Unique Properties of PathHunter™ β-Arrestin Assays for GPCR Antagonist Discovery and Characterization

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Abstract

A common feature of 7TM receptors is that upon activation, the receptors are bound by cellular Arrestin proteins. DiscoveRx has pioneered the PathHunter™ β-Arrestin assay platform for monitoring these events using enzyme fragment complementation (EFC) and applied this technique to develop assays for more than 170 human GPCR targets. Unlike second messenger assays that are amplified through downstream signaling events, each arrestin protein binds to a single activated GPCR. The PathHunter Arrestin assay is unique among arrestin assays in that it preserves the one-to-one nature of arrestin binding and directly translates the binding events to signal generation. This eliminates the receptor reserve effect and enables the interrogation of all available receptors. The work presented here focuses on how this property of the PathHunter Arrestin platform makes it ideal for the discovery and characterization of GPCR antagonists.

PathHunter β-Arrestin GPCR Assay System

Figure 1. PathHunter β-Arrestin Assays monitor the interaction of β-Arrestin with activated GPCRs using **E**nzyme **F**ragment **C**omplementation (EFC). In this system, a small 42 AA enzyme fragment (called ProLink™) is appended to the C-terminus of the GPCR. Arrestin is fused to the larger enzyme fragment (called EA or Enzyme Acceptor). Activation of the GPCR stimulates binding of Arrestin and forces complementation of the two enzyme fragments, resulting in an increase in enzyme activity that is measured using chemiluminescent PathHunter Detection Reagents.

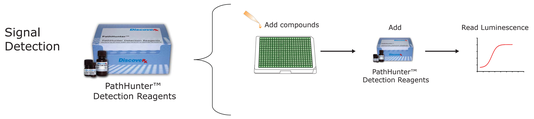

Figure 2. The PathHunter™ Detection Reagents consist of a lysis buffer and substrate mixture that is added as a single solution to stimulated cells. After a short incubation, the PathHunter chemiluminescent signal can be detected using any standard benchtop luminometer.

Methods

1. For all 384-well assays, 5,000 cells per well were seeded in 20 μL of optimized Cell Plating (CP) reagent and incubated at 37°C, 5% CO_2 for 48 hours prior to running the PathHunter assay.
2. 5 μl of 5X test compound was added to cells and incubated at 37°C for 90 minutes.
3. 13 μl of PathHunter Detection Reagents were added and the plates were incubated for 90 min at room temperature.
4. Data was read on a multimode plate reader (chemiluminescence) and analyzed using GraphPad Prism® 4.

Amplified vs Non-Amplified GPCR Assays

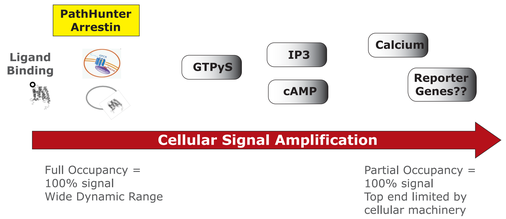

Figure 3. The available GPCR screening assays have various levels of intra-cellular signal amplification. Until recently, the system with the lowest level of amplification was ligand binding. Unfortunately ligand binding is not a functional assay that can be used to determine the different efficacies of GPCR ligands. The PathHunter Arrestin assay is unique in that it is a functional read-out which operates in a stoichiometric fashion with receptor activation. Each activated GPCR provides one unit of signal thus the maximum signal is reached with 100% occupancy which makes the system ideal for efficacy measurements and the application to antagonist discovery and characterization.

