Development of novel assays and application of innovative screening approaches for improving hit discovery efficiency of G protein-coupled receptor targets

Ph.D. Theses

László Kiss

Semmelweis University

Doctoral School of Pharmaceutical Sciences

Supervisor:	András Visegrády, Ph.D.
Official Reviewers:	Gábor Turu, M.D. Ph.D.
	László Molnár, Ph.D.
Head of the complex examination committee:	Dr. habil. István Antal, Ph.D.
Members of the complex examination committee:	András Balla, Ph.D.
	Róbert Kiss, Ph.D.

Budapest 2021

Gedeon Richter Plc.

1. INTRODUCTION

Target-based drug discovery is a long process with many different phases and due to attrition in each phase, on average 1 out of 50 projects delivers a single drug. Accordingly, the overall developmental costs of a drug can amount to around ~1.8-2.5 billion dollars. Comprehensibly, any improvement in any step of the discovery and development process would contribute to an increase in the productivity of Pharmaceutical R&D. In our work we focused on the hit discovery part.

The goal of hit identification in drug discovery is to deliver high quality and diverse chemical starting points for lead optimization and the subsequent development of lead molecules into candidate. The high structural similarity between hit, lead and candidate implies that properties of the candidate highly depend on the initial hits, placing hit identification into a central position in the drug discovery process. Besides the analogue-based approach, screening-based approaches including high-throughput screening (HTS), fragment screening and virtual screening are applied for the identification of chemical starting points. In HTS a high number of compounds are tested in an *in vitro* assay adapted to an automated environment with the aim to identify those molecules that interact with the selected target. Besides the mass testing of lead-like and drug-like molecular libraries in HTS it is also possible to screen lower complexity compounds, called fragments. Due to their lower binding affinity, fragment hits are typically identified using sensitive biophysical techniques instead of biochemical and cellular assays. In addition to the experimental screening approaches above, it is also possible to screen and predict the activity of compounds with computational models, the approach called virtual screening. In today's drug discovery, ideally the different hit discovery approaches are applied in combination, in an integrated manner to maximize the chance of success of a project. Unfortunately, for G protein-coupled receptors (GPCRs), the largest integral membrane protein family targeted by ca. 35% of the FDA-approved drugs, such integrated drug discovery solutions have limitations as GPCRs due to their high level of conformational flexibility are difficult to isolate and stabilize in native form for the biophysical studies primarily applied in fragment screening or 3D structural determination needed for structure-based virtual screening. On the other hand, a high number of *in vitro* biochemical and

functional HTS assays have been developed in the last decades for GPCRs, therefore, HTS represents the most prominent hit discovery tool for the GPCR target class in general. Despite the abundance of HTS assays and the availability of large compound collections, the success rate of HTS lies only around 50%. The two main reasons considered to leading to failure of hit discovery by HTS and some solutions proposed for them are described below.

(1) The first reason is the use of compound libraries covering the chemical space only fractionally. Typically, HTS libraries contain up to a couple of million compounds whereas the size of the chemical space is estimated to be around 10 to the power of 60. Fragment screening could represent a solution as lower complexity molecules sample the chemical space more efficiently than higher molecular weight compounds. Unfortunately, GPCRs, as integral membrane proteins in general are not well suited for biophysical investigations primarily applied for fragment hit identification. Therefore, high concentration biological screening of fragment size molecules was proposed and successfully applied for GPCRs in a few cases. However, in these reports exclusively binding assays were applied; no studies about the application of functional high concentration screening assays, that compared to binding assays are more generally applicable, have been reported up to date.

(2) The second bottleneck is the limited sensitivity of the screening assays. False negatives can emerge from technical errors or assay specific reasons, which sources can to a certain extent be handled by optimization, quality control and proper selection of the assay. However, in the case of GPCRs, this is worsened by false negatives in functional assays originating as a consequence of functional selectivity characteristic for this target class, namely that GPCRs ligands can activate different downstream signaling pathways with different efficacy. In routine HTS typically only a single pathway is investigated, so ligands that act on other pathways will be lost. Furthermore, often subtle changes in the structure can substantially change the activity profile of molecules, which implies that not only single molecules but whole chemotypes can be lost.

