Molecular Pharmacology Fast Forward. Published on May 24, 2011 as doi: 10.1124/mol.111.072801

MOL #72801

Quantifying Ligand Bias at Seven-Transmembrane Receptors

Sudarshan Rajagopal, Seungkirl Ahn, David H. Rominger, William Gowen-McDonald, Christopher M. Lam, Scott M. DeWire, Jonathan D. Violin and Robert J. Lefkowitz

Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA (SR, SA, CML, RJL), Trevena Inc, 1018 West 8th Avenue, Suite A, King of Prussia PA 19406, USA (DHR, WGM, SMD, JDV), Department of Biochemistry and Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA (RJL).

Molecular Pharmacology Fast Forward. Published on May 24, 2011 as DOI: 10.1124/mol.111.072801
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Running Title: Quantifying Ligand Bias at 7TMRs

Corresponding Author: Robert J. Lefkowitz, Duke University Medical Center, P.O. Box 3821, Durham, North Carolina 27710, email: lefk001@receptor-biol.duke.edu

Number of Text Pages: 33

Number of Tables: 2

Number of Figures: 6

Number of References: 36

Number of words in Abstract: 209

Number of words in Introduction: 945

Number of words in Discussion: 878

Abbreviations: Seven transmembrane receptors (7TMRs), G protein-coupled receptors (GPCRs), β2 adrenergic receptor (β2AR), angiotensin II type 1A receptor ($AT_{1A}R$), cyclic AMP (cAMP),

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Abstract

Seven transmembrane receptors (7TMRs), commonly referred to as G protein-coupled receptors (GPCRs), form a large part of the druggable genome. 7TMRs can signal through parallel pathways simultaneously, such as through heterotrimeric G proteins from different families, or, as more recently appreciated, through the multifunctional adapters, β-arrestins. Biased agonists, which signal with different efficacies to a receptor's multiple downstream pathways, are useful tools for deconvoluting this signaling complexity. These compounds may also be of therapeutic utility as they have distinct functional and therapeutic profiles from 'balanced agonists'. Although some methods have been proposed to identify biased ligands, no comparison of these methods applied to the same set of data has been performed. Therefore, at this time there are no generally accepted methods to quantify the relative bias of different ligands, making studies of biased signaling difficult. Here, we use complementary computational approaches for the quantification of ligand bias and demonstrate their application to two well-known drug targets, the β2 adrenergic (β2AR) and angiotensin II type 1A ($AT_{1A}R$) receptors. The strategy outlined here allows a quantification of ligand bias and the identification of weakly biased compounds. This general methodology should aid in deciphering complex signaling pathways and may also be useful for the development of novel biased therapeutic ligands as drugs.

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Introduction

For more than two decades it has been appreciated that a 7TMR can signal through parallel pathways simultaneously, such as through heterotrimeric G proteins from different families (Abramson et al., 1988; Fargin et al., 1989). It was soon discovered that ligands can have different efficacies for these different signaling pathways (Kenakin, 1995), a characteristic referred to as biased agonism or functional selectivity (Roth, 2009). Compared to "balanced agonists" that signal with equal efficacy to available downstream pathways, biased agonists have different efficacies for signaling to different G proteins (Kenakin, 1995) or to G proteins and the multifunctional adapter proteins β-arrestins (Gesty-Palmer et al., 2006; Wei et al., 2003). Unlike heterotrimeric G proteins, which classically act through the activation of second messengers such as cAMP, diacylglycerol or calcium, β-arrestins act as scaffolds for a number of signaling proteins, such as MAP kinases and E3 ubiquitin ligases (DeWire et al., 2007). Biased agonists are currently being developed as tools to dissect the signaling complexity downstream of 7TMRs and as novel therapeutics, as they appear to have different functional and physiological consequences from conventional balanced agonists (Rajagopal et al., 2010). For example, a β-arrestin-biased ligand of the parathyroid hormone receptor results in increased bone density without activating treatment-limiting catabolic pathways (Gesty-Palmer et al., 2009) and the novel AT_1R agonist TRV120027 selectively signals via β-arrestins, leading to increased cardiac performance with a reduction in blood pressure (Violin et al., 2010).

