DiscoverX

EBOOK

INSIGHTS INTO GPCR DRUG DISCOVERY & DEVELOPMENT

Exploring GPCR–Ligand Interactions and Signaling Pathways with Binding and Functional Assays

"… Examples of β-arrestin and G protein-biased ligands demonstrate how our new understanding of these two types of signaling pathways, gained initially at a biochemical level, can potentially be harnessed for therapeutic benefit."

—Dr. Robert J. Lefkowitz, Awardee 2012 Nobel Prize in Chemistry for Groundbreaking Discoveries on GPCR Receptors

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INTRODUCTION

GPCR Biology and the Drug Discovery & Development of New Therapeutics

GPCRs IMPORTANCE

G protein-coupled receptors (GPCRs) represent the largest family of validated therapeutic targets with over 800 known human GPCRs. Their physiological and pathological involvements are vast, ranging from regulation of hunger to the development of fetal structures, and from roles in cardiovascular disorders to rare diseases (see Table 1.). Therapeutics targeting GPCRs represent over 40% of all currently marketed drug therapeutics acting on GPCRs either directly or indirectly.

The importance of GPCRs was elevated in 2012 with the awarding of the Nobel Prize in Chemistry to American Drs. Robert Lefkowitz and Brian Kobilka for their efforts on the inner workings of GPCRs, and revealing some of the mystery about how cells could sense their environment. Tremendous interest in the discovery and development of drugs targeting GPCRs continues, spurred on by advances in information, tools, technologies, additional GPCR crystal structures, and the identification of numerous orphan GPCRs. As the largest family of validated therapeutic targets, GPCRs continue to be of interest for drug and treatment development for diseases, including cancer, Alzheimer's disease, and diabetes.

Physiological		Pathological	
Addiction	Pain tolerance	Cancers	Fertility disorders
Behavioral/mood regulation	Pheromone response	Cardiovascular disorders (e.g. high blood pressure)	Neurological diseases (e.g., Alzheimer's, schizophrenia, depression, Parkinson's disease)
Embryonic development	Regulation of heart rate	Endocrine and metabolic disorders (e.g. diabetes, obesity, hypo- and hyperthyroidism, and nephrogenic diabetes insipidus)	Ocular disorders (e.g. Retinitis pigmentosa)
Homeostasis	Regulation of cellular machinery		Rare diseases
Hunger regulation	Smell (olfactory)		Respiratory disorders (e.g. asthma)
Learning and memory	Taste		
Metabolism	Vision		

Table 1. GPCR physiological and pathological involvements

GPCR SIGNALING PATHWAYS

GPCRs are 7-transmembrane domain receptors with a broad array of ligands, including growth factors, ions, lipids, nucleotides, hormones, and neurotransmitters. Upon activation by ligands, GPCRs undergo a conformational change that translates the extracellular signal to an intracellular response through G protein-dependent and -independent pathways (β-arrestin recruitment) (see Figure 1.).

These ligands are classified by their mechanism-of-action (MOA) and function as agonists, antagonists, inverse agonists, or allosteric modulators (positive or negative). When the ligand behaves as an agonist, several downstream effects are produced. These effects include up- or down-regulation of G protein-dependent second messenger signaling (via cAMP or calcium), and G protein-independent β-arrestin recruitment with subsequent receptor internalization.

In the G protein-dependent pathway (Figure 1. A.), heterotrimeric (α/β/γ) G-proteins contain a GDP molecule bound to the Gα subunit, which holds the trimer together. Upon activation, GDP is exchanged for GTP, leading to the dissociation of the Gβ/Gγ dimer from Gα. Both parts remain anchored to the membrane and become free to act upon their downstream effectors and initiate unique intracellular signaling responses. The activated Gα subunit interacts with and regulates many effector molecules such adenylyl cyclase that can ultimately lead to the accumulation of cAMP (a second messenger) seen in this example.

GPCR Biology and the Drug Discovery & Development of New Therapeutics (Continued)

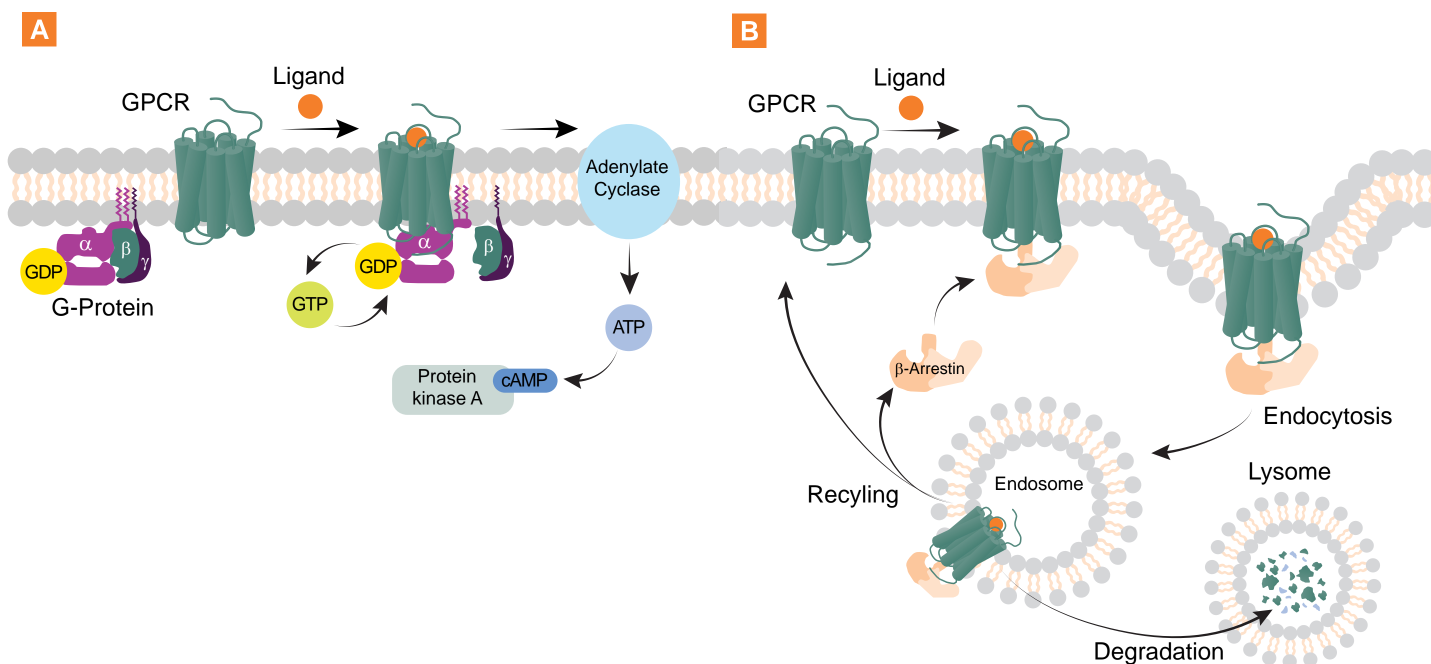

Figure 1. GPCR signaling pathways. A. G protein-dependent and B. G protein-independent pathways.

In the G protein-independent pathway (Figure 1. B.), the ligand-activated GPCR is phosphorylated by a specific GPCR kinase (GKRs, not shown here), leading to β-arrestin recruitment. This β-arrestin binding blocks G protein-mediated signaling and results in the internalization (or endocytosis) of the GPCR and ending of the attenuation of GPCR signaling (known as desensitization). Subsequently, the GPCR is recycled back to the plasma membrane or degraded in the lysosome.

A key difference between both pathways is the GPCR/G-protein interaction is catalytic (i.e., one receptor can sequentially activate multiple G proteins) leading to an amplified signal. In contrast, the GPCR β-arrestin interaction “arrests” the signaling leading to a non-amplified, one-to-one signal. Overall, GPCRs can signal through one or both pathways as well as internalize/recycle without the presence of a ligand.

GPCRS IN DRUG DISCOVERY AND DEVELOPMENT

What needs to be considered are both the GPCR drug MOA and which downstream transduction pathway is triggered: These can have key consequences in the development of drugs targeting GPCRs. Depending on the objective of the drug developer, there may be a need to study a ligand or drug effect on more than one of these signaling pathways. For example, a drug developer may need compound-profiling data from β-arrestin recruitment and second messenger assays to measure possible ligand bias. Additionally, there may be a need to test effects on receptor internalization to examine drug tolerance or unwanted side effects.

Investigating how small molecule or biologic therapeutics bind to GPCRs and modulate their downstream cellular signal pathways provides insights into the therapeutic MOA and resulting phenotypic responses. Drug discovery programs for developing therapeutics targeting GPCRs go through several steps, from the identification and characterization of the GPCR through pre-clinical testing (see Figure 2. for a detailed breakdown of these steps).

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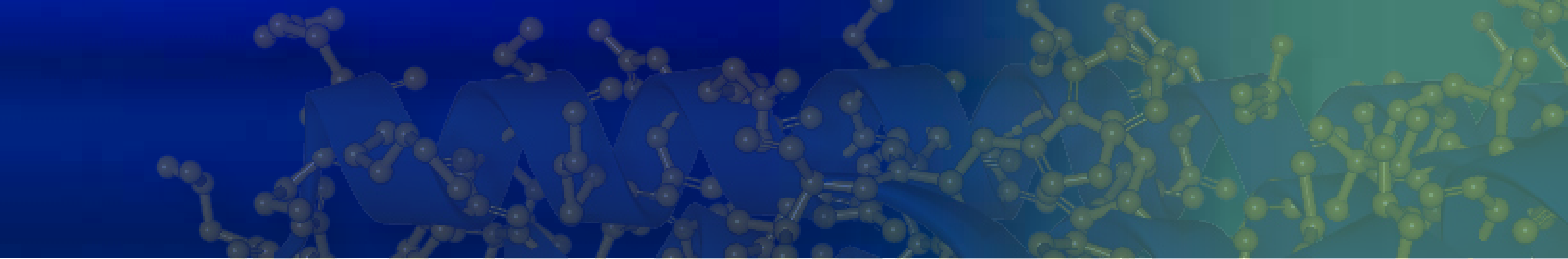


GPCR target hunting & characterization MOA determination Deorphanization Multiple GPCR pathways analysis Mutation / disease analysis Assay development	High-throughput screening Secondary & orthogonal screening Hit-to-lead Rank order hits Pharmacochaperone discovery	Lead characterization & optimization Ligand bias studies Structure activity relationship for potency & efficacy Multiple species (orthologs) analysis Internalization studies	Quality Control lot release studies Stability testing of biologics

Figure 2. GPCR drug discovery and development applications

As drug development progresses, hundreds of potential therapeutic agents targeting GPCRs continue to enter into clinical trials. These drug candidates include small molecule agonists and antagonists that target known GPCRs. Over the past decade, there has been an increased number of biological drugs, allosteric modulators, and biased agonists as well as candidates targeting orphan GPCRs without a known ligand or approved drug. Additionally, there has been a shift towards a focus on metabolic and neurological disorders as well as cancer. Future developments of basic tools to study GPCRs and greater understanding of GPCRs, as well as in the number and quality of therapeutics, will go hand-in-hand to advance the GPCR space.

Learn more about GPCRs via this GPCR eBook and see discoverx.com/GPCRs to learn how Eurofins DiscoverX®
can help you along your journey to fully understanding your GPCR and GPCR ligand/therapeutic of interest.

EXPERT INSIGHT

Q&A GPCR Drug Discovery & Development Insights with Dr. Terry Kenakin

Q&A with Terry Kenakin, Ph.D., Professor of Pharmacology at University of North Carolina School of Medicine, key opinion leader in GPCRs and drug discovery, author of several publications on pharmacology in drug discovery, and collaborator and consultant for many global biotechnology and pharmaceutical companies.

Q: WHAT IS THE IMPORTANCE OF GPCRS IN DRUG DISCOVERY AND DEVELOPMENT?

A: GPCRs have long been regarded as a low-hanging fruit drug target as they are conveniently located on the cell surface and control a myriad of cell functions, but at various times over the decades, they have been the most utilized target for new therapies. An important theme in drug discovery is the realization that GPCRs are Nature's prototypical allosteric protein linking the internal signaling machinery in the cell cytosol with the outside world (receptor compartment extracellular space). What makes GPCRs especially attractive as drug targets is their versatility. Specifically, GPCRs are pleiotropically coupled to many signaling systems in the cell and with the advent of methods to discern biased signaling, we can customize the cellular signal emerging from the activated receptor through different molecular scaffolds. In addition to drugs having efficacies of varying strength, they also yield ligands with different qualities of efficacy presented to the cell. In this regard, GPCRs, historically thought to be switches, are now known to be micro-circuits taking different signals in and yielding different signals out. Through the application of quantitative pharmacology, biologists and medicinal chemists are learning to control these signaling patterns to provide more focused drugs with fewer unwanted effects.

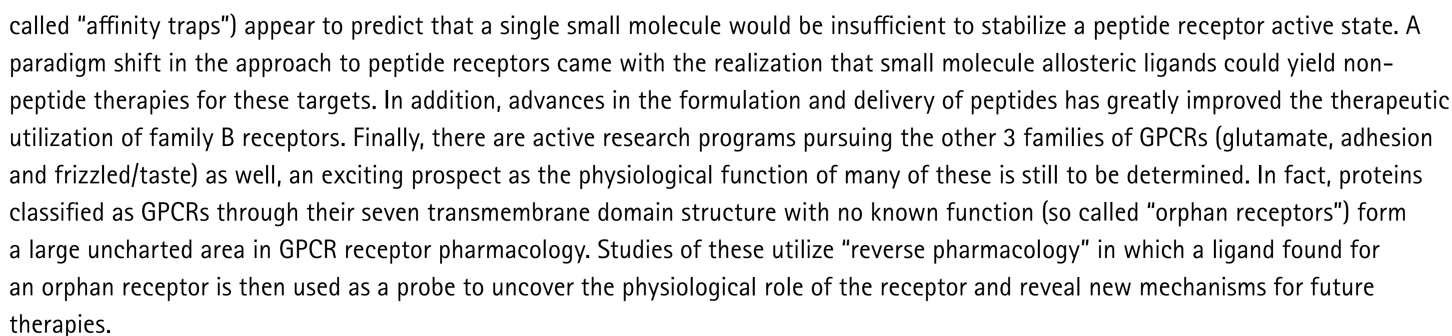
Q: HOW HAS THIS IMPORTANCE EVOLVED IN THE LAST DECADE?

A: Arguably, there has been a perfect storm in pharmacology and drug discovery in the past 15 years brought on from two sources: molecular dynamics and the increasing availability of new functional assays to detect the outcome of drug-receptor interactions. The appreciation of receptor proteins as dynamic systems of varying micro-conformations (ensembles) has enabled the understanding of GPCR signaling bias (the ability to dissect multiple signals from the receptor to emphasize some at the expense of others). The increased availability of functional assays has allowed us to quantify these varying signals; in early years when the response was considered a monotonic signal from the receptor to the cell, there was little opportunity to visualize the texture of signaling produced by receptor activation. Now with "more eyes to see," we can discern subtleties of receptor signaling and observe differences in drugs that previously could not be detected.

In addition, the understanding of receptor allostery has advanced with the advent of the discovery of increasing numbers of allosteric ligands. Historically, new drug screening had been conducted in an orthosteric mode (binding) thereby understandably providing orthosteric lead molecules and few allosteric ones. With increased screening in functional systems (more suited to detect allosteric molecules) has come a concomitant increase in allosteric lead molecules. As we study these new allosteric ligands, we are seeing the versatility of allosteric drug ligands through the properties of saturation of effect and practice of probe dependence, and these ideas have opened new therapeutic vistas. So, the emphasis for small molecule drug development has shifted from copies of natural neurotransmitters and hormones and molecules that sterically block them to a flexible array of allosteric molecules capable of blocking, enhancing or otherwise modifying natural signals in drug therapy. In addition, biologic molecules are emerging onto the therapeutic horizon in the form of replacement proteins, peptides, vaccines, antibodies, and nucleotide-based therapies (gene therapy, RNAi therapy). The exciting aspect of this new class is that many biologics can do things small molecules cannot do due to an interactive conversation with the human immune system.

Q: WHAT ARE THE COMMONLY STUDIED GPCR FAMILIES BEING RESEARCHED WITHIN THE LAST DECADE?

A: Historically family A GPCRs (rhodopsin family - monoamines, neurotransmitters) were the bread and butter of receptor therapies. From there, family B (secretin family - peptide) receptors emerged, but this led to an appreciation of the difficulties encountered trying to find small drug-like molecules for them. Theoretical ideas around the requirement of multiple binding domains on these receptors (so



called “affinity traps”) appear to predict that a single small molecule would be insufficient to stabilize a peptide receptor active state. A paradigm shift in the approach to peptide receptors came with the realization that small molecule allosteric ligands could yield non-peptide therapies for these targets. In addition, advances in the formulation and delivery of peptides has greatly improved the therapeutic utilization of family B receptors. Finally, there are active research programs pursuing the other 3 families of GPCRs (glutamate, adhesion and frizzled/taste) as well, an exciting prospect as the physiological function of many of these is still to be determined. In fact, proteins classified as GPCRs through their seven transmembrane domain structure with no known function (so called “orphan receptors”) form a large uncharted area in GPCR receptor pharmacology. Studies of these utilize “reverse pharmacology” in which a ligand found for an orphan receptor is then used as a probe to uncover the physiological role of the receptor and reveal new mechanisms for future therapies.

Q: WHAT INSIGHTS DO YOU KNOW ABOUT FOR FUTURE THERAPEUTIC GPCR TARGETS?

A: The most obvious change in the field has come about with the discovery of biased signaling for GPCRs. At first thought to be a new phenomenon, it is now known simply to be standard probe dependent allostery practiced by all receptor proteins. It was detected when new functional assays became available to dissect the signaling emerging from activated receptors. The fact that biased signaling is a natural behavior of standard proteins predicts that synthetic ligands will more often than not produce signaling of a different quality than natural hormones and neurotransmitters. Gone is the belief that a synthetic analog of a natural agonist will produce the same quality of signal to a cell, but only of greater or lesser magnitude. This both opens new avenues for better therapy, but also places a burden on researchers to fully characterize the effects of synthetic molecules aimed for therapy. In addition, with the great advances in screening technologies over the past years (high throughput screening, DNA-encoded libraries, virtual screening, structure-assisted screening, fraction-based screening), the present general notion indicates it is possible to “drug” (find a ligand for) any defined target. This puts the onus on the choice of protein target as being the critical and weak link in the discovery process and target validation, and thus having the target taking the center stage in discovery programs.

> “What makes GPCRs especially attractive as drug targets is their versatility. Through the application of quantitative pharmacology, biologists and medicinal chemists are learning to control these signaling patterns to provide more focused drugs with fewer unwanted effects.”
>
> *– Terry Kenakin, Ph.D., UNC School of Medicine*

Q: WHAT ROLES DO GPCRS PLAY IN VIRAL THERAPEUTICS?

A: There are basically three mechanisms GPCRs could be involved in viral function:

1. The first mechanism is typified by HIV-1 and AIDs. Specifically, the HIV-1 virus utilizes a GPCR (the CCR5 chemokine receptor) directly as the portal allowing cell infection. Allosteric blockers of CCR5-HIV interaction such as maraviroc are used as a therapy in this setting.
2. A second viral GPCR association is virally encoded GPCRs whereby the viral genome encodes for GPCRs that are then set free in the host to support viral replication and growth. Examples of these are various herpesvirus-encoded GPCRs and US28 of human cytomegalovirus (HCMV). Many of these GPCRs are constitutively active (spontaneously form active states that signal with no need of an agonist) and thus become pathological entities in the host. Interestingly, these pose unique therapeutic problems as only an inverse agonists can be used to quell the viral GPCR signal.
3. A third association is a heightened pathology resulting from the virus targeted to GPCRs in the host; in these cases, the GPCRs are targeted for symptomatic treatment. With regard to COVID-19, the pro-inflammatory GPR4 mediating leukocyte infiltration in vascular endothelium and the C5a-C5a receptor mediating platelet hyperactivity have been implicated as targets for GPCR-based therapy in COVID-19 treatment.

Q: WHAT ARE SOME OF THE LARGEST CHALLENGES RESEARCHERS ENCOUNTER WHEN STUDYING GPCRS?

A: There are two areas of heightened importance with the changing landscape of GPCR therapeutic research:

The first is accepting the premise that screening technologies may be not be such a limiting step in the discovery chain (i.e., we can find ligands for nearly all GPCR targets). The problem area becomes which target to choose? Considering that of the 30,000 genes in the human genome, there are approximations that about 3,000 are "druggable" (small molecules will change their behavior) and another 3,000 are associated with disease. This leaves an intersection of between 800- to -1,500 viable drug targets and a great many of these are GPCRs. Currently, industry and academic programs target ~300-350 targets leaving a great many still to be explored. Incorrect choice of target minimally costs programs years of precious discovery time and resources.

A second hurdle is translation of *in vitro* observed activity to complex systems (sometimes under the control of pathophysiological processes). The advent of biased signaling which predicts complex signaling patterns from GPCRs that may be altered by cell type and the relative stoichiometry of receptors to signaling components of the cell, means that predictions of *in vivo* GPCR-mediated effects are not straightforward. In this regard, the rise of phenotypic screening is leading to identification of new mechanisms of GPCR control of cell function, which may be exploited by discovery.

Q: HOW FAR ALONG HAS GPCR RESEARCH EVOLVED FROM THE TRADITIONAL *IN VIVO* ANIMAL MODELS TO *IN VITRO* CELL-BASED ASSAY?

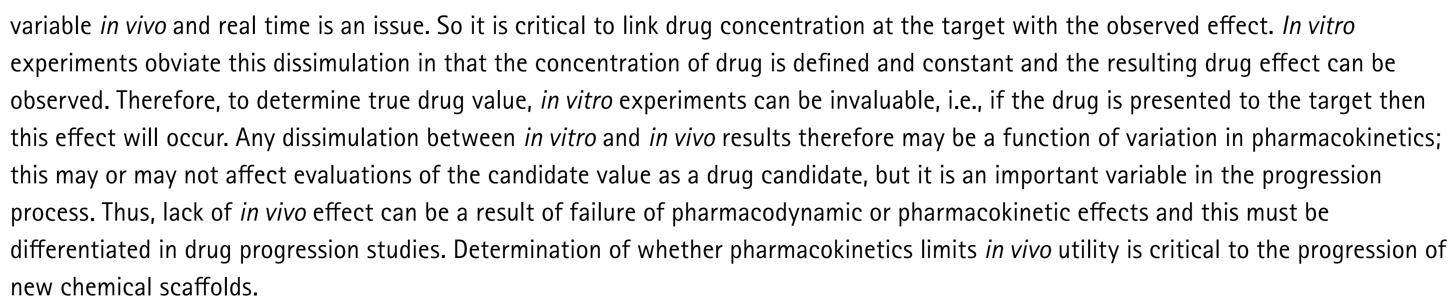
A: The history of drug discovery has been rooted in the phenomenological observation of drug effect in whole systems (animal models, isolated organs). A major stumbling block in this process has been dissimulation between human and animal material, i.e. correspondence of GPCR activity in animal test systems to humans. Biochemical binding technology followed by cellular signaling studies bridged this gap as the genome provided human drug targets and test systems devoid of species variance. This led to a "biochemical age" of discovery where simple system pharmacology (so called "synoptic pharmacology") was de-emphasized and recombinant systems took the lead. However, with the realization that the complete system is greater than the sum of its parts, there is now a return to whole system pharmacology. Cellular systems and label-free technology enable observation of whole system pharmacology in human tissue. Ironically, this describes nearly a full circle from whole system to whole system, but with unique differences in the systems.

Q: WHY IS IT NECESSARY TO CONSIDER MULTIPLE ASSAY TYPES TO CHARACTERIZE GPCRS?

A: GPCRs are interactive systems whose behavior is intimately associated with other proteins and molecules in the cell and extracellular space. The allosteric nature of GPCRs dictates a bidirectional flow of allosteric energy between the extracellular ligand binding site and the binding sites in the cytoplasm for signaling molecules. The absence of these partners leads to differences in observed activity. Thus, the binding of efficacious agonists to bare GPCR proteins devoid of G protein partners is hugely different from systems where those G proteins are present. This extends to many other signaling components present in the cell (i.e. β-arrestin) thereby dictating the need for the complete system to reflect pharmacological activity, i.e., functional cell systems. In addition, this question can be applied to new drug screening. Specifically, when a single probe of the receptor is used to detect ligand interaction with that receptor (i.e., radioligand), then any ligands that interact with the receptor but do not report that interaction to the screening probe, will be missed (i.e., many allosteric ligands). In contrast, a functional assay utilizes the full cadre of signaling proteins in the cell interrogating the receptor and reporting alteration of receptor conformation and function when ligands bind, i.e., this is a much broader *brush to paint* for detection of compound detection. It can be likened to a music box with a full complement of tuning forks vs a music box with only one tuning fork.

Q: WHAT IS THE INDUSTRY TREND TO MOVE FORWARD WITH *IN VITRO* ASSAYS VERSUS *IN VIVO* EXPERIMENTATION?

A: Clearly, *in vivo* drug effects are preferable since this is a view of what drugs do therapeutically. However, drug concentration is



variable *in vivo* and real time is an issue. So it is critical to link drug concentration at the target with the observed effect. *In vitro* experiments obviate this dissimulation in that the concentration of drug is defined and constant and the resulting drug effect can be observed. Therefore, to determine true drug value, *in vitro* experiments can be invaluable, i.e., if the drug is presented to the target then this effect will occur. Any dissimulation between *in vitro* and *in vivo* results therefore may be a function of variation in pharmacokinetics; this may or may not affect evaluations of the candidate value as a drug candidate, but it is an important variable in the progression process. Thus, lack of *in vivo* effect can be a result of failure of pharmacodynamic or pharmacokinetic effects and this must be differentiated in drug progression studies. Determination of whether pharmacokinetics limits *in vivo* utility is critical to the progression of new chemical scaffolds.

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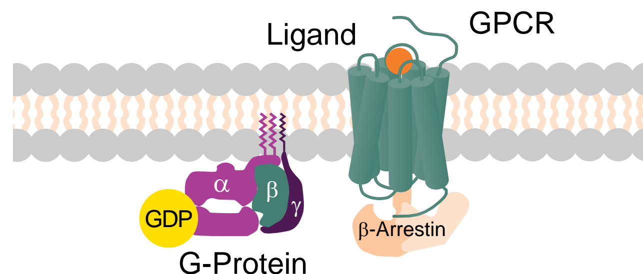
ARTICLE

GPCR Innovations from Eurofins DiscoverX

To help researchers fully characterize their specific GPCR and GPCR-binding small molecule or biologic of interest, Eurofins DiscoverX® encourages exploring all possible scenarios with a variety of available biologically relevant cell-based functional and binding assays and membrane preparations. These products are target specific, sensitive, and robust for detecting receptor-mediated second messenger signals, β-arrestin recruitment, receptor internalization, and ligand binding.



