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TITLE
Fragment-based lead discovery on GPCRs.

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ARTICLE HIGHLIGHTS

- Recent advances in structural and biophysical characterization of GPCRs lead to improved efficacy of in vitro and in silico fragment-based lead discovery for GPCRs
- Virtual fragment screening is a feasible approach for GPCR lead discovery
- Multiple receptor conformations (including both experimental and theoretical models) might enhance the success rate of virtual fragment screening
- Relevance of biophysical methods for fragment screening and evaluation on GPCRs has increased significantly
- In vitro biological assays are suitable for functional screening on GPCRs

ABSTRACT

Introduction
G-protein-coupled receptors form one of the largest groups of potential targets for novel medications. Low druggability of many GPCR targets and inefficient sampling of chemical space in high throughput screening expertise however often hinder discovery of drug discovery leads for GPCRs. Fragment-based drug discovery is an alternative approach to the conventional strategy and has proven its efficiency on several enzyme targets. Based on developments in biophysical screening techniques, receptor stabilization and *in vitro* assays, virtual and experimental fragment screening and fragment-based lead discovery recently became applicable for GPCR targets.

Areas covered
Biophysical as well as biological detection techniques suitable to study GPCRs are reviewed, together with their applications to screen fragment libraries and identify fragment-size ligands of cell surface receptors. Several recent examples are presented, including both virtual and experimental protocols for fragment hit discovery and early hit to lead progress.

Expert opinion
With the recent progress in biophysical detection techniques the advantages of fragment-based drug discovery could be exploited for GPCR targets. Structural information on GPCRs will be more abundantly available for early stages of drug discovery projects, providing information on the binding process and efficiently supporting the progression of fragment hit to lead. *In silico* approaches in combination with biological assays can be used to address structurally challenging GPCRs and confirm biological relevance of interaction early in the drug discovery project.

1. INTRODUCTION

The superfamily of G-protein-coupled receptors (GPCRs) consists of a large number of potential drug targets and their role in numerous diseases has been proposed (1). Discovery of suitable drug candidates targeting specific GPCRs has been often hindered by various factors like achieving sufficient potency and selectivity and lead to numerous failures in this target class (2). In one hand, a number of technical challenges encumbered the experimental investigation of their atomic level structure (3-4) that limits the success of their *in silico* targeting. On the other hand, dynamic intramolecular organization (5) of these receptors lends high degree of complexity between ligand binding and functional efficacy and phenomena like signalling bias (6-8). Despite these difficulties, GPCRs represent a family with high potential to serve as targets of novel pharmaceutical agents.

Fragment-based drug discovery has proven as an efficient approach as demonstrated on various enzyme targets (5). The basic concept of this paradigm lies in identification of efficiently binding low molecular weight fragments of druglike structures to serve as starting points for lead discovery. This way a more efficient sampling of chemical space is possible in addition to exploring and enthalpy-lead targeting of protein "hot spots" (10) expected to result in higher developmental potential for fragment-derived ligands. Although fragment-based drug discovery in the last decade has lead to several clinical candidates on enzyme targets, its utility for integral membrane proteins has been regarded limited. Recently, significant advance has been achieved in structural investigation methods of GPCRs as well as receptor stabilization and presentation techniques required for development of screening assays (11). Several very recent reports for as diverse techniques as NMR screening on immobilized receptors (12) to live cell binding studies (13) with the aim to identify and develop fragment-size GPCR ligands have been reported. It is expected that wide spread application of fragment-based drug discovery to GPCR targets could provide novel chemical matter previously inaccessible for random screening.

2. FRAGMENT HIT DISCOVERY ON GPCRS

2.1 Virtual screening

Virtual screening has become an industry standard for the identification of chemical starting points for a wide variety of targets. Although ligand-based methods were found to be useful for structurally not characterized targets high throughput docking is clearly the most popular approach used in structure based virtual screening (14). The success of such a screen basically depends on multiple factors including the accuracy of the protein structure used for docking, the docking algorithm and the scoring function used for the prediction of binding mode and energetics, and also the characteristics of the library screened.