At this time, there are no widely accepted methods for quantifying ligand bias and most groups have relied on comparing the maximal effects (E_{max}) and potencies (EC_{50}) of ligands for different signaling pathways (Galandrin and Bouvier, 2006). However, these parameters cannot account for differences in receptor reserve and amplification of different assays (Rajagopal et al., 2010). In assays with significant amplification, such as second messenger assays, e.g., cyclic AMP formation, both full and partial agonists can reach the same maximal response (Fig. 1A), while in assays with little amplification, such as assays

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that monitor recruitment of β-arrestin to a receptor by enzyme complementation (Eglen et al., 2007), partial agonists have significantly lower maximal responses than full agonists (Rajagopal et al., 2010) (Fig. 1B). Therefore, a partial agonist that reaches maximal effect in one assay and half-maximal effect in another assay would be incorrectly identified as being biased compared to a full agonist, which reaches maximal response in both assays. A comparison of potencies is likewise limited by differences in receptor reserve between assays; as shown, the difference in potencies between the full agonist and partial agonist may be smaller in assays with less receptor reserve (Fig. 1C and D) (Rajagopal et al., 2010). Recent studies that have attempted to identify biased agonists using such comparisons (Galandrin and Bouvier, 2006; Molinari et al., 2010) may be confounded by these problems, although a reversal in rank order of efficacies or potencies would be evidence for ligand bias (Berg et al., 1998; Kenakin, 2007). More recently, a few approaches have been proposed for overcoming these problems (Figueroa et al., 2009; Gregory et al., 2010; Kenakin and Miller, 2010; Koole et al., 2010), but they have not been tested rigorously against one another and may have limitations (see below). With the rising interest in the development of biased agonists, a robust method for identifying weakly biased ligands and for quantifying ligand bias in 7TMR drug development is sorely needed.

Here we modify these approaches to develop a general methodology for identifying biased ligands and validate it at two well-characterized 7TMR drug targets, the β2AR and $AT_{1A}R$. This methodology utilizes complementary approaches that are based on comparisons of: 1) responses at the same ligand concentrations (*equimolar*) (Gregory et al., 2010); 2) ligand concentrations that result in *equiactive* responses (Figueroa et al., 2009); and 3) estimates of coupling efficiency derived from the *operational* model (Black and Leff, 1983; Evans et al., 2010; Kenakin and Miller, 2010) using experimentally determined dissociation constants. The first two approaches can allow the identification of weakly biased ligands with concentration-response data alone but are not as robust as the operational model, which, while requiring an experimentally determined dissociation constant, allows

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an estimate of ligand efficacy and a calculation of ligand bias. Thus, these complementary approaches can serve in a general strategy for the development of biased ligands.

Materials and Methods

Materials

The β2AR ligands isoproterenol, epinephrine, dobutamine, dichloroisoproterenol, fenoterol, salbutamol, norepinephrine, formoterol, clenbuterol, salmeterol and pindolol were all obtained from Sigma (St. Louis, MO). The $AT_{1A}R$ ligands Angiotensin II, SGG, S1C4 and A1 were custom synthesized by Genscript (Piscataway, NJ). The ligands TRV120026 (Sar-Arg-Val-Tyr-Tyr-His-Pro-NH_2), TRV120055 (Sar-Arg-Val-Tyr-Val-His-NH_2), TRV120056 (Asp-Arg-Val-Tyr-Ile-His-Pro-Gly), TRV120044 (NMAla-Arg-Val-Tyr-Ile-His-Pro-D-Ala), TRV120045 (Sar-Arg-Val-Tyr-Arg-His-Pro-NH_2) and TRV120034 (NMAla-Arg-Val-Tyr-Ile-His-Pro-Ala) were custom synthesized (Sar denotes sarcosine, NMAla denotes N-methyl-L-alanine and NH_2 denotes an amino group at the C-terminus) by Trevena, Inc. (King of Prussia, PA). Bright-Glo and Glosensor reagents were obtained from Promega (Madison, WI). Reagents for the IP-One HTRF assay were obtained from Cisbio Bioassays (Bedford, MA). Reagents for the DiscoveRx PathHunter β-arrestin assay were obtained from DiscoveRx (Fremont, CA). The Tango construct for the β2AR was provided by Gilad Barnea and Richard Axel.