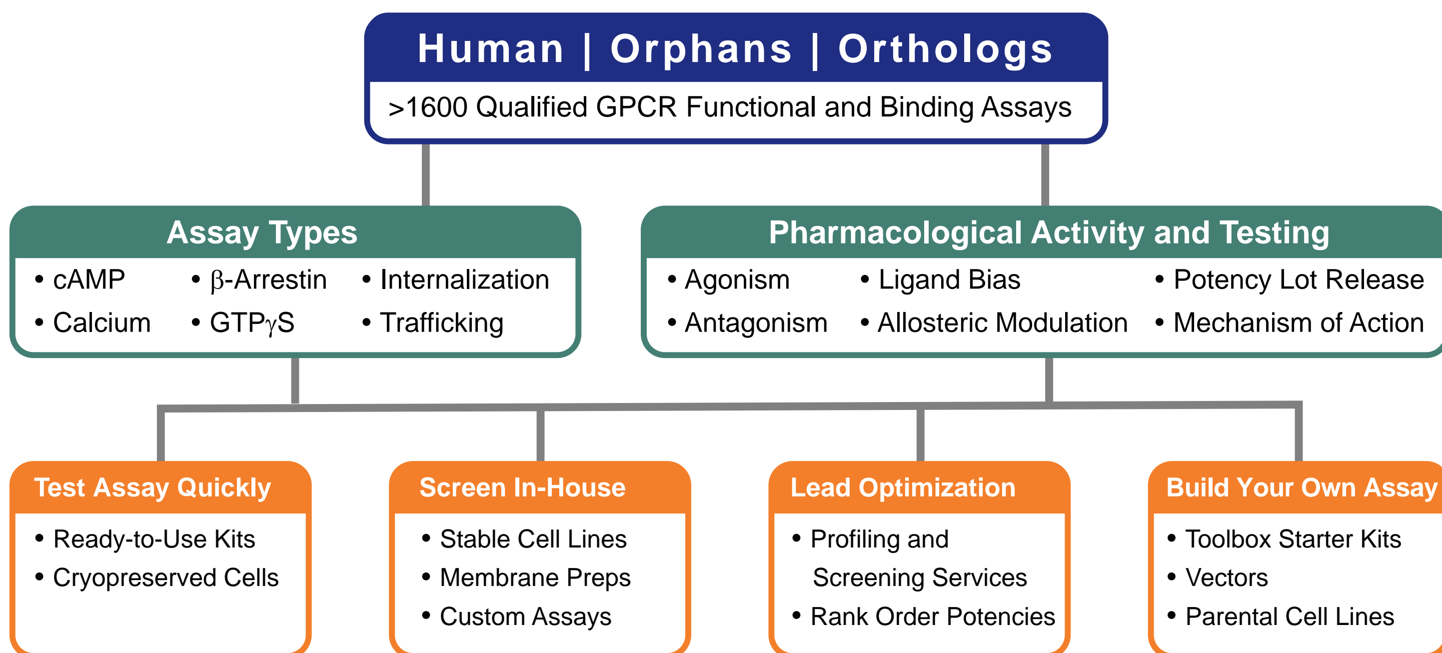
GPCR INNOVATIONS FROM Eurofins DiscoverX

Highlights of GPCR Product Solutions			
Applications	Benefits	Product Portfolio	Credibility
Biologically relevant functional assays Broad coverage of human GPCRome, including both liganded and orphan GPCRs Ortholog receptor assays Multiple modalities (second messenger, β-arrestin, and internalization) for ligand bias studies Pharmacological profiling (e.g., agonist, antagonist, allosteric modulation) Ability to multiplex various readouts (in one cell line)	Homogeneous assays with easy-to-follow, rapid, no-wash protocols Luminescent and fluorescent readouts Sensitive detection Robust dynamic range Low false positive rate Reproducible Suitable for miniaturization and high-throughput screening	Cell lines and ready-to-use assay kits Flexible toolbox (do-it-yourself) cell-based assay development products Membrane preps suitable for radio- and fluorescent ligand binding assays Qualified, optimized, and highly reproducible bioassays for development through QC lot-release applications Custom product development services	Accepted by industry and the scientific community (e.g., 100s of peer-reviewed publications, billions of data points screened) Thorough technical documentation Helpful and experienced customer support

CHOOSE THE SOLUTION THAT BEST MEETS YOUR GPCR PROGRAM NEEDS

Whether you are developing small molecule or biologic therapeutic drugs, Eurofins DiscoverX® provides you with a variety of drug discovery and development products and services with multiple assay types to meet your specific GPCR research needs.

- Broad Coverage – >90% targets covered across the human GPCRome to always find an assay for your target of interest
- Highly Applicable – Ideal for the development of small molecule and biologic therapeutics, from target ID through pre-clinical drug discovery programs
- Various Assay Options – Multiple assay types (e.g., β-arrestin, cAMP, calcium, internalization, ligand binding, trafficking) offering a diverse array of GPCR assay solutions for maximum flexibility
- Largest Collection – >1,600 qualified functional and binding assays through products and services seen in >800 peer-reviewed publications, giving you the most comprehensive GPCR assay solutions available for the confidence you need



GPCR PRODUCT TYPES OVERVIEW

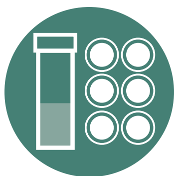
Eurofins DiscoverX® offers a variety of product types for quickly testing through thorough characterization of your small molecule or biologic GPCR ligand or therapeutics for multiple applications.

Stable Cell Lines

- Qualified cryopreserved, target-specific cell lines
- Unlimited culture ready for all drug discovery and development programs
- High functional expression
- Optimal performance with Eurofins DiscoverX detection kits

Membrane Preparations

- Applicable for competitive radioligand binding and GTPγS functional studies
- Simple assay, single-use preps
- Robust performance with reproducible results
- Provides high total binding

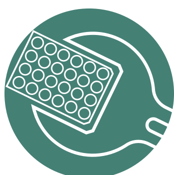


Assay Ready Cells and Kits

- Complete kits with ready-to-use cryopreserved cells, plates, required reagents, and simple homogeneous protocols
- Applicable for target identification, screening, proof-of-concept, rank-order potencies, and more
- Amenable for miniaturization and high-throughput screening
- Bioassays developed for potency testing in QC lot release to meet robustness, precision, linearity, and reproducibility

Toolbox "Do-it-Yourself" Products

- Create your own cell-based assays to evaluate mutants, orthologs, unique targets, and more
- Cost saving assay development kits, retroparticles (retrovirus), and expression vectors
- Time saving engineered parental cells
- Enables development of nearly any target in most cell types



Custom Assays & Bulk Lot Production

- Custom cell pools, clonally-derived lines, membrane preps, or cell-based assays
- Leverage internal expertise (with decades of experience) and flexible milestone structure
- Assay protocol development and 100% method transfer

DECIDING ON THE BEST GPCR SOLUTION

Achieving full understanding of your GPCR and/or GPCR-ligand/therapeutics is possible through using multiple cell-based assays and evaluating GPCR families, targets, variants (e.g., orthologs, isotypes), biological function, or signaling pathway as well as ligand/therapeutic binding, functional status, MOAs, and pharmacological profiles. By knowing the applications and benefits for each GPCR cell-based assay type and detection kit, you can chose the appropriate assay for your GPCR research goals and drug discovery and development program.

GPCR Assay Types	Cell-Based Assay Applications & Benefits	Eurofins DiscoverX® Solutions
β-Arrestin Functional Assays	G protein-independent assays engineered to detect GPCR activation via recruitment of β-arrestin by ligand activated GPCRs Study virtually any Class A or B GPCR, including orphan receptors Less likely to miss partial agonists or get false agonist response with positive allosteric modulators (PAMs) Superior for screening antagonists (IC_{50} more reflective of ligand binding affinity) and partial agonists Use with any GPCR regardless of coupling status	PathHunter® arrestin cell lines and assay ready kits (eXpress™ & bioassay) Toolbox products PathHunter detection kit
cAMP Functional Assays	Monitor the functional status of GPCRs directly by quantifying cellular cAMP accumulation levels in a dose-dependent manner Characterize ligand pharmacology with precision (superior agonist detection) Evaluate ligand bias with complementary assays (e.g. β-arrestin assay) Reproducible performance with large assay windows and broad sensitivity ranges Use with cell lines or ready-to-assay cells expressing naturally G_s- or G_i-coupled GPCRs	cAMP Hunter™ cell lines and assay ready kits (eXpress & bioassay) HitHunter® cAMP assays (detection kits)
Calcium Functional Assays	Measure intracellular calcium mobilization (flux) in cells based on the GPCR activation status Qualified for use with FLIPR® and fluorescent dye-based assays Robust and flexible assays with broad sensitivity ranges and large assay windows Use with cell lines or ready-to-assay cells expressing naturally G_q-coupled or force-coupled (non-G_q) GPCRs	Native calcium cell lines ChemiSCREEN™ and ChemiBRITE cell lines and assay ready cells HitHunter Calcium assay (detection kit)
Internalization Assays	Measure GPCR internalization in live cells Secondary and orthogonal screening tool to help in identifying safer drugs (e.g., evaluating drug tolerance, unwanted side effects, and diseases) Quantify effect of ligand on internalization of GPCRs localized to intracellular endosomes Monitor your activated GPCR without expensive microscopy or antibodies Use with most GPCRs regardless of coupling status	PathHunter Internalization cell lines and assay ready kits (eXpress) Toolbox products PathHunter detection kit
Pharmacotrafficking Assays	Identify small molecule compounds called pharmacological chaperones or pharmacochaperones Characterize pharmacochaperones that enter the cell and function by promoting proper folding of misfolded mutant receptors and enable their trafficking to the cell membrane and further to the endosome Interrogate your compounds' function in disease processes associated with protein trafficking and internalization due to protein misfolding Quantitative chemiluminescent results without the need for antibodies or imaging	PathHunter Pharmacotrafficking cell lines Toolbox products PathHunter detection kit
Radioligand Binding and GTPγS Functional Assays	Utilize high-quality membrane preparations to identify ligands that bind your GPCR For saturation and competitive radioligand binding and GTPγS functional studies Obtain consistent, reproducible results and high total binding values with large signal-to-background ratio	ChemiSCREEN membrane preparations

GPCR Innovations from Eurofins DiscoverX (Continued)

GPCR Detection Kits	Applications & Benefits
HitHunter® cAMP Assays	Detect cAMP accumulation with a luminescent read-out Robust, highly sensitive, no-wash, and easy-to-use high-throughput assay for monitoring GPCR activation based on cAMP production in cells Enzyme Fragment Complementation (EFC)-based detection system for small molecule and biologics testing applications Reduce optimization steps by using the detection kit qualified for use with cAMP Hunter™ GPCR cell lines Compatible for use with most GPCR cell lines Available in multiple sizes; based on the number of data-points
HitHunter Calcium Assays	Easily measure intracellular calcium to the determine activation status of GPCR Dye-based assay with a fluorescent read-out and single-step, no-wash (homogeneous) protocol
PathHunter® Detection Kit	Luminescent detection kit for EFC-based cell lines engineered to study protein-protein interactions, protein translocation, receptor trafficking, and receptor internalization Available in multiple sizes

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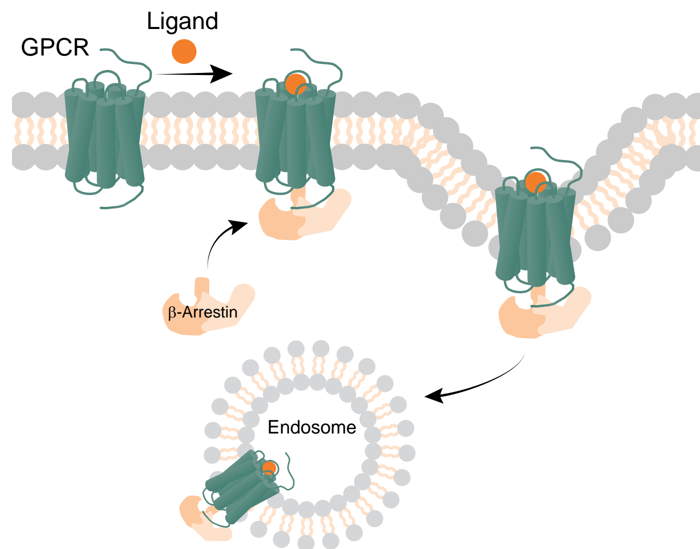
ARTICLE
Why Study GPCR Arrestin Recruitment?

GPCR β-ARRESTIN SIGNALING



Arrestins are ubiquitously expressed and function in the activation of GPCRs, desensitization of most 7-transmembrane receptors (7-TMs), and regulation of other signaling molecules such as protein kinases.

There are currently four known arrestins:

- Visual and cone arrestins play a major role in photoreceptor cell regulation
- β-arrestin1 and 2 interact with the majority of commonly targeted 7-TMs receptors

For GPCRs, ligand-induced β-arrestin (1 or 2) recruitment activates signaling cascades independently of G-protein signaling to provide a stoichiometric, non-amplified signal. β-arrestin recruitment assays offer an easy-to-use alternative to second messenger cAMP and calcium G-protein dependent pathways to enable enhanced profiles of compound pharmacology – a universal assay that expands opportunities for development of novel drugs.

BENEFITS OF A NON-AMPLIFIED SYSTEM

The β-arrestin signaling pathway is an ideal system for discovering antagonists, studying GPCR deorphanization, and dissecting ligand pharmacology differences.

cAMP SECOND MESSENGER G-PROTEIN DEPENDENT SIGNALING PATHWAY

An amplified system that can lead to rapid saturation using low levels of GPCR activating ligands. This is a more sensitive system based on partial receptor occupancy giving a full signal due to the amplified signaling event (receptor reserve phenomena) after ligand activation. Agonist can easily be detected since it does not take much agonist to obtain a full signal, but differentiating partial from full agonists can be difficult, and cAMP assays tend to have lower sensitivities to antagonists than do arrestin assays.

β-ARRESTIN SIGNALING PATHWAY

A stoichiometric (one receptor: one ligand), non-amplified system that requires full ligand occupancy of the ligand bound to the receptor to give a full signal. This leads to a lower sensitivity to agonists, but improved ability to detect differences of efficacy between agonists, and superior sensitivity for antagonists (compared to second messenger systems). This makes β-arrestin an ideal system for fine-tuning GPCR biology when screening antagonists as well as for deorphanizing GPCRs and distinguishing between full, super, and partial agonists.

ARTICLE

Why Study GPCR Arrestin Recruitment? (Continued)

MULTIPLE APPLICATIONS OF β-ARRESTIN ASSAYS

- Perform multiple pathway analysis in the same cell line
- Uncover unique pharmacology
- Correctly rank order ligands
- Deorphanize GPCRs
- Evaluate difficult GPCRs
- Compare ligand responses in different species receptors (orthologs)
- Study mutant or isoform differences
- Investigate tissue-specific variations using different cell types
- Enable ligand bias studies

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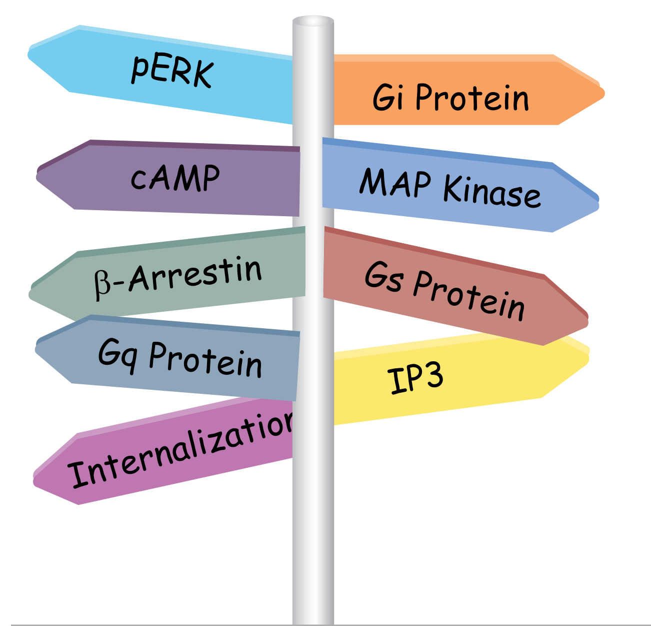
Learn more at discoverX.com/arrestin.

Four Reasons Why You Should Measure Signaling Bias in Your Molecules

Most GPCRs are pleiotropically coupled to multiple signaling pathways in cells; the availability of functional assays to separately measure agonist-induced activation of these pathways has revealed that agonists do not activate all pathways in a uniform manner, i.e., some activate selected pathways at the expense of others and thus are "biased" toward some signals in the cell. The way in which cells mix these signals for an overall cellular response also reveals that efficacy has quality as well as quantity for different agonists. This has led to a revolution in GPCR candidate selection since an important further selection criterion for agonism, in the form of bias determination, now offers new ways to advance molecules.

THERE ARE FOUR DISTINCT REASONS WHY BIAS MEASUREMENTS ARE IMPORTANT

1. Bias can make better drugs: The identification of biased ligands can lead to molecules that (a) emphasize beneficial signaling pathways, (b) de-emphasize harmful pathways, and (c) de-emphasize harmful pathways and preclude the natural signaling system from activating those same harmful pathways. In addition, bias can expand the pursuit of targets otherwise thought excluded from consideration due to activation of harmful signaling (i.e., dysphoria for κ-opioid agonists).
2. Bias identifies molecules for progression from high-throughput screens: Counter-screens of hits found in high throughput screens in biased assays can identify hits that are truly different on a molecular level, thereby optimizing the subsequent identification of useful therapeutic phenotypes in animal models and more complex therapeutic assays.
3. Bias reduces efficacy fingerprints to measurable scales that may then be optimized through medicinal chemistry: It is now known that efficacy has quality as well as quantity, which arises from the mixture of elemental signaling pathways in the cytosol. Radar plots or clustering have been used to represent these efficacy types, but bias measurements further allow the reduction of these complex phenotypes to graded activation of signaling pathways. Therefore, an identified favorable efficacy in therapeutic cells could be deconstructed to a combination of these scales to allow medicinal chemists to optimize the pattern and amplify the favorable phenotype.
4. Bias can more effectively quantify selectivity: Selective agonism on more than one signaling pathway can identify possibly false levels of selectivity or at least offer a rational scheme for determining selectivity based on all known signaling for a given molecule.



In general, and at little extra cost, the measurement of signaling bias increases value in already known lead compounds and can assist in the prediction of the activity of these compounds in other, including therapeutic, assays.

BIAS DETERMINATION

Eurofins DiscoverX® provides the largest portfolio of GPCR assays and cell lines and a unique service that utilizes state-of-the-art tools developed by Professor Terry Kenakin at the UNC School of Medicine for the characterization of ligand bias. With the appropriate β-arrestin, internalization, and second messenger assays to quantify selective response and statistical tools to scale these effects, harnessing bias to produce selective ligands is now made simple.

Four Reasons Why You Should Measure Signaling Bias in Your Molecules (Continued)

EMPHASIS OF BENEFICIAL SIGNALING PATHWAYS

Parathyroid hormone (PTH) alternately builds and degrades bone physiologically; it has been shown that PTH does not build bone in β-arrestin knockout mice (Ferrari *et al.* 2005; Gesty-Palmer *et al.* 2006; 2009) suggesting that PTH agonists biased toward β-arrestin signaling would be optimally beneficial in the treatment of osteoporosis.

DE-EMPHASIS OF HARMFUL SIGNALING PATHWAYS

Opioid agonists such as morphine provide useful analgesia but debilitating respirator depression. It has been shown in β-arrestin knockout mice that morphine produces less respiratory depression thereby suggesting that an opioid agonist biased toward G protein signaling and away from β-arrestin may be a superior analgesic (Raehal *et al.* 2005; DeWire *et al.* 2013; Charfi *et al.* 2015).

DE-EMPHASIS OF HARMFUL PATHWAYS WITH INHIBITION OF NATURAL ACTIVATION OF THOSE SAME HARMFUL PATHWAYS

The increase of angiotensin signaling in congestive heart failure, resulting in elevation of arterial pressure, is a normal response to decreased organ perfusion; this leads to short-term improvement in organ perfusion. However, long-term elevation in arterial pressure is deleterious to a failing myocardium; therefore, decreased angiotensin responsiveness, in the form of angiotensin receptor blockade with drugs such as losartan, is the indicated therapy. However, angiotensin also provides beneficial effects through β-arrestin signals and these are lost during standard losartan therapy. New angiotensin antagonists such as TRV120027 block the deleterious effects of angiotensin, but also provide beneficial β-arrestin signals through biased signaling leading to an improved profile for congestive heart failure therapy (Violin *et al.* 2006; 2010).

MAKING κ-AGONISM A VIABLE THERAPEUTIC OPTION

κ-Opioid agonist activity has been implicated in the modulation of reward, mood, cognition, and perception making these molecules of possible benefit as antidepressants and anxiolytics in affective disorders, drug addiction, and psychotic disorders. However, κ-opioid agonists also produce serious dysphoria thereby precluding their application to these therapies. Biased κ-opioid agonists may circumvent these limitations by reducing dysphoric effects and emphasizing beneficial effects (White *et al.* 2014).

LIGANDS WITH DIFFERENT BIAS IN SIGNALING ARE DIFFERENT ON A MOLECULAR LEVEL

Biased signaling is the result of the stabilization of unique ensembles of receptor states by agonists (Kenakin and Morgan, 1989; Kenakin, 1995; 2002). Therefore, if two agonists are found to produce signaling with different bias, it is likely they are producing different receptor active states, which may behave very differently *in vivo*. Under these circumstances, these two agonists would also likely produce different signaling patterns in complex assays such as *in vivo* animal models.

RADAR PLOTS DISPLAY UNIQUE PATTERNS OF EFFICACY

Multiple agonist activities can be displayed on multiple ordinate axes in the form of radar plots to show two dimensional arrays of "webs of efficacy" to distinguish agonists, i.e., see such webs for β-adrenoceptor agonists (Evans *et al.* 2010) and κ-opioid receptors (Zhou *et al.* 2013). Interestingly, each agonist tested reveals a different web, thereby indicating the uniqueness of agonist efficacy quality profiles; these may relate to the unique therapeutic phenotypic responses to these agonists.

CLUSTERING DISPLAYS AGONIST EFFICACY PHENOTYPES

Through testing agonists in multiple functional assays, measures of efficacy for each signaling pathway (in the form of transducer coefficients, (Kenakin *et al.* 2012)) can be used in clustering programs to group agonists in terms of their efficacy (Kenakin, 2015). Such clusters may enable simple manipulation of these phenotypes through medicinal chemistry.

THERAPEUTICALLY RELEVANT RECEPTOR SELECTIVITY

β2-adrenoceptor agonists for agonist therapy require high activity at β2-adrenoceptors and correspondingly low activity at cardiac β1-adrenoceptors. The β2-adrenoceptor bronchodilator clenbuterol has a 500-fold selectivity of β2– over β1– activating capability for cyclic AMP, indicating a favorable profile (Casella *et al.* 2011). However, this selectivity reduces to 5.7-fold when the receptor-mediated β-arrestin effects for these same receptors are measured. The measurement of selectivity for different signaling pathways may be critical to the assessment of true selectivity for new agonists for therapy.

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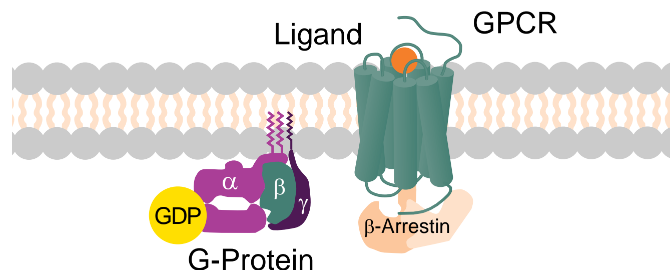
ARTICLE

Ligand Bias: Increasing Specificity and Reducing Side Effects of Drug Compounds

Ligand bias is the ability of compounds to exert differential signal transduction responses on activation of a GPCR. This ligand bias effect is a relatively new area for drug discovery and offers novel approaches to develop pharmaceuticals that have enhanced specificity and reduced side effects.



When a ligand binds to a GPCR, it alters the conformation of the protein, which is transduced through the plasma membrane to induce cell signaling. There are multiple ways signal transduction is mediated through GPCRs:

- G-proteins to modulate second messengers such as cAMP or calcium
- β-arrestins

β-arrestins are involved in regulating the duration of receptor signaling by associating with activated receptors and blocking further G-protein binding and signaling. β-arrestins subsequently form complexes with kinases such as MAPK and Erk to provide alternate signaling events.

APPLICATIONS FOR LIGAND BIAS

Potential applications for ligand bias are highlighted by observations that these distinct signaling pathways can have significantly diverse effects either as a result of a medical condition, such as hypertension, or due to unforeseen consequences of drug action, such as opiates.

ANGIOTENSIN II LIGAND BIAS

Drug candidate TRV027, currently in Phase IIb trials for acute heart failure from Trevena Pharmaceuticals, is one such drug that uses ligand bias in GPCR signaling to tease out the positive effects from the negative effects of receptor activation.

Angiotensin II plays a central role in heart failure by regulating blood pressure, cardiac cell function, and renal function. In cases of acute heart failure, angiotensin II has been shown to make the symptoms worse via second messenger signaling that results in vasoconstriction, reducing blood flow to the heart. Therefore, a drug that blocks this deleterious effect of angiotensin II has proven to show positive results for patients with acute heart failure.

In contrast, angiotensin II also promotes cardiac health and protects cardiac cells from dying through signaling via β-arrestin. Blocking this positive effect of angiotensin II activity through the β-arrestin response can make the heart condition worse. The development of drugs, such as TRV027, that specifically activate the β-arrestin pathway while simultaneously inhibiting second messenger signaling from angiotensin II type 1 receptor (AT1R) can block the negative effects of angiotensin II while promoting its positive effects.

OPIOID LIGAND BIAS

G proteins are not always the bad guys in signaling. For pain sensation, the body has the ability to naturally suppress pain sensation by downregulating the transmission of pain signals to the brain. Naturally occurring endorphins released by inhibitory neurons activate opioid receptors on synaptic terminals. This dampens neuronal signal transmission by reducing cAMP levels in the terminal.

Opiate drugs, such as morphine, produce pain relief by mirroring the actions of endorphins. Unfortunately, since opiate drugs are systemically applied, they also act on opioid receptors present in other parts of the body, such as the gut and lungs. The effects are significant with constipation and cramping in the gut and respiratory suppression in the lungs. These perceived negative effects are largely driven via β-arrestin signaling. Therefore, while opiates provide effective pain control, the side effects limit dosage and produce significant complications.

The next generation of opiate drugs in development are biased ligands that target the second messenger pathway and minimize β-arrestin engagement, thus reducing the negative side effects of opioid pain treatment. Oliceridine (TRV130), a Phase 3 drug for intravenous treatment for the management of moderate-to-severe acute pain, is one such drug that activates the second messenger response, and not the β-arrestin response, using biased GPCR signaling.

ASSAYS TO MEASURE LIGAND BIAS

The development of biased ligands requires tools to measure signaling via different pathways plus methods for analyzing results in order to quantify bias.

- Assays for β-arrestin recruitment and signaling via cAMP modulation or calcium mobilization are needed to define compound activity in both potency and efficacy terms since bias can be presented in either pathway.
- Quantifying bias between pathways that accounts for natural system bias is important. The method proposed by Dr. Terry Kenakin at the University of North Carolina achieves this by utilizing the Black and Leff operational model and normalizing compound activity to reference ligand responses.

Clearly, biased ligand development is opening a new chapter in drug discovery by taking compound specificity a step further to address not only receptor specificity but also downstream signaling selectivity to ensure the most positive outcome for the patient with minimum side effects

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ARTICLE

Can We Use Assay "Volume Control" to Unveil Efficacies in Allosteric Lead Molecules for GPCRs?

Drugs interact with living physiology; an *in vitro* assay of drug activity can be considered a snapshot of the full movie seen *in vivo* of the drug exposed to a range of tissues of varying sensitivities. An assay measures signaling at one sensitivity, namely that of the particular *in vitro* assay being utilized, but the more of these snapshots we have, the better will be our understanding of what drugs will do *in vivo*.

SIGNALING AND TISSUE VOLUME CONTROL

It is well known that physiological tissues control signal magnitude through adjustment of receptor expression levels and the relative stoichiometry of receptors to signaling components. This natural control of signaling is operative for all tissues *in vivo*. Such adjustment of tissue sensitivity can have a dominating control on the effects of agonists. For example, a low efficacy agonist such as prenalterol can produce nearly full agonism in thyroxine-treated guinea pig atria and no agonism (instead become an antagonist) in skeletal muscle such as the digitorum longus (Kenakin, 1985). An *in vitro* assay of drug activity measures signaling at one defined sensitivity, but if the sensitivity of the *in vitro* assay differs from that of the relevant therapeutic tissues *in vivo*, then a possibly costly dissimulation can occur. leading to an incorrect choice of drug candidate moving forward. This is why testing drugs in an array of assays of differing sensitivity can be a valuable tool to correctly characterizing hidden efficacies of new molecules.