For rescuing false negatives due to functional selectivity in diversity screening, running several HTS campaigns in parallel or in sequence would be a theoretically sound solution, but unfortunately it is not financially feasible. In turn, multiplexing different

readouts into a single assay could support the idea of multiple probe screening, and indeed, several successful attempts were reported in the literature for combining labeled assays, in some cases even labeled assays with label-free assays. Without questioning the place and usefulness of the already established solutions, all suffer from limitations either due to the use of recombinant constructs for the sake of the detection or because of restricted applicability in terms of signaling pathways and intracellular analytes.

2. OBJECTIVES

To improve the efficiency of hit and lead generation by high-throughput screening for the GPCR target class, in line with and as an extension to the possible solutions described above in Introduction, we defined the following objectives.

1.) Our first objective was to explore whether *in vitro* cellular and biochemical assays can efficiently support hit expansion activities following hit identification by a functional high concentration screening campaign against α_{2C} receptor. We tested fragment hits and their fragment-size close structural analogues in radioligand displacement binding assay for the determination of binding affinity of the ligands as well as in cell-based Ca^{2+} release assay for the characterization of their functional activity. It was assessed if ligand efficiency (as opposed to affinity *per se*) and functional profile of the compounds derived from these studies can guide hit follow-up in lack of accurate structural information on binding.

2.) Our second objective was to develop more generally applicable multiplex assays combining readout technologies that can monitor cellular signaling events in a more versatile manner than solutions established so far.

2A.) In our first endeavor we aimed at combining the fluorescent Ca^{2+} measurement with the HTRF technology following activation of recombinant human parathyroid hormone 1 receptors (PTH1R) expressed in HEK-293 cells, which offers an ideal system to monitor parallel G protein pathways without the need for genetic engineering in favor of the detection method.

2B.) To further increase the versatility of the approach, our next goal was to multiplex the label-free Dynamic Mass Redistribution (DMR) technology with the HTRF readout using CHO-K1 cells expressing α_{2C} adrenergic receptors, a system suitable for monitoring changes in cAMP with the labeled HTRF technology in addition to recording the whole-cell label-free DMR response.

In order to evaluate the developed assay combinations from hit discovery perspective, a pilot screen was also performed in both cases with a suitable validation library.

3. MATERIALS AND METHODS

3.1. Materials

The pre-assembled core set Rule of Three (Ro3) compliant fragment collection was acquired from Maybridge, whereas the fragment library consisting of 3,071 fragments was purchased from Albany Molecular Research Inc. Reference compounds were purchased from Sigma or Bachem. Buffer components and cell culture reagents were acquired from Sigma-Aldrich (Merck) or Thermo Fischer Scientific.

3.2. Cell lines

For the functional Ca^{2+} assays during fragment hit expansion Chinese hamster ovary (CHO-K1) cells stably expressing human α_{2C} adrenergic receptors and the chimeric $G_{\alpha qi5}$ protein were used, whereas for the displacement binding experiments membrane homogenates were prepared from the same parental CHO-K1 cells recombinantly expressing human α_{2C} receptor (Perkin Elmer) without chimeric $G_{\alpha qi5}$ protein.
For the realization of fluorometric Ca^{2+} - HTRF cAMP multiplex assay HEK-293H-CNG cells recombinantly expressing the human PTH1 receptor and the cyclic-nucleotide gated channel (BD Biosciences) were applied, whereas for the development of the label-free DMR - HTRF cAMP multiplex assay CHO-K1 cells stably expressing the human α_{2C} adrenergic receptors were used (Perkin Elmer).
In all cases cells were maintained in complete culture medium and were passaged 2 to 3-times a week by trypsinization.