β-arrestin recruitment assays

For the β2AR, β-arrestin recruitment to receptor was assessed by the Tango assay, as previously described by Barnea et al. (Barnea et al., 2008). In this assay, the C-terminus of the human β2AR is replaced with the C-terminal tail of the V2 vasopressin receptor tail (to increase signal-to-noise) followed by a TEV protease cleavage site and a tTA transcription factor. This construct was stably transfected in HEK293 cells along with a construct encoding β-arrestin 2 fused to TEV protease. Upon

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ligand stimulation, the recruitment of β-arrestin to the receptor results in the cleavage tTA from the receptor. The tTA translocates to the nucleus where it transcribes a stably expressing luciferase reporter gene. HEK293 cells stably transfected with these constructs were seeded at 25,000 cells per well in a 96 well plate. The next day, compounds diluted in PBS were added to the wells to their final concentration followed by incubation at 37 degrees for 14-20 hours. The next day, the plate was cooled to room temperature, and an equal amount of Bright Glo luciferase assay reagent (Promega) was added to each well. After 5 min, luminescence was read in a NOVOstar microplate reader (BMG Labtech, Durham, NC). To ensure that the results obtained using this technology were not an artifact of the overnight incubation with ligand or the V2R tail, we also used the PathHunter β-arrestin assay from DiscoveRx (see below), which uses the human β2AR (with a Prolink peptide added to the C-terminus) with a shorter incubation time with ligand (~ 30 minutes), representative data of which is shown in Figure S5.

For the $AT_{1A}R$, we used the PathHunter β-arrestin assay from DiscoveRx and read for chemiluminescent signaling on a PheraStar reader (BMG Labtech, Durham, NC) as previously described (Violin et al., 2010). Briefly, complementary halves of β-galactosidase were genetically fused to the carboxyl termini of the human AT_1R and β-arrestin2. When co-transfected, the two fusion proteins serve as a proximity sensor; when β-arrestin 2 translocates to active receptor, the β-galactosidase fragments interact to form a functional enzyme, which is detected by a chemoluminescent substrate.

cAMP assay

The GloSensor™ cAMP biosensor (Promega) uses a modified form of firefly luciferase containing a cAMP-binding motif (Fan et al., 2008). Upon cAMP binding a conformational change leads to enzyme complementation and incubation with a luciferase substrate results in a luminescence readout. Analysis of cAMP accumulation was performed in HEK293 cells stably transfected with the Glosensor construct and the human β2AR. Cells were seeded in 96-well white, clear-bottomed plates at 80,000 cells/well, in

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MEM supplemented with 10% fetal bovine serum (10% v/v). The next day the GloSensor reagent (Promega; 4% v/v) was incubated at room temperature for 2 h. Cells were then stimulated with a range of β_2 AR agonists for 5 min and increases in luminescence read on a NOVOstar microplate reader (BMG Labtech, Durham, NC). These assays were repeated in the Tango cell lines used for the β-arrestin recruitment assays with transient transfection of the Glosensor construct, which demonstrated the same behavior, albeit with poorer signal-to-noise (Figure S6).

IP_1 assay

IP_1, a downstream metabolite of IP_3 that is downstream of signaling by G_q, was detected by the IP-One Tb HTRF kit (Cisbio, Bedford, MA) as previously described (Violin et al., 2010). Plates were read on a PheraStar reader using a time-resolved fluorescence ratio (665nm/620nm).