POSITIVE ALLOSTERIC MODULATION

A case in point are positive allosteric modulators (PAMs) for GPCRs. These unique molecules are becoming important therapeutic entities as they have many beneficial features for agonist signal modification. Specifically, they can augment natural responses and preserve the way in which physiological systems are wired (not alter the complex patterns as they function in Nature). In addition, they produce saturable effects that allow them to change the operational sensitivity of systems without signal overload. PAMs may have quite different patterns of activity in tissues of varying sensitivity and these patterns can be used to quantify their efficacies and predict effects *in vivo*. One pattern is sensitization of tissues to agonist response as shown in Figure 1. A. for a tissue of moderate sensitivity. This single assay yields important information such as, in this case, the fact that the PAM sensitizes the tissue to the agonist. However, there are two important questions left unanswered; (1) in a more sensitive tissue, will direct agonism to the PAM be seen? And (2) does the potentiation of agonist effect occur because of effects on agonist affinity, efficacy, or both? Answers to these questions can be obtained by testing the PAM in two more assays of varying sensitivity.

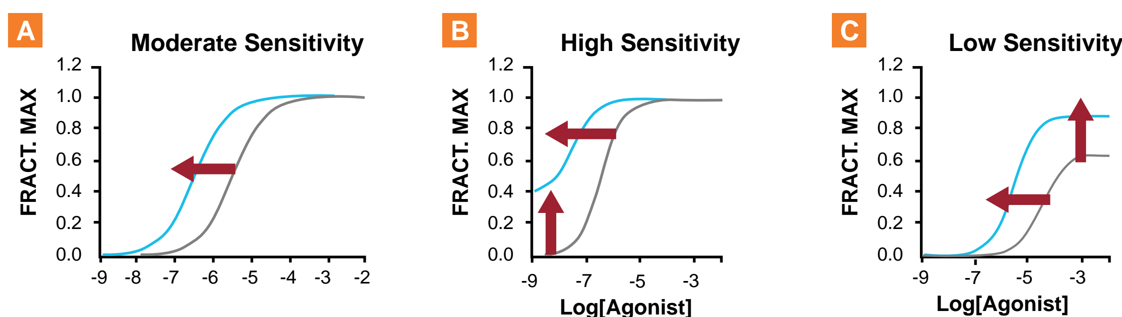

Figure 1. The effects of a PAM possessing direct efficacy and potentiating activity for efficacy. A. In a tissue of moderate sensitivity, sinistral displacement of the agonist dose response curve is observed. B. In a sensitive tissue, the direct agonism to the PAM is shown by the elevated baseline. C. In a tissue of low sensitivity where the agonist is a partial agonist, the efficacy increase produced by the PAM is shown by the increased maximal response to the agonist.

DIRECT PAM AGONISM

Figure 1. B. shows the same PAM in an assay of higher sensitivity. There are many chemical series whereby PAM activity transfers to direct agonism in highly sensitive tissues; presumably, this is related to the fact that PAMs stabilize the formation of receptor active states. Therefore, the data this figure predicts that if this PAM encounters a sensitive tissue, it would produce direct agonism. In many cases PAMs are used to avoid direct agonism as these may lead to side effects. Thus, a PAM without agonism would produce effects only when the system is activated naturally and otherwise will produce no effect (thus reducing side effects). The detection of direct efficacy may be a harbinger of the side effects of a therapeutic PAM.

DO PAMs INCREASE AGONIST AFFINITY, EFFICACY, OR BOTH?

PAMs can potentiate responses through elevation of agonist affinity, efficacy, or both of those; it can be very important which of these activities is operative for a therapeutic PAM. If the PAM is aimed to rejuvenate a failing system, i.e. neurotransmission in Alzheimer's disease, then an increase only in agonist affinity may not produce increased signaling as the original signal is weak and after PAM treatment would still be weak. Instead, what is needed is an increase in efficacy whereby a low level signal is magnified. Under these circumstances, an inoperative receptor system could be revitalized by the efficacy-based PAM. For example, a $D112^{3.32}$ mutation of the muscarinic M4 receptor makes it completely refractory to the natural agonist acetylcholine yet application of efficacy-active PAM LY2033298 can completely restore the receptor sensitivity to the agonist (Leach *et al.* 2011). In general, efficacy-based PAMs would be predicted to produce more robust sensitization of signaling systems.

PAMs CAN INCREASE EFFICACY

It is not possible to differentiate whether sensitization to a full agonist is due to increased affinity or efficacy as both of those will produce a leftward shift of the agonist dose response curve. However, if the agonist is a partial agonist, then increases in efficacy will be shown as an increased maximal response, i.e. increases in affinity will not change the maximal response. Therefore, if the PAM could be tested in an assay where the agonist is a partial agonist, then increased efficacy effects can be unveiled, i.e. see Figure 1. C. Once efficacy involvement has been identified, then any possible role for changes in affinity can be detected through a comparison of dose-response curves to the functional allosteric model (Bdioui *et al.* 2018). Thus, testing of the PAM in the three systems would fully characterize the activity as PAM agonism with increased efficacy; an example of such a molecule can be found in the muscarinic M2 receptor PAM BQCA (benzylquinolone carboxylic acid) (Bdioui *et al.* 2019).

WHAT IS TISSUE "VOLUME CONTROL"?

Assay volume control can be attained through various means such as differences in receptor expression levels, alkylation of surface receptors, choice of host cell, and augmentation of signal disposition (i.e., GRK co-expression for β-arrestin). Exploration of these approaches could yield valuable information for the full characterization of candidate efficacies. It has been estimated that 50% of new drug candidate failures occur because of therapeutic efficacy (Arrowsmith, 2011); this is in addition to issues of safety that add to this high rate of attrition. Demonstration of such failure usually occurs in the most complex systems showing pathology-controlled pharmacology at the end stage of discovery and development, a costly point in the process. While complete examination of all of the efficacies of a candidate molecule may not prevent such failures at the outset, it may prevent follow-up failures, i.e. why follow up a failed molecule with one that has the same profile of efficacies? Full candidate characterization also can help identify which collection of efficacies are important therapeutically as an effective treatment may require multiple activities, not a single one. For example, the blockade of β-adrenoceptors has been identified as an important treatment for heart failure. However, the testing of 16 β-blockers in clinical trials shows an unequal spectrum of benefit with the multiple efficacy molecule carvedilol emerging as the best (Metra *et al.* 2004). In general, investment into determining a full characterization of the efficacies of new candidate molecules may yield dividends in late-stage development; thus, an array of *in vitro* assays can be a valuable asset in this regard.

Can We Use Assay "Volume Control" to Unveil Efficacies in Allosteric Lead Molecules for GPCRs? (Continued)

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ARTICLE

Unlocking Orphan GPCRs: Application of GPCR Pharmacotrafficking and Cell-Based Assays

Orphan GPCRs comprise one-third of the druggable GPCR space[1]. Despite their pharmacotherapeutic potential, they remain underexploited owing to difficulties in interrogating their physiological roles and signaling pathways. Further, the lack of chemical probes or antibodies needed for elucidating their roles makes oGPCRs largely untapped for pharmaceutical interventions in a variety of diseases.

To address this gap, a systematic approach has been developed to identifiy chemical probes of oGPCRs using a recently developed novel platform called the Ligand-Induced Forward Trafficking (LIFT) assay. Established to circumvent impediments to the application of automated high-throughput technologies to pursue oGPCR screening, the innovative LIFT assay design features:

- A universal assay system suitable for high throughput screening (HTS)
- No requirement of a *priori* knowledge of receptor pharmacology
- Universal positive control
- Reasonably cost-effective and integrative platform for lead ID
- Rapid ability to validate utility

Scientists at the Conrad Prebys Center for Chemical Genomics at Sanford Burnham Prebys (SBP) Medical Discovery Institute in San Diego, Calif., have conducted a full HTS campaign to identify chemical probes of the orphan GPCR, GPR20. They used a highly curated library of compounds in the LIFT approach, thereby providing a new approach to determine compound:target engagement.

Development and overall validation of the LIFT assay platform, as well as the utility of hits emerging from this HTS campaign was conducted in collaboration with Eurofins DiscoverX® using the GPCR PathHunter® Pharmacotrafficking assay, PathHunter β-arrestin recruitment assay, and HitHunter® cAMP assay platforms, as well as the *gpcr*MAX panel services.

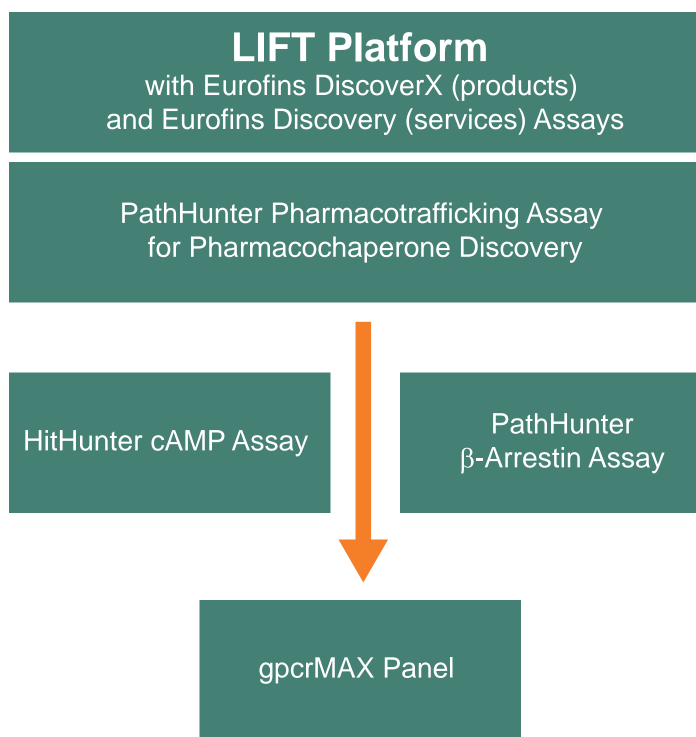

Figure 1. Flow schematic of the LIFT assay platform for ligand identification. The LIFT platform uses DiscoverX's pharmacochaperone, cAMP, and β-arrestin assays along with Eurofins Discovery services *gpcr*MAX panel to validate the forward trafficking approach to identify chemical probes against oGPCRs.

IDENTIFICATION OF CHEMICAL PROBES FOR oGPCRS

Overview of the LIFT Assay

The LIFT assay platform was developed to enable the identification of chemical probes for oGPCRs using the concept of pharmacochaperone-induced trafficking of mutant receptors. Pharmacochaperones are cell-permeable small molecules that bind to mutant receptors, stabilizes them, and enables their trafficking to the membrane[2]. Assays that report on receptor trafficking, such as the cell-based pharmacotrafficking assays from DiscoverX, have been valuable in the identification of target-specific ligands, and the interrogation of their mechanisms-of-action (MOA). When applied to oGPCRS, the assay consists of a target orphan receptor harboring a synthetic point mutation that causes retention of the target in the endoplasmic reticulum (ER) due to improper folding. (Note that the cellular protein synthesis machinery can accommodate point mutations usually found in conserved Cholesterol Consensus Motif (CCM) residues among many class A oGPCRs[3].) Upon binding of

a chemical ligand (pharmacochaperone), the mutated receptor adopts a stabilized conformation in the ER that is forward-trafficked through the Golgi, to the plasma membrane, and ultimately to the early endosome. The Eurofins DiscoverX® 's Enzyme Fragment Complementation (EFC) platform, which is the base technology of these assays, reports the forward trafficking of the receptor yielding a robust luminescent signal that can be measured. The overview of the general oGPCR LIFT lead discovery strategy is shown in Figure 1.

As proof of concept, LIFT was applied to the human orphan GPR20, and a large compound collection (~450,000 compounds) was screened with the goal of identifying small molecule probes of GPR20. Since the approach involves mutating the target receptor, it is to be noted that the induced mutation is not related to human disease, but only as a means to retain the receptor in the ER. Hence, assays for wild-type receptor including cAMP and β-arrestin recruitment assays are required for hit validation. Given the orphan nature of GPR20, compound selectivity to GPR20 was also assessed using *gpcr*MAX panel assays. Overall, applying a wide array of technologies, such as second messenger, receptor internalization, and cellular impedance assays formed a comprehensive strategy for probe discovery, delineating functional cell-based assays to serve as critical tools for interrogating the MOA in drug discovery therapeutic characterization.

HOW PHARMACOCHAPERONE ASSAY CAN MONITOR RECEPTOR TRANSLOCATION?

Eurofins DiscoverX's PathHunter® cell-based pharmacotrafficking assays provide a simple and quantitative tool for broad pharmacological characterization and interrogation of compound function in disease processes associated with receptor protein trafficking and internalization due to protein misfolding. Featured as part of the repertoire of cell-based assays surrounding translocation and trafficking of membrane proteins, the benefits of its use over other methods are described here.

These pharmacotrafficking assays provide a validated, cell-based chemiluminescent assay platform that enables identification and optimization of compounds that function as pharmacochaperones by promoting proper folding of misfolded receptor proteins and assisting in trafficking to their intended location.

Figure 2. illustrates the EFC-based PathHunter® pharmacotrafficking assay in two formats wherein the binding of a chemical ligand (pharmacochaperone) to a misfolded, tagged receptor protein stabilizes the protein in a conformation that enables its trafficking from the ER through the golgi, then onward to the cell membrane, and further internalized to the endosome. Both formats involve two inactive β-galactosidase (β-gal) enzyme fragments (a small enzyme donor and a large enzyme acceptor (EA)) to come together to form an active β-gal enzyme that hydrolyzes a substrate to generate a chemiluminescent signal that can be measured on any standard luminometer. In these assays, the membrane protein (GPCR in this case) is tagged with the small enzyme donor called ProLink™ (PK) and the EA is localized to either the cell membrane or endosome. In the Membrane-EA format (Figure 2. (A)), complementation of the two β-gal enzyme fragments (EA & PK) occurs at the membrane. In contrast, in the Endosome-EA format (Figure 2. (B)), the complementation of the fragments occur in endosomes after receptor internalization. In the LIFT platform strategy, the Endosome-EA assay format has been employed for monitoring mutant receptor translocation.

Using the fully optimized LIFT assay on β2-adrenergic receptor (ADRB2), a screening of a Library of Pharmacologically Active Compounds (LOPAC) typically used for demonstrating HTS feasibility of an assay was performed as LOPAC contains several compounds with known activity at ADRB2, as well as many compounds with no effect for this receptor. (www.sigmaaldrich.com/life-science/cell-biology/bioactive-small-molecules/lopac1280-navigator.html)[4]. The ADRB2 LIFT assay performed well with a Z' factor = 0.5 and having identified all ADRB2 ligands in the LOPAC library. The complete results of this pilot study are reported in (Morfa CJ *et al.* 2018).

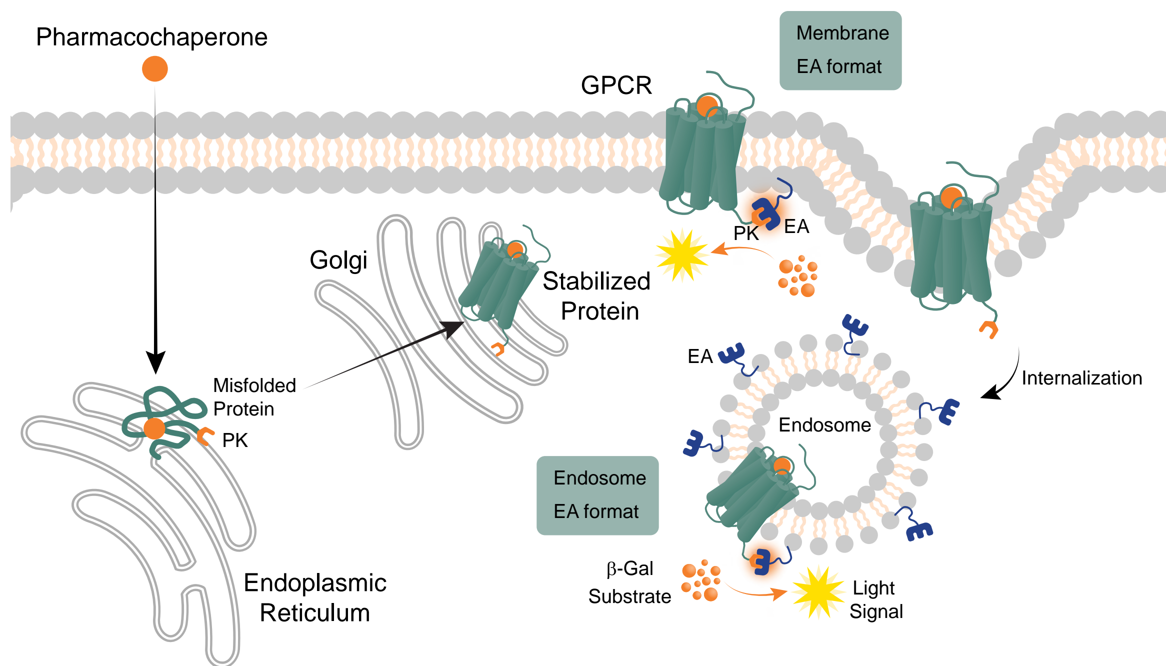

Figure 2. Pharmacochaperone assay principle using the EFC technology. Membrane-EA format: Cell lines co-express the PK-tagged receptor protein that is retained in the ER (due to protein misfolding) and EA localized to the cell membrane. Endosome-EA format: Cell lines co-express the PK-tagged receptor protein and the EA localized to the early endosomes. In both formats, the complementation of the two β-gal enzyme fragments (EA & PK) at either the transmembrane or endomes results in an active β-gal enzyme that hydrolyzes the substrate to generate a chemiluminescent signal.

COMPOUND SCREENING & HIT CONFIRMATION

HTS screening and hit confirmation using the LIFT assay platform was employed to identify novel ligands of GPR20. The assay performed well exhibiting a Z factor = 0.5′ [4]. Among the hits (> 300 compounds), compound SBI-6630 emerged as a confirmed hit showing a robust concentration response in the GPR20 LIFT assay (EC_{50} = 0.067 µM) and a high degree of selectivity for GPR20 over ADRB2 (Figure 3. A.). SBI-6630 was further validated as a potent and selective GPR20 agonist using wild-type human GPR20 (hGPR20) expressed in HEK-293 cells. The ability of SBI-6630 to reduce forskolin-stimulated intracellular cAMP was measured using the HitHunter® cAMP assay. The compound exhibited robust activity with a similar potency to that observed in the primary LIFT assay (EC_{50} = 0.150 µM). As before, SBI-6630 showed a high degree of selectivity for hGPR20 with no activity observed in cells lacking GPR20 (parental HEK-293).

Unlocking Orphan GPCRs: Application of GPCR Pharmacotrafficking and Cell-Based Assays (Continued)

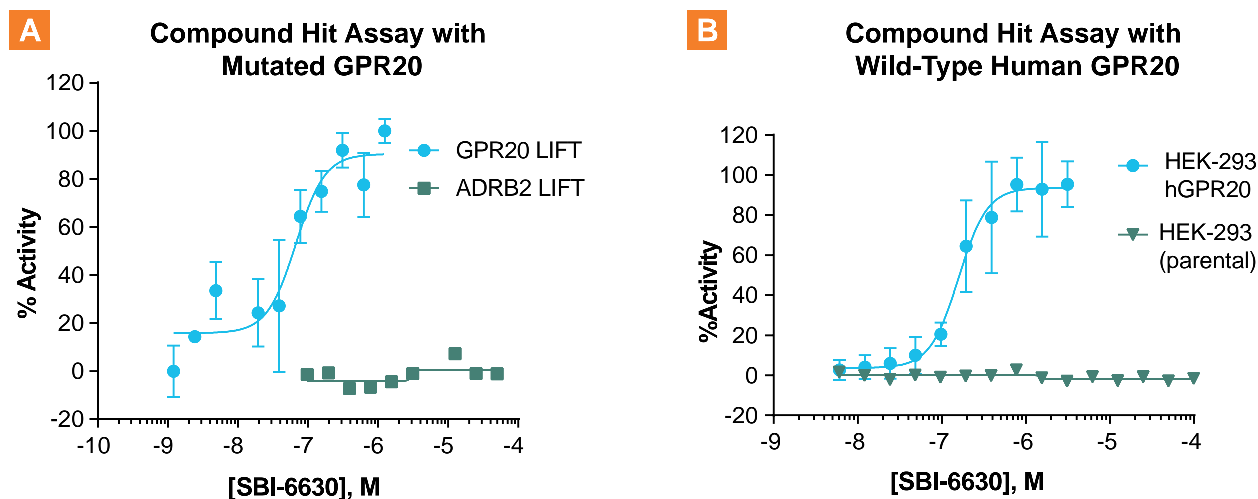

Figure 3. SBI-6630 shown as a validated hit agonist of GPR20. A. Dose-response activity of SBI-6630 against mutated GPR20 and ADBR2. B. Dose-response activity of SBI-6630 against wild-type GPR20 and parental cells lacking GPR20.

CONCLUSIONS

A novel approach that addresses critical limitations surrounding deorphanization of GPCRs is presented as a solution. Further, employing functional downstream assays to validate hits, and confirm probe compound selectivity and receptor signaling specificity, underscores the overall value of the strategy encompassing the forward trafficking approach. Representative data shown here, in addition to data from previous work[4], verifies the capability of the pharmacotrafficking assay platform to identify chemical ligands of oGPCRs using existing high-throughput screening automation and compound libraries. The implementation of Eurofins DiscoverX®'s EFC technology in the adoption of a forward trafficking approach not only ensures specificity, but also exerts suitability for automated screening platforms at an economical and time-sensitive standpoint, especially with the availability of target- and MOA-selective ready-to-run assays.

> "While GPCRs have been historically one of the most successful sources of drug targets, there is still a number of unexploited therapeutic opportunities in the GPCRome ... Opportunities to deorphanize GPCRs with no known ligand by taking advantage of the many cell-based assays available. "
>
> *– Layton Smith, Ph.D., VP at Fate Therapeutics & Executive Director, Medical Discovery Institute*

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Uses and Advantages of Membrane Preparations for GPCRs

Cellular membrane proteins play important functions in many biological processes. Understanding their roles often requires that these proteins be isolated from their cellular context. This can be achieved by the disruption of the cell membrane using various mechanical methods and differential centrifugation (Roy, A. 2015). Membrane preps generated through such methods result in small fragments of cell membranes expressing large quantities of receptor proteins.

Eurofins DiscoverX® membrane preparations (aka membrane preps) are purified membranes derived from stable cell lines optimized for high expression levels of the targeted GPCR protein. They are excellent for studying binding affinities of GPCR targets, without the requirement for cell culture.

MEMBRANE PREPARATIONS' USAGE

Eurofins DiscoverX membrane preps are intended for two main purposes: (1) to screen for ligand binding affinities using a radiolabeled ligand approach; and (2) to screen for GPCR activity using a radiolabeled GTPγS approach. For binding affinities and GPCR activation levels, these can be calculated based on changes in the resulting amount of radioactive signal in the assay. An increase in signal is seen for GTPγS and saturation binding assays, while a decrease in signal is seen for competition binding assays.

RADIOLABELED BINDING ASSAYS

Radiolabeled ligand studies can be performed using DiscoverX's membrane preps in two formats – saturation binding and competition binding. The differences between these formats are described below.

SATURATION BINDING

The saturation binding format is achieved by adding increasing doses of radiolabeled ligand in the presence (non-specific binding curve) or absence (total binding curve) of extremely excessive amounts of non-labeled ligand and subtracting non-specific from total binding to get a saturation binding curve. Saturation binding assays can be used in combination with information on IC_{50} or EC_{50} from other sources to determine the K_d (dissociation equilibrium constant or binding affinity) of the ligand.

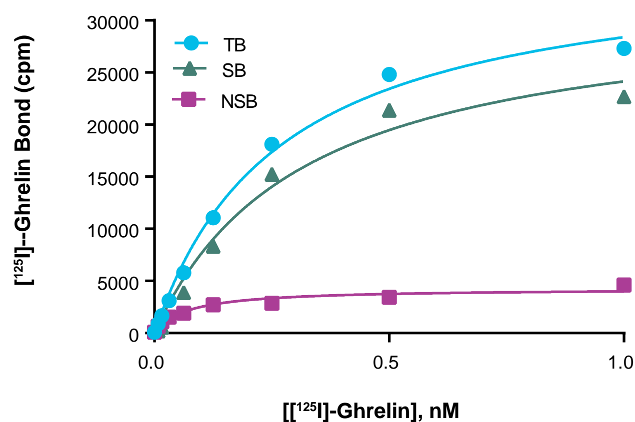

Figure 1. Saturation binding experiment for the ghrelin receptor (Cat. No. HTS187M). Ghrelin receptor membrane prep (5.0 µg/well) was incubated with increasing amount of $[^{125}I]$-ghrelin in the absence (total binding, TB) or presence (non-specific binding, NSB) of 200-fold excess unlabeled ghrelin. Specific binding (SB) was determined by subtracting NSB from TB. The K_d of ghrelin and B_{max} (total number of receptors) of the system can be calculated from the specific binding curve.

COMPETITION BINDING

The competition binding format involves using one low dose of radiolabeled ligand, and increasing concentrations of non-labeled ligand. This will produce a binding-characteristic-dependent competition curve showing how the amount of radiolabeled ligand-bound decreases as the concentration of non-labeled ligand increases. Competition binding assays can be used to determine the K_i (inhibitory constant) of the test ligand, as well as the IC_{50} of the unlabeled ligand with respect to the radiolabeled ligand.

GTPγS ACTIVITY ASSAYS

GTPγS assays work by using a radiolabeled non-hydrolyzable GTP analog. This analog is added into the buffer with the membrane prep. Then the membrane is provided with the known or experimental ligand for the GPCR expressed on the membrane. If the GPCR is activated, the now-available Gα protein will exchange its GDP for GTP. Due to the non-hydrolyzable nature of GTPγS, the Gα protein will then remain permanently bound to the GTPγS. After the removal of excess unbound GTPγS, radioactive detection methods can then be used to determine how much GTPγS remains bound by Gα, and therefore how much of the target GPCR was activated by the applied ligand. This assay is a useful alternative approach to checking agonism vs. antagonism and determining potency or rank order of experimental ligands in an easy-to-use format that doesn't require cell culture steps.

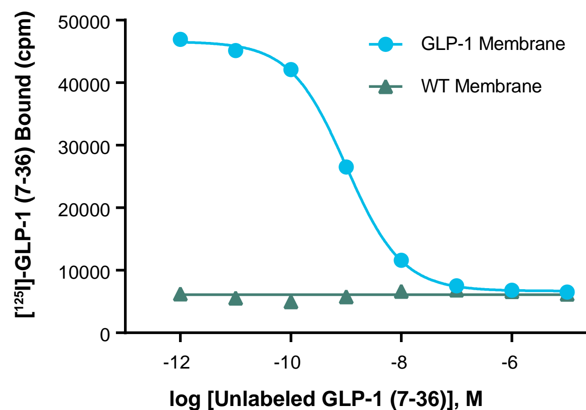

Figure 2. Competition binding experiment for glucagon-like peptide-1 GLP-1 receptor (Cat. No. HTS020M). GLP-1 membrane preparation or wild-type (WT) Chem- 1 membrane prep (Cat. No. HTS000MC1) was incubated with 0.5 nM [^{125}I]-GLP-1 (7-36) and increasing concentrations of unlabeled GLP-1 (7-36), producing a competition curve. Using this curve, Ki and IC_{50} of the unlabeled GLP-1 (7-36) can be calculated. More than 7-fold signal to background was obtained.

MEMBRANE PREPS VALIDATION

The Eurofins DiscoverX® membrane preps have been tested for and provide reference data in either GTPγS or binding assay applications, not both. They have also been validated with radioligands and not been specifically tested using fluorescent or other ligand labeling methods. These membrane preps are intended for filtration-based ligand labeling strategies and were not validated using scintillating plates or bead-bound methods. Attempting to use these other strategies will require some optimization. Overall, if you are interested in studying binding affinities of GPCR targets, without a requirement for cell culture, pre-prepared membrane preps are an excellent assay tool to have in your toolbox.

