Identify Effective Therapeutics with Simple, Quantitative Cell-Based Assays That Measure Receptor Internalization

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Abstract

Both multi- and single-pass membrane receptors constitutively exhibit complicated and dynamic membrane receptor trafficking as well as internalization induced by ligand or compound binding. Measuring how therapeutic molecule binding to membrane receptors affects receptor internalization can provide insight into drug tolerance, unwanted side effects, diseases, and ultimately help with identifying safer drugs. Additionally, antibody-based therapeutics that target specific receptors such as the immuno-oncology checkpoint receptors can induce high levels of internalization and serve as candidates for conjugation to cytotoxic compounds. These therapeutic Antibody-Drug Conjugates (ADCs) take advantage of both antibody specificity for tumor antigens and antibody induction of receptor internalization to deliver cytotoxic drugs to cancer cells effectively.

We present here a collection of cell-based assays that use the industry-validated Enzyme Fragment Complementation (EFC) technology to monitor receptor internalization activated by small molecules or biologics. These assays are easy-to-use and offer a quantitative means of measuring receptor internalization that is amenable to high-throughput screening. PathHunter® Internalization Assays provide an effective alternative to more cumbersome, less quantitative techniques using imaging, antibodies or dyes/labels to determine internalization. Assays for both single-pass membrane receptors such as receptor tyrosine kinases (RTK) and checkpoint receptors and multi-pass membrane receptors like GPCRs have been developed using this generic platform. An additional assay format for GPCRs specifically measures activated GPCR internalization using β-arrestin recruitment. Results from such membrane receptor internalization assays can provide important safety and efficacy information during drug development.

PathHunter Receptor Internalization Assays

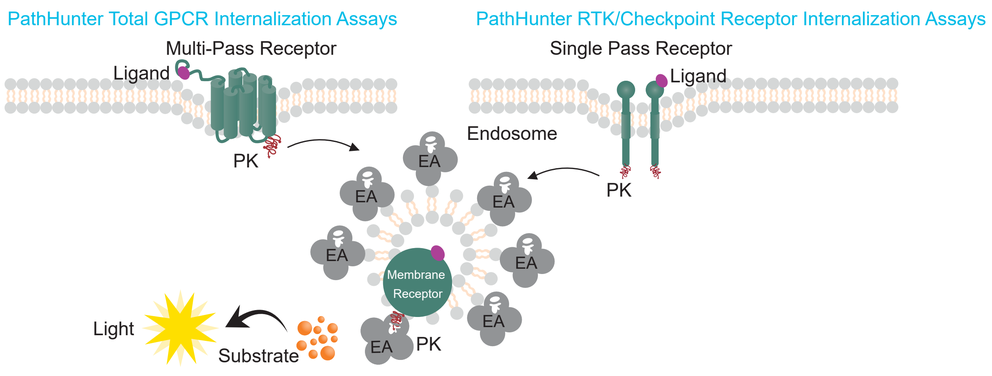

Figure 1. PathHunter Receptor Internalization Assays detect membrane receptor translocation to the endosome. Cell lines are engineered to co-express the ProLink™ (PK; EFC enzyme donor)-tagged membrane receptors, and an EFC enzyme acceptor (EA) tag localized to the endosomes. Internalization assays for both multi-pass membrane receptors (GPCRs) and single-pass membrane receptors (RTKs or immune checkpoint receptors) have been developed for this assay platform. Small molecules or biologics (e.g. antibodies) that induce activation of the receptor-PK fusion protein leads to internalization of the receptor to the EA-tagged endosomes, forcing complementation of the two β-galactosidase (β-gal) enzyme fragments (EA and PK). The resulting functional β-gal enzyme hydrolyzes a substrate to generate a chemiluminescent signal.

