Detection of Endogenous Ligand Bias: A Case Study Using the Chemokine Receptor Family

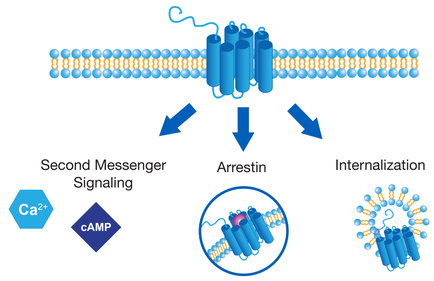
Tom S. Wehrman[1], Daniel L. Bassoni[1], James J. Campbell[2], Norma P. Gerard[3], Craig Gerard[3] and Sudarshan Rajagopal[4]

[1]DiscoveRx Corporation, [2]Department of Dermatology, Brigham and Women's Hospital, [3]Division of Respiratory Diseases, Department of Pediatrics, Children's Hospital, [4]Department of Medicine, Duke University Medical Center

Abstract

The pattern of intracellular signaling downstream of GPCR activation is now known to be specific to the ligand-receptor pair as opposed to an inherent, rigid property of the receptor. In a drug discovery setting, this property of GPCRs is now being exploited to develop designer molecules that have tailored intracellular responses. Although pathway bias is typically associated with synthetic molecules, we hypothesized that receptors having multiple endogenous ligands could use the same principles to tailor their cellular responses.
The chemokine receptor family was selected due to the ability of several ligands to activate multiple targets and vice versa. The receptor responses were profiled in second messenger, β-arrestin and internalization assays. Although many ligands signal equally well in all three systems, a surprising number of ligands exhibited significant bias. Importantly, these biased molecules also had differential effects when assayed on primary cells, confirming the differences observed using the recombinant systems. Taken together these results suggest that functional selectivity is a generally applicable phenomenon of synthetic as well as endogenous ligands.

DiscoveRx High-Content GPCR Characterization

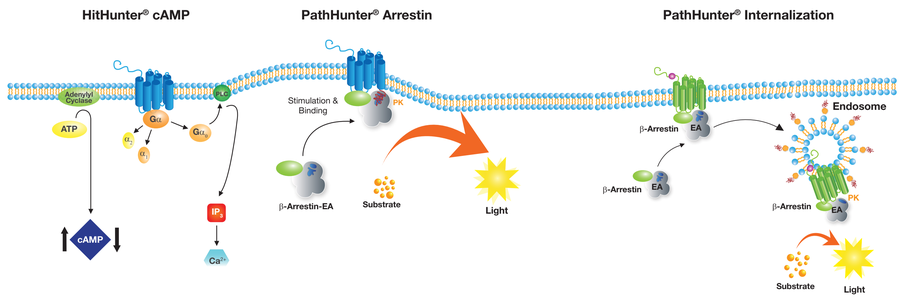


High-Content Compound Characterization

	2nd Messenger		Arrestin		Internalization	
	Potency	Efficacy	Potency	Efficacy	Potency	Efficacy
Compound X	10nM	Full	5nM	Partial	5nM	Partial
Compound Y	50nM	Full	35nM	Partial	35nM	Full
Compound Z	20nM	Partial	100nM	Full	100nM	Full

Characterization of GPCR agonism can differentially activate disparate receptor signaling events. This phenomenon of ligand bias has been observed across dozens of receptors. Importantly, the differential activation of selected pathways can have profound effects on the *in vivo* activity of the agonists. This realization of subtleties in GPCR activation necessitates observing their activity using different measures of GPCR activity in order to thoroughly characterize their activation patterns.