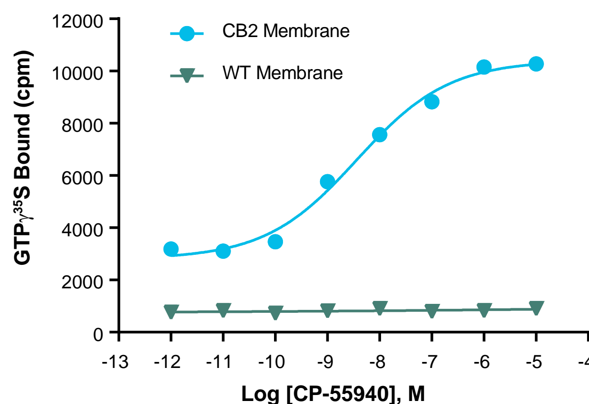

Figure 3. GTPγS assay for cannabinoid-2 (CB2) receptor (Cat. No. HTS020M). 5 µg/well CB2 membrane prep or WT Chem-1 membrane prep (Cat. No. HTS000MC1) were incubated with 0.3 nM [^{35}S]-GTPγS, 10 µM GDP, and increasing amounts of unlabeled CP-55940. Bound radioactivity was determined by filtration and scintillation counting. An EC_{50} of 3.7 pmol/mg and signal to background of > 3.4 fold were obtained from this curve.

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ARTICLE

Ideal Methods to Study Cellular Transport of Membrane Proteins

TRANSLOCATION AND TRAFFICKING OF MEMBRANE PROTEINS

Membrane proteins (such as receptors like GPCRs and ion channels) are translated and undergo proper folding in the endoplasmic reticulum (ER) and then transported to the cell surface (plasma membrane). Additionally, as part of the normal recycling and receptor activation processes, membrane proteins internalize from the plasma membrane into endosomes. The appropriate localization of membrane proteins is essential to maintain their biological function, but in some cases, these protein transport events are altered leading to a non-functional membrane protein and undesirable effects that are often associated with serious human diseases (e.g. cystic fibrosis, Alzheimer's disease, and Huntington's disease).

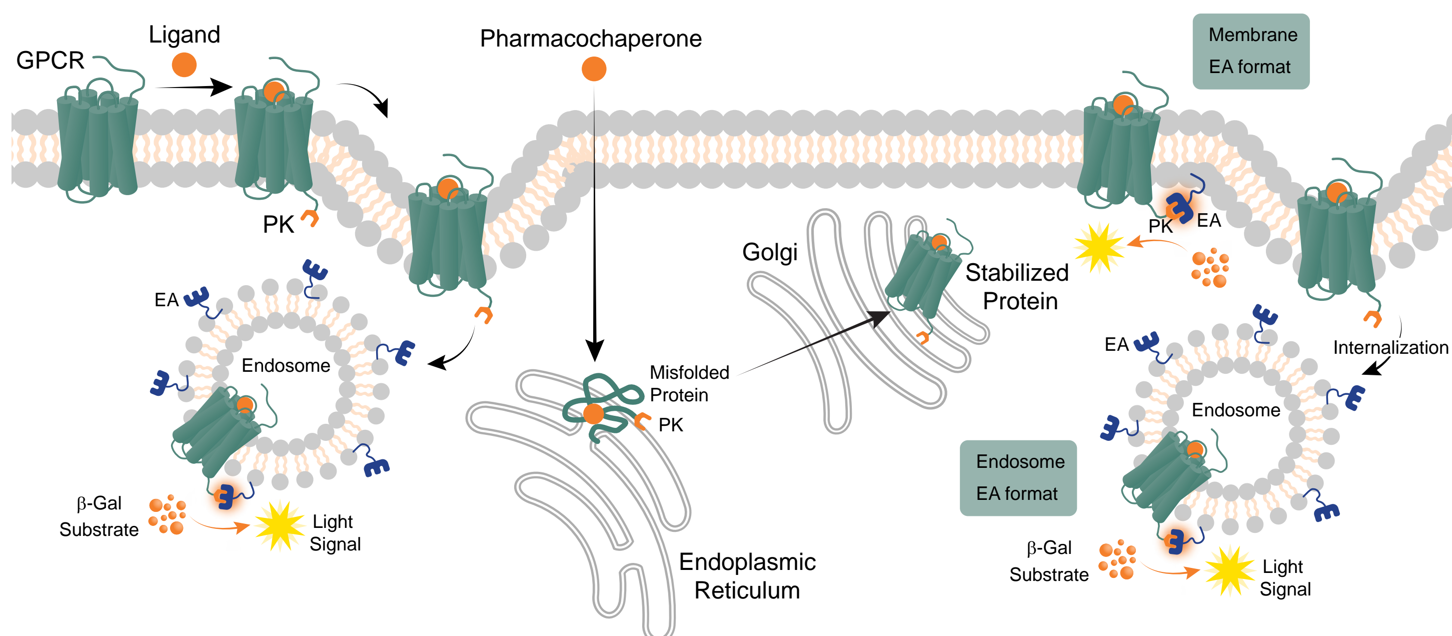

Figure 1. PathHunter® internalization and pharmacotrafficking assays.

Explore Eurofins DiscoverX® products and solutions related to these methods: Translocation assays, GPCR Internalization assays, and Pharmacotrafficking assays.

PREVENTING UNDESIRABLE TRANSLOCATION EVENTS

Membrane protein defects that are caused by mutations, deletions, and truncations can lead to protein misfolding in the ER and prevent their trafficking to the plasma membrane. This mislocalization renders the membrane protein non-functional and leads to diseases like cystic fibrosis. Many endogenous and synthetic receptor ligands (like morphine) can change the rate of membrane protein internalization and recycling. Drug-induced receptor internalization and recycling are important pharmacological properties since they can limit their clinical effectiveness and result in unwanted side effects, and tachyphylaxis drug tolerance problems.

The detrimental cellular events caused by protein mislocalization and excessive protein internalization can be reversed by the development of compounds called pharmacochaperones (short for pharmacological chaperones) and better drugs (such as partial agonists). Pharmacochaperones, correct, or stabilize the folding of the abnormally misfolded proteins allowing them to translocate properly from the ER to the plasma membrane. Once at the cell surface, a partial agonist can bind to the membrane protein initiating a partially efficacious response compared to a full agonist, while also reducing unwanted side effects and preventing other internalization issues.

Ideal Methods to Study Cellular Transport of Membrane Proteins (Continued)

METHODS OF MEASURING SUBCELLULAR TRANSLOCATION OF MEMBRANE PROTEINS

Researchers currently monitor membrane protein translocation events using high content imaging methods.

- Immunohistochemistry (IHC) or immunocytochemistry (ICC) – Provides visualization of membrane protein localization
- Fluorescence-activated cell sorting (FACS) – Provides quantitative detection of membrane protein localization

PROBLEMS WITH HIGH CONTENT IMAGING METHODS:

- Low throughput
- Requires antibodies or fluorescent tags
- Uses specialized equipment that can often be expensive

In contrast, cell-based assays for studying protein function, trafficking, and internalization are becoming more mainstream for screening and profiling potential drugs to prevent translocation issues.

BENEFITS OF USING CELL-BASED ASSAYS FOR STUDYING TRANSLOCATION ISSUES:

- High throughput
- Quantitative
- Affordable with no need for specialized equipment; assays can be read using standard luminometer

These methods are complementary to each other and should all be considered when studying cellular membrane protein translocation events.

Author Dana Haley-Vicente, Ph.D.,
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for Eurofins DiscoverX®

KNOWLEDGE-BASED VIDEOS

GPCR Assays: Choosing the Best Assay for Your Programs

Learn more about multiple GPCR assays, and choose the best ones to use and why for your research and drug discovery and development program.

Find out what GPCR product types are available and discover assays including cAMP and calcium accumulation, β-arrestin recruitment, internalization, pharmacotrafficking, radioligand binding, and GTPyS assays to expedite your GPCR research and therapeutic development. Video covers GPCR families, targets, assay technology and protocol, and applications for target ID through QC lot release. (30 min.)
Short Recording (5 min.)

View Related Products at discoverx.com/gpcrs.

Internalization Assays: Identify Safer Therapeutics with Cell-Based Assays

Learn about PathHunter® Internalization assays developed to measure translocation of both single-pass and multi-pass receptors (like receptor tyrosine kinases, checkpoint receptors, and GPCRs) to intracellular endosomes.

Identify safer therapeutics with simple, quantitative cell-based assays for measuring receptor internalization. (13 min.)

View Related Products at discoverx.com/translocation.

Pharmacotrafficking Assays: Discover Pharmacological Chaperones that Rescue Disease-Relevant Proteins

Learn more about PathHunter pharmacotrafficking assays to identify small molecule compounds that rescue disease relevant mutant membrane proteins like GPCRs, ion channels, and transporters.

Discover how these quantitative, antibody- and image-free cell-based assays can be used to screen compounds (called pharmacological chaperones) that stabilize mutated membrane proteins allowing for their proper trafficking to the cell membrane. Applications discussed include rescuing conditions like retinitis pigmentosa, obesity, diabetes, cystic fibrosis, and cardiac arrhythmias. (30 min.)

View related products at discoverx.com/pharmacotrafficking.

Uncover Novel G-Protein or Arrestin-Biased Ligands Using a Suite of GPCR Signaling Cell-Based Assays: A Study of Biased Agonism on Opioid Receptors

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INTRODUCTION

It is well understood that GPCR activation results in G-protein dependent, as well as G-protein independent signaling events such as β-arrestin recruitment and receptor internalization. For the G-protein dependent pathway, agonist binding promotes a conformational change of the receptor that results in the activation of receptor-associated heterotrimeric G-proteins. In turn, these G-proteins activate downstream second messenger molecules such as cyclic AMP (cAMP), inositol triphosphate, diacylglycerol, and calcium. For the G-protein independent pathway, agonist binding promotes G-protein receptor kinases (GRKs) to phosphorylate the C-terminal tail of most GPCRs, resulting in the recruitment of β-arrestin. Bound arrestin then serves to sterically block further G-protein activation, limiting the length of G-protein signaling and results in receptor desensitization followed by internalization into clathrin coated pits[1]. In addition to regulating G-protein signaling, β-arrestins can also initiate a distinct second set of G-protein independent signals such as the activation of several downstream protein kinases. Overall, the complex relationship that exists between G-proteins and β-arrestin signaling determines both the efficacy and potential side effects of GPCR targeted drugs[2,3]. Research from the last two decades has shown that different agonists of the same GPCR can induce different signaling responses, a phenomenon termed as biased agonism. For example, one agonist may trigger G-protein dependent signaling events such as cyclic AMP production. In contrast, another agonist may show a preference for G-protein independent events such as arrestin recruitment. Therefore, in the context of therapeutics, quantitatively examining these signaling pathways can aid in defining the compound function, and can lead to the discovery of novel biased ligands with unique efficacy and safety profiles.

Several therapeutically relevant examples of biased agonism have been described with the opioid system[3, 4, 5] that is involved in the body's response to pain. Activation of the δ human opioid receptor (OPRD1, hDOR) alleviates persistent pain leading to the desired analgesic effect. However, sustained or repeated receptor activation results in receptor desensitization that is thought to be the main cause of opioid tolerance *in vivo*[6]. Despite having similar potencies and efficacies *in vitro*, δ opioid receptor agonists can differ dramatically in their desensitization and receptor internalization profiles. Importantly, in the context of pain, it has now been shown that δ agonist-induced analgesic effects are retained when receptors remain at the cell surface and are lost following receptor activation and internalization[6, 7]. Similar to hDOR, there are many disease-associated GPCRs that cannot be maximally utilized for effective drug development due to adverse side effects of downstream receptor signaling. Therefore, there is a need to characterize and develop novel ligands that target only the pathways that lead to beneficial therapeutic effects, thus increasing efficacy while decreasing unwanted side effects.

To address this need, Eurofins DiscoverX offers a portfolio of cell-based assays for GPCR drug discovery research. Whether the goal of an experiment is to quantify 2nd messenger accumulation, measure arrestin recruitment, or understand receptor internalization, an assay is available for drug characterization. While these assays are designed to capture different signaling events, all of the assays are based on the Enzyme Fragment

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Complementation (EFC, discoverx.com/efc) technology for generating a chemiluminescent signal that can be read on any standard luminometer. EFC involves tagging the receptor of interest with an inactive β-galactosidase (β-gal) small enzyme fragment called ProLink or PK (also called Enzyme Donor or ED).
Its complementary inactive larger fragment, Enzyme Acceptor (EA), is either tagged with a relevant signaling protein (e.g. β-arrestin) or added in with the detection reagents. Ligand-mediated signaling events bring the PK/ED and EA fragments together, resulting in the formation of an active β-gal enzyme. This active enzyme hyrdrolyzes an added substrate to generate a chemiluminescent signal. Figure 1 illustrates the working principles of these cell-based assays.

In this paper, we demonstrate that the PathHunter® β-arrestin, PathHunter GPCR Internalization, and HitHunter® cAMP second messenger assays can be used in parallel to uncover novel, biased ligands with specific receptor activation and internalization profiles. We systematically characterized the activation and internalization profiles of OPRD1 in response to multiple hDOR agonists using three functional readouts. Our results demonstrate that compound potency and efficacy differences that exist between endogenous and synthetic ligands can be easily uncovered using a combination of arrestin recruitment, internalization, and second messenger assays. Thus, Eurofins DiscoverX®'s suite of functional, cell-based assays is an ideal platform for ligand bias discovery and the characterization of compounds using empirically derived guidelines.

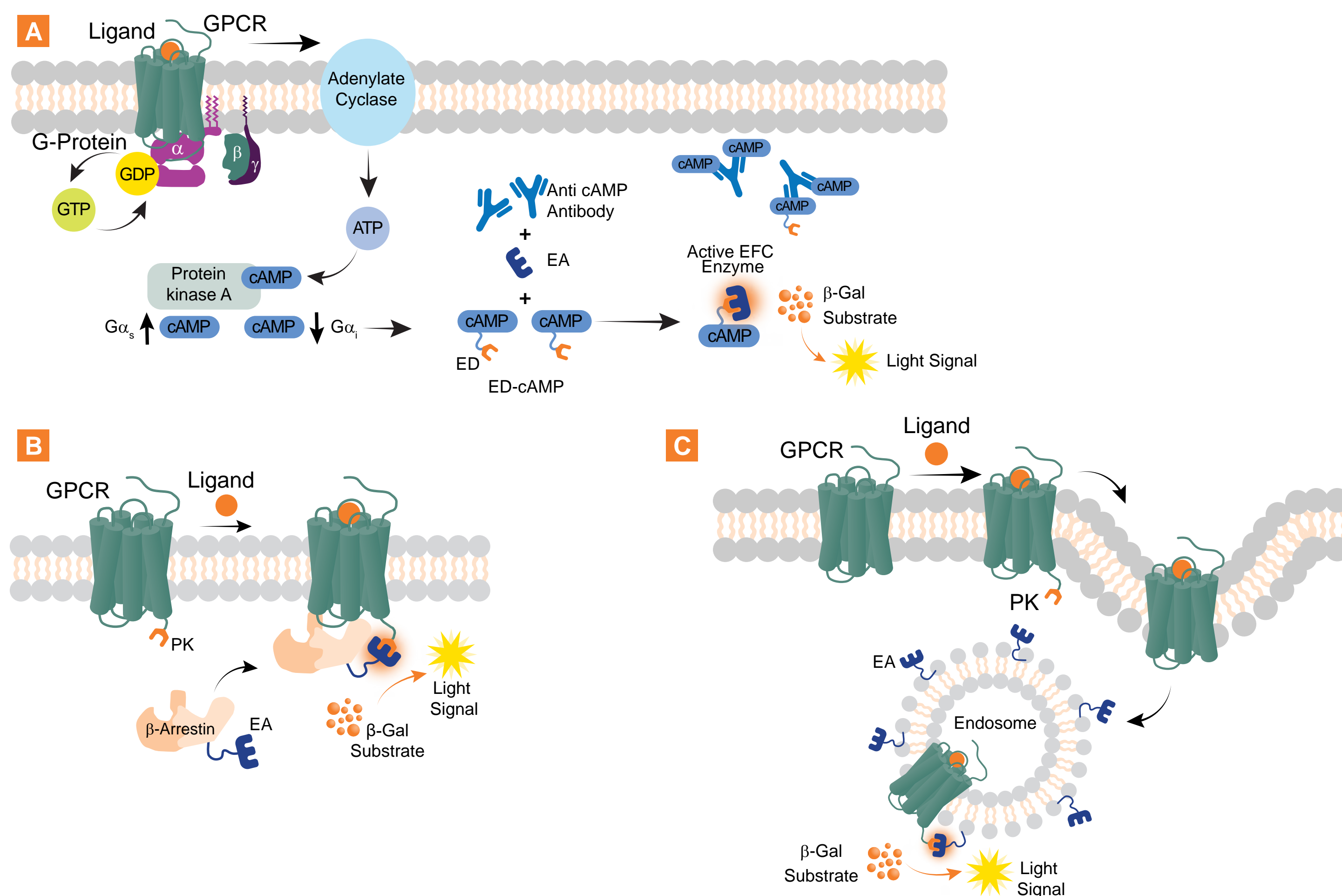

Figure 1. Functional cell-based assays by Eurofins DiscoverX. A. cAMP accumulation, B. β-arrestin recruitment, and C. GPCR internalization.

For a complete list of assays, visit discoverx.com/gpcrs.

METHODS

Clonal Cell Lines

Cells stably overexpressing hDOR in the cAMP (cAMP Hunter™, Cat. No. 95-0108C2), arrestin recruitment (PathHunter® Arrestin, Cat. No. 93-0241C2), and GPCR internalization (PathHunter Activated GPCR Internalization, Cat. No. 93-0673C3) formats were plated at 5,000 cells per well of a 384-well plate and incubated overnight at 37°C and 5% CO_2.

HitHunter cAMP assay

hDOR cAMP Hunter cells were treated with increasing concentrations of known compounds for 30 minutes at 37°C and 5% CO_2. cAMP signal was detected using the HitHunter® cAMP Detection Kit (Cat. No. 90-0075) according to the recommended protocol. HitHunter cAMP assays are competitive immunoassays, where free cAMP from cell lysates competes with ED-tagged cAMP for binding to the anti-cAMP antibody. In the presence of ample cellular cAMP, fewer ED-labeled cAMP molecules are able to bind to the antibody. As a result, there is a high level of ED-labeled cAMP that is free to complement with EA from the detection reagent. The resulting active full β-gal enzyme hydrolyzes a substrate to produce a chemiluminescent signal. A positive signal is directly proportional to the amount of cellular cAMP. (Figure 1. A.).

PathHunter β-Arrestin Assay

hDOR PathHunter β-Arrestin cells were treated with increasing concentrations of the compounds for 90 minutes at 37°C and 5% CO_2. In this system, the PK fragment is appended to the C-terminus of the GPCR. β-arrestin is fused to the EA fragment. Activation of a single GPCR stimulates the binding of arrestin, forcing complementation of the two enzyme fragments. The resultant increase in β-gal enzyme activity is measured by addition of chemiluminescent PathHunter Detection Reagents (Cat. No. 93-0001). (Figure 1. B.)

PathHunter Activated GPCR Internalization Assay

hDOR PathHunter GPCR Internalization cells were treated with increasing concentrations of the compounds for 180 minutes at 37°C and 5% CO_2. PathHunter Internalization assays employ the PK tag that is localized to the surface of intracellular endosomes and the EA fused to β-arrestin. Stimulation of the untagged receptor results in β-arrestin binding to the activated GPCR, internalization of the receptor, and trafficking to cellular endosomes. The resultant enzyme complementation leads to β-gal enzymatic activity that is measured by addition of chemiluminescent PathHunter Detection Reagents (Cat. No. 93-0001). (Figure 1. C.)

Agonists

Four hDOR agonists (DADLE, [Met^5]-enkephalin, [Leu]-enkephalin, and SNC80, a strongly internalizing compound and functional antagonist), were analyzed by the three types of GPCR functional assays. For comparison, the data was normalized to [Met^5]-enkephalin in potency (set equal to 1) and efficacy (set equal to 100%).

All data was read on a multi-mode plate reader and analyzed using GraphPad Prism®.

RESULTS AND DISCUSSION

Our experiments demonstrate that potency and efficacy differences exist between endogenous enkephalin peptides and synthetic analogs for the human δ opioid receptor. Furthermore, these differences can be easily uncovered using a combination of second messenger, arrestin recruitment, and internalization assays, as shown in Figure 2. According to the internalization assay, SNC80 was clearly defined as a super agonist. Although the hDOR receptor undergoes rapid internalization, distinct ligand-specific differences were observed during re-sensitization when the GPCR became available. These results correlate with published literature that indicates that receptor internalization, as determined by a large reduction in cell surface receptors, influences the efficacy of an agonist[6, 7]. Therefore, using a single pathway approach to GPCR analysis *in vitro* could lead to incorrect prediction of compound activity *in vivo*. From a drug development standpoint, using multiple GPCR signaling readouts during the screening and lead optimization process can facilitate the identification of novel biased ligands with unique activation and internalization profiles.

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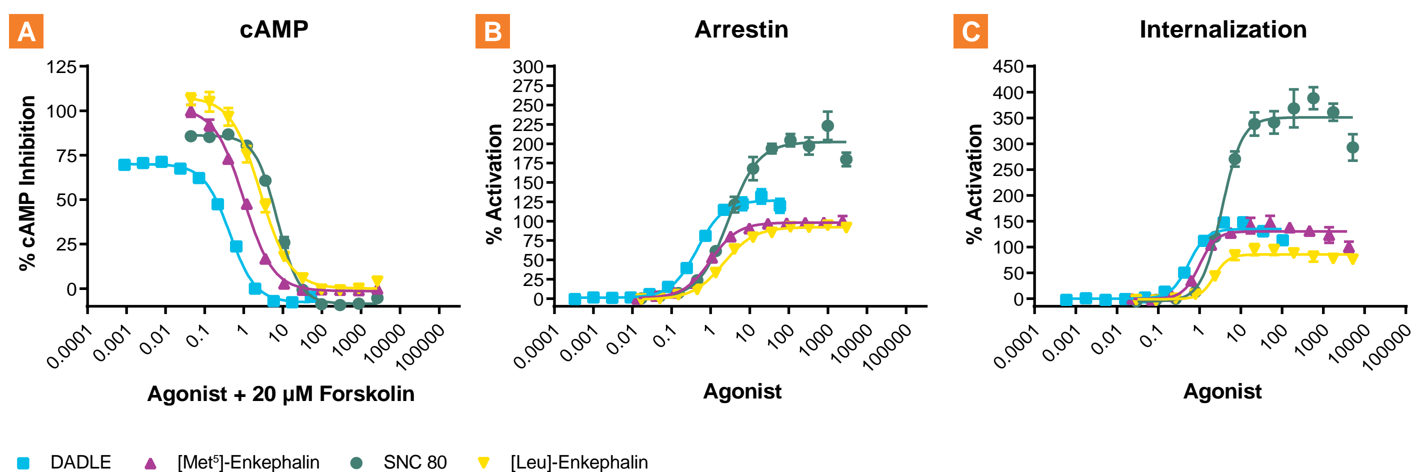

Figure 2. Comparison of human δ opioid receptor cell-based assays. A. cAMP, B. β-arrestin recruitment, and C. GPCR internalization assays of the human δ opioid receptor (OPRD1, hDOR) receptor.

CONCLUSIONS

Understanding G-protein and arrestin biased signaling has important implications in the discovery and development of novel therapeutics. Using Eurofins DiscoverX®'s suite of functional cell-based assays, our experiments indicate that novel biased ligands can be identified and characterized for multiple activities *in vitro* using the same robust, reproducible, and high throughput-friendly chemiluminescent format. We have completed a systematic analysis of many GPCRs and their related ligands to study the relationship between different readouts of GPCR activation. More than half of the receptors tested showed significant pharmacological differences depending on the pathway studied, indicating that functional selectivity is not an isolated event, but is prevalent across multiple receptor classes and ligand types. We have embarked on an era of GPCR drug discovery that will enable researchers to develop smarter, safer, and more effective drugs based on a deeper understanding of receptor activity and compound mechanism. With the largest and most comprehensive menu of over 1600 functional and binding GPCR assays for screening and profiling, Eurofins DiscoverX delivers simple, functional, quantitative, and high throughput cell-based assays for small molecule and biologics drug discovery analysis.

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Quantify GPCR Endocytosis and Recycling with PathHunter® GPCR Internalization Assays – Analyzing Therapeutics for Opioid and Cholecystokinin Receptors

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INTRODUCTION

G-protein coupled receptors (GPCRs) are the largest and most druggable class of cell surface receptors[1]. Following agonist-mediated receptor activation and subsequent G-protein signaling, GPCRs are phosphorylated by G-protein receptor kinases (GRKs), which results in the binding of β-arrestin to the activated receptor.

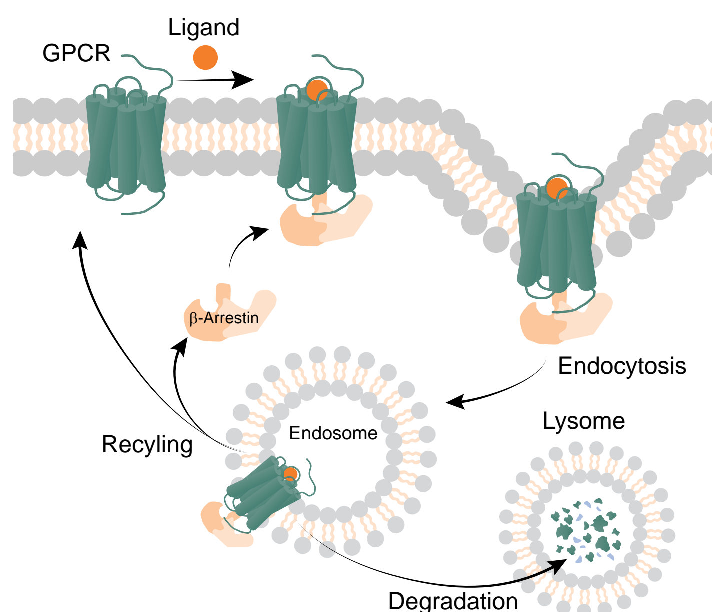

Figure 1. The life cycle of a GPCR includes activation, endocytosis, and recycling back to the plasma membrane. Repeated or prolonged exposure to a ligand can re-route receptors to the lysosome, thus contributing to the downregulation of receptors.

Bound arrestin sterically blocks further G protein activation, thus limiting the length of G-protein signaling and leading to receptor desensitization and internalization into clathrin coated pits[2]. Once internalized, GPCRs are either targeted to lysosomes for degradation, retained within the endosomal compartment, or recycled back to the cell membrane[3], as seen in Figure 1.

The rate and extent of receptor internalization can dramatically impact the biological efficacy of a ligand or therapeutic. Studies have shown that agonist treatment can lead to tachyphylaxis, a decrease in responsiveness to the drug with repeated dosing[4]. Moreover, GPCR agonists can induce tolerance, whereby a higher amount of drug is required with repeated doses to obtain the same therapeutic effect[5,6]. Both tachyphylaxis and drug tolerance, which can limit the clinical effectiveness of a drug, have been shown to correlate with the tendency of the ligand to induce receptor internalization following prolonged or repeated receptor activation[4-6]. Using a mouse model of inflammatory pain, Pradhan *et al.* showed that despite having similar *in vitro* potencies and efficacies, δ-opioid receptor (DOR) agonists were only able to retain full analgesic efficacy when receptors remained at the cell surface[6].

Although various approaches for monitoring receptor internalization have been developed, these techniques are often difficult to quantify, rely on the availability of highly specific antibodies and expensive imaging instruments, and are not amenable to a high-throughput format[7]. As a result, internalization profiles of candidate drugs may not be investigated until very late in the discovery process following expensive and labor-intensive animal testing. Therefore, there is a need for a simple, scalable, and quantitative internalization assay that can be used to characterize therapeutic activity earlier in the drug discovery process in order to elucidate the mechanism by which a GPCR-targeted drug exerts its effect *in vivo*. As a solution, PathHunter GPCR Internalization Assays by Eurofins DiscoverX are specifically designed to

quantitatively measure internalization and recycling patterns of GPCR drug targets to enable the discovery and characterization of novel agonists. These assays assist in differentiating between strongly and weakly internalizing agonist ligands or therapeutics, and provide a simple, non-imaging, non-antibody based chemiluminescent technique that is amenable to high throughput screening.

