Interrogating Allosteric Interactions Using Multiple Readouts for GPCRs

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Abstract
Compounds that bind to allosteric sites offer many benefits over those which interact orthostERICally. These include enhanced specificity, ability to operate only on receptor activation and reduced risk of overdose. A number of allosteric compounds have been identified in multiple disease areas including CNS (physiological and neuropathic disorders), metabolic disorder (diabetes and weight control), immunomodulation and cardiovascular indications. Allosteric approaches provide opportunities for new drug discovery by overcoming ligand incompatibility with certain drug scaffolds and improving the ability of small molecules drugs to modulate peptide ligand activity. In this study, we investigate the efficacy of a range of allosteric compounds using multiple GPCR readouts including arrestin recruitment, calcium mobilization, cAMP modulation and receptor internalization. Compounds were tested for ability to potentiate or down-regulate agonist responses using different approaches including EC50 shift analysis and residual agonist activity. Interactions were obtained in all three GPCR classes. Responses were obtained for multiple pathways demonstrating the utility for readouts involving either arrestin or second messenger signaling. Allosteric activity was also determined for receptor internalization and the means to examine the effect of allosterics to alter receptor cell surface population and activation kinetics.

Methods
All cell lines used were from DiscoverX. For Arrestin recruitment assays, cell lines stably express various GPCR tagged with ProLink and EA-Beta-Arrestin. For second messenger assays, cell lines stably express GPCR with no additional modification. In some cases, Calcium mobilization assays were performed in Arrestin cell lines. For Beta-Arrestin recruitment and internalization assays, 5000 cells per well were seeded in 20 µL media and incubated overnight prior to assay. Agonist responses were induced by addition of 5µL 5 X compound. Positive Allosteric Modulator (PAM) responses were obtained by co-incubation of 5µL 6 X compound plus 5µL 6 X EC50 ligand. EC50 shift analysis was achieved by performing agonist dose responses in the presence and absence of compound at varying concentrations. Incubation time was 120 minutes for Arrestin and 180 minutes for Internalization assays. Beta-Arrestin recruitment or Internalization was detected after 1 hour room temperature incubation with 50 % (v/v) of PathHunter Detection Reagent (Dx 93-0001) and chemiluminescence read using a PerkinElmer Envision reader. Data was plotted with GraphPad Prism using sigmoidal dose-response (variable slope) or Allosteric EC50 shift fits.

For second messenger assays, 10,000 cells per well were seeded in 20 µL media and incubated overnight prior to assay. For cAMP modulation assays, media was exchanged with 10 µL 1:1 HBSS/10mM Hepes / cAMP Ab reagent. Agonist responses were induced by addition of 10µL 3 X compound. PAM responses were obtained by co-incubation of 5µL 4 X compound plus 5µL 4 X EC50 ligand. EC50 shift analysis was achieved by performing agonist dose responses in the presence and absence of compound at varying concentrations. Incubation time was 120 hours for Arrestin and 180 minutes for Internalization assays. Beta-Arrestin recruitment or Internalization was detected after 1 hour room temperature incubation with 50 % (v/v) of PathHunter Detection Reagent (Dx 93-0001) and chemiluminescence read using a PerkinElmer Envision reader. Data was plotted with GraphPad Prism using sigmoidal dose-response (variable slope) or Allosteric EC50 shift fits.

Functional GPCR Signaling

2nd Messenger
Events following ligand binding
Arrestin
Internalization

DiscoverX offers multiple approaches for interrogating compound activity for GPCRs. This enables the ability to examine compound efficacy from receptor activation to internalization and transport to the endosomes.

Positive Allosteric Modulators (PAM)

A number of known allosteric compounds were screened for activity in agonist and PAM mode. Compounds ranged in level of allosteric agonist activity and produced significant potentiation in the presence of EC50 ligand.

Negative Allosteric Modulators (NAM)

Negative allosteric modulators were also identified by the ability to reduce ligand efficacy. In these examples EC50 responses are sufficient to pick up potential NAM activity.

Summary
Allosteric modulators provide an alternative approach for controlling GPCR activity. Here we demonstrate that allosteric regulation can be identified using multiple functional readouts from secondary messenger signaling, arrestin recruitment and receptor internalization. DiscoverX offers the largest commercially available library of functional cell-based assays for GPCRs, including Calcium, cAMP, β-Arrestin, and receptor internalization. These assays are available to clients as continuous culture cell lines, ready-to-assay kits, and PathHunter Services. The ability to test these assays with a wide variety of readouts such as these enables the identification of allosteric compounds as well as biased ligands.

References