GPCR-target-class-directed platform approach: establishment of GPCR-specific biological assay panels and creation of computational chemistry methods for finding and optimizing small molecules modulating the activity of GPCRs.

G-protein-coupled receptor-focused drug discovery using a target class platform approach

Ralf Heilker, Michael Wolff, Christofer S. Tautermann and Michael Bieler

Department of Lead Discovery, Boehringer Ingelheim Pharma GmbH & Co. KG, Birkendorfer Strasse 65, D-88397 Biberach, Germany

In recent years, several large pharmaceutical companies have taken a novel approach to drug discovery biology and chemistry in that they channel their efforts with respect to particular target classes, such as G-protein-coupled receptors (GPCRs), toward dedicated, specialized teams. Benefits of such an organizational structure are the prospects of establishing several target-family-specific experimental techniques and skill sets, thereby enabling a comprehensive functional profiling of drug candidates in different pharmacological respects. In this context, the recently increased number of reports on GPCR ligand-biased signaling has further spurred the efforts in the pharmaceutical industry toward broader biological characterization of the test compounds, for example employing high-content screening to analyze different GPCR ligand-induced signaling pathways. The knowledge of the disease-relevant functional properties of the small molecule GPCR ligands enables target-specific chemical optimization and GPCR-subclass-directed library design. In the case of GPCRs, where little – although at present slowly expanding – structural information on the targets is available, the modeling of GPCR structures crucially depends on biological validation (typically supported by site-directed mutagenesis of the GPCR ligand binding site).In this review, we aim to recapitulate efforts in the pharmaceutical industry to address GPCR-directed drug discovery in a target-class-directed platform approach: establishing GPCR-specific biological assay panels and creating computational chemistry methods for finding and optimizing small molecules modulating the activity of GPCRs.

Target class platform approach

Combining drug discovery activities on the basis of a common target class such as G-protein-coupled receptors (GPCRs) [1,2] provides several benefits, for example the establishment of

Corresponding author: Heilker, R. (Ralf.Heilker@bc.boehringer-ingelheim.com)

Ralf Heilker studied Biochemistry at the University of Tübingen and at the ETH Zürich. He prepared his diploma thesis with Prof. Rolf Zinkernagel and obtained his degree in Biochemistry from the ETH Zürich. After research stays at Kyoto University and at the Basel Institute for Immunology, he gained his PhD at the Biocenter Basel with Prof. Martin Spiess, studying 'clathrin-mediated intracellular transport'. Following an employment at Novartis Pharma in Basel, he joined Boehringer Ingelheim Pharma in 1999, where his current responsibilities include the management of an HTS team, a High Content Screening team and a GPCR target class module.

Michael Wolff studied Biology at the Johannes-Gutenberg-University of Mainz and prepared his diploma thesis at the DKFZ Heidelberg in 1999. He then joined the Max-von-Pettenkofer Institute where he worked on virus–host cell interactions, and received his PhD from the Ludwig-Maximilians-University Munich in 2004. After a post-doc training at the University of Ulm he joined Boehringer Ingelheim, Biberach (Germany) as a principal scientist in 2005. His research interests include confocal fluorescence microscopy and image-based analysis of cellular protein translocations for compound screening and characterization.

Christofer S. Tautermann obtained a degree in Chemistry in 1999 and in Mathematics in 2001 at the University of Innsbruck, Austria. After his doctorate about tunneling and reaction path investigations on hydrogen bonded networks in Innsbruck, he joined the research group of David C. Clary in Oxford (UK) in 2003, focussing on computational investigations on heterogenous catalysis. Since 2005 he has been employed at the Computational Chemistry group at Boehringer Ingelheim, Biberach (Germany). His research interests are in the field of homology modeling, especially the structure prediction of GPCRs.

Michael Bieler studied Pharmacy in Braunschweig. During his trainee period at Solvay Pharmaceuticals in Hannover he prepared his Diploma with the title 'Modeling of the human NK1-receptor in consideration of mutagenesis and SAR of selected ligands'. In 1998 he worked for Solvay Pharmaceuticals and the University of Marburg within the BMBF-project 'SOL – Search and Optimisation of Lead Structures' and since October 2002 he has been working as a scientist for Boehringer Ingelheim. His current work comprises data mining, QSAR modeling virtual screening and library design. His main focus is on GPCR and chemogenomics related aspects.

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.11.011

integrated technological solutions, the gain of 'institutional memory', cross-target comparability for chemogenomic analyses and options for isoform selectivity profiling.

In the case of the GPCR target class, there are some particular challenges based upon the ligand-modulated conformational diversity and functionality of these transmembrane receptors. The allosteric variety calls for a broad and multiplexed pharmacological profiling platform to analyze GPCR ligands and to predict the function of these ligands in a particular disease context. Technologically, this has been enabled by the establishment of numerous medium- or high-throughput-screening-compatible assay formats to measure ligand affinities, G-protein modulation, second messenger production and downstream signaling events, as described below.

In recent years, the *in vitro* assessment of bioactive compounds with regard to features such as toxicity, adverse side effects, bioavailability or aqueous solubility has been shifted to earlier stages of the drug discovery process. Advantageously, this leads to a higher probability of drug candidates passing later developmental hurdles. On the downside, the increased side-effect-based attrition in earlier drug discovery brings about the need for a higher number of test compounds to enter the 'post-HTS' drug discovery stages. In the case of GPCR projects, an extensive pharmacological profiling of bioactives, as described below, should support the medicinal chemists in lead optimization by putting the compounds into a disease-relevant rank order of potencies and efficacies.

A challenge in this context is that it is often unknown, in the earlier phases of a project, which signaling pathway(s) correspond(s) to the disease-relevant processes that are modulated by the target GPCR. Hereby, the more recently developed paradigm of ligand-biased signaling [3,4] and *in vivo* evidence for receptor crosstalk through heterodimerization [5] make the picture even more complex. Ultimately, single parameters or particular patterns from a multifaceted experimental profiling matrix may be selected, where a specific *in vitro* signaling/phenotypic response matches best with the *in vivo* disease-relevant parameters. A brilliant example for the applicability of ligand-biased signaling to drug discovery is the work of Richman *et al.* [6], which identified agonists of GPR109A that are capable of selectively eliciting the therapeutic, antilipolytic pathway while avoiding the activation of the parallel flush-inducing pathway. In contrast to flushing agonists, exposure of cells expressing GPR109A to the nonflushing agonists failed to induce internalization of the receptor or to activate ERK 1/2 mitogen-activated protein kinase phosphorylation.

Another challenge in the area of GPCRs is that a significant number of these receptors have not yet been matched with biologically relevant ligands. Accordingly, several large pharmaceutical companies have established strategies either to 'deorphanize' the respective GPCRs or to deal with these so-called 'orphan receptors' without identifying the physiological ligands [7]. For deorphanization, the cellularly overexpressed orphan receptor is typically tested for a functional response not only to 'orphan' biological mediators, natural peptides, lipids, metabolic intermediates but also complex biological mixtures such as tissue lysates.

If the receptor of interest cannot be deorphanized and no suitable reference agonist is available, drug discovery should be driven by a generic functional readout that bears a high probability of responding to an agonistic ligand. In this context, promiscuous G-proteins that couple the majority of GPCRs to Ca signaling have been of major importance. More recently, the use of arrestin redistribution assays has been described as a format applicable to the majority of GPCRs independent of their specific G-protein coupling. Deorphanization is usually done in dedicated, specialized functional units within companies, establishing the required techniques and skill sets.