PathHunter GPCR Internalization Assays

Eurofins DiscoverX® has developed two different formats of the PathHunter® GPCR Internalization Assays – the activated internalization assay and the total internalization assay. While both formats measure receptor internalization in whole cells, the activated internalization assay measures receptor internalization mediated by β-arrestin recruitment. On the other hand, the total internalization assay measures GPCR endocytosis brought about by all relevant mechanisms including the natural recycling of GPCRs. Both of these formats are based on the established Enzyme Fragment Complementation (EFC; discoverx.com/efc) technology. EFC is based on two recombinant β-galactosidase (β-gal) enzyme fragments – a large enzyme acceptor (EA) fragment and a smaller enzyme donor (ED, also called ProLink™ (PK) fragment. Separately, these fragments are inactive, but when combined, they form an active β-gal enzyme that hydrolyzes its substrate to produce a chemiluminescent signal.

As illustrated in Figure 2, the PathHunter Activated GPCR Internalization Assays combine engineered clonal cell lines stably expressing the wild-type (untagged) GPCR, PK-tagged endosomes, and a β-arrestin/EA fusion protein. Activation of the untagged GPCR induces β-arrestin recruitment and internalization of the GPCR/arrestin-EA complex to the PK-tagged endosomes, resulting in the complementation of the PK-EA fragments to form the active β-gal enzyme. In comparison (not shown here), the PathHunter Total GPCR Internalization Assays combine engineered clonal cell lines stably expressing a PK-tagged GPCR and EA-tagged endosomes. Activation of the PK-tagged GPCR induces internalization to the EA-tagged endosomes, bringing about the formation of the active β-gal enzyme. Overall, both assays result in a positive, chemiluminescence gain-of-signal with large signal-to-noise ratios using a simple, mix-and-read detection system for optimal assay performance. Additionally, these internalization assays, like most EFC assays, can be used to screen small molecules or biologics in a fast, scalable, and highly quantitative manner.

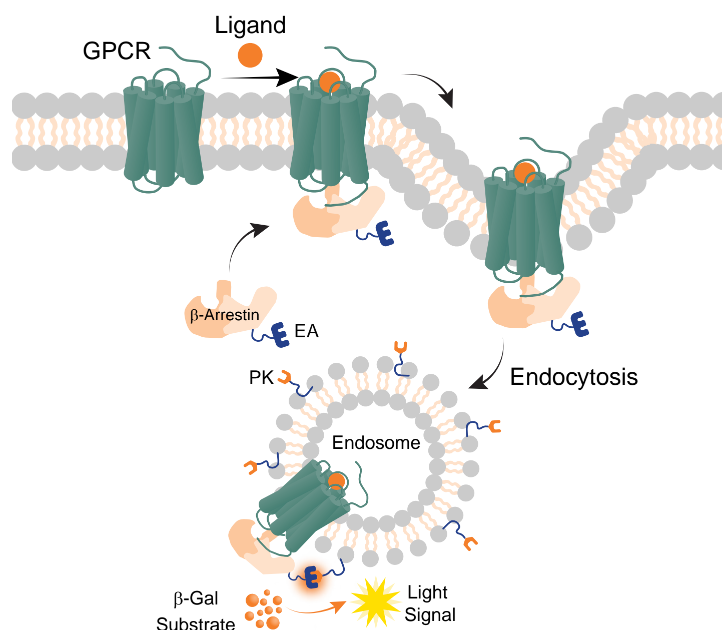

Figure 2. PathHunter Activated GPCR Internalization Assays provide a novel, non-imaging approach for measuring GPCR activation and internalization in live cells. In this system, enzyme fragment complementation occurs following β-arrestin recruitment and receptor internalization and localization at the early endosome.

To demonstrate that the PathHunter Activated GPCR Internalization Assays can be used as a powerful tool to measure receptor internalization, we characterized the receptor trafficking properties of agonists of two different classes of GPCRs—DOR and Cholecystokinin-A receptor (CCKAR) agonists using various GPCR cell lines. DORs are involved in pain modulation as mentioned above, while CCKAR plays an active role in various parts of the digestive system including the pancreas, gallbladder, and stomach. Using a combination of activation and internalization assays for the same GPCR target, we also demonstrate that these assays can be used to uncover potency and efficacy differences between ligands.

METHODS

Clonal Cell Lines

PathHunter® Activated GPCR Internalization Cell Lines expressing the receptors CCR5 (Cat. No. 93-0653C3), CHRM5 (Cat. No. 93-0657C3), SSTR2 (Cat. No. 93-0676C2), FPRL1 (Cat. No. 93-0666C3), DOR (Cat. No. 93-0673C3) and CCKAR (Cat. No. 93-0650C3) and PathHunter β-Arrestin Cell Line expressing CCKAR (93-0190C2) and cAMP Hunter™ Cell Line expressing DOR (Cat. No. 95-0108C2) were cultured as recommended in the cell line-specific datasheets. Prior to running the assays, cells were plated at 5,000 cells per well of a 384-well plate and incubated overnight at 37°C and 5% CO_2.

Ligands

Reference ligands CCL3 (Cat. No. 92-1002), Oxotremorine-M (Cat. No. 92-1106), Somatostatin-28 (Cat. No. 92-1068), WKYMVm-NH2 (Cat. No. 92-1075), DADLE (Cat. No. 92-1055), [Met[5]]-Enkephalin (Cat. No. 92-1086) were obtained from Eurofins DiscoverX®. SNC-80 (Cat. No. 0764) and A-71623 (Cat. No. 2411) were obtained from Tocris Bioscience (Minneapolis, MN). Cholecystokinin (CCK) Fragment 26-33 Amide, Non-sulfated, sCCK8 (Cat. No. C2901) and [Leu]-Enkephalin (Cat. No. L9133) were obtained from Sigma Aldrich (St. Louis, MO).

PathHunter Activated GPCR Internalization Assays

Cells were treated with increasing concentrations of ligands for 180 minutes at 37°C and 5% CO_2. The resultant EFC after agonist treatment leads to an increase in enzyme activity that is measured by addition of chemiluminescent PathHunter Detection Reagents (Cat. No. 93-0001). All data was read on a multi-mode plate reader and analyzed using GraphPad Prism®.

Calcium Flux Assays

Naturally Gq-coupled PathHunter β-Arrestin CCKAR cells were loaded for intracellular calcium flux measurement with a Calcium No Wash[PLUS] Kit (Cat. No. 90-0091L) assay solution supplemented with 2.5 mM probenecid to avoid calcium dye leakage from the cells. The cells were then incubated for 1 hour at 37°C and 5% CO_2. The agonist was added after 10 seconds of baseline acquisition. Increase of intracellular calcium concentration was monitored using the FLIPR® Tetra detection system from Molecular Devices (Sunnyvale, CA). Visit discoverx.com/calcium for more details.

HitHunter cAMP Assays

Human DOR cAMP Hunter cells were treated with increasing concentrations of known ligands for 30 minutes at 37°C and 5% CO_2. The cAMP signal was detected using the HitHunter® cAMP XS+ Detection Kit (Cat. No. 90-0075) according to the recommended protocol. HitHunter cAMP assays are competitive immunoassays, where free cAMP from cell lysates competes for antibody binding against labeled cAMP (ED-cAMP conjugate). Unbound ED-cAMP is free to complement EA to form an active enzyme by EFC which hydrolyzes substrate to produce signal. A positive signal is directly proportional to the amount of cellular cAMP. Visit discoverx.com/cAMP for more details.

Data Normalization

Where indicated, data was normalized to the reference agonist in potency (set equal to 1) and efficacy (set equal to 100%) to enable a graphic representation of the agonist comparisons.

RESULTS AND DISCUSSION

At first, a series of experiments were run using the GPCR internalization assay across a range of different GPCR receptors. Figure 3 shows representative dose-response curves for the human CCR5, M5, SSTR2, and FPRL1 receptors, demonstrating that these assays are broadly applicable to a large number of GPCR families and receptor types to provide quantitative comparisons of ligand activity.

To determine whether PathHunter GPCR Internalization assays can be used to differentiate between strongly and weakly internalizing agonist ligands, we characterized the receptor trafficking properties of four DOR agonists (DADLE, [Met[5]]-enkephalin, [Leu]-enkephalin, and SNC-80, a known strongly internalizing compound and functional antagonist). For comparison, the data was normalized to [Met[5]]-enkephalin in potency (set equal to 1) and efficacy (set equal to 100%). Our results demonstrate that treatment with SNC-80 leads to high levels of DOR receptor internalization (Figure 4. A.). Moreover, SNC-80 could be defined as a superagonist in

the assay based on a maximal response greater than that of the endogenous agonist, [Met[5]]-enkephalin. We also examined the inhibitory effects of the four agonists on forskolin-stimulated cAMP accumulation using the DOR cAMP Hunter™ Cell Line. In contrast to the internalization profiles, we observed similar ligand potencies and efficacies for both SNC-80 and enkephalins using the cAMP assay (Figure 4. B.).

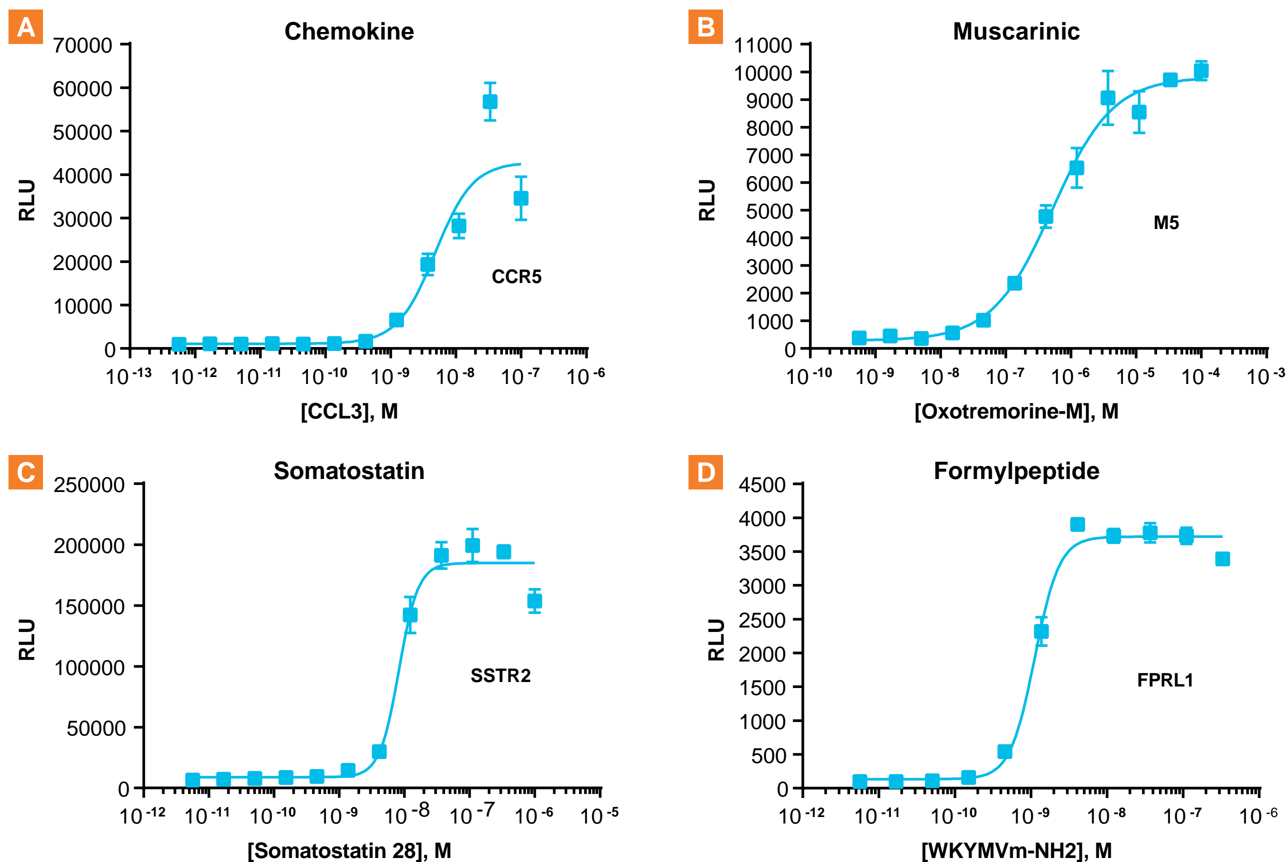

Figure 3. The PathHunter® GPCR Activated Internalization Assay is broadly applicable to a large number of GPCR families. Cells expressing the wild type untagged A. Chemokine CCR5, B. Cholinergic Muscarinic CHRM5, C. Somatostatin SSTR2, and D. Formylpeptide FPRL1 were treated with increasing concentrations of control agonist and assayed using PathHunter detection reagents. In all cases, the results show a positive, gain-of-signal assays with large signal-to-noise ratios for the agonists tested.

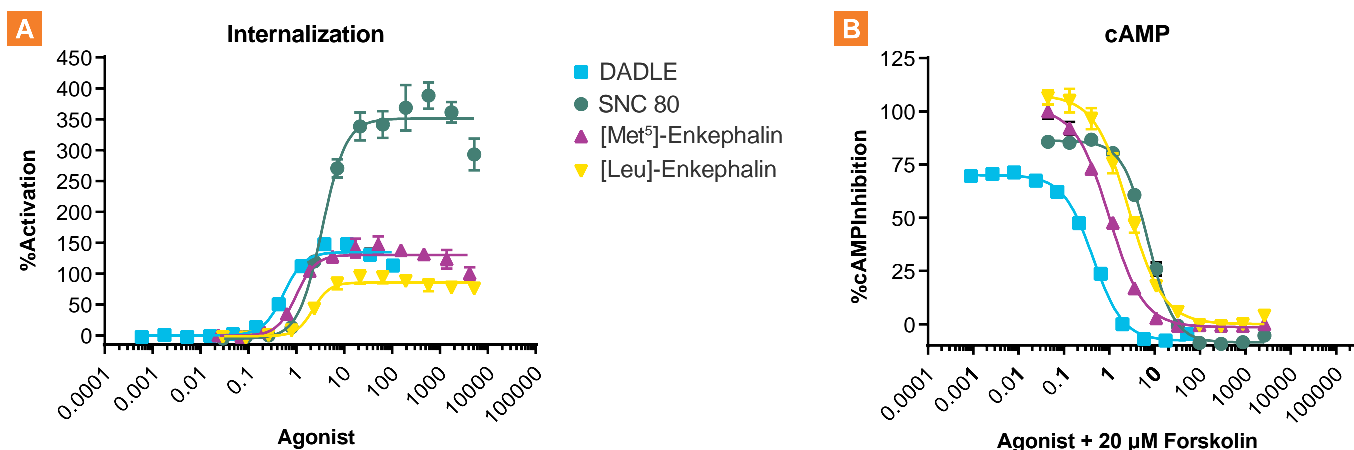

Figure 4. Comparison of DOR internalization versus cAMP accumulation by known receptor agonists. Cells overexpressing the DOR receptor in the PathHunter® Activated GPCR Internalization A. and cAMP Hunter™ B. assay formats were treated with agonists and assayed using PathHunter and HitHunter detection reagents, respectively, as described in Methods. Despite similar ligand potencies and efficacies based on the inhibition of cAMP accumulation, SNC-80 which is a known strongly internalizing compound and functional antagonist, is clearly defined as a superagonist in the internalization assay only.

To help explain these results, we associated these finding with other studies. Lecoq *et al.* first studied the desensitization and internalization patterns of the DOR receptor following exposure to endogenous enkephalin ligands or the synthetic opioid agonist, SNC-80. Their results showed that δ opioid agonists differed dramatically in their desensitization and receptor internalization profiles despite having similar potencies and efficacies *in vitro*. Moreover, DORs were more rapidly desensitized by SNC-80 resulting in high levels of internalization via clathrin-coated pits, whereas exposure to the natural enkephalin ligands led to internalization followed by sorting to recycling endosomes[5]. Using a mouse model of inflammatory pain, Pradhan *et al.* established a direct link between receptor localization and function by demonstrating that δ opioid agonist-induced analgesic effects are retained when receptors remain at the cell surface, but lost following receptor activation and internalization[6]. Overall, the PathHunter internalization assays results correlated well with the aforementioned experiments.

The PathHunter Activated GPCR Internalization assays could be broadly applied to other therapeutically relevant targets, as demonstrated through our experiments on the receptor trafficking properties of CCKAR and related agonists. Asin *et al.* described the *in vivo* behavior of a highly selective CCKAR agonist, A-71623. Despite being less potent compared to the endogenous ligand cholecystokinin (CCK), A-71623 showed improved potency and prolonged duration by suppressing food intake and locomotor activity in food deprived rats[10]. Using a combination of activation (calcium flux) and receptor internalization assays, our results demonstrate that agonist treatment with the sulfated version of CCK (sCCK8) leads to high levels of receptor internalization, whereas treatment with A-71623 does not (Figure 5. A.). When we compared the potency of the ligands using a calcium flux assay, our results confirmed that A-71623 is less potent than the endogenous sCCK8 ligand. In the case of CCKAR, one hypothesis is that the lack of receptor internalization following A-71623 treatment in the food deprived rats could explain the greater therapeutic benefit of the less potent synthetic analog compared to the endogenous ligand by increasing the length of time the drug can interact with its therapeutic target.

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Our results demonstrate that these assays can be used to directly measure internalized GPCRs localized to intracellular endosomes in a dose-response dependent manner, and identify strongly internalizing ligands that appear as superagonists. Furthermore, we saw that both DOR and CCKAR receptors undergo rapid internalization. We observed dramatic differences in the internalization profiles between endogenous agonists and synthetic analogs that were easily uncovered using our simple, mix-and-read chemiluminescent readout. Additionally, we completed a systematic pharmacologic analysis of a set of chemokine receptors and their related endogenous ligands using assays for G-protein signaling, β-arrestin recruitment, and receptor internalization. For chemokines targeting the same receptor, differences in the efficacies for G-protein- or arrestin-mediated signaling or receptor internalization were observed. From a drug development standpoint, using PathHunter® GPCR Internalization assays as orthogonal screening tools during the hit-to-lead and lead optimization stages can provide novel information about a ligand's functionality early in the drug discovery process that cannot be inferred using a simple receptor activation readout alone (2nd messenger signaling or β-arrestin recruitment). Understanding differences in receptor trafficking and how localization of the GPCR impacts the biological efficacy of a ligand could have important implications for drug discovery in many therapeutic areas.

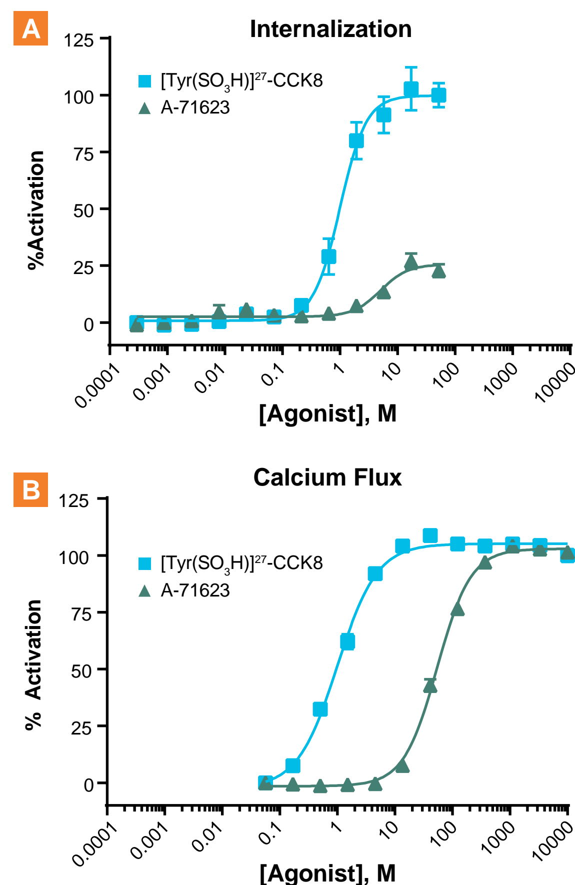

Figure 5. Comparison of CCKAR internalization versus calcium flux by known receptor agonists. Cells overexpressing the CCK-A receptor in the PathHunter Activated GPCR Internalization A. and PathHunter β-Arrestin B. formats were treated with agonists and assayed using PathHunter and Calcium No Wash^{PLUS} reagents, respectively, as described in Methods. A-71623 showed a lower potency in calcium flux as well as ~25% efficacy relative to the sulfated version of the endogenous CCK ligand (sCCK8) in the GPCR internalization assay.

CONCLUSIONS

GPCR activation and internalization patterns in response to ligand activation have important implications in the discovery and development of novel therapeutics. Although integral to GPCR pharmacology, quantification of receptor internalization has proven cumbersome and difficult. Frequently applied tools to study GPCR trafficking include a combination of low-throughtput fluorescence microscopy with complex automated image analysis software and expensive antibodies[7]. To overcome these challenges in monitoring cell surface receptor biology, we developed a generic, quantitative, cellular assay based on the EFC[8, 9] technology.

Eurofins DiscoverX®'s comprehensive menu of more than 100 PathHunter® GPCR Internalization assays offers a simple, non-imaging, non-antibody based chemiluminescent assay that is amenable to high-throughput screening. By providing routine analysis of receptor internalization at an early point in the drug discovery process, Eurofins DiscoverX delivers on the promise of a deeper understanding of ligand activity and the development of novel small molecule or biologic drug candidates with better safety and efficacy profiles.

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Ligand Characterization of Mas-Related GPCR-X2 via Cell-Based Assays: Assessing the Regulation of Mast Cell Function and Implications in Safety Pharmacology

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INTRODUCTION

Effectors of mast cell degranulation (MCD) such as histamine, serotonin, heparin, and certain cytokines principally drive IgE-mediated allergic reactions. On the other hand, non-immune mediated activation of mast cells has been identified to cause pseudo-allergic Adverse Drug Reactions (ADR), due in part to multiple receptor expression profiles on their surfaces. Furthermore, the recognition of a wide range of activating ligands, including certain classes of therapeutics targeting mast cell receptors warrants a closer examination into their safety pharmacological roles. Among other receptor classes, orphan GPCRs have emerged as active proponents in non-immunogenic mast cell activation profiles with possible roles in pseudo-allergic or anaphylactoid reactions. As a result, efforts in drug discovery and development have focused on developing safer therapeutics aimed at reducing anaphylactoid reactions. For instance, a study by McNeil *et al.* (2015) has formed the basis of exploring the role of orphan receptors in non-IgE mediated occurrence of histamine-like symptoms associated with secretagogues and therapeutic drugs.

A relatively new subfamily of GPCRs associated with the MAS1 oncogene was identified based on transcriptome analysis by Dong *et al.* (2001). The encoding genes were termed as Mas-Related genes (MRGs) and the receptors as MAS-related GPCRs (MRGPR). One of its members, MrgprX2 encoded by the gene MRGPRX2, has been identified to be promiscuous and activated by several synthetic small molecules and peptides including U.S. Food and Drug Administration (FDA) approved drugs. While a role for the former "orphan" MrgprX2 was established in 2015, subsequent studies have identified MrgprX2 receptor-mediated agonism to be implicated in causing adverse reactions associated with clinical candidate molecules during their development (Grimes *et al.* 2019).

Overall, these studies have since necessitated more complex risk assessments and targeted safety testing for drugs in development to minimize ADRs, including anaphylaxis and allergic reactions.

RECEPTOR INTERACTIONS AND CLINICAL RELEVANCE

Mast cells are principal players in IgE-, and more recently, MrgprX2-mediated anaphylactoid reactions. They can be activated by immunogenic effectors and by several types of receptors including Toll-like receptors (TLR), protease-activated receptors (PARs), opioid receptors, c5a complement, and T-cells depending on localization and type of mast cell (Hennino *et al.* 2006, Spoerl *et al.* 2017) opening up routes along non-immunogenic activation related pathways (Figure 1.).

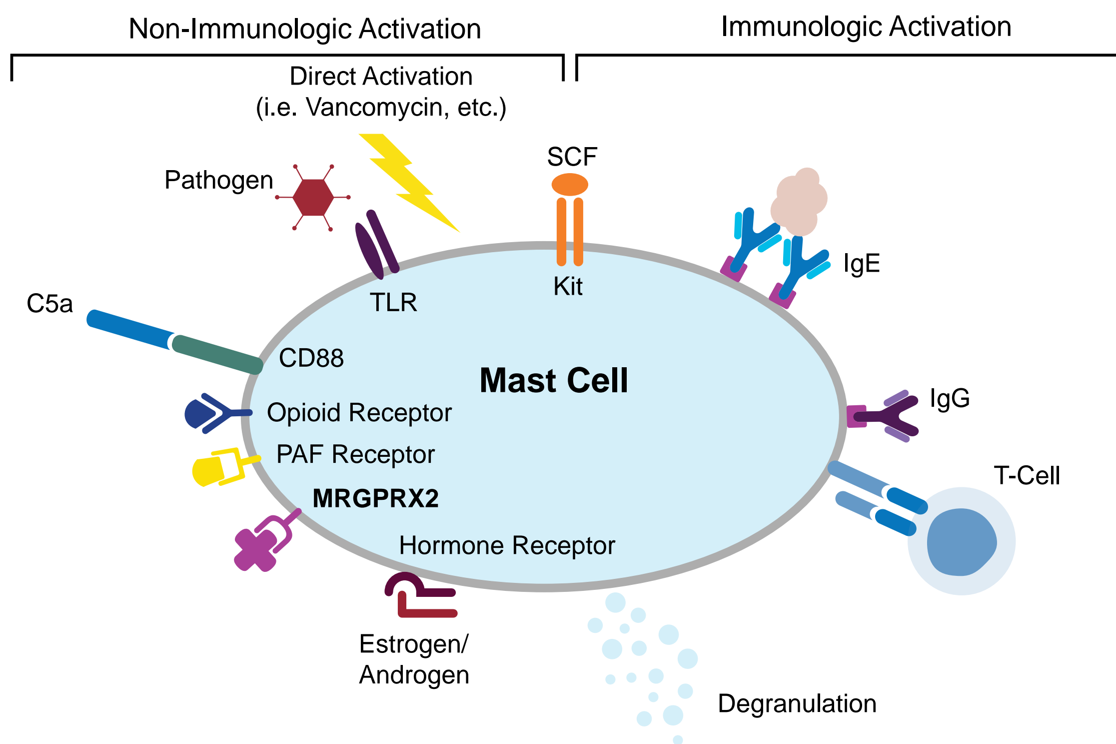

Figure 1. Mast cell degranulation including non-immunological activation route (adapted from Spoerl *et al.* 2017).

Historically and despite significant efforts, the exact function and role of the MrgprX2 orphan receptor remained elusive given its extensive mast cell expression profile. Previously, studies with the human MrgprX2 receptor had revealed surrogate ligand pairings, but were unsuccessful in linking the receptor to its biological function; primarily due to the lack of understanding of orthologue receptor genetics. The catalyst for the recent focus on this receptor was a study from Johns Hopkins University (McNeil *et al.* 2015) that elucidated a physiological role for the receptor for the first time. The group focused on the mouse orthologue gene, recapitulating the same pharmacology observed with the human MrgprX2 receptor and their knockout studies confirmed a link between receptor expression to mast cell function and anaphylaxis.

A few other studies emerged to better understand the receptor's link to drug-induced anaphylaxis. Of particular mention is a study led by Dr. Andrew Brown's team at GlaxoSmithKline (GSK), which identified a series of orthologue receptors, typically of pharmacological interest during drug development, with a bioinformatics approach (Grimes *et al.* 2019). Subsequently, recombinant expression studies and compound profiling, including Eurofins DiscoverX®'s β-arrestin recruitment and calcium signaling assays, provided a rigorous characterization platform of compound pharmacology.

In retrospect, a series of drug discovery programs, where anaphylaxis in animals had impeded development, the (drug) candidates were shown to exhibit MrgprX2 pharmacology at the corresponding orthologue gene. Notably, these programs studied diverse therapeutic types. Further analysis and insight correlated diverse pharmacology to a ligand molecule's net charge or pH rather than any specific peptide sequence or structural features. Indeed, the promiscuity of the MrgprX2 receptor supported Dr. Brown's hypothesis that it functions as a sensor for exogenous and potentially harmful (viral) peptides, invoking a mast cell host response (Grimes *et al.* 2019). This is analogous to the function of the formyl peptide receptor (FPR1), a promiscuous receptor without an established cognate ligand, sensing patterns of bacterial peptides.