Angiotensin II Type IA receptor competition membrane radioligand binding assays.

HEK293 cells with stable expression of the rat (r) AT1 receptor were harvested by centrifugation at 400xg for 30min at 4°C, washed once with a balanced salt solution, re-pelleted, and the pellet flash frozen in liquid nitrogen. The cell pellets were stored at -80°C until processed for membranes. Pellets were resuspended in buffer (50 mM HEPES, 2 mM EDTA pH 7.4 containing fresh protease inhibitors - Complete Brand protease tablets from Roche Diagnostics (Indianapolis, IN) and subjected to nitrogen cavitation with a Parr Cell Disruption Bomb (Parr Instrument Co.,Moline, IL) at 1000 psi for 20 min on ice. Ruptured cells were sedimented at 500g for 10 min at 4°C and the supernatant containing cellular membranes was washed twice at 48,000g for 15 min. cell pellets were re-suspended at 4°C in 10 volumes of ice-cold buffer A and cavitation, placed on ice. To remove large particles, a low speed centrifugation (500xg for 30 min at 4°C) was performed, followed by high-speed centrifugation (48,000xg for 45 min at 4°C), re-suspension in buffer plus protease inhibitor cocktail, and a final high

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speed centrifugation at (48,000g for 45 min at 4°C). A dounce homogenizer was used to resuspend the final pellet using ice-cold buffer. The membrane suspension was passed through a 23G needle, aliquots made, and stored at -80°C. Total protein concentration of the membrane preparation was determined with a Coomassie Plus Reagent Kit from Pierce Biotechnology (Rockford, IL) using bovine serum albumin as the standard.

Membranes were diluted in assay buffer (50 mM Hepes, 150 mM NaCl, 5mM $MgCl_2$, Gpp(NH)p 10 µM pH 7.2 at 23°C) to a concentration of 1-3 µg protein/well. Assays were initiated by the addition of 94 µl of membrane suspension to 200 µl of [^{125}I]-Sar^{1}Ile^8-Angiotensin II ([^{125}I]- Sar^{1}Ile^8-ANGII, specific activity 2200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), at 0.4-1 times K_d and various concentrations of inhibitors in buffer plus a cocktail of protease inhibitors and 0.02% BSA to reduce non-specific radioligand binding. Compounds were diluted in DMSO and tested at a final concentration of 1% DMSO (determined to be non-detrimental to the assay). Competition binding with compounds (11 point concentrations) was performed in polypropylene 96 well plates (Costar Corp., Cambridge, MA). Nonspecific binding was defined in the presence of 10µM losartan. Competition assays were performed at 23°C for 4 hours to allow adequate time for compounds and radioligand to reach equilibrium for binding. The separation of bound from free radioligand was accomplished by rapid vacuum filtration of the incubation mixture over GF/B uni-filter (polyethylenamine-treated) plates (Perkin Elmer, Waltham, MA) using a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were washed 2 times with 0.3 ml of ice-cold phosphate buffered saline pH 7.0 containing 0.01% Triton X100. Radioactivity on the filters was quantified using a MicroBeta TriLux Liquid Scintillation Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

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Data Analysis

For radioligand binding, calculation of apparent binding affinities, $K_i = IC_{50}/ (1+ [Radioligand]/K_d)$ was performed using the nonlinear iterative curve-fitting computer program GraphPad PRISM (San Diego, CA). All fitting using the equiactive approach and operational model was performed using GraphPad PRISM (San Diego, CA). For the β2AR, reported dissociation constants for ligands were used from del Carmine et al. (Del Carmine et al., 2002).

