DOI:10.14753/SE.2021.2563

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

Ph.D. Dissertation

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.

DOI:10.14753/SE.2021.2563

TABLE OF CONTENTS

DOI:10.14753/SE.2021.2563

DOI:10.14753/SE.2021.2563

DOI:10.14753/SE.2021.2563

LIST OF ABBREVIATIONS

α_{2C}R, Adrenergic alpha 2c receptor

AC, Adenylate cyclase

ADME, Absorption, distribution, metabolism and excretion

AngII, Angiotensin II

AP-2, Adaptor protein-2

ATP, Adenosine triphosphate

AT_1R, Angiotensin II receptor type I

BRET, Bioluminescence resonance energy transfer

cAMP, Cyclic adenosine monophosphate

CHO, Chinese hamster ovary

clogP, Calculated logP

CNG, Cyclic nucleotide-gated ion channel

CNS, Central nervous system

CRC, Concentration-response curve

DAG, Diacylglycerol

DMR, Dynamic mass redistribution

DMSO, Dimethyl sulfoxide

EC_{50}, Half maximal effective concentration

ECL, Extracellular loop

EGTA, Ethyleneglycoltetraacetic acid

ERK1/2, Extracellular signal-regulated kinase 1 and 2

FBLD, Fragment-based lead discovery

FCS, Fetal calf serum

FDA, U.S. Food and Drug Administration

FLIPR, Fluorescent Imaging Plate Reader

FRET, Fluorescence resonance energy transfer or Förster resonance energy transfer

FS, Fragment screening

FSK, Forskolin

GAP, GTPase-activating proteins

GDP, Guanosine diphosphate

GEF, Guanine nucleotide exchange factor

GFP, Green fluorescent protein

GPCR, G protein-coupled receptor

GRK, G protein-coupled receptor kinases

GTP, Guanosine-5'-triphosphate

HBA, H-bond acceptor count

HBD, H-bond donor count

HCS, High concentration screening

HEK, Human embryonic kidney

HTRF, Homogenous time-resolved fluorescence

HTS, High-throughput screening

H2L, Hit-to-lead

IBMX, 3-isobutyl-1-methylxanthine

$IC_{50,}$ Half maximal inhibitory concentration

ICL, Intracellular loop

IP_1, Inositol monophosphate

IP_3, Inositol 1,4,5- trisphosphate

iPSC, Induced pluripotent stem cell

K_d/K_i, Equilibrium dissociation or inhibition constant

LE, Ligand efficiency

LF, Label-free

LBVS, Ligand-based virtual screening

logP, logarithm of the n-octanol-water partition coefficient

LOPAC, Library of Pharmacologically Active Compounds

MAPK, Mitogen-activated protein kinase

MoA, Mechanism of action

MW, Molecular weight

N_{heavy}, Heavy atom count (non-hydrogen atom count)

NMR, Nuclear magnetic resonance

PDL, Poly-D-lysine

PIP_2, Phosphatidylinositol 4,5-bisphosphate

PKA, Protein kinase A

PKC, Protein kinase C

PLC-β, Phospholipase C-β

PTH, Parathyroid hormone

PTH1R, Parathyroid hormone 1 receptor

R&D, Research and Development

Ro3, Rule of Three

SAR, Structure-activity relationship

SBVS, Structure-based virtual screening

SD, Standard deviation

SFSR, Structure-functional selectivity relationship

SPR, Surface plasmon resonance

S/B, Signal-to-background ratio

TBDD, Target-based drug discovery

TR-FRET, Time-resolved FRET

TRPM8, Transient receptor potential cation channel subfamily M member 8

VS, Virtual screening

7-TM receptor, Seven-transmembrane receptor

$\Delta F/F_b$, Ratio used to express signal window in Ca^{2+} fluorometry measurements: peak fluorescence minus baseline fluorescence (ΔF) divided by baseline fluorescence (F_b)

DOI:10.14753/SE.2021.2563

1. INTRODUCTION

1.1. Hit discovery approaches in drug discovery

1.1.1. Overview of target-based drug discovery

The primary aim of pharmaceutical research is to discover effective and safe treatments against pathophysiological conditions. Over thousands of years medicaments originated exclusively from natural sources and their application was based mainly on observations and experiences. Rational drug discovery could only start with the advances in organic and analytical chemistry in the 2nd half of the 19th century that enabled the structural analysis of natural substances as well as the synthesis and testing of novel organic compounds against the symptoms of diseases. In this era of classical pharmacology testing of small molecule samples was predominantly performed in tissues or whole organisms as model systems often without knowing the underlying mechanism of action (MoA).[1,2] It was not until the early 20th century that progresses in the field of enzymology and the birth of receptor theory concept revealed that xenobiotics exert their effect through binding to biomolecules in the body via molecular recognition. The subsequent fundamental discoveries in molecular biology progressively enabled the study of these interactions at molecular and functional level that slowly induced a paradigm change from earlier phenotypic approaches towards target-based drug discovery (TBDD).[2–4]

The first step in target-based drug discovery for a given indication is the exploration of possible target biomolecules that are hypothesized to play a role in the pathomechanism of the disease and whose modulation in the right direction could grant therapeutic value.[5] The most actively pursued drug targets in small molecule drug discovery fall in one of the following protein families: (1) G protein-coupled receptors (2) protein kinases (soluble enzymes or receptor tyrosine kinases) (3) ion channels (ligand- and voltage-gated) and (4) nuclear hormone receptors.[6]

Once a target is selected and validated, identification of small molecules exhibiting the desired modulatory activity at the target is initiated. Chemical starting points considered to be worth further exploration are optimized first into leads and then to developmental

drug candidate, whose safety and toxicity are tested in experimental animals (e.g. mice, rat, dog, primate).[5] The candidate that survives the preclinical phase can enter the highly regulated clinical trials for assessing safety and efficacy in human (Phases I-III). Drugs successfully passing all three phases can be registered and receive market authorization after approval.[7]

The whole discovery and development process of a single drug (Figure 1) takes approximately 10-15 years and costs around 250-300 million dollars. Due to attrition present in each phase, on average only 1 out of 50 projects delivers an approved drug[8,9] and accordingly, the overall developmental costs of a drug might amount to around 1.8-2.5 billion dollars.[7,10] Comprehensibly, decreasing attrition rate at any stage would contribute to the improvement of the overall R&D productivity.[7]

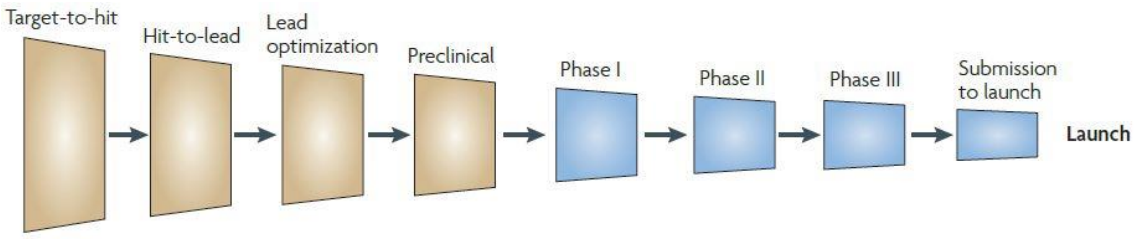


Figure 1. Phases of target-based drug discovery[7]

1.1.2. Identification of chemical starting points

The discovery phase of drug research starts with the identification of compounds that show specific activity against the target in an *in vitro* model system. These chemical starting points are then developed through iterative design and testing cycles to lead compounds. At this early stage the pharmacodynamic properties of the compounds are most typically characterized by binding affinity and/or *in vitro* potency, whereas the pharmacokinetic liabilities are assessed through *in vitro* ADME parameters (absorption, distribution, metabolism and excretion). Although the exact definition of lead varies slightly across the industry, the minimum criteria for a lead are favorable affinity/potency and ADME profile as well as a suitable chemical structure that holds the potential to be optimized to developmental drug candidate.[5,11] Because of the usually high degree of structural similarity between leads and candidates, the pharmacodynamic and pharmacokinetic properties of the final drug compounds are highly dependent on the initial lead.[12–14] Due to this direct linkage of the starting

molecules to the future investigational new drug and thus to the overall success of the drug discovery project, identification of chemical starting points has a central role in the discovery process.

The quest for chemical starting points is realized by two main approaches, namely analogue-based and screening approaches which are often applied in combination rather than being mutually exclusive.[11,14] The analogue-based approach relies on drug-like ligands already disclosed in the literature (known natural substances or compounds identified by other investigators) showing activity against the target in question. In screening approaches, in contrast, starting points, called hits are identified by testing large number of molecules (compound libraries) without prior knowledge about their biological activity. Screening can be exercised using experimental or virtual screening methods.[5,11,14] From the experimental screening approaches high-throughput screening has been the dominant hit discovery strategy since its inception in the early 90's,[14,15] but in recent years fragment-based lead discovery (FBLD) has also grown to be a pertinent source of clinical candidates.[14,16,17] In addition to experimental approaches, hit discovery based on computational models (virtual screening) is also widely applied in drug discovery.[11,14,18] In the next sections the different screening strategies will be introduced in more detail, whereas the analogue-based approach will not be discussed further in this dissertation.

1.1.2.1. Hit discovery with high-throughput screening (HTS)

HTS is a highly interdisciplinary hit identification approach where a large number of compounds are tested in an *in vitro* assay adapted to high-density microtiter plates and an automated environment.[19] The birth of HTS was due to advances in molecular biology (purified proteins, monoclonal antibodies, heterologous expression of enzymes and receptors), automation, data informatics and finally, high throughput analytical and combinatorial synthetic chemistry in the 80's and early 90's.[20,21] Nowadays, thanks to further developments in sensitive molecular detection techniques and high precision liquid handling devices, assays are routinely run in 384- or 1536-well formats with a throughput of 10,000-100,000 compounds/day and assay volumes miniaturized to the microliter volume range (Figure 2).[22,23] The HTS assays are executed either as biochemical or cell-based assays with typical screening concentration lying in the 10-30

μM range. In biochemical assays the isolated molecular target, usually a soluble protein or protein fragment is directly used for the identification of modulators or ligands, whereas in cell-based assays predominantly the functional effect of compounds are assessed in a more complex environment reflecting the physiological conditions more closely.[19,21,23,24]

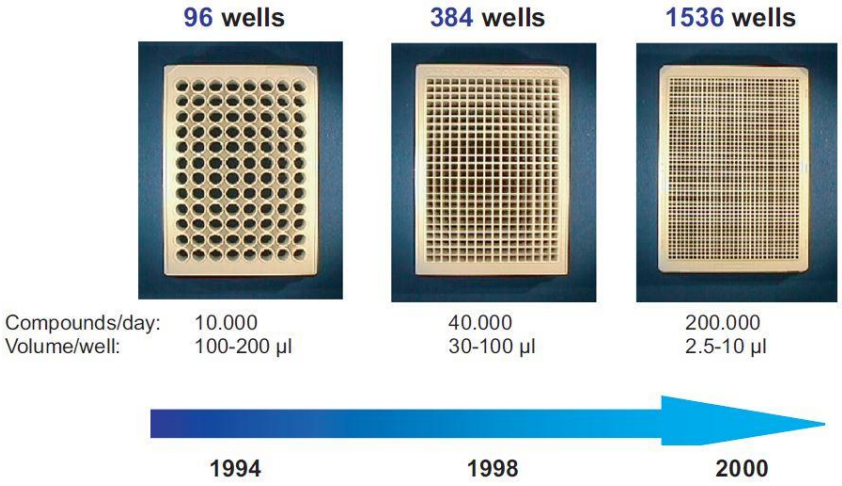


Figure 2. Miniaturization and increase in the throughput of screening[25]

Depending on the purpose and the available resources, HTS can be performed as diversity screening on the whole compound collection of an institution, or as focused screen only on a subset of the library preselected specifically for the target.[11,15,22] Comprehensibly, due to extensive investment in the establishment and maintenance of an automated assay platform and a compound library, as well as the expenses accompanying the high reagent and consumable needs of the individual campaigns, HTS is a highly resource-intensive undertaking.[21] Accordingly, compounds are usually tested only once (singlet) which highlights the importance of sufficient sensitivity and accuracy of the screening assay as well as rigorous process monitoring to avoid false positive and false negative results as much as possible. In the quality control of HTS pass/fail judgment is handled at plate-level, based mainly on Z' statistical parameter[26] which shows the extent of the separation between the positive and negative signals taking into account the standard deviations (SD) and the means:

$$Z' = 1 - 3 * \frac{SD_+ + SD_-}{|mean_+ - mean_-|}$$

Z' value below 0.4 is considered to reflect poor assay quality and compounds on such plates are usually marked for retesting.[5,26]

At the end of an HTS campaign compounds that exceed the activity threshold defined either on statistical basis (most typically mean activity + 3-times the standard deviation) or arbitrarily (e.g. a predefined activity limit or ratio of actives) are selected as primary actives. These primary actives need to undergo and pass confirmation, specificity testing and chemical integrity analysis to be qualified as hits, the chemical starting points that form the bases of the medicinal chemistry activities of the hit-to-lead (H2L) phase.[5,11,27]

Briefly, in H2L hit compounds are first grouped into chemical families called clusters or chemotypes based on their structural elements, and then, compounds in selected chemotypes are systematically altered through various substitutions and/or expansions to improve affinity/potency and ADME properties.[5,11,27,28] The importance of hit identification, proper execution of H2L and careful selection of the lead compound(s) can be highlighted by literature examples that show that key structural elements of the drug candidate can often be recognized in the lead structure[13] or even already in the initial hit molecule[14]. The significance of HTS in the hit identification process cannot be overemphasized as HTS has been constantly delivering approx. 30% of clinical candidates or approved drugs since the early 90's.[14,15] For illustration, selected hit-lead-drug series are depicted in Figure 3 below.

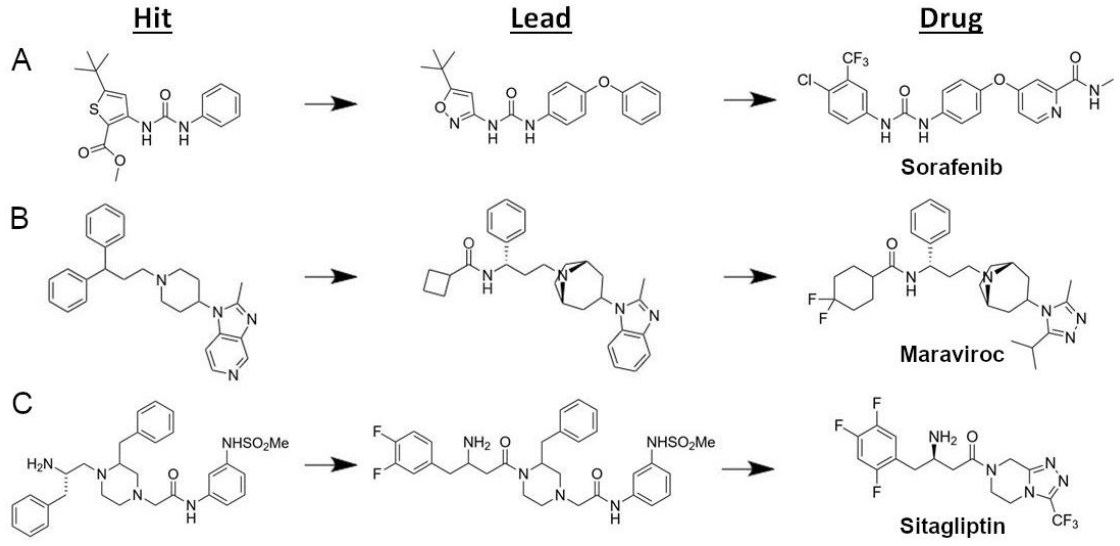


Figure 3. Exemplary drugs originating from HTS. Hit-lead-drug series for (A) Sorafenib[29,30], (B) Maraviroc[31] and (C) Sitagliptin[32]

1.1.2.2. Hit discovery with fragment screening (FS)

The concept of fragment screening as another screening-based hit identification tool besides HTS was contrived at the beginning of the 2000s based on the complexity model which projected that molecules with lower number of structural moieties and pharmacophore features have higher chance of establishing specific interactions with the target proteins than highly complex ('drug-like') molecules[33], e.g. those overrepresented in the HTS libraries of the 1990s.[15,34–36] In addition, molecules fulfilling the criteria of lower complexity defined originally by the 'Rule of Three' (Ro3; i.e. MW<300, clogP<3, HBD<3, HBA<3)[37], called fragments, due to their smaller size and advantageous physicochemical properties might provide larger operational freedom during hit-to-lead activities.[38] Finally, screening performed with fragments allows a much more efficient sampling of chemical space than conventional HTS libraries (Figure 4).[39] As an illustration, the chemical universe of molecules up to 17 heavy atoms (N_{heavy}) is approximated to contain ~166 billion compounds[40], much lower than the chemical space of drug-like (N_{heavy} up to ~35-40) molecules estimated[41] to fall between 10^{23} and 10^{60}.

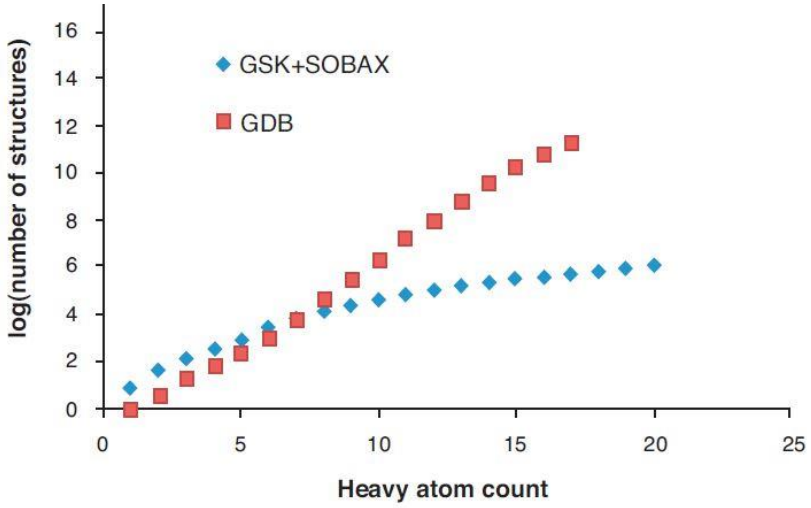


Figure 4. Coverage of chemical space as a function of molecular size (GDB=database of molecules fulfilling drug-likeness criteria up to 17 N_{heavy}; GSK+SOBAX: physically available compounds in GlaxoSmithKline library + SOBAX vendor database). Note: as no filters were applied for GSK+SOBAX collection, at N_{heavy}<7 the blue symbols run slightly above the red squares due to the presence of molecules not passing drug-likeness criteria.[39]

The obvious disadvantage of the lower complexity of fragments is the fewer number of interactions with the target that results in weaker binding affinity. To overcome this, fragments are typically screened at much higher concentration compared to HTS, normally in the 100-1000 μM range, with highly sensitive biophysical detection technologies like surface plasmon resonance (SPR), NMR spectroscopy or X-ray crystallography. Unfortunately, such investigational techniques limit the type of targets to soluble proteins or protein fragments, and moreover, do not reveal the molecular mechanism of action of the ligands.[37,42]

In line with the principle of the fragment approach, the hit rates in fragment screens are typically markedly higher than those of conventional HTS campaigns (1-10%[43–45] compared to 0.1-1%[46]),[47] which also means that screening as few as a couple of thousand compounds can deliver sufficient quantity of starting points. Unfortunately, as fragment hits have low affinity and represent individual islands in the chemical space (rather than archipelagos as in the case of HTS), affinity alone, without taking into account factors like molecular size or lipophilicity, might not be sufficient for their initial evaluation. The ligand efficiency (LE) parameter that relates the affinity (K_d) of a ligand with the number of its non-hydrogen atoms[48] on the other hand characterizes the efficiency of fragment binding better than K_d alone and is therefore widely used for prioritization of fragments[49]:

$$LE = -RT\frac{\ln K_d}{N_{heavy}}$$

Ligands with the highest LE values are preferred for follow-up, whereas, fragments with LE<0.3 are usually not considered worth pursuing in H2L.[38,48]

Strategies for the elaboration of prioritized fragment hits include fragment optimization and fragment evolution.[38,49] Optimization of fragment hits is performed by small structural changes around the original hit at nearly constant size with the aim to improve LE.[49,50] Evolution of fragments can be performed by adding new structural elements in the optimal vectorial direction, called ‘growing’ (Figure 5A),[16,49] but in addition to this, given the low size of fragments, linking of fragments (Figure 5B) occupying adjacent positions in the same binding cavity or merging of partially overlapping fragments have also been successfully exercised.[42,51] Due to the only partial occupation of the binding surface by the small-size hits, fragment elaboration substantially relies on structural

information about the binding mode generated by X-ray crystallography or NMR spectroscopy.[38,42,45] Nevertheless, although it is certainly a slower and highly challenging undertaking, it is possible to initiate fragment hit expansion and generate preliminary SAR (structure-activity relationship) in early fragment elaboration without structural information:[38,52] most typically, close structural analogues of the fragment hits are searched for in commercial or internal databases and SAR is explored by testing the available analogues ('SAR-by-catalog').[44,45,49,50]

Successful application of fragment-based hit identification and lead generation has already materialized in four FDA-approved drugs[14,16,17] as well as a number of candidates at various stages in the clinical pipeline.[14,17]

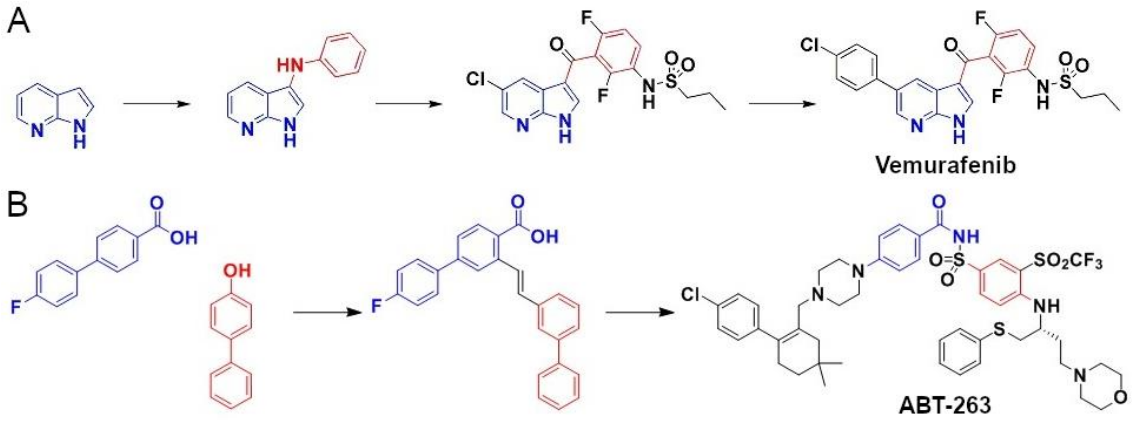


Figure 5. Fragment elaboration illustrated by drugs originating from fragment-based lead discovery. (A) Fragment growing approach in case of Vemurafenib[53]; (B) fragment linking strategy leading to ABT-263, the non-selective predecessor candidate of Venetoclax[54]

1.1.2.3. Hit discovery with Virtual Screening (VS)

Screening of molecules can also be performed with quantitative models using high performance computational methods. Besides speed, the main advantage of this '*in silico*' approach, called virtual screening, is that the chemical space sampled is not limited to physically available chemical compounds, implying that even molecules not actually available can be assessed in a cost-effective way.[18,55]

Virtual screening methods can be categorized into the ligand-based virtual screening (LBVS) and structure-based virtual screening (SBVS). In the case of ligand-based approach, activity estimation of compounds are performed by evaluating their structural

similarity, pharmacophore features or other molecular descriptors using models built upon a set of reference ligands with known biological activity.[18,55]

In SBVS the computational work is supported by 3D structural information, whereby in ideal case the crystal structure of the drug target alone or in complex with a suitable ligand is available for the assessment of the molecular interactions in the binding site. For many therapeutically interesting targets, most notably for integral membrane proteins, however, experimentally solved structures are not available. In these cases, as a surrogate solution, homology modeling can be used for building the spatial model of the target protein based on already solved structures of a related protein with sufficient sequence homology.[18,55] Following library preparation and validation of the SBVS model, molecules are docked into the binding site with a computer program and ranked according to a mathematical algorithm (scoring function) that estimates the goodness of fit.[18] As final step in virtual screening campaigns, top-ranking compounds are experimentally assessed for biological activity in an *in vitro* pharmacological assay, analogously to HTS. However, thanks to the computational pre-filtering, the number of compounds to be tested and thus the resources required are substantially lower in this case.[55]

1.2. G protein-coupled receptors as drug targets

G protein-coupled receptors (GPCRs) or seven transmembrane (7-TM) receptors with around 800 members are the largest superfamily of cell surface receptors.[56,57] By transmitting signals from outside the cells, GPCRs are involved in a wide range of vital physiological processes that is also reflected in their prevalence as drug targets: approximately 35% of the drugs currently approved by the U.S. Food and Drug Administration (FDA) target in total more than 100 different members of the GPCR superfamily.[6,56,57] The significance of 7-TM receptors was also recognized at the highest scientific level as the Nobel Prize in chemistry was awarded to Brian Kobilka and Robert Lefkowitz in 2012 for their discoveries in GPCR structure and function.[58]

1.2.1. Activation and signaling cascades of GPCRs

GPCRs are integral membrane proteins with seven transmembrane (TM) helices, an extracellular N-terminal part as well as an intracellular C-terminal (Figure 6). Ligand

binding to the extracellular part triggers conformational changes of the receptor that promote the binding of heterotrimeric G proteins ('G protein-coupling') to the intracellular side of the receptor.[59] The guanine nucleotide exchange factor (GEF) activity of the conformationally reorganized intracellular loops leads to GDP-GTP exchange in the α subunit of the G protein which in turn triggers the dissociation of the βγ subunit and the activation of second messenger-mediated signal transduction pathways that converge towards the final physiological response (e.g. change in cell metabolism, activation/repression of gene expression).[60–63] Based on the sequence similarity and function of the α subunit, heterotrimeric G proteins are classified into four main types ($G_{\alpha q/11}$, $G_{\alpha i/o}$, $G_{\alpha s}$, $G_{\alpha 12/13}$)[64,65], each with a separate sequence of signaling events (Figure 6).[61,63,66] The effector molecule of the G_s pathway is adenylate cyclase (AC) which catalyzes the formation of the second messenger cAMP from ATP. cAMP in turn activates Protein kinase A (PKA), a serine/threonine kinase with diverse physiological functions. G proteins with $G_{\alpha i}$ or $G_{\alpha o}$ subunits on the other hand inhibit adenylate cyclase and its downstream mediators. $G_{\alpha q/11}$ subunits activate phospholipase C-β (PLC-β) which then cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds IP_3 receptors on the endoplasmic reticulum leading to Ca^{2+} release from these intracellular stores. DAG activates various Protein kinase C (PKC) isoforms that in turn regulate target proteins through phosphorylation.[67,68] $G_{\alpha 12/13}$-coupling leads to the activation of Rho GTPases and other downstream effectors through the interaction with RhoGEF proteins.[69,70] Besides the G_α subunits, the $G_{\beta\gamma}$ heterodimers released upon G protein activation might also participate in signaling, in particular by regulating a variety of ion channels through direct interactions.[71,72] The activation cycle of G proteins is terminated with the hydrolysis of bound GTP to GDP catalyzed by the GTPase activity of the α subunit and the subsequent regeneration of the trimeric complex that is then ready to participate in the next round of activation. The rate of GTP hydrolysis can be accelerated by GTPase-activating proteins (GAPs).[60,61,63]

1.2.2. Desensitization and arrestin-mediated signaling

There are regulatory mechanisms to protect the cells from extensive activation elicited by sustained or repeated exposure to an activator ligand. In homologous desensitization,

following receptor activation G protein-coupled receptor kinases (GRKs) phosphorylate the serine/threonine residues on the intracellular loops (ICLs) as well as the C-terminal of the receptor[73] that results in the recruitment of β-arrestin-1,2 (in other names arrestin 2 and arrestin 3, respectively) to the activated receptor.[61,74,75] The high-affinity binding of arrestins to the receptor imparts a sequence of events (Figure 6).[76,77] First of all, β-arrestin sterically hinders G protein binding and thereby uncouples G protein-mediated downstream signaling. In parallel or subsequently, it can also facilitate the binding of adaptor protein-2 (AP-2) and clathrin needed for receptor internalization through endocytosis. After internalization, the receptor can be targeted for proteolytic degradation in lysosomes or can be recycled to the plasma membrane following resensitization by dephosphorylation of the receptor in the endocytic compartment.[76–78]

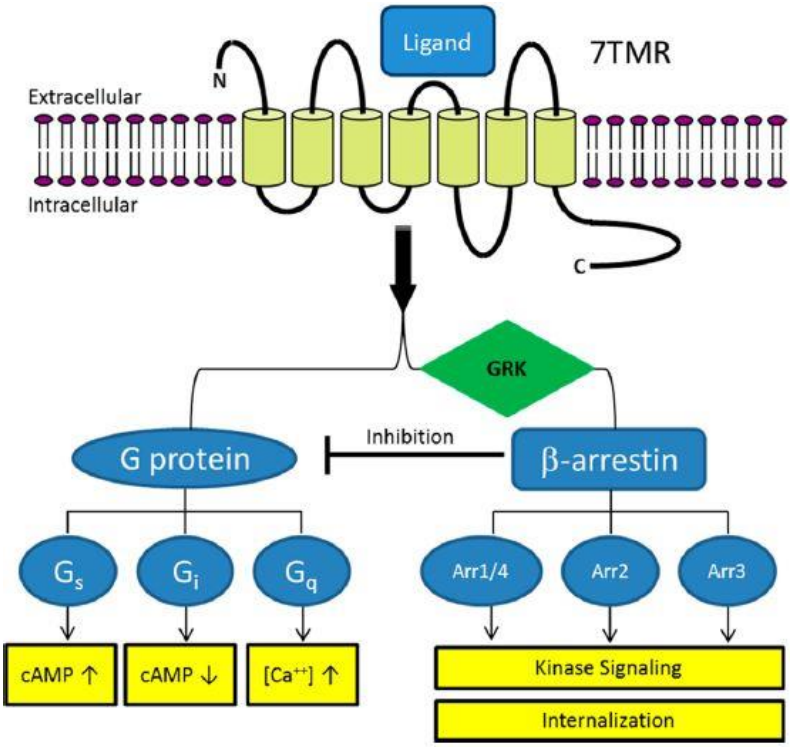


Figure 6. Overview of signaling cascades of GPCRs and the role of β-arrestin[79] Note: Arr1/4 are visual arrestins involved in phototransduction.[80] Reprinted (adapted) with permission from *Correll and McKittrick 2014*[79]. Copyright (2014) American Chemical Society.