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NEED FOR SAFETY LIABILITY SCREENS

Receptor activation towards regulating mast cell degranulation has a purported key safety liability, as observed in studies such as that of Lafleur *et al.* 2020. Since assays with mast cells are challenging (principally due to access to insufficient cells and difficulty creating viable cultures), their study characterized an extended panel of established and novel ligands with an emphasis on investigating the pharmacology of compounds (peptide) charge.

Several targets can represent pharmacological panels in safety liability screens. With appropriate testing methodologies, improved understanding of their pharmacological relevance can be met, which in turn can show the impact of a particular target (and its mechanism-of-action) on the safety liability of the drug at a much earlier stage in the discovery process. In general, it was observed that the outcomes of MrgprX2 activation bear a strong correlation with histamine release in CD34+ stem cell-derived mature human mast cells (La Fleur *et al.* 2020). For that study, the Eurofins DiscoverX® 's PathHunter® platform was employed to analyze MrgprX2 for both calcium release and β-arrestin recruitment. The study endorsed the use of the cell-based functional assays in safety liability screens as a predictive and phenotypically representative model of mast cell activation.

MRGPRX2 is part of Mas-related gene (MRG) family of GPCRs comprising almost 50 members sub-divided into subfamilies (A-H and X), of which the X-species is specific to primates including humans, macaques, and rhesus monkeys. Transcriptomic analyses have shown that MRGPRX2 to be one of the highest expressed receptors in human mast cells (Motakis *et al.* 2014). Studies related to ligand activation of the MrgprX2 receptor have brought forth its promiscuous nature of exerting unexpected/un-intended histamine-like allergenic effects, necessitating the investigation of interaction of potential ligands with MrgprX2.

In this application note, we independently generated a new set of data with selected agonists, previously identified as MrgprX2 agonists, using calcium mobilization and β-arrestin recruitment assays feasible with the application of the PathHunter MrgprX2 cell line. The goal of this study was to show the high-throughput and time-savings value of using engineered, cell-based assays to study its activation through these screening assays.

MATERIALS

Compound 48/80 (Sigma Aldrich, UK), mastoparan (Tocris Biosciences, Bristol, UK), cortistatin-14 (Genscript, USA), octreotide, indolicidin, and vancomycin (Sigma Aldrich, UK); U2OS (ATCC Cat. No. HTB-96, RRID:CVCL_0042) or HEK 293-MSRII cells; and Eurofins DiscoverX PathHunter CHO-K1 MRGPRX2 β-Arrestin Cell Line (Cat. No. 93-0309C2), AssayComplete™ Cell Plating 0 Reagent (CP0, Cat. No. 93-0563R0A), and PathHunter Detection Kit (Cat. No. 93-0001).

METHODS

Calcium Mobilization Assay

Assay comprised of cell line expansion and propagation, dye loading, and agonist activity assay to determine the mobilization of calcium in the cell.

Cell Handling

U2OS or HEK 293-MSRII cell lines were expanded from freezer stocks according to standard procedures. Cells were seeded in a total volume of 20 µL into black-walled, clear-bottom, Poly-D-lysine coated 384-well microplates, and then incubated at 37°C and 5% CO_2 for the appropriate time prior to testing.

Dye Loading

Assays were performed in 1X Dye Loading Buffer consisting of 1X dye, 1X Additive A, and 2.5 mM Probenecid in HBSS / 20 mM HEPES. Probenicid was prepared fresh. Cells were loaded with dye prior to testing. Media was aspirated from cells and replaced with 20 µL Dye Loading Buffer. Cells were incubated for 45 minutes at 37°C and 5% CO_2.

Agonist Format

For agonist determination, cells were incubated with ligand samples (compound 48/80, mastoparan, cortistatin-14, octreotide, indolicidin, and vancomycin) to induce a response. After dye loading, cells were

removed from the incubator, and 10 µL HBSS / 20 mM HEPES was added. Cells were incubated for 15 minutes at room temperature in the dark to equilibrate plate temperature. Intermediate dilution of sample stocks was performed to generate 4X sample in assay buffer. Compound agonist activity was measured on a FLIPR Tetra (MDS). Calcium mobilization was monitored for 2 minutes and 10 µL 4X sample in HBSS / 20 mM HEPES was added to the cells 5 seconds into the assay. Refer to Figure 2. for the assay principle.

β-ARRESTIN RECRUITMENT ASSAY

5000 Cells/well of the PathHunter® CHO-K1 MRGPRX2 β-arrestin cells were seeded with 20 µL AssayComplete CP0 reagent into white 384-well plates (Corning) and incubated overnight (37°C and 5% CO_2). Test agents were serially diluted in DMSO, added to OptiMEM/0.1% BSA (to 5x final), and incubated with 5 µL of cells for 90 minutes at 37°C and 5% CO_2 followed by incubation for 30 minutes at room temperature. Luminescence was detected using an Envision plate reader (Perkin Elmer) after the addition of 12.5 µL PathHunter detection reagent at room temperature for 2 hours. Refer to Figure 3. for the assay principle.

RESULTS

Several agonists (cortistatin-14, indolicidin, compound 48/80, mastoparan, octreotide, and vancomycin) were assessed in PathHunter MRGPRX2 cell lines for β-arrestin recruitment and calcium release using Eurofins DiscoverX® assays.

Measuring intracellular calcium mobilization based on the activation status of specific GPCRs can provide information that uncovers pharmacological rank-orders of lead agonists and calcium signaling flux. The calcium mobilization assay (Figure 2.) uses an inactive dye probe that, when it penetrates the cell membrane, switches to an active form, whose intensity can be measured via fluorescence.

For measuring β-arrestin recruitment, the PathHunter β-arrestin assay (Figure 3.) was used. This assay is based on DiscoverX's proprietary Enzyme Fragment Complementation (EFC) technology, which involves two recombinant β-galactosidase (β-gal) enzyme fragments that act as an enzyme acceptor (EA) and an enzyme donor (ED). Separately, the fragments are inactive, but when combined, they form an active enzyme. In this assay, MrgprX2 cell lines were engineered to co-express the ED, called ProLink™ (PK), tagged to the receptor, MrgprX2, and the EA tagged to β-arrestin. Activation of the MrgprX2-PK by agonist treatment (cortistatin-14) induces β-arrestin-EA recruitment, forcing complementation of the two β-gal enzyme fragments. The resulting functional enzyme hydrolyzes substrate to generate a chemiluminescent signal.

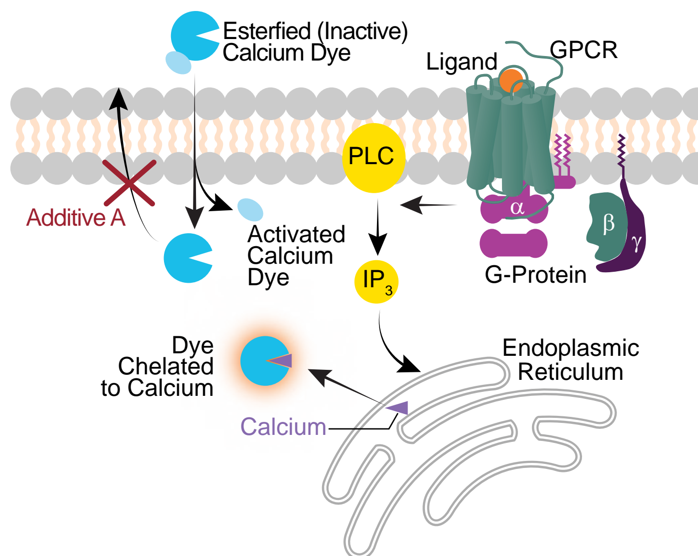


Figure 2. Calcium mobilization assay principle. The calcium mobilization assay (see DiscoverX.com/calcium) is a Calcium No Wash^{PLUS} Assay wherein an esterified (inactive) calcium dye (probe) penetrates the cell membrane and becomes active once inside the cell. The active form of the dye becomes fluorescent after binding to intracellular calcium. Additive A is added to prevent the dye from being released out from the cell. Ligand binding stimulates GPCR activation, resulting in the release of intracellular calcium stores from the endoplasmic reticulum (ER), which leads to an increase in fluorescence in the presence of the activated calcium dye.

Published studies using a wide range of chemotypes were previously characterized using calcium release and β-arrestin recruitment assays for MrgprX2. The studies adopted the same clone used in this application note and have been useful in ascribing the role of receptor activation to its purported effect of non-immune mediated mast cell activation (Grimes *et al.* 2019 and Lafleur *et al.* 2020). Results herein are in good agreement with those previously published with respect to rank orders of potency agonists (Grimes *et al.* 2019) maintained relative to cortistatin-14 used as reference agonist for both assays (Figure 4.). Vancomycin activity is negligible with the concentration range used in these assays, but consistent with its expected low potency. These studies also highlight the potential for the β-arrestin assay to report higher levels of efficacy relative to cortistatin-14 that was not observed in the calcium mobilization assay. Our recent data set is limited, but these apparent differences in efficacy may warrant further consideration.

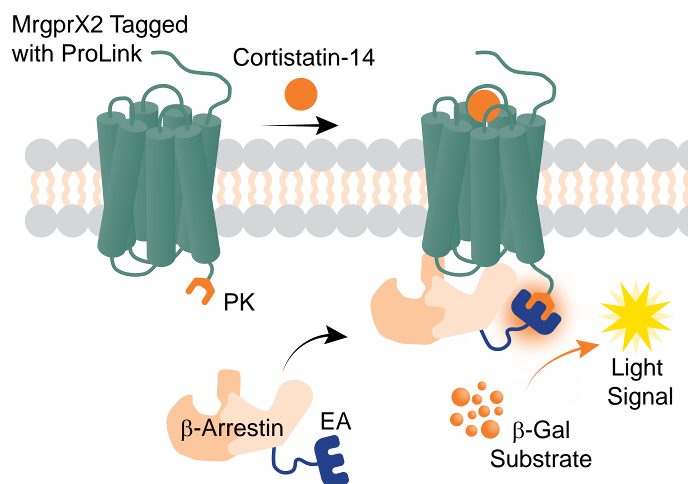
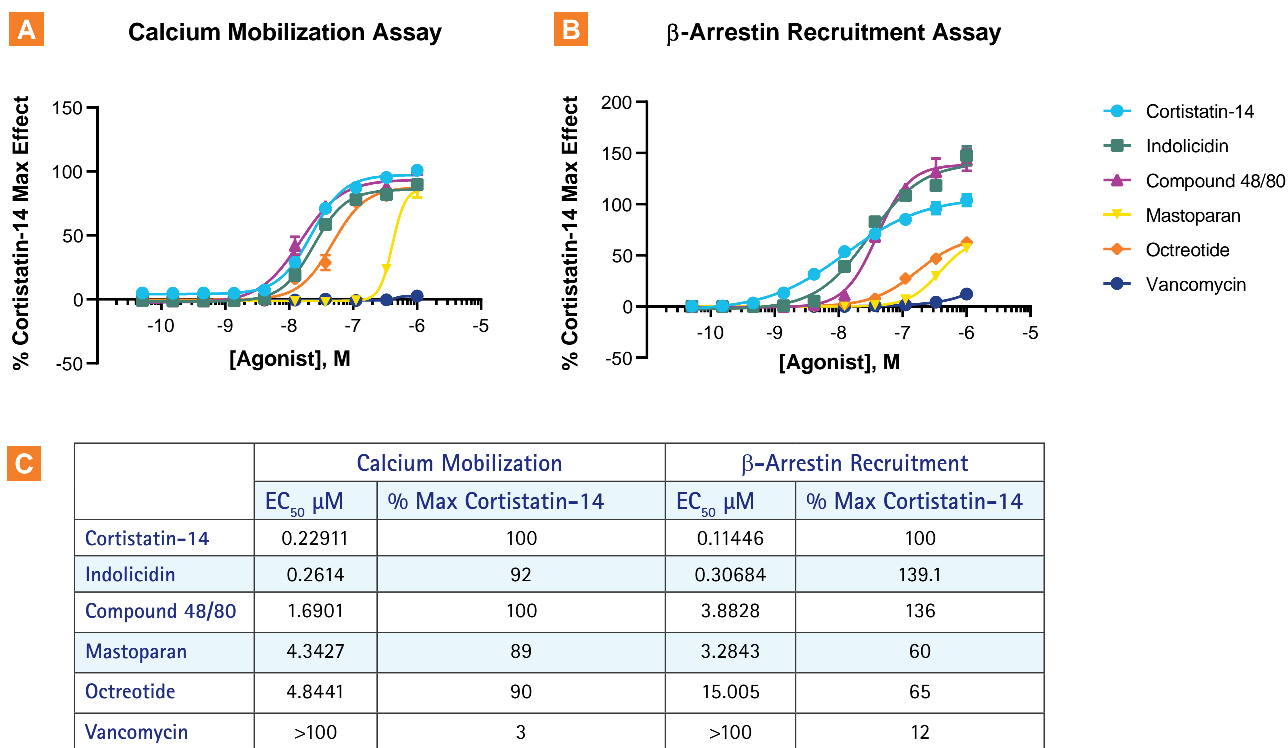


Figure 3. Eurofins DiscoverX®'s EFC-based β-arrestin recruitment assay principle. The PathHunter® MrgprX2 β-arrestin recruitment assay principle is based on the EFC platform. Binding of cortistatin-14 results in MrgprX2 activation thereby stimulating recruitment of β-arrestin-EA fusion protein to MrgprX2-PK. The resulting complementation of the two enzyme fragments results in an active β-gal enzyme whose activity can be measured using chemiluminescent PathHunter detection reagents.



	Calcium Mobilization		β-Arrestin Recruitment	
	EC_{50} µM	% Max Cortistatin-14	EC_{50} µM	% Max Cortistatin-14
Cortistatin-14	0.22911	100	0.11446	100
Indolicidin	0.2614	92	0.30684	139.1
Compound 48/80	1.6901	100	3.8828	136
Mastoparan	4.3427	89	3.2843	60
Octreotide	4.8441	90	15.005	65
Vancomycin	>100	3	>100	12

Figure 4. Dose response and rank order potency for calcium mobilization and β-arrestin recruitment assays. Readouts showing select agonists effect on (A.) calcium mobilization of intracellular calcium and on (B.) β-arrestin recruitment upon binding to MrgprX2 receptors. Both dose response curves are the mean of two replicates and show normalized data calculated from the maximum effect obtained with cortistatin-14. C. Table showing potency and efficacy of agonist relative to cortistatin-14.

CONCLUSION

Several targets are given consideration to represent pharmacological panels in safety liability screens as it is crucial to identify any specific molecular interactions at profiling stages that may cause ADRs in humans. Hence, an improved understanding of the pharmacological relevance of targets, including establishment of in vitro testing methodologies that can show the impact of a particular target (and its mechanism-of-action) on the safety liability of the drug at a much earlier stage in the discovery process is needed.

In an effort to support the characterization and possible role of MrgprX2 in non-classical mast cell-mediated anaphylactoid reactions, Eurofins DiscoverX® collaborated with Andrew Brown (GSK) and helped identify compounds/peptides that activated MrgprX2 via calcium mobilization and β-arrestin recruitment assays (Grimes *et al.* 2019). Further, assessing MrgprX2 activation in an engineered cell model can provide value as a rapid, high-throughput, economical mechanism-based screening tool for early mast cell degranulation or hazard identification during preclinical safety evaluation of therapeutics.

The importance and role of MrgprX2 as an emerging activator of non-IgE mediated MCD underscores its value in being a top panelist in safety pharmacology. Besides, its potential as a drug target for conditions/diseases related to mast cell function and regulation, including anaphylaxis, attests to its position as central to safety liability testing. Overall, MrgprX2 antagonism promotes a fresh interest as an emerging therapeutic target for treating pseudo-allergic reactions, and Eurofins DiscoverX qualified assays are ideal for supporting research programs centered on MrgprX2 activation and mast cell regulation.

For more information, visit discoverx.com/gpcrs.

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Automating Cell-Based Bioassays on Tecan®'s Fluent Automation Workstation: Measuring β-Arrestin Recruitment of Cannabinoid Receptor 1

Bringing Higher Throughput, Improved Assay Consistency, and Reproducibility for Easy Implementation in Quality Testing Programs

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INTRODUCTION

Through early discovery, screening, characterization, and particularly for market release and commercialization of biologics, drug candidates require physiologically relevant, functional cell-based assays as characterization tools that attest to their therapeutic activity. This biological activity is a critical attribute for drug candidates, and can be accurately measured using cell-based potency assays. Moreover, analyzing potency is a key measurement for the final market release of a therapeutic product such as a biologic or a biosimilar.

Cell-based assays implemented in quality controlled (QC) lot-release testing programs of biologics are often indicators of any altered potency or the presence of impurities in the manufactured drug products intended for clinical use. Hence, such programs demand highly consistent and reproducible cell-based assays. There are specific requirements associated with implementing cell-based potency assays, including cell culture and handling expertise, skilled pipetting, and precise plate layout. These abilities directly contribute to an operator's ability to handle live cells and routinely run sensitive assays with a high degree of precision and accuracy. The operator is expected to execute the assay consistently and uniformly across experiments performed on different plates and across different days. QC testing typically includes multiple operators adding another variable in attaining consistent results. Thus, it can be challenging for biopharma, contract research organizations (CROs), and contract development and manufacturing organizations (CDMOs) to achieve desirable assay reproducibility, particularly with borderline assays with less reproducible results. For these reasons, bringing automation to cell-based assays can provide better assay consistency and increased throughput that can result in decreased operational costs. Introducing assay automation can also bring consistent method execution when assays are transferred between different testing sites with automation capabilities.

This Application Note presents collaborative work of Eurofins DiscoverX, the leader in cell-based assays for drug discovery and development, and Tecan, a leader in robotic lab automation. It compiles and discusses the results obtained during the automation of a Eurofins DiscoverX cell-based bioassay on Tecan's Fluent® Automation Workstation. In summary, the automation produced highly consistent and reproducible results with minor optimization. In addition, the results obtained from this automation were comparable to those obtained by an experienced QC scientist.

PathHunter β-ARRESTIN ASSAY

β-Arrestin recruitment is considered a hallmark mechanism for GPCR activation upon ligand binding. The recruitment of β-arrestin activates signaling cascades to provide a non-amplified signal ideal for antagonist mode screening, studying ligand pharmacologies, and de-orphanizing GPCRs. Using PathHunter® β-arrestin cell-based assays, researchers can run cAMP or calcium second messenger signaling assays in parallel using the same β-arrestin cell line. The benefit is this allows for easily distinguishing ligand pharmacological differences for multiple ligand mechanism-of-actions.

PathHunter β-arrestin assays utilize the well-established Enzyme Fragment Complementation (EFC) technology (Figure 1. A.) pioneered and patented by Eurofins DiscoverX.

This robust technology is based on the complementation of two inactive β-galactosidase (β-gal) enzyme fragments – a large enzyme acceptor (EA) fragment and a small enzyme donor (ED) fragment. Separately, these fragments are inactive, but they form an active enzyme that hydrolyzes a substrate to produce a chemiluminescence signal when combined. For this study, we selected the PathHunter® CHO-K1 CNR1 Bioassay that measures β-arrestin recruitment (Figure 1. B.) to demonstrate the automation of the Eurofins DiscoverX® bioassays on the Tecan Fluent® instrument. CNR1, a type 1 cannabinoid receptor (CB1), encoded by the CNR1 gene, is expressed in the peripheral and central nervous systems. Endocannabinoids and phytocannabinoids such as THC, an active ingredient of cannabis, are known to activate CB1. In this bioassay, CNR1 is fused in frame with an ED fragment called ProLink™ (PK). The CNR1-PK fusion protein is then stably co-expressed in cells stably expressing a fusion protein of β-arrestin and EA. Binding of a ligand (CP 55,940, a synthetic cannabinoid) to CNR1 results in its activation stimulates recruitment of β-arrestin-EA fusion protein to CNR1-PK. This recruitment forces the complementation of the two enzyme fragments that results in the formation of an active β-galactosidase enzyme. This interaction leads to an increase in enzyme activity that can be measured using chemiluminescent PathHunter detection reagents.

For this study, the bioassay format of the assay was performed. Eurofins DiscoverX bioassays are derived from qualified stable cell lines and provide a thaw-and-use approach that alleviates any assay variability associated with cells derived from continuous culture (passage-to-passage variability). Bioassays are further optimized with a ready-to-use protocol where cells are thawed and plated directly into assay plates, thus not requiring cell culture. These bioassays are ideal for comparability studies, QC lot release testing, and are critical for accelerating the drug release into the market.

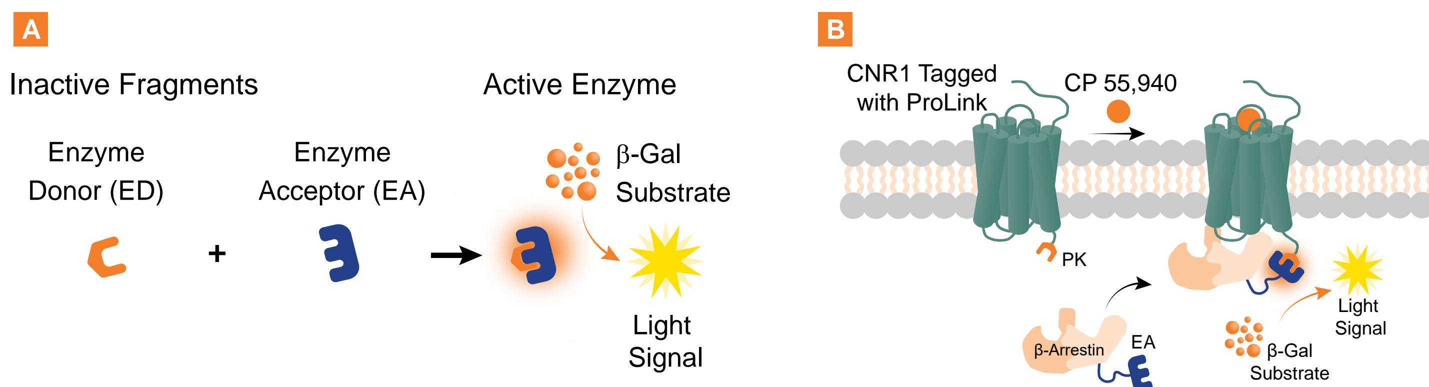


Figure 1. Eurofins DiscoverX's EFC assays. A. The EFC assay principle is based on two recombinant β-galactosidase (β-gal) enzyme fragments - an enzyme acceptor (EA) and an enzyme donor (ED). Separately, the fragments are inactive, but when combined, they form an active enzyme that hydrolyzes its substrate to produce a chemiluminescence signal. B. The PathHunter CNR1 β-arrestin recruitment assay principle: Binding of CP 55,940 results in CNR1 activation thereby stimulating recruitment of β-arrestin-EA fusion protein to CNR1-PK (noting PK corresponds to the ED of β-gal). The resulting complementation of the two enzyme fragments results in an active β-gal enzyme whose activity can be measured using chemiluminescent PathHunter detection reagents.

MATERIALS

Assay Components

PathHunter® CHO-K1 CNR1 Bioassay Kit (Cat. No. 93-0959Y2-00120), containing ready-to-use PathHunter CHO-K1 CNR1bioassay cells, 96-well assay plate, AssayComplete™ Cell Plating 2 Reagent (CP2), protein dilution buffer, reference agonist, and detection reagent, was used from Eurofins DiscoverX®.

INSTRUMENTATION

Automation Workstation

Experiments were conducted on a Tecan Fluent® 780 system that includes an eight-channel Air Flexible Channel Arm™ (Air FCA), a Multiple Channel Arm™ (MCA) with an extended volume adapter for pipetting up to 500 µl with 96 tips in parallel, and a long Robotic Gripper Arm™ (RGA) to reach below the Dynamic Deck™ (Figure 2. A.). A vertical laminar flow HEPA hood with UV light (Bigneat) was integrated with the Fluent system to ensure a clean environment. Liquid handling tasks were conducted with the Air FCA using 1000 µL and 200 µL filtered disposable tips, and with the MCA used150 µL filtered disposable tips. Sterile 15 mL Falcon™ tubes and 2 mL Eppendorf tubes were placed into tube runners for pipetting into sterile 96-well white, clear flat-bottom, TC-treated microplates with lid, and 96-well green V-bottom microplates (Eurofins DiscoverX, Cat. No. 92-0011). A Cytomat™ 2 C450-LiN Automated Incubator with two stackers (for 21 and 10 microplates, respectively) was integrated with the Fluent system; enabling the storage and incubation of CHO-K1 cells in microplates during the 48-hour incubation period with the agonist CP 55,940 (Figure 2. B.). The automated incubator parameters were set at 37°C and 5% CO_2 to provide an ideal and stable environment for the cryopreserved ready-to-use cells.

Individual scripts were developed using the FluentControl™ software for cell seeding on Day 1 and sample preparation of the agonist's serial dilutions with the subsequent addition of the detection reagents on Day 3. The chemiluminescence signal was measured with Tecan's Spark® multimode reader.

A

B

Figure 2. Tecan's Fluent Automation system. A. The Fluent Automation Workstation used to automate the PathHunter CHO-K1 CNR1 bioassay, and B. the Cytomat 2 C450-LiN Automated Incubator that was integrated with the Fluent system.

METHODS

Automation and manual experiments were performed independently. The protocol for experiments run on the Fluent® automation system is outlined in the protocol schematic (Figure 3.). For manually performed experiments, the protocol in the bioassay user manual was followed.

Cell Culture

The PathHunter® CHO-K1 CNR1 Bioassay cells were manually thawed and plated in AssayComplete™ Cell Plating 2 Reagent (CP2) at 5,000 cells-per-well in a 96-well plate and incubated overnight at 37°C and 5% CO_2.

Agonist

A 10 mM stock of CP 55,940 was prepared in DMSO as detailed in the user manual. An intermediate stock of 100 µM CP 55,940 was prepared in DMSO. Using this intermediate stock, an 11-point 1:3 dilution series of CP 55,940 was prepared with a top dose of 5 µM at 5X using CP2 as the diluent. 20 µL of the above serial dilutions were subsequently added to cells plated in 80 µL CP2 to obtain a final dose of 1 µM of CP 55,940 at 1X.

PathHunter β-Arrestin Assay

PathHunter CHO-K1 CNR1 bioassay cells were treated with increasing concentrations of CP 55,940 for 120 minutes at 37°C and 5% CO_2. Activation of a single GPCR stimulates the binding of arrestin, forcing complementation of the two enzyme fragments (ED and EA) fused with the GPCR or β-arrestin, respectively. The resultant increase in β-gal enzyme activity is measured by the addition of the chemiluminescent PathHunter Bioassay Detection Kit (Cat. No. 93-0933). Increased complementation of the two β-gal fusion subunits is directly proportional to higher chemiluminescent signal.

