Discovery of Novel G-Protein or Arrestin-Biased Ligands Using a Suite of GPCR Signaling Platforms

Daniel Bassoni, Elizabeth Quinn, Ph.D. and Tom Wehrman, Ph.D.

Introduction

As more is learned about the intricacies of GPCR signaling, the harder it becomes to accurately describe ligand activity using a single functional readout. It is now well appreciated that GPCR activation results in G-protein dependent as well as G-protein independent signaling events such as β-arrestin recruitment and internalization. The complex relationship that exists between G-proteins and β-Arrestin signaling determines both the efficacy and potential side effects of GPCR-targeted drugs. Therefore, quantitatively examining these pathways can aid in defining compound function and can lead to the discovery of novel biased ligands with unique attributes. DiscoveRx® has developed a suite of assays designed to detect GPCR signaling through second messenger activation, Arrestin binding, and receptor internalization. These assays can be used in parallel as a simple, rapid, and quantitative approach to discover novel, biased ligands with unique attributes that could result in greater therapeutic efficacy and more favorable side effect profiles.

Importance of GPCR Pathways in Discovery Therapeutics

GPCR activation is mediated through two different signaling pathways: G-protein dependent activation and G-protein independent β-Arrestin recruitment and receptor internalization. First, ligand binding activates heterotrimeric G-proteins that, in turn, activate downstream, second-messenger signaling, which can lead to an increase in cyclic AMP (cAMP). Once activated, G-protein receptor kinases (GRKs) phosphorylate the C-terminal tail of these receptors, which results in binding of β-Arrestin. Bound Arrestin sterically blocks 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]. As β-Arrestin turns off G-protein signaling, β-Arrestin also serves as a molecular switch to activate a second set of G-protein independent signals. The complex relationship between G-protein and β-Arrestin signaling determines both the efficacy and potential side effects of GPCR-targeted drugs [2,3].

Biased Ligands Activate Alternate Signaling Pathways

Scientists originally thought that most ligands binding to GPCRs signal equally through both G-protein and β-Arrestin pathways in a balanced or "unbiased" manner. During the last ten years, however, it has been shown that these pathways can operate independently of one another. Depending on the type of ligand used for stimulation, the GPCR receptor can be activated or inhibited selectively, resulting in a biased response to different signaling pathways downstream of the GPCR. Such ligands, termed "biased ligands," stabilize a certain conformation of a receptor, thereby stimulating certain responses, but not others (Figure 1). There is now a growing list of documented Arrestin-biased and G-protein biased ligands covering a diverse list of receptors [2].

One therapeutically relevant example of biased agonism has been described with the opioid system, which is involved in the body's response to pain. Activation of the d Opioid receptor (hDOR) 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 [4]. Despite having similar potencies and efficacies in vitro, delta agonists can differ dramatically in their desensitization and receptor internalization profiles. Importantly, in the context of pain, it has now been shown that delta agonist-induced analgesic effects are retained when receptors remain at the cell surface and are lost following receptor activation and internalization [4,5]. 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.

DiscoveRx® Assays: Exploring Differences in GPCR Signaling Pathways

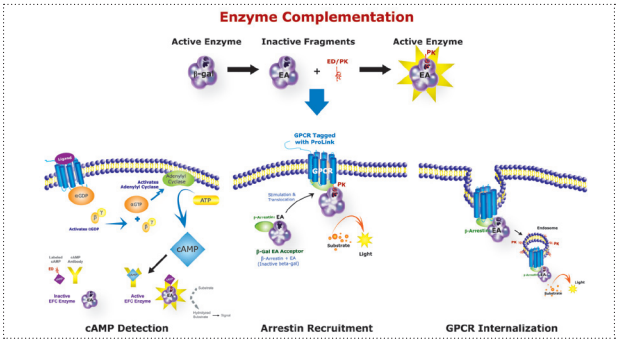
DiscoveRx Corporation (www.discoverx.com) has developed an innovative, suite of validated, functional whole cell assays: a universal, G-protein-independent platform utilizing recruitment of β-Arrestin to monitor GPCR activation, second messenger assays, and GPCR internalization assays. Access to the same GPCR target in different technology formats allows multiple signaling pathways to be assayed in parallel using the same robust, reliable and high throughput friendly chemiluminescent format. Using a combination of all 3 technology platforms enables the identification of G-protein or Arrestin biased ligands and provides a unique opportunity to determine mechanism of action, potency in vitro, and uncover potential side effect profiles of novel drug candidates.

