioactives, and natural products
Prestwick Chemical, Inc.: Prestwick Chemical Library	**http://www.prestwickchemical.com/**	1,120 off-patent medications, >85% of which are marketed
Sequoia Research Products	**http://www.seqchem.com/**	Many available on- and off-patent medications
NIH Clinical Collection Library	**http://www.nihclinicalcollection.com/**	450 small molecules with a history of use in human clinical trials
NIMH Chemical Synthesis and Drug Supply Program	**http://nimh-repository.rti.org/**	320 CNS-active drugs, metabolites, and bioactives

CNS, central nervous system; NIH, National Institutes of Health; NIMH, National Institute of Mental Health.

treat Parkinson's disease) as potent 5-HT_{2B} agonists (100) and warned that they, too, might be associated with valvulopathy. Subsequent clinical studies demonstrated that these two medications are significantly associated with cardiac-valve disease (103, 104), leading to the withdrawal of pergolide and cabergoline in the European Union and elsewhere. These clinical studies reinforce the idea of a causal association between 5-HT_{2B} agonism and VHD (105).

More recently, we systematically screened several commercial, governmental, and in-house libraries against 5-HT_{2B} (see **Table 2** for sources of libraries containing approved medications) to comprehensively determine whether other FDA-approved medications might be likely to induce VHD of the fenfluramine type by virtue of 5-HT_{2B} receptor agonism (56). We assayed a total of 2,200 approved or investigational drugs for 5-HT_{2B} agonism using calcium mobilization assays. The identified hits were validated and additionally profiled using multiple functional readouts including β-arrestin translocation, ERK phosphorylation, reporter gene expression, and cell proliferation assays (56). We identified 27 compounds as 5-HT_{2B} receptor agonists (hits); 14 of these had previously been identified as agonists, including seven bona fide valvulopathogens. Six of these newly identified hits (guanfacine, quinidine, xylometazoline, oxymetazoline, fenoldopam, and ropinirole) are approved medications.

The receptorome screen that identified the 5-HT_{2B} receptor as the target for the side effects of norfenfluramine also identified the 5-HT_{2C} receptor as the site likely responsible for the appetite-suppressing actions of norfenfluramine. Indeed, for many years, it had been appreciated that 5-HT_{2C} receptor agonists are anorectic (106, 107) and that the anorectic actions of fenfluramine in vivo are produced, in part, by 5-HT_{2C} receptor agonism (108). These observations have also led to the proposal that 5-HT_{2C} receptor-selective agonists that are devoid of 5-HT_{2B} receptor activity will be safe and effective appetite suppressants (109). Indeed, many pharmaceutical companies now have programs for developing 5-HT_{2C} receptor agonists absent of activity at 5-HT_{2B} receptors; one of these, Arena Pharmaceuticals, has recently reported that lorcaserin (APD356) suppresses appetite and induces weight loss without detectable echocardiographic changes in obese humans (110, 111). Therefore, identifying this off-target side effect of valvulopathogens at 5-HT_{2B} receptors by GPCR profiling has facilitated the development of selective and safer drugs for 5-HT_{2C} receptors.

Based on the finding that 5-HT_{2B} agonism is associated with potentially lethal side effects, we have recommended more comprehensive counterscreening of candidate drugs against the entire "druggable genome," as technology is generally available for this. For example, profiling activity

of a lead compound against the known GPCR receptorome early in preclinical development should identify potentially serious off-target actions of compounds. Preclinical counterscreening of candidate drugs against all known "toxic targets" will also enable early identification of potential drug toxicities; once identified by screening, toxicity can be closely monitored as compounds are advanced into clincal trials, or development can be halted at the preclinical stage. Such counterscreening for new candidate drugs is a far safer, and cheaper, alternative to drug failure during clinical trials or possible withdrawal of an approved medication.