Antagonists in PathHunter™ Arrestin vs cAMP

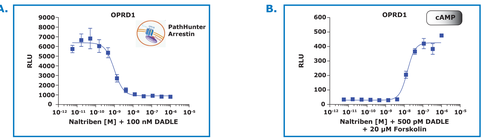

Figure 4. The Gi-coupled Delta Opioid Receptor (OPRD1) PathHunter **(A)** and cAMP Hunter cell lines **(B)** were pre-treated with fixed concentrations of Naltriben antagonist for 30 minutes prior to agonist treatment. The agonist concentration used for stimulation of OPRD1 was higher in the PathHunter assay compared to the cAMP assay; however, the lowest detectable dose of antagonist was lower in the PathHunter assay. The Ki values derived from the Cheng-Prusoff equaton show that Naltriben exhibits similar Ki values regardless of the assay measurement.

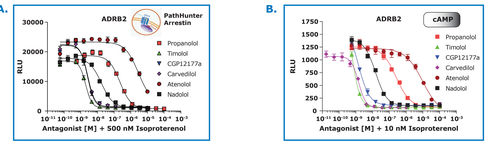

Figure 5. A panel of antagonists were tested against both PathHunter **(A)** and cAMP Hunter cell lines **(B)** expressing the ADRB2 (Gs-coupled) receptor. The IC_{50} values in both assay formats were similar reflecting the same rank order potency of the antagonists regardless of the format.

Antagonist Character in Arrestin and cAMP

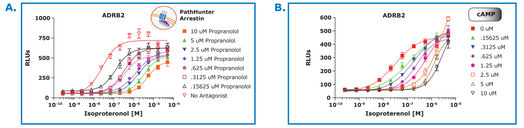

Figure 6. Schild analysis of Propanolol antagonism of the ADRB2 receptor. The cAMP Hunter **(B)** and PathHunter **(A)** assays were incubated in the presence of the indicated fixed concentration of antagonist and then challenged with a dose response of agonist (isoproterenol). In both cases, increasing doses of propanolol caused progressive right shifting of the dose response curves indicating a competitive antagonism.

Gi-Coupled Receptors

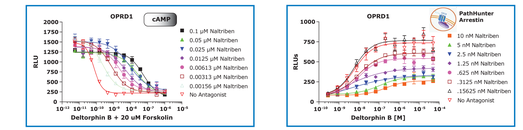

Figure 7. Schild analysis performed similar to the above ADRB2 receptor shows a distinct difference in the antagonist character for the Gi-coupled OPRD1 receptor. In the cAMP measurement, increasing the concentration of the antagonist caused a right shifting of the dose curve. However, in the arrestin assay, increasing the dose of antagonist causes a decrease in the max agonist signal, suggestive of a non-competitive antagonist.

Summary

In non-amplified systems, agonist curves can be right shifted compared to typical second messenger assays. In our experience, this is commonly seen versus calcium flux assays where the target is highly over expressed and there exists a significant receptor reserve.

In this study, we tested how antagonist curves compared between the PathHunter Arrestin and cAMP Hunter assay formats. Our data demonstrates that unlike agonist sensitivity, the sensitivity of the non-amplified arrestin assay to antagonists is similar if not more sensitive than second messenger assays even when challenging with higher levels of agonist.

We also found specific instances, such as for OPRD1, where the PathHunter Arrestin assay can reveal novel pharmacology of antagonists. It has been noted previously that in systems where there exists significant receptor reserve, antagonists may take on a competitive phenotype upon Schild analysis where the endogenous expression levels will reveal a non-competitive nature of the antagonist (Hopkinson, Latif and Hill 2000. Br. J. of Pharm. 131,124-130).

Studies are now ongoing to determine the causative nature of the non-competitive phenotype and its relationship to compound function and G-protein coupling.

Technology Access Section

Cell Lines — Finished cell lines containing the Arrestin-EA and GPCR-ProLink fusions as heterogeneous pools or clonal cell lines. More than 150 cell lines available.

Tool Box — A clonal PathHunter parental cell line and a ProLink vector are available for build-your own cell lines (7 cell backgrounds available)

Kits — Cells, detection reagents, and plates ready-to-go right out of the freezer. Each kit contains enough cells for 2 X 96 well plates

Discovery Partnerships — Custom screening and profiling against PathHunter GPCRs

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