In parallel to the advantages on the biological side of organizing drug discovery in target-family-focused teams, there are also several benefits on the side of target-class-directed chemistry [1,2]. While single-target-focused medicinal chemistry remains an important objective in drug discovery projects, combinatorial libraries directed toward families or subfamilies of GPCRs, protein kinases, nuclear hormone receptors and proteases, have begun to pour into the compound collections of big pharmaceutical companies. This approach integrates the above-described biological target class focus with chemoinformatic strategies of privileged structures, Bayesian filters and bioanalogous design strategies, as discussed below.

Privileged structures [8] may address a target-family-wide common feature in terms of molecular recognition elements. Protein kinases constitute an early example in this context. This chemogenomic 'pioneer role' of the protein kinase family is based upon the structurally straightforward access to the ATP-binding site. The high homology between these ATP pockets enables the generation of, for example, combinatorial libraries that 'hit' several protein kinase targets with numerous compounds. The relative ease of producing general protein-kinase-directed chemotypes is, however, a curse at the same time, because the need for protein kinase selectivity of the drug candidate compound is a frequently encountered challenge in protein-kinase-directed pharmaceutical research. Obviously, a differentiation between promiscuous binders and compounds with a restricted selectivity, for example against some GPCR subfamily isoforms, is required.

This exemplifies a more general discussion point with regard to target-class-directed chemotypes. In an ideal target-class-focused combinatorial library, for each target class representative some library representatives should act as ligands, whereby an individual library compound should be more or less selective for a single target class representative. The fact that this is an achievable goal has meanwhile been established for the GPCR target class (see below).

GPCRs

GPCRs constitute a large superfamily of cell surface receptors that is classified into more than 100 subfamilies, typically according to sequence homology, ligand structure and receptor function. Members of the GPCR superfamily share a common membrane topology: an extracellular N-terminus, an intracellular C-terminus and seven transmembrane (TM) helices, which are connected by three intracellular loops (ICLs) and three extracellular loops (ECLs). On the basis of their shared topological structure, GPCRs are also referred to as 7TM receptors. The more frequently employed name 'GPCRs' is derived from their typical functional association with a heterotrimeric G-protein complex, through which the ligand-activated GPCRs initiate some of their intracellular signaling processes.

Ligands matched with the different receptor representatives of this superfamily are remarkably diverse, including biogenic amines, amino acids, peptides, proteins, prostanoids, phospholipids, fatty acids, nucleosides, nucleotides, Ca^{2+} ions, odorants, bitter and sweet tastants, pheromones and protons. Classically, the mammalian members of this superfamily have been divided into three families: A, B and C [9]. Meanwhile, novel less well-explored mammalian families have been added to the classification scheme [10].

Family A includes the aminergic and some of the peptidergic GPCRs. Nucleotides, lipids and other small molecules serve as ligands for some other family A receptors. A second branch of the peptidergic GPCRs is assigned to the family B GPCRs. Finally, family C GPCRs contains the metabotropic glutamate receptors, γ-aminobutyric acid receptors and the Ca^{2+} sensing receptor.

Human genome sequence analysis predicts ~865 GPCR genes, ~500 of which are estimated to be olfactory receptors and the residual GPCRs are considered to act by stimulation via an endogenous, physiological ligand [11]. Presently, ~100 of the nonolfactory GPCRs have not yet been matched with a ligand and are categorized as orphan receptors.

GPCRs are the most frequently addressed drug targets in the pharmaceutical industry. Approximately 30% of all marketed prescription drugs address GPCRs, which makes this protein family the pharmaceutically most successful target class [1]. Fuelled by these accomplishments, G-protein-coupled receptors are taking a prominent place in the target portfolios of many pharmaceutical companies.

'Classical' drug discovery techniques to analyze GPCR–ligand interactions and GPCR signaling

GPCR ligand binding

The interaction between GPCRs and their extracellular ligands has proven to be an attractive point of interference for therapeutic agents. For that reason, the pharmaceutical industry has developed biochemical drug discovery assays to investigate these ligand–GPCR interactions, such as the scintillation proximity assays (SPAs) [12] or the less frequently employed fluorescence polarization (FP) assays [13] and fluorescence intensity distribution analysis (FIDA) assays [14]. Binding assays enable equilibrium binding or kinetic displacement measurements so that K_D-type and k_{on}/k_{off}-type values can be derived. Because biochemical data are less prone to falsification by 'off-target effects' than cellular data, the ligand binding data are typically a robust first basis for computational chemistry approaches.

All the above-mentioned biochemical binding assays rely on the competition of the test compound with a labeled reference ligand. An obvious disadvantage of these binding assays is the risk of missing noncompetitive, allosteric ligands. Thus, the binding assay does not elucidate functional aspects of test compound activity such as full/partial agonism, inverse agonism versus neutral antagonism, negative or positive modulation. To expand compound testing toward measuring GPCR activity, there was a need for functional medium-to-high-throughput assays.

G-protein function and second messenger generation

Interaction of an activated GPCR with a heterotrimeric G-protein catalyzes the exchange of guanosine diphosphate (GDP) by guanosine triphosphate (GTP) enabling Gα-GTP and βγ-dimers to interact with a variety of downstream effectors [15]. The respective nucleotide exchange process on the Gα-subunit can be monitored by measuring the binding of [^{35}S]GTPγS [16], for example in an SPA-based format.

Signaling downstream of the Gα subunit is dependent on the Gα isoform [17] that is preferred by the GPCR of interest: proteins of the $G\alpha_{q/11}$ family stimulate phospholipase C (PLC), representatives of the $G\alpha_{i/o}$ and $G\alpha_s$ families mostly modulate adenylate cyclase (AC) activity. The more recently described $G\alpha_{12/13}$ family acts via stimulation of the small GTPase Rho, omitting a small molecule second messenger. If the GPCR of interest signals via PLC, the most broadly applied cell-based technique to measure GPCR activation is the Ca^{2+} release assay, either measured in a fluorescent format using Ca^{2+}-sensitive fluorophores (fluors) [18] or in a luminescent format using aequorin and a chemiluminescent substrate [19]. If the GPCR of interest signals via AC, the cytosolic cyclic adenosine monophosphate (cAMP) content may be determined using various detection technologies [20].

GPCR downstream signaling

For several years, bioluminescence-based reporter gene assays have been employed to measure functional activity of GPCRs [21]. This assay format is very sensitive owing to the low signal background of the bioluminescent readout and the signal amplification steps between GPCR activation and the cumulative reporter gene expression. A cAMP response element (CRE) in the promoter of the reporter gene enables the specific monitoring of $G_{s/i}$-dependent signaling; elevated cytoplasmic cAMP levels activate protein kinase A (PKA), which turns on the CRE binding protein and initiates CRE-dependent reporter expression. A nuclear factor of activated T cells (NFATs) response element (RE) in the promoter of the reporter gene monitors G_q-dependent signaling; in response to elevated cytoplasmic calcium levels, NFAT is dephosphorylated by the Ca-dependent phosphatase calcineurin and induces NFAT–RE-dependent reporter expression [22]. Because reporter gene assays typically require a few hours of incubation to accumulate the reporter gene product in the cells, potential side effects of a test compound with regard to, for example, toxicity or GPCR desensitization should be taken into account.