Protein structures might be available from both experimental and theoretical sources. Experimental structures are usually better suited for virtual screening; however, the flexibility of the protein is often considered using theoretical approaches such as docking to conformational ensembles typically generated by molecular dynamics simulations (15). Comparative modeling represents another option for targets having sequential homologues with known 3D structure. Although homology models were used successfully in a number of cases their usefulness depends strongly on the level of sequence identity and the character of the target (16). The limited availability of experimental GPCR structures made homology models popular for virtual screening applications. On the other hand, however, recent developments in GPCR structural biology resulted in a high number of GPCR structures initiating a significant number of virtual screening studies (18-22). Fast and often parallelized docking algorithms allow the prediction of the binding mode for hundreds of thousands of potential ligands in reasonable time. There are, however, several limitations of these approaches since (i) they are typically neglecting the flexibility of the protein that limits the accuracy of the pose prediction and (ii) they estimate the binding affinity by empirical scoring functions that limit the accuracy of ranking docked ligands. In general, proteins with limited flexibility are better suited for virtual screening studies. Membrane proteins, such as GPCRs might therefore be advantageous in virtual screening applications. It is interesting to see that the most popular docking tools show pretty similar performance on different target classes (23) scoring functions should be optimized or even tailored for the actual problem.

The character of the screened ligands might also influence the outcome of virtual screening. In routine applications typically druglike libraries are screened virtually. More recently, however, fragment based approaches seem to be more and more popular in the virtual world that is indicated by the increasing number of virtual fragment screens reported (24-25). There are, however, a number of challenges associated to these applications. First, fragments are small and polar compounds that might interact with a relatively large number of interaction sites on the protein surface. Second, small volume of fragments relative to that of the binding site could result multiple alternative binding modes or even incorrect poses. Third, estimation of their binding affinity might be less accurate since (i) most of the scoring functions are optimized for druglike compounds (ii) fragments form less interactions with the target that are more challenging to estimate. Despite of these factors virtual fragment screening is a viable alternative of experimental approaches if the experimental structure of the target is available. In this special case low resolution homology models are less feasible since poses of small fragments could only be estimated with relatively large errors that impacts the screening efficacy negatively.

In the next section we review several case studies on virtual fragment screening against GPCRs that includes histamine, adenosine and dopamine receptors. Here we discuss the impact of experimental structures and homology models as well as screening on active and inactive receptor conformations.

2.1.1. H_1 antagonist screening

One of the first published virtual fragment screening has been performed on the recently available X-ray structure of the human histamine H_1 receptor (26). The authors started from 757,728 fragment like compounds with less than 22 non-hydrogen atoms and generated plausible tautomers and protonation states. Next they selected only those carrying a formal charge of +1 (108,790 fragments) ensuring that these fragments might form ionic interactions with the crucial and highly conserved D107 residue located in helix 3. Then these fragments were docked to the binding pocket of hH_1 receptor by the docking program PLANTS. Fragments with binding modes contacting to D107 were considered further (95,147). These poses were post-processed by interaction fingerprints generated from the interactions identified for doxepine co-crystallized with hH_1 receptor. The resulted 354 fragments were first subjected to the Tanimoto based novelty filter that compares the virtual hits to known hH_1 antagonists. The last round of visual inspection gave 30 fragments from which 26 were available for experimental testing. Interestingly, 19 fragments showed hH_1 affinity in the range of 10 μM to 6 nM resulting in an exceptionally high hit rate of 73%.

2.1.2. H_3 antagonist

In addition to structure-based methods ligand-based approaches might also be useful identifying reduced complexity low molecular weight starting points. In a recent paper Sirci and coworkers reported the identification of new histamine H_3 receptor antagonist fragments using ligand-based and protein-based molecular fingerprints (27). Their methodology termed as FLAP (Fingerprint for Ligands And Protein) first identifies 4-point pharmacophores by interacting molecular fields (MIFs) that are used to align the compounds with specific biological activity. In the final step linear discrimination analysis (LDA) is used to generate probe scores discriminating compounds with different affinity. This approach has been tested against conventional ligand-based and structure-based virtual screening. In a retrospective study FLAP outperformed both Tanimoto ECFP-4 similarity, ROCS shape similarity and also docking methods including PLANTS and GOLD, for virtually all test set compounds. These positive results prompted the authors to screen 156,090 fragment-like compounds collected from ZINC by FLAP. LDA-R scores calculated for both ligand-based and structure-based FLAP approaches gave 1,292 and 28,973 fragments with probe score larger than 0.5. The authors then selected the 202 consensus hits and also the top 200 fragments identified by the ligand-based FLAP approach. After visual inspection 29 of them were selected and measured to yield 19 compounds (63%) with H_3 affinity between 0.5 and 10 μM as measured in a radioligand binding assay.