In contrast to homologous desensitization, during heterologous desensitization it is the extensive stimulation of 'Receptor A' that reduces the responsiveness of 'Receptor B'. This is achieved through phosphorylation of the intracellular surface of 'Receptor B' by cytosolic protein kinases, such as PKA or PKC as a consequence of 'Receptor A'

activation.[81,82] Case studies showing that β-arrestins might also play a role in the following internalization event have also been reported.[81,82]

Besides the role of β-arrestins in desensitization, in the last decades it was also discovered that arrestins through acting as a scaffolding protein might initiate diverse signaling activities independent from G proteins, most notably the regulation of cellular kinase pathways (e.g. MAP kinases, AKT).[76,77,83,84] At this point overlaps between G protein-dependent and independent signaling might be encountered, as e.g. the MAP kinases ERK1/2 can also be activated by G proteins through PKA and PKC; nevertheless, it was reported that G protein-mediated and β-arrestin-mediated ERK1/2 activation are typically temporarily as well as spatially different.[76,84–86]

1.2.3. Molecular pharmacology of GPCRs – classical concepts

The molecular pharmacology of GPCR ligands can be characterized by affinity, efficacy and potency, and the interconnection between these pharmacological parameters are visually shown in Figure 7 below.[3]

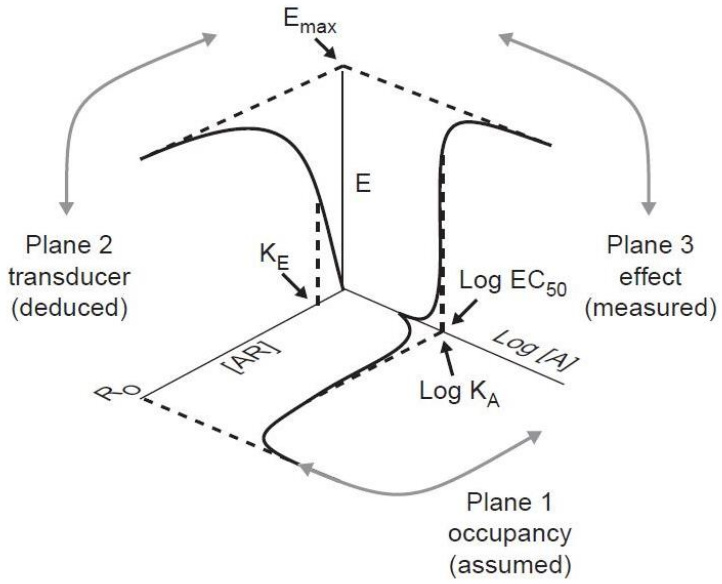


Figure 7. Illustration of pharmacological parameters of GPCR ligands according to the operational model: Plane 1 shows ligand binding; Plane 2 describes stimulation, the response as a function of receptor occupancy; and Plane 3 is the concentration-response function[3]

Affinity reflects the strength of binding (K_A on Plane 1 in Figure 7) whereas efficacy (τ, see below in equation) measures the ability of the ligands to stimulate a response, with

E_{max} representing the maximal response in the system. Efficacy has an agonist-specific component, called intrinsic efficacy and also a system-dependent component related to receptor density.[87] Potency is the ligand concentration [A] that produces 50% response (EC_{50} on the concentration-response curve on Plane 3 in Figure 7) and it is a complex function of efficacy and affinity.[3] This description of GPCR pharmacology is based on the operational model of receptor theory formulated by Black and Leff[88] in 1983 that establishes the mathematical relationship between ligand concentration and functional response in the following form[3]:

$$Response\ (E) = \frac{[\mathrm{A}] * \tau * \mathrm{E_{max}}}{[\mathrm{A}] * (\tau + 1) + \mathrm{K_A}}$$

The operational model is widely used even today, however, with the recognition of constitutive receptor activity in the era of recombinant expression of receptors, new models that were able to accommodate basal activity were needed to be introduced. According to these allosteric models, the extended ternary complex model and the cubic ternary complex model,[3,89] receptors exist either in active or inactive state and there is equilibrium between these conformations.[3,89,90] Ligands that bind to the site of the endogenous ligand, that is the orthosteric binding site, and activate the receptor by shifting the equilibrium towards the active receptor conformation are called agonists. Inverse agonists[91] on the other hand push the receptor conformational distribution towards the inactive state and thus abolish constitutive activity of the receptor occurring under agonist-free ('basal') conditions. Neutral antagonists do not influence the equilibrium; they purely hinder the binding of other ligands, e.g. agonists.[3] Partial agonists are a subgroup within agonists that can elicit only submaximal system response.

Besides orthosteric binders there are also ligands that have affinities to binding sites other than the orthosteric site. These secondary binding sites are called allosteric sites and ligands showing affinity to these sites are referred to as allosteric modulators. A positive allosteric modulator (PAM) increases the affinity and/or efficacy of the orthosteric ligand, whereas negative allosteric modulators (NAM) have the opposite effect. Those compounds that do not influence the ligand binding and activation at the orthosteric site are silent allosteric modulators (SAM).[92] Allosteric interactions are

probe-dependent, meaning that the same allosteric ligand might have different modulatory effect on the efficacy and/or affinity of different orthosteric ligands.[3,89,92]

1.2.4. Pluri-dimensional efficacy and functional selectivity

With the advances in functional assays it became obvious that GPCR signaling in most cases is not restricted to a single G protein, instead, GPCRs can couple to multiple types of G proteins as well as other intracellular effectors.[65,90,93,94] Agonists were originally considered to activate all these connected downstream pathways with the same efficacy. However, over the last two decades it was discovered that instead of two states, receptors exist in multiple active and inactive conformational states[95,96] and these different conformations can be stabilized by ligands selectively binding to them[59,61] either through conformational selection or conformational induction or both.[87] The resulting ligand-receptor pairs in turn have different efficacies to couple to and activate the different effectors and the connected cellular signaling pathways (Figure 8)[63,97,98], which phenomenon is known as functional selectivity or ligand bias and compounds exhibiting functional selectivity are called biased ligands.[99]

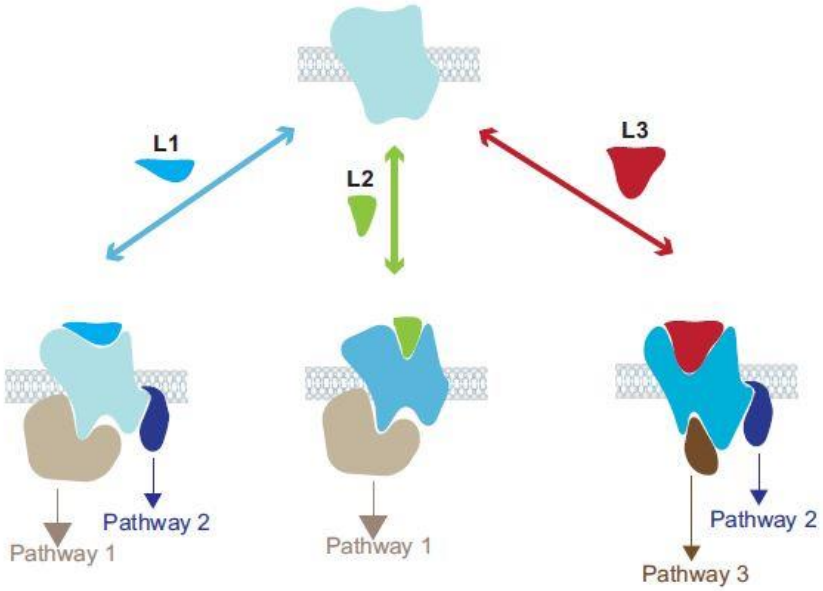


Figure 8. Functional selectivity: different ligands (L1, L2, L3) activate different subsets of all signaling pathways with different efficacy[100]

Ligand bias can manifest between different G protein pathways[90,94,101,102] as well as between G protein-mediated and arrestin-mediated signaling.[63,90,94] For the

conformational origin of functional selectivity experimental evidence has been collected in a number of structural studies over the years. Fluorescence-based[103], ^{19}F NMR[104], X-ray[105–107], and cryo-EM[108] studies revealed both in the case of secretin family[103,108] as well as rhodopsin family GPCRs[104–107] that binding of biased-ligands is accompanied by ligand-specific conformational changes in the transmembrane and extracellular regions, primarily in TM6, TM7 and ECL2 regions. These and other structural studies[65,96,109–112], some also focusing on the G-protein part of the complex[65,113–115] have substantially supported our comprehension of the coupling preferences of different receptors and different receptor-ligand pairs that would eventually materialize in different functional outcomes. However, probably due to the lack of a general and complete understanding of the whole receptor activation process[110], these revolutionary structural discoveries have not yet been translated into more refined mathematical models. Currently, the phenomenon of functional selectivity can best be interpreted mathematically by allosteric models like the extended ternary complex model and the cubic ternary complex model, considering GPCRs as allosteric proteins with regards to Ligand–Receptor–Signaling protein (e.g. heterotrimeric G protein) ternary complex. Along this line, the probe dependency of the allosteric interaction between the ligand and the signaling protein can explain the different efficacy of different ligands toward the different signaling partners.[89]

In addition or in combination with the conformational basis of receptor activation and ligand efficacy, the differences in the apparent efficacy of ligands for the different signaling pathways and thus the perception of signaling bias, too, might be affected by temporal effects as well: differences in ligand binding kinetics, in the rate of transition between conformational states, in the nucleotide exchange rate and in the time-course of the parallel signaling pathways might all influence the observed response.[60,87,116,117]

1.2.4.1. Quantification of bias

For the quantification of bias different approaches and philosophies evolved in parallel in the field. The ‘transduction coefficient’ (τ/K_A) approach favored by Kenakin [99,118] is based on the Black-Leff operational model[88] and in cases where the slope of the concentration-response curves (CRC) equals unity, it is reduced to the ‘relative activity’ method (ratios of E_{max}/EC_{50} values)[99] proposed by Ehlert[119,120]. The σ_{lig} (and β_{lig})

method praised by Rajagopal[121] also has its roots in the operational model; however, it is only applicable if the 'functional' affinity of the ligand does not change across the different pathways.[99,121] The common amongst all approaches is that parameters chosen for the quantification of bias (τ/K_A; E_{max}/EC_{50}, σ_{lig}) are normalized to an arbitrarily chosen reference ligand for each pathway to avoid observational and system bias caused by the differing sensitivities of the different signaling pathways, and then these pathway-specific values are subtracted (in log scale) or divided (in anti-log scale).[99,119–121] Although there is some theoretical debate as to the best approach to quantify bias[118,122], in most cases the bias factors delivered by the different methods are in general comparable.[90]

1.2.4.2. Therapeutic relevance of functional selectivity

Biased agonists have been observed for many therapeutically interesting GPCRs, including μ-opioid, β_2-adrenergic, D_2 dopamine, H_4 histamine, AT_1, PTH1, 5-HT_{2A} and 5-HT_{2C} receptors.[63,79,94,100] There is growing evidence that different downstream signaling pathways lead to different physiological effects suggesting that functionally selective engagement of certain pathway(s) could grant therapeutic advantages by separating therapeutic effect from side effect[63,79,123–125], as exemplified below.

μ-opioid receptor represents an instance where G protein-controlled signaling is deemed to confer the analgesic effect whereas arrestin-mediated signaling is surmised to be responsible for the unwanted adverse events like respiratory suppression and constipation.[126] Through spectacular medicinal chemistry work applying structure-functional selectivity relationship (SFSR)[127], Trevena Inc. developed a G protein-biased agonist (TRV-130) against moderate-to-severe acute pain. In clinical trials the compound showed marked analgesic effect with a lower incidence of adverse events[128] that led to FDA approval in 2020.[129] Interestingly, the most recent studies argued that the *in vivo* profile of TRV-130 is conferred by the low intrinsic efficacy rather than the biased nature of the ligand.[130]

Antagonists against angiotensin II receptor type I (AT_1R) are used in therapy for decades for the treatment of high blood pressure, unfortunately, not without adverse events.[79] In the early 2000s, however, in laboratory experiments it was observed that AT_1R ligands that block only G protein-dependent signaling have diminished side

effects. Thus, it was hypothesized that ligands biased towards β-arrestin can have therapeutic advantage over not biased antagonists.[63,79,123–125,131] The arrestin-biased compound TRV-027 developed by Trevena Inc. after showing promising results in preclinical setup (lowered blood pressure and increased cardiac contractility and performance) entered clinical phase. Although it was entirely safe in Phase I and Phase IIb clinical trials, finally it turned out to be ineffective in the indication of acute heart failure.[132] It is hypothesized that in chronic treatment biased AT_1R agonists might still be beneficial.[82,132] Interestingly, the possibility of repurposing TRV-027 against COVID-19 is currently under evaluation by Imperial College London and Trevena Inc. The compound is being tested for its ability to correct the imbalance of the renin-angiotensin system (RAS) caused by SARS-CoV-2 that in many cases leads to acute lung injury which can progress to acute respiratory distress syndrome.[133]

1.3. Hit discovery for GPCRs

In modern drug discovery the different hit identification approaches are ideally applied in combination to exploit their advantages and potential in a synergistic manner.[11,14] GPCRs, however, being conformationally highly rich membrane-bound protein targets are typically less amenable to such integrated drug discovery solutions than soluble drug targets. Firstly, difficulties with the solubilization and stabilization of GPCRs, just like other integral membrane protein targets, hinder their in-depth investigation using biophysical techniques like SPR, NMR or X-ray. Although thermostabilization through genetic modifications can facilitate biophysical screening of fragments against detergent-solubilized receptors[134,135], this is an extensive and iterative optimization process with uncertain outcome.[136] Besides, even if successful, stabilization in a single, usually inactive conformation as well as the lack of intracellular interacting proteins (especially G proteins) that might allosterically influence the energetic landscape and ligand binding prevents this approach from identifying ligands with diverse activity profile which is especially problematic for agonists that occupy an active, higher energy conformational state of the receptor.[96,111,137–140] Isolation of the receptors in native form would be preferable; unfortunately, the number of such successful attempts reported is scarce.[141,142] The second limitation is that experimental structure determination of GPCRs is much more complicated than that of soluble protein targets. To date, crystal

structure of only ~100 unique[143] receptors have been solved (e.g. β_2-adrenergic receptor) out of ~800 proteins in total[57]. Furthermore, even in case experimental structure is available, the poor representation of the conformational richness of 7-TM receptors in the structural models limits the contribution of structure-based virtual screening initiatives. Although examples of active state structures have started to emerge recently[143–145] and various refinement methods for homology models have also been proposed leading to some success stories [144–146], the current performance of structure-based approaches represents a bottleneck in hit identification for GPCRs in general and for fragment-based lead discovery in particular.

High-throughput screening assays based on a wide variety of assay technologies on the other hand are generally available for the purpose of GPCR hit discovery. A non-exhaustive overview of higher throughput biochemical and functional assays routinely run for GPCR targets[23,68,147] is given in more detail in the next section.

1.3.1. Screening assays for GPCRs

1.3.1.1. Biochemical assays

Biochemical assays in the GPCR field are typically competition binding assays that measure the ability of compounds to displace a tracer ligand from the receptor.[67,147] Besides the classical filtration-based radioligand binding assays[148,149] having only limited throughput due to the mandatory separation step, homogeneous alternatives (coined no-wash assays) allowing higher throughput, like scintillation proximity assays[150] or fluorescent alternatives (Tag-lite by Cisbio)[151] have also been developed in the last two decades. Beyond delivering single concentration activity results from the screen, displacement binding assays in concentration-response mode can also be used for the determination of equilibrium dissociation constant (reciprocal of affinity; in this case inhibition constant K_i) of ligands indirectly through IC_{50} values and the Cheng-Prusoff equation[152]:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

, where [L] is the tracer ligand concentration and K_d is the equilibrium dissociation constant of the tracer ligand.[3,152,153]

Assays monitoring GTP binding to G proteins (usually with non-hydrolysable radiolabeled GTPγS or Europium-labeled GTP analogues) are also cell-free assays but they record the functional event closest to the receptor activation enabling also accurate assessment of the efficacy of ligands in concentration-response mode.[67,154] Although in scintillation proximity or fluorescent format they are adaptable to HTS, it has to be noted that the applicability of GTPγS assays is restricted mainly to $G_{i/o}$ coupled receptors, usually recombinantly expressed in established cell lines, due to the high abundance and the higher nucleotide exchange rate of the $G\alpha_{i/o}$ protein type.[67,154]

1.3.1.2. Cell-based functional assays

Cell-based assays enable the investigation of diverse functional activities of GPCR ligands, implying that in concentration-response mode these complex assays can deliver the potency values of the compounds expressed as EC_{50}/IC_{50} values (or their negative logarithmic values pEC_{50}/pIC_{50}) as well as the efficacy parameter (E_{max} %) usually expressed relative to a reference compound representing 100% response of the system.

For the investigation of GPCRs in HTS mode primarily mammalian cell lines, like CHO-K1 and HEK-293 cells are used with native or recombinantly expressed receptors. In certain cases, for the sake of the detection principle, cells are further engineered to express stably or transiently (1) promiscuous G proteins (e.g. $G\alpha_{16}$ or $G\alpha_{qi5}$) for investigation of GPCRs irrespective of their natural-coupling preferences[67,147,155] or (2) other heterologous proteins as biosensor constructs for monitoring various signaling molecules and events.[147,156]

In cellular HTS assays G protein-dependent signaling following GPCR activation can most conveniently be monitored through the detection of changes in the level of second messengers (cytoplasmic Ca^{2+}, cAMP, IP_3) based on various biophysical principles (fluorescence intensity, fluorescence anisotropy, FRET, BRET, AlphaScreen).[21,23,147,153]

Ca^{2+} release is most commonly studied in kinetic mode with Fluo-4-AM-based cell permeable fluorophores, the fluorescence quantum yield of which highly increases upon binding to Ca^{2+}. In the case of FLIPR Calcium assay kits (e.g. Calcium 5) by Molecular Devices membrane-impermeable proprietary red-colored food dye additives enhance signal window by quenching background fluorescence (e.g. excreted Ca^{2+} dye, media components, test compounds) and thus eliminate the need for a washing step.[155,157–159]

Alternatively, recombinant expression of the Ca^{2+}-sensitive aequorin photoprotein can also be used for $[Ca^{2+}]_i$ detection.[160]

Changes in cAMP level can be detected in homogeneous format either with proximity-based immunoassays (HTRF or AlphaScreen) in cell lysates[147,161,162] or with recombinant cAMP-sensitive biosensor constructs in live cells (e.g GloSensor)[163]. In the particularly attractive TR-FRET-based HTRF technology, two appropriately selected (donor and acceptor) fluorophores form FRET pair. If biomolecules tagged with these fluorophores are brought into close proximity, in dilute samples predominantly due to molecular interactions then FRET is observed. The long lifetime of donor fluorophore (Eu^{3+} or Tb^{3+}-cryptate complex) allows time-gated reading that improves signal-to-background and the ratiometric detection confers better repeatability and robustness (Figure 9A). Yet, the most important advantage of HTRF is the range of kits available for a wide variety of second messengers (e.g. cAMP, IP_3 through its degradation product IP_1) and intracellular kinases (e.g. ERK1/2, AKT) that offers enormous versatility in studying GPCR signaling.[164] The detection of cAMP in competition assay design by HTRF is illustrated in Figure 9B below.

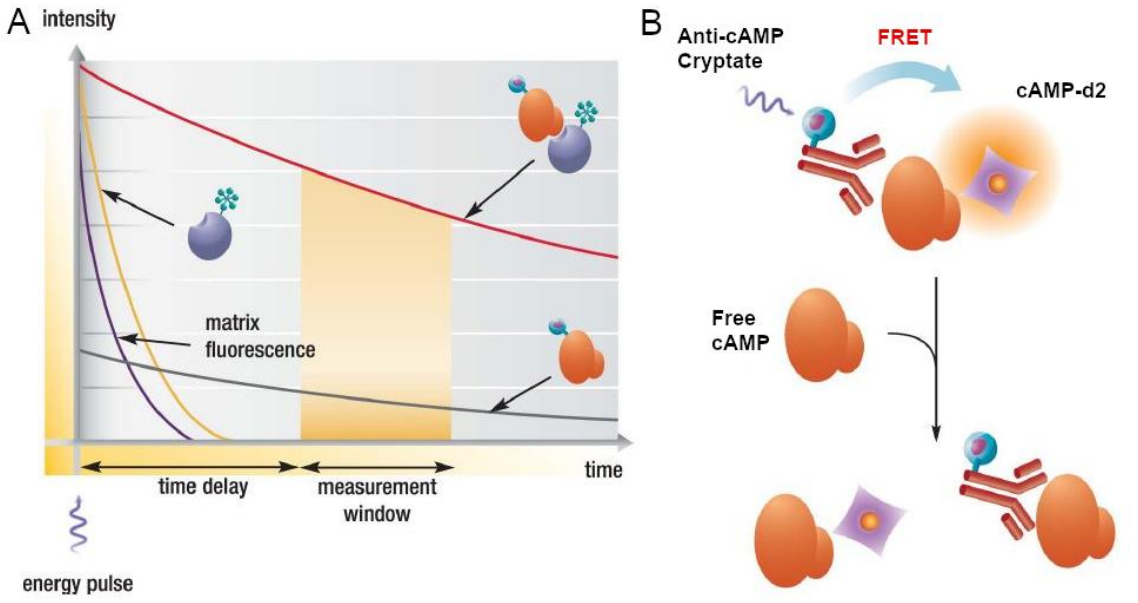


Figure 9. Principle of HTRF technology: (A) detection principle of the TR-FRET-based HTRF technology; (B) cAMP detection with HTRF is based on competition between the acceptor-coupled cAMP (cAMP-d2) and the cAMP released from the cells after lysis[164]

Besides the direct detection of second messengers, reporter gene assays based on the observations that G proteins selectively activate various transcription factors are also available for monitoring G protein activation: the G_q pathway activates the NFAT transcription factor through the Ca^{2+}-calmodulin-calcineurin axis whereas cAMP accumulation activates the CREB transcription factor.[165]

In contrast to G protein-dependent pathways, direct monitoring of the signaling events mediated by β-arrestin is currently not possible. Therefore, arrestin involvement in GPCR activation is typically monitored through the translocation of cytosolic arrestin to the receptor using proximity-based biosensors most commonly constructed based on the principle of structural complementation (e.g. PathHunter[166]), or bioluminescence resonance energy transfer (BRET) between the donor-tagged receptor (e.g. Rluc tag) and the acceptor-tagged arrestin (e.g. GFP/YFP-tag).[167] In addition, just recently, TR-FRET-based detection of the β-arrestin-2–AP-2 interaction in sandwich immunoassay format became also available for studying the first steps in internalization (β-arrestin recruitment kit by Cisbio).

All the above described assay technologies utilize labeled proteins or external reagents for highly sensitive and specific detection of changes in a single, predefined intracellular component. In the last two decades an alternative approach, termed ‘label-free’ technology based on either optical or impedance-based detection principle became also available. These detection methods monitor the changes in subcellular structural organization upon receptor activation and thus are not committed to any particular downstream pathway.[168] The optical label-free technology developed by Corning utilizes special plates with resonant waveguide grating (RWG technology) and detects the change in the wavelength of the reflected light due to a change in the refraction index near the plate surface (penetration depth ~200 nm) caused by the so called dynamic mass redistribution (DMR) within the cells adhered to the plate surface triggered by receptor activation (Figure 10).[169] The resulting DMR signal is an integrated cellular response which can be recorded in kinetic or end-point mode.[170] The kinetic mode although at lower throughput can provide higher pharmacological information content as in certain cases the response can be deconvoluted to components of the individual signal transduction pathways[171], whereas in end-point mode the technology is amenable to larger scale screening campaigns, however, in this case one

can expect to observe activity primarily in the main anticipated pathway as only a snapshot of the signaling events are recorded at the selected time-point after receptor stimulation.[169,170,172–174]

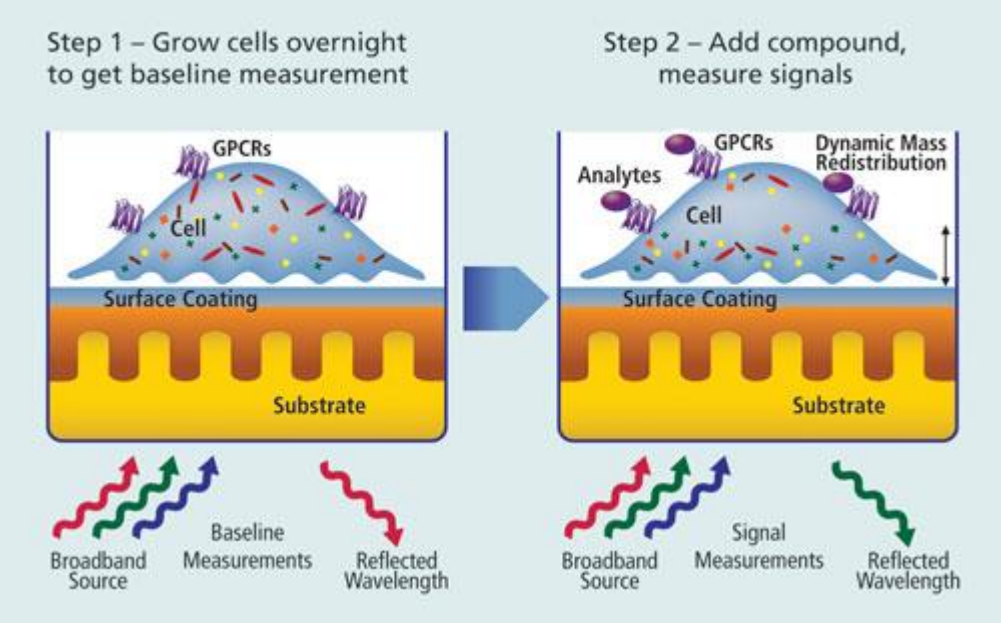


Figure 10. Principle of the optical label-free dynamic mass redistribution technology[175]

1.3.2. Considerations for the selection of screening assays

As illustrated above there is a broad repertoire of assays and detection technologies available for GPCR screening. For the selection of a primary screening assay for a given mechanism of action, from the numerous different aspects to be taken into account, some general considerations are outlined below focusing primarily on functional assays:

1.) Assay quality and throughput: pharmacologically sensitive and low variability simple ‘mix-and-read’ assays that can be miniaturized to at least 384-well plate format are better suited for HTS applications than heterogeneous assays with multiple fluid exchange steps.[21,153]

2.) Proximity to the event of receptor activation: multiplication of the signal in the signal transduction cascade can saturate the detection system which in turn can hinder the ability of the assay to resolve efficacy of compounds in a broad range.[99,100] Readouts monitoring events closer to the receptor activation (e.g. at the second messenger level) are thus favored to far downstream events like reporter gene activation. As additional

benefit, this also serves the elimination of primary actives that act on components of the signaling pathways other than the receptor (i.e. false positives).

