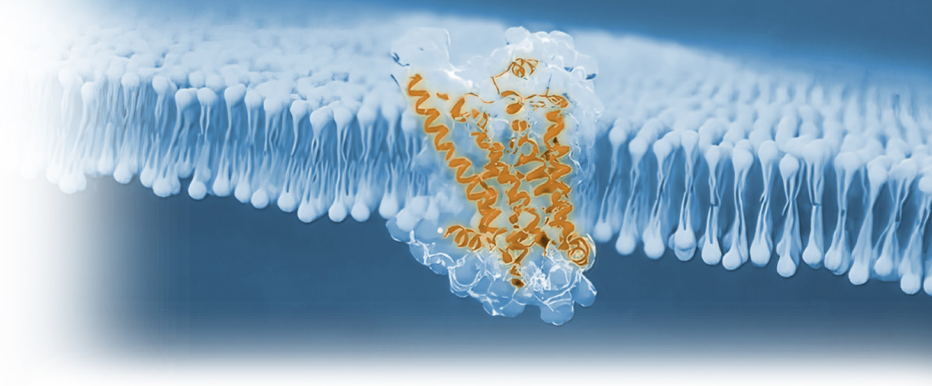

APPLICATION NOTE

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.

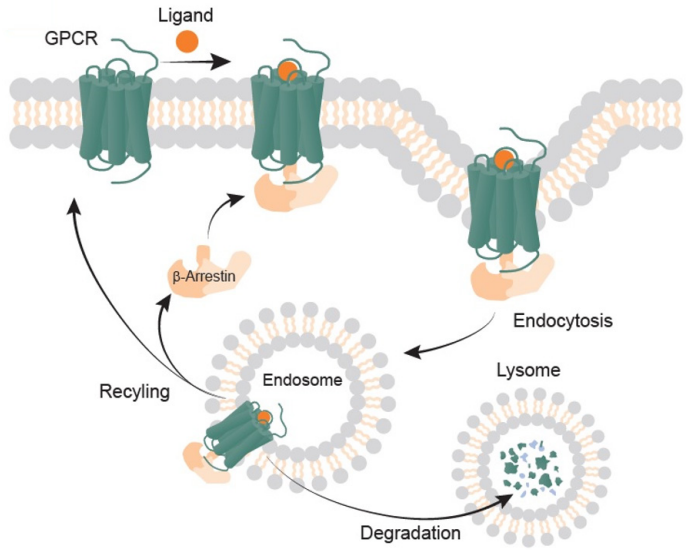

Figure 1. The life cycle of a GPCR includes activation, endocytosis, and recycling back to the plasma membrane. Repeated or prolonged exposure to a ligand can re-route receptors to the lysosome, thus contributing to the downregulation of receptors.

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.

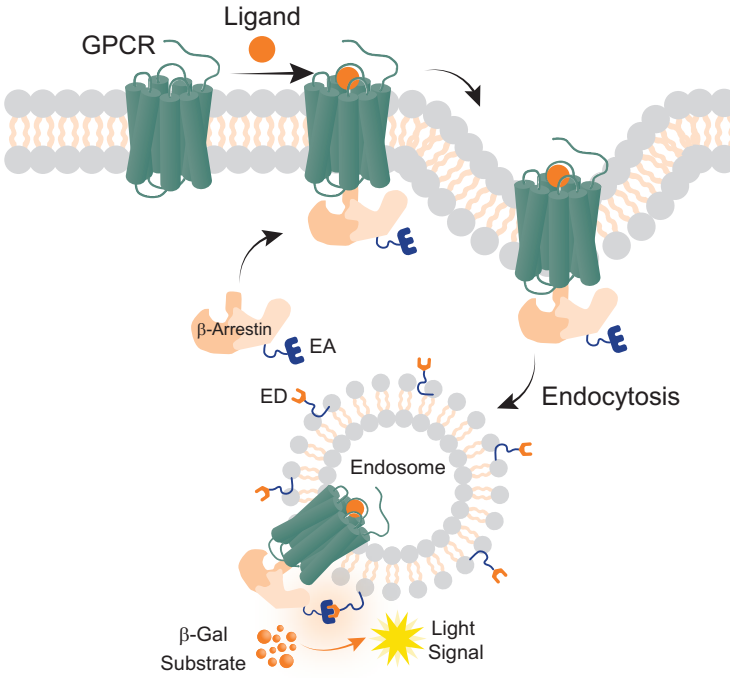

Figure 2. PathHunter Activated GPCR Internalization Assays provide a novel, non-imaging approach for measuring GPCR activation and internalization in live cells. In this system, enzyme fragment complementation occurs following β-arrestin recruitment and receptor internalization and localization at the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