Salvinorin A: a highly potent, naturally occurring hallucinogen and psychoactive component of the mint family plant *Salvia divinorum*; also a selective agonist of the κ opioid receptor

KOR: κ opioid receptor

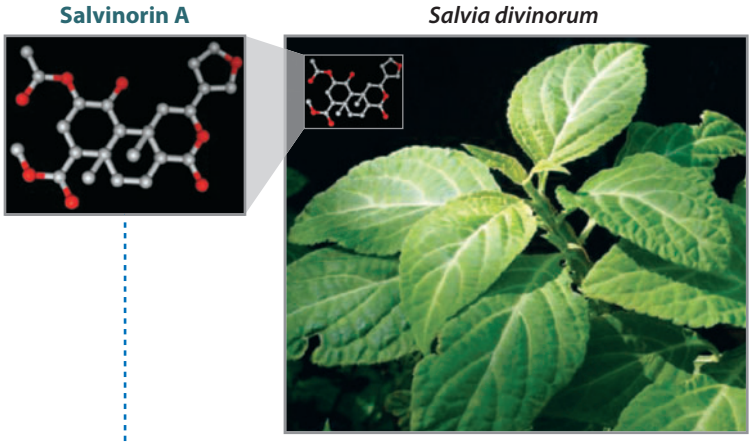
Receptorome Profiling Reveals κ Opioid Receptor as Site of Hallucinogen Action

The hallucinogenic "magic mint" leaves of the mint family plant *Salvia divinorum* have long been used as an entheogen (a plant or drug taken to bring on a spiritual experience) in divination and shamanistic ceremonies by the Mazatec peoples of Oaxaca, Mexico (112). *Salvia* leaves are also used as a recreational drug throughout Europe and North America; in several U.S. states, the drug is now regulated and illegal. The active component of *Salvia divinorum* is salvinorin A, a neoclerodane diterpene (112a, 113). The lipid-like salvinorin A molecule represents the only known psychoactive diterpene, was the first nonnitrogenous hallucinogen, and is the most potent naturally occurring hallucinogen that has been identified. The effective dose of salvinorin A (200–500 μg) in humans is similar to that of the synthetic hallucinogens lysergic acid diethylamide (LSD) and 4-bromo-2,5-dimethoxy-phenylisopropylamine (DOB) (114).

In 2002, we identified salvinorin A's specific molecular target as the κ opioid receptor (KOR) (115); salvinorin A has no activity at the human 5-HT_{2A} receptor, the principal molecular target of classical hallucinogens such as LSD (115, 116), and no activity at other opioid receptors (115) (**Figure 5**). Functional studies measuring inhibition of forskolin-stimulated cAMP accumulation and [^{35}S]GTPγS binding confirmed salvinorin A as a potent agonist at a cloned KOR and at the native KOR expressed in guinea pig brain (115, 117). Salvinorin A thus became the first identified naturally occurring, nonnitrogenous KOR-selective agonist with psychotomimetic properties. This discovery has suggested new directions in opioid research and has fueled the search for other diterpenes with similar pharmacological properties.

Several recent reports have verified that salvinorin A exerts its effects via KOR activation in vivo. Thus, intraperitoneal injections of salvinorin A (1.0–4.0 mg kg^{-1}) result in a dose- and time-dependent increase in tail-flick latencies in mice, with the maximal response occurring within 10 minutes of administration (118, 119). The antinociceptive effects of salvinorin A and the 2-propionate derivative have also been examined in wild-type mice and a novel strain of KOR-knockout mice (119). In these studies, salvinorin A and 2-salvinorinyl propionate produced concentration- and time-dependent antinociception in wild-type mice but not in the KOR-knockout mice. Similar to other KOR-selective agonists (120), salvinorin A and the 2-propionate derivative reduce rectal body temperature in wild-type mice, but this effect is absent in KOR-knockout mice (120). Salvinorin B tested at the same concentrations failed to produce antinociception or hypothermic effects in wild-type mice, which is consistent with in vitro observations demonstrating that salvinorin B is inactive at the KOR (115, 117).