3.) Detection technology: detection approaches requiring no or minimal perturbation of the cellular physiology is preferred (HTRF, AlphaScreen, Ca^{2+} dyes, label-free) over systems that are engineered in favor of the detection mode. GloSensor technology for cAMP measurements, PathHunter enzyme fragment complementation for studying arrestin recruitment and aequorin-based Ca^{2+} assays all require recombinant expression of the detection biosensor or the interacting partners whereby the stoichiometry of the components occurring naturally can be disturbed which might lead to false interpretations.[99,176] Sometimes even the choice between technologies detecting the same analyte is difficult, like in the case of the proximity detection-based HTRF and AlphaScreen immunoassays. As an example, the experience of the HTS team at AstraZeneca shows that HTRF is more successful than AlphaScreen at least for cAMP detection.[177] Also, in our practice, the extreme light sensitivity of AlphaScreen detection reagents renders this technology rather unfavored.

4.) Economic aspects: detection reagents and in some cases special types of consumables (e.g. DMR plates) are relatively costly. Considering that in an HTS campaign hundreds of thousands of assay points are measured, assays amenable to cost reduction (like miniaturization of assay volume, dilution of reagents) are appreciated.

5.) Biological relevance: systems more closely reflecting the physiological/pathological background of the disease state are regarded as preferred especially in conditions with complex pathomechanisms (e.g. use of cells differentiated from induced pluripotent stem cells (iPSC) in psychiatric or cardiovascular diseases).[178–180] This goal is the most difficult to achieve taken into account the above listed aspects as well as the complications in generating and maintaining such close to natural systems.

1.4. Challenges and perspectives of hit discovery against GPCRs by HTS

As seen in the previous section, for the GPCR target class, due to scarcity of experimental structures needed for virtual and fragment screening as well as to difficulties with sensitive biophysical screening techniques, only conventional high-throughput screening can be considered as a generally applicable hit discovery approach. Regrettably, the success rate of HTS for GPCRs as well as for other target

classes is reported to be only around 50%.[15,181] In general, failure to deliver a sufficient quantity of high quality hits is considered to lie in two major factors: one is the low sampling efficiency of chemical space and the other is the limited sensitivity of the HTS assays. These shortcomings and the reported attempts to provide solutions for them focusing specifically on the GPCR target class are described below.

1.4.1. Sampling efficiency of the chemical space

1.4.1.1. Limited coverage of chemical space

The library size of industrial R&D collections usually lies between 10^5 and a couple of million samples whereas the chemical space of drug-like compounds is estimated[41] to contain molecules in the range of 10^{23} to 10^{60}. It is obvious that even the largest existing libraries represent only a minor portion of the exploitable chemical space. The main limitation of HTS for any target class in general is therefore the low coverage of chemical space provided by the HTS libraries. Although fragment-screening offers a solution for the sampling inefficiency of diversity screening by providing a superior coverage of the chemical space, as discussed above, efficient fragment screening with biophysical techniques have technical and conceptual obstacles for GPCRs[44,141].

1.4.1.2. High concentration screening to improve sampling efficiency of chemical space

In contrast to biophysical fragment screening, testing fragment size compounds at high concentration in *in vitro* biochemical and cell-based assays (i.e. in HTS-like setting) might provide a generic solution for GPCRs. Although such biological assays is expected to have lower analytical sensitivity and the higher screening concentration can yield an increased rate of false positive results due to non-specific effects (precipitation/aggregation, cytotoxicity, optical interference etc.), the approach has already proven its applicability for soluble targets[38,182,183] and there are also a few reports on successful attempts in the GPCR field as follows.

Using radioligand displacement assays, screening of 660 fragments on MC_4 receptor at 1 mM screening concentration[44] as well as 1,010 fragments on H_4 receptor at 10 μM concentration[184] were reported. Both groups applied 50% activity threshold for hit selection and both screens delivered hit rates between 5-10% validating the choice of

screening concentration for the respective type of receptor (peptide hormone receptor vs. biogenic amine receptor).[44,184] The team working on the MC_4 receptor also performed as part of the hit follow-up near neighbor search in the in-house database for generating preliminary SAR, and several compounds with improved potency could be identified. In another work, 248 fragments were tested against adenosine A_3 receptor in a fluorescent ligand displacement assay on live CHO cells at 1 mM.[140] The imaging assay delivered hits with affinities ranging from pK_i=3.97 to 6.44, which was followed by hit elaboration whereby the team demonstrated that by careful exploration of SAR around the best hit, compounds with improved affinity and selectivity can successfully be developed even without accurate structural insights into the binding interactions.[140]

1.4.1.3. Further perspectives on high concentration screening

In all the publications available high concentration screening (HCS) for GPCRs was performed by studying exclusively binding interactions. Although competition binding assays are widely used for established targets, many therapeutically exciting GPCRs lack radiolabeled or fluorescent ligands suitable for hit discovery purposes. Functional assays on the other hand could allow the interrogation of a broader range of targets, and in addition compounds could directly be screened for the desired mechanism of action. At the outset of our work reports on screens actually performed using functional assays were only available for non-GPCR targets[50,185]; HCS utilizing functional cellular assays for hit discovery of GPCRs was still subject to further exploration.

1.4.2. Sensitivity of the screening assay

Besides the sampling problematic of the chemical space, another major factor contributing to the failure of hit and eventually lead generation by HTS is the loss of potential actives due to the insufficient sensitivity of the given screening assay to identify them in the library. The sensitivity (or true positive rate, TPR) of an assay is directly dependent on the false negative rate (FNR; TPR=1-FNR).[55] The occurrence of false negatives due to random factors or systematic technical errors, like instrument errors or other operational factors, can be minimized through careful optimization of experimental conditions, resource allocation and quality control measures.[26,186] However, there are other sources of errors that are inherent to a given screening

technology and cannot be entirely controlled or eliminated in single point screening (e.g. interference with the components of the signaling pathway, with assay reagents or the detection technology). Even in case the same molecular event is detected, different detection technologies (e.g. AlphaScreen, TR-FRET) tend to deliver different hit lists as it was experienced by a Novartis team investigating a nuclear receptor family target[187–190] and also by AstraZeneca HTS scientists in the case of GPCRs[177]. This emphasizes the significance of careful selection of the screening technology (see also 1.3.2. in the case of GPCRs) in minimizing the number of assay-specific false negatives.

1.4.2.1. Ligand bias as extra source of false negatives in the case of 7-TM receptor target class

In addition to the origins of false negatives introduced above in general, in the case of GPCRs the phenomenon of functional selectivity represents an additional target class-specific major source of false negatives in functional assays that cannot be managed by the above described strategies.

Biased agonism has transformed our perception of the functional profile of ligands including those originally classified as antagonists. Indeed, several β-blocker drugs used in the therapy for the treatment of hypertension turned out to exhibit a wide variety of efficacy profiles in various functional assays.[93,97,100,191] It is now increasingly recognized that ligands can activate parallel signaling cascades with different efficacy which alone has a huge impact on the discovery of novel ligands using selected functional readouts[63,97,98,147]: in typical cell-based HTS campaigns for GPCR modulators only a single pathway is monitored of the whole signaling repertoire, which implies that potential actives present in the library that act preferentially on other pathways remain silent as false negatives. This is exacerbated by observations coming from SAR studies on a number of receptors (e.g. D_2 dopamine, H_4 histamine, μ-opioid and β-adrenergic receptors) that demonstrate that even minor differences in chemical structure can cause extreme changes in the functional activity of ligands.[79,100,127,192] In the example highlighted in Figure 11 taken from the structure-functional selectivity-based optimization of TRV-130, it is astonishing to see how moving the methyl group round in the thiophene ring turns the G protein-biased compound **(R)-23** into ‘unbiased’

compound (**R)-24** that loses β-arrestin activity again by shifting the methyl-group to position 5 in **(R)-25**.[127]

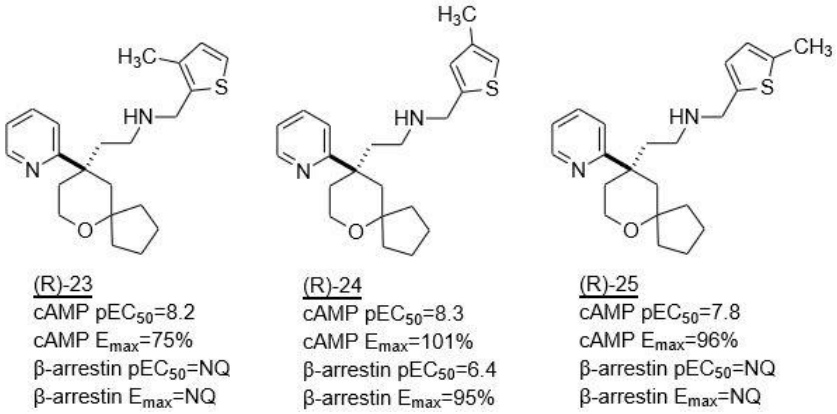


Figure 11. Changes in bias profile caused by small structural changes applied during the development of TRV-130[127]; NQ: not quantifiable

This rather steep and unpredictable structure-functional selectivity relationship combined with the fact that compounds in HTS libraries are typically not evenly dispersed in chemical space, but due to accessibility, economic reasons and early SAR considerations form clusters of structurally similar molecules[46], can have serious consequences on the outcome of HTS: in case none of the representatives of an otherwise active chemotype is identified as active in the very pathway selected for the screen then not only single compounds but complete chemotypes can be lost.[97,100,177]

1.4.2.2. Multiplex assays for reducing the false negative rate in GPCR screening campaigns

Solutions that lower the chance of losing compounds as false negatives in GPCR hit discovery due to functional selectivity are vital to be identified. Apparently, monitoring multiple parallel pathways using functional assays[97,100], analogously to the suggestive illustration in Figure 12 could proffer the best solution. Routinely running separate HTS campaigns, however, is not an economically realistic option: except for a study by the AstraZeneca hit discovery team describing the execution of two HTS campaigns parallel to each other[173], no other such intentional attempts have been reported so far to our knowledge.

Combining orthogonal cellular assays into a single ‘multiplexed’ assay, on the other hand offers the opportunity to perform parallel functional readouts from the same well (selecting specific keys in Figure 12) within a single screening campaign in a more affordable manner.[97,147,193]

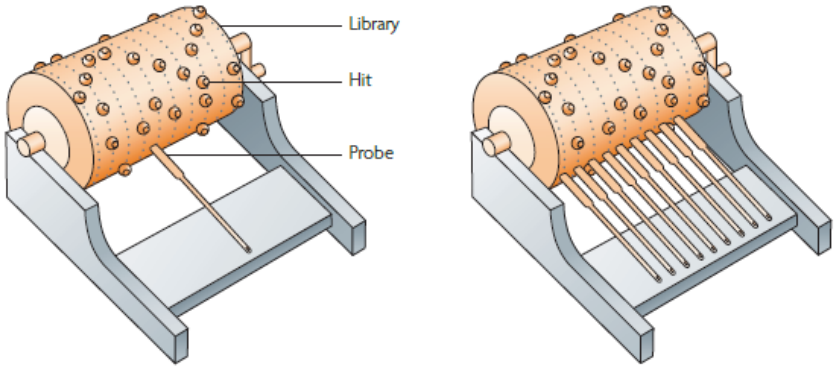


Figure 12. Illustration of the rationale behind multiple-probe screening[97]

Such multiplex assays are supposed to identify ligands showing functional activity in any of the readouts investigated and in addition to recover assay-specific false negatives discussed in 1.4.2., too. By increasing the probability of identifying hit series, this approach is expected to deliver a wealthier pool of starting points for medicinal chemistry for the H2L phase. Furthermore, multiple parallel readouts especially from relevant biological assays could deliver valuable pharmacological information on functional selectivity of complete hit series that might eventually be leveraged as a potential therapeutic advantage, too.[125] Although it is comprehensibly more challenging to optimize an assay with two readouts than two assays separately, the burdens on the resources (except for the detection reagents) and screen-related operations like cell culturing, automation and compound handling are not necessarily additive.

A handful of HTS compatible cellular multiplex assays have already been reported in the literature in the GPCR field as follows. (1) Fluorescent Ca^{2+} measurement was compliant to be combined with a FRET-based beta-lactamase reporter gene assay (GeneBLAzer) to study the activation of muscarinic M_1[194] or melatonin receptor (MT_1) activation[195]. As the two readouts actually represent subsequent downstream steps of the same event, namely the activation of the G_q pathway, the resulting assay can be considered and was indeed published as a semi-multiplex assay serving to eliminate false positives. (2) Researchers at DiscoveRx demonstrated the possibility to read

fluorescent Ca^{2+} responses and subsequently to detect translocation of tagged β-arrestin through enzyme complementation from the same well.[196] (3) Aequorin-based luminometric detection of Ca^{2+} transients enabled the successful multiplexing of G_q pathway with the $G_{i/o}$ or G_s pathways: cAMP signals of $G_{i/o}$ coupled GPR43 receptor[197] were detected by HTRF technology, whereas G_s activation of PTH1 receptors was monitored with GloSensor technology[198]. (4) In another study the GloSensor technology was also combined with FLIPR Calcium 5-based Ca^{2+} detection.[199] (5) An additional multiplex system introduced only recently is the NOMAD by Innoprot[200] that offers recombinant constructs for detection of Ca^{2+}, cAMP, DAG or β-arrestin and any two can be combined into a 2-color green/red fluorescent multiplex.[201]

As an alternative to multiplexing labeled technologies, label-free whole cell assays could also address multiple-probe screening[169,172], as e.g. the dynamic mass redistribution (DMR) technology itself represents a pathway agnostic readout that in end-point mode is even applicable to larger scale screening.[169,172–174] Although label-free assays due to the integrated nature of the response are expected to deliver lower false negative rate, DMR assays, too, suffer from assay-type specific false negatives (and false positives, too). First, in cases where multiple pathways with opposing DMR signal characteristics are triggered, in the resulting DMR signal the underlying signaling events might neutralize each other, leading to false negative results.[147] Second, although our knowledge is sparse yet, investigation of certain biological processes, in particular capturing β-arrestin translocation upon receptor activation is apparently not possible using label-free technology.[202] Setting up and running subsequent full-scale HTS campaigns utilizing labeled assays to reveal false negatives and the mechanism of action of the DMR hits, therefore seems to be desired, unless a label-free assay is directly combined with labeled methods in an analogous way to the multiplexing strategy of selected labeled methods. So far there are only two examples of multiplexing a label-free and a conventional labeled readout: Zhong et al. reported on the combination of fluorescent Ca^{2+} measurement with DMR assay for testing positive allosteric modulators against mGluR4 receptor[203]; whereas Jacobson presented in a poster the multiplexing of DMR with the cAMP measurement on ACTOne cells expressing 5-HT_7 receptors and the CNG channel through the change in membrane potential[204].

It is worth mentioning that aside from functional assays, competition binding assays could theoretically report on all compounds showing binding affinity towards the receptor in a pathway-unbiased manner, however, the difficulties in obtaining suitable, high-affinity tracer ligands for arbitrary GPCRs as well as limitations in the throughput especially for radioligand binding assays restricts their general applicability.[67]

1.4.2.3. Further perspectives on multiplex assays

The few functional assays developed so far based on multiplexing of labeled orthogonal readouts (examples (1) to (5) above) all require genetic engineering in favor of the detection method. Even in the case of the Euroscreen approach that is particularly attractive due to its versatility provided by the use of HTRF technology as second readout, the Ca^{2+} readout relies on the recombinantly expressed aequorin photoprotein. Unfortunately, recombinant biosensor constructs represent a difficulty in system development and their application definitely precludes screening on native or more native-like systems (e.g. iPSC-derived cells). In addition, heterologous protein expression might disturb the normally occurring detection events ultimately leading to false interpretations.

The label-free DMR-based multiplex assays developed up to now[203,204], despite being technically bravura solutions are limited to applications where changes in the intracellular [Ca^{2+}] is expected to occur; furthermore, the cation channel-based CNG technology is particularly liable to extensive compound interference (our unpublished results).

Development of multiplex assays with more generally applicable assay components (fluorescent Ca^{2+}, HTRF, label-free) that do not necessitate heterologous expression of biosensor constructs for the readouts is still missing. Multiplexing strategy with such assays seems to be particularly well-suited for the identification of novel GPCR agonists, whose detection is less straightforward with other approaches like biophysical and structure-based methods.

DOI:10.14753/SE.2021.2563

2. OBJECTIVES

Given their ubiquitous presence and central role in numerous physiological and pathological processes, GPCRs represent an untapped source of potential in drug discovery even today. To improve the efficiency of hit and lead generation by high-throughput screening, in line with and as an extension to the possible solutions described in section 1.4.1.2. and 1.4.2.2., we defined the following objectives.

1.) The Hit Discovery team at Gedeon Richter Plc. set out to investigate if high concentration screening of fragment size compounds using a cell-based functional assay against a GPCR target can form the basis of hit identification activities. Agonists for adrenergic α_{2C} receptors might have therapeutic benefit in various CNS indications, however, selectivity required towards the highly homologous other subtypes of the receptor family (α_{2A} and α_{2B}) is difficult to achieve[205]. In order to discover and develop novel chemotypes, a high concentration cell-based fragment screen was performed using CHO-K1 cells expressing the α_{2C} receptor and a chimeric $G\alpha_{qi5}$ protein enabling Ca^{2+} release to be applied as readout of the functional assay.
Our first objective was to explore whether *in vitro* cellular and biochemical assays can efficiently support hit expansion activities following hit identification by the 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 compounds 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. We considered the following readouts as suitable components of novel multiplex assays:

- Detection of changes in intracellular Ca^{2+} concentration using fluorescent Ca^{2+}-binding dyes offers a non-invasive tool for monitoring activation of GPCRs, in particular but not restricted to those that couple to $G_{\alpha q/11}$ proteins.
- HTRF technology based on TR-FRET principle is available for a broad panel of second messengers and cellular signaling events (IP_1, cAMP, p-ERK, p-AKT etc.) accompanying GPCR activation.
- The optical label-free dynamic mass redistribution (DMR) detection technology reflects an integrated whole cell response following GPCR activation.

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. PTH1R belongs to the family B GPCRs (secretin receptor family) and agonists of this receptor type are applied in the therapy of osteoporosis (e.g. Teriparatide)[206]. As PTH1 receptor activation triggers cAMP accumulation as well as intracellular Ca^{2+} release through natural coupling to both G_s and G_q proteins[207], respectively, it 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 multiplex approach, our next goal was to multiplex the label-free DMR technology with the HTRF readout. For this multiplex assay development CHO-K1 cells expressing α_{2C} adrenergic receptors were used without heterologous expression of additional signaling or detection components. α_{2C} receptor couples primarily to $G_{\alpha i/o}$ protein, thus the system appears to be 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 combinations introduced above from hit discovery perspective, a pilot screen was also performed in both cases with a suitable validation library.

DOI:10.14753/SE.2021.2563

3. MATERIALS AND METHODS

3.1. Hit expansion following high concentration screening against α_{2C} adrenergic receptor

3.1.1. Materials

The pre-assembled core set Rule of Three (Ro3) compliant fragment collection was acquired from Maybridge (Cambridge, UK), whereas the fragment library consisting of 3,071 fragments was purchased from Albany Molecular Research Inc. (AMRI, Albany, NY, USA). All fragments in the AMRI library fulfilled the criteria of $9 \leq N_{heavy} \leq 22$, clogP < 3, HBD ≤ 3 (at pH 7.4), HBA ≤ 3 (at pH 7.4) and containing at least one ring. The fragments were stored at -20 °C dissolved at 50 mM in dimethyl sulfoxide (DMSO). UK 14,304, phentolamine and buffer components were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany).

3.1.2. Cell culture

Generation of Chinese hamster ovary (CHO-K1) cells stably expressing human α_{2C} adrenergic receptors and the chimeric $G_{\alpha qi5}$ protein was described previously.[208] Cells were maintained in Ham's F12 Nutrient Mixture supplemented with L-glutamine containing 10% fetal calf serum (FCS) and 400 µg/mL G418 (Geneticin®) (all purchased from Gibco; Thermo Fisher Scientific, Waltham, MA, USA), 200 µg/mL hygromycin (Invitrogen; Thermo Fisher Scientific, Carlsbad, CA, USA), 2.5 µg/mL amphotericin B, 100 U/mL penicillin G, 100 µg/mL streptomycin, 1x RPMI-1640 vitamin solution and 1x non-essential amino acid mixture (all from Sigma-Aldrich; Merck, Darmstadt, Germany).

3.1.3. Displacement binding assay using [^{3}H]UK 14,304

For the displacement binding assay aliquots of the membrane homogenate prepared from CHO-K1 cells recombinantly expressing human α_{2C} receptor[209] were thawed and diluted in binding assay buffer (Tris 50 mM, pH 7.4 at 22 °C): for each well of a 96-well microplate 40 µg total protein was added in 160 µL final volume. Then, 20 µL of

radioligand [^{3}H]UK 14,304 (Perkin Elmer) diluted in binding assay buffer to a concentration around K_d (5.3 nM) was added in the presence or absence of 20 µL test compound diluted to 10-fold concentration in binding assay buffer. For non-specific binding, the α-adrenergic antagonist phentolamine at 10 µM was used. The plates with 200 µL reaction mixtures were then incubated for 30 min at 22 °C. After incubation the content of the wells was filtered through UniFilter GF/C using a Filtermate Harvester (Perkin Elmer) and the plates were washed 5-times each with 1 mL binding assay buffer. The plates were then dried for 60 min at 50 °C and finally 20 µL Microscint-20 (Perkin Elmer) scintillation cocktail was added to the wells before reading in a MicroBeta counter (Perkin Elmer).

3.1.4. Fluorometric Ca^{2+} measurement (agonist and antagonist mode)

Fluorometric measurements of cytoplasmic calcium concentration ($[Ca^{2+}]_i$) were carried out in $\alpha_{2C}/G_{\alpha qi5}$ expressing CHO-K1 cells. 25 µL per well of a $5x10^5$ cells/mL cell suspension was plated in culture medium in standard tissue culture-treated 384-well microplates (Corning, Corning, NY, USA) and maintained overnight in a tissue culture incubator at 37 °C and 5% CO_2. Next day the wells were washed with assay buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose) complemented with 2 mM probenecid (wash buffer) using a BioTek ELx405 automated plate washer (BioTek, Winooski, VT, USA) leaving the cells in 20 µL wash buffer. The cells were then loaded with 20 µL/well FLIPR Calcium 5 dye (FLIPR Calcium 5 Assay kit, catalog number R8186; Molecular Devices, San José, CA, USA) diluted 2-fold in wash buffer for 25 min at 37 °C and 5% CO_2. After the incubation the plates were put into a FLIPR^{TETRA} plate reader (Molecular Devices) set at 37 °C. Baseline fluorescence was recorded for 15 s followed by addition of 20 µL 3-fold concentrated reference agonist/test compound (in assay buffer with 3% DMSO) by the in-built 384-channel pipettor head and the evoked fluorescent signal was detected for further 55 s at 1 s intervals. In the FLIPR^{TETRA} instrument the excitation at 470–495 nm is provided by a LED bank pair whereas the emitted light band-filtered at 515–575 nm is collected with a sensitive CCD camera.

In the case of the α_{2C} antagonist measurements, following dye loading for 10 minutes, the cells were pre-treated with 20 µL 4-fold concentrated test compounds/vehicle (assay

buffer with 4% DMSO) for 15 min before stimulation with 20 µL 4-fold concentrated reference agonist UK 14,304 (in 1% DMSO) at 30 nM final concentration, corresponding to an approx. EC_{80} concentration. Inhibitory activity of the compounds was expressed as percent inhibition of the response of UK 14,304 after correcting for the vehicle effect.

3.1.5. Data analysis

In the radioligand binding assay the activity of test compounds (inhibition %) at a given concentration was determined by normalizing to the average of the positive control wells after subtraction of the average of the wells reserved for non-specific binding. The concentration dependent displacement of the tracer ligand by the compounds was determined using a minimum of six concentrations in duplicates and experiments were repeated twice. IC_{50} values were obtained from displacement curves by sigmoidal fitting of the data points using Origin 6.0 software (OriginLab, Northampton, MA, USA). 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.

For the fluorometric Ca^{2+} measurements raw fluorescence data were transformed to $\Delta F/F_b$ values in Microsoft Excel (Microsoft, Redmond, WA, USA): baseline fluorescence (F_b) was subtracted from peak fluorescence and the difference (ΔF) was divided by baseline fluorescence. EC_{50}/IC_{50} values were obtained by sigmoidal fitting of the data points using Origin 6.0 software.

3.2. Fluorometric Ca^{2+} - HTRF cAMP multiplex against PTH1 receptor

3.2.1. Materials

Human PTH (1-34) and PTH (2-38) peptide ligands were obtained from Bachem (Bubendorf, Switzerland); probenecid, 3-isobutyl-1-methylxanthine (IBMX), sodium-pyruvate, antibiotic-antimycotic solution, puromycin and buffer components were from Sigma-Aldrich (Merck), whereas DMEM medium, FCS and trypsin solution were purchased from Gibco (Thermo Fisher Scientific). In the assay development phase the reference agonist PTH (1-34) was dissolved in H_2O at 200 µM concentration and serial dilutions were applied for the preparation of concentration-response curves. For

pharmacological validation of the finalized assay, analogously to the composition of the compound library, peptides were dissolved in DMSO at 600 μM and diluted further in DMSO. The final DMSO concentration applied was 0.5%.

3.2.2. Cell culture

HEK-293H-CNG cells stably expressing the human PTH1 receptor and the cyclic-nucleotide gated channel (CNG channel, component of ACTOne technology) obtained from Atto Bioscience (currently BD Biosciences, San Jose, CA, USA) were cultured in DMEM supplemented with 10% FCS, 1% sodium-pyruvate, 1% antibiotic-antimycotic solution and 1 mg/L puromycin.

3.2.3. Fluorometric Ca^{2+} measurement

For the low volume measurements applied in the final multiplex protocol, 8 μL per well of a 10^6 cells/mL cell suspension in cell culture medium without FCS was seeded into low volume PDL-coated (poly-D-lysine) 384-well plates (Corning) with a Multidrop Combi dispenser (Thermo Scientific) and the plates were incubated overnight in a tissue culture incubator at 37 °C and 5% CO_2. On the day of the measurement 8 μL of FLIPR Calcium 5 dye (FLIPR Calcium 5 Assay kit, catalog number R8186; Molecular Devices) in assay buffer was added to the wells with a Multidrop Combi dispenser. To eliminate interference due to CNG-mediated calcium influx[210], the assay buffer (140 mM NaCl, 5 mM KCl, 10 mM D-glucose, 10 mM HEPES, 2 mM probenecid, pH=7.4) contained no divalent cations and was supplemented with 2 mM EGTA (ethyleneglycoltetraacetic acid) that chelates Ca^{2+} present in cell culture medium. In addition, the phosphodiesterase inhibitor IBMX at 100 μM concentration was also included in the assay buffer for the sake of the subsequent cAMP determination by HTRF in the multiplex setup.

After an incubation step of 60 min at 37 °C and 5% CO_2, the plates were put into a $FLIPR^{TETRA}$ instrument where baseline fluorescence was recorded for 10 s followed by the addition of 4 μL 5-fold concentrated reference agonist/test compound by the in-built 384-channel pipettor head and the evoked fluorescent signal was detected for further 100 s at 1 s intervals.

For conventional Ca^{2+} measurements, 20 µL per well of a 10^6 cells/mL cell suspension was seeded into standard volume PDL-coated 384-well plates (Corning). On the day of the measurement both the FLIPR Calcium 5 dye as well as the 3-fold concentrated reference agonist were added at a volume of 20 µL. Other circumstances were identical.