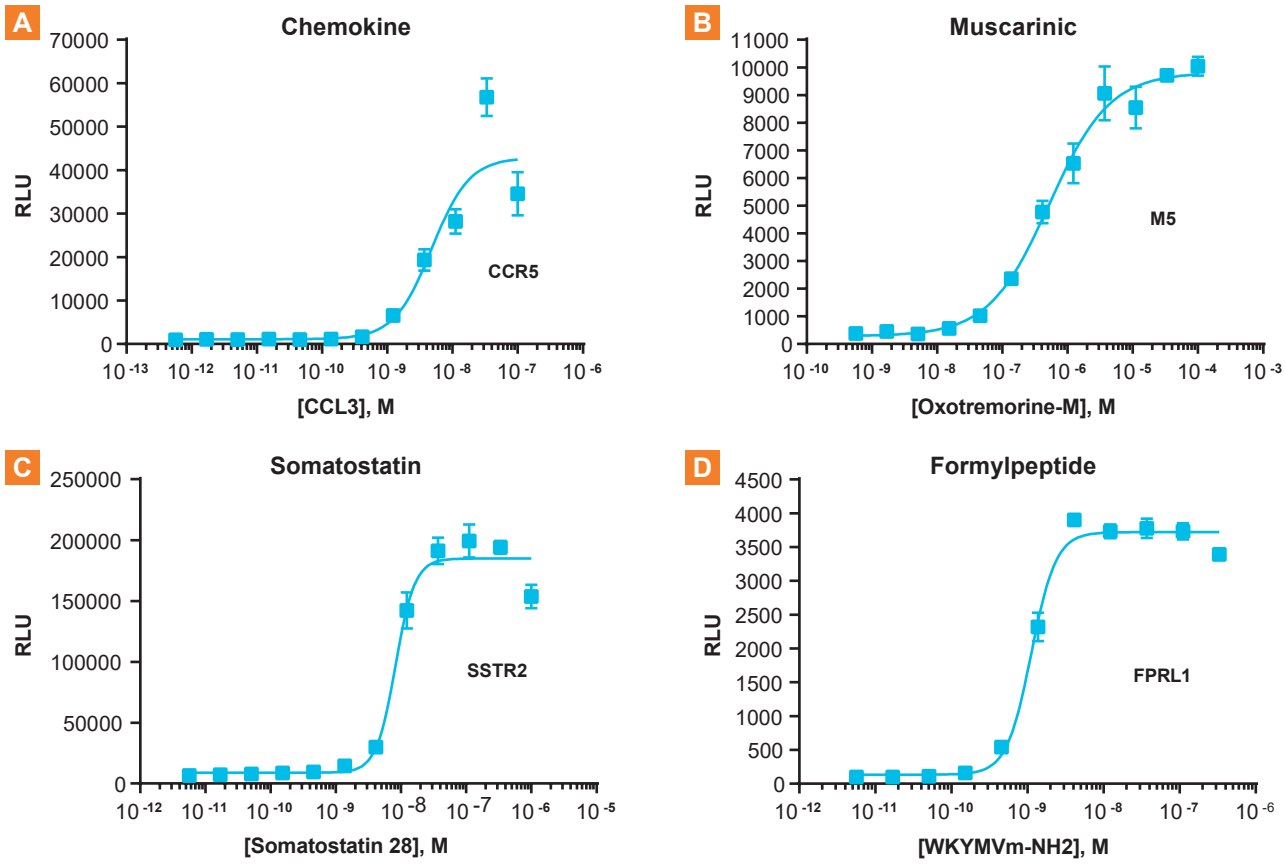

Figure 3. The PathHunter® GPCR Activated Internalization Assay is broadly applicable to a large number of GPCR families. Cells expressing the wild type untagged A. Chemokine CCR5, B. Cholinergic Muscarinic CHRM5, C. Somatostatin SSTR2, and D. Formylpeptide FPRL1 were treated with increasing concentrations 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.