Another marker, which has been applied in drug discovery to monitor the activation of G-protein signaling more recently, is the phosphorylation status of ERK1/2 [23]. Advantageously, from a practical perspective, $G\alpha_q$, $G\alpha_s$ and $G\alpha_i$ signaling converge on the level of ERK phosphorylation. ERK phosphorylation may be quantified in the lysate of GPCR-stimulated cells by the so-called SureFire™ assay (TGR BioSciences Pty Ltd, Thebarton, Australia) or by high-content screening (HCS), as described below.

Arrestins are cytoplasmic proteins that are recruited to the plasma membrane by ligand-activated, GRK-phosphorylated GPCRs [24]. Arrestins then uncouple the GPCR from the cognate G-protein and target the desensitized receptors to clathrin-coated pits for endocytosis. Apart from this indirect influence on G-protein signaling by the physical interceding between GPCR and G-protein, the arrestins have more recently been found to mediate G-protein-independent signaling directly [25,26]. The latter mechanism regulates aspects of cell motility, chemotaxis, apoptosis and probably other cellular functions through a rapidly

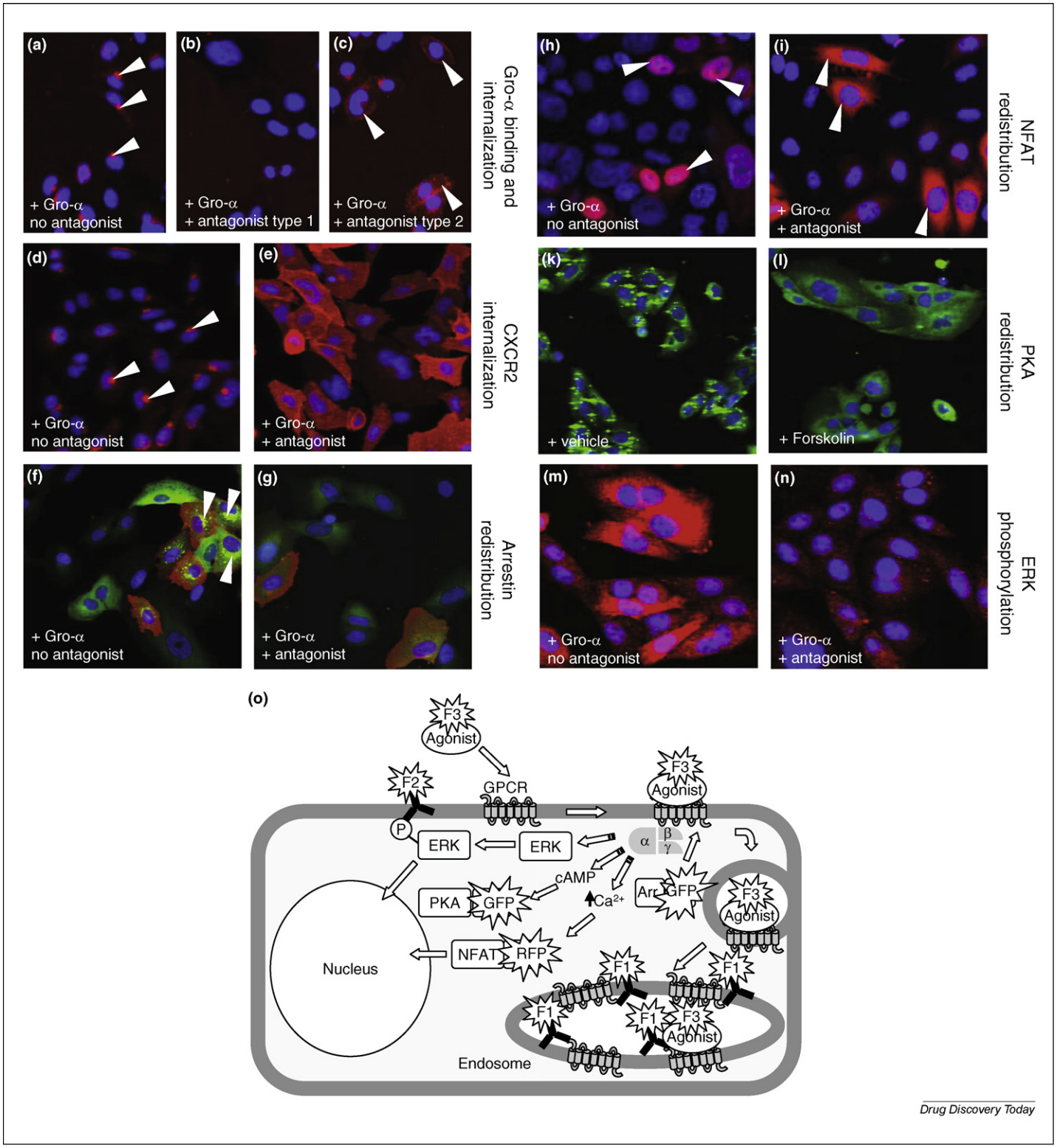

FIGURE 1

HCS to monitor diverse aspects of GPCR activation. The nuclei of the formaldehyde-fixed cells were always stained with Hoechst 33342™ (shown in blue). **(a–c)** Fluor-labeled ligand binding and internalization. 10 nM fluor-labeled Gro-α (shown in red) was added to the supernatant of CXCR2/Gα16 stably transfected Chinese Hamster Ovary (CHO) cells. (a) In the absence of an antagonist, the fluor-labeled Gro-α was accumulated in endosomal compartments; indicated by filled arrowheads. (b) A ligand-displacing antagonist prevented binding of fluor-labeled Gro-α to CXCR2. (c) An allosteric, not ligand-displacing, antagonist allowed fluor-labeled Gro-α to bind to CXCR2 at the plasma membrane (indicated by open arrowheads) but inhibited Gro-α/CXCR2 internalization. **(d)** and **(e)** Fluor-labeled anti-CXCR2 antibody to detect CXCR2 internalization. 15 nM Gro-α was added to the supernatant, CXCR2 was immune-stained in the permeabilized cells with a fluor-labeled anti-CXCR2 antibody (shown in red). (d) In the absence of an antagonist, CXCR2 was accumulated in endosomal compartments; indicated by filled arrowheads. (e) An antagonist – independent if ligand-displacing or not – prevented Gro-α-stimulated CXCR2 internalization. Owing to the permeabilization of the cells, the anti-CXCR2 antibody also detected steady-state intracellular CXCR2. **(f)** and **(g)** Arrestin redistribution. A human osteosarcoma cell line (U2-OS)

expanding list of signaling pathways. Arrestin signaling may be monitored by HTS-applicable techniques such as enzyme fragment complementation [27] or the Transfluor™ technique as described below.

HCS in the GPCR target family approach: one technology platform to analyze all aspects of GPCR ligand function

HCS

In recent years, a novel technique generally referred to as HCS has been introduced to the early pharmaceutical drug discovery process [28]. HCS combines high-resolution fluorescence microscopy with automated image analysis. HCS provides several advantages over 'classical' HTS as described in the above chapter. Cellular HTS conventionally monitors the mean response of the whole cell population of a microtiter plate (MTP) well. By contrast, HCS can distinguish the individual response of many cells in an MTP well, which may differ with respect to the differentiation, the stage of the cell cycle, the state of transfection or owing to natural variability.

As a result, heterogeneous pharmaceutical drug effects on mixed cell populations may be analyzed in a single MTP well. 'On-target' drug effects may be cross-correlated with other phenomena, such as cellular toxicity. Compound artifacts such as cell lysis or compound autofluorescence may be detected. HCS permits work with endogenous targets and/or primary cells [29] using specific antibodies or morphological image analysis. In this way, HCS enables novel assay formats that do not depend on an overall change of fluorescence or luminescence intensity from the whole MTP well. In addition to these general benefits in the area of drug discovery, HCS is well suited to cover all aspects of GPCR ligand binding and function.