3.2.4. HTRF cAMP assay

During the assay development phase, for suspension measurements untreated low volume solid white 384-well plates (ProxiPlate-384 Plus; Perkin Elmer) and tissue culture-treated low volume clear bottom black 384-well plates (Corning) were used. In order to harmonize the protocol with the fluorometric Ca^{2+} measurement, first 6 µL/well assay buffer was added to 6 µL/well of a 10^6 cells/mL cell suspension in either assay buffer or culture medium and the plates were incubated for 60 min incubation at 37 °C 5% CO_2, followed by the addition of 3 µL 5-fold concentrated PTH (1-34). After 15 min stimulation time, cells were lysed by the addition of 4 µL cAMP-d2 and 4 µL Anti-cAMP-Eu^{3+}-Cryptate solution diluted 1:20 in cAMP & cGMP conjugates and lysis buffer (cAMP dynamic 2 kit, catalog number 62AM4PEC; Cisbio Bioassays, Codolet, France; Perkin Elmer). Finally, after 60 min incubation at room temperature, the plates were put into an EnVision Xcite (Perkin Elmer) multimode plate reader equipped with a Xenon flash lamp and HTRF filters. After excitation at 320 nm, fluorescence was detected at 665 nm and at 620 nm with 100 µs delay and 300 µs time window. For solid white plates top reading was applied, whereas for clear bottom black plates a bottom reading protocol was developed and applied.

3.2.5. Final multiplex assay

In the final multiplex setup, initial steps of the protocol were identical to the miniaturized Ca^{2+} measurement described above. For the adherent cAMP measurements, 15 min after the reference agonist/test compound addition and reading by $FLIPR^{TETRA}$, 5 µL cAMP-d2 and 5 µL Anti-cAMP-Eu^{3+}-cryptate HTRF detection reagents diluted 1:20 in cAMP & cGMP conjugates and lysis buffer were added to the wells and incubated for 60 min at room temperature followed by reading in the EnVision Xcite reader. Quality of the assay was assessed by calculating the Z' statistical parameter for both readouts, as well as the $\Delta F/F_b$ for the Ca^{2+} assay and the

signal-to-background value (S/B) for the HTRF readout using the vehicle controls and maximal tested concentration of the reference compound PTH (1-34). The pharmacological sensitivity of the assay was monitored through the pEC_{50} value of the PTH (1-34) derived from its concentration-response curve.

3.2.6. Pilot screening

In total 1,895 compounds on 6 low volume 384-well plates were screened with the final multiplex protocol for their agonist activity at 10 μM final concentration with 0.5% DMSO present. 1,255 compounds originated from the LOPAC library (Library of Pharmacologically Active Compounds; Sigma-Aldrich; Merck) and further 640 diverse compounds were selected from the corporate compound collection. Wells in columns 2 and 23 on each plate were spared for negative control containing only vehicle as well as positive controls with PTH (1-34) either at supramaximal (1 μM) or at submaximal (200 nM approximating EC_{50} concentration) concentration. Z' was calculated from the supramaximal control wells and the negative control wells. Activity of test compounds in the pilot screen was determined by normalizing to the average $\Delta F/F_b$ and HTRF ratio values of positive and negative control wells (activity%). Activity threshold of Mean+3×SD was calculated from activity values of the complete tested compounds set, and compounds exceeding this threshold (i.e. primary actives) were confirmed on 3 separate plates in 4 parallels with the same multiplex assay protocol.

3.2.7. Specificity testing

Specificity of confirmed hits was tested on HEK-293 cells expressing human recombinant TRPM8 ion channels. This counter-screen was performed separately for the Ca^{2+} and the HTRF cAMP readouts in 3 parallels on 2 separate days in standard volume clear-bottom black plates and low volume solid white plates, respectively. For the Ca^{2+} measurements 20 μL per well of a 10^6 cells/mL TRPM8 HEK-293 cell suspension was seeded on standard volume 384-well plates (Corning) and the plates were incubated overnight in a humidified atmosphere at 37 °C and 5% CO_2. Next day 20 μL FLIPR Calcium 5 dye in assay buffer was added to the wells with a Multidrop Combi dispenser. The plates were incubated with the dye for 45 min at 37 °C and 5% CO_2. Then, the plates were put into the $FLIPR^{TETRA}$ instrument, where baseline

fluorescence was detected for 10 s that was followed by the addition of 20 µL 3-fold concentrated positive control/test compounds by the in-built 384-channel pipettor head and the evoked fluorescent signal was detected for further 120 s at 1 s intervals. As positive control, ATP (adenosine triphosphate) known to act on endogenous purinoreceptors in HEK-293 cells was applied at a final concentration of 50 µM. As negative control 0.5% DMSO in assay buffer was used.

The cAMP measurements were carried out in suspension in low volume white 384-well plates (ProxiPlate-384 Plus; Perkin Elmer). First, 6 µL assay buffer was added to 6 µL of a 10^6 cells/mL cell suspension freshly prepared in assay buffer and right after that, cells were treated with 3 µL 5-fold concentrated positive control/test compounds and were incubated for 20 min at 37 °C and 5% CO_2. After 20 min incubation at 37 °C and 5% CO_2 cells were lysed with 4 µL cAMP-d2 and 4 µL Anti-cAMP-Eu^{3+}-cryptate solution diluted 1:20 in cAMP & cGMP conjugates and lysis buffer and were incubated at room temperature for 60 min. Finally, the plates were put into the EnVision Xcite multimode plate reader and after excitation at 320 nm, fluorescence was detected at 665 nm and at 620 nm with 90 µs delay and 300 µs time window. As positive control forskolin was applied at a final concentration of 100 µM. As negative control 0.5% DMSO in assay buffer was used.

3.2.8. Data analysis

$\Delta F/F_b$ values for data evaluation of the Ca^{2+} assay were calculated in Microsoft Excel as described earlier. For the cAMP measurement HTRF 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 (GraphPad Software, San Diego, CA, USA). Unless otherwise indicated, data are plotted as Mean±SD calculated from 2-3 independent experiments run in quadruplicates.

3.3. Label-free DMR - HTRF cAMP multiplex against α_{2C} receptor

3.3.1. Materials

G418 was obtained from Calbiochem (Merck Millipore, Billerica, MA, USA); Forskolin (FSK), UK 14,304, 3-isobutyl-1-methylxanthine (IBMX), antibiotic-antimycotic solution, MEM Non-essential Amino Acid Solution, RPMI 1640 Vitamins Solution and buffer components were purchased from Sigma-Aldrich (Merck), whereas Ham's F12 medium, FCS and trypsin solution were procured from Gibco (Thermo Fisher Scientific).

3.3.2. Cell culture

CHO-K1 cells stably expressing the human α_{2C} adrenergic receptors (Perkin Elmer; formerly Euroscreen, Brussels, Belgium) were cultured in complete medium (Ham's F12 medium containing 10% FCS, 1% MEM Non-essential Amino Acid Solution, 1% RPMI 1640 Vitamins Solution, 1% antibiotic-antimycotic solution and 400 µg/ml G418).

3.3.3. HTRF cAMP assay

For cAMP measurements on adherent culture 10 µL per well of a $8x10^5$ cells/mL cell suspension in complete medium was plated on 384-well low volume white tissue culture-treated plates (Greiner Bio One, Kremsmünster, Austria) and the plates were incubated overnight at 37 °C and 5% CO_2. To mimic the special conditions required for the label-free DMR measurement[211,212], the next day cell culture medium was exchanged for 10 µL assay buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, 20 mM HEPES pH=7.4, complemented with 100 µM 3-isobutyl-1-methylxanthine). Cells were then stimulated with 2.5 µL 5-fold concentrated UK 14,304 reference agonist in concentration-dependent manner in the presence of different concentrations of forskolin. After 5-45 min stimulation time cells were lysed with 3 µL cAMP-d2 and 3 µL Anti-cAMP-Eu^{3+}-cryptate solution previously diluted 1:10 in cAMP & cGMP conjugates and lysis buffer (cAMP dynamic 2 kit, catalog number 62AM4PEC; Cisbio Bioassays). After 60 min incubation at room temperature,

plates were read in an EnVision Xcite. After excitation at 320 nm, fluorescence was detected at 665 nm and at 615 nm with 100 μs delay and 400 μs time window.

3.3.4. Label-free DMR assay

30 μL per well of a $6.66x10^5$ cells/mL cell suspension in complete medium were seeded manually on top of 10 μL complete medium (used to pre-soak the wells before seeding) in the wells of fibronectin-coated EnSpire-LFC 384-well cellular assay microplates (Perkin Elmer) and the plates were incubated overnight in a humidified atmosphere at 37 °C and 5% CO_2. On the day of the measurement the complete medium was replaced with assay buffer containing 1% DMSO with a Biomek FX (Beckman Coulter, Brea, CA, USA) automated pipettor leaving 40 μL assay buffer in the wells. The plates were then incubated for 2 h at 28 °C[211,212] inside an EnSpire multimode plate reader (Perkin Elmer) equipped with label-free technology module, followed by baseline reading by the instrument (4 readings; 1 reading cycle of an entire DMR plate takes approximately 90 s). Then 10 μL 5-fold concentrated agonist solution in assay buffer (with 1% DMSO present) complemented with FSK (final concentration 2.5 μM) was added to the wells and the plates were read for further 30 min.

3.3.5. Final multiplex assay

For the multiplex assay the cell seeding step was unchanged, however, the assay was performed with reduced volume compared to regular DMR assay (described above). Thus, in the medium exchange step following overnight incubation, the volume remaining in the wells was 20 μL and the plates were then incubated for 2 h at 28 °C inside the EnSpire reader. Following baseline reading (4 readings, one per 90 s) 5 μL 5-fold concentrated reference agonist/test compounds were added to the wells and the DMR signal was read every 90 s for further 15 min. After DMR reading 12.5 μL cAMP-d2 and 12.5 μL Anti-cAMP-Eu^{3+}-cryptate HTRF cAMP detection reagents diluted 1:20 in cAMP & cGMP conjugates and lysis buffer were added to the wells. The plates were then incubated for 60 min at room temperature followed by reading in the EnVision Xcite plate reader in top reading mode optimized for the label-free DMR assay plate. In the final multiplex setup, except for the addition of the HTRF detection reagent (cAMP-d2 and Anti-cAMP-Eu^{3+}-cryptate), all steps in the protocol were

automated: cell seeding was performed with a Multidrop Combi dispenser (Thermo Scientific), whereas medium exchange and agonist stimulation/test compound addition were carried out with a Biomek FX. Quality of the developed assay was evaluated by calculating the Z' statistical parameter for both readouts, as well as the dynamic range (Δpm) for the DMR readout and the S/B for the HTRF readout using the maximum and minimum signals of the concentration-response curve of UK 14,304. The pharmacological sensitivity of the assay was monitored through the EC_{50} value of UK 14,304 derived from its concentration-response curve.

3.3.6. Pilot screening

In total 1,575 compounds on five 384-well fibronectin-coated label-free DMR assay plates were screened with the final multiplex protocol for their agonist activity at 4 μM final concentration with 1% DMSO present. 1,255 compounds originated from the LOPAC library and further 320 diverse compounds were selected from the corporate compound collection. Columns 2 and 23 on each plate contained negative control wells (vehicle) and positive control wells with UK 14,304 at supramaximal (3 μM) as well as submaximal (20 nM approximating EC_{50} concentration) concentration. Z' was calculated from the supramaximal as well as negative control wells. Pharmacological sensitivity of the assay was monitored by evaluation of the concentration-response curves of UK 14,304 placed into columns 1 and 24 on each plate. Activity of test compounds in the pilot screen was determined by normalizing to average DMR and HTRF ratio values of positive and negative control wells.

3.3.7. Data analysis

In the case of the DMR readout, the signal (e.g. the shift in the wavelength of the reflected light) is expressed in 'pm' values. First, the raw 'pm' values were transferred from the EnSpire software into Microsoft Excel in plate layout mode and then the 'pm' values corresponding to the time-point of the peak response of UK 14,304 were extracted from the kinetic traces. Peak response was observed at ca. 3 min after stimulation which corresponded to the 2^{nd} readout cycle when measuring whole plates (e.g. during the pilot screen).

DOI:10.14753/SE.2021.2563

For the cAMP measurement HTRF ratio was calculated by dividing raw fluorescence values measured at 665 nm by 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. Unless otherwise indicated, data are plotted as Mean±SD derived from 3-4 replicate wells.

DOI:10.14753/SE.2021.2563

4. RESULTS

4.1. Hit expansion following a high concentration screening campaign for the identification of α_{2C} receptor agonists

High concentration screening of 3,071 fragments from the AMRI library and 160 diverse compounds from the Maybridge Rule of Three core set at 250 μM in a functional receptor activation assay previously undertaken on CHO-K1 cells expressing the human α_{2C} receptor and chimeric $G\alpha_{qi5}$ protein identified in total 17 hits. Following K_i and EC_{50} determination, hit compounds were also characterized with ligand efficiency parameters to assist with the prioritization of fragment hits. In addition, subsequent to the experimental screening, virtual screening was also performed on the same compound set by CADD (computer-aided drug design) experts using a homology model of the α_{2C} receptor built upon the agonist-activated β_2-adrenergic receptor X-ray structure as template[213]. The first 1% compounds (corresponding to 30 fragments) based on docking scores were selected as actives and 2 out of them were concluded as VS hits as they were present in the original hit list, too.[209]

As hit follow-up after the screening campaign, hit expansion was undertaken: the corporate compound library was scanned for close structural analogues ('neighbors') of the most promising primary actives (19 'seed' compounds with $K_i < 10$ μM, $N_{heavy} \leq 22$, LE > 0.3) using the Tanimoto similarity metric[214]. With the molecule size limited to $N_{heavy} \leq 26$ (corresponding to ~350 Da), and the number of analogues per seed molecule maximized at 5, in total 90 compounds were selected (one 1-member, one 4-member, and seventeen 5-member groups) and were then tested in radioligand binding assay at 20 μM. 25 compounds showed higher than 50% displacement of the [^{3}H]UK 14,304 tracer ligand, out of which 9 ligands were sole actives within their respective groups, whereas 16 actives belonged to 4 seed groups (each with 3 to 5 active representatives). Based on the single point determination, estimated displacement IC_{50} values were calculated for the analogues using the logistic function below:

$$inh\% = \frac{Top}{1 + \frac{x_0}{x}}$$

that can be reformulated to

$$x_0 = \frac{x * (Top - inh\%)}{inh\%}$$

where *inh%* is the inhibition percent at 20 μM, *x* is the actual test concentration (20 μM), *Top* is maximum asymptote (100%) of the displacement curve and x_o is the IC_{50} of the ligand in the displacement assay. Compounds predicted to excel their seed counterpart either in affinity (K_i calculated using the Cheng-Prusoff equation) or ligand efficiency (LE) were then selected and investigated in concentration-response manner as well for exact K_i determination. Encouragingly, of the 16 neighbors 12 indeed demonstrated improvement either in K_i or LE and only 4 turned out to be slightly inferior to their seed compound in both metrics (Table 1).

Table 1. Comparison of K_i and LE values of the analogues with that of the seed compounds. The red-green background coloration indicates superiority (*green*) or inferiority (*red*) of the analogue in K_i or LE compared to the respective seed compound. Analogues marked with asterisk (*) were chosen for evaluation in antagonist mode in the functional assay (see also in text).

Seed compound				Analogue compound				
ID	K_i (μM)	N_{heavy}	LE	ID	K_i (μM)	N_{heavy}	LE	IC_{50} (μM)
A	6.2	19	0.37	A1*	0.07	21	0.46	
				A2*	0.83	20	0.41	
				A3*	0.25	21	0.43	10.59
B	1.5	14	0.57	B1*	0.46	16	0.54	
				B2*	0.56	14	0.61	17.24
				B3*	0.24	14	0.65	8.88
C	10.3	17	0.40	C1*	1.46	19	0.42	
				C2	14.9	14	0.47	
D	7.3	22	0.32	D1	28.1	17	0.36	
				D2	13.8	18	0.37	
E	10.4	16	0.42	E1	16.8	16	0.41	
				E2	23.2	20	0.32	
F	0.083	19	0.51	F1*	0.41	16	0.54	
G	5.4	20	0.36	G1	5.40	22	0.33	
H	5.3	20	0.36	H1	7.6	22	0.32	
I	5.8	17	0.42	I1	10.1	19	0.36	

Parallel to the binding studies, the 90 analogues were also run in the functional Ca^{2+} assay at 20 μM. 2 compounds displayed borderline activity (~50%) and 3 compounds showed strong activity but none of these high activity compounds exhibited displacement of the radioligand, therefore these compounds were assumed non-specific

and were not investigated further. Considering also possible alteration in the functional activity, the analogues with the best K_i values (K_i < 1.5 μM, IDs marked with asterisk in Table 1) derived from the binding experiments were also run in antagonist mode in the functional Ca^{2+} assay (against UK 14,304 stimulation) and interestingly, all 8 compounds showed inhibition >40% at 20 μM. Therefore, the compounds were also tested in concentration-response manner but full inhibition curves and thus functional IC_{50} values could only be obtained for 3 of them (analogues A3, B2, B3). The rank order of the 3 compounds remained the same in the functional assay; however, when comparing the functional IC_{50} results of the compounds with the respective K_i values, an approx. 30-fold difference was observed (Table 1 and Figure 13). The origin of this rightward shift might be explained by the substantial differences in affinity of the reference agonist and the antagonist fragments magnified under the hemi-equilibrium conditions characteristic for the fast and transient Ca^{2+} measurement. Compounds B2 and B3 (corresponding to #18 and #19 in Szőllősi et al.[209], respectively) were the analogues of the original partial agonist hit compound B (Table 1 and Figure 13; corresponding to hit #17 in Szőllősi et al.[209]). Interestingly, as it can be seen in Figure 13B, simply by moving the amine group from 'para' to either 'ortho' or 'meta' position, the functional profile of the seed compound changed from agonist to antagonist with parallel increase in affinity.

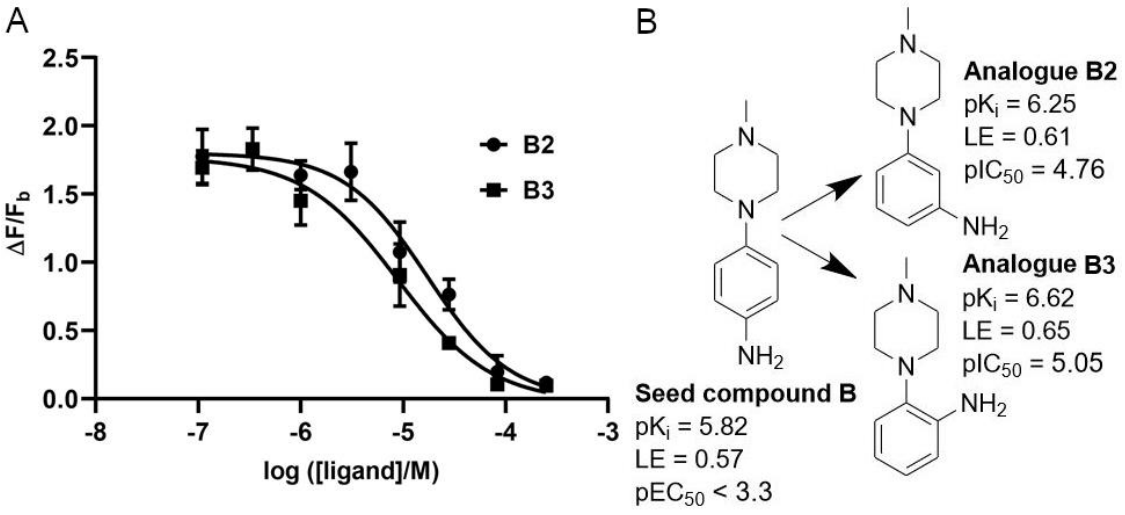


Figure 13. Change of functional profile between seed compound B and its structural analogues. (A) Representative inhibition curves of the analogues B2 and B3; (B) Structure of the partial agonist seed compound B (#17 in Szőllősi et al.[209]) and its analogues B2 and B3 (#18 and #19, respectively in Szőllősi et al.[209]).

Taken together, analogues with increased K_i or LE values were successfully identified, however, they were not pursued further due to their functional antagonist profile.

4.2. Fluorescent Ca^{2+} measurement multiplexed with HTRF technology

For the proof of concept of a multiplex assay combining fluorometric Ca^{2+} measurement and HTRF-based cAMP technology, HEK-293 cells expressing the human PTH1 receptor (naturally coupled to both G_q and G_s) were chosen. This HEK-293 cell line used also expressed a recombinant cyclic nucleotide-gated channel (CNG, component of ACTOne technology) originally designed to facilitate fluorometric Ca^{2+}-based monitoring of G_s or $G_{i/o}$-coupled GPCRs through cAMP-mediated Ca^{2+} influx. However, in our generic multiplex assay principle we intended to detect the natural coupling ability of PTH1R to both $G_{\alpha q}$ and $G_{\alpha s}$ proteins, therefore, interference of the cAMP dependent Ca^{2+} influx with the G_q signaling was eliminated by running the assay in Ca^{2+}-free assay medium complemented with EGTA. This step would not be required when using a generic cell line expressing only the receptor of interest.

As described in detail below, for the development of a HTS compatible multiplex assay, beyond tackling methodological challenges due to the profoundly different assay parameter preferences of the two measurement technologies, pharmacological and economic aspects also needed to be taken into account.

4.2.1. Miniaturization of the fluorometric Ca^{2+} measurement

Fluorometric Ca^{2+} measurements in plate readers with epifluorescent design are recommended to be performed in black plates with clear bottom using adherent cells in a volume of 40-80 µL in 384-well format.[155,157–159] By contrast, HTRF assays are usually run in 10-20 µL reaction volume with top reading in white 384-well plates to avoid signal attenuation. In order to minimize the increased expenses implied by the introduction of HTRF as second readout, it was desirable to keep the assay volume of the Ca^{2+} assay as low as possible. Due to our preference to simple mix-and-read protocols, insertion of an extra liquid aspiration step after the Ca^{2+} readout was not intended (especially at a very low residual volume) as this could have led to compromised reliability and precision. Therefore, the standard Ca^{2+} assay protocol had to be adapted to low volume plate format, which miniaturization work included the

identification of the low volume (LV) plate suitable for the Ca^{2+} assay (black, clear bottom, PDL-coated plates), the optimization of the cell number and cell seeding parameters in the LV plate as well as the adjustment of the readout parameters of the FLIPR^{TETRA} reader. As Figure 14 illustrates, the optimized protocol with 20 µL final volume instead of 60 µL did not alter the assay window or the potency of the reference agonist PTH (1-34) peptide.

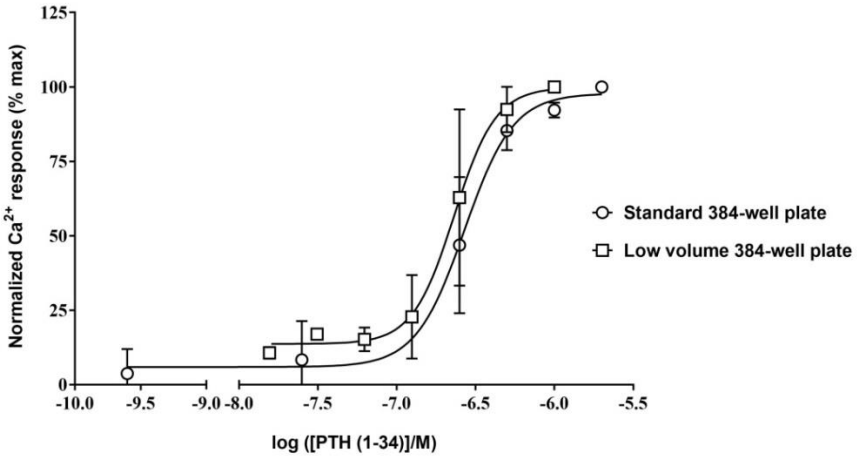


Figure 14. Comparison of the performance of FLIPR Ca^{2+} assay in different plate types and different assay volumes. Concentration-response curves for PTH (1-34) in standard 384-well plate with 60 µL final assay volume (*clear circle*) and in low volume 384-well plate with 20 µL assay volume (*clear square*). Data points represent MEAN±SD from at least two independent measurements.

4.2.2. Development of HTRF cAMP assay under conditions used for FLIPR Ca^{2+} measurement

In TR-FRET applications the time-resolved reading confers specificity due to the complete decay of the background fluorescence by the time of signal detection but at the same time due to the time-delay inserted the target signal is also attenuated (see also Figure 9). The HTRF signal in general can therefore be maximally collected from white plates, and in addition, according to the manufacturer's recommendations the cAMP measurement itself is ideally performed with cells added in suspension in clear assay buffer. Thus, in order to combine HTRF cAMP with the FLIPR Ca^{2+} assay, measurement in adherent mode, detection from black plates and additional interference caused by the colored cell culture medium and calcium assay detection reagent had to be investigated for the cAMP assay.

Preparation of cell suspension in cell culture medium instead of assay buffer did not alter the quality of the cAMP determination in white plates (Figure 15A). Then, in order to minimize the detrimental effect of the black plates on the signal window due to absorption of part of the emitted signal by the walls of the wells, a bottom reading TR-FRET protocol was established and optimized for the black LV plates implemented for the miniaturized Ca^{2+} assay. Although the HTRF signal window in black plate in line with the anticipations was compressed to approximately half of that observed in white plate (Figure 15A), the resulting signal window of approximately 4 was still suitable for further assay development. Thus in the next step, detection of cAMP responses from adherent culture in black plates with or without the interfering Calcium 5 dye was investigated (Figure 15B). Reassuringly, apart from a slight downward shift of the PTH (1-34) curve, the measurements with adherent cells delivered similar results (Figure 15B) to those obtained with cell suspension in the same plate type (without PDL treatment) (Figure 15A and 15B). Unfortunately, in the presence of the Calcium 5 fluorometric dye, the HTRF ratio curve shifted upwards and thus assay quality was seriously compromised by the significant drop in assay window (S/B approx. from 4 to 2, Figure 15B).

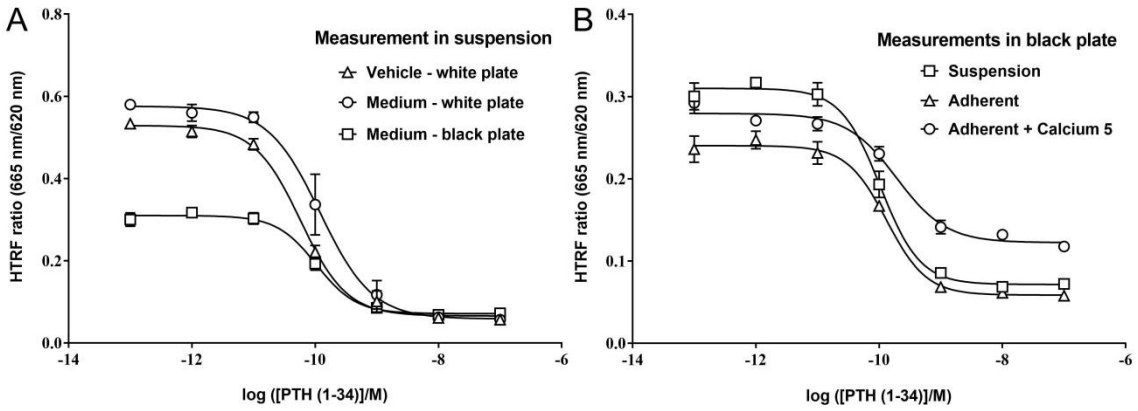


Figure 15. HTRF cAMP measurement in different assay conditions: evaluation of the effect of assay matrix, plate type and suspension vs. adherent mode on HTRF cAMP measurement. (A) Representative concentration-response curves generated by PTH (1-34) with a cell suspension in clear buffer (*clear triangle*), in the presence of cell culture medium in white plates (*clear circle*) and finally in black low volume plates (*clear square*). (B) Comparison of the performance of the HTRF cAMP assay run in suspension mode in black plates (*clear square*, also on Figure 15A) with measurements performed on adherent cells in black PDL-coated low volume plates in the absence (*clear triangle*) and presence of Calcium 5 dye (*clear circle*). Data points represent results of a representative measurement.

4.2.3. Key adjustment step for the realization of the multiplex assay

Recognizing the calcium dye as the major source of interference with the HTRF readout, optimization of the Calcium 5 dye concentration was assessed in both readouts. Unlike the 4-fold dilution, 2-fold diluted Calcium 5 could improve the HTRF assay window without dramatically affecting the signal window of the Ca^{2+} assay, as judged by the trends in the concentration-response curves (Figure 16A and 16B) and by the performance parameters $\Delta F/F_b$, S/B, Z' as well as EC_{50} of PTH (1-34) (Table 2).