PROTOCOL SCHEMATIC

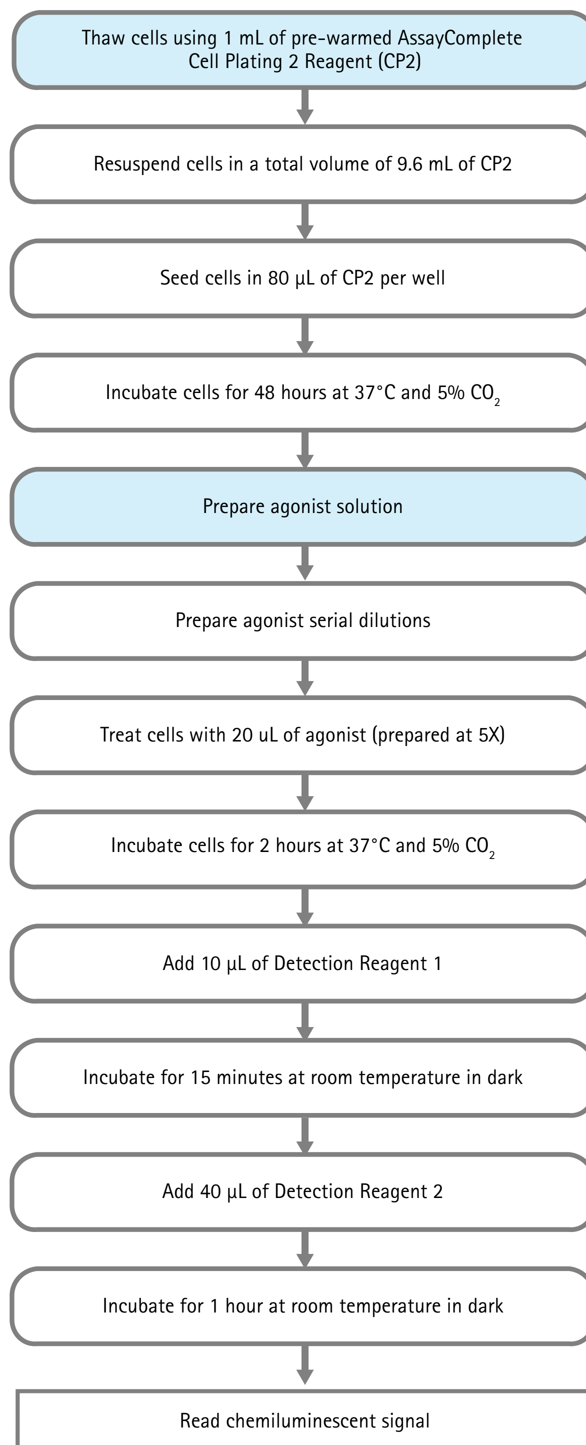


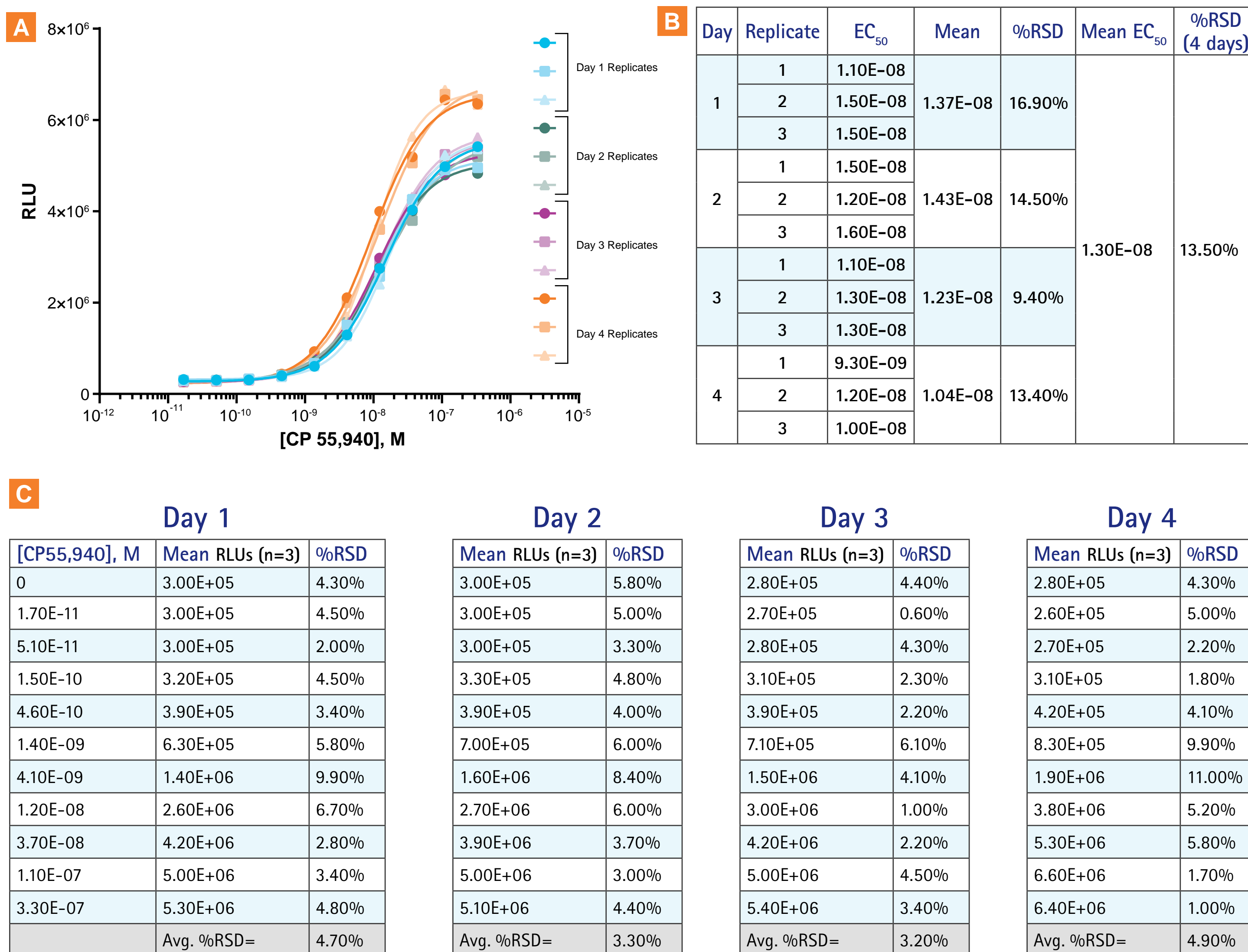
Figure 3. Protocol schematic for PathHunter CHO-K1 CNR1 Bioassay performed on Tecan's Fluent 780 System – steps highlighted in blue required minimal human intervention.

RESULTS

Automated

Inter-day results show comparable EC_{50} values across four independent experiments performed on four individual days with an average relative standard deviation (%RSD) of 13.5% (Figure 4.). Each experiment was performed in triplicate, and each replicate is plotted as a separate curve as shown below in Figure 4. A. All three curves show a high degree of overlap for every experiment, thereby demonstrating high intra-day intermediate precision.

In order to evaluate inter-day replicate consistency, the raw luminescence data from all four experiments were analyzed. As shown in Figure 4. C., the replicates for each data-point are visibly tight, with an average %RSD of less than 5%. These results demonstrate a high inter-day replicate consistency.



Day	Replicate	EC_{50}	Mean	%RSD	Mean EC_{50}	%RSD (4 days)
1	1	1.10E-08	1.37E-08	16.90%	1.30E-08	13.50%
	2	1.50E-08				
	3	1.50E-08				
2	1	1.50E-08	1.43E-08	14.50%		
	2	1.20E-08				
	3	1.60E-08				
3	1	1.10E-08	1.23E-08	9.40%		
	2	1.30E-08				
	3	1.30E-08				
4	1	9.30E-09	1.04E-08	13.40%		
	2	1.20E-08				
	3	1.00E-08				

Day 1

[CP55,940], M	Mean RLUs (n=3)	%RSD
0	3.00E+05	4.30%
1.70E-11	3.00E+05	4.50%
5.10E-11	3.00E+05	2.00%
1.50E-10	3.20E+05	4.50%
4.60E-10	3.90E+05	3.40%
1.40E-09	6.30E+05	5.80%
4.10E-09	1.40E+06	9.90%
1.20E-08	2.60E+06	6.70%
3.70E-08	4.20E+06	2.80%
1.10E-07	5.00E+06	3.40%
3.30E-07	5.30E+06	4.80%
	Avg. %RSD=	4.70%

Day 2

Mean RLUs (n=3)	%RSD
3.00E+05	5.80%
3.00E+05	5.00%
3.00E+05	3.30%
3.30E+05	4.80%
3.90E+05	4.00%
7.00E+05	6.00%
1.60E+06	8.40%
2.70E+06	6.00%
3.90E+06	3.70%
5.00E+06	3.00%
5.10E+06	4.40%
Avg. %RSD=	3.30%

Day 3

Mean RLUs (n=3)	%RSD
2.80E+05	4.40%
2.70E+05	0.60%
2.80E+05	4.30%
3.10E+05	2.30%
3.90E+05	2.20%
7.10E+05	6.10%
1.50E+06	4.10%
3.00E+06	1.00%
4.20E+06	2.20%
5.00E+06	4.50%
5.40E+06	3.40%
Avg. %RSD=	3.20%

Day 4

Mean RLUs (n=3)	%RSD
2.80E+05	4.30%
2.60E+05	5.00%
2.70E+05	2.20%
3.10E+05	1.80%
4.20E+05	4.10%
8.30E+05	9.90%
1.90E+06	11.00%
3.80E+06	5.20%
5.30E+06	5.80%
6.60E+06	1.70%
6.40E+06	1.00%
Avg. %RSD=	4.90%

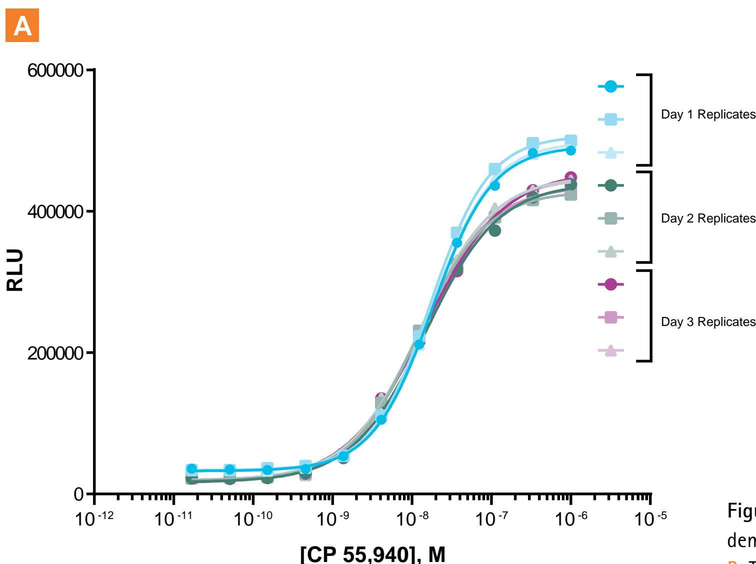
Figure 4. Results from the Tecan Fluent® 780 Automation Workstation. A. Dose response curves for CP 55,940 demonstrating β-arrestin recruitment in a dose-dependent manner for four days. B. Table summarizing EC_{50} values and %RSD for each day; and mean EC_{50} and %RSD across four days. C. Table illustrating %RSD of raw RLUs for each day. RLU = Relative Luminescence Units

Return to TOC

MANUAL

To compare assay consistency and reproducibility, the same set of experiments were performed manually by a QC scientist with more than six years of experience running similar assays. These experiments were run across three separate days in triplicate. The team plotted data of each replicate per experiment as an individual curve (Figure 5.). Data analysis of the assays performed revealed that the results obtained were very close to the automation results with the %RSD for each day being below 20%. Overall, the inter-day intermediate precision for the manually performed data was 17.5% that is slightly lower than that obtained from the Fluent® system (13.5%).

A



B

<table>
<tr><th>Day</th><th>Replicate</th><th>EC_{50}</th><th>Mean EC_{50}</th><th>%RSD</th><th>Mean EC_{50} (3 days)</th><th>%RSD (3 days)</th></tr>
<tr><td rowspan="3">1</td><td>1</td><td>1.80E-08</td><td rowspan="3">1.77E-08</td><td rowspan="3">3.30%</td><td rowspan="9">1.47E-08</td><td rowspan="9">17.50%</td></tr>
<tr><td>2</td><td>1.70E-08</td></tr>
<tr><td>3</td><td>1.80E-08</td></tr>
<tr><td rowspan="3">2</td><td>1</td><td>1.50E-08</td><td rowspan="3">1.35E-08</td><td rowspan="3">15.70%</td></tr>
<tr><td>2</td><td>1.20E-08</td></tr>
<tr><td>3</td><td>1.30E-08</td></tr>
<tr><td rowspan="3">3</td><td>1</td><td>1.40E-08</td><td rowspan="3">1.30E-08</td><td rowspan="3">7.70%</td></tr>
<tr><td>2</td><td>1.20E-08</td></tr>
<tr><td>3</td><td>1.30E-08</td></tr>
</table>

Figure 5. Results from manual experiments. A. Dose response curves for CP 55,940 demonstrating β-arrestin recruitment in a dose-dependent manner for three days. B. Table summarizing EC_{50} values and %RSD for each day; and mean EC_{50} and %RSD across three days.

The assay design of the CNR1 bioassay used in this study was automation-friendly and performed optimally out-of-the-box with minimal optimization needed for running on Tecan's Fluent system. Overall, analyzing the data obtained from automation and manually performed experiments show comparable results. Thus, assay automation can help ease congested QC testing pipelines that often result in delayed market release of critical drugs by sharing the workload without compromising the quality of results.

CONCLUSION

The work described in this Application Note demonstrates that automation of Eurofins DiscoverX®'s cell-based assays can consistently produce reproducible results, which is key for cell-based potency assays implemented in QC lot-release programs. The results from the assays run using automation were remarkably similar compared to the manually executed assays run by an experienced QC scientist with several years of experience. Automating cell-based potency assays in quality testing programs can play a significant role in streamlining assay workflows, maintaining assay consistency, and ultimately saving considerable time and cost by dramatically reducing the handling of repetitive tasks and increasing assay throughput. This automation is particularly helpful when operators are running several assay plates with complex plate layouts. Overall, automating assay is key in circumventing challenges and minimizing day-to-day and operator-to-operator differences producing high-quality data.

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For more information on bioassays, visit discoverX.com/bioassays.

WHITE PAPER

The New World of GPCR Allosteric Modulation: Another Shot on Goal

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Although historically GPCRs have been a rich source of new drug molecules, the discovery of unique drug types for this target class has waned in the last 3 decades. In the same time frame, the emergence of functional screening and the appreciation of the allosteric nature of GPCRs has revitalized the field and led to an explosion of activity that has transformed GPCR discovery.

Thus allosteric screening and development are seen as another shot on goal for many mined out and/or intractable GPCR targets. It is useful to consider the particular mechanistic interactions of allosteric ligands with receptors, the unique molecules that emerge from these interactions, and finally the key role of pharmacological assays in their discovery and development.

THE EMERGENCE OF RECEPTOR ALLOSTERY IN PHARMACOLOGY

The emergence of allostery in pharmacology supports the notion that whether a discovery program seeks an allosteric ligand or not, it is increasingly likely that allosteric hits and leads will emerge from functional screening campaigns. Thus it is incumbent upon pharmacologists to at least be aware of how to study these unique molecules and develop their full potential in discovery programs.

The study of allosterism was pioneered with enzymes, but the pursuit of this mechanism for receptors has lagged behind. One important reason for this is the historical reliance of high-throughput screening on basically orthosteric assays (binding). In all probability, many allosteric ligands were not detected leading to a paucity of these molecules for development. The advent of functional assay screening, where allosteric effects are readily seen, has led to a precipitous increase in the publication of papers on receptor allosterism beginning in 1992 and continuing through to the present day. With this trend has come a resurgence of interest in allosteric receptor ligands (and its offshoot, biased receptor signaling) and an increase in the number of molecules available to study allosteric receptor mechanisms.

ALLOSTERIC MECHANISMS AND NEW DRUG DISCOVERY

GPCRs are nature's prototypical allosteric protein (everything the receptor does is allosteric) since these proteins simply act as energy conduits for extracellular molecules and intracellular signaling proteins. The molecular dynamics of the receptor suggests that they can form a multitude of active and inactive conformations through selective stabilization. These concepts also support the notion that efficacy and affinity are thermodynamically linked since binding is not a passive process. Therefore, the pursuit of possible signaling effects (pluridimensional efficacy[1]) for all molecules detected in a screening assay could lead to unique therapeutic profiles. In this regard, the assay assumes a key role in GPCR drug discovery.

WHAT MAKES ALLOSTERIC MOLECULES SO SPECIAL?

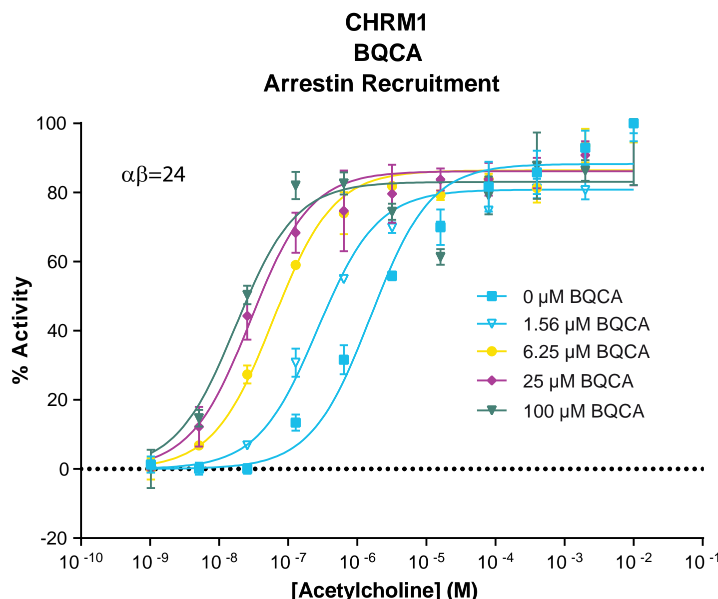
There are at least three unique features of allosteric ligand-receptor interactions that lead to potentially valuable therapeutic behaviors[2].

- Allosterism can change the very nature of receptors (efficacy, functional signaling)
- The fact that allosteric molecules bind to separate sites on the receptor allows them to modulate or potentiate receptor effects (re-set physiology)
- Allosteric effects are probe dependent, allowing precise discrimination between endogenous molecules and signaling proteins (effects can be molecule-specific)

ALLOSTERIC MODULATION CAN CREATE A NEW RECEPTOR

Allosteric modulators have the unique capability of allowing the natural endogenous agonist to concomitantly bind to the receptor and, therefore, they can potentiate physiological response. An active research area in the pharmacology of drug discovery is the search for Positive Allosteric Modulators (PAMs) to revitalize failing physiological systems in disease. PAMs possess unique advantages over orthosteric agonists in that the systems affected respond only when activated. For example, a current therapy for diabetes employs GLP-1 agonists that potentiate the release of insulin. However, a limitation of this approach is that the constant activation of the GLP-1 receptors with an agonist causes intractable nausea in patients. In contrast, a GLP-1 PAM potentiates GLP-1 enhancement of insulin release in diabetes only when the system is activated, such as when the patient has a meal but otherwise produces no effect. This intermittent activation will eliminate the current GLP-1 based nausea seen with agonist therapies for diabetes. The potentiation of muscarinic response by the PAM BQCA is illustrated with the Eurofins Discovery services, M1 receptor β-arrestin recruitment assay shown in Figure 1. Patterns of concentration-response curves such as these can be fit to the functional allosteric model[3-5] to yield universal parameters that characterize allosteric function in a system-independent manner.

Such analyses yield parameters for modification of endogenous agonist affinity (through a cooperativity term α) and efficacy (through a cooperativity term β) to guide medicinal chemistry structure-activity studies. For the data shown in Figure 1, the cooperativity factor for BQCA effect is 24 indicating that BQCA will produce a 24-fold potentiation of acetylcholine β-arrestin response in all systems.



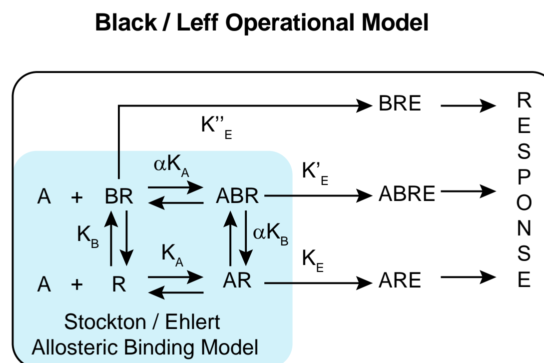


Figure 1. Concentration-response curves to acetylcholine for the receptor recruitment of β-arrestin in the absence and presence of a range of concentrations of the PAM BQCA. Concentration-dependent shifts of the CR curves to the left indicate allosteric potentiation of acetylcholine response. Fitting these curves to the functional allosteric model, schematically shown on the right, yields universal parameters quantifying the allosteric effect in terms of the co-operative effects on acetylcholine binding and efficacy. Data from Eurofins Discovery.

ALLOSTERIC MODULATORS RE-SET TARGET PHYSIOLOGY

Another feature of allosteric molecules is that they can produce a redefinition of target responsiveness including complete inhibition, reduced sensitivity, increased sensitivity, and full activation. Figure 2 shows the effect of the negative allosteric modulator (NAM) UCB35625 on the binding of the chemokine CCL3. It can be seen that the receptor can still bind CCL3, but with a 2-fold reduced affinity; such fine-tuning of responsiveness can be useful therapeutically.

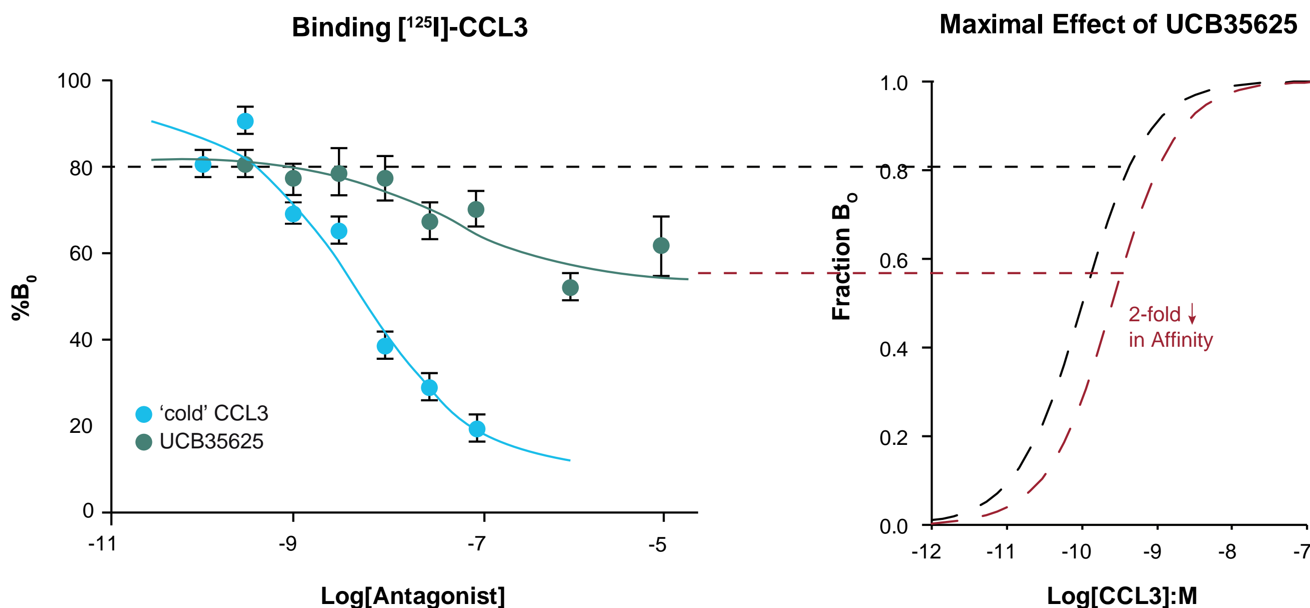


Figure 2. Displacement binding of [125I]-CCL3 by non-radioactive CCL3 (blue circles) and the allosteric NAM UCB35625 (green circles). It can be seen that the maximal concentrations of UCB35625 do not displace bound [125I]-CCL3, but rather re-set the receptor affinity for the radioligand by a factor of 2. Data are redrawn from [6].

ALLOSTERIC MODULATORS PRACTICE PROBE-DEPENDENCE

Finally, allosterism is probe dependent, that is, an allosteric molecule can produce an effect for one probe of the receptor (i.e. agonist, radioligand), but have no effect on another (a different agonist, radioligand, etc.). This can be extremely valuable therapeutically as shown in Figure 3 for the allosteric HIV-1 entry inhibitors TAK779 and TAK652 in the prevention of HIV-1 infection and AIDS. Specifically, these molecules demonstrate probe dependence in the form of a 10-fold differential activity for blocking HIV-1 vs. the blockade of a beneficial effect for AIDS patients (CCL3-L1-induced CCR5 receptor internalization found to be correlated with increased survival[7]). It can be seen that while TAK779 is 10-fold more potent at blocking CCR5 internalization over HIV-1 infection, TAK652 reverses this profile to yield a beneficial 10-fold margin for blockade of HIV-1 infection while sparing the beneficial CCL3L1-induced internalization effect[8]. In general, the judicious application of different target probes in assays can uncover such therapeutically relevant probe dependence.

In general, allosteric modulators can re-format receptor sensitivity (including the revitalization of failing systems) and alter responses to agonists. They can also make targets "smart" in that they will discern different activators and respond to some but not others. These effects re-define the drug discovery playing field.

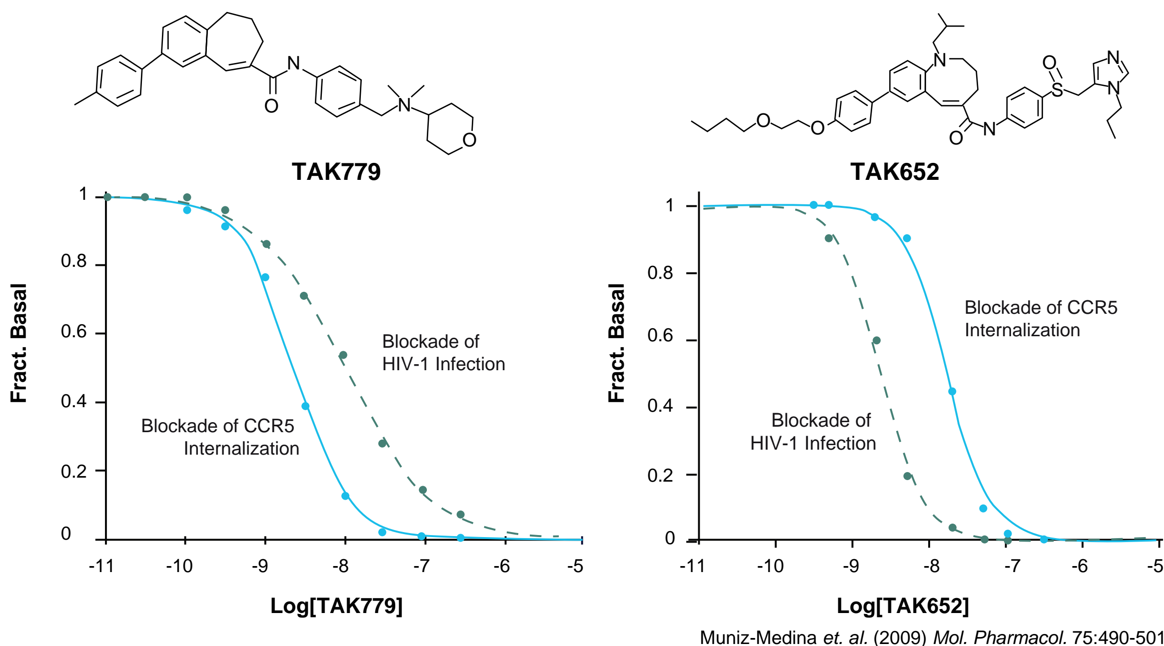


Figure 3. Inhibition of HIV-1 infection of HOS cells (green circles, dotted line curves) and blockade of CCL3L1-induced CCR5 internalization (blue circles, solid line curves). Data are shown for two allosteric inhibitors of HIV-1 entry, TAK779 and TAK652-redrawn from [8].

NEW PLAYERS AS DRUG TARGET MOLECULES IN PHARMACOLOGICAL THERAPY

Allosteric mechanisms have spawned new types of molecules for therapeutic application. These new players can generally be classified as PAMs that increase an endogenous agonist response and thus can be used to augment failing physiology in disease. NAMs (Negative Allosteric Modulators) decrease endogenous agonism (and are basically antagonists, but with some very special qualities that differentiate them from standard competitive blockers. In addition, a special type of PAM that augments receptor-signaling protein interaction (and thus is an agonist) practices probe dependence for cytosolic signaling proteins; these are a new and important class of ligand referred to as the biased agonist. Considering two probes of the receptor as being two points of interaction with different cytosolic signaling proteins (i.e. G protein, and β-arrestin), biased agonists differentially activate one probe at the expense of another to produce a biased cell signal. These types of effects can be of enormous value in that beneficial signals can be emphasized (i.e. β-arrestin PTH response for osteoporosis[9]), deleterious signals can be de-emphasized (respiratory depression and addiction for opioids[10]) and de-emphasized with blockade of natural activation of the same pathway (G_q protein-mediated vasoconstriction by angiotensin in heart failure[11]). In addition, the editing of pleiotropic signaling may allow pursuit of previously forbidden drug targets for therapeutic advantage (i.e. κ-opioid receptors[12]).