Recent chemistry efforts have produced new salvinorin A analogs that exhibit previously unprecedented high-affinity and wash-resistant binding (121). Two analogs, 22-thiocyanatosalvinorin A (RB-64) and 22-chlorosalvinorin A (RB-48), were found to be extraordinarily potent KOR agonists in vitro and in vivo (121). RB-64 represents the most potent, selective KOR agonist thus far identified, exhibiting efficacy and subnanomolar affinity. No significant activity ($K_i \gg 10{,}000$ nM) at μ or δ opioid receptors was revealed for these compounds. We



	K_i (nM)
5-HTT	>10,000
5-HT1A	>10,000
5-HT1B	>10,000
5-HT1D	>10,000
5-HT1E	>10,000
5-HT2A	>10,000
5-HT2B	>10,000
5-HT2C	>10,000
5-HT3	>10,000
5-HT5A	>10,000
5-HT6	>10,000
5-HT7	>10,000
α-1A	>10,000
α-1B	>10,000
β-1	>10,000

	K_i (nM)
β-2	>10,000
mAChR1	>10,000
mAChR2	>10,000
mAChR3	>10,000
mAChR4	>10,000
mAChR5	>10,000
Nic. AChR	>10,000
D1	>10,000
D2	>10,000
D3	>10,000
D4	>10,000
D5	>10,000
DAT	>10,000
GABA A	>10,000
NMDA	>10,000

	K_i (nM)
Histamine 1	>10,000
mGluR1	>10,000
mGluR2	>10,000
mGluR4	>10,000
mGluR5	>10,000
mGluR6	>10,000
mGluR8	>10,000
δ opioid R	>10,000
μ opioid R	>10,000
κ opioid R	4*
V1	>10,000
V2	>10,000
V3	>10,000

Figure 5

Identification of the κ opioid receptor (KOR) as the molecular target for the plant-based hallucinogen salvinorin A via G protein–coupled receptorome profiling. Salvinorin A is a psychoactive hallucinogen produced from the plant *Salvia divinorum*. Salvinorin A has been screened against a large number of GPCRs and is shown to have high nanomolar (nM) affinity and selectivity for KORs (115). K_i values at the KOR range from 4 to 45 nM, depending on receptor species and tissue source (see the K_i Database at **http://pdsp.med.unc.edu/pdsp.php**). Modified from Reference 112a with permission.

also recently used RB-64 as a high-affinity probe to gain insights into the agonist-activated state and structure of KOR. Molecular modeling suggested that RB-64 required a free cysteine for wash-resistant binding to KOR, and mass spectrometry studies revealed that a free cysteine at position 315 anchored RB-64 to KOR (121). This suggests that a free cysteine may exist in the agonist-bound state of KOR and has provided novel insights into the mechanism of KOR

activation. Therefore, these novel salvinorin-A-derived agonists have emerged as exceptional high-affinity probes for both pharmacological and structural studies of KOR.

G Protein–Coupled Receptor Profiling Reveals that Antipsychotic Drugs Exhibit Robust Polypharmacology

Even though the atypical antipsychotic clozapine was discovered nearly 50 years ago (122), it remains the gold-standard antipsychotic drug because of the absence of debilitating extrapyramidal side effects and demonstrated clinical superiority in treating schizophrenia (123) and reducing suicidality (124). However, clozapine is also associated with severe and potentially life-threatening side effects, including an increased risk of agranulocytosis, seizures, weight gain, and diabetes, and is therefore typically prescribed only for individuals with treatment-resistant schizophrenia. Screening many GPCRs and other CNS targets reveals that clozapine has a highly complex pharmacological profile, with high affinity for a number of serotonin (5-HT_{2A}, 5-HT_{2C}, 5-HT_6, 5-HT_7), dopamine (D_4), muscarinic (M_1, M_2, M_3, M_4, M_5), adrenergic (α_1 and α_2 subtypes), and other biogenic amine receptors (35). Clozapine is thought to normalize glutamatergic and dopaminergic neurotransmission in schizophrenia, thereby ameliorating symptoms, via complex interactions with a large number of molecular targets. These pleiotropic actions of clozapine may be responsible for its beneficial actions in schizophrenia and related disorders (123, 124). In this case, clozapine's polypharmacology at GPCRs appears beneficial, although interaction with H_1-histamine and 5-HT_{2C} serotonin receptors likely contributes to its adverse metabolic side effects (125).