2.1.3. H_3/H_4 antagonist

A combined approach using both ligand and protein information has been published by Evotec (28). First the structure of the human histamine H_3 receptor was modeled using comparative modeling, next the binding pocket was optimized by fully flexible docking of known H_3 ligands. This resulted in bioactive conformations for druglike H_3 compounds that were used in a subsequent ROCS shape similarity search performed on 4 million compounds. Results of experimental fragment screening served as an alternative source of ROCS queries using both H_3 specific and dual H_3/H_4 fragment hits. ROCS searches provided 2500 primary hits from each type of queries that were finally docked into the optimized binding pocket of the H_3 homology model. Best scored virtual hits have been visually inspected and 62 of them have been selected for biological testing.

In parallel the authors developed a homology model for the human histamine H_4 receptor that was further optimized using H_4 selective fragment hits. Dual H_3/H_4 and H_4 specific fragment hits were then docked into the optimized binding site to obtain bioactive conformations for

the subsequent ROCS shape similarity search that identified 1200 virtual hits for each type of queries. This set of hits was extended by further 12,000 compounds identified by searching known 2D H_4 substructures in the database of 4.8 million compounds. The total of 14,400 primary hits was then docked into the optimized binding pocket of the H_4 homology model. Best scored virtual hits have been visually inspected and 110 of them have been selected and combined with the previously identified 62 H_3 hits for biological testing. The total of 172 H_3/H_4 hits were evaluated at 20 μM concentration in functional tests on histamine H_3 and H_4 receptors providing 79 (54%) and 58 (40%) hits, respectively.

2.1.4. H_4 antagonist

As a continuation of our work aiming to identify novel chemotypes for the H_4 receptor (29) we developed a molecular dynamics-based protocol for virtual fragment screening. We first created a homology model for the human H_4 receptor and its complex with JNJ7777120 was subjected to explicit membrane simulations with TIP3P waters in NAMD 2.7. After 20 ns NpγT equilibration at 310 K we run five independent 5 ns long NpγT simulations and the resulting trajectories were analyzed to identify structurally diverse frames for fragment docking. Frame selection was based on the calculation of RMSD values for the interacting residues around the JNJ7777120 ligand. RMSD based clustering by average linkage methodology provided 12 frames in total that were used for virtual fragment screening. Comparing the results provided by docking to MD frames with that obtained by the homology model-based virtual screening we concluded that virtual screening of 13,000 fragments gave 8 and 5 hits with larger than 20% displacement at 10 μM (30). These results revealed that MD based ensemble approach provides much higher enrichment (20%) that that of the single structure screen (11%).

2.1.5. D_3 antagonist

A similar MD based strategy has been applied to human D_3 receptor having an X-ray structure available for virtual screening. Docking the 13,000 fragments to the experimental structure yielded 9 hits with displacement larger than 20% as measured at 10 μM that gave the hit rate of 18%. Contrary, docking the same set of fragments to 29 frames identified by the analysis of MD trajectories resulted in 18 hits in total having displacement larger than 20% at 10 μM yielding the hit rate of 32% (31). Again, this study demonstrated that MD based ensembles might provide better enrichment than single structure virtual fragment screening.