DiscoveRx Cell-Based GPCR Assay Formats

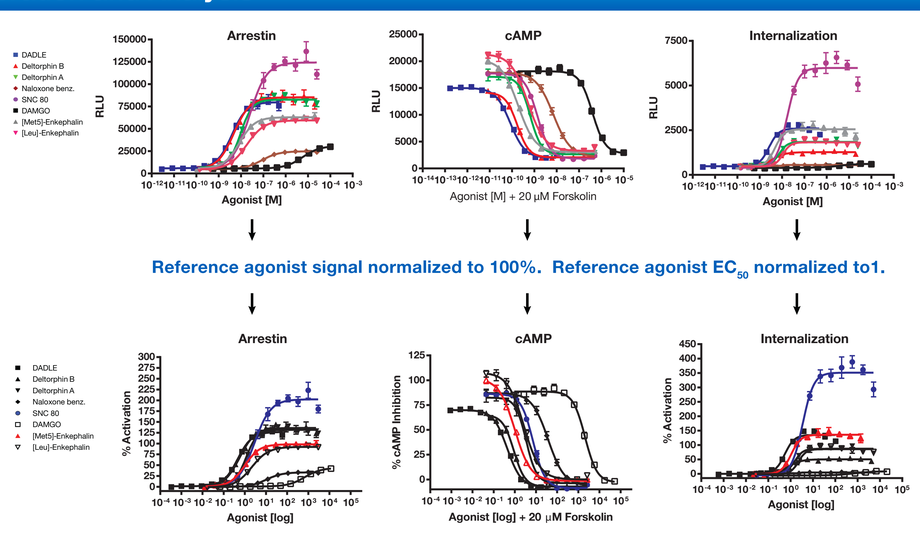


HitHunter® cAMP Assays. Free cAMP from cell lysates competes for antibody binding against labeled cAMP (ED-cAMP conjugate). Unbound ED-cAMP is free to complement EA to form active enzyme which subsequently hydrolyzes substrate to produce signal. A positive signal generated is directly proportional to the amount of free cAMP bound by the binding protein.

PathHunter® Arrestin Assays. Cell lines are engineered to co-express an optimized ED peptide, termed ProLink™ (PK), that weakly binds the complementary EA fragment, and the EA-tagged β-Arrestin protein. Activation of the GPCR-PK induces β-Arrestin-EA recruitment, forcing complementation of the two β-galactosidase enzyme fragments (EA and PK). The resulting functional enzyme hydrolyzes substrate to generate a chemiluminescent signal.

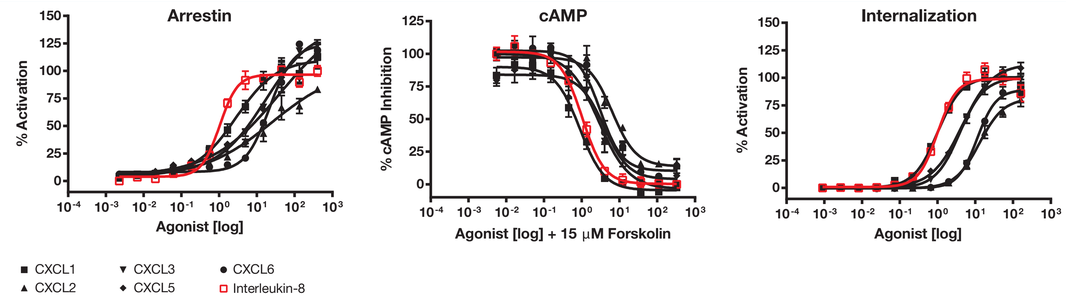
PathHunter® GPCR Internalization Assays. Cell lines co-express an untagged GPCR, an EA-tagged β-Arrestin and PK-tag linked to endofin, which is localized to endosomes. Activation of the untagged GPCR induces β-Arrestin recruitment to and internalization of the receptor/β-Arrestin-EA complex in PK-tagged endosomes. Therefore, this assay measures recruitment of receptor/β-Arrestin complexes to endosomes.