Equiactive Comparison

The equiactive comparison is analogous to the method employed by Furchgott (Furchgott, 1966) to determine the dissociation constant of agonists. In this approach, the concentrations of ligand required for an equiactive response for pathway 1 ($[A_1]$) and 2 ($[A_2]$) are extrapolated from fits of each concentration-response curve (Fig. 1C). A linear relationship between the inverse of these concentrations is then given by (see Supplementary Methods):

$$\frac{1}{[A_2]} = \frac{a_2}{a_1}\frac{\varepsilon_2}{\varepsilon_1} * \frac{1}{[A_1]} + \frac{1}{K_D}\left(\frac{a_2}{a_1}\frac{\varepsilon_2}{\varepsilon_1}\left(1+\varepsilon_1\right) - \left(1+\varepsilon_2\right)\right)$$

A bias factor, which quantifies the relative stabilization of one signaling state over another compared to the reference agonist, can then be calculated as:

$$\beta = \log\left(\frac{RA_{12,lig}}{RA_{12,ref}}\right) = \log\left(\left(\frac{E_{max,1}}{EC_{50,1}}\frac{EC_{50,2}}{E_{max,2}}\right)_{lig} * \left(\frac{E_{max,2}}{EC_{50,2}}\frac{EC_{50,1}}{E_{max,1}}\right)_{ref}\right)$$

Operational Model

We chose to use the operational model of Black and Leff (Black and Leff, 1983) to quantify the effective signaling by receptors. In the operational model, the response of the system to ligand stimulation is based on receptor occupancy alone, as the ligand:receptor complex is coupled to downstream signaling

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pathways without any allosteric component. The response of the system is then related to ligand concentration (when the Hill coefficient is 1):

$$\frac{E}{E_m} = \frac{\tau\,[A]}{\tau\,[A] + ([A] + K_D)}$$

where E_m is the maximal response of the system to a full agonist, K_D is the agonist dissociation constant and τ is "coupling efficiency" between the agonist:receptor complex and its downstream signaling partners. This coupling efficiency τ can be considered to be composed of two components ($\tau=\tau^*\varepsilon$), where the τ^* term accounts for the amplification inherent to the downstream signaling pathway that is the same for all ligands in the same assay, and the other component (ε) accounts for a ligand's efficacy at generating a signaling-competent agonist:receptor conformation. The ability of an agonist to signal to downstream pathways can then be compared to a reference agonist by the effective signaling (σ_{lig}):

$$\sigma_{lig} = \log\left(\frac{\tau_{lig}}{\tau_{ref}}\right) = \log\left(\frac{\varepsilon_{lig}}{\varepsilon_{ref}}\right)$$

A bias factor, β, equal to the distance from the point (σ_{path1}, σ_{path2}) for a ligand to the line of unity for balanced ligands, can then be calculated as the difference between the effective signaling factors (Fig. 1C) in relation to balanced agonists:

$$\beta_{lig} = \frac{\sigma_{lig}^{path1} - \sigma_{lig}^{path2}}{\sqrt{2}}$$

Results

Development of Approaches for the Quantification of Ligand Bias

We used three general approaches to identify biased ligands (Fig. 2). The first of these is a qualitative approach to identify biased ligands originally proposed by Christopoulos and coworkers (Gregory et al., 2010), which we refer to as an 'equimolar comparison'. As shown in Fig. 2A, data for a single ligand is collected in 2 different assays, such as those for G protein and β-arrestin signaling. The responses of

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these 2 different assays at the same concentration of ligand are then plotted against each other; therefore, the shape of this curve is a direct comparison of the signaling through the two different pathways. The shape of this curve may vary depending on the assays compared; for example, two assays based on biosensors for the same second messenger may have different sensitivities to the messenger, one with nanomolar and the other with picomolar sensitivity. If their concentration-response data were plotted against each other, a hyperbolic curve would be obtained, suggesting bias towards the assay with picomolar sensitivity although no true underlying bias would actually be present. Therefore, to identify biased ligands using this method, the shape of the equimolar curve for a test ligand must be qualitatively compared to that of a reference balanced agonist (Fig. 2A, right panel). In the example shown, the test agonist is biased towards response 1 compared to the reference agonist (dashed line), which by definition is balanced.

