Quantifying GPCR Signaling Bias: A Simple Approach to Quantify Functional Selectivity and Agonist Bias

Dana Haley-Vicente, Poonam Kaul, Lakshmi Anantharaman, and Neil Charter

DiscoverX Corporation, Fremont, CA 94538

Abstract

Interest in GPCR ligand bias has increased in recent years due to evidence that positive and negative aspects of drug activity can be driven by differential pathway signaling. As a result, it is possible to develop drugs with reduced side effects that enhance positive effects through favoring one pathway over another. The availability of assays that can measure signaling events such as calcium mobilization, cAMP modulation, arrestin recruitment, and receptor internalization provides the ability to characterize compound action in multiple pathways. Here, we provide examples of ligand bias and how quantification of receptor potency and efficacy in different pathways can be used to generate a ligand bias index.

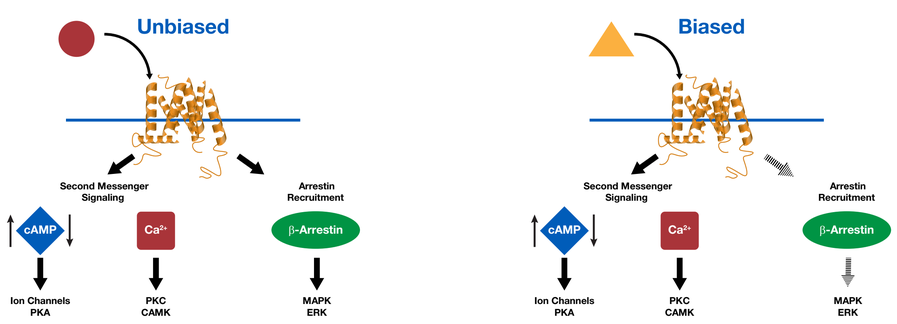
Methods

All cells lines used were from DiscoverX Corporation. For arrestin recruitment assays, cell lines stably express various GPCR tagged with ProLink and protein. 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. (1,2) For beta-arrestin recruitment assays, 5000 cells per well were seeded in 20 µL media and incubated overnight prior to assay. Agonist responses were induced by addition of 5 µL 5X compound. Incubation time was 120 minutes for arrestin. 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 on a Perkin Elmer Envision reader. Total arrestin recruitment was determined by incubating cells with 5 µL PathHunter Lysis buffer and 5 µL 10X EA reagent followed by 15 uL chemiluminescent substrate.

For second messenger assays, 10000 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 5 µL buffer plus 5 µL 4X compound. cAMP was detected after induction by one hour incubation with 20 µL of ED-cAMP / CL lysis buffer followed by an one hour incubation with 20 µL EA reagent. Chemiluminescence signal was read on a Perkin Elmer Envision reader. For calcium mobilization assays, media was exchanged with 20 µL dye loading buffer (Calcium No Wash™ kit). Agonist responses were induced by addition of 10 µL buffer and 10 µL 4X compound. Calcium mobilization was monitored for 2 minutes using a FLIPR Tetra (MDS, CA) after compound addition to determine agonist responses. Ligand bias was determined using tools kindly provided by Dr. Terry Kenakin (UNC School of Medicine). Compounds were tested in quadruplicate and singlicate curves were plotted with GraphPad Prism using the Black and Leff Operational Model (3,4) to derive values for log(τ/KA). Δlog(τ/KA) between reference agonist and test samples and ΔΔlog(τ/KA) between pathways were calculated to obtain a ligand bias value. Pooled results were used to calculate variability and determine 95% confidence levels (95% c.l.). Responses +/- 95% c.l. that are greater or less than zero were deemed to be significantly different and therefore to have induced a biased response. Relative Intrinsic activity was calculated as the maximal response G protein/maximal response beta-arrestin.

Ligand Bias

Ligand bias occurs when an agonist generates differential responses for one signaling pathway over another. In the example below, a biased ligand produces muted signaling via the arrestin pathway. Biased response can affect potency, efficacy or both.