HCS to measure GPCR ligand binding, internalization and arrestin signaling

One means to monitor ligand binding and ligand-induced internalization of a GPCR [30] is to cointernalize a specific, fluor-labeled ligand [31] (Fig. 1a,b: displaced ligand, cointernalized ligand). Similar to the classic radioligand-binding assay, the fluor-labeled ligand protocol investigates test compounds with regard to reference ligand displacement. Beyond the possibilities of the radioligand displacement, however, the HCS protocol enables the identification of test compounds that allow for the binding of the fluor-labeled ligand but prevent its internalization (Fig. 1c: surface-bound ligand). Image analysis allows us to distinguish inactive test compounds from displacing or from the nondisplacing antagonists. In the case of inactive test compounds (Fig. 1a) an image analysis algorithm can be established to search for ligand fluorescence in perinuclear, granular spots. For ligand-displacing antagonists (Fig. 1b) there should be no ligand fluorescence associated with the cells. For nondisplacing antagonists of internalization (Fig. 1c) the ligand fluorescence is typically associated with the plasma membrane. In the latter case, an image analysis algorithm can be established to search for cell peripheral ligand fluorescence.

Using a fluor-labeled antibody against the GPCR of interest [32], test compounds can additionally be qualified as agonists or antagonists of internalization (Fig. 1d,e). If combined in multiplexed or parallel experiments, the use of fluor-labeled ligand and antibody may serve to analyze test-compound-induced/antagonized intracellular receptor internalization, recycling or degradation. Depending on the specific disease biology and target, these data may help to predict test-compound-induced receptor desensitization and tachyphylaxis issues.

In parallel to receptor internalization, it has been demonstrated for numerous GPCRs [33] that the so-called arrestins undergo an intracellular redistribution process as described in the above chapter. In the Transfluor™ technology [34], the redistribution of an arrestin-fluorescent protein (ArrFP) conjugate is monitored by HCS (Fig. 1f,g). In nonstimulated cells, ArrFP is uniformly distributed across the cytoplasm. Upon receptor stimulation, ArrFP is recruited to the activated GPCR, accompanies the GPCR to clathrin-coated pits and, for some GPCRs, even to the endocytic vesicle level [35]. In HCS, the corresponding change from uniform cytoplasmic fluorescence to granular fluorescence may be quantified.

HCS to measure second messenger generation

To detect the rise of cytoplasmic Ca^{2+} in response to the stimulation of a G_q-coupled receptor, NFAT may be employed in an HCS assay. In response to elevated cytoplasmic calcium levels, NFAT is dephosphorylated by calcineurin and migrates from the cytoplasm to the nucleus [22]. If NFAT is expressed as a fusion protein with a fluorescent protein, this nuclear translocation may be monitored and quantified as a measure of GPCR activation. In the given example (Fig. 1h,i), Gro-α was administered to CXCR2/Gα16 stably transfected CHO cells. Of course, all data generated in receptor-overexpressing systems and/or systems employing nonphysiological signaling chain components (e.g. a promiscuous Gα16) should be handled with care and, if available, compared to other signaling assays.

Protein kinase A (PKA) may be employed to monitor intracellular cAMP levels. PKA is a heterotetrameric complex of two

stably transfected with arrestin-green-fluorescent protein (ArrGFP; cell line is a kind gift of Molecular Devices/now part of MDS Analytical Technologies, Concord, ON, Canada) was transiently transfected with CXCR2. 15 nM Gro-α was added to the supernatant, ArrGFP is shown in green, CXCR2 was immune-stained (shown in red). (f) In the absence of an antagonist, the cells expressing CXCR2 showed granular spots in proximity to the nuclei (green channel); indicated by filled arrowheads. (g) In the presence of an antagonist, the green fluorescence was uniformly distributed across the cytosol. **(h)** and **(i)** NFAT nuclear translocation. CXCR2/Gα16 stably transfected CHO cells were transiently transfected with an NFAT-red-fluorescent protein (RFP) fusion construct. 15 nM Gro-α was added, NFAT–RFP is shown in red. (h) In the absence of an antagonist, the cells expressing NFAT–RFP displayed NFAT to be in the nuclei; indicated by filled arrowheads. (i) In the presence of an antagonist, the red NFAT fluorescence was localized to the cytosol. **(k)** and **(l)** PKA–GFP redistribution. PKAcat–EGFP transfected CHO cells were purchased from BioImage/ThermoFisher Scientific. (k) In vehicle-stimulated cells, a PKA–GFP fusion protein (shown in green) was associated with various intracellular membranes. (l) Upon forskolin stimulation, the cytosolic cAMP level increased and PKA–GFP was redistributed homogeneously across the cytoplasm. **(m)** and **(n)** ERK phosphorylation. CXCR2/Gα16 stably transfected CHO cells were stimulated with 15 nM Gro-α, phosphoERK was immune-stained (shown in red). (m) In the absence of an antagonist there was a high level of ERK phosphorylation. (n) In the presence of an antagonist ERK phosphorylation was reduced to background levels. **(o)** Schematic illustration of the GPCR activation pathways as exemplified in (a)–(n). Small molecule fluorophores to label anti-CXCR2 antibody, anti-pERK antibody and Gro-α are designated F1, F2 and F3, respectively.

regulatory and two catalytic subunits. At low cAMP concentrations, the PKA holoenzyme is associated with various intracellular membranes through the so-called A kinase anchor proteins (AKAPs) [36]. With rising cAMP levels, the PKA holoenzyme is dissociated and releases the catalytic subunit (PKAcat) into the cytoplasm. In an HCS application of this cAMP-dependent process (Fig. 1k,l), the release of PKAcat is monitored by employing a fusion protein of PKAcat and GFP (http://www.bioimage.com) .

HCS to measure various GPCR downstream signaling events

The monitoring of the ERK pathway is a broadly applicable assay format, because numerous activated GPCRs – independent of their G-protein coupling – induce ERK signaling [23]. In an HCS application of this principle (Fig. 1m,n) a phosphoERK-specific antibody is employed in an immune stain procedure [32]. Advantageously, ERK phosphorylation is detectable by HCS already a few minutes after GPCR stimulation, so that the relatively long test compound incubation times as required in reporter gene assays may be avoided.

$G_{12/13}$-coupled GPCRs signal by the activation of the small monomeric GTPase Rho. In an HCS application of this process, the cytosol-to-plasma membrane translocation of a GFP-tagged RhoGEF is employed to monitor and quantify GPCR/$G_{12/13}$ activation [37]. Alternative experimental techniques to quantify $G_{12/13}$-signaling are less pathway-specific (e.g. serum response element [SRE]-dependent reporter gene assays) or of lower throughput (e.g. specific affinity precipitation of GTP-bound Rho and subsequent immunoblot).

These (scheme in Fig. 1o) and many further aspects of G-protein signaling biology may be monitored in the HCS format, for example reorganization of the cytoskeleton, morphological changes or various kinase phosphorylation cascades. The pharmacological profiling of ligands against various GPCRs is a good basis for the below described chemogenomic analyses.