2.1.6. A_{2A} agonist

Despite the fact that most of the available GPCR structures are in inactive state several screening programs aim the identification of activators. Successful virtual screening for agonists typically requires active state conformations. The high resolution structure of the activated adenosine A_{2A} receptor provided a new opportunity for virtual agonist screening and structure-based fragment design (32). The authors compared the performance of virtual screening on three different receptor conformations including the active and inactive X-ray structures and an agonist optimized model. The screening library consists of substituted adenosine derivatives, 10 derivatives with known active substituent available from ChEMBL and further 200 derivatives as decoys. Decoys were generally low molecular weight fragments (M_W<130) from commercial databases. It was rather obvious that the activated experimental structure provided the best enrichment of AUC=93%. Screening efficacy on the inactive experimental structure was much inferior (AUC=57%), and finally the agonist-optimized inactive model served as an intermediate source of new fragment chemotypes. This study indicates that virtual fragment screening for agonists is not an easy task. Enrichment factors

are typically higher for active receptor conformations from which experimental structures might be somewhat better relative to agonist optimized inactive conformations.

2.2. Biophysical screening

The major obstacles of applying fragment-based drug discovery are specific identification of substantially lower affinity on-target interactions and assessment of development propensity of low complexity hits. Thus, in contrast to conventional drug discovery often carried out based on indirect evidence, fragment-based approach is seriously limited in lack of direct observation of interactions with the molecular target. NMR spectroscopy as well as X-ray crystallography allow for direct and sensitive detection of ligand-target interaction less prone to downstream artifacts of detection (33), but historically GPCRs, just like other membrane targets, fell out of scope of structure-based approaches. Owing to very recent achievements in stabilization techniques of membrane proteins as well as direct detection methods to monitor low affinity interactions GPCRs became accessible for biophysical detection techniques (12,34). The advent of this approach was demonstrated by successful structure-based drug discovery on a GPCR under industrial settings (35). Not only more refractory to downstream signal interference compared to biological assays, biophysical techniques in theory could provide structural or kinetic information on ligand binding that could contribute to efficient hit validation and evaluation. In the subsequent sections, recent applications of biophysical detection techniques in fragment screening on GPCRs and hit to lead transition are presented.

2.2.1. Nuclear magnetic resonance spectroscopy

Capable to detecting both orthosteric and allosteric binding events of loosely bound ligands, ligand-observed ^{1}H NMR spectroscopy techniques like saturation transfer difference (STD) (36) or waterLOGSY (37) proved powerful in the study of protein-ligand interactions (38). However, owing to the surface transfer of proton resonances, these methods are sensitive to aggregation and nonspecific binding, and thus, their utility diminish when studying complex heterogeneous systems like membrane protein preparations. Target immobilized NMR screening (TINS) (39) overcomes this problem by utilizing flow-injection NMR spectroscopy on immobilized protein preparations in a dual cell sample holder with spatially selective detection. Elimination of signals originating from nonspecific binding is achieved by subtraction of the ^{1}H spectrum of the reference channel which contains preparation of a protein with low druggability. In addition to the resulting improved specificity, also lower protein consumption and sensitivity of TINS are favorable compared to traditional ^{1}H NMR methods (40). Successful application of the technique for GPCRs has been reported with stabilized β_1 (12) and A_{2A} receptors (41), where signals from a preparation of the rather nondruggable OmpA protein served for correction of nonspecific binding. In these proof-of concept studies, 579 and 531-membered libraries, respectively, were screened as 3-8 component mixtures at 500 μM. A rather conservative hit selection resulted in hit rates of 18% and 14% respectively, and more than 10 fragment hits in each case (ca. 2% overall hit rate) whose binding could be confirmed using a biochemical assay. As detailed investigation of screening hits for A_{2A} revealed, both orthosteric and allosteric ligands were identified using the TINS method, the latter ones characterized by higher selectivity over close homologue A_1 (41).

Although the SAR by NMR principle by 2D protein-observed NMR was developed especially for fragment-based lead discovery (42), routine use of ^{15}N or ^{13}C correlated NMR spectroscopy is limited due to deconvolution of complex 2D spectra and resource intensive isotope-labeling of the protein sample, the limit for which currently lies near the size of GPCRs. Although this approach currently falls out of reach for primary fragment screening

for GPCRs, liquid phase protein NMR spectroscopy could in the future support studying conformational or dynamic changes of the receptor upon ligand binding without the need for immobilization (43).