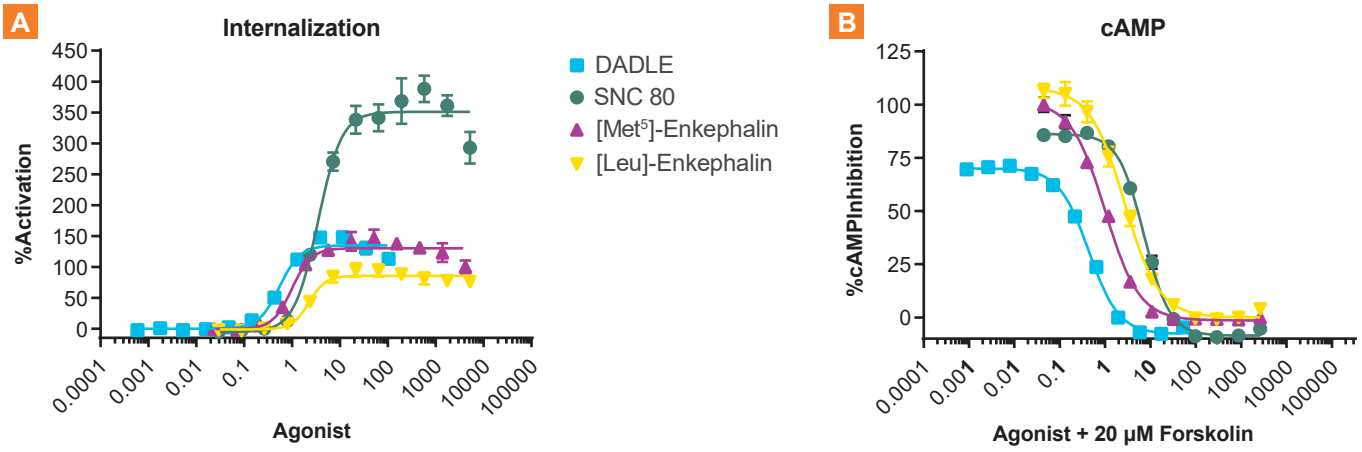

Figure 4. Comparison of DOR internalization versus cAMP accumulation by known receptor agonists. Cells overexpressing the DOR receptor in the PathHunter Activated GPCR Internalization A. and cAMP Hunter™ B. assay formats were treated with agonists and assayed using PathHunter and HitHunter detection 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.

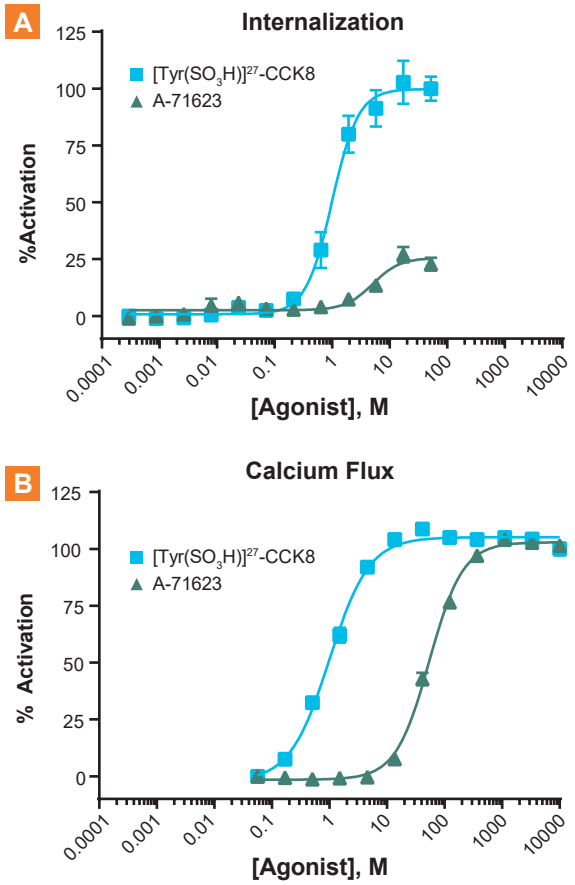

Figure 5. Comparison of CCKAR internalization versus calcium flux by known receptor agonists. Cells overexpressing the CCK-A receptor in the PathHunter Activated GPCR Internalization A. and PathHunter β-Arrestin B. formats were treated with agonists and assayed using PathHunter and Calcium No Wash^{PLUS} 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.

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.

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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