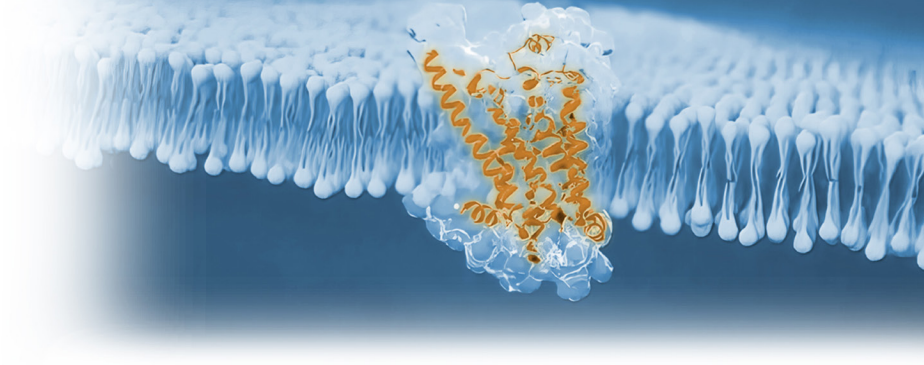

APPLICATION NOTE

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

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.

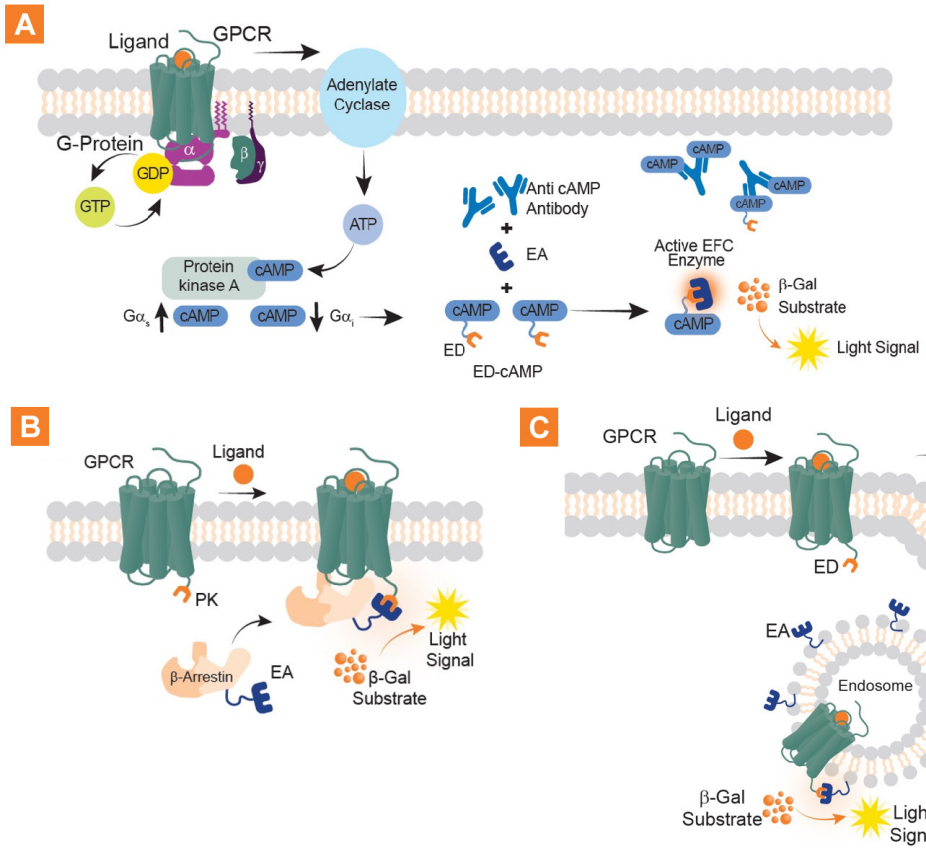

Figure 1. Functional cell-based assays by Eurofins DiscoverX. A. cAMP accumulation, B. β-arrestin recruitment, and C. GPCR internalization. For a complete list of assays, visit discoverx.com/gpcrs.

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.

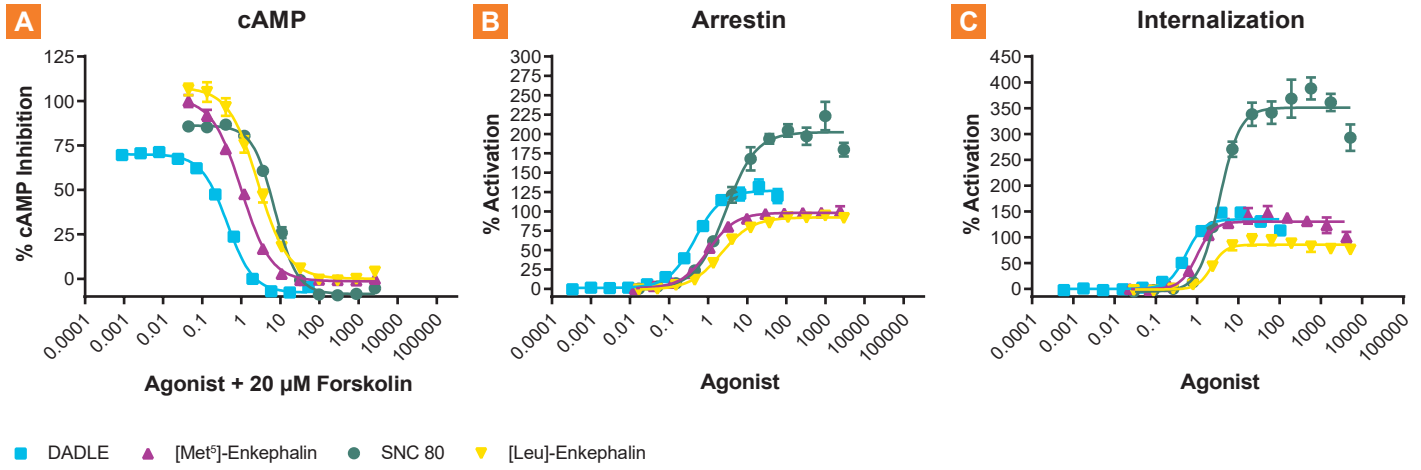

Figure 2. Comparison of human δ opioid receptor cell-based assays. A. cAMP, B. β-arrestin recruitment, and C. GPCR internalization assays of the human δ opioid receptor (OPRD1, hDOR) receptor.

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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