2.2.2. X-ray crystallography

Around 2007, a breakthrough in crystallographic investigation GPCR structure and function has been achieved, enabled by advanced protein isolation and stabilization techniques in addition to availability of improved X-ray sources, culminating in resolving the crystal structures of numerous GPCRs as well as illuminating various phases of the receptor activation cycle (44-45). For crystallization purposes, receptor stabilization is achieved either by fusion with a highly crystallizable protein, like T4 bacteriophage lysozyme, addition of a stabilizing antibody or site-directed mutations (11). Although the approach is very informative on determining binding mode of an actual fragment hit, various limitations are also obvious for this method to be used in mass screening. Crystal soaking with ligands provides an approach more suited for screening studies, where however, crystal packing or the crystallized conformation of the receptor might hinder proper binding to occur (46). Moreover, high resolution structural determination of low affinity ligands, more stable receptors might be required compared to alternative biophysical techniques (47), demonstrated by the co-crystallization of several leadlike compounds with the adenosine A_{2A} receptor (48, see latter). X-ray crystallography on GPCR targets could in the near future serve as a powerful tool to validate and evaluate screening hits on GPCRs and provide valuable support in efficient elaboration of fragment hits.

2.2.3. Surface plasmon resonance

Binding of GPCR ligands to receptors attached to biosensors was detected by surface plasmon resonance for tagged wildtype (49) as well as stabilized receptors (50). Captured onto antibody or NTA surfaces of a sensor chip, purified proteins or even crude extracts could be applied for detecting small molecule binding (50). Although ligand mass-dependent effect size hints at potential limitations of SPR for fragment screening (51), Congreve et al. successfully screened a library of 136-194-Da fragments on an A_{2A} receptor construct stabilized in the antagonist mode (12). A hit rate of 10% was achieved at a screening concentration of 200 μM and binding affinity as low as 5 mM could be detected, demonstrating the utility of SPR for primary screening of weak ligands. Moreover, this technique allows for experiments to be run in multiple formats, capable to extract both equilibrium and kinetic data on the binding event.

2.3. Biological screening assays

Irrespectively of primary screening strategy, high concentration biochemical assays are regularly used in the secondary phase of fragment-based drug discovery projects. Their utility is warranted by the need to demonstrate biological relevance of the binding event before initializing an extensive and demanding chemistry program for lead identification. However, apart from fulfilling this supportive role, traditional *in vitro* biological assays also offer an easily accessible repertoire for screening fragment libraries at high concentration. In addition to the relative ease of application a further benefit is the quantitative data acquired, as fragment hit evaluation relies more on calculated ligand efficiency than on absolute activity (52). Lastly, GPCRs are known to adopt multiple active conformations that might bear therapeutic relevance from a drug discovery perspective (4, 53). Biochemical assays presenting the target in a dynamic fashion and in proper supramolecular context might thus also support fragment screening against membrane targets.

2.3.1. Binding assays

High concentration screening of fragment libraries against GPCR targets can be performed utilizing *in vitro* biochemical assays developed for conventional drug discovery programs. As an example, researchers from AstraZeneca reported discovery of melanocortin 4 receptor ligands using a radioligand binding assay (54). Screening at 1 mM concentration resulted in 9% hit rate, followed by rapid hit expansion on druglike derivatives of fragment hits. Fragment screening of several histamine receptors subtypes and adrenergic β_2 receptor using radioligand has been reported (55, 56). The somewhat low test concentration in the binding assays (10 μM) was justified by the 0.4-6% hit rate on the particular library. Contradictory to expectations on fragment binding, lower level of selectivity has been observed among more complex fragment hits, a phenomenon that could not be explained based upon sequence homology.

Finally, an interesting approach utilizing a fluorescent ligand of adenosine A_3 receptor was reported recently, where fluorescence intensity of a xanthine amine congener analogue reported on binding to intact cells (13). Fragment screening was performed on a high content imaging platform and resulted in 15% hit rate with activity as low as pK_i=3.97 reliably detected. Not confined to high content instrumentation, this live cell approach could in theory be pursued with time-resolved fluorescence spectroscopy, fluorescence anisotropy or fluorescence correlation spectroscopy (FCS) as well.