Black & Leff Operational Model

$$Response = \frac{[A]^n \ \tau^n \ E_m}{[A]^n \ \tau^n + ([A] + K_A)^n}$$

The Black-Leff operational model (3,4) can be used to fit experimentally derived concentration response curves (CRC) to derive the parameters tau and K_A, where:

- A = Agonist
- KA = the equilibrium constant of the agonist-receptor complex
- tau (τ) = efficacy parameter
- n = slope coefficient for the CRC
- E_m = maximal response capability for the system

Quantifying Signal Bias

- Kenakin has demonstrated a model for comparing responses between different pathways by deriving a "transduction coefficient" that utilize the ligand and pathway specific parameter tau and the ligand-receptor specific parameter K_A (5).

$$Transduction\ Coefficient = \log\left(\frac{\tau}{K_A}\right)$$

- The efficiency of agonist relative to a reference agonist for a particular receptor and given pathway was defined as:

$$Normalized\ Transduction\ Coefficient = \Delta\log\left(\frac{\tau}{K_A}\right)$$

- Finally, two signaling pathways (j1, j2) can be compared for the same ligands to determine potential bias:

$$Bias = 10^{\Delta\Delta\log\left(\frac{\tau}{K_A}\right)_{j1-j2}}$$

where

$$10^{\Delta\Delta\log\left(\frac{\tau}{K_A}\right)_{j1-j2}} = \log bias = \Delta\log\left(\frac{\tau}{K_A}\right)_{j1} - \Delta\log\left(\frac{\tau}{K_A}\right)_{j2}$$

Acknowledgments

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- All cell lines were generated at DiscoverX and we want to thank everyone who contributed to the development.

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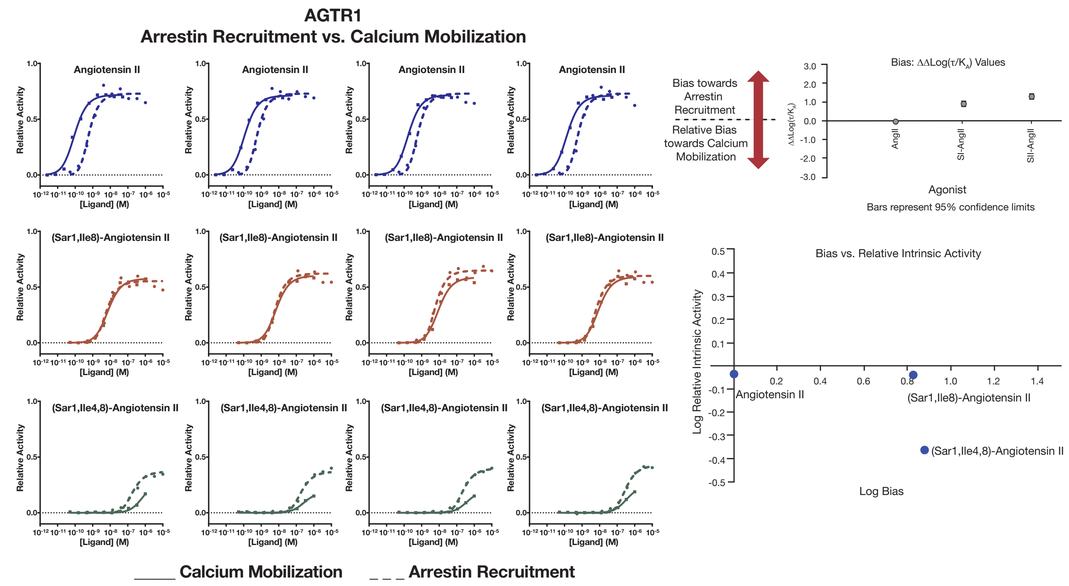
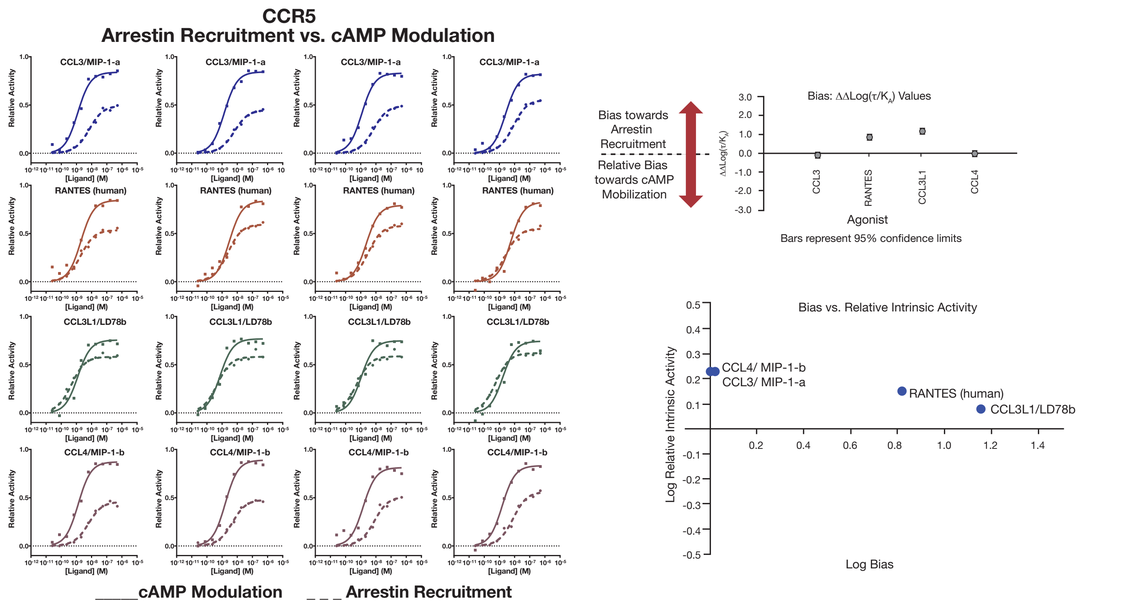
AT_1 Receptor