Chemogenomics strategies to address the GPCR target family

One of the first companies using the term 'chemogenomics' was Vertex Pharmaceuticals, in a press release in February 2000 where it was described as an approach to 'rapidly and simultaneously design multiple lead classes of drugs directed at protein targets in gene families'. Meanwhile, several reviews in recent literature prove that chemogenomics has become a well-established concept [38,39], especially in the field of the GPCR target family [40]. The chemogenomics approach describes a procedure of linking information about several biological targets and chemical compounds [41]. The idea behind this bridging of the chemical space and the biological space is that closely related compounds should have similar biological profiles and closely related targets should share similar compounds – or in other words: similar receptors bind similar ligands [42]. But the crucial question is: what does 'similar' mean? It is not necessarily the similarity of the chemical graph, but the similarity of those features of the compounds that are responsible for the respective ligand–target interactions. And, therefore, the description of ligand similarity can differ from target to target.

The chemogenomics concept can be applied in different ways; phylogenetically similar binding sites of GPCRs should accommodate ligands with similar features. So, known ligands are good sources for identifying ligands for closely related, maybe even orphan, targets [43]. Ligands for a target should share common features that correspond to characteristic binding site attributes. Phylogenetically different binding sites with similar attributes should be addressed by these ligands in a similar way. This is useful for finding new targets for known compounds or for the prediction of potential side effects [44,45]. If two compounds have similar activities at N targets they shall have similar activities at the target $N+1$, even if they differ significantly in their chemical graph. This is helpful in identifying new lead compounds. For somatostatin receptor subtype 5 (SST5R), Guba *et al.* were able to identify an antagonist via a chemogenomics strategy based on affinity fingerprint similarity [46]. The knowledge of the essential properties of the ligands for a target subfamily was shown to be useful for library design [42]. Other chemogenomic applications compare and classify receptors based on binding sites by using sequence motifs or (putative) 3D information, mostly focusing on those amino acids that are known to be essential for ligand recognition from site-directed mutagenesis studies. One of the first applications using sequence motifs was that of Crossley *et al.*, whose 'thematic analysis' comprises groups of amino acids that are extremely likely to be involved in ligand binding, on the basis of experimental data [47].

Because real life restricts the chemogenomics approach – usually information about binding affinities is limited to the related ligands of the targets – attempts have been made to predict binding affinities of ligands for new targets based on known ligands of these targets, using methods of virtual screening. Bock and Gough used a support vector machine approach [48] while Martynova *et al.* used a Bayesian approach to predict activity spectra [49]. We have employed Bayesian filters to screen the company compound collection 'virtually' for ligands of muscarinic acetylcholine receptors (hMx; Fig. 2) and to profile different chemotypes against five peptidergic GPCRs from the same GPCR subfamily (Fig. 3). Selective and cross-active chemotypes could be identified. The chemogenomics approach vitally depends on reliable and crosstarget comparable biological data collections, as may be provided by the thorough pharmacological profiling described above.

Structural knowledge on GPCRs and GPCR modeling

Modeling of GPCRs was hampered by the lack of template structures until the year 2000, when the structure of bovine rhodopsin was resolved to a high resolution by X-ray crystallography by Palczewski *et al.* [50]. Bovine rhodopsin stayed the only GPCR structure resolved to an atomic level for another few years until new methods for GPCR crystallization emerged. Recently, the structures of human β2 adrenergic [51], turkey β1 adrenergic [52] and human A_{2A} adenosine [53] receptors were resolved, all three GPCRs of therapeutic interest. In addition to these, structures of squid rhodopsin and the retinal-free (presumably active) form of rhodopsin (opsin) have recently been resolved [54].

In 2000, the emerging X-ray structure of rhodopsin made the use of homology modeling approaches for GPCRs feasible. The sequence identity of numerous GPCRs of therapeutic interest to rhodopsin is, however, very low (<25%), which directs straightforward homology modeling preferentially to class A GPCRs (of which also rhodopsin is a member). For these, at least a sequence alignment to rhodopsin can be done owing to highly conserved

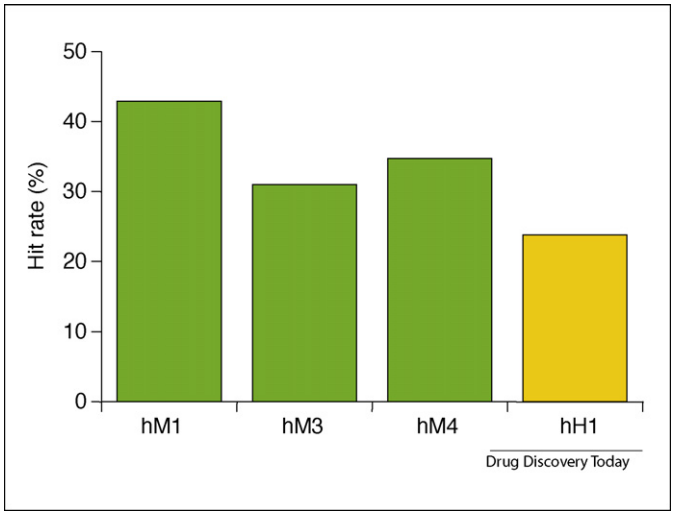

FIGURE 2

Hit rates of a set of compounds, tested at the human muscarinic acetylcholine receptors hM1, hM3 and hM4 and the human histamine receptor hH1. A Bayesian model for the binary classification of compounds into muscarinic acetylcholine receptor (hMx) hits and nonhits was trained using MDDR™ data. This model was used to screen the Boehringer Ingelheim compound pool 'virtually'. The resulting virtual hits were sorted according to their Bayesian index of hMx ligand likeness and clustered, using the pairwise Tanimoto similarity of their Daylight™ fingerprints. From the first clusters, up to 3 compounds were selected, so that 1000 compounds were in the final set. The compounds were tested in radioligand displacement assays against the four receptors in duplicate at a ligand concentration of 10 μM. A compound was defined as a hit, if both measurements were below 50% inhibition of binding. The hit rate is the ratio of hits per assay to the number of compounds. These results show the feasibility of target hopping. By virtual screening supported selection of ligands for the hMx receptors, also ligands for the hH1 receptor could be found.