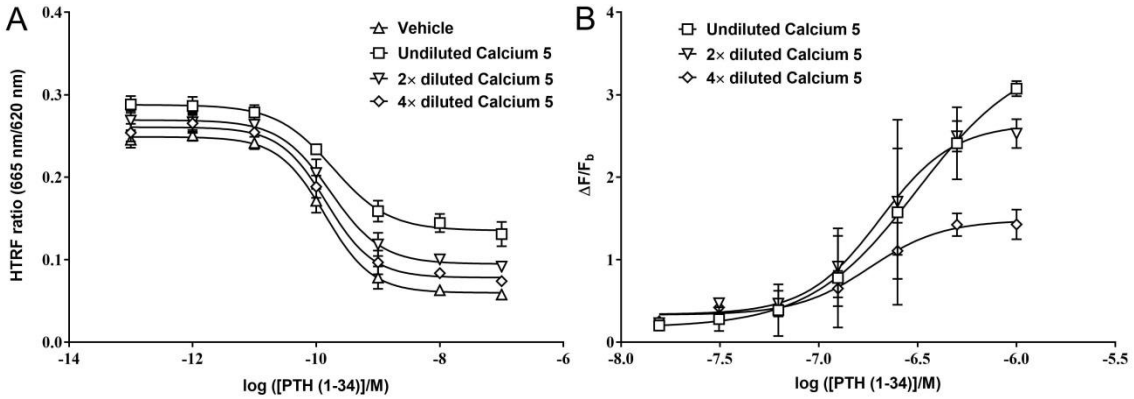


Figure 16. Adjustment of Calcium 5 dilution for the multiplex assay. (A) Concentration-response curves generated by PTH (1-34) in the HTRF assay without Calcium 5 dye (*clear triangle*), with undiluted Calcium 5 (*clear square*), 2× diluted Calcium 5 (*clear inverted triangle*) or 4× diluted Calcium 5 (*clear diamond*) in PDL-coated low volume black plates on adherent cells. (B) Concentration-response curves generated by PTH (1-34) in the fluorometric Ca^{2+} assay with undiluted Calcium 5 dye (*clear square*), 2× diluted Calcium 5 (*clear inverted triangle*) or 4× diluted Calcium 5 (*clear diamond*) in black low volume plates. Data points represent MEAN±SD from at least three independent determinations.

Table 2. Assay window ($\Delta F/F_b$ and S/B), signal separation (Z') and potency (pEC_{50}) of PTH (1-34) obtained in the multiplex assay run at different dilutions of the Calcium 5 dye. The values in the table were calculated from the respective concentration-response curves of PTH (1-34) and represent MEAN values from at least 3 independent measurements.

	Ca^{2+} measurement			**HTRF cAMP measurement**		
	$\Delta F/F_b$	**Z'**	**pEC_{50}**	**S/B**	**Z'**	**pEC_{50}**
Calcium 5, undiluted	3.07	0.89	6.50	2.04	0.54	9.73
Calcium 5, 2× diluted	2.53	0.74	6.69	2.85	0.77	9.77
Calcium 5, 4× diluted	1.43	0.55	6.74	3.54	0.80	9.84
Vehicle				4.16	0.83	9.86

4.2.4. Assay validation

Prior to library screening, pharmacological validity of the final multiplex assay with 2-fold diluted Calcium 5 dye was assessed with two known agonists of differing signaling profile at the PTH1 receptor, PTH (1-34) and PTH (2-38).[215] While PTH (1-34) exerted full agonist activity in both readouts, PTH (2-38) showed pronounced efficacy and potency comparable to PTH (1-34) only in the HTRF readout (G_s pathway). The pEC_{50} values were in accordance with reported values of the respective readout[216,217] and the partial (~30%) efficacy of PTH (2-38) relative to PTH (1-34) in the G_q pathway verified the biased nature of this ligand (Figure 17A and 17B).[215]

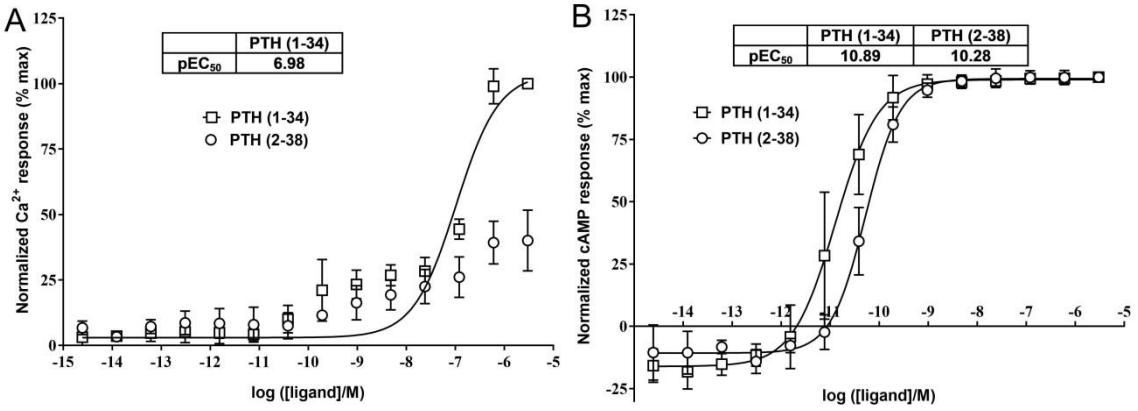


Figure 17. Pharmacological validation of the multiplex assay. Concentration-response curves of PTH (1-34) (*clear square*) and PTH (2-38) (*clear circle*) in fluorometric Ca^{2+} measurement (A) and in subsequent HTRF cAMP readout (B) using the final multiplex protocol. Potency values of the ligands are indicated in table inserts. Data points represent MEAN±SD of at least three independent determinations.

4.2.5. Pilot screen of a validation library

In order to demonstrate the HTS applicability of the multiplex assay, a pilot screen was undertaken with 1,895 small molecules at 10 μM on 6 low volume 384-well plates, including 1,255 compounds from the LOPAC library and 640 diverse compounds from the corporate collection of Gedeon Richter Plc. The LOPAC compound set by Merck is a commercially available library of bioactive compounds widely used in HTS assay validation due to the high probability of finding actives against various pathways and targets. Z' values for every plate exceeded 0.50 in both readouts with an average of 0.66 for the Ca^{2+} and 0.62 for the cAMP readout. Activity thresholds calculated from the activity distribution of the compound set (Mean+3×SD) resulted in 41% and 40% for

the Ca^{2+} and HTRF cAMP readout, respectively, comprehensibly due to the high number of pharmacologically active ligands in the LOPAC library. These threshold values resulted in 18 actives in the FLIPR Ca^{2+} readout and 28 actives in the cAMP assay, with no overlapping hits. Upon retesting them in three independent experiments, all 18 actives in the Ca^{2+} assay were confirmed, while in the cAMP readout 21 compounds reached the threshold (100% and 75% confirmation rates, respectively). All confirmed actives were known bioactive ligands originating from the LOPAC subset thus it was possible to explain the apparent activity of most of the hits through their interference with specific biochemical mechanisms or described activity on receptors reported to be expressed endogenously by HEK-293 cells.[218,219] Not surprisingly, in the Ca^{2+} readout several muscarinic and purinergic receptor agonists were identified, whereas in the cAMP readout besides adrenergic and adenosine agonists, forskolin as direct activator of adenylate cyclase or cAMP itself were also identified. The nonspecific nature of confirmed actives was tested in a counter-screen separately in fluorometric Ca^{2+} as well as HTRF cAMP assays on another HEK-293 cell line expressing TRPM8 ion channels but lacking PTH1 receptors and CNG channels. Except for 3 primary actives in the cAMP readout (activity between 45 and 70%), all other compounds showed activity in the respective readouts of this specificity screen (1 additional sample was found impure). The explanation for the inability of the counter-assay to detect the activity of these 3 documented adenosine ligands might be the decreased sensitivity of HEK-293-TRPM8 cells possibly due to slightly altered adenosine signaling (expression level of the endogenous receptors or G proteins, coupling efficiency to $G_{i/o}$ vs. G_s). This assumption is corroborated by (1) a fourth adenosine ligand that despite being highly active in the primary PTH1R assay (95% activity in cAMP), showed only 30% activity in the specificity testing, as well as by (2) all the other confirmed actives that in contrast to adenosine ligands maintained their ‘cAMP activity’ in the specificity screen.

4.3. Label-free DMR measurement multiplexed with HTRF technology

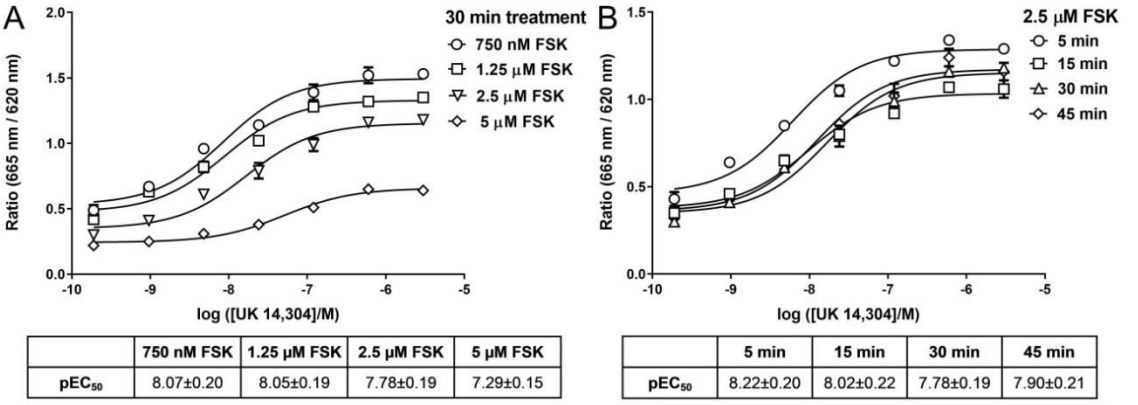
As a model system for the combination of the label-free DMR assay and the HTRF technology, CHO-K1 cells expressing adrenergic α_{2C} receptor with native $G_{i/o}$-coupling were used (i.e. without chimeric G_{α} protein). Although there is only marginal overlap in the optimal parameter space (e.g. plate type, assay volume, adherence) of DMR and HTRF technologies, fortunately, the experiences collected in the Ca^{2+}-HTRF cAMP multiplex assay development could already be exploited here. The key question for the success of the multiplex assay development was thus the interrelation between the kinetics of the DMR whole cell response and the dynamic changes in intracellular cAMP concentration reflected in the HTRF response.

4.3.1. HTRF cAMP assay development

Adrenergic α_{2C} receptor is primarily coupled to $G\alpha_{i/o}$ protein that in turn inhibits the formation of the second messenger cAMP. As basal cAMP level is usually low, in order to obtain a sufficient assay window, its level has to be elevated using the natural product forskolin (FSK), a direct activator of adenylate cyclase. Assay development therefore started with titration of FSK followed by the optimization of the measurement time after stimulation with α_{2C} receptor agonist UK 14,304.

First, CHO-K1 cells expressing α_{2C} receptors were stimulated with UK 14,304 for 30 min with concentrations of FSK ranging from 0.75 to 5 μM. Except for 5 μM, all concentrations of FSK resulted in similar concentration-response curves and UK 14,304 potency values (Figure 18A). As 2.5 μM FSK generated the largest assay window (S/B of 4.1), this concentration was chosen for further experiments. In the next step the dependence of the cAMP assay performance on stimulation time was investigated. Although a slight rightward shift of the concentration-response curve was observed by increasing the stimulation time from 5 to 45 min (Figure 18B), the potency values remained in the range reported in the literature[220] for UK 14,304.

DOI:10.14753/SE.2021.2563



	750 nM FSK	1.25 µM FSK	2.5 µM FSK	5 µM FSK
pEC_{50}	8.07±0.20	8.05±0.19	7.78±0.19	7.29±0.15

	5 min	15 min	30 min	45 min
pEC_{50}	8.22±0.20	8.02±0.22	7.78±0.19	7.90±0.21

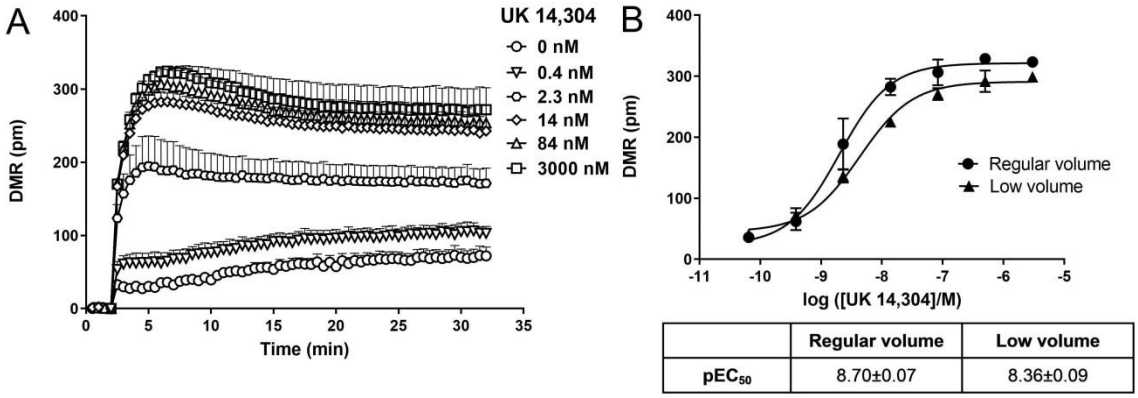
Figure 18. Development of the HTRF cAMP assay: optimization of forskolin concentration and investigation of stimulation time. (A) HTRF cAMP response of α_{2C} receptor-expressing CHO-K1 cells upon stimulation with UK 14,304 for 30 min with different concentrations of FSK (0.75-5 µM) present. (B) cAMP response was monitored at different time-points between 5 and 45 min after α_{2C} receptor stimulation with UK 14,304 in the presence of 2.5 µM FSK. Data points are results of a representative measurement.

4.3.2. Label-free DMR assay development – kinetics and miniaturization

Activation of $G_{i/o}$ pathway normally leads to a transient DMR response.[212] As first priority, it was investigated if upon activation of α_{2C} receptor in the presence of 2.5 µM FSK, a sufficiently high DMR assay window can be obtained within the 40 min timeframe that was tested above for the HTRF cAMP readout. As can be seen in Figure 19A, the supramaximal DMR response was transient with a fast signal increase reaching plateau phase after a few minutes followed by a slowly decreasing signal.

Next, considering that the standard DMR assay volume is relatively high (ca. 50-60 µL) compared to volumes of HTRF cAMP assays (10-20 µL), substantial miniaturization of the DMR assay was undertaken to lower the extra reagent need of the HTRF kit components. Fortunately, leaving only half the volume (20 µL instead of 40 µL) after the buffer exchange step and adjusting the volume of the stimulation step accordingly caused neither a decrease in assay quality nor a dramatic shift in the EC_{50} of UK 14,304 (Figure 19B).

DOI:10.14753/SE.2021.2563



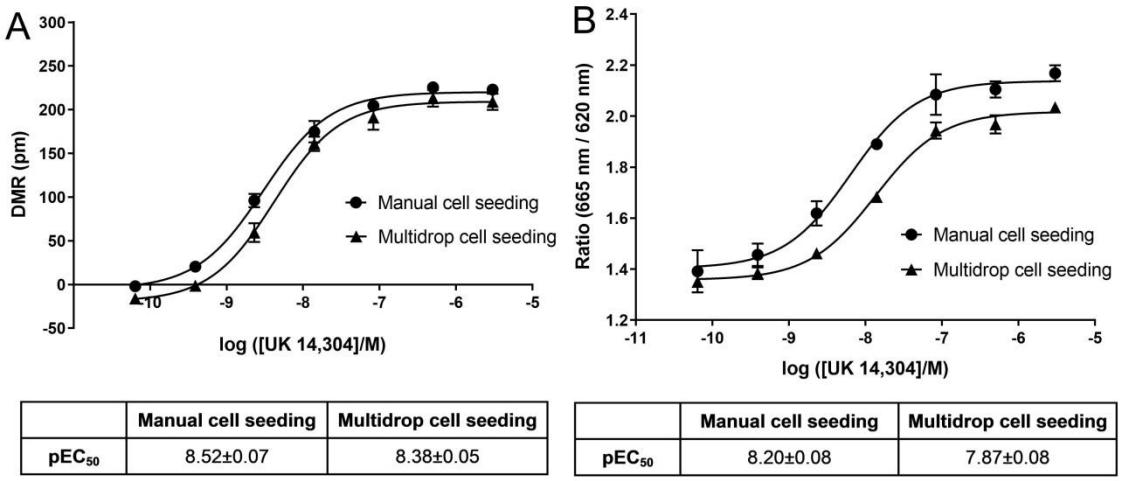
	Regular volume	Low volume
pEC_{50}	8.70±0.07	8.36±0.09

Figure 19. Kinetics and miniaturization of the label-free DMR assay. (A) Typical DMR kinetic traces upon α_{2C} receptor activation with different concentrations of UK 14,304 in the presence of 2.5 μM FSK. (B) Label-free DMR concentration-response curves generated by the agonist UK 14,304 with the standard protocol (*black circle*) as well as with the miniaturized protocol (*black triangles*). Data points are results of a representative experiment.

4.3.3. Development of the multiplex assay

Based on the kinetic traces of the DMR readout, 15 min stimulation time was chosen for the HTRF cAMP assay before the cell lysis step. In order to maximize the HTRF signal from the black-walled DMR plate not designed and intended for HTRF reading, first the amount and concentration of HTRF detection reagents were adjusted which was followed by the optimization of the parameters of the detection protocol (e.g. detection height, number of flashes, delay and measurement time window) of the EnVision reader to the special well-dimensions of the DMR plate.

Finally, in order for the multiplex assay to be suitable for larger scale screening purposes, steps of the assay had to be adapted for automated laboratory equipment. Automated protocols for cell culture medium exchange and the stimulation step for DMR assays were previously established in our laboratory. The adaptation of the cell seeding using an automated dispenser on the other hand required meticulous adjustment (e.g. dispensing height and speed) due to the special well design of the DMR plate. The automated cell seeding protocol finally led to comparable results to manual cell seeding in both readouts, as can be seen in Figure 20 below.



	Manual cell seeding	Multidrop cell seeding
pEC_{50}	8.52±0.07	8.38±0.05

	Manual cell seeding	Multidrop cell seeding
pEC_{50}	8.20±0.08	7.87±0.08

Figure 20. Performance of the final multiplex assay. Label-free DMR (A) and HTRF cAMP (B) readout following α_{2C} receptor activation on CHO-K1 cells seeded manually (*black circle*) or automated with Multidrop Combi (*black triangle*). Data points are results of a representative experiment.

The final multiplex protocol was also characterized for assay window, Z' as well as potency, as validation parameters (Table 3). Although the assay window of the HTRF readout was moderate, Z' and EC_{50} values in both readouts confirmed sufficient signal separation and pharmacological sensitivity of the multiplex assay for screening purposes.

Table 3. Results of the validation of the final multiplex assay

		Label-free DMR	**HTRF cAMP**
Signal window (n=8)	**Dynamic range (Δpm)**	**237**	**-**
	S/B ratio	**-**	**1.46**
Z' (n=8)		**0.84**	**0.66**
pEC_{50} (n=2-3)		**8.38**	**7.87**

4.3.4. Pilot screen of a validation library

To test the ability of the multiplex assay to identify compounds with different activities in the two readouts, a pilot screen on a small validation set including the LOPAC library and 320 diverse library compounds was run at 4 μM final concentration. In total, five 384-well plates were screened with an average Z' of 0.74 and 0.67 for the DMR and cAMP readouts, respectively. EC_{50} values of UK 14,303 calculated from concentration-response wells included on each plate fell in a range from 2.7 to 11.3 nM in the DMR

assay and 4.6 and 14.8 nM in the cAMP readout, confirming the consistent and comparable pharmacological sensitivity of the assay. In addition to screening controls, the sample of UK 14,304 present also in the LOPAC set acted as an internal control and its activity above 90% in both readouts further validated the quality of the screen.

The activity thresholds calculated from the whole compound set were fairly high (DMR: 54%; cAMP: 57%), again due to the high representation of bioactive ligands in the LOPAC library. Thresholds calculated from the negative controls on the other hand, were absolutely in line with expectations (DMR: 8%; cAMP: 9%).

The overall activity of the screened compounds was assessed through a correlation plot. The activity of the 320 diverse library compounds (clear dots in Figure 21A) lay below 10% and 30% in the DMR and in the cAMP readout, respectively. In turn, the 1,255 compounds representing the LOPAC library (filled dots in Figure 21A) exhibited activities covering the entire range between 0 and 100% in both readouts. It is of note, that Ruthenium red, a LOPAC library representative reached 277% activity in the DMR readout (and -3% activity in the cAMP readout), a finding in line with a similar study performed earlier on A431 cells endogenously expressing β_2 adrenergic receptor.[172] This unusually high activity was considered unspecific in nature and this compound was omitted from further analysis. Based on the analysis of the correlation plot (Figure 21A) and frequency distribution (Figure 21B) the multiplex assay was able to identify ligands with distinct activities above 20% in DMR and 30% in the cAMP readout with reasonably good correlation (slope=0.86; R^2=0.73).

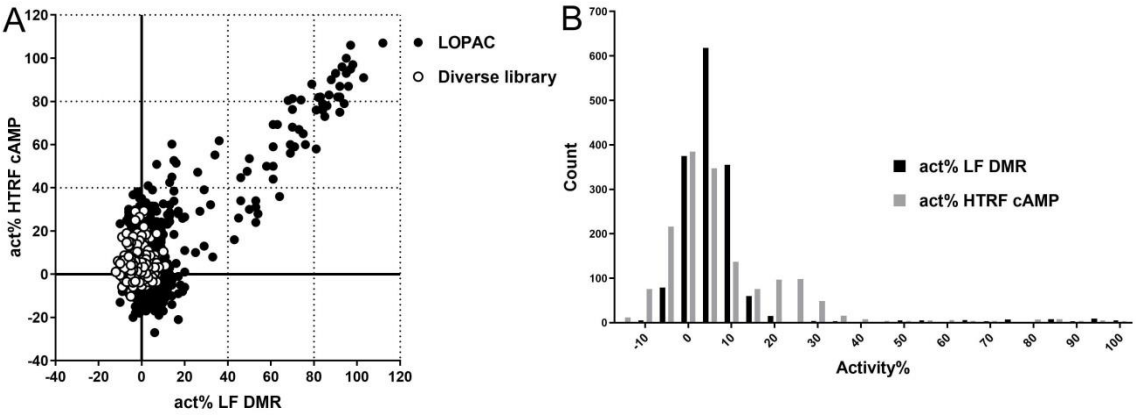


Figure 21. Visualization of the outcome of the DMR - HTRF cAMP pilot screen. (A) Correlation plot of the activities in the DMR and HTRF readouts (LOPAC: *filled dots*; corporate compounds: *clear dots*). (B) Frequency distribution of the activities in the DMR (black bars) and HTRF (grey bars) readout.

DOI:10.14753/SE.2021.2563

Taking e.g. the arbitrarily chosen 40% activity threshold, out of the 57 DMR as well as 58 HTRF actives 48 were shared. The described pharmacological profile of these ‘LOPAC’ actives was consulted and predominantly (>92% in the DMR whereas 86% in the HTRF cAMP) compounds with known adrenergic activity or structurally similar catecholamine analogues were identified in both readouts.

DOI:10.14753/SE.2021.2563

5. DISCUSSION

Hit discovery approaches for the therapeutically exceptionally important GPCR target class are predominantly constrained to HTS as applicability of biophysical fragment screening and structure-based approaches is limited for cell surface receptors in general. Unfortunately, the success rate of high-throughput screening for delivering viable chemical starting points for GPCRs only lies around 50%.[15,181] This unsatisfactory performance might be attributed primarily to the inefficient sampling of chemical space in HTS campaigns and additionally also to a GPCR target class specific factor, namely the incomplete coverage of the 'signaling event space' with the currently used single probe screening approaches resulting in the potential loss of diverse and novel hits as false negatives in functional assays. Our objective was to provide forward-looking solutions for both challenges in order to improve the success rate of experimental screening against 7-TM receptors.

5.1. Fragment hit expansion following high concentration screening against α_{2C} adrenergic receptor

The poor coverage of the chemical space provided by HTS libraries limits the diversity and versatility of starting points for drug discovery programs. The sampling efficiency could be improved by applying fragment screening, as fragments due to their low size offer a much better sampling of the chemical space.[39] Unfortunately, GPCRs as integral membrane protein targets are difficult to isolate and stabilize in native form for the biophysical studies primarily applied in fragment screening and thus, the general applicability of these technologies for the identification of novel GPCR ligands is limited. Biochemical and cell-based assays on the other hand could more generally be used for GPCR targets. Studies describing high concentration screening using competition binding assays have been reported for GPCR targets[44,140,184], however, no reports on HCS based on functional assays for the identification of GPCR ligands with various activity profiles (e.g. agonists) were available at the outset of our work.

The hit discovery team at Gedeon Richter Plc. was determined to explore identification and follow-up of fragment size hits with high concentration functional and binding

assays. The adrenergic α_{2C} receptor expressed in CHO-K1 cells was chosen as model system, which is particularly interesting from FBLD perspective as identification of agonists that show selectivity over the α_{2A} and α_{2B} isoforms is notoriously difficult.[205] The prior high concentration screening of 3,071 fragments in a functional Ca^{2+} assay provided in total 17 hits, two of which were also identified as top 1% in a virtual screening approach run in parallel using a homology model derived from the active state of the β_2-adrenergic receptor.[209]

We set out to evaluate a proposed workflow for the hit expansion of the identified fragment hits.[44,45,49,50] In order to establish early SAR, fragment-size structural analogues of the hits with highest affinity were searched for in the corporate library and were tested in competitive binding experiments. Compounds with the potential to possess improved affinity or LE values were also tested in concentration-response manner and several of these analogues indeed demonstrated superior affinity and/or ligand efficiency (LE). In parallel, all analogues were also tested for agonist activity in the functional Ca^{2+} assay and surprisingly, none of them showed pronounced and specific agonistic activity. Therefore, the best analogues from the K_i and LE profiling studies were also run in antagonist mode in the Ca^{2+} assay. Interestingly, all selected compounds turned out to exhibit functional antagonism in the Ca^{2+} assay, which is striking taking into account the high level of structural similarity of the analogues to the original compounds. The very steep structure-activity relationship (SAR) emphasizes the importance of applying cell-based assays that closely monitor the functional activity of compounds in the hit follow-up and optimization phase. Although the compounds with antagonist profile were not pursued further due to their incompatibility with the original aim of the project, it has to be noted that selective antagonist ligands for α_{2C} adrenergic receptor represent an attractive therapeutic option in the treatment of Alzheimer's disease.[221]

In this work we demonstrated that LE-driven hit expansion following hit identification by high concentration functional screening against a GPCR target can be successfully performed using conventional biochemical and functional assays even in the lack of accurate structural information of the target protein. This experience is consistent with future hit discovery directions suggested by the AstraZeneca global screening operations, namely that HTS and the fragment screening approach can be reconciled by

screening lower molecular weight libraries (MW~300-350 Da) at higher concentration (30-100 μM).[222]

5.2. Development of cell-based multiplex assays

The sensitivity of a screening campaign is directly linked to the false negative rate. False negatives can emerge from technical reasons as well as due to the different propensity of the different screening technologies to identify actives, even when monitoring the same signaling event.[187–190,223] Even if significant efforts are put in sufficient assay quality as well as in selecting the most appropriate assay or detection technology, in the case of the GPCR target class an extra major source of false negatives still remains in functional assays, the phenomenon called functional selectivity. Due to the pleiotropic signaling characteristic of this target class, ligands are able to preferentially activate distinct signaling pathways downstream of receptor activation.[99] Besides, it has been demonstrated in case studies that subtle changes in a compound's structure can cause dramatic changes in the functional activity profile of ligands.[100,127,224] Functional selectivity combined with this intricate and unpredictable structure activity relationship has consequences for the success of the entire lead discovery as compounds active only in pathways not actually monitored in a diversity screen might be missed, leading to the potential loss of complete chemotypes.[97,100,147,192,225] For antagonists the issue seems to be less relevant at first glance, however, given the multitude of different GPCR conformations as well as the multidimensionality of GPCR signaling, the occurrence of compounds exhibiting neutral antagonist profile uniformly in all signaling pathways is rare.[91,226] This all emphasizes the importance of finding any ligand showing activity on any of the pathways first and sorting out the less interested series later on. Displacement binding assays could support this strategy by identifying any compound showing affinity toward the receptor; however, this approach is impeded due to the availability of a tracer ligand in general as well as the limitations around throughput. The apparently obvious solution of running separate functional HTS campaigns for several pathways is unfortunately financially not feasible in industrial settings. On the other hand, monitoring multiple pathways within a single multiplexed assay, although technically highly challenging, might increase the chance of identifying actives present in the library. Successful

attempts in developing combinations of assays for different analytes or signaling pathways both using labeled and label-free technologies have been reported; however, all these approaches have inherent constraints by the dependence on recombinant biosensor constructs or limitations in the diversity of signaling events that can be investigated.[194–201,203,204]

The aim of our work was the development of generally applicable versatile multiplex systems by combining widely used assay technologies: (1) fluorescent measurement of the change in intracellular [Ca^{2+}] that is a general feature of GPCR activation not restricted to $G_{q/11}$-coupled receptors; (2) the TR-FRET-based HTRF that enables maximal flexibility in terms of the signaling molecule monitored; and (3) label-free DMR that offers integrated cellular response on its own.