Data Generation and Analysis



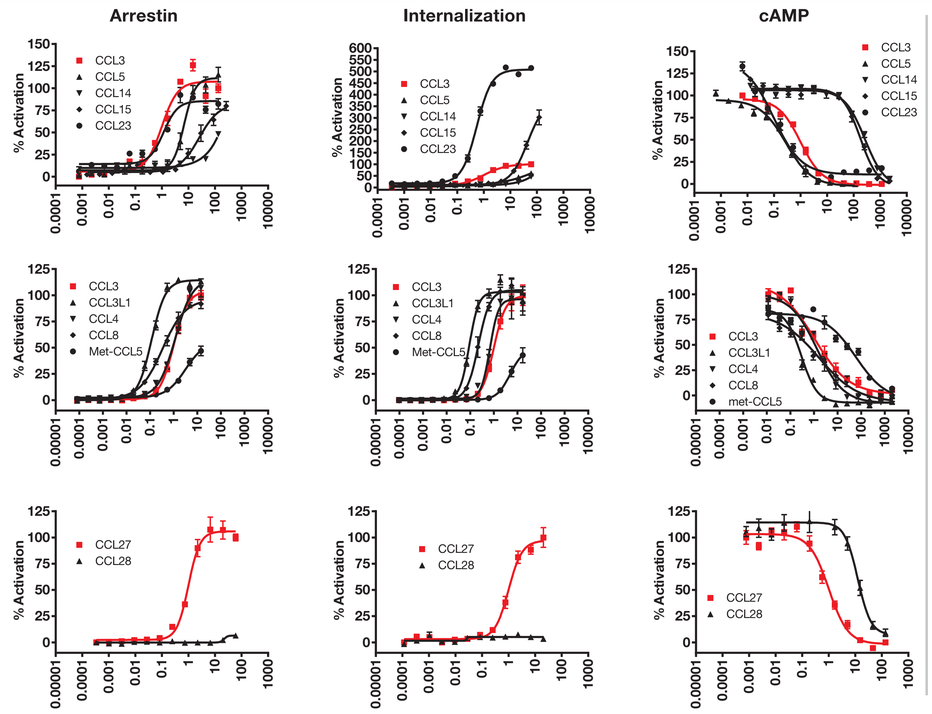
Cell lines overexpressing OPRD1 in the cAMP, Arrestin, and internalization formats were treated with the compounds listed at the top left. The signals for each assay are shown in the series. In order to enable a graphical representation of the data for determination of bias behavior, the data was normalized to the reference agonist [Met^5]-Enkephalin in potency (set equal to 1) and efficacy (set equal to 100%). In this example, SNC80 (blue curves), a known, strongly internalizing compound and functional antagonist, is clearly defined as a super agonist in the internalization assay.

Investigating Functional Selectivity



Cell lines overexpressing CXCR2 in the Arrestin, cAMP and Internalization formats were tested with six known chemokine agonists. Interleukin-8 (red curves) served as the reference ligand for this receptor. The relative potencies and efficacies of these ligands are similar in all three assay formats.

Discovery of Bias With Endogenous Ligands

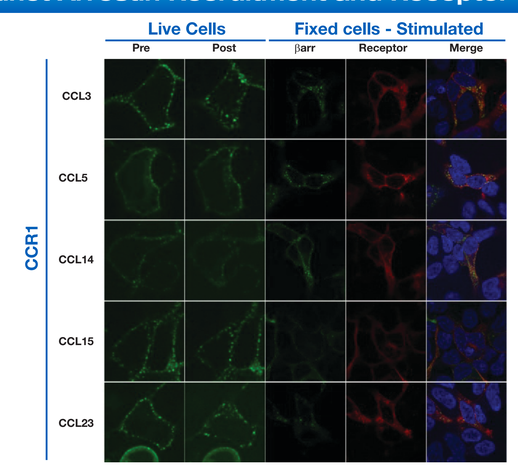


Ligands Scoring Positive for Bias

Target	Ligand	Bias
CCR1	CCL23, 15	Internalization
CCR1	CCL5, 23	G-protein
CCR5	CCL8	Arr/Int
CCR10	CCL28	G-Protein

Potency values are relative to the normalized potency (set equal to 1) of the reference ligand (in red). A potency value >1 indicates a ligand that is less potent than the reference ligand. Conversely, a potency value <1 indicates that the ligand is more potent than the reference ligand. Efficacy is expressed as a percentage, relative to the efficacy of the reference ligand (set at 100%).

Distinct Arrestin Recruitment and Receptor Internalization Profiles



Ligand stimulation results in a change in localization pattern of β-arrestin. The biased ligands identified in the PathHunter assays are shown to cause differential patterns of activation. Notably the compounds showing the highest efficacy in the internalization assay also exhibit a more pronounced internalization phenotype (βarr2-GFP –green; receptor –red; DNA - blue).

Summary & Conclusions

The ability to more accurately tailor cellular responses to GPCR ligands by exploiting the phenomenon of functional selectivity has opened up a number of new opportunities in drug discovery. Given the prevalence of ligand bias and the seemingly ubiquitous nature of this behavior it seemed likely that endogenous ligands could exploit the same pathways to design ligands with different behaviors. In order to address this question we developed assays for a number of chemokine receptors in three different formats: receptor internalization, G-protein signaling, and arrestin recruitment. The chemokine family presented an ideal case study with a wide range of ligands for each of the targets. A survey of these ligands against a handful of the targets showed that pathway selective activity could be readily identified in both a G-protein dependent and independent manner. These results highlight the added information that can be obtained from utilizing 2nd messenger in addition to arrestin and internalization for compound characterization of natural and synthetic molecules.

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tel | 1.866.448.4864 tel | +44.121.260.6142 e | info@discoverx.com

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