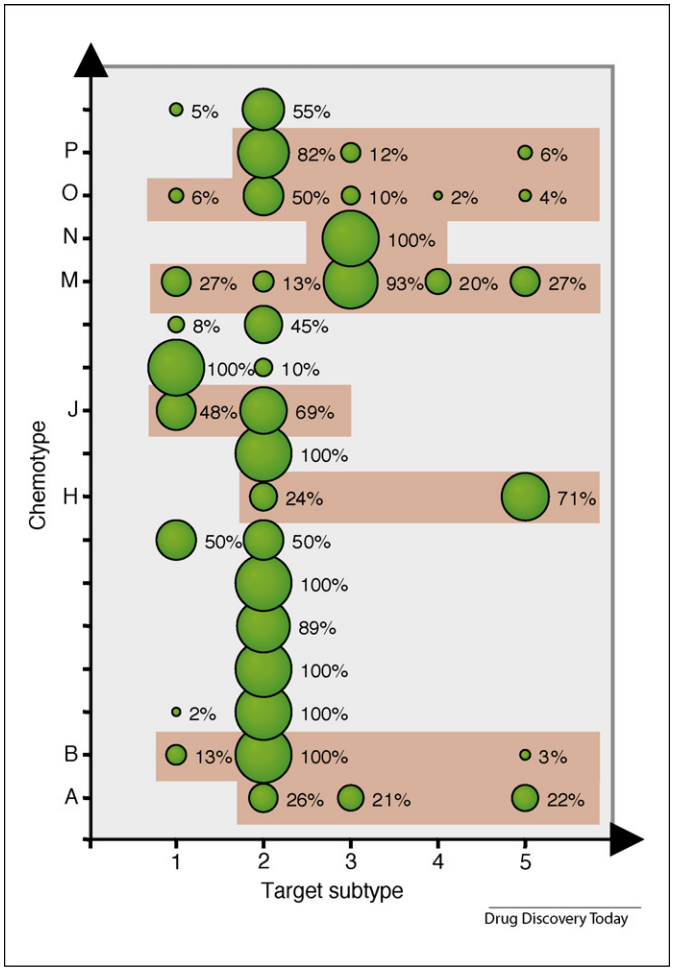

FIGURE 3

Bioprofile of different chemotypes. A Bayesian model was trained for the recognition of hits against a certain GPCR target. The training data were taken from the Aureus™ database. A set of 1123 compounds was taken from the Boehringer Ingelheim compound pool by virtual screening, using this Bayesian model. The classification of the compounds into different chemotypes was done by structure-based clustering and visual inspection of the results, the latter process considering the feasibility of combinatorial synthesis for each chemotype. That means, each chemotype could be used as a scaffold for a combinatorial compound library. IC_{50} determination was done using radioligand displacement assays for five subtypes (1–5) of the GPCR target class, the model was built for. For each chemotype, only compounds with an IC_{50} less than or equal to 3 μM were considered as hits. The bubble size reflects the percentage of hits among the number of compounds in the respective chemotype class. For the chemotype A, 26% of all compounds of that chemotype are hits at the subtype 2, 21% at subtype 3 and 22% at subtype 5. Most chemotypes have different distributions of hits and therefore represent different bioprofiles (compare chemotype A, B, H, J, M, N). Chemotypes A and P as well as M and O, however, address the same spectrum of target subtypes – even if the distribution differs. These are candidates for directed combinatorial libraries that aim at multiple GPCRs. Obviously, for chemotype P 82% of its compounds are hits at subtype 2, while only 6% hit subtype 5. This shows that the majority of hits of chemotype P are specific for subtype 2; however, there is a chance to address three different GPCRs by only one combinatorial library.

sequence motifs known to occur in the TM regions in class A GPCRs. In several modeling studies, it was realized that the rhodopsin template suffers from various drawbacks (i.e. the very narrow retinal binding and the very distinct structure of the extracellular loop 2 (ECL2), which deeply plunges into the TM-domain). To overcome the problems encountered with the rhodopsin template, several methods for *ab initio* prediction of GPCR structures were developed. Two methods should be mentioned in this context, the Predict algorithm and the Membstruk method. Both methods use hydrophobicity analysis rather than conserved pattern matching for TM-helix prediction, making these methods also suitable for GPCRs lacking the well-known sequence motifs (especially classes B and C). While the Predict algorithm may be seen as a real *ab initio* method (no findings from the crystal structure of rhodopsin are used), the Membstruk method uses the fold of a known GPCR structure for helix arrangement. Both algorithms have been shown to be able to reproduce the TM-structure of rhodopsin well and were successfully applied to the structure prediction of many therapeutically important GPCRs. All these methods are well described in recent reviews by Martinelli and Tuccinardi [55] as well as Mobarec and Filizola [56].

With the availability of the β2 structure it was seen that the overall fold is very similar to that of rhodopsin, with a slightly widened binding site and some small displacements of the helices. The ECL2, however, was seen to be completely different, including a small helical part being much higher above the TM-binding site than the ECL2 in rhodopsin. These differences provide invaluable information to the modeler, in a way that different templates can be chosen and the degree of conformational flexibility may be estimated. This, of course, applies only to the inactive state of GPCRs and can only be taken as a convenient guideline. Modeling

attempts directed toward activated GPCRs are still an unresolved problem as the structures are shown to undergo strong helical movement upon activation [57]. Some computational methods emerged that use agonist information to reorient the helices to mimic the activated state. The very recently resolved structure of opsin, which corresponds to one of the activated states in the rhodopsin activation cascade, may open a door to active state modeling.

The most crucial and cumbersome step in any model building process is the experimental validation. A receptor model – whatever method has been used to derive it – has to be validated by experimental results, which is also clearly pointed out by Martinelli and Tuccinardi [55]. There are several experimental methods that provide results that can be used for model validation, such as mutagenesis studies, spin labeling experiments, SAR studies with homologous ligands, cysteine scans and many more. Many results of experimental evidence have to be put together carefully to refine receptor models. This procedure is not straightforward, because most experimental findings cannot be directly incorporated into the model. For example, a decreased ligand binding affinity of a ligand to a mutated receptor could result from a change of the direct interaction between the ligand and the mutated amino acid or it could come from a mutation induced shape change in the receptor. Therefore, the experiments must be designed carefully to provide a solid basis for structural model validation.

Validated receptor models (example in Fig. 4) have been widely used for SAR explanation and for gaining new ideas for ligand modification as well as virtual screening purposes and library design [58].

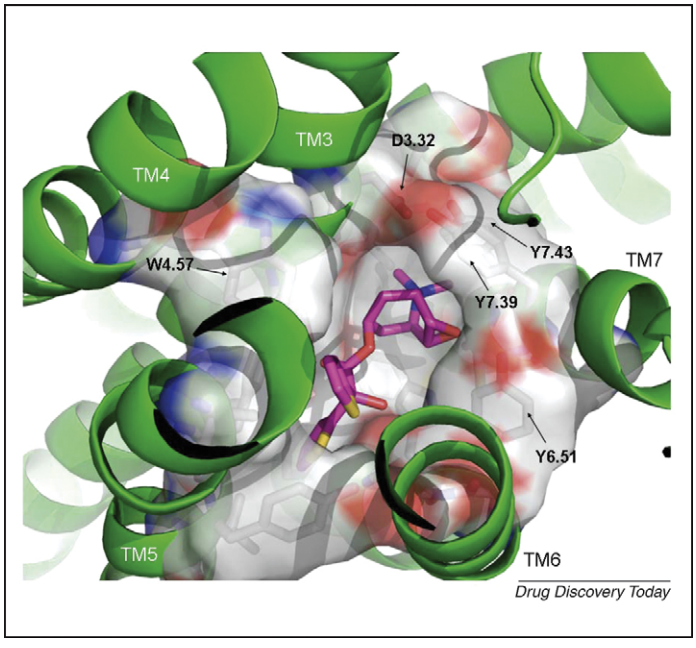

FIGURE 4

Tiotropium™ placed in a homology model of the M3-receptor, on the basis of the β2-crystal structure. Owing to the close homology of the two receptors, a direct modeling approach is feasible and validation of the model could be achieved by mutagenesis data from literature. Five amino acid residues crucial for ligand binding are labeled by arrows.

Discussion

GPCR platform approaches in drug discovery profit from establishing target-family-specific techniques and skill sets in a dedicated team or institution. On the biology side, this means the generation of GPCR-related assay formats to measure diverse aspects of GPCR ligand pharmacology. In a GPCR target class family dedicated team, it is easier to standardize particular bioassay formats, specific bioreagents and similar buffer conditions. Such assay standardization facilitates and shortens assay development for new target class representatives and improves the comparability of biological data between different GPCR targets for chemogenomic analyses or isoform selectivity studies, among other analyses.