PathHunter Activated Receptor Internalization Assays for GPCRs

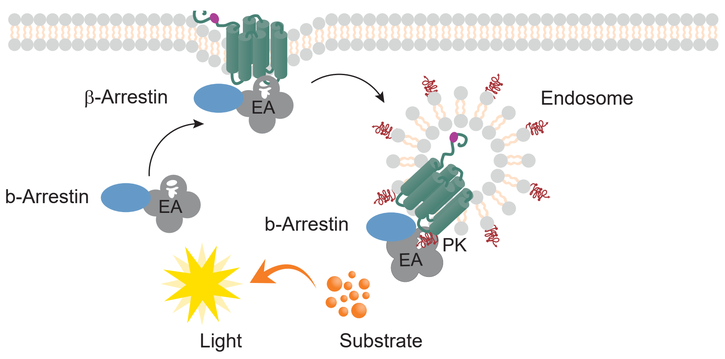

Figure 2. Activated PathHunter GPCR Internalization Assays measure internalization of the GPCR-β-arrestin complex to the endosome. An alternate GPCR internalization format changes the location of the β-gal enzyme fragments to allow for detection of a GPCR/Arrestin complex. These cell lines are engineered to co-express an untagged GPCR, β-arrestin tagged with the EA, and a ProLink (PK) fragment that is localized to the endosomes. Activation of the untagged GPCR induces β-arrestin recruitment, and the resulting GPCR/β-arrestin-EA complex is internalized in the PK-tagged endosomes. Similar to the more general internalization format described in Figure 1, internalization forces complementation of the two β-gal enzyme fragments, forming a functional enzyme that hydrolyzes a substrate to generate a chemiluminescent signal.