- Several ligands were tested with the angiotensin receptor and activity for calcium mobilization and arrestin recruitment determined. Data was normalized to activity relative to the Emax for each system. For calcium, ionomycin was used to determine the maximal response for the system. For arrestin, maximal EFC activity of tagged GPCR was determined by adding exogenous EA protein to cell lysates.
- By comparing responses relative to the natural ligand using the Operational Model, a value for ligand bias can be obtained. SI-AngII and SII-AngII both exhibited significant bias towards the arrestin pathway. 95% c.l. limits for the angiotensin derivatives were greater than 0 indicating that bias towards the arrestin pathway was significant. These results are consistent with published activity for these two peptides (6).
- Bias was plotted against relative intrinsic activity to identify key components in the biased response. SI-AngII exhibited significant bias but did not differ from AngII in terms of relative activity. This indicated that the primary component of bias for SI-AngII resulted from a shift in potency rather than efficacy. In contrast SII-AngII differed from AngII in both bias and intrinsic activity. This established that SII-AngII effected bias by shifting potency and efficacy in favor of the arrestin pathway.

CCR5 Receptor



- A number of chemokines were tested with the CCR5 receptor and activity for cAMP Gi signaling and arrestin recruitment determined. Data was normalized to activity relative to the Emax for each system. For cAMP, basal response was used to determine the maximal response for the system. For arrestin, maximal EFC activity of tagged CCR5 was determined by adding exogenous EA protein to cell lysates.
- By comparing responses relative to the natural ligand using the Operational Model, a value for ligand bias can be obtained for the chemokines. RANTES and CCL3L1 both exhibited significant bias towards the arrestin pathway. A third chemokine, CCL4, did not generate a biased response. 95% c.l. limits for RANTES and CCL3L1 were greater than 0 indicating the bias towards the arrestin pathway was significant. These results are consistent with published activity for these two peptides (5).
- Bias was plotted against relative intrinsic activity to identify key components in the biased response. RANTES and CCL3L1 exhibited significant bias and also resulted in a shift in relative activity towards the arrestin pathway. Therefore these chemokine effected bias through increased potency as well as improved efficacy in the arrestin pathway.

Summary

- Biased ligands offer the ability to develop novel drugs that selectively target the beneficial aspects of receptor signaling.
- Compound responses can be quantified by deriving tau and KA using the Black-Leff operational model.
- Kenakin has demonstrated that it is possible to characterize agonist selectivity using "transduction coefficient" term, log(τ/KA) and effectively compare effects between signaling pathways.
- This approach was tested for several GPCRs and ligands were evaluated for biased signaling between second messenger and arrestin pathways. Ligands were identified with statistically significant bias for one pathway over another. The results were consistent with published activity and demonstrate the ability to provide a quantitative assessment of ligand bias.
- Over 160 GPCR targets are available for biased ligand discovery at PathHunter Services. The majority of targets are available for second messenger and arrestin recruitment as well as receptor internalization.
- The gpcrBIAS service uses this established approach for defining the relative activities of samples in different signaling pathways. This method provides the means to characterize biased receptor signaling, independent of the cell background or expression level, to provide a statistically relevant value for ligand bias.

w discoverx.com usa 1.866.448.4864 eu +44.121.260.6142 e info@discoverx.com