The above-described variety of GPCR-directed drug discovery assay technologies serves to measure ligand–receptor binding, receptor activation/desensitization and receptor signaling. The individual assay formats differ with respect to information content, ease of assay development, cost and throughput. Several assay formats deliver partially complementary information: a binding assay typically produces a data set that is focused on the ligand–receptor interaction itself, the cellular assay provides a view on the cellular context of the ligand-induced receptor activation, and various functional assays provide different facets of the pharmacological effect spectrum.

The establishment and maintenance of a reasonably broad GPCR-specific assay panel may overburden a single therapeutic area team with respect to the particular technological skills and required instrumentation. As long as the particular assays on GPCR ligand function are not highly disease-specific, it makes good sense to bundle respective activities in a central GPCR target-class-specific unit that serves several therapeutic areas. Hereby, it should be noted that many GPCR signaling pathways are identical in different cell types and redundantly important across diverse disease contexts.

The power of HCS assays lies in the potential for monitoring diverse aspects of GPCR function (e.g. ligand binding, G-protein signaling and receptor internalization) based upon a single technology platform. HCS allows us to generate additional information by observing additional readouts in the same or parallel assays, such as rounding or loss of cells in case of a toxic compound effect, enhancement of plasma membrane GPCR levels by an inverse agonist or the enhancement of pERK levels by a compound with a stress-inducing side effect.

HCS, however, also bears certain limitations. A technical challenge in transferring an HCS signaling assay from one GPCR to another is that not all the bioreagents are generic. Thus, it may for example be cumbersome to screen for an HCS-suitable antibody directed against a GPCR of interest. Further, fluor-labeled ligands are not always commercially available. Another challenge is that particular signaling events may display differences in different model cell lines, based for example upon diverse protein levels of the GPCR or the signaling chain proteins.

Owing to the relatively fast signaling kinetics of some readouts and the relatively long measurement times for imaging, most medium-throughput HCS assays are carried out as endpoint assays using formaldehyde fixation. In addition, endpoint assays simplify the assay protocols because they decouple the liquid handling steps from the imaging process. Because different signaling readouts have different kinetics, only particular signaling events can be

multiplexed with one another (e.g. ERK phosphorylation and PKA redistribution at approximately 5 min after stimulation; or ligand/receptor internalization, arrestin and NFAT redistribution at 30–60 min after stimulation).

High-content assay formats are feasible for high throughput, but are generally used as secondary assays owing to the relatively high costs and the often more complex assay protocols. With regard to the assay reagents, the antibodies and the imaging-suitable MTPs are typically rather expensive. In particular, when an immune staining procedure is required the assay protocols are labor-intensive and time-consuming. Currently, HCS is not often used for primary screening but rather employed to add new data after a primary uHTS campaign in a classic HTS format. The establishment of the HCS technology requires relatively high capital investment and depends upon the generation of specialized technical skills, making a particularly good case to launch HCS activities preferentially in a central servicing unit.

In summary, all discussed biochemical and cellular assays on GPCR ligands have their individual merits, with each approach providing advantages over the other under particular circumstances. The diversity of assay formats to characterize GPCR ligands delivers partially overlapping information, thereby cross-validating experimental answers and reducing the probability of technology-based assay artifacts. In our opinion, an appropriate assay panel for an extended pharmacological characterization of GPCR ligands should include data on binding, G-protein signaling via various pathways and GPCR internalization.

Likewise, on the side of computational chemistry, the formation of dedicated units provides numerous advantages with respect to specific skill sets, GPCR ligand pharmacological databases or GPCR 3D modeling softwares. Thus, an important basis for all virtual screening approaches, and especially for those in the context of chemogenomics, is databases linking chemical structures with described biological activities against different targets. In this context, the Wombat Database (http://www.sunsetmolecular.com) and the MDL Drug Data Report (MDDR™) (http://www.mdl.com) are useful collections of data on the structures and properties of GPCR ligands. Another database, which bridges between the biological and chemical space, has recently been developed by Aureus Pharma (http://www.aureus-pharma.com). The strength of this database is its comprehensive coverage of known chemicals interacting with native and mutated GPCRs, including SARs, as well as functional and pharmacological activities resulting from ligand–receptor interactions.

With regard to GPCR modeling, various successful studies have been reported, however, with a surprisingly high degree of variability in the success in virtual screening applications. A recent study by Bissantz *et al.* [59] on three different class A receptors shows that it is much easier to obtain good results with rhodopsin-based models when searching for receptor antagonists. In particular, it has been shown that the likelihood of obtaining reliable models is improved when including ligand information from known receptor ligands in the modeling process. Along these lines, the chances for success in targeted library design are much higher when already some high-affinity ligands that bind in the TM region are known. In addition to this, combining ligand-based approaches with receptor modeling efforts are the most successful approaches to GPCR-targeted libraries.

Although there are already several elaborate receptor modeling algorithms available, the next challenge will be to model the activated state in a reliable way and to extend the modeling process to receptor dimers and oligomers, because biological evidence has been found that many GPCRs form functional homo-/hetero-oligomers. Another open question is the mechanism of the activation of the G-protein itself through the GPCR. Once this is clarified to an atomic level, it is thinkable to design agonist or antagonist libraries that target several steps of the activation cascade rather than just the mere binding of a ligand to the receptor.

Currently, most modeling efforts aim toward a better understanding of SARs for GPCR ligands to explain differences in binding affinities to a receptor. Discrepancies between functional readout and binding affinity are therefore a big problem for receptor-model-driven lead optimizations. This means that a close interaction of receptor modeling, chemogenomic approaches and screening methods is needed to get a more comprehensive picture of the receptor binding and signaling behavior. This knowledge is needed to be able to plan libraries that include potent, selective molecules that show the requested function on the receptor (potency can be optimized with the help of receptor models, selectivity can be achieved through chemogenomic approaches and the required functionality has to be tested by different screening methods).

For this, we believe a combined GPCR platform approach, where computational chemists, combinatorial chemists and biologists cooperate very closely, can lead to shortened timelines in lead identification and lead optimization processes.

Acknowledgements

We are grateful to Ulrike Küfner-Mühl, Martin Valler, Herbert Koeppen, Jürgen Mack, Ingo Mügge, Christoph Hoenke, Barbara Kistler and Paola Casarosa for helpful advice and discussions. We thank Susanne Schmidt and Michael Sulger for technical support using the HCS technology platform.

References

1 Jacoby, E. *et al.* (2006) The 7 TM G-protein-coupled receptor target family. *ChemMedChem* 1, 761–782

2 Klabunde, T. and Hessler, G. (2002) Drug design strategies for targeting G-protein-coupled receptors. *Chembiochem* 3, 928–944

3 Drake, M.T. *et al.* (2008) {beta}-Arrestin-biased agonism at the {beta}2-adrenergic receptor. *J. Biol. Chem.* 283, 5669–5676

4 Strange, P.G. (2008) Signaling mechanisms of GPCR ligands. *Curr. Opin. Drug Discov. Dev.* 11, 196–202

5 Gonzalez-Maeso, J. *et al.* (2008) Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452, 93–97

6 Richman, J.G. *et al.* (2007) Nicotinic acid receptor agonists differentially activate downstream effectors. *J. Biol. Chem.* 282, 18028–18036

7 Parmentier, M. and Detheux, M. (2006) Deorphanization of G-protein-coupled receptors. *Ernst Schering Found. Symp. Proc.* 2, 163–186

8 Evans, B.E. *et al.* (1988) Methods for drug discovery: development of potent, selective, orally effective cholecystokinin antagonists. *J. Med. Chem.* 31, 2235–2246