3.3. Instrumentation

Fluorometric Ca^{2+} measurements were performed with FLIPR^{TETRA} from Molecular Devices. For the HTRF cAMP measurement an EnVision (Perkin Elmer), whereas for the label-free DMR measurement an EnSpire multimode plate reader was used (Perkin Elmer). Radioligand displacement assays were read with a MicroBeta counter (Perkin Elmer). For liquid handling and liquid dispensing steps Multidrop Combi from

ThermoFisher and Biomek FX automated workstation from Beckman Coulter were used. Plate washing was completed by a BioTek ELx405 microplate washer.

3.4. Biological assays

3.4.1. Binding assay

Displacement binding assays were performed on membrane homogenates using [^{3}H]UK 14,304 radioligand at concentration around its K_d value (5.3 nM) in the presence or absence of test compounds. For non-specific binding, the α-adrenergic antagonist phentolamine at 10 μM was used. After incubation of the reaction mixtures, the content of the wells was filtered through UniFilter GF/C (Perkin Elmer) and the plates were washed. The plates were then dried and finally scintillation cocktail was added to the wells before reading in a MicroBeta counter.

3.4.2. Fluorometric Ca^{2+} measurements

Fluorometric Ca^{2+} measurements were performed with FLIPR Calcium 5 dye in 384-well black clear-bottom microplates with FLIPR^{TETRA} instrument. Except for the Ca^{2+} measurements in agonist or antagonist modes during fragment hit expansion, all Ca^{2+} assays were performed in a homogeneous format. Miniaturization of the Ca^{2+} assay to one third of the standard volume for the Ca^{2+}-HTRF cAMP multiplex was performed with a suitable low volume plate from Corning.

3.4.3. HTRF cAMP measurements

HTRF cAMP assays were performed in white or black low volume plates with suspension or adherent cells. Briefly, cells in suspension (or in adherent mode following overnight incubation) were challenged with agonist or test compounds and after sufficient stimulation time, the cells were lysed with the addition of HTRF cAMP detection reagents. The TR-FRET signal detection was performed in an EnVision reader. For the multiplex combinations the reader parameters as well as the volume and concentration of the detection reagents had to be adjusted to attain sufficient assay quality.

3.4.4. Label-free DMR measurement

Label-free DMR assays were performed according to the manufacturer's instructions. Briefly, after overnight incubation of the cells in fibronectin-coated DMR plates a buffer exchange as well as an equilibration incubation step was performed which was followed by the stimulation step by the agonist or test compounds. Readout was carried out in kinetic mode in an EnSpire reader. Miniaturization of the DMR assay was achieved by decreasing the remaining volume after the buffer exchange step in the protocol.

3.5. Pilot screening

For both multiplex assays developed, a pilot screening campaign was performed with a validation library containing the LOPAC set of pharmacologically active compounds. The screen was run at 10 μM for Ca^{2+}-HTRF multiplex and 4 μM for the DMR-HTRF combination. Assay quality and pharmacological sensitivity of the assays were monitored through Z' and EC_{50} values, respectively. In the case of Ca^{2+}-HTRF, confirmation and specificity testing in repeated experiments were also performed.

3.6. Data analysis

In the radioligand displacement binding experiments IC_{50} values were obtained from displacement curves by sigmoidal fitting of the data points using Origin 6.0 software. K_i values (i.e. inhibition constants) were calculated from the IC_{50} values using the Cheng-Prusoff equation with the K_d value of tracer ligand determined previously.

In the case of fluorometric Ca^{2+} measurements raw fluorescence data were transformed to $\Delta F/F_b$ values in Microsoft Excel. For the HTRF cAMP measurements TR-FRET ratio was calculated by dividing raw fluorescence values measured at 665 nm with raw signal intensity measured at 620 nm. To create sigmoidal concentration-response curves, data points were fitted to four-parameter logistic function using the GraphPad Prism software.