Experimental Methods

Cell lines overexpressing the Opioid Receptor δ (OPRD1, 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% CO_2.

HitHunter® cAMP Assay. Cells were treated with increasing concentrations of known compounds for 30 minutes at 37OC, 5% CO2. cAMP signal was detected using the HitHunter® cAMP XS+ Kit (part # 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

FIGURE 1



Functional whole cell assays for cAMP (Left panel), Arrestin recruitment (Middle panel) and GPCR Internalization (Right panel).

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 (Figure 1, left panel).

PathHunter® β-Arrestin Assay. Cells were treated with increasing concentrations of the compounds for 90 minutes at 37°C, 5% CO_2. 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 an arrestin protein, 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). (Figure 1, middle panel).

PathHunter® Activated GPCR Internalization Assay. Cells were treated with increasing concentrations of the compounds for 180 minutes at 37°C, 5% CO_2. PathHunter Internalization assays employ a ProLink 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). Figure 1, right panel).

All data was read on a multimode plate reader and analyzed using GraphPad Prism® [4].

Results

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 ligands with specific receptor activation and internalization profiles. Seven agonists (DADLE, Deltorphin A, Deltrophin B, Naloxone benzoylhydrazone, DAMGO, [Met5]-enkephalin, and [Leu]-enkephalin) and the SNC80, a strongly internalizing compound and functional antagonist, were analyzed by the three types of DiscoveRx assays to the hDOR receptor. 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 clearly was 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 clearly indicates that receptor internalization, as determined by a large reduction in cell surface receptors, influences the efficacy of an agonist [4,5]. Thus, using a single pathway approach to GPCR analysis in vitro can 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.

Conclusion

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. Parallel analysis of more than 30 GPCRs have been completed to obtain 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. We have now embarked on an era of GPCR drug development that will enable drug discoverers to develop smarter and more effective drugs. With the largest and most comprehensive menu of over 400 GPCR assays for screening and profiling, DiscoveRx can deliver on the promise of high content imaging using a simple, high content compound analysis approach.

References

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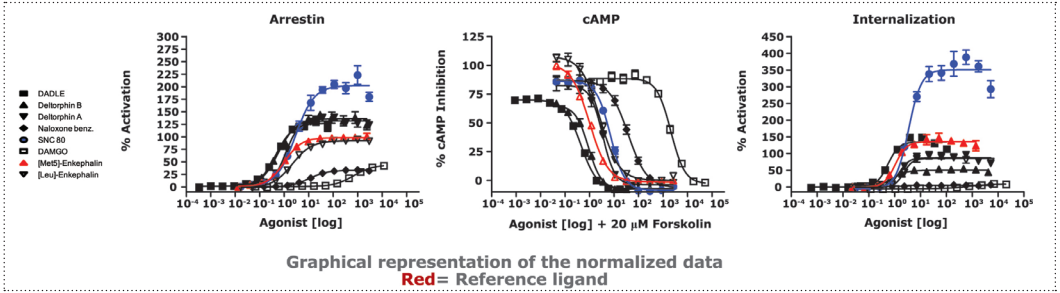
FIGURE 2



Comparison of Arrestin recruitment, cAMP, and GPCR Internalization assays at the Opioid Receptor δ (OPRD1, hDOR) receptor.