5.2.1. Fluorescent Ca^{2+} measurement combined with the HTRF technology

In our proof of concept first attempt, we developed the combination of fluorescent Ca^{2+} and the HTRF-based cAMP measurement in HEK-293 cells exogenously expressing the PTH1 receptor that is naturally coupled to both the G_q and G_s pathways. Looking at the substantial differences in the optimal assay conditions of the individual assay technologies highlights the challenge of the undertaking. Fluorescent Ca^{2+} measurements are well established live cell measurements carried out typically with adherent cells in clear bottom black plates with assay volumes in the 40-80 µL range for 384-well plate format[155,157–159], while cAMP measurements using commercially available HTRF kits are usually performed with cells suspended in clear buffer in low volume white plates with markedly lower assay volumes. Increasing the volume of the costly HTRF detection reagents to the volume of the Ca^{2+} measurement was not considered as economically favorable, therefore the miniaturization of the Ca^{2+} assay was addressed. After extensive vendor search and considerable optimization work, the imaging plate reader-based Ca^{2+} assay was successfully miniaturized to low volume plate with a final assay volume of 20 µL. Next, the HTRF cAMP assay was developed for adherent cultures in black plates in the presence of colored medium and the Calcium 5 dye. Thanks to thorough adjustment of the HTRF readout protocol, the detrimental effect of black plates on the HTRF signal was still acceptable and the assay proved to perform well with adherent cells, too. Unfortunately, the presence of the red-colored

Calcium 5 dye rendered the HTRF signal window insufficient that in turn made adjustment of the concentration of the Calcium 5 dye necessary resulting in the end in an assay with acceptable performance in both readouts.

The pharmacological relevance of the optimized multiplex assay was validated with two reference peptides, PTH (1-34) and PTH (2-38). While PTH (1-34) was found to be a full agonist for both pathways with respective EC_{50} values in line with those reported in the literature earlier[216,217], we could also reproduce previous findings which showed that PTH (2-38) acted as a biased agonist towards the G_s pathway; even the concentration-response relationship of PTH (2-38) in our Ca^{2+} mobilization assay agreed remarkably well with the IP_1 results of Cupp et al.[215]

Following pharmacological and assay validation, the multiplex assay was successfully implemented in a pilot screen. The average activity of the compound set due to the high number of pharmacologically active compounds in the LOPAC library was fairly high, around 40% in both readouts; however, if calculating activity threshold (Mean±3×SD) from negative controls, the corresponding threshold values were completely in the range normally obtained in diversity screening campaigns (11% and 27% for the Ca^{2+} and cAMP readouts, respectively). Based on the described biological activity of the LOPAC compounds most of the primary actives were supposed to act either on respective receptors endogenously expressed in HEK-293 cells (muscarinic and purinergic receptors for Ca^{2+} readout as well as adrenergic and adenosine receptors for cAMP readout)[218,219] or interfered with cellular/biochemical events associated with changes in $[Ca^{2+}]_i$ or cAMP levels.

Not surprisingly, no real and specific actives (i.e. hits) emerged from this small scale screen as the LOPAC library does not contain molecules active on the PTH system; moreover, finding small molecule ligands for PTH receptors, similarly to other peptidergic receptors in the secretin receptor family like GLP-1[227], is generally highly challenging even when screening large libraries. Excitingly, just recently PCO371, an orally active non-peptide PTH1R agonist ligand was identified in a cell-based functional screen[228] and characterization of this non-peptidic ligand showed similar pharmacological profile as the recently approved osteoporosis drug Abaloparatide[229].

5.2.2. Label-free DMR measurement combined with the HTRF technology

As our next objective in developing multiplex assays we set out to establish the combination of label-free DMR detection with the HTRF technology. As compared to the technically closest multiplex assay reported so far in the literature that combines the DMR technology with Ca^{2+} fluorometry[203], HTRF as a labeled second readout would allow the detection of a much wider panel of signaling molecules and events (e.g. cAMP, IP_1, MAPK cascade members, etc.).

As a model system, CHO-K1 cells expressing the human α_{2C} adrenergic receptor was chosen, the activation of which through coupling to $G_{i/o}$ proteins inhibits the adenylate cyclase[230] which reduces the intracellular cAMP production that can be detected with HTRF reagents after forskolin pretreatment. At the same time, activation of GPCRs is accompanied by subtle movements of cellular components and the resulting mass redistribution within the cells can be detected by changes in the label-free DMR index.[171]

Beyond technical challenges posed by the different conditions of the two assay technologies, the key factor determining the feasibility of the multiplex approach was the kinetic compatibility of the two measurements. As DMR measurement is performed on living cells, whereas HTRF cAMP is measured from the lysate of the cells, the sequence of the readouts was predefined. Fortunately, the HTRF cAMP signal window was found stable long enough after the peak of the transient DMR signal following α_{2C} receptor activation in the presence of previously determined 2.5 μM forskolin, allowing subsequent readouts in the combination. In the next step, to minimize reagent cost/well for the HTRF part, miniaturization of the DMR assay to half of the relatively high original volume was realized with no change in DMR assay quality. This paved the way for the adjustment of the amount of HTRF detection reagents as well as for the fine-tuning of the HTRF readout from the unexpendable DMR plate with attributes far from optimal for HTRF reading.

Applicability of the final automated assay for high-throughput screening was assessed in a small scale screen on a validation library containing the LOPAC set. The relatively low number of tested compounds enabled the kinetic recording of the responses; however, for the evaluation of the primary activities, just like in the case of a putative large scale screening campaign, end-point measurement defined by the peak signal of

the reference compound UK 14,304 was used, a choice certainly biasing the DMR results towards compounds activating pathways with fast positive DMR responses (e.g. $G_{i/o}$ or G_q pathways). We found that despite the relatively low signal-to-background of the HTRF readout, the average Z' was excellent in both readouts and the potency of the reference compound was also in good agreement both between readouts and between the different plates. Relative to a naïve screen, a substantially higher number of actives were observed which was again the consequence of the LOPAC collection comprising biologically active ligands. Besides the identification of a high number of compounds with described adrenergic activity, many functionally and structurally related catecholamines also displayed a wide range of activities in both readouts with good correlation. As a comparison, LOPAC compounds with the same profile were reported to be detected in previous DMR case studies performed on A431 cells endogenously expressing β_2 adrenergic receptor.[172,231] The latter study by Corning is particularly interesting as the LOPAC library in this case was screened (at 1 μM concentration) not only in a label-free DMR but also separately in a cAMP assay. While both assays identified specific β_2 agonists, direct comparison of the activities in the two assays seemed to be problematic as the cAMP assay delivered much lower number of actives then the DMR assay, most probably due to substantial difference in pharmacological sensitivity between the two readouts. This assumption is further confirmed by the ~400-fold difference in the EC_{50} values of the reference compound epinephrine in favor of the DMR assay.[231]

5.2.3. Outcomes of multiplex assay development

The experiences collected from the two developed multiplex assays are numerous. To start the evaluation with critical comments, first of all contrasting results were obtained regarding the overlap of the hits between the readouts. In the Ca^{2+}-HTRF multiplex system on PTH1R the lack of overlap amongst confirmed actives between the two readouts demonstrated the ability of the approach to monitor and detect separate signaling events, which forecasts the possibility of obtaining improved sensitivity also in large scale screening. On the other hand, in the case of the DMR-HTRF combination on α_{2C} receptor, a high proportion of shared hits between the readouts were observed. The putative reason behind the different outcomes is that in the case of Ca^{2+}-HTRF in

the PTH1R system the pathways/physiological events investigated were indeed orthogonal, whereas in the DMR-HTRF system the detected end-points were not completely independent as the DMR signal linked to α_{2C} receptor activation also reflects the characteristics of the activation of the $G_{i/o}$ pathway. At the same time, the good correlation in the DMR-HTRF combination might be regarded as orthogonal confirmation within a single multiplex assay. Second, it has to be admitted that the success of developing a fit for purpose multiplex assay is naturally dependent on a number of factors, like the particular target (e.g. peptidergic versus monoaminergic GPCRs), the mechanism of action (agonism vs. inverse agonism vs. allosteric modulation), the nature of the analyte, as well as on the available cellular backgrounds to mention but just a few. For example in the case of the Ca^{2+}-HTRF multiplex on PTH1R, the significant difference in the potencies of the peptide agonist for the two pathways could make an antagonist or positive allosteric modulator multiplex screen highly challenging. And finally, the development of multiplex assays usually takes longer, the combination can result in compromised signal separation in one or both readouts, and running a multiplex screen is also more expensive mainly due to the costs incurred by the double-readout (e.g. costly Ca^{2+} dye or HTRF reagents, special DMR plates).

Acknowledging these potential difficulties and limitations, the multiplex solutions combining generic and widely applied assay technologies developed by us offer multiple advantages. The biggest benefit lies in the generic applicability of the combinations in terms of signaling analytes and readout technologies. Especially the HTRF component of the combinations enables the detection of a wide variety of intracellular signaling molecules opening the way for the detection of G protein-independent pathways as well. In particular, to date detection of β-arrestin involvement in GPCR signaling is only possible with recombinant constructs at the level of arrestin recruitment[166,167,232,233]; even the theoretically pathway-unbiased label-free DMR fails to detect arrestin-mediated signals[202]. At the same time, the involvement of β-arrestins as scaffolding proteins in MAP kinase pathways is well-described[85,234,235], therefore, with the HTRF technology[164] offering kits optimized for the cell-based detection of phospho-MAP kinases (like p-ERK1/2, p38, p-AKT), it might be possible to indirectly study the functional effects mediated by β-arrestin, too.

Thus, the above presented multiplex assays are expected to enable the detection of the broadest repertoire of simultaneous intracellular signaling processes reported up to date in the GPCR realm. This perfectly suits our primary objective of increasing the sensitivity of HTS through decreasing the ratio of false negatives. First, it is conceivable that one of the two screening technologies applied in the multiplex is blind to specific compounds that the other readout can rescue; and more importantly, such screening assays are able to identify compounds exerting activity in only one of the monitored signaling pathways (like in the case of the PTH1R multiplex) due to functional selectivity. Certainly, bias profile might not seem to have relevance at an early stage in discovery, yet, the rich pharmacological information collected by the multiplex approach might turn out to be extremely useful in later stages of development[125] and in such cases medicinal chemistry work might directly profit from the multiplex design as concentration-response determination in multiplex manner also delivers bias factors for the quantification of bias[99,121] that can serve as a guide in SFSR studies[100,236] to optimize for the therapeutically useful activity[89], like during the development of TRV130[127].

In addition, applying generic assay technologies that do not require genetic manipulation for the detection step makes the multiplex approach well suitable for more delicate cells as *in vitro* model systems, including primary cells with endogenous targets as well as differentiated cells of stem cell origin, where the cell quantity is usually a serious limiting factor. Several research groups reported on successfully running fluorescent Ca^{2+} measurement[237], HTRF and label-free DMR assays in primary cells and even in iPSCs.[212,238–240] This is of particular importance for diseases with highly complex biology and molecular mechanisms where only delicate and valid *in vitro* disease models are expected to enable the identification of unique and promising ligands.[180]

The approach has significance from chemical genomics perspective, too. With over 100 non-sensory GPCRs with no documented endogenous ligand, 7-TM receptors represent an immense reservoir of orphan receptors[156,241], many of which bear the potential to become an exciting therapeutic target.[174,242] For the identification of ligands and activation pathways for such receptors ideally multiple parallel pathways should be monitored simultaneously.[241] Consequently, multiplex assays would not only be perfectly suited for drug discovery in this undiscovered area, but also represent huge

advantages over the use of various single end-point assays in deorphanization campaigns where often the amount of highly valuable biological material (tissue extracts etc.) is limited for sequential and iterative investigations.[241]

Finally, as an outlook beyond the 7-TM receptor world, thanks to the versatility in detecting possible signaling intermediates, the presented combinations might be applicable to target classes other than GPCRs: (1) the FLIPR assay enables interrogation of Ca^{2+} permeable ion channels[159]; (2) HTRF detection is also available for transcription factors and kinases involved in the complex signaling mechanism of ion channels[243,244], and is also widely used for the investigation of receptor tyrosine kinase signaling[245]; (3) the label-free DMR technology was reported to be responsive to Ca^{2+} signaling, ion channel as well as receptor tyrosine kinase activation[168,246]. Therefore, with the creative and careful combination of the presented detection technologies versatile multiplex assay platforms can be established for the study of a number of pharmacologically relevant targets and physiological processes.

DOI:10.14753/SE.2021.2563

6. CONCLUSIONS

In order to improve the hit discovery efficiency of high-throughput screening for GPCR targets, we set out to (1) explore if hit expansion, following a pioneering high concentration functional screening of a fragment library against a GPCR target, supported by cell-based and biochemical assays is a viable approach and to (2) develop multiplex assays to increase the sensitivity of HTS assays by decreasing the number of false negatives. The main findings of our work are summarized below:

1.) In our first objective the follow-up of active fragments with outstanding K_i and LE originating from a cell-based high concentration screen against the α_{2C} adrenergic receptor was explored. Analogues selected by neighbor-search of the corporate compound collection were tested for binding and functional activity in an iterative way. Ligands with superior affinity and/or LE values were successfully identified, however, all of them turned out to demonstrate antagonist behavior in functional Ca^{2+} assay. 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 binding. Applying this approach 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.

2.) As our second objective we successfully developed and evaluated multiplex assays based on the combination of generally applicable cell-based assay technologies:

2A.) Fluorometric Ca^{2+} measurement was successfully combined with consecutive TR-FRET-based HTRF technology in cells stably expressing PTH1 receptor for simultaneous monitoring of the G_q and G_s pathways. During pharmacological validation, the 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 distinct sets of pharmacologically active compounds (not PTH1R specific ligands) were identified in the two readouts with no overlap. The complementary nature of the active list 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.

2B.) The dynamic mass redistribution-based optical label-free integrated whole cell readout that alone can be considered a multiple-probe screening technology was multiplexed with the HTRF readout using cells expressing α_{2C} adrenergic receptor. The developed assay tested in a validation screen exhibited proper assay quality and delivered specific ligands with activities in good agreement. 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.

2C.) Development of multiplex assays alone from technical perspective would be a noteworthy achievement. Beyond the methodological aspects, however, the main significance of these 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 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.

3.) The outcome of our work might also point towards considering the integrated use of the two main objectives, as we do not see any obstacle to run lower molecular size compounds at elevated concentrations in multiplex assays. Such a combination is expected to maximize the probability of identifying structurally and functionally diverse compounds with high ligand efficiency for a successful lead discovery.

4.) 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.

DOI:10.14753/SE.2021.2563

7. SUMMARY

The goal of hit identification in drug discovery is to deliver high quality and diverse chemical starting points. The most prominent hit discovery tool is high-throughput screening (HTS) whereby a high number of compounds with sufficient diversity are tested in an *in vitro* model with the aim to identify compounds that interact with the selected target. G protein-coupled receptors (GPCRs) are the largest integral membrane protein family targeted by ca. 35% of the FDA-approved drugs. Despite the abundance of *in vitro* biochemical and functional HTS assays developed in the last decades against GPCRs and the availability of large compound collections, the success rate of HTS remained around 50%. The main reasons considered to lead to failure of hit discovery by HTS are (1) the use of compound libraries covering the chemical space only fractionally and (2) the limited sensitivity of the screening assays. In the case of GPCRs, the latter is worsened by false negatives in functional assays originating as a consequence of functional selectivity characteristic for this target class.

To improve hit discovery efficiency for GPCRs, first a hit expansion workflow following high concentration cellular screening of a fragment library was explored. 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.

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 biased agonism 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.

In the future, combining our two main objectives by screening smaller size compounds in multiplex assays might synergistically improve the efficiency of hit discovery by high-throughput screening.

DOI:10.14753/SE.2021.2563

8. ÖSSZEFOGLALÁS

A gyógyszerkutatás felfedező szakaszának célja gyógyszermolekulává fejleszthető kémiai kiindulópontok azonosítása. A kiindulópont-keresés legjelentősebb eszköze a nagy áteresztőképességű szűrés (röviden HTS), amelynek során egy kellően nagy elemszámú és diverz molekulatárat egy alkalmas *in vitro* modellben letesztelünk, és azonosítjuk azokat a vegyületeket, amelyek a kiválasztott célponttal kölcsönhatásba lépnek. A G-fehérjéhez kapcsolt receptorok (GPCR) a legnagyobb membránfehérje-család, amelynek gyógyszeripari jelentősége vitathatatlan. Annak ellenére, hogy nagy méretű vegyületkönyvtárak állnak rendelkezésre a tesztelésre illetve számos *in vitro* biokémiai és funkcionális HTS teszt vált elérhetővé az elmúlt évtizedekben a GPCR-ek vizsgálatára, a HTS sikerrátája 50% körül maradt. Ennek fő okai (1) a kémiai teret korlátozottan lefedő vegyületkönyvtárak használata és (2) a szűrővizsgálatok mérsékelt érzékenysége. A GPCR-ek esetén ez utóbbit tovább csökkentik a célpontcsaládra jellemző funkcionális szelektivitás jelenségéből fakadó hamis negatív eredmények.

A kiindulópont azonosítás hatékonyságának javítása érdekében először azt vizsgáltuk meg, hogy nagy koncentrációjú funkcionális szűrésből származó fragmens találatok kiterjesztése hatékonyan kivitelezhető-e. A fragmensek kisebb méretüknek köszönhetően a kémiai tér hatékonyabb mintavételezését teszik lehetővé, ezzel újszerű és ígéretes kiindulópontokat kínálva a vezérmolekula felfedezéséhez. Bemutattuk az α_{2C} adrenerg receptor példáján, hogy sejtalapú és biokémiai vizsgálatokkal megfelelően támogatható a fragmens találatok ligandum hatékonyság alapú expanziója.

Második célként általánosan alkalmazható multiplexált sejtes funkcionális szűrési teszteket dolgoztunk ki annak érdekében, hogy csökkentsük a funkcionális szelektivitásból vagy a szűrési technikából származó hamis negatívok arányát. Az irodalomban elsőként sikeresen kombináltuk a fluoreszcens Ca^{2+} mérést és a jelölésmentes DMR technológiát a HTRF technikával. Ezek az új multiplex platformok egyszerre képesek nyomon követni az egymással párhuzamos GPCR útvonalak gyakorlatilag bármely kombinációját, ami a HTS kampányok érzékenységének javításával bíztat. A jövőben a két fő célkitűzés kombinálása, vagyis a kisebb méretű vegyületek multiplex vizsgálatban emelt koncentrációban történő szűrése szinergikus módon javíthatja a HTS általi találat azonosítás hatékonyságát.

9. REFERENCES

(1) Sneader, W. Drug discovery: a history, 1st ed.; Wiley, 2005.

(2) Eder, J.; Sedrani, R.; Wiesmann, C. (2014) The discovery of first-in-class drugs: origins and evolution. Nat. Rev. Drug Discov. 13 (8), 577–587.

(3) Kenakin, T. A pharmacology primer: theory, application and methods.; Elsevier: Burlington, 2009.

(4) Croston, G. E. (2017) The utility of target-based discovery. Expert Opin. Drug Discov. 12 (5), 427–429.

(5) Hughes, J.; Rees, S.; Kalindjian, S.; Philpott, K. (2011) Principles of early drug discovery. Br. J. Pharmacol. 162 (6), 1239–1249.

(6) Santos, R.; Ursu, O.; Gaulton, A.; Bento, A. P.; Donadi, R. S.; Bologa, C. G.; Karlsson, A.; Al-Lazikani, B.; Hersey, A.; Oprea, T. I.; Overington, J. P. (2017) A comprehensive map of molecular drug targets. Nat. Rev. Drug Discov. 16 (1), 19–34.

(7) Paul, S. M.; Mytelka, D. S.; Dunwiddie, C. T.; Persinger, C. C.; Munos, B. H.; Lindborg, S. R.; Schacht, A. L. (2010) How to improve R&D productivity: the pharmaceutical industry's grand challenge. Nat. Rev. Drug Discov. 9 (3), 203–214.

(8) Vasaikar, S.; Bhatia, P.; Bhatia, P.; Chu Yaiw, K. (2016) Complementary approaches to existing target based drug discovery for identifying novel drug targets. Biomedicines 4 (4), 27.

(9) Pammolli, F.; Righetto, L.; Abrignani, S.; Pani, L.; Pelicci, P. G.; Rabosio, E. (2020) The endless frontier? The recent increase of R&D productivity in pharmaceuticals. J. Transl. Med. 18 (1), 162.

(10) DiMasi, J. A.; Grabowski, H. G.; Hansen, R. W. (2016) Innovation in the pharmaceutical industry: new estimates of R&D costs. J. Health Econ. 47, 20–33.

(11) Holenz, J.; Stoy, P. (2019) Advances in lead generation. Bioorg. Med. Chem. Lett. 29 (4), 517–524.

(12) Fejzo, J.; Lepre, C. A.; Peng, J. W.; Bemis, G. W.; Ajay; Murcko, M. A.; Moore, J. M. (1996) The SHAPES strategy: an NMR-based approach for lead generation in drug discovery. Chem. Biol. 6 (10), 755–769.

(13) Perola, E. (2010) An analysis of the binding efficiencies of drugs and their leads in successful drug discovery programs. J. Med. Chem. 53 (7), 2986–2997.

(14) Brown, D. G.; Boström, J. (2018) Where do recent small molecule clinical development candidates come from? J. Med. Chem. 61 (21), 9442–9468.

(15) Macarron, R.; Banks, M. N.; Bojanic, D.; Burns, D. J.; Cirovic, D. A.; Garyantes, T.; Green, D. V. S.; Hertzberg, R. P.; Janzen, W. P.; Paslay, J. W.; Schopfer, U.; Sittampalam, G. S. (2011) Impact of high-throughput screening in biomedical research. Nat. Rev. Drug Discov. 10 (3), 188–195.

(16) Erlanson, D. A.; Fesik, S. W.; Hubbard, R. E.; Jahnke, W.; Jhoti, H. (2016) Twenty years on: the impact of fragments on drug discovery. Nat. Rev. Drug Discov. 15 (9), 605–619.

(17) Fragments in the clinic: 2020 Edition; https://practicalfragments.blogspot.com/2020/03/fragments-in-clinic-2020-edition.html (accessed on 06/08/2021)

(18) Lavecchia, A.; Giovanni, C. (2013) Virtual screening strategies in drug discovery: a critical review. Curr. Med. Chem. 20 (23), 2839–2860.

(19) Inglese, J.; Johnson, R. L.; Simeonov, A.; Xia, M.; Zheng, W.; Austin, C. P.; Auld, D. S. (2007) High-throughput screening assays for the identification of chemical probes. Nat. Chem. Biol. 3 (8), 466–479.

(20) Pereira, D. A.; Williams, J. A. (2007) Origin and evolution of high throughput screening. Br. J. Pharmacol. 152 (1), 53–61.

(21) Janzen, W. P. (2014) Screening technologies for small molecule discovery: the state of the art. Chem. Biol. 21 (9), 1162–1170.

(22) Mayr, L. M.; Bojanic, D. (2009) Novel trends in high-throughput screening. Curr. Opin. Pharmacol. 9 (5), 580–588.

(23) Blay, V.; Tolani, B.; Ho, S. P.; Arkin, M. R. (2020) High-throughput screening: today’s biochemical and cell-based approaches. Drug Discov. Today 25 (10), 1807–1821.

(24) Michelini, E.; Cevenini, L.; Mezzanotte, L.; Coppa, A.; Roda, A. (2010) Cell-based assays: fuelling drug discovery. Anal. Bioanal. Chem. 398 (1), 227–238.

(25) Mayr, L. M.; Fuerst, P. (2008) The future of high-throughput screening. J. Biomol. Screen. 13 (6), 443–448.

(26) Zhang, J.-H.; Chung, T. D. Y.; Oldenburg, K. R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening Assays. J. Biomol. Screen. 4 (2), 67–73.

(27) Keserű, G. M.; Makara, G. M. (2006) Hit discovery and hit-to-lead approaches. Drug Discov. Today 11 (15–16), 741–748.

(28) Erlanson, D. A.; McDowell, R. S.; O'Brien, T. (2004) Fragment-based drug discovery. J. Med. Chem. 2004, 47 (14), 3463–3482.

(29) Smith, R. A.; Barbosa, J.; Blum, C. L.; Bobko, M. A.; Caringal, Y. V.; Dally, R.; Johnson, J. S.; Katz, M. E.; Kennure, N.; Kingery-Wood, J.; Lee, W.; Lowinger, T. B.; Lyons, J.; Marsh, V.; Rogers, D. H.; Swartz, S.; Walling, T.; Wild, H. (2001) Discovery of heterocyclic ureas as a new class of Raf kinase inhibitors: identification of a second generation lead by a combinatorial chemistry approach. Bioorg. Med. Chem. Lett. 11 (20), 2775–2778.

(30) Wilhelm, S.; Carter, C.; Lynch, M.; Lowinger, T.; Dumas, J.; Smith, R. A.; Schwartz, B.; Simantov, R.; Kelley, S. (2006) Discovery and development of Sorafenib: A multikinase inhibitor for treating cancer. Nat. Rev. Drug Discov. 5 (10), 835–844.

(31) Xu, G.; Guo, J.; Wu, Y. (2014) Chemokine receptor CCR5 antagonist Maraviroc: medicinal chemistry and clinical applications. Curr. Top. Med. Chem. 14 (13), 1504–1514.

(32) Parmee, E. R.; Sinharoy, R.; Xu, F.; Givand, J. C.; Rosen, L. A. Discovery and development of the DPP-4 inhibitor Januvia™ (Sitagliptin). In Case Studies in Modern Drug Discovery and Development; Huang, X., Aslanian, R. G., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2012; pp 10–44.

(33) Hann, M. M.; Leach, A. R.; Harper, G. (2001) Molecular complexity and its impact on the probability of finding leads for drug discovery. J. Chem. Inf. Comput. Sci. 41 (3), 856–864.

(34) Teague, S. J.; Davis, A. M.; Leeson, P. D.; Oprea, T. I. (1999) The design of leadlike combinatorial libraries. Angew. Chem. Int. Ed Engl. 38 (24), 3743–3748.

(35) Oprea, T. I.; Davis, A. M.; Teague, S. J.; Leeson, P. D. (2001) Is there a difference between leads and drugs? A historical perspective. J. Chem. Inf. Comput. Sci. 41 (5), 1308–1315.

(36) Hann, M. M.; Oprea, T. I. (2004) Pursuing the leadlikeness concept in pharmaceutical research. Curr. Opin. Chem. Biol. 8 (3), 255–263.

(37) Congreve, M.; Carr, R.; Murray, C.; Jhoti, H. (2003) A 'Rule of Three' for fragment-based lead discovery? Drug Discov. Today 8 (19), 876–877.

(38) Ferenczy, G. G.; Keserű, G. M. (2013) How are fragments optimized? A retrospective analysis of 145 fragment optimizations. J. Med. Chem. 56 (6), 2478–2486.

(39) Leach, A. R.; Hann, M. M. (2011) Molecular complexity and fragment-based drug discovery: ten years on. Curr. Opin. Chem. Biol. 15 (4), 489–496.

(40) Ruddigkeit, L.; van Deursen, R.; Blum, L. C.; Reymond, J.-L. (2012) Enumeration of 166 billion organic small molecules in the chemical universe database GDB-17. J. Chem. Inf. Model. 52 (11), 2864–2875.

(41) Polishchuk, P. G.; Madzhidov, T. I.; Varnek, A. (2013) Estimation of the size of drug-like chemical space based on GDB-17 data. J. Comput. Aided Mol. Des. 27 (8), 675–679.

(42) Lamoree, B.; Hubbard, R. E. (2017) Current perspectives in fragment-based lead discovery (FBLD). Essays Biochem. 61 (5), 453–464.

(43) Schuffenhauer, A.; Ruedisser, S.; Marzinzik, A.; Jahnke, W.; Selzer, P.; Jacoby, E. (2005) Library design for fragment based screening. Curr. Top. Med. Chem. 5 (8), 751–762.