Applications for Internalization Assays

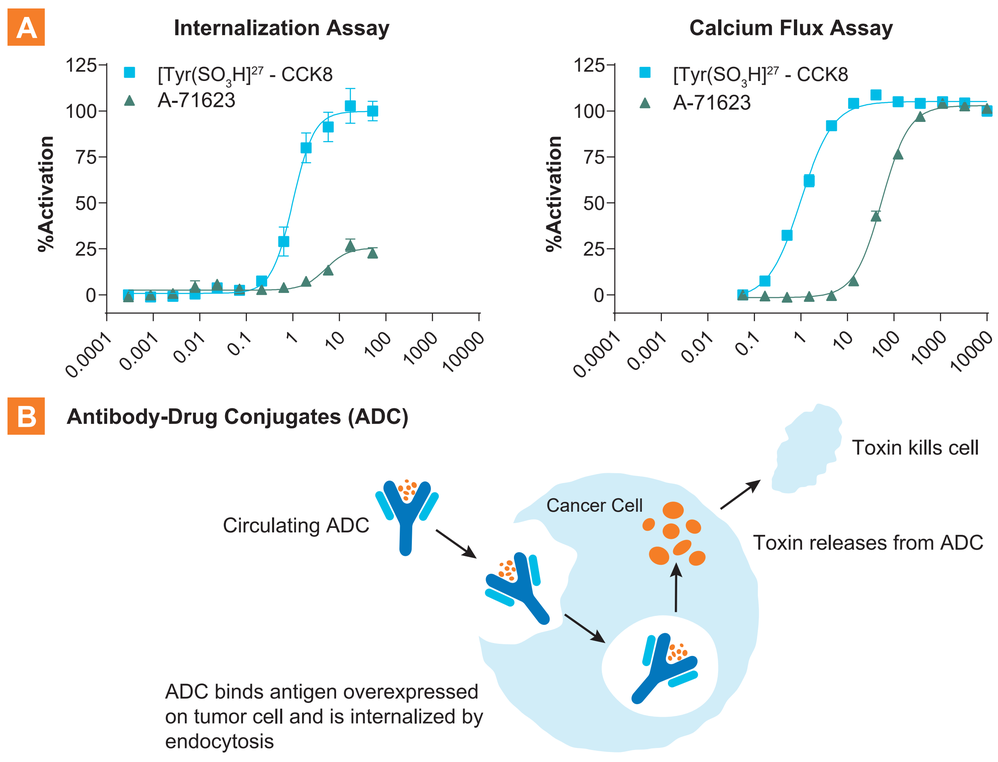

Figure 3. Compounds binding membrane receptors can be profiled for their effects on receptor internalization. A. Data from the PathHunter GPCR CCKAR (cholecystokin A receptor) Internalization Assay can be used for comparison to results from other GPCR CCKAR Assays (β-arrestin recruitment, cAMP accumulation, or calcium flux) evaluating receptor signal activity in response to the same ligands. Combining knowledge of how ligand/compound binding affects receptor signaling events, as well as receptor localization and internalization, can provide insights into the overall effects on efficacy and safety. B. PathHunter Internalization assays can also be used to effectively characterize antibodies targeted for specific receptors such as the immuno-oncology checkpoint receptors for their ability to activate receptor internalization. Antibodies identified as inducing high levels of internalization may serve as candidates for conjugation to cytotoxic compounds in order to selectively target and kill tumor cells. Many ADCs are already in various phases of clinical trials for the treatment of cancer.

Comparison of GPCR Internalization vs. Signaling Efficacy

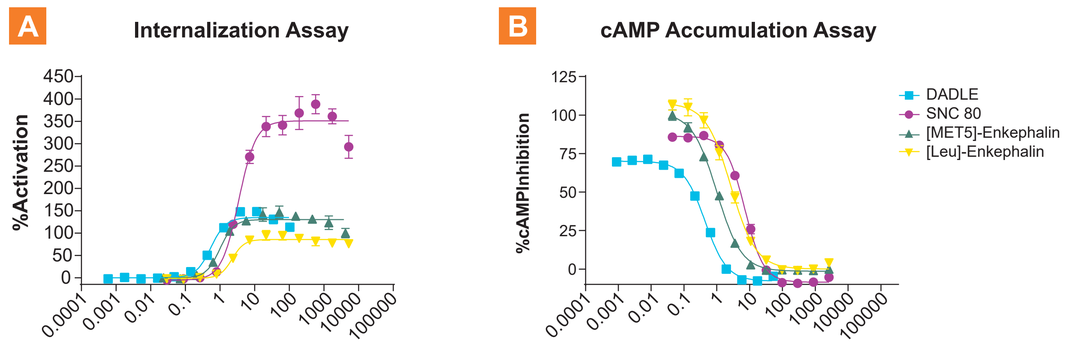

Figure 4. Four human δ-Opioid receptor (DOR) agonists were characterized for their activation of receptor internalization and the inhibition of forskolin-stimulated cAMP accumulation. Cells overexpressing the hDOR receptor in the PathHunter Activated GPCR Internalization A. and cAMP Hunter B. formats were treated with known agonists and assayed using PathHunter and HitHunter® Detection reagents respectively. For comparison, the data was normalized to [Met^5]-enkephalin in potency (value set to 1) and efficacy (set to 100%). Despite similar compound potencies and efficacies based on the inhibition of cAMP accumulation in the cAMP assay, SNC-80, which is known as a strongly internalizing compound and functional antagonist is clearly defined as a super-agonist in the internalization assay. The super-agonist effect suggests that SNC80 may lead to higher levels of internalized receptor, and subsequently lower levels of receptor reserve – thus behaving as a "functional antagonist."