2.3.2. Functional assays

The interaction of the relatively featureless interfaces CXCR4 and CXCL12 has proved challenging to target using conventional drug discovery tools. Mysinger and colleagues reported a virtual screening-based approach, followed by high concentration testing of hits in a functional assay (20). In this case leadlike (logP<3.5, molar weight<350 Da, number of rotatable bonds<8) compounds were virtually screened followed by testing hits at high concentration (100 μM) in a calcium mobilization and subsequently in a cell migration assay. Several hits displayed activity in both *in vitro* models with no sign of acute toxicity assessed via Trypan Blue exclusion (20). Interestingly, when assessed for binding activity, two of the five hits lacked the ability to displace labeled CXCL12, emphasizing at the complexity of the chemokine-receptor interaction.

The first reported functional agonist fragment screen has been presented by Szőllősi and colleagues on adrenergic α2c receptor (57). High concentration screening of more than 3000 fragment-sized compounds using a calcium assay lead to the identification of 16 validated fragment hits, several of which displayed submicromolar affinity and micromolar potency. Counterscreening in this case was performed using a non-related target, however, owing to the lower specificity of fragment hits versus druglike compounds (58), this approach might have underestimated specificity of actives. Similar to the above example, however, no strong correlation between agonist potency and binding affinity could be observed, as several close neighbors of agonist fragment hits turned out to act as potent antagonists of α2c receptor.

3. FOLLOW UP OF HITS

Owing to necessarily multicomponent preparations of GPCRs in a hydrophobic environment and the usually low affinity of fragment hits, validation of hits is crucial as the first step in hit to lead activity. Biophysical screening hits can turn out to be nonspecific aggregators (38, 59) or intractably low affinity binders, while biological screening assays are prone to be misled by assay interference or poor solubility (60). Typically, demonstration of target specific or direct interaction as well as pharmacologically relevant binding should be required for an active to qualify as a true hit. Selectivity, although not impossible to obtain, is not a requirement at the fragment hit level (61), thus counterscreening low complexity hits using a homologous target should be used in extreme situations only.

It is unequivocal that evaluation of developmental potential of fragment hits can be more adequately assessed based on ligand binding efficiency than absolute affinity (e.g. 52). Multiple approaches are available to pursue a particular fragment hit (52), either by increasing ligand efficiency (hit optimization) or by increasing size at near constant ligand efficiency (hit evolution) (62). Hit expansion using fragment hits for substructure searching have resulted in several slightly more potent, but disproportionally less efficient ligands on MC4 receptor (54), not unlike results obtained after high concentration functional fragment screening against ASIC3 channel (63). Szőllősi et al. (57) reported adrenergic α2c agonist fragment hits, several size-matching neighbors of which behaved as more potent and more efficient ligands, albeit, as functional testing revealed, acting as antagonists of the target. This example underlines the importance of testing functional activity early on in the lead discovery process.
Stoddart et al. report on a more successful case of fragment screening and early hit to lead activity on a GPCR target (13). Their initial screen using a live cell binding assays resulted in several highly efficient hits on the adenosine A_3 receptor. After unsuccessful initial efforts on optimization of the most potent hit DP 01095 while maintaining low molecule size, careful and systematic hit evolution was undertaken. Small scale extension of the scaffold resulted in several analogues of higher potency and improved selectivity versus the main antitarget A_1 receptor and demonstrated thus, that careful elaboration of an initial fragment hit, although very demanding can be applied successfully for lead generation.

Although not yet accessible for a generic GPCR target, X-ray crystallography can prove exceptionally effective for obtaining structural information about fragment binding. Crystallography was applied successfully in practice to complement biochemical and SPR data in discovery of A_{2A} antagonists starting from virtual screening hits (48). At a crucial point of lead optimization, two fragment-size key compounds were crystallized with the receptor to gain valuable structural information on their binding mode and optimization potential. Eventually a close analogue displaying beneficial pharmacological properties, including long residence time at the receptor as measured with SPR was progressed into in vivo studies (48). This excellent piece of work showed that utilizing highly demanding biophysical approaches can indeed efficiently support lead generation on GPCRs (35).

4. EXPERT OPINION

Drugs targeting GPCRs form a substantial part of clinically successful therapeutic agents and this target class stills bears opportunities for drug discovery. In the past, however, many GPCR targets have been targeted in vain as suboptimal affinity or selectivity has hindered successful drug discovery (2, 64). It can be assumed that, in lack of structural information for *in silico* screening and insufficient coverage of chemical space for random *in vitro* screening, inefficient hit discovery might have contributed substantially to the failure of discovery efforts on promising GPCR targets.