(44) Albert, J.; Blomberg, N.; Breeze, A.; Brown, A.; Burrows, J.; Edwards, P.; Folmer, R.; Geschwindner, S.; Griffen, E.; Kenny, P.; Nowak, T.; Olsson, L.-L.; Sanganee, H.; Shapiro, A. (2007) An integrated approach to fragment-based lead generation: philosophy, strategy and case studies from AstraZeneca's drug discovery programmes. Curr. Top. Med. Chem. 7 (16), 1600–1629.

(45) Fuller, N.; Spadola, L.; Cowen, S.; Patel, J.; Schönherr, H.; Cao, Q.; McKenzie, A.; Edfeldt, F.; Rabow, A.; Goodnow, R. (2016) An improved model for fragment-based lead generation at AstraZeneca. Drug Discov. Today 21 (8), 1272–1283.

(46) Saha, A.; Varghese, T.; Liu, A.; Allen, S. J.; Mirzadegan, T.; Hack, M. D. (2018) An analysis of different components of a high-throughput screening library. J. Chem. Inf. Model. 58 (10), 2057–2068.

(47) Chessari, G.; Woodhead, A. J. (2009) From fragment to clinical candidate—a historical perspective. Drug Discov. Today 14 (13–14), 668–675.

(48) Hopkins, A. L.; Groom, C. R.; Alex, A. (2004) Ligand efficiency: a useful metric for lead selection. Drug Discov. Today 9 (10), 430–431.

(49) Orita, M.; Ohno, K.; Warizaya, M.; Amano, Y.; Niimi, T. Lead generation and examples: opinion regarding how to follow up hits. In Methods in Enzymology; Elsevier, 2011, 493, 383–419.

(50) Wolkenberg, S. E.; Zhao, Z.; Mulhearn, J. J.; Harrison, S. T.; Sanders, J. M.; Cato, M. J.; Jovanovska, A.; Panigel, J.; Cook, S. P.; Henze, D. A.; Kane, S. A.; Hartman, G. D.; Barrow, J. C. (2011) High concentration electrophysiology-based fragment screen: discovery of novel Acid-sensing ion channel 3 (ASIC3) inhibitors. Bioorg. Med. Chem. Lett. 21 (9), 2646–2649.

(51) Rees, D. C.; Congreve, M.; Murray, C. W.; Carr, R. (2004) Fragment-based lead discovery. Nat. Rev. Drug Discov. 3 (8), 660–672.

(52) Erlanson, D. A.; Davis, B. J.; Jahnke, W. (2019) Fragment-based drug discovery: advancing fragments in the absence of crystal structures. Cell Chem. Biol. 26 (1), 9–15.

(53) First fragment-based drug approved; http://practicalfragments.blogspot.com/2011/08/first-fragment-based-drug-approved.html (accessed on 06/08/2021)

(54) Second fragment-based drug approved; http://practicalfragments.blogspot.com/search?q=+venetoclax (accessed on 06/08/2021)

(55) Gimeno, A.; Ojeda-Montes, M.; Tomás-Hernández, S.; Cereto-Massagué, A.; Beltrán-Debón, R.; Mulero, M.; Pujadas, G.; Garcia-Vallvé, S. (2019) The light

and dark sides of virtual screening: what is there to know? Int. J. Mol. Sci. 20 (6), 1375.

(56) Hauser, A. S.; Attwood, M. M.; Rask-Andersen, M.; Schiöth, H. B.; Gloriam, D. E. (2017) Trends in GPCR drug discovery: new agents, targets and indications. Nat. Rev. Drug Discov. 2017, 16 (12), 829–842.

(57) Sriram, K.; Insel, P. A. (2018) G protein-coupled receptors as targets for approved drugs: how many targets and how many drugs? Mol. Pharmacol. 93 (4), 251–258.

(58) Lefkowitz, R. J. (2013) A brief history of G-protein coupled receptors (Nobel Lecture). Angew. Chem. Int. Ed. 52 (25), 6366–6378.

(59) Weis, W. I.; Kobilka, B. K. (2018) The molecular basis of G protein-coupled receptor activation. Annu. Rev. Biochem. 87 (1), 897–919.

(60) Grundmann, M.; Kostenis, E. (2017) Temporal bias: time-encoded dynamic GPCR signaling. Trends Pharmacol. Sci. 38 (12), 1110–1124.

(61) Hilger, D.; Masureel, M.; Kobilka, B. K. (2018) Structure and dynamics of GPCR signaling complexes. Nat. Struct. Mol. Biol. 25 (1), 4–12.

(62) Oldham, W. M.; Hamm, H. E. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. Nat. Rev. Mol. Cell Biol. 9 (1), 60–71.

(63) Smith, J. S.; Lefkowitz, R. J.; Rajagopal, S. (2018) Biased signalling: from simple switches to allosteric microprocessors. Nat. Rev. Drug Discov. 17 (4), 243–260.

(64) Simon, M.; Strathmann, M.; Gautam, N. (1991) Diversity of G proteins in signal transduction. Science 252 (5007), 802–808.

(65) Kim, H. R.; Xu, J.; Maeda, S.; Duc, N. M.; Ahn, D.; Du, Y.; Chung, K. Y. (2020) Structural mechanism underlying primary and secondary coupling between GPCRs and the Gi/o family. Nat. Commun. 11 (1), 3160.

(66) Galandrin, S.; Oligny-Longpré, G.; Bouvier, M. (2007) The evasive nature of drug efficacy: implications for drug discovery. Trends Pharmacol. Sci. 28 (8), 423–430.

(67) Eglen, R. M.; Bosse, R.; Reisine, T. (2007) Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening. ASSAY Drug Dev. Technol. 5 (3), 425–452.

(68) McLoughlin, D.; Bertelli, F.; Williams, C. (2007) The A, B, Cs of G-protein-coupled receptor pharmacology in assay development for HTS. Expert Opin. Drug Discov. 2 (5), 603–619.

(69) Siehler, S. (2009) Regulation of RhoGEF proteins by $G_{12/13}$-coupled receptors. Br. J. Pharmacol. 158 (1), 41–49.

(70) Ritchie, B. J.; Smolski, W. C.; Montgomery, E. R.; Fisher, E. S.; Choi, T. Y.; Olson, C. M.; Foster, L. A.; Meigs, T. E. (2013) Determinants at the N- and C-termini of $G\alpha_{12}$ required for activation of Rho-mediated signaling. J. Mol. Signal. 8, 3.

(71) Smrcka, A. V. (2008) G protein βγ subunits: central mediators of G protein-coupled receptor signaling. Cell. Mol. Life Sci. 65 (14), 2191–2214.

(72) Smrcka, A. V.; Fisher, I. (2019) G-Protein βγ subunits as multi-functional scaffolds and transducers in G-protein-coupled receptor signaling. Cell. Mol. Life Sci. 76 (22), 4447–4459.

(73) Komolov, K. E.; Benovic, J. L. (2018) G protein-coupled receptor kinases: past, present and future. Cell. Signal. 41, 17–24.

(74) Zhou, X. E.; He, Y.; de Waal, P. W.; Gao, X.; Kang, Y.; Van Eps, N.; Yin, Y.; Pal, K.; Goswami, D.; White, T. A.; Barty, A.; Latorraca, N. R.; Chapman, H. N.; Hubbell, W. L.; Dror, R. O.; Stevens, R. C.; Cherezov, V.; Gurevich, V. V.; Griffin, P. R.; Ernst, O. P.; Melcher, K.; Xu, H. E. (2017) Identification of phosphorylation codes for arrestin recruitment by G protein-coupled receptors. Cell 170 (3), 457-469.e13.

(75) Scheerer, P.; Sommer, M. E. (2017) Structural mechanism of arrestin activation. Curr. Opin. Struct. Biol. 45, 160–169.

(76) Luttrell, L. M.; Gesty-Palmer, D. (2010) Beyond desensitization: physiological relevance of arrestin-dependent signaling. Pharmacol. Rev. 62 (2), 305–330.

(77) Gurevich, V. V.; Gurevich, E. V. (2019) GPCR signaling regulation: the role of GRKs and arrestins. Front. Pharmacol. 10, 125.

(78) Mohan, M. L.; Vasudevan, N. T.; Gupta, M. K.; Martelli, E. E.; Naga Prasad, S. V. (2012) G-protein coupled receptor resensitization - appreciating the balancing act of receptor function. Curr. Mol. Pharmacol.

(79) Correll, C. C.; McKittrick, B. A. (2014) Biased ligand modulation of seven transmembrane receptors (7TMRs): functional implications for drug discovery: miniperspective. J. Med. Chem. 57 (16), 6887–6896.

(80) Gurevich, V. V.; Hanson, S. M.; Song, X.; Vishnivetskiy, S. A.; Gurevich, E. V. (2011) The functional cycle of visual arrestins in photoreceptor cells. Prog. Retin. Eye Res. 30 (6), 405–430.

(81) Carmona-Rosas, G.; Alcántara-Hernández, R.; Hernández-Espinosa, D. A. The role of β-arrestins in G protein-coupled receptor heterologous desensitization: a brief story. In Methods in Cell Biology; Elsevier, 2019, 149, 195–204.

(82) Tóth, A. D.; Turu, G.; Hunyady, L.; Balla, A. (2018) Novel mechanisms of G-protein-coupled receptors functions: AT1 angiotensin receptor acts as a signaling hub and focal point of receptor cross-talk. Best Pract. Res. Clin. Endocrinol. Metab. 32 (2), 69–82.

(83) Beaulieu, J.-M.; Sotnikova, T. D.; Marion, S.; Lefkowitz, R. J.; Gainetdinov, R. R.; Caron, M. G. (2005) An Akt/β-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell 122 (2), 261–273.

(84) Peterson, Y. K.; Luttrell, L. M. (2017) The diverse roles of arrestin scaffolds in G protein–coupled receptor signaling. Pharmacol. Rev. 69 (3), 256–297.

(85) Ahn, S.; Shenoy, S. K.; Wei, H.; Lefkowitz, R. J. (2004) Differential kinetic and spatial patterns of β-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. J. Biol. Chem. 279 (34), 35518–35525.

(86) Eishingdrelo, H. (2013) Minireview: targeting GPCR activated ERK pathways for drug discovery. Curr. Chem. Genomics Transl. Med. 7, 9–15.

(87) Zhao, P.; Furness, S. G. B. (2019) The nature of efficacy at G protein-coupled receptors. Biochem. Pharmacol. 170, 113647.

(88) Black, J. W.; Leff, P. (1983) Operational models of pharmacological agonism. Proc. R. Soc. Lond. B Biol. Sci. 220 (1219), 141–162.

(89) Kenakin, T. Emergent concepts of receptor pharmacology. In Concepts and Principles of Pharmacology; Barrett, J. E., Page, C. P., Michel, M. C., Eds.; Handbook of Experimental Pharmacology; Springer International Publishing: Cham, 2019; Vol. 260, pp 17–41.

(90) Wisler, J. W.; Xiao, K.; Thomsen, A. R.; Lefkowitz, R. J. (2014) Recent developments in biased agonism. Curr. Opin. Cell Biol. 2014, 27, 18–24.

(91) Kenakin, T. (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. Mol. Pharmacol. 65 (1), 2–11.

(92) Conn, P. J.; Christopoulos, A.; Lindsley, C. W. (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nat. Rev. Drug Discov. 8 (1), 41–54.

(93) Galandrin, S.; Bouvier, M. (2006) Distinct signaling profiles of β_1 and β_2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. Mol. Pharmacol. 70 (5), 1575–1584.

(94) Rajagopal, S.; Rajagopal, K.; Lefkowitz, R. J. (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat. Rev. Drug Discov. 9 (5), 373–386.

(95) Kenakin, T. (2002) Efficacy at G-protein-coupled receptors. Nat. Rev. Drug Discov. 1 (2), 103–110.

(96) Deupi, X.; Kobilka, B. K. (2010) Energy landscapes as a tool to integrate GPCR structure, dynamics, and function. Physiology 25 (5), 293–303.

(97) Kenakin, T. P. (2009) Cellular assays as portals to seven-transmembrane receptor-based drug discovery. Nat. Rev. Drug Discov. 8 (8), 617–626.

(98) Reiter, E.; Ahn, S.; Shukla, A. K.; Lefkowitz, R. J. (2012) Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. Annu. Rev. Pharmacol. Toxicol. 52 (1), 179–197.

(99) Kenakin, T.; Christopoulos, A. (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat. Rev. Drug Discov. 12 (3), 205–216.

(100) Shonberg, J.; Lopez, L.; Scammells, P. J.; Christopoulos, A.; Capuano, B.; Lane, J. R. (2014) Biased agonism at G protein-coupled receptors: the promise and the challenges--A medicinal chemistry perspective. Med. Res. Rev. 34 (6), 1286–1330.

(101) Evans, B. A.; Broxton, N.; Merlin, J.; Sato, M.; Hutchinson, D. S.; Christopoulos, A.; Summers, R. J. (2011) Quantification of functional selectivity at the human α_{1A} -adrenoceptor. Mol. Pharmacol. 79 (2), 298–307.

(102) Gesty-Palmer, D.; Luttrell, L. M. (2011) 'Biasing' the parathyroid hormone receptor: a novel anabolic approach to increasing bone mass? Br. J. Pharmacol. 164 (1), 59–67.

(103) Rahmeh, R.; Damian, M.; Cottet, M.; Orcel, H.; Mendre, C.; Durroux, T.; Sharma, K. S.; Durand, G.; Pucci, B.; Trinquet, E.; Zwier, J. M.; Deupi, X.; Bron, P.; Baneres, J.-L.; Mouillac, B.; Granier, S. (2012) Structural insights into biased G protein-coupled receptor signaling revealed by fluorescence spectroscopy. Proc. Natl. Acad. Sci. 109 (17), 6733–6738.

(104) Liu, J. J.; Horst, R.; Katritch, V.; Stevens, R. C.; Wuthrich, K. (2012) Biased signaling pathways in β_2-adrenergic receptor characterized by ^{19}F-NMR. Science 335 (6072), 1106–1110.

(105) Warne, T.; Edwards, P. C.; Leslie, A. G. W.; Tate, C. G. (2012) Crystal Structures of a stabilized β_1-adrenoceptor bound to the biased agonists bucindolol and carvedilol. Structure 20 (5), 841–849.

(106) Wacker, D.; Wang, C.; Katritch, V.; Han, G. W.; Huang, X.-P.; Vardy, E.; McCorvy, J. D.; Jiang, Y.; Chu, M.; Siu, F. Y.; Liu, W.; Xu, H. E.; Cherezov, V.; Roth, B. L.; Stevens, R. C. (2013) Structural features for functional selectivity at serotonin receptors. Science 340 (6132), 615–619.

(107) Wang, C.; Jiang, Y.; Ma, J.; Wu, H.; Wacker, D.; Katritch, V.; Han, G. W.; Liu, W.; Huang, X.-P.; Vardy, E.; McCorvy, J. D.; Gao, X.; Zhou, X. E.; Melcher, K.; Zhang, C.; Bai, F.; Yang, H.; Yang, L.; Jiang, H.; Roth, B. L.; Cherezov, V.; Stevens, R. C.; Xu, H. E. (2013) Structural basis for molecular recognition at serotonin receptors. Science 340 (6132), 610–614.

(108) Liang, Y.-L.; Khoshouei, M.; Glukhova, A.; Furness, S. G. B.; Zhao, P.; Clydesdale, L.; Koole, C.; Truong, T. T.; Thal, D. M.; Lei, S.; Radjainia, M.; Danev, R.; Baumeister, W.; Wang, M.-W.; Miller, L. J.; Christopoulos, A.; Sexton, P. M.; Wootten, D. (2018) Phase-plate cryo-EM structure of a biased agonist-bound human GLP-1 receptor–Gs complex. Nature 555 (7694), 121–125.

(109) Kim, T. H.; Chung, K. Y.; Manglik, A.; Hansen, A. L.; Dror, R. O.; Mildorf, T. J.; Shaw, D. E.; Kobilka, B. K.; Prosser, R. S. (2013) The role of ligands on the equilibria between functional states of a G protein-coupled receptor. J. Am. Chem. Soc. 135 (25), 9465–9474.

(110) Latorraca, N. R.; Venkatakrishnan, A. J.; Dror, R. O. (2017) GPCR dynamics: structures in motion. Chem. Rev. 117 (1), 139–155.

(111) Manglik, A.; Kim, T. H.; Masureel, M.; Altenbach, C.; Yang, Z.; Hilger, D.; Lerch, M. T.; Kobilka, T. S.; Thian, F. S.; Hubbell, W. L.; Prosser, R. S.; Kobilka, B. K. (2015) Structural insights into the dynamic process of β_2-adrenergic receptor signaling. Cell 161 (5), 1101–1111.

(112) Mary, S.; Damian, M.; Louet, M.; Floquet, N.; Fehrentz, J.-A.; Marie, J.; Martinez, J.; Baneres, J.-L. (2012) Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor. Proc. Natl. Acad. Sci. 109 (21), 8304–8309.

(113) Du, Y.; Duc, N. M.; Rasmussen, S. G. F.; Hilger, D.; Kubiak, X.; Wang, L.; Bohon, J.; Kim, H. R.; Wegrecki, M.; Asuru, A.; Jeong, K. M.; Lee, J.; Chance, M. R.; Lodowski, D. T.; Kobilka, B. K.; Chung, K. Y. (2019) Assembly of a GPCR-G protein complex. Cell 177 (5), 1232-1242.e11.

(114) Ma, X.; Hu, Y.; Batebi, H.; Heng, J.; Xu, J.; Liu, X.; Niu, X.; Li, H.; Hildebrand, P. W.; Jin, C.; Kobilka, B. K. (2020) Analysis of β_2AR-G_s and β_2AR-G_i complex formation by NMR spectroscopy. Proc. Natl. Acad. Sci. 117 (37), 23096–23105.

(115) Westfield, G. H.; Rasmussen, S. G. F.; Su, M.; Dutta, S.; DeVree, B. T.; Chung, K. Y.; Calinski, D.; Velez-Ruiz, G.; Oleskie, A. N.; Pardon, E.; Chae, P. S.; Liu, T.; Li, S.; Woods, V. L.; Steyaert, J.; Kobilka, B. K.; Sunahara, R. K.; Skiniotis, G. (2011) Structural flexibility of the Gαs α-helical domain in the β_2-adrenoceptor Gs complex. Proc. Natl. Acad. Sci. 108 (38), 16086–16091.

(116) Furness, S. G. B.; Liang, Y.-L.; Nowell, C. J.; Halls, M. L.; Wookey, P. J.; Dal Maso, E.; Inoue, A.; Christopoulos, A.; Wootten, D.; Sexton, P. M. (2016) Ligand-dependent modulation of G protein conformation alters drug efficacy. Cell 167 (3), 739-749.e11.

(117) Klein Herenbrink, C.; Sykes, D. A.; Donthamsetti, P.; Canals, M.; Coudrat, T.; Shonberg, J.; Scammells, P. J.; Capuano, B.; Sexton, P. M.; Charlton, S. J.; Javitch, J. A.; Christopoulos, A.; Lane, J. R. (2016) The role of kinetic context in apparent biased agonism at GPCRs. Nat. Commun. 7 (1), 10842.

(118) Kenakin, T.; Christopoulos, A. (2013) Measurements of ligand bias and functional affinity. Nat. Rev. Drug Discov. 12 (6), 483–483.

(119) Ehlert, F. J. (2008) On the analysis of ligand-directed signaling at G protein-coupled receptors. Naunyn. Schmiedebergs Arch. Pharmacol. 377 (4–6), 549–577.

(120) Ehlert, F. J. (2005) Analysis of allosterism in functional assays. J. Pharmacol. Exp. Ther. 315 (2), 740–754.

(121) Rajagopal, S.; Ahn, S.; Rominger, D. H.; Gowen-MacDonald, W.; Lam, C. M.; DeWire, S. M.; Violin, J. D.; Lefkowitz, R. J. (2011) Quantifying ligand bias at seven-transmembrane receptors. Mol. Pharmacol. 80 (3), 367–377.

(122) Rajagopal, S. (2013) Quantifying biased agonism: understanding the links between affinity and efficacy. Nat. Rev. Drug Discov. 12 (6), 483–483.

(123) Whalen, E. J.; Rajagopal, S.; Lefkowitz, R. J. (2011) Therapeutic potential of β-arrestin- and G protein-biased agonists. Trends Mol. Med. 17 (3), 126–139.

(124) Violin, J. D.; Crombie, A. L.; Soergel, D. G.; Lark, M. W. (2014) Biased ligands at G-protein-coupled receptors: promise and progress. Trends Pharmacol. Sci. 35 (7), 308–316.

(125) Luttrell, L. M.; Maudsley, S.; Bohn, L. M. (2015) Fulfilling the promise of 'biased' G protein–coupled receptor agonism. Mol. Pharmacol. 88 (3), 579–588.

(126) Schmid, C. L.; Kennedy, N. M.; Ross, N. C.; Lovell, K. M.; Yue, Z.; Morgenweck, J.; Cameron, M. D.; Bannister, T. D.; Bohn, L. M. (2017) Bias factor and therapeutic window correlate to predict safer opioid analgesics. Cell 171 (5), 1165-1175.e13.

(127) Chen, X.-T.; Pitis, P.; Liu, G.; Yuan, C.; Gotchev, D.; Cowan, C. L.; Rominger, D. H.; Koblish, M.; DeWire, S. M.; Crombie, A. L.; Violin, J. D.; Yamashita, D. S. (2013) Structure–activity relationships and discovery of a G protein biased μ opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridin-

2-yl)-6-oxaspiro-[4.5]decan-9-yl]ethyl})amine (TRV130), for the treatment of acute severe pain. J. Med. Chem. 56 (20), 8019–8031.

(128) Bond, R. A.; Lucero Garcia-Rojas, E. Y.; Hegde, A.; Walker, J. K. L. (2019) Therapeutic potential of targeting β-arrestin. Front. Pharmacol. 10, 124.

(129) https://www.fda.gov/news-events/press-announcements/fda-approves-new-opioid-intravenous-use-hospitals-other-controlled-clinical-settings (accessed on 06/08/2021)

(130) Gillis, A.; Gondin, A. B.; Kliewer, A.; Sanchez, J.; Lim, H. D.; Alamein, C.; Manandhar, P.; Santiago, M.; Fritzwanker, S.; Schmiedel, F.; Katte, T. A.; Reekie, T.; Grimsey, N. L.; Kassiou, M.; Kellam, B.; Krasel, C.; Halls, M. L.; Connor, M.; Lane, J. R.; Schulz, S.; Christie, M. J.; Canals, M. (2020) Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. Sci. Signal. 13 (625), eaaz3140.

(131) Violin, J. D.; Lefkowitz, R. J. (2007) β-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol. Sci. 28 (8), 416–422.

(132) Pang, P. S.; Butler, J.; Collins, S. P.; Cotter, G.; Davison, B. A.; Ezekowitz, J. A.; Filippatos, G.; Levy, P. D.; Metra, M.; Ponikowski, P.; Teerlink, J. R.; Voors, A. A.; Bharucha, D.; Goin, K.; Soergel, D. G.; Felker, G. M. (2017) Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, Phase IIB, dose ranging trial (BLAST-AHF). Eur. Heart J. 38 (30), 2364–2373.

(133) https://www.trevena.com/pipeline/trv027 (accessed on 06/08/2021)

(134) Congreve, M.; Rich, R. L.; Myszka, D. G.; Figaroa, F.; Siegal, G.; Marshall, F. H. Fragment screening of stabilized G-protein-coupled receptors using biophysical methods. In Methods in Enzymology; Elsevier, 2011, 493, 115–136.

(135) Chen, D.; Errey, J. C.; Heitman, L. H.; Marshall, F. H.; IJzerman, A. P.; Siegal, G. (2012) Fragment screening of GPCRs using biophysical methods: identification of ligands of the adenosine A_{2A} receptor with novel biological activity. ACS Chem. Biol. 7 (12), 2064–2073.

(136) Navratilova, I. H.; Aristotelous, T.; Bird, L. E.; Hopkins, A. L. (2018) Surveying GPCR solubilisation conditions using surface plasmon resonance. Anal. Biochem. 556, 23–34.

(137) Magnani, F.; Shibata, Y.; Serrano-Vega, M. J.; Tate, C. G. (2008) Co-evolving stability and conformational homogeneity of the human adenosine A_{2A} receptor. Proc. Natl. Acad. Sci. 105 (31), 10744–10749.

(138) Serrano-Vega, M. J.; Magnani, F.; Shibata, Y.; Tate, C. G. (2008) Conformational thermostabilization of the β_1-adrenergic receptor in a detergent-resistant form. Proc. Natl. Acad. Sci. 105 (3), 877–882.

(139) Kenakin, T. P. (2012) Biased signalling and allosteric machines: new vistas and challenges for drug discovery: 7TMRs as allosteric proteins. Br. J. Pharmacol. 165 (6), 1659–1669.

(140) Stoddart, L. A.; Vernall, A. J.; Denman, J. L.; Briddon, S. J.; Kellam, B.; Hill, S. J. (2012) Fragment screening at adenosine-A_3 receptors in living cells using a fluorescence-based binding assay. Chem. Biol. 19 (9), 1105–1115.

(141) Aristotelous, T.; Ahn, S.; Shukla, A. K.; Gawron, S.; Sassano, M. F.; Kahsai, A. W.; Wingler, L. M.; Zhu, X.; Tripathi-Shukla, P.; Huang, X.-P.; Riley, J.; Besnard, J.; Read, K. D.; Roth, B. L.; Gilbert, I. H.; Hopkins, A. L.; Lefkowitz, R. J.; Navratilova, I. (2013) Discovery of β_2 adrenergic receptor ligands using biosensor fragment screening of tagged wild-type receptor. ACS Med. Chem. Lett. 4 (10), 1005–1010.

(142) Igonet, S.; Raingeval, C.; Cecon, E.; Pučić-Baković, M.; Lauc, G.; Cala, O.; Baranowski, M.; Perez, J.; Jockers, R.; Krimm, I.; Jawhari, A. (2018) Enabling STD-NMR fragment screening using stabilized native GPCR: a case study of adenosine receptor. Sci. Rep. 8 (1), 8142.

(143) GPCRdb; https://gpcrdb.org/structure/statistics (accessed on 06/08/2021)

(144) Coudrat, T.; Simms, J.; Christopoulos, A.; Wootten, D.; Sexton, P. M. (2017) Improving virtual screening of G protein-coupled receptors via ligand-directed modeling. PLOS Comput. Biol. 13 (11), e1005819.

(145) Basith, S.; Cui, M.; Macalino, S. J. Y.; Park, J.; Clavio, N. A. B.; Kang, S.; Choi, S. (2018) Exploring G protein-coupled receptors (GPCRs) ligand space via cheminformatics approaches: impact on rational drug design. Front. Pharmacol. 9, 128.

(146) Jaiteh, M.; Rodríguez-Espigares, I.; Selent, J.; Carlsson, J. (2020) Performance of virtual screening against GPCR homology models: impact of template

selection and treatment of binding site plasticity. PLOS Comput. Biol. 16 (3), e1007680.

(147) Zhang, R.; Xie, X. (2012) Tools for GPCR drug discovery. Acta Pharmacol. Sin. 33 (3), 372–384.

(148) Bylund, D. B.; Toews, M. L. (1993) Radioligand binding methods: practical guide and tips. Am. J. Physiol.-Lung Cell. Mol. Physiol. 265 (5), 421–L429.

(149) Bylund, D. B.; Toews, M. L. Radioligand binding methods for membrane preparations and intact cells. In Receptor Signal Transduction Protocols; Willars, G. B., Challiss, R. A. J., Eds.; Methods in Molecular Biology; Humana Press: Totowa, NJ, 2011; Vol. 746, pp 135–164.

(150) Glickman, J. F.; Schmid, A.; Ferrand, S. (2008) Scintillation proximity assays in high-throughput screening. ASSAY Drug Dev. Technol. 6 (3), 433–455.

(151) Zwier, J. M.; Roux, T.; Cottet, M.; Durroux, T.; Douzon, S.; Bdioui, S.; Gregor, N.; Bourrier, E.; Oueslati, N.; Nicolas, L.; Tinel, N.; Boisseau, C.; Yverneau, P.; Charrier-Savournin, F.; Fink, M.; Trinquet, E. (2010) A fluorescent ligand-binding alternative using Tag-Lite® technology. J. Biomol. Screen. 15 (10), 1248–1259.

(152) Cheng, Y.-C.; Prusoff, W. H. (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22 (23), 3099–3108.

(153) Williams, C.; Sewing, A. (2005) G-protein coupled receptor assays: to measure affinity or efficacy that is the question. Comb. Chem. High Throughput Screen. 8 (4), 285–292.