Finally, the application of functional and binding assays may be used to identify a unique new class of antagonist, namely the PAM-Antagonist. These are a special subset of NAMs that actually become more potent upon activation of the functional system by the agonist allowing them to seek and destroy signaling agonist-bound receptors[13]. PAM-Antagonists can reverse persistent pathological signaling (e.g. endothelin-based pre-eclampsia) and have extraordinarily high target coverage properties *in vivo* (and long $t_{1/2}$ for clearance) due to the cooperative binding with endogenous hormones and neurotransmitters as seen for the 5-HT_3 receptor antiemetic palonosetron[14]). Figure 4 shows Eurofins Discovery services data used to characterize Org27569, a PAM Antagonist for the cannabinoid CB1 receptor[5]. The key to detecting these unique profiles is the orthogonal application of functional and binding assays.

Specifically, this behavior emanates from a positive α cooperativity to increase affinity and a fractional β activity to decrease efficacy. Thus, the presence of the agonist increases the affinity of the receptor for Org27659 that then becomes incapable of signaling once Org27659 is bound.

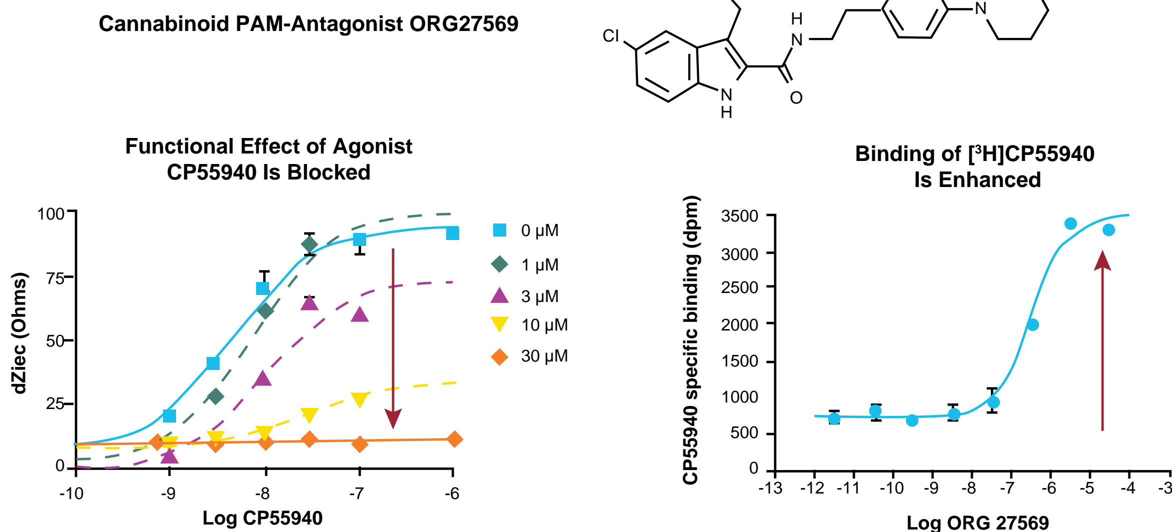


Figure 4. Opposing effects of the cannabinoid CB1 receptor PAM-Antagonist Org27659 on function (non-competitive blockade) and binding (increased agonist binding). Data from Eurofins Discovery.

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PHARMACOLOGICAL ASSAYS AS THE WINDOW INTO PLURIDIMENSIONAL EFFICACY

The key to the discovery of ligand diversity is to have multiple views into pharmacological molecular activity. Classification of molecules into simple bins of agonist and antagonist has ceased to be relevant as molecules can be agonists for some signaling pathways and antagonists in others[15]. A more sophisticated classification system to characterize efficacy must be employed. For example, the multiple testing of ligand functional response yields textured patterns of ligand activity such as the display of μ-opioid signaling activity determined by clustering of data for sixteen opioid agonists in six functional assays shown in Figure 5[16]. The hope is that such detailed profiling of new molecules will lead to more informed choices for compound progression.

In general, the key to unlocking complex and potentially useful efficacy and affinity fingerprints in new molecules is the pharmacological assay. Data from multiple assays allows comparison to quantitative pharmacological models to yield universal and system-independent scales of activity for use in medicinal chemistry efforts to optimize activity.

50% of new molecules in clinical testing fail due to lack of efficacy[17]. While some of this is due to lack of knowledge of what needs to be corrected in some diseases, some of this may also be due to the inadequate characterization of the efficacy of the candidate molecules put forward in the clinic. With more informed characterization of ligand efficacy fingerprints, perhaps better targeting for progression will result in a reduction in compound late-stage attrition.

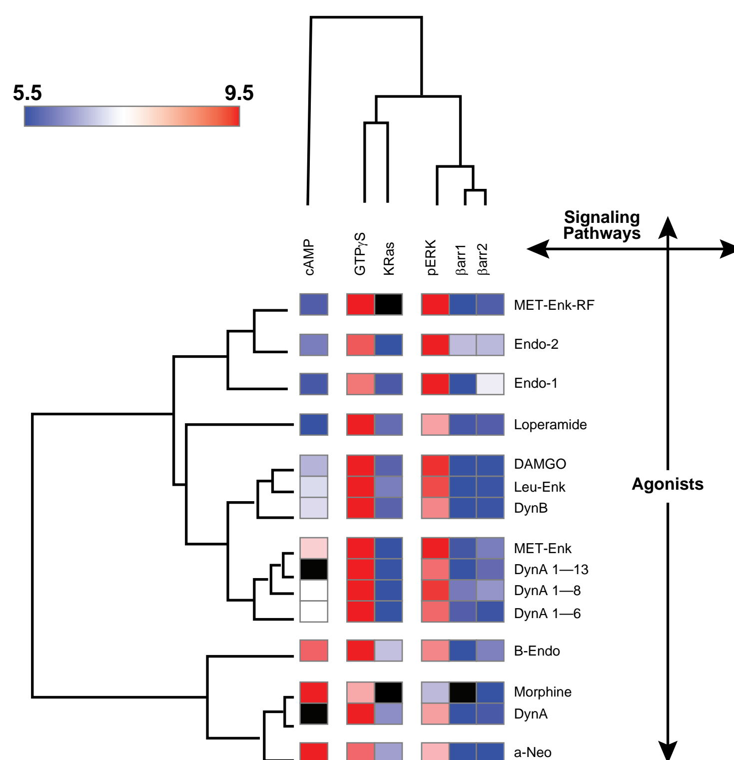

Figure 5. Cluster analysis of sixteen μ-opioid agonists in six different functional assays. The gene cluster program GENE-E groups the agonists according to their Log(τ/KA) values in each assay thereby grouping agonists according to their signaling profiles-redrawn from [16].

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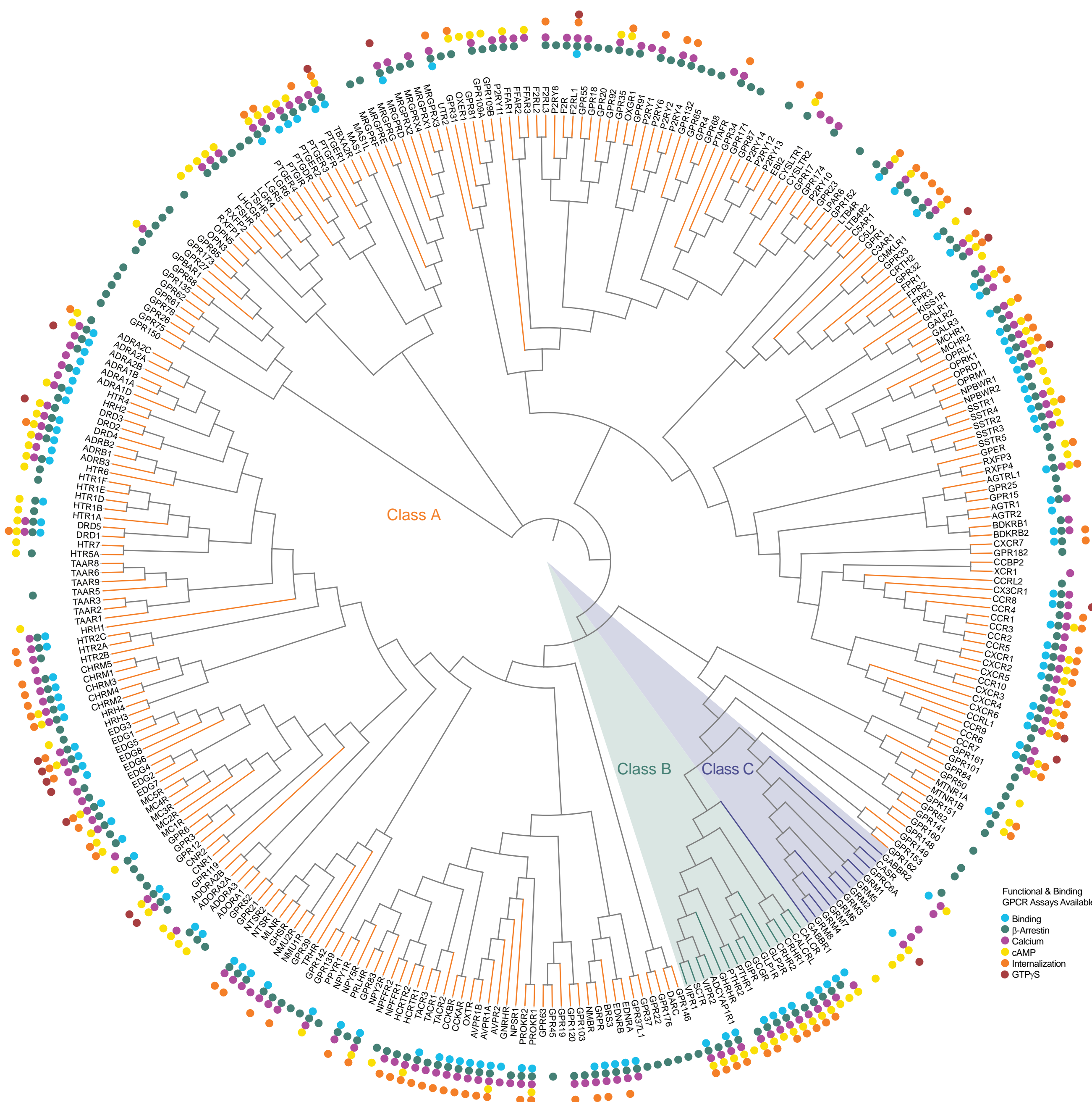
The Human GPCRome Dendrogram and Assays for GPCR Targets

Eurofins DiscoverX® offers GPCR cell-based assays covering ~90% of the human GPCRome to meet your specific GPCR research and drug discovery & development needs. Using the dendrogram below for class A, B & C GPCRs, determine the availability of binding, second messenger activation, β-arrestin recruitment, and receptor internalization assays for your favorite GPCR target. Additional assays (or products) for GPCR targets not represented in the dendrogram are available at discoverx.com/gpcrs and through custom development requests via email at: customdevelopment@eurofins.com.



GPCR Families, Targets, and Couplings

Class A GPCRs

Family	Target Gene ID (Common)	Coupling
5-Hydroxytryptamine (Serotonin)	HTR1A (5HT1A)	G_i/G_o
	HTR1B (5HT1B)	G_i/G_o
	HTR1E (5HT1E)	G_i/G_o
	HTR1F (5HT1F)	G_i/G_o
	HTR2A (5HT2A)	G_q
	HTR2B (5HT2B)	G_q
	HTR2C (5HT2C)	G_q
	HTR5A (5HT5A)	G_i/G_o
	HTR6 (5HT6)	G_s
	HTR7A (5HT7A)	G_s
	HTR7B (5HT7B)	G_s
	HTR7D (5HT7D)	G_s
Acetylcholine (Muscarinic)	CHRM1 (M1)	G_q
	CHRM2 (M2)	G_i/G_o
	CHRM3 (M3)	G_q
	CHRM4 (M4)	G_s & G_i/G_o
	CHRM5 (M5)	G_q
Adenosine	ADORA1 (A1)	G_i/G_o
	ADORA2A (A2A)	G_s & G_q
	ADORA2B (A2B)	G_s
	ADORA3 (A3)	G_i/G_o
Adrenoceptor	ADRA1A (α1A)	G_q
	ADRA1B (α1B)	G_q
	ADRA1D (α1D)	G_q
	ADRA2A (α2A)	G_i/G_o
	ADRA2B (α2B)	G_i/G_o
	ADRA2C (α2C)	G_i/G_o
	ADRB1 (β1AR)	G_s
	ADRB2 (β2AR)	G_s
	ADRB3 (β3AR)	G_s
Angiotensin	AGTR1 (AT1)	G_q
Apelin	AGTRL1 (APJ)	G_i/G_o
Bile Acid (GPBA)	GPBAR1 (GPR131)	G_s
Bombesin	BRS3 (BB3)	G_q
	GRPR (BB2)	G_q
	NMBR (BB1)	G_q
Bradykinin	BDKRB1 (B1)	G_q
	BDKRB2 (B2)	G_q
Cannabinoid	CNR1 (CB1)	G_i/G_o
	CNR2 (CB2)	G_i/G_o
Chemerin	CMKLR1 (ChemR23)	G_i/G_o

Class A GPCRs

Family	Target Gene ID (Common)	Coupling
Chemokine	CCR1	G_i/G_o
	CCR2	G_i/G_o
	CCR3	G_i/G_o
	CCR4	G_i/G_o
	CCR5	G_i/G_o
	CCR6	G_i/G_o
	CCR7	G_i/G_o
	CCR8	G_i/G_o
	CCR9	G_i/G_o
	CCR10	G_i/G_o
	CCRL1	
	CX3CR1	G_i/G_o
	CXCR1 (IL8RA)	G_i/G_o
	CXCR2 (IL8RB)	G_i/G_o
	CXCR3	G_i/G_o
	CXCR4	G_i/G_o
	CXCR5	G_i/G_o
	CXCR6	G_i/G_o
	CXCR7 (CMKOR1)	
	XCR1 (XC1)	G_i/G_o
Cholecystokinin	CCKAR (CCK1)	G_q
	CCKBR (CCK2)	G_q
Complement Peptide (Anaphylatoxin)	C3AR1 (C3A)	G_i/G_o
	C5AR1 (C5A)	G_i/G_o
	C5AR2 (C5L2/GPR77)	
Dopamine	DRD1 (D1)	G_s
	DRD2 (D2)	G_i/G_o
	DRD3 (D3)	G_i/G_o
	DRD4 (D4)	G_i/G_o
	DRD5 (D5)	G_s
Endothelin	EDNRA (ETA)	G_q
	EDNRB (ETB)	G_s & G_i/G_o
Formylpeptide	FPR1 (FPR)	G_i/G_o
	FPR2 (FPRL1/ALX)	G_i/G_o
	FPR3 (FPRL2)	G_i/G_o
Free Fatty Acid	FFAR1 (FFA1/GPR40)	G_q
	FFAR2 (FFA2/GPR43)	G_q & G_i/G_o
	FFAR3 (FFA3/GPR41)	G_i/G_o
	FFAR4 (FFA4/GPR120)	
Galanin	GALR1 (GAL1)	G_i/G_o
	GALR2 (GAL2)	G_q & G_s

Class A GPCRs

Family	Target Gene ID (Common)	Coupling
Ghrelin	GHSR (GRLN)	G_q
Glycoprotein Hormone	FSHR (FSH)	G_s
	LHCGR (LH)	G_s
	TSHR (TSH)	G_s & G_q
Gonadotrophin-Releasing Hormone	GNRHR (GnRH)	G_q
Histamine	HRH1 (H1)	G_q
	HRH2 (H2)	G_q & G_s
	HRH3 (H3)	G_i/G_o
	HRH4 (H4)	G_i/G_o
Hydroxycarboxylic Acid	HCAR1 (HCA1/GPR81)	G_i/G_o
	HCAR2 (HCA2/GPR109A/HM74A/NIC1)	G_i/G_o
	HCAR3 (HCA3/GPR109B/NIC2)	G_i/G_o
Kisspeptin	KISS1R (GPR54)	G_q
Leukotriene	CYSLTR2 (LTC4)	G_q
	LTB4R (BLT1)	G_i/G_o & G_q
	OXER1 (GPR170)	G_i/G_o
Lysophospholipid (LPA & S1P)	LPAR1 (LPA1/EDG2)	G_i/G_o
	LPAR2 (LPA2/EDG4)	G_q
	LPAR3 (LPA3/EDG7)	G_q
	LPAR5 (LPA5/GPR92)	G_q
	S1PR1 (S1P1/EDG1)	G_i/G_o
	S1PR2 (S1P2/EDG5)	G_s
	S1PR3 (S1P3/EDG3)	G_q
	S1PR4 (S1P4/EDG6)	G_q
	S1PR5 (S1P5/EDG8)	G_i/G_o
Melanin-Concentrating Hormone	MCHR1 (MCH1)	G_i/G_o
	MCHR2 (MCH2)	G_q
Melanocortin	MC1R (MC1)	G_s
	MC3R (MC3)	G_s
	MC4R (MC4)	G_s
	MC5R (MC5)	G_s
Melatonin	MTNR1A (MT1)	G_i/G_o
	MTNR1B (MT2)	G_i/G_o
Motilin	MLNR (Motilin)	G_q
Neuromedin U	NMU1R (NMU1)	G_q
Neuropeptide B & W	NPBWR1 (GPR7)	G_i/G_o
	NPBWR2 (GPR8)	G_i/G_o
Neuropeptide FF	NPFFR1 (GPR147)	G_i/G_o
Neuropeptide S	NPSR1 (NPS)	G_q

Class A GPCRs

Family	Target Gene ID (Common)	Coupling
Neuropeptide Y	NPY1R (Y1)	G_i/G_o
	NPY2R (Y2)	G_i/G_o
	NPY4R (Y4/PPYR1)	G_i/G_o
Neurotensin	NTSR1 (NTS1)	G_q
Opioid	OPRD1 (δ)	G_i/G_o
	OPRK1 (κ)	G_i/G_o
	OPRL1 (NOP)	G_i/G_o
	OPRM1 (μ)	G_i/G_o
Orexin	HCRTR1 (OX1)	G_q
	HCRTR2 (OX2)	G_q
Oxoglutarate	OXGR1 (GPR99/GPR80)	G_q
P2Y	P2RY1 (P2Y1)	G_q
	P2RY2 (P2Y2)	G_q
	P2RY4 (P2Y4)	G_q
	P2RY6 (P2Y6)	G_q
	P2RY11 (P2Y11)	G_s & G_q
	P2RY12 (P2Y12)	G_i/G_o
Peptide P518	QRFPR (QRFP/GPR103)	G_q
Platelet-Activating Factor	PTAFR (PAF)	G_i/G_o & G_q
Prokineticin	PROKR1 (PKR1/GPR73)	G_s & G_q
	PROKR2 (PKR2)	G_s
Prolactin-Releasing Peptide	PRLHR (PRRP)	G_q
Prostanoid	PTGDR (DP1)	G_s
	PTGDR2 (DP2/CRTH2)	G_i/G_o
	PTGER1 (EP1)	G_q
	PTGER2 (EP2)	G_s
	PTGER3 (EP3)	G_i/G_o & G_q
	PTGER4 (EP4)	G_s
	PTGFR (FP)	G_q
	PTGIR (IP)	G_s
	TBXA2R (TP)	G_q
Proteinase-Activated	F2R (PAR1)	G_i/G_o
	F2RL1 (PAR2)	G_i/G_o & G_q
	F2RL3 (PAR4)	G_i/G_o & G_q
Relaxin Family Peptide	RXFP1 (LGR7)	G_s
	RXFP2 (LGR8)	G_s
	RXFP3 (SALPR)	G_i/G_o
	RXFP4	G_i/G_o

RESOURCES

GPCR Families, Targets, and Couplings (Continued)

Class A GPCRs

Family	Target Gene ID (Common)	Coupling
Somatostatin	SSTR1 (SST1)	G_i/G_o
	SSTR2 (SST2)	G_i/G_o
	SSTR3 (SST3)	G_i/G_o
	SSTR4 (SST4)	G_i/G_o
	SSTR5 (SST5)	G_i/G_o
Succinate	SUCNR1 (GPR91)	
Tachykinin	TACR1 (NK1)	G_q
	TACR2 (NK2)	G_q
	TACR3 (NK3)	G_s & G_q
Thyrotropin-Releasing Hormone	TRHR (TRH1)	G_q
Trace Amine	TAAR1 (TA1)	G_s
Urotensin	UTS2R (UTR2)	G_q
Vasopressin & Oxytocin	AVPR1A (V1A)	G_q
	AVPR1B (V1B)	G_q
	AVPR2 (V2)	G_s
	OXTR (OT)	G_q

Class B GPCRs

Family	Target Gene ID (Common)	Coupling
Calcitonin	CALCR (CT)	G_s
	CALCR-RAMP1 (AMY1)	G_s
	CALCR-RAMP2 (AMY2)	G_s
	CALCR-RAMP3 (AMY3)	G_s
	CALCRL-RAMP1 (CGRP1)	G_s
	CALCRL-RAMP2 (AM1)	G_s
	CALCRL-RAMP3 (AM2)	G_s
Corticotropin-Releasing Factor	CRHR1 (CRF1)	G_s
	CRHR2 (CRF2)	G_s
Glucagon	GCGR (Glucagon)	G_s
	GHRHR (GHRH)	G_s
	GIPR (GIP)	G_s
	GLP1R (GLP-1)	G_s
	GLP2R (GLP-2)	G_s
	SCTR (Secretin)	G_s
Parathyroid Hormone	PTH1R (PTH1)	G_s & G_q
	PTH2R (PTH2)	G_s
VIP & PACAP	ADCYAP1R1 (PAC1)	G_s & G_q
	VIPR1 (VPAC1)	G_s & G_q
	VIPR2 (VPAC2)	G_s

Class C GPCRs

Family	Target Gene ID (Common)	Coupling
Calcium-Sensing	CASR (CaS)	G_q & G_i/G_o
$GABA_B$	GABBR1-GABBR2	G_i/G_o
Metabotropic Glutamate	GRM1 (mGlu1)	G_q
	GRM2 (mGlu2)	G_i/G_o
	GRM3 (mGlu3)	G_i/G_o
	GRM4 (mGlu4)	G_i/G_o
	GRM5 (mGlu5)	G_q
	GRM6 (mGlu6)	G_i/G_o
	GRM7 (mGlu7)	G_i/G_o

Liganded Orphans

Family	Target Gene ID (Common)	Coupling
Liganded Orphans	GPR1	
	GPR17	
	GPR35	G_i/G_o
	GPR39	G_q
	GPR55	
	GPR84	G_i/G_o
	GPR119	
	GPR139 (PGR3)	
	GPR183 (EBI2)	G_i/G_o
	MRGPRD	
	MRGPRX1 (MRGX1)	G_q
	MRGPRX2 (MRGX2)	G_q

Note: GPCR nomenclature and classification was determined using IUPHAR (guidetopharmacology.org), GPCR phylogenetic analysis was performed using ClustalW2 (ebi.ac.uk/Tools/msa/clustalw2), and dendrogram creation was done using iTOL (itol.embl.de). ~70 Non-liganded orphan receptors (not shown in table) and over 100 GPCR orthologs (other species) assays (not shown in dendrogram) are available. Refer to our websites for the most recent list of available assays including additional GPCR products and services not shown in the dendrogram.

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RESOURCES
Product Types

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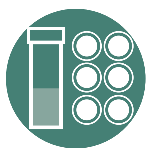
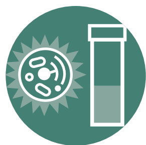


Assay Kits
Complete off-the-shelf, cell-based functional assays with high reproducibility for rapid therapeutic assessment to QC lot release testing

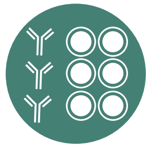
Cells
Stable cell lines, cell pools, frozen cells, & effector cells for a broad range of target classes & applications

Membrane Preparations
Qualified GPCR & Ion Channel membrane preps for saturation and competitive radioligand binding & GTPγS functional studies



Detection Kits & Reagents
Chemiluminescent & fluorescent readout kits & reagents to suit the diverse needs of your drug discovery & development programs

Cell Culture Kits & Reagents
Optimized cell culture kits & reagents for consistent, reliable, & high quality results



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Highly purified, pre-validated reference ligands & inhibitors for use with Eurofins DiscoverX cell lines

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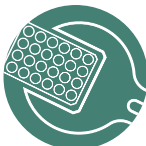
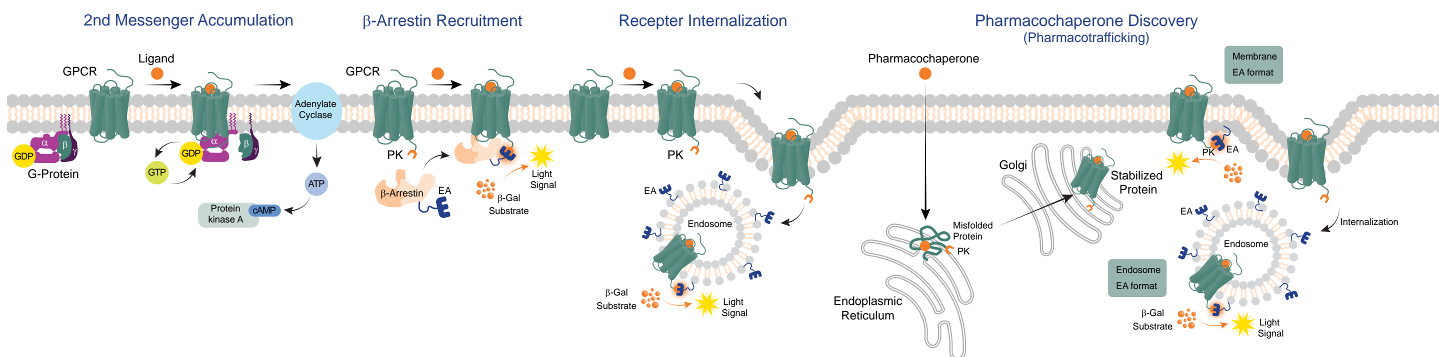
Complete set of vector & retroparticle-based tools & protocols to build your own stable cell lines & cell-based assays



Custom Capabilities
Custom cell lines, assays, & protein development capabilities optimized to your requirements

GPCR Product Topics

Whether you are developing small molecule or biologic therapeutic drugs, Eurofins DiscoverX® provides you with a variety of drug discovery and development products with multiple assay types and applications to meet your specific GPCR research needs.



GPCR Solutions

Discover the largest collection of GPCR assay solutions for developing small molecule or biologic therapeutics

HitHunter® cAMP Assays

Accurately detect cellular cAMP levels for a variety of biologics and small molecules ligands and applications

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Easily measure intracellular calcium within cells after activation status GPCRs and ion channels

PathHunter® β-Arrestin Assays

Universal screening & profiling platform that can be used for virtually any G_i-, G_s-, or G_q-coupled GPCR

GPCR Internalization Assays

Simple orthogonal screening assays for monitoring GPCR internalization without microscopy to ultimately identify safer therapeutics

Pharmacotrafficking Assays

Identify pharmacochaperones that promote proper folding of misfolded protein & assist in trafficking to their intended location

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Tools and References

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Access discoverx.com for the most recent list of available products and to learn more about the Enzyme Fragment Complementation (EFC) technology that is behind most of the Eurofins DiscoverX GPCR products.

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Assay guide and list of available GPCR assays, cell lines, membrane preps, & other products

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GPCR β-Arrestin Publications
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Publications (see RESOURCES | Publications)
Peer-reviewed publications covering Eurofins DiscoverX products focused on GPCRs, kinases, ion channels, & more targets

The Druggable GPCRome Poster
Downloadable poster showcasing the human GPCRome dendrogram, available assays & families, targets & coupling table

EFC Platform
Robust, homogeneous detection assay system used for a wide variety of drug discovery & development applications

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