The paradigm of fragment-based drug discovery has proven its efficiency against soluble targets (9) and recent developments in biophysical investigational methods bear the promise to efficiently tackle membrane proteins as well. The major obstacle for application of a fragment-based approach to cell surface receptors lie in the difficulty to present an arbitrary GPCR ready for structural and biophysical studies, to sensitively and specifically identify fragment ligands and to efficiently evolve low complexity screening hits. Moreover, bridging binding phenomena to functional consequences like proper downstream signaling events or receptor modulation might be crucial to achieve pharmacological relevance.

Although limited number of case studies has been reported, virtual fragment screening seems to be a promising approach for the identification of low complexity starting points for GPCR targets. Considering that the number of potential fragments with maximum 17 non-hydrogen atoms is still about 166 billion (65) virtual fragment screening might contribute to the design of screening libraries and would be useful prioritizing fragments for acquisition and screening. On the other hand, early results suggest that it might be a viable strategy for the identification of GPCR hits. Analyzing the experimental activity of virtual fragment hits we think that their potency is typically higher than that reported for other target classes. In fact, fragments identified for aminergic GPCR in the case studies reviewed here show micromolar potency. Since endogenous ligands of these receptors are also polar, low complexity compounds fragment hits fit well to their binding site of similar size and form interactions similar to natural ligands. Although scoring schemes designed for druglike compounds might limit the docking accuracy for fragments (17) these characteristic polar interactions formed at the hot spot seem to be adequately described. This is reflected in the unusually high hit rates reported for this subclass of GPCR targets. Although the binding cavity of peptidergic GPCRs and lipid receptors might also contains hot spots with similar characteristics we think that virtual fragment screening on these subclasses would provide lower hit rates. Virtual screening results obtained for druglike compounds are in line with this hypothesis (26) since hit rates for aminergic receptors are usually much higher than other GPCRs (20-40% vs. 0-10%, respectively). Considering the relatively high hit rates found in the reviewed virtual fragment screens one consider this approach as a viable alternative for peptidergic GPCRs and lipid receptors. Based on these conclusions we argue that similar to the druglike situation integration of virtual and experimental fragment screening would be a synergistic approach for maximizing the output of fragment approaches applied for GPCR targets.

Either after virtually screening a large library of potential binders or a moderate to small collection of real samples, experimental detection of protein-ligand interaction has to be demonstrated *in vitro* (Figure 1). Recently, successful examples have been reported for fragment screening against GPCR targets using surface plasmon resonance (12) and target immobilized NMR screening (12, 41). NMR screening is typically performed on mixtures and both methods can be run against libraries of several hundred to thousand samples and to

identify both orthosteric and allosteric ligands. Quality control for non plate-based fragment screening methods is not as standardized yet as for conventional *in vitro* assays, still, Congreve et al. included regularly a positive control at SPR testing to demonstrate reproducibility and stability of the assay (12).

Lately, independently from ^{1}H techniques, ^{19}F NMR has been proposed as an efficient tool for fragment screening based on its high sensitivity and specificity suitable for screening mixtures (66). In theory, combination of TINS with ^{19}F NMR spectroscopy could yield a method of improved throughput and detection sensitivity to be applied for screening reasonable size fragment libraries against GPCRs and other membrane targets. Yet another novel method, preliminary data on capillary electrophoresis point at the potential of upcoming developments of this analytical technique aiming at screening of fragment ligands (35).

Conventional *in vitro* screening assays have been also reported for fragment screening on GPCRs and considering the pharmacological complexity of G-protein-coupled receptor function, these assays play a major role in a fragment or structure-based drug discovery program on a receptor target. Low specificity can indeed compromise data from high concentration biological testing, but this can be countered with novel more specific readouts of cellular events (e.g. 67). Although follow up of hits might prove challenging in lack of structural information (54), careful hit expansion on adenosine A_3 receptor has demonstrated that biological testing can still form the basis for a fragment-based lead discovery approach (13).

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Figure 1. Fragment based hit discovery for GPCRs