Using similar GPCR-profiling approaches, we also discovered that the atypical antipsychotic aripiprazole has a robust pharmacological profile with partial agonism at several 5-HT (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_7) and dopamine (D_2, D_3, D_4) receptors (126). Thus its complex pharmacology precludes us from concluding that the beneficial actions of aripiprazole in schizophrenia arise solely from partial agonism of D_2 receptors. It is more likely that the balance of partial agonism and antagonism at a multiplicity of receptors is responsible for its efficacy in schizophrenia and related disorders. Taken together, these findings have potentially important implications for CNS drug discovery because not only do they imply that simply developing selective low-efficacy D_2 partial agonists will not yield effective antipsychotic drugs, but they also imply that D_2 partial agonists that functionally interact with various 5-HT and dopamine receptors might be effective (35).

Given that selectively nonselective drugs may be more beneficial than single-action agents in many CNS disorders, how might one best develop them? Clearly, conventional approaches relying on HTS of cloned human molecular targets and the subsequent optimization of these single-target agents are not likely to yield selectively nonselective agents, except, perhaps, by chance. Structure-based drug design approaches in which ligands are designed to interact with the correct subset of molecular targets are also not likely to be successful: Because many of the molecular targets selected have a high degree of structural similarity, designing drugs to target a subset of them is not likely to succeed (5, 35).

As we have previously discussed in detail (35), the implication of these findings for CNS discovery efforts is that the screening of small molecules by nonconventional approaches should be considered. Conceptually, at least two nonconventional approaches for discovering "magic shotguns" can be envisioned: behavior-based screening and genomic approaches. The first, which has been termed HTS-based behavioral screening (127, 128), relies on the semiautomated screening of candidate drugs in behavioral assays. At least two novel antidepressants—YKP10A and INN 00835—were discovered using this approach. Neither drug seems to have appreciable affinity for any known antidepressant drug target, including various biogenic amine receptors and transporters (128), and both have demonstrated effectiveness in clinical trials (128, 129). Based on this,

it is probable that the large-scale, automated and random screening of libraries of compounds enriched for activity at CNS targets, using mainly behavioral assays, will yield compounds with novel—and possibly improved—efficacies for a variety of CNS diseases. These approaches carry with them the advantage of examining responses to drugs in intact organisms rather than in overly simplified experimental systems, such as isolated in vitro binding studies.

The second of the two nonconventional approaches for discovering "magic shotguns" is a genomic one, in which compounds are screened solely on the basis of their abilities to modify the expression of genes. In this approach, compounds with known beneficial actions and pleiotropic actions (for example, drugs such as lithium or clozapine) are screened in vivo and in vitro for their effects on gene expression and determined using DNA microarray technology. Once these gene expression profiles are discovered, compound libraries are subsequently screened to discover small molecules that, when administered in vitro and in vivo, yield similar gene expression signatures; such an approach has been undertaken by Psychiatric Genomics, Inc. (130). Lead compounds can then be optimized to eliminate interactions with potentially toxic molecular targets, such as H_1 receptor for weight gain (125), hERG for arrhythmias (131), and the 5-HT_{2B} receptor for fenfluramine-like VHD (99). In addition, it might prove possible to use combinations of compounds to fine-tune these gene regulatory signatures.

Network pharmacology: an approach to drug discovery and design that encompasses systems biology, drug network analysis, connectivity, redundancy and pleiotropy

SUMMARY AND CONCLUSIONS

Studies to understand the pharmacology of drugs targeted at GPCRs will remain an area of intensive research for the foreseeable future. The fact that at least 150 GPCRs remain orphan receptors suggests that much work is required. Using HTS, HCS, and related approaches to physically screen the GPCR receptorome for drug activity (studies that can be guided and made efficient using cheminformatics) will likely uncover novel targets for drugs and help clarify mechanisms of therapeutic action for existing drugs. It is also likely that these efforts will identify additional receptors whose off-target interactions result in toxicity.