(154) Strange, P. G. (2010) Use of the GTPγS ([^{35}S]GTPγS and Eu-GTPγS) binding assay for analysis of ligand potency and efficacy at G protein-coupled receptors. Br. J. Pharmacol. 161 (6), 1238–1249.

(155) Emkey, R.; Rankl, N. B. Screening G protein-coupled receptors: measurement of intracellular calcium using the Fluorometric Imaging Plate Reader. In High Throughput Screening; Janzen, W. P., Bernasconi, P., Eds.; Methods in Molecular Biology; Humana Press: Totowa, NJ, 2009; Vol. 565, pp 145–158.

(156) Yasi, E. A.; Kruyer, N. S.; Peralta-Yahya, P. (2020) Advances in G protein-coupled receptor high-throughput screening. Curr. Opin. Biotechnol. 64, 210–217.

(157) Chambers, C.; Smith, F.; Williams, C.; Marcos, S.; Liu, Z.; Hayter, P.; Ciaramella, G.; Keighley, W.; Gribbon, P.; Sewing, A. (2003) Measuring intracellular calcium fluxes in high throughput mode. Comb. Chem. High Throughput Screen. 6 (4), 355–362.

(158) Xin, H.; Wang, Y.; Todd, M. J.; Qi, J.; Minor, L. K. (2007) Evaluation of no-wash calcium assay kits: enabling tools for calcium mobilization. J. Biomol. Screen. 12 (5), 705–714.

(159) Vetter, I. Development and optimization of FLIPR high throughput calcium assays for ion channels and GPCRs. In Calcium Signaling; Islam, Md. S., Ed.; Advances in Experimental Medicine and Biology; Springer Netherlands: Dordrecht, 2012; Vol. 740, pp 45–82.

(160) Mithöfer, A.; Mazars, C. (2002) Aequorin-based measurements of intracellular Ca^{2+}-signatures in plant cells. Biol. Proced. Online 4 (1), 105–118.

(161) Gabriel, D.; Vernier, M.; Pfeifer, M. J.; Dasen, B.; Tenaillon, L.; Bouhelal, R. (2003) High throughput screening technologies for direct cyclic AMP measurement. ASSAY Drug Dev. Technol. 1 (2), 291–303.

(162) Williams, C. (2004) cAMP detection methods in HTS: selecting the best from the rest. Nat. Rev. Drug Discov. 3 (2), 125–135.

(163) Binkowski, B. F.; Butler, B. L.; Stecha, P. F.; Eggers, C. T.; Otto, P.; Zimmerman, K.; Vidugiris, G.; Wood, M. G.; Encell, L. P.; Fan, F.; Wood, K. V. (2011) A luminescent biosensor with increased dynamic range for intracellular cAMP. ACS Chem. Biol. 6 (11), 1193–1197.

(164) Degorce, F. (2009) HTRF: a technology tailored for drug discovery - a review of theoretical aspects and recent applications. Curr. Chem. Genomics 3 (1), 22–32.

(165) Cheng, Z.; Garvin, D.; Paguio, A.; Stecha, P.; Wood, K.; Fan, F. (2010) Luciferase reporter assay system for deciphering GPCR pathways. Curr. Chem. Genomics 4, 84–91.

(166) Zhao, X.; Jones, A.; Olson, K. R.; Peng, K.; Wehrman, T.; Park, A.; Mallari, R.; Nebalasca, D.; Young, S. W.; Xiao, S.-H. (2008) A homogeneous enzyme fragment complementation-based β-arrestin translocation assay for high-throughput screening of G-protein-coupled receptors. J. Biomol. Screen. 13 (8), 737–747.

(167) Hamdan, F. F.; Audet, M.; Garneau, P.; Pelletier, J.; Bouvier, M. (2005) High-throughput screening of G protein-coupled receptor antagonists using a bioluminescence resonance energy transfer 1-based β-arrestin2 recruitment Assay. J. Biomol. Screen. 10 (5), 463–475.

(168) Scott, C. W.; Peters, M. F. (2010) Label-free whole-cell assays: expanding the scope of GPCR screening. Drug Discov. Today 15 (17–18), 704–716.

(169) Fang, Y.; Frutos, A.; Verklereen, R. (2008) Label-free cell-based assays for GPCR screening. Comb. Chem. High Throughput Screen. 11 (5), 357–369.

(170) Fang, Y. (2014) Label-free drug discovery. Front. Pharmacol. 5.

(171) Schröder, R.; Janssen, N.; Schmidt, J.; Kebig, A.; Merten, N.; Hennen, S.; Müller, A.; Blättermann, S.; Mohr-Andrä, M.; Zahn, S.; Wenzel, J.; Smith, N. J.; Gomeza, J.; Drewke, C.; Milligan, G.; Mohr, K.; Kostenis, E. (2010) Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. Nat. Biotechnol. 28 (9), 943–949.

(172) Tran, E.; Ye Fang. (2008) Duplexed label-free G protein-coupled receptor assays for high-throughput screening. J. Biomol. Screen. 13 (10), 975–985.

(173) Dodgson, K.; Gedge, L.; Murray, D. C.; Coldwell, M. (2009) A 100K well screen for a muscarinic receptor using the Epic® label-free system – a reflection on the benefits of the label-free approach to screening seven-transmembrane receptors. J. Recept. Signal Transduct. 29 (3–4), 163–172.

(174) Deng, H.; Hu, H.; He, M.; Hu, J.; Niu, W.; Ferrie, A. M.; Fang, Y. (2011) Discovery of 2-(4-methylfuran-2(5H)-ylidene)malononitrile and thieno[3,2-b]thiophene-2-carboxylic acid derivatives as G protein-coupled receptor 35 (GPR35) agonists. J. Med. Chem. 54 (20), 7385–7396.

(175) Cloutier, T. Combining labeled and label-free tools. Genetic Engineering & Biotechnology News, April 15, 2011 (Vol. 31, No. 8)

https://www.genengnews.com/magazine/156/combining-labeled-and-label-free-tools/ (accessed on 06/08/2021)

(176) Seifert, R. (2013) Functional selectivity of G-protein-coupled receptors: from recombinant systems to native human cells. Biochem. Pharmacol. 86 (7), 853–861.

(177) Greasley, P. HTS - Developing future success from previous experience; SMi - High throughput screening conference, 27th April 2010, London, United Kingdom; http://www.smi-online.co.uk/pharmaceuticals/uk/conference/high-throughput-screening#day_2 (accessed on 06/08/2021)

(178) Engle, S. J.; Vincent, F. (2014) Small molecule screening in human induced pluripotent stem cell-derived terminal cell types. J. Biol. Chem. 289 (8), 4562–4570.

(179) Horvath, P.; Aulner, N.; Bickle, M.; Davies, A. M.; Nery, E. D.; Ebner, D.; Montoya, M. C.; Östling, P.; Pietiäinen, V.; Price, L. S.; Shorte, S. L.; Turcatti, G.; von Schantz, C.; Carragher, N. O. (2016) Screening out irrelevant cell-based models of disease. Nat. Rev. Drug Discov. 15 (11), 751–769.

(180) Avior, Y.; Sagi, I.; Benvenisty, N. (2016) Pluripotent stem cells in disease modelling and drug discovery. Nat. Rev. Mol. Cell Biol. 17 (3), 170–182.

(181) Fox, S.; Farr-Jones, S.; Sopchak, L.; Boggs, A.; Nicely, H. W.; Khoury, R.; Biros, M. (2006) High-throughput screening: update on practices and success. J. Biomol. Screen. 11 (7), 864–869.

(182) Barker, J.; Courtney, S.; Hesterkamp, T.; Ullmann, D.; Whittaker, M. (2006) Fragment screening by biochemical assay. Expert Opin. Drug Discov. 1 (3), 225–236.

(183) Boettcher, A.; Ruedisser, S.; Erbel, P.; Vinzenz, D.; Schiering, N.; Hassiepen, U.; Rigollier, P.; Mayr, L. M.; Woelcke, J. (2010) Fragment-based screening by biochemical assays: systematic feasibility studies with trypsin and MMP12. J. Biomol. Screen. 15 (9), 1029–1041.

(184) Verheij, M. H. P.; de Graaf, C.; de Kloe, G. E.; Nijmeijer, S.; Vischer, H. F.; Smits, R. A.; Zuiderveld, O. P.; Hulscher, S.; Silvestri, L.; Thompson, A. J.; van Muijlwijk-Koezen, J. E.; Lummis, S. C. R.; Leurs, R.; de Esch, I. J. P. (2011) Fragment library screening reveals remarkable similarities between the G

protein-coupled receptor histamine H_4 and the ion channel serotonin 5-HT_{3A}. Bioorg. Med. Chem. Lett. 21 (18), 5460–5464.

(185) Artis, D. R.; Lin, J. J.; Zhang, C.; Wang, W.; Mehra, U.; Perreault, M.; Erbe, D.; Krupka, H. I.; England, B. P.; Arnold, J.; Plotnikov, A. N.; Marimuthu, A.; Nguyen, H.; Will, S.; Signaevsky, M.; Kral, J.; Cantwell, J.; Settachatgull, C.; Yan, D. S.; Fong, D.; Oh, A.; Shi, S.; Womack, P.; Powell, B.; Habets, G.; West, B. L.; Zhang, K. Y. J.; Milburn, M. V.; Vlasuk, G. P.; Hirth, K. P.; Nolop, K.; Bollag, G.; Ibrahim, P. N.; Tobin, J. F. (2009) Scaffold-based discovery of Indeglitazar, a PPAR pan-active anti-diabetic agent. Proc. Natl. Acad. Sci. 106 (1), 262–267.

(186) Malo, N.; Hanley, J. A.; Cerquozzi, S.; Pelletier, J.; Nadon, R. (2006) Statistical practice in high-throughput screening data analysis. Nat. Biotechnol. 24 (2), 167–175.

(187) Glickman, J. F.; Wu, X.; Mercuri, R.; Illy, C.; Bowen, B. R.; He, Y.; Sills, M. (2002) A comparison of ALPHAScreen, TR-FRET, and TRF as assay methods for FXR nuclear receptors. J. Biomol. Screen. 7 (1), 3–10.

(188) Wu, X.; Glickman, J. F.; Bowen, B. R.; Sills, M. A. (2003) Comparison of assay technologies for a nuclear receptor assay screen reveals differences in the sets of identified functional antagonists. J. Biomol. Screen. 8 (4), 381–392.

(189) Wu, X.; Sills, M. A.; Zhang, J.-H. (2005) Further comparison of primary hit identification by different assay technologies and effects of assay measurement variability. J. Biomol. Screen. 10 (6), 581–589.

(190) Zhang, J.; Wu, X.; Sills, M. A. (2005) Probing the primary screening efficiency by multiple replicate testing: a quantitative analysis of hit confirmation and false screening results of a biochemical assay. J. Biomol. Screen. 10 (7), 695–704.

(191) Baker, J. G.; Hill, S. J.; Summers, R. J. (2011) Evolution of β-blockers: from anti-anginal drugs to ligand-directed signalling. Trends Pharmacol. Sci. 32 (4), 227–234.

(192) Rankovic, Z.; Brust, T. F.; Bohn, L. M. (2016) Biased Agonism: An emerging paradigm in GPCR drug discovery. Bioorg. Med. Chem. Lett. 26 (2), 241–250.

(193) Slack, M.; Winkler, D.; Krämer, J.; Hesterkamp, T. (2009) A multiplexed approach to hit finding. Curr. Opin. Drug Discov. Devel. 12 (3), 351–357.

(194) Hanson, B. J. (2006) Multiplexing Fluo-4 NW and a GeneBLAzer® transcriptional assay for high-throughput screening of G-protein-coupled receptors. J. Biomol. Screen. 11 (6), 644–651.

(195) Sheth, H. (2013) A multiplexed fluorescent calcium and NFAT reporter gene assay to identify GPCR agonists. Curr. Chem. Genomics Transl. Med. 7, 1–8.

(196) T. Wehrmann, C.-Y. Loh, L. Anantharaman, et al., Dual purpose cell lines for analysis of GPCR/arrestin interaction using PathHunter and natural Gq-coupling using the improved calcium No-Wash PLUS (CaNWPLUS) Kit, in: Poster Presented at Society for Biomolecular Sciences 14th Annual Conference and Exhibition, 2008 (St. Louis, MO). https://www.discoverx.com/tools-resources/document-resource-library/documents/dual-purpose-cell-lines-for-analysis-of-gpcr-arres (accessed on 06/08/2021)

(197) S. Brogniet, L. Meeus, J. Bernard, Multiplexing approach for GPCR signaling pathways in HTS format. http://www.ontarget-ddss.co.jp/EUS/EUS_Multiplexing%20approach%20for%20GPCR%20signaling%20pathways%20in%20HTS%20format.pdf (accessed on 06/08/2021)

(198) M. Robers, P. Stecha, N. Karassina, et al., Multiplexed analysis of second messenger signaling in live cells using aequorin and GloSensor cAMP on the Hamamatsu μCell, in: Paper Presented at 10th European Functional Drug Screening Symposium, 2014 (Allschwil, Switzerland). https://fdss.hamamatsu.com/sp/hpe/community/fdss/meeting/2014/poster/No9_2014_MR.pdf (accessed on 06/08/2021)

(199) https://cdn.technologynetworks.com/TN/Resources/PDF/P101-%20MIPTEC_GloSensor_2011.pdf (accessed on 06/08/2021)

(200) Mella, R. M.; Kortazar, D.; Roura-Ferrer, M.; Salado, C.; Valcárcel, M.; Castilla, A.; Villacé, P. (2018) Nomad biosensors: a new multiplexed technology for the screening of GPCR ligands. SLAS Technol. Transl. Life Sci. Innov. 23 (3), 207–216.

(201) https://innoprot.com/technology/ (accessed on 06/08/2021)

(202) Grundmann, M.; Merten, N.; Malfacini, D.; Inoue, A.; Preis, P.; Simon, K.; Rüttiger, N.; Ziegler, N.; Benkel, T.; Schmitt, N. K.; Ishida, S.; Müller, I.; Reher, R.; Kawakami, K.; Inoue, A.; Rick, U.; Kühl, T.; Imhof, D.; Aoki, J.;

König, G. M.; Hoffmann, C.; Gomeza, J.; Wess, J.; Kostenis, E. (2018) Lack of beta-arrestin signaling in the absence of active G proteins. Nat. Commun. 9 (1), 341.

(203) Zhong, H.; Huang, X.; Doller, D. Multiplexing label-free and fluorescence-based methods for pharmacological characterization of GPCR ligands. In Label-Free Biosensor Methods in Drug Discovery; Fang, Y., Ed.; Methods in Pharmacology and Toxicology; Springer New York: New York, NY, 2015; pp 215–231.

(204) http://codexbiosolutions.com/wp-content/uploads/Applications-of-multiplex-cAMP-and-label-free-assays-using-FDSS7000-and-EPIC-BT-for-studying-potential-biased-signaling-through-the-5-HT7-receptor.pdf (accessed on 06/08/2021)

(205) Gyires, K.; Zádori, Z. S.; Török, T.; Mátyus, P. (2009) α_2-adrenoceptor subtypes-mediated physiological, pharmacological actions. Neurochem. Int. 55 (7), 447–453.

(206) Tella, S. H.; Gallagher, J. C. (2014) Biological agents in management of osteoporosis. Eur. J. Clin. Pharmacol. 70 (11), 1291–1301.

(207) Vilardaga, J.-P.; Romero, G.; Friedman, P. A.; Gardella, T. J. (2011) Molecular basis of parathyroid hormone receptor signaling and trafficking: a family B GPCR paradigm. Cell. Mol. Life Sci. 68 (1), 1–13.

(208) Kurko, D.; Bekes, Z.; Gere, A.; Baki, A.; Boros, A.; Kolok, S.; Bugovics, G.; Nagy, J.; Szombathelyi, Z.; Ignácz-Szendrei, G. (2009) Comparative pharmacology of adrenergic α_{2C} receptors coupled to Ca^{2+} signaling through different Gα proteins. Neurochem. Int. 55 (7), 467–475.

(209) 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.

(210) Kaupp, U. B.; Seifert, R. (2002) Cyclic nucleotide-gated ion channels. Physiol. Rev. 82 (3), 769–824.

(211) Fang, Y.; Ferrie, A. M.; Tran, E. Resonant waveguide grating biosensor for whole-cell GPCR assays. In G Protein-Coupled Receptors in Drug Discovery;

Leifert, W. R., Ed.; Methods in Molecular Biology; Humana Press: Totowa, NJ, 2009; Vol. 552, pp 239–252.

(212) Schröder, R.; Schmidt, J.; Blättermann, S.; Peters, L.; Janssen, N.; Grundmann, M.; Seemann, W.; Kaufel, D.; Merten, N.; Drewke, C.; Gomeza, J.; Milligan, G.; Mohr, K.; Kostenis, E. (2011) Applying label-free dynamic mass redistribution technology to frame signaling of G protein-coupled receptors noninvasively in living cells. Nat. Protoc. 6 (11), 1748–1760.

(213) Rasmussen, S. G. F.; DeVree, B. T.; Zou, Y.; Kruse, A. C.; Chung, K. Y.; Kobilka, T. S.; Thian, F. S.; Chae, P. S.; Pardon, E.; Calinski, D.; Mathiesen, J. M.; Shah, S. T. A.; Lyons, J. A.; Caffrey, M.; Gellman, S. H.; Steyaert, J.; Skiniotis, G.; Weis, W. I.; Sunahara, R. K.; Kobilka, B. K. (2011) Crystal structure of the β_2 adrenergic receptor–Gs protein complex. Nature 477 (7366), 549–555.

(214) Bajusz, D.; Rácz, A.; Héberger, K. (2015) Why is tanimoto index an appropriate choice for fingerprint-based similarity calculations? J. Cheminformatics 7 (1), 20.

(215) Cupp, M. E.; Nayak, S. K.; Adem, A. S.; Thomsen, W. J. (2013) Parathyroid hormone (PTH) and PTH-related peptide domains contributing to activation of different PTH receptor–mediated signaling pathways. J. Pharmacol. Exp. Ther. 345 (3), 404–418.

(216) Appleton, K. M.; Lee, M.-H.; Alele, C.; Alele, C.; Luttrell, D. K.; Peterson, Y. K.; Morinelli, T. A.; Luttrell, L. M. Biasing the parathyroid hormone receptor. In Methods in Enzymology; Elsevier, 2013, 522, 229–262.

(217) Mamidanna, G.; Cole, J. (2015) Comparison of Parathyroid Hormone (PTH) (1-34) and PTH (7-34) effects on cell signaling and proliferation in Saos-2 osteoblast-like cells; The FASEB Journal 29, 772.10.

(218) Thomas, P.; Smart, T. G. (2005) HEK293 cell line: a vehicle for the expression of recombinant proteins. J. Pharmacol. Toxicol. Methods 51 (3), 187–200.

(219) Atwood, B. K.; Lopez, J.; Wager-Miller, J.; Mackie, K.; Straiker, A. (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12 (1), 14.

(220) Umland, S. P.; Wan, Y.; Shah, H.; Billah, M.; Egan, R. W.; Hey, J. A. (2001) Receptor reserve analysis of the human α_{2C}-adrenoceptor using [^{35}S]GTPγS and cAMP functional assays. Eur. J. Pharmacol. 411 (3), 211–221.

(221) Rinne, J. O.; Wesnes, K.; Cummings, J. L.; Hakulinen, P.; Hallikainen, M.; Hänninen, J.; Murphy, M.; Riordan, H.; Scheinin, M.; Soininen, H.; Rouru, J. (2017) Tolerability of ORM-12741 and effects on episodic memory in patients with Alzheimer's disease. Alzheimers Dement. Transl. Res. Clin. Interv. 3 (1), 1–9.

(222) Wigglesworth, M. J.; Murray, D. C.; Blackett, C. J.; Kossenjans, M.; Nissink, J. W. M. (2015) Increasing the delivery of next generation therapeutics from high throughput screening libraries. Curr. Opin. Chem. Biol. 26, 104–110.

(223) Sills, M. A.; Weiss, D.; Pham, Q.; Schweitzer, R.; Wu, X.; Wu, J. J. (2002) Comparison of assay technologies for a tyrosine kinase assay generates different results in high throughput screening. J. Biomol. Screen. 7 (3), 191–214.

(224) Nijmeijer, S.; Vischer, H. F.; Rosethorne, E. M.; Charlton, S. J.; Leurs, R. (2012) Analysis of multiple histamine H_4 receptor compound classes uncovers $G\alpha_i$ protein- and β-arrestin2-biased ligands. Mol. Pharmacol. 82 (6), 1174–1182.

(225) Conroy, J. L.; Free, R. B.; Sibley, D. R. (2015) Identification of G protein-biased agonists that fail to recruit β-arrestin or promote internalization of the D_1 dopamine receptor. ACS Chem. Neurosci. 6 (4), 681–692.

(226) Nelson, C. P.; Challiss, R. A. J. (2007) 'Phenotypic' pharmacology: the influence of cellular environment on G protein-coupled receptor antagonist and inverse agonist pharmacology. Biochem. Pharmacol. 73 (6), 737–751.

(227) Kawai, T.; Sun, B.; Yoshino, H.; Feng, D.; Suzuki, Y.; Fukazawa, M.; Nagao, S.; Wainscott, D. B.; Showalter, A. D.; Droz, B. A.; Kobilka, T. S.; Coghlan, M. P.; Willard, F. S.; Kawabe, Y.; Kobilka, B. K.; Sloop, K. W. (2020) Structural basis for GLP-1 receptor activation by LY3502970, an orally active nonpeptide agonist. Proc. Natl. Acad. Sci. 117 (47), 29959–29967.

(228) Tamura, T.; Noda, H.; Joyashiki, E.; Hoshino, M.; Watanabe, T.; Kinosaki, M.; Nishimura, Y.; Esaki, T.; Ogawa, K.; Miyake, T.; Arai, S.; Shimizu, M.; Kitamura, H.; Sato, H.; Kawabe, Y. (2016) Identification of an orally active

small-molecule PTHR1 agonist for the treatment of hypoparathyroidism. Nat. Commun. 7 (1), 13384.

(229) Hattersley, G.; Dean, T.; Corbin, B. A.; Bahar, H.; Gardella, T. J. (2016) Binding selectivity of Abaloparatide for PTH-Type-1-Receptor conformations and effects on downstream signaling. Endocrinology 157 (1), 141–149.

(230) Quaglia, W.; Del Bello, F.; Giannella, M.; Piergentili, A.; Pigini, M. (2011) α_{2C} -adrenoceptor modulators: a patent review. Expert Opin. Ther. Pat. 21 (4), 455–481.

(231) Improved cell-based screening of an endogenously expressed gs-coupled GPCR using the Epic® system over a conventional cAMP tehnology; SnAPPShots - a brief technical report from the corning applications group. https://www.yumpu.com/en/document/read/32703000/improved-cell-based-screening-of-an-endogenously-expressed-gs- (accessed on 06/08/2021)

(232) Ghosh, R. N.; DeBiasio, R.; Hudson, C. C.; Ramer, E. R.; Cowan, C. L.; Oakley, R. H. (2005) Quantitative cell-based high-content screening for vasopressin receptor agonists using Transfluor® technology. J. Biomol. Screen. 10 (5), 476–484.

(233) Barnea, G.; Strapps, W.; Herrada, G.; Berman, Y.; Ong, J.; Kloss, B.; Axel, R.; Lee, K. J. (2008) The genetic design of signaling cascades to record receptor activation. Proc. Natl. Acad. Sci. 105 (1), 64–69.

(234) Shenoy, S. K.; Drake, M. T.; Nelson, C. D.; Houtz, D. A.; Xiao, K.; Madabushi, S.; Reiter, E.; Premont, R. T.; Lichtarge, O.; Lefkowitz, R. J. (2006) β-arrestin-dependent, G protein-independent ERK1/2 activation by the β_2 adrenergic receptor. J. Biol. Chem. 281 (2), 1261–1273.

(235) Shukla, A. K.; Xiao, K.; Lefkowitz, R. J. (2011) Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. Trends Biochem. Sci. 36 (9), 457–469.

(236) Shonberg, J.; Herenbrink, C. K.; López, L.; Christopoulos, A.; Scammells, P. J.; Capuano, B.; Lane, J. R. (2013) A structure–activity analysis of biased agonism at the dopamine D_2 receptor. J. Med. Chem. 56 (22), 9199–9221.

(237) Dage, J. L.; Colvin, E. M.; Fouillet, A.; Langron, E.; Roell, W. C.; Li, J.; Mathur, S. X.; Mogg, A. J.; Schmitt, M. G.; Felder, C. C.; Merchant, K. M.;

Isaac, J.; Broad, L. M.; Sher, E.; Ursu, D. (2014) Pharmacological characterisation of ligand- and voltage-gated ion channels expressed in human iPSC-derived forebrain neurons. Psychopharmacology (Berl.) 231 (6), 1105–1124.

(238) Pai, S.; Verrier, F.; Sun, H.; Hu, H.; Ferrie, A. M.; Eshraghi, A.; Fang, Y. (2012) Dynamic mass redistribution assay decodes differentiation of a neural progenitor stem cell. J. Biomol. Screen. 17 (9), 1180–1191.

(239) https://www.cisbio.eu/media/asset/l/s/ls-pr-slas2015-tutorial-htrf-assays-with-ipsc-derived-cell-types.pdf (accessed on 06/08/2021)

(240) https://fujifilmcdi.com/assets/CDI140121SLAS06.pdf (accessed on 06/08/2021)

(241) Civelli, O.; Reinscheid, R. K.; Zhang, Y.; Wang, Z.; Fredriksson, R.; Schiöth, H. B. (2013) G protein–coupled receptor deorphanizations. Annu. Rev. Pharmacol. Toxicol. 53 (1), 127–146.

(242) Hennen, S.; Wang, H.; Peters, L.; Merten, N.; Simon, K.; Spinrath, A.; Blattermann, S.; Akkari, R.; Schrage, R.; Schroder, R.; Schulz, D.; Vermeiren, C.; Zimmermann, K.; Kehraus, S.; Drewke, C.; Pfeifer, A.; Konig, G. M.; Mohr, K.; Gillard, M.; Muller, C. E.; Lu, Q. R.; Gomeza, J.; Kostenis, E. (2013) Decoding signaling and function of the orphan G protein-coupled receptor GPR17 with a small-molecule agonist. Sci. Signal. 6 (298), ra93.

(243) Bradley, J. (2006) Splice variants of the NR1 subunit differentially induce NMDA receptor-dependent gene expression. J. Neurosci. 26 (4), 1065–1076.

(244) Paul, S.; Connor, J. A. (2010) NR2B-NMDA receptor-mediated increases in intracellular Ca^{2+} concentration regulate the tyrosine phosphatase, STEP, and ERK MAP kinase signaling. J. Neurochem. 1107–1118.

(245) Caruso, M.-E.; Clément, P.; Parent, S.; Dupriez, V.; Bossé, R.; Rouleau, N. (2013) Integrated cell-based platform to study EGFR activation and transactivation. ASSAY Drug Dev. Technol. 11 (7), 423–434.

(246) Ye Fang; Ferrie, A. M.; Fontaine, N. H.; Po Ki Yuen. Optical biosensors for monitoring dynamic mass redistribution in living cells mediated by epidermal growth factor receptor activation. In 2005 IEEE Engineering in Medicine and Biology 27th Annual Conference; IEEE: Shanghai, China, 2005; pp 666–669.

DOI:10.14753/SE.2021.2563

10. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

10.1. 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.

10.2. 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.

DOI:10.14753/SE.2021.2563

11. ACKNOWLEDGEMENTS

I am grateful beyond words to my supervisor **Dr. András Visegrády**, who as a professional mentor introduced me to the mysteries and wonders of pharmacology, shaped my scientific thinking and always encouraged me over the years, never sparing time or energy for my support.

I am also immensely grateful to **my loving wife** who supported my ambitions even in the middle of our engagement period and during the first year of our marriage and who never got tired of asking what an agonist or antagonist is.

My heart always misses a beat when I remember the way **my mother** and **my father** raised me with love and care, guaranteeing the best education for me and giving me all the opportunities I needed.

I also wish to say thank you to my dearest colleagues, **Éva Horváth** for technical assistance in cell culture work, **Attila Bielik** for support in laboratory automation exercises and **Dr. Attila Cselenyák** for IT support throughout the years.

Furthermore, special thanks should go to **Amrita Bobok** for training me in radioligand binding assays and **Dr. Eszter Riethmüller** and **Dr. Árpád Könczöl** for sample integrity determinations.

As the presented work was performed in the Lead Discovery Unit of Gedeon Richter Plc., I also acknowledge the former and present management of the R&D Directorate, **Prof. György M. Keserű** and **Dr. István Greiner** who enabled my research activity beside the daily work.