4. RESULTS

In our first objective the follow-up of active fragments with outstanding K_i and LE originating from a functional high-concentration screen against the α_{2C} adrenergic receptor was explored. The corporate compound library was scanned for close structural analogues ('neighbors') of the most promising primary actives and the 90 selected analogues were directly tested in competition binding assay at a single 20 μM concentration. The most active 16 compounds were then also tested in concentration dependent manner for exact K_i determination so that LE values could also be calculated. Except for 4 compounds all the others displayed improvement either in K_i or the calculated LE values, so the hit expansion was successful in this respect. In the meantime, however, the 90 analogues were also run in the functional Ca^{2+} assay at 20 μM and surprisingly none of them displayed robust and specific agonist activity. Considering also potential switch in the activity profile, 8 analogues with the best K_i were run in the Ca^{2+} assay also in antagonist mode and all these fragments turned out to be functional antagonists. As antagonists did not fit in the project scope, they were not pursued further. With no similar reports available in the literature for GPCRs, we demonstrated for the first time that fragment-based hit identification and the subsequent ligand efficiency-driven hit expansion can successfully be supported by biochemical and cell-based assays for GPCRs as membrane targets even in the lack of accurate structural information about the receptor-ligand interactions.

As our second main goal, we successfully developed and evaluated multiplex assays based on the combination of generally applicable cell-based assay technologies.

First, the fluorometric Ca^{2+} measurement was combined with consecutive TR-FRET-based HTRF technology in cells stably expressing the human PTH1R receptor for simultaneous monitoring of the G_q and G_s pathways. The differences in the optimal assay conditions of the two technologies represented ample amount of optimization and adjustment work until the multiplex assay with proper assay quality could be established: most importantly, the Ca^{2+} assay had to be miniaturized whereas the HTRF cAMP readout had be adapted for adherent cells in black plates in the presence of red colored Ca^{2+} detection dye. In the course of pharmacological validation, the developed multiplex assay was able to differentiate between ligands with distinct pharmacological

profiles in line with previous reports in the literature. In a pilot validation screen on 6 plates including the LOPAC library of pharmacologically active compounds and diverse compounds from the corporate library, distinct sets of LOPAC compounds acting on endogenous receptors and biochemical pathways of the applied HEK-293 cells were identified in the two readouts with no overlap: in the Ca^{2+} readout mostly compounds with purinergic or muscarinic activity, whereas in the HTRF cAMP adenosine and adrenergic ligands were identified. The complementary nature of the active lists obtained suggests that our multiplex approach is successful in detecting compounds activating different intracellular signaling pathways and thus has the potential to improve the sensitivity of large scale screening campaigns.

As a second task, the dynamic mass redistribution-based optical label-free integrated whole cell readout, that alone can be considered a multi-probe screening technology, was multiplexed with the HTRF readout using CHO-K1 cell line recombinantly expressing the human α_{2C} adrenergic receptor, which primarily couples to $G_{\alpha i/o}$ proteins. In this system the kinetic compatibility of the two readouts raised the most crucial question as the lysis-based HTRF cAMP readout could only be run after the live DMR measurement. Fortunately, the DMR response reached peak value earlier than the optimal time-point for the HTRF readout, which opened the way for the development of the HTRF cAMP assay in the DMR plate with suboptimal design for TR-FRET detection. Finally, the HTRF reading was successfully optimized in DMR plate by adjusting the concentration and volume of the HTRF detection reagents as well as the reader parameters. The developed assay was tested in a validation screen using the LOPAC library where the multiplex demonstrated proper assay quality and delivered specific, mainly adrenergic ligands with activities in good agreement between the readouts. The high degree of overlap between the actives in the two readouts can be regarded as internal orthogonal confirmation and can be attributed to the ability of DMR assay to identify ligands activating the $G_{i/o}$ pathway with high confidence. To our knowledge our work represents the first demonstration of combining a miniaturized DMR assay with HTRF technology in a single screening assay delivering comparable pharmacology in both readouts.

Beyond the methodological aspects, the main significance of these multiplex assay systems is that they enable the simultaneous monitoring of a diverse array of receptor-

mediated signaling events, which at the end is expected to increase the chance of providing promising chemical starting points for successful lead discovery by rescuing functional selective and/or screening technology-specific false negative ligands. Finally, with multiplex assays the investigation of targets and ligands with complex and less well characterized pharmacology (functional selectivity, orphan receptors) might become possible, too.