The increased understanding that many drugs exhibit polypharmacology at GPCRs may open an entirely new systems-based approach to GPCR drug discovery that incorporates the concept of network pharmacology. Network pharmacology is an emerging systems approach to drug design that encompasses systems biology, network analysis, target connectivity, target redundancy, and drug pleiotropy (34). Network pharmacology seeks to understand the role of networks for drug action in biological systems and uses this information to inform and guide drug development. Because many effective drugs targeted at GPCRs exhibit pleiotropic actions with multiple receptors, approaches of network pharmacology could help elucidate favorable and unfavorable combinations of GPCRs (and other targets) that would be subject to modulation by drugs. Translating a network-level understanding of drug action to the field of GPCR drug development will rely heavily on cheminformatic methods that themselves are informed by screening strategies. The integration of in silico methods, combined with ligand-biological profiling against receptors and other targets including gene expression arrays, can provide drug designers with a new toolbox that they can use to assess GPCR polypharmacology. Studies of polypharmacology and network information have the potential to facilitate identification of drugs that interact with multiple targets, and they may eventually be used for rational, multitarget design of GPCR drugs (35, 132, 133).

SUMMARY POINTS

1. GPCRs regulate nearly all physiological processes, are the focus of intense study in both academia and industry, and remain major targets in drug discovery efforts.

2. Many clinically effective drugs targeted toward specific GPCRs exhibit activity across multiple receptors—that is, they exhibit polypharmacology. Although lack of drug selectivity can increase off-target drug actions that result in toxicity, surprisingly, many clinically effective drugs (especially ones that work in the CNS) appear to exert superior clinical actions via the modulation of many GPCRs (i.e., they are "magic shotguns"). These seemingly contrasting outcomes of drug polypharmacology (i.e., side-effect liability versus enhanced efficacy) suggest that efficient but comprehensive approaches to define sites of drug action(s) at GPCRs are essential.
3. Drug activity at GPCRs can be defined by physical screening methods and cheminformatic approaches; these approaches illuminate drug activity across GPCRs and other targets.
4. Through the use of cheminformatics and SEA, off-target drug actions can be predicted and subsequently validated at GPCRs and other proteins. Such in silico predictions speed the process of defining drug activity at GPCRs and guide efforts toward testing specific drugs at specific GPCRs.
5. Profiling drugs at the GPCR receptorome using screening and cheminformatic approaches has revealed unexpected insights. These include the identification of toxic targets to avoid, discovery of specific receptor sites for drug activity, and clarification of the polypharmacology of clinically effective drugs. Drug-GPCR profiling has indentified GPCR targets that explain both favorable and adverse drug mechanisms of action.
6. Successful case studies of GPCR profiling include identification of the 5-HT_{2B} receptor as a site of cardiac-valve toxicity; this receptor is now routinely avoided by screening during drug development. Profiling the hallucinogen salvinorin A at GPCRs identified the KOR as the essential site of drug action; this has allowed the development of a new class of potent KOR agonists.
7. Profiling atypical antipsychotic drugs has determined that these drugs display complex polypharmacology across many GPCRs, which may explain their superior clinical efficacy. Importantly, evidence for polypharmacology challenges the idea that magic-bullet drugs (drugs designed to modulate a specific target) will be successful for treating complex CNS disorders such as schizophrenia and depression. Rather, magic-shotgun drugs that exhibit multiple actions and polypharmacology may be better candidates for complex diseases.
8. The evidence that many drugs exhibit polypharmacology at GPCRs may open a new systems-based approach to GPCR drug discovery that incorporates the concept of network pharmacology. This approach would seek to understand the role of receptor networks of drug action in biological systems and use this information to inform and guide drug development. Discovering drug activity among the GPCRs will remain an essential tool for such future efforts.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health (NIH) research grants PHS RO1MH61887, U19MH82441, RO1DA017204, and HHSN-271-2008-00025-C to B.L.R. J.A.A. is supported by NIH National Research Service Award training grant T32HD040127–07 and the UNC Carolina Institute for Developmental Disabilities.

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