PathHunter Receptor Tyrosine Kinase Internalization Assays

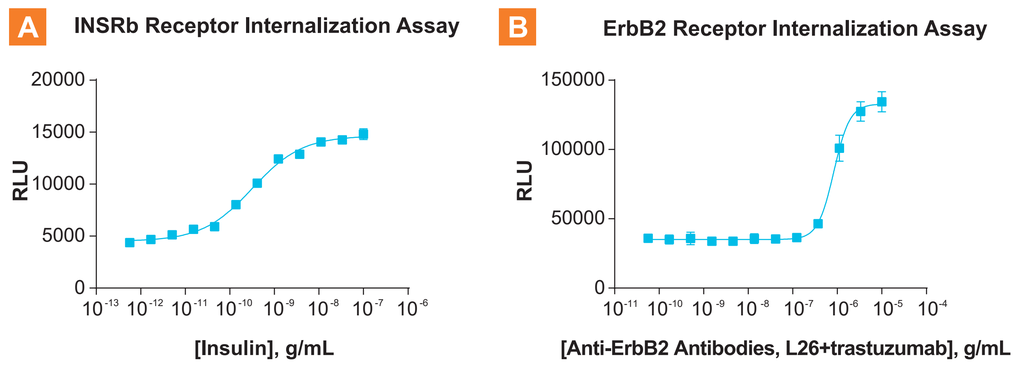

Figure 5. PathHunter RTK Internalization Assays can be activated with ligands or receptor antibodies. A. Insulin receptor b (INSRb) internalization in response to incubation with increasing concentrations of insulin. B. Epidermal growth factor B2 (ErbB2) receptor internalization is detected when cells are incubated with two different ErbB2 antibodies including the therapeutic antibody trastuzumab (Herceptin®). Similar robust ErbB2 internalization was observed when cells were incubated with Herceptin and pertuzumab (Perjeta®; data not shown). Herceptin and Perjeta are registered trademarks of Genentech USA, Inc. Graphs' x-axes use log-scale.

PathHunter Checkpoint Receptor Internalization Assays for ADC Development

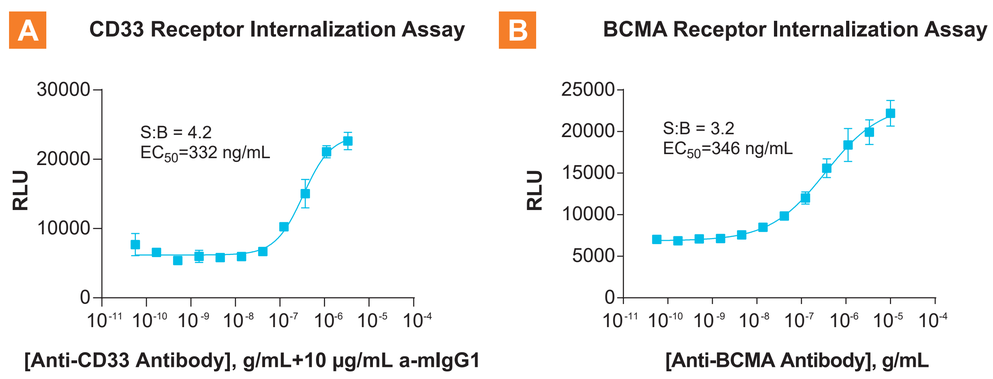

Figure 6. Checkpoint receptor antibodies activate receptor internalization. A. PathHunter internalization assay for the CD33 checkpoint receptor was tested with a commercial antibody to CD33. CD33 antibody was pre-incubated with a secondary antibody to cluster the CD33 receptor and then added to cells to monitor internalization. With higher cross-linked antibody concentrations, greater amounts of PK-tagged CD33 complemented EA-tagged protein localized to the endosome. A number of ADCs for CD33 are in clinical trials for the treatment of acute myeloid leukemia.[1] B. B-cell maturation antigen (BCMA) receptor internalization was activated with a commercial BCMA antibody. Several BCMA ADCs are in development as a therapeutic for multiple myeloma. Graphs' x-axes use log-scale.

Summary

- PathHunter Internalization Assays allow direct and quantitative measurements of internalized single- and multi-pass receptors localized to intracellular endosomes. For GPCRs, an additional Activated GPCR Internalization Assay format allows for specifically measuring arrestin-dependent GPCR internalization.
- Unlike other imaging and antibody-based internalization techniques, PathHunter assays are simple, quantitative chemiluminescent assays that can be used effectively for high throughput screening.
- These cell-based assays can be applied to study receptor activation kinetics, identify novel inhibitors, confirm compound pharmacology following a primary screen, and for the development of therapeutic ADCs.
- For GPCR targets, the availability of PathHunter assays for the same GPCR target in multiple pathway readouts such as second messenger, β-arrestin recruitment, and GPCR internalization allows for a more comprehensive understanding of receptor biology and the potential discovery of novel ligands that are biased toward one signaling pathway or another.

Reference

1. Yu B, Liu D. Gemtuzumab ozogamicin and novel antibody-drug conjugates in clinical trials for acute myeloid leukemia. *Biomark Res.* 2019;7:24. https://doi.org/10.1186/s40364-019-0175-x

Learn more about the PathHunter Internalization Assays by visiting discoverx.com/translocation.