5. CONCLUSIONS

In our work we focused on developing solutions to improve the hit discovery efficiency of high-throughput screening for G protein-coupled receptor targets.

First a hit expansion workflow following high concentration functional screening of a fragment library was explored, as fragments thanks to their smaller size show improved sampling efficiency of the chemical space and can provide novel and attractive starting points for lead discovery. Using the α_{2C} adrenergic receptor expressed in CHO-K1 cells we have provided an example of ligand efficiency-guided fragment hit expansion using cell-based and biochemical assays. Applying functional and biochemical assays in fragment screening and hit expansion is supposed to substantially increase the sampling efficiency of the chemical space and thereby the chance of delivering novel and intriguing chemical starting points for challenging GPCR targets.

As second objective, generally applicable multiplexed cell-based functional assays were developed and established to reduce the loss of actives as false negatives, originating due to functional selectivity or from assay-specific sources. For the first time, both the fluorometric Ca^{2+} assay and the label-free DMR technology were successfully combined with HTRF technology. These novel multiplex platforms offer simultaneous monitoring of practically any combination of collateral GPCR pathways thereby holding the promise of improving sensitivity of high-throughput screening campaigns.

The outcome of our work might also point towards considering the integrated use of the two main objectives, as screening smaller size compounds at elevated concentrations in multiplex assays is expected to maximize the probability of identifying structurally and functionally diverse compounds with high ligand efficiency for a successful lead discovery. Although our proof-of-concept work both on fragment hit expansion and multiplex assay development was done with GPCRs using mammalian cell lines recombinantly expressing a target receptor, we believe that our results might be extrapolated to integral membrane proteins other than the GPCR target class and also beyond the usage of stable cell lines.

6. PUBLICATIONS

6.1. Publications related to the thesis

Szőllősi, E.; Bobok, A.; **Kiss, L.**; Vass, M.; Kurkó, D.; Kolok, S.; Visegrády, A.; Keserű, G. M. (2015) Cell-based and virtual fragment screening for adrenergic α_{2C} receptor agonists. Bioorg. Med. Chem. 23 (14), 3991–3999.

Kiss, L.; Cselenyák, A.; Varga, Á.; Visegrády, A. (2016) A novel cell-based duplex high-throughput screening assay combining fluorescent Ca^{2+} measurement with homogeneous time-resolved fluorescence technology. Anal. Biochem. 507, 33–39.

Kiss, L.; Cselenyák, A.; Visegrády, A. (2019) Label-free drug screening assay multiplexed with an orthogonal time-resolved fluorescence labeled assay. Anal. Biochem. 566, 126–132.

6.2. Further scientific publications

Nasiri, H. R.; **Kiss, L**.; Krämer, J. (2016) A fluorescence polarization based assay for the identification and characterization of polymerase inhibitors. Bioorg. Med. Chem. Lett. 26 (18), 4433–4435.

Ledneczki, I.; Horváth, A.; Tapolcsányi, P.; Éles, J.; Molnár-Dudás, K.; Vágó, I.; Visegrády, A.; **Kiss, L.**; Szigetvári, Á.; Kóti, J.; Krámos, B.; Mahó, S.; Holm, P.; Kolok, S.; Fodor, L.; Thán, M.; Kostyalik, D.; Balázs, O.; Vastag, M.; Greiner, I.; Lévay, Gy.; Lendvai, B.; Némethy, Zs. (2021) HTS-based discovery and optimization of novel positive allosteric modulators of the α7 nicotinic acetylcholine receptor. Eur. J. Med. Chem. 222, 113560

Behr-Gross, M-E.; Siklodi, B.; Le Tallec, D.; Halder, M.; Manou, I.; Sinitskaya, N.; Bruckner, L.; Dalmadi, B.; **Kiss, L.**; Redhead, K. (2021) Collaborative study for the validation of cell line assays for in-process toxicity and antigenicity testing of

Clostridium septicum vaccine antigens - Part 2: Optimisation of cell line assays. Pharmeuropa bio & scientific notes, 101-156.