9 Kolakowski, L.F., Jr (1994) GCRDb: a G-protein-coupled receptor database. *Receptors Channels* 2, 1–7
10 Kristiansen, K. (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol. Ther.* 103, 21–80
11 Fredriksson, R. and Schioth, H.B. (2005) The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Mol. Pharmacol.* 67, 1414–1425
12 Alouani, S. (2000) Scintillation proximity binding assay. *Methods Mol. Biol.* 138, 135–141
13 Banks, P. and Harvey, M. (2002) Considerations for using fluorescence polarization in the screening of g protein-coupled receptors. *J. Biomol. Screen.* 7, 111–117
14 Zemanova, L. *et al.* (2003) Confocal optics microscopy for biochemical and cellular high-throughput screening. *Drug Discov. Today* 8, 1085–1093
15 Cabrera-Vera, T.M. *et al.* (2003) Insights into G protein structure, function, and regulation. *Endocr. Rev.* 24, 765–781
16 Milligan, G. (2003) Principles: extending the utility of [^{35}S]GTP gamma S binding assays. *Trends Pharmacol. Sci.* 24, 87–90
17 Kostenis, E. *et al.* (2005) Techniques: promiscuous Galpha proteins in basic research and drug discovery. *Trends Pharmacol. Sci.* 26, 595–602
18 Sullivan, E. *et al.* (1999) Measurement of [Ca^{2+}] using the fluorometric imaging plate reader (FLIPR). *Methods Mol. Biol.* 114, 125–133
19 Dupriez, V.J. *et al.* (2002) Aequorin-based functional assays for G-protein-coupled receptors, ion channels, and tyrosine kinase receptors. *Receptors Channels* 8, 319–330
20 Gabriel, D. *et al.* (2003) High throughput screening technologies for direct cyclic AMP measurement. *Assay Drug Dev. Technol.* 1, 291–303
21 Hill, S.J. *et al.* (2001) Reporter-gene systems for the study of G-protein-coupled receptors. *Curr. Opin. Pharmacol.* 1, 526–532
22 Berridge, M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature* 361, 315–325
23 Osmond, R.I. *et al.* (2005) GPCR screening via ERK 1/2: a novel platform for screening G protein-coupled receptors. *J. Biomol. Screen.* 10, 730–737
24 Lefkowitz, R.J. (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* 273, 18677–18680
25 Lefkowitz, R.J. and Shenoy, S.K. (2005) Transduction of receptor signals by beta-arrestins. *Science* 308, 512–517
26 Shenoy, S.K. *et al.* (2006) Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J. Biol. Chem.* 281, 1261–1273
27 Eglen, R.M. (2005) Functional G protein-coupled receptor assays for primary and secondary screening. *Comb. Chem. High Throughput Screen.* 8, 311–318
28 Hoffman, A.F. and Garippa, R.J. (2007) A pharmaceutical company user's perspective on the potential of high content screening in drug discovery. *Methods Mol. Biol.* 356, 19–31
29 Wolff, M. *et al.* (2008) Activation and translocation of glucokinase in rat primary hepatocytes monitored by high content image analysis. *J. Biomol. Screen.* epub ahead of print
30 Heilker, R. (2006) High content screening to monitor G-protein coupled receptor internalisation. *Ernst Schering Found. Symp. Proc.* 2, 229–248
31 Haasen, D. *et al.* (2006) G protein-coupled receptor internalization assays in the high-content screening format. *Methods Enzymol.* 414, 121–139
32 Haasen, D. *et al.* (2008) Pharmacological profiling of chemokine receptor-directed compounds using high-content screening. *J. Biomol. Screen.* 13, 40–53
33 Ferguson, S.S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* 53, 1–24
34 Oakley, R.H. *et al.* (2002) The cellular distribution of fluorescently labeled arrestins provides a robust, sensitive, and universal assay for screening of G protein-coupled receptors. *Assay Drug Dev. Technol.* 1, 21–30
35 Oakley, R.H. *et al.* (2001) Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor–beta-arrestin complexes after receptor endocytosis. *J. Biol. Chem.* 276, 19452–19460
36 Feliciello, A. *et al.* (2001) The biological functions of A-kinase anchor proteins. *J. Mol. Biol.* 308, 99–114
37 Siehler, S. (2008) Cell-based assays in GPCR drug discovery. *Biotechnol. J.* 3, 471–483
38 Caron, P.R. *et al.* (2001) Chemogenomic approaches to drug discovery. *Curr. Opin. Chem. Biol.* 5, 464–470
39 Schuffenhauer, A. and Jacoby, E. (2004) Annotating and mining the ligand-target chemogenomics knowledge space. *Drug Discov. Today: Biosilico* 2, 190–200
40 Rognan, D. (2007) Chemogenomic approaches to rational drug design. *Br. J. Pharmacol.* 152, 38–52
41 Caron, P.R. *et al.* (2001) Chemogenomics, a gene family approach to parallel drug discovery. *Drug Discov. World Fall* 57–62
42 Klabunde, T. (2007) Chemogenomic approaches to drug discovery: similar receptors bind similar ligands. *Br. J. Pharmacol.* 152, 5–7
43 Surgand, J.S. *et al.* (2006) A chemogenomic analysis of the transmembrane binding cavity of human G-protein-coupled receptors. *Proteins* 62, 509–538
44 Bender, A. *et al.* (2007) Analysis of pharmacology data and the prediction of adverse drug reactions and off-target effects from chemical structure. *ChemMedChem* 2, 861–873
45 Azzaoui, K. *et al.* (2007) Modeling promiscuity based on in vitro safety pharmacology profiling data. *ChemMedChem* 2, 874–880
46 Guba, W. *et al.* (2007) From astemizole to a novel hit series of small-molecule somatostatin 5 receptor antagonists via GPCR affinity profiling. *J. Med. Chem.* 50, 6295–6298
47 Crossley, R., *et al.*, (2003) Compound libraries. *Patent WO-03004147*
48 Bock, J.R. and Gough, D.A. (2005) Virtual screen for ligands of orphan G protein-coupled receptors. *J. Chem. Inf. Model.* 45, 1402–1414
49 Martynova, N.B. *et al.* (2000) Computer prediction of biological activity spectra for low-molecular peptides and peptidomimetics. *Bioorg. Khim.* 26, 330–339
50 Palczewski, K. *et al.* (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289, 739–745
51 Rasmussen, S.G. *et al.* (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450, 383–387
52 Warne, T. *et al.* (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* 454, 486–491
53 Jaakola, V.P. *et al.* (2008) The 2.6 Angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322, 1211–1217
54 Park, J.H. *et al.* (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* 454, 183–187
55 Martinelli, A. and Tuccinardi, T. (2006) An overview of recent developments in GPCR modelling: methods and validation. *Expert Opin. Drug Discov.* 1, 459–476
56 Mobarec, J.C. and Filizola, M. (2008) Advances in the development and application of computational methodologies for structural modeling of G-protein-coupled receptors. *Expert Opin. Drug Discov.* 3, 343–355
57 Schwartz, T.W. *et al.* (2006) Molecular mechanism of 7TM receptor activation – a global toggle switch model. *Annu. Rev. Pharmacol. Toxicol.* 46, 481–519
58 Mannhold, R. *et al.* (2006) *Ligand Design for G Protein-coupled Receptors* (Rognan, D., ed.),
59 Bissantz, C. *et al.* (2003) Protein-based virtual screening of chemical databases. II. Are homology models of G-protein coupled receptors suitable targets? *Proteins* 50, 5–25