Introduction

It is now 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. Agonist binding first promotes a conformational change that results in the activation of receptor-associated heterotrimeric G-proteins that, in turn, activate downstream second messenger molecules such as cyclic AMP (cAMP), inositol triphosphate, diacylglycerol and calcium. Once activated, G-protein receptor kinases (GRKs) phosphorylate the C-terminal tail of these receptors, which results in the binding of β-Arrestin. Bound Arrestin serves to sterically block further G-protein activation, which limits 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 are also capable of initiating a distinct second set of G-protein independent signals. 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]. Therefore, quantitatively examining these pathways can aid in defining compound function and can lead to the discovery of novel biased ligands with unique efficacy and safety profiles.

To demonstrate that PathHunter® 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 the δ Opioid receptor (OPRD1, hDOR) 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, DiscoveRx’s suite of functional whole cell assays is an ideal platform for ligand bias discovery and the characterization of compounds using empirically derived guidelines.

Abstract

As more is learned about the intricacies of GPCR signaling, the harder it becomes to accurately describe ligand activity using a single functional readout. This creates the need for a comprehensive suite of naturally coupled cell-based assays that allows quantitative examination of multiple signaling pathways in parallel using the same robust, reliable and high-throughput screening friendly format. DiscoveRx® has developed an industry leading menu of functional assays designed to detect GPCR signaling through second messenger activation, Arrestin binding and receptor internalization. Access to the same target in multiple readouts provides a unique opportunity to identify novel biased ligands that selectively target different aspects of GPCR activation in hopes of developing new and improved drugs with greater therapeutic benefit.
Experimental Methods

Clonal cell lines. Cells overexpressing hDOR in the cAMP (cAMP Hunter™, Cat.# 95-0108C2), Arrestin recruitment (PathHunter® Arrestin, Cat.# 93-0241C2) and GPCR Internalization (PathHunter® Activated GPCR Internalization, Cat. #93-0673C3) formats were plated at 5,000 cells per well of a 384-well plate and incubated overnight at 37°C, 5% CO₂.

HitHunter® cAMP assay. hDOR cAMP Hunter cells were treated with increasing concentrations of known compounds for 30 minutes at 37°C, 5% CO₂. cAMP signal was detected using the HitHunter cAMP XS Detection Kit (Cat.# 90-0075) according to the recommended protocol. HitHunter cAMP assays are competitive immunoassays. 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 active enzyme by enzyme fragment complementation which hydrolyzes substrate to produce signal. A positive signal is directly proportional to the amount of cellular cAMP (Fig. 1, left panel).

PathHunter β-Arrestin Assay. hDOR PathHunter Arrestin cells were treated with increasing concentrations of the compounds for 90 minutes at 37°C, 5% CO₂. In this system, a small 42 amino acid enzyme fragment called ProLink is appended to the C-terminus of the GPCR. Arrestin is fused to the larger enzyme fragment, called Enzyme Acceptor (EA). Activation of a single GPCR stimulates the binding of arrestin, forcing complementation of the two enzyme fragments. The resultant increase in enzyme activity is measured by addition of chemiluminescent PathHunter® Detection Reagents (Cat. # 93-0001). (Fig 1, right panel).

PathHunter Activated GPCR Internalization Assay. hDOR PathHunter GPCR Internalization cells were treated with increasing concentrations of the compounds for 180 minutes at 37°C, 5% CO₂. PathHunter Internalization assays employ a ProLink tag that is localized to the surface of intracellular endosomes and the EA is fused to β-Arrestin. Stimulation of the untagged receptor results in arrestin binding to the activated GPCR, internalization of the receptor and trafficking to cellular endosomes. The resultant enzyme complementation leads to an increase in enzyme activity that is measured by addition of chemiluminescent PathHunter Detection Reagents (Cat. # 93-0001). Fig 1, right panel).

All data was read on a multi-mode plate reader and analyzed using GraphPad Prism® 6.

Results & Discussion

Several therapeutically relevant examples of biased agonism have been described with the opioid system [3 and references therein,4,5], which is involved in the body’s response to pain. Activation of the hDOR receptor alleviates persistent pain leading to the desired analgesic effect. However, sustained or repeated receptor activation results in receptor desensitization which is thought to be the main cause of opioid tolerance in vivo [6]. Despite having similar potencies and efficacies in vitro, δ 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, the need exists 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.

Figure 2 demonstrates that a combination of DiscoveRx’s HitHunter second messenger, PathHunter β-Arrestin recruitment, and PathHunter Activated GPCR Internalization assays can be used in parallel to uncover novel, biased receptor activation and internalization. Four hDOR agonists (DADLE, [Met⁵]-enkephalin, [Leu]-enkephalin and SNC80, a strongly
Conclusions
Understanding G-protein and Arrestin biased signaling has important implications in the discovery and development of novel therapeutics. Using DiscoveRx's suite of functional whole cell assays, this data indicates that novel biased ligands can be identified and characterized for multiple activities in vitro using the same robust, reliable and high throughput friendly chemiluminescent format. We have completed a systematic analysis of greater than 30 GPCRs and their related ligands to study the relationship between the different read-outs 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 now 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 600 functional GPCR assays for screening and profiling, DiscoveRx can deliver on the promise of high content imaging using a simple, quantitative, high content compound analysis approach.

References

internalizing compound and functional antagonist), were analyzed by the three types of hDOR functional assays. For comparison, the data was normalized to [Met⁵]-enkephalin in potency (set equal to 1) and efficacy (set equal to 100%). 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. Our data demonstrates that potency and efficacy differences exist between endogenous enkephalin peptides and synthetic analogs that can be easily uncovered using a combination of second messenger, arrestin recruitment and internalization assays. 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 read-outs during the screening and lead optimization process can facilitate the identification of novel biased ligands with unique activation and internalization profiles.

Application Note | GPCR Signaling

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