The second approach is a quantitative 'equiactive comparison' between two different assays for a ligand (Fig. 2B). This is analogous to pharmacologic methods for the estimation of agonist affinity (Furchgott, 1966), but by comparing the various assays downstream of the receptor, a quantification of bias can be obtained. In most cases, this comparison can be performed using a simplified formula with intrinsic relative activities proposed by Ehlert (Ehlert, 2008), which can be calculated from maximal effects and potencies (Figueroa et al., 2009) (Fig. 1B, middle panel) (see Suppl. Materials). A 'bias factor' (denoted β) is calculated as the logarithm of the ratio of intrinsic relative activities for a ligand at two different assays compared to a reference agonist (Fig. 2B, right panel) (Materials and Methods). This bias factor is an estimate for the molecular efficacy of pathway 1 versus pathway 2 on a logarithmic scale, e.g., a bias factor of 1 between 2 pathways means that a ligand is 10 times better at generating the active receptor conformation for one pathway over the other pathway compared to the reference balanced agonist (Equation 1).

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$$\beta = \log\left(\left(\frac{E_{max,P1}}{EC_{50,P1}}\frac{EC_{50,P2}}{E_{max,P2}}\right)_{lig} * \left(\frac{E_{max,P2}}{EC_{50,P2}}\frac{EC_{50,P1}}{E_{max,P1}}\right)_{ref}\right) \quad (1)$$

where P1 and P2 denote signaling through pathways 1 and 2 respectively.

The third approach is based on classic pharmacologic models that were originally developed to account for receptor reserve and shifts in agonist concentration-response curves, the first of which was proposed by Stephenson (Stephenson, 1956). We chose to use the operational model of Black and Leff (Black and Leff, 1983), which allows the calculation of a coupling efficiency to each downstream signaling pathway, and has recently been proposed as a method to quantify bias (Evans et al., 2010; Kenakin and Miller, 2010). To calculate this coupling efficiency, referred to as τ, concentration-response data is fit by equation 2 (Materials and Methods) using the dissociation constant of the ligand for the receptor from a separate binding experiment (McPherson et al., 2010) (Fig. 2C, left panel). By comparing these coupling efficiencies to that of a reference compound, the effective signaling (σ) by a ligand in each assay can be calculated (Equation 3). A comparison of effective signaling between different pathways can be performed by the calculation of bias factors (β) (Equation 4), equal to the distance from the point (σ_{path1}, σ_{path2}) to the line of unity for each ligand (resulting in division by the square root of 2), thereby allowing the identification of biased ligands (Fig. 2C, right panel).

$$\frac{E}{E_m} = \frac{\tau\,[A]}{\tau\,[A]+([A]+K_D)} \quad (2)$$

$$\sigma_{lig} = \log\left(\frac{\tau_{lig}}{\tau_{ref}}\right) \quad (3)$$

$$\beta = \frac{\sigma_{path1}-\sigma_{path2}}{\sqrt{2}} \quad (4)$$

If there are errors in the dissociation constants used in the operational model, such as those associated with different conditions used for ligand binding and functional assays, they would be expected to largely cancel out in a calculation of bias factors as the higher or lower effective signaling associated with those errors should affect estimations of both pathways similarly. If the dissociation constants are

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left-shifted relative to the EC_{50}s, an observation which cannot be accounted by any pharmacologic model, it should be obvious from the poor fits to the data (which were not observed in this study).

Identification of Biased Ligands at the β2AR

The β2AR is a prototype for 7TMRs and is a drug target in the treatment of heart failure and asthma. At this receptor, the identification of a partially β-arrestin-biased agonist may allow for the development of more strongly biased agonists with possible therapeutic utility. Upon stimulation by its endogenous agonists epinephrine and norepinephrine, the β2AR signals to G proteins, which increase cAMP formation by adenylate cyclase, and β-arrestins, which signal to a wide range of intracellular targets (DeWire et al., 2007). No strongly biased ligands have been identified at this receptor, although the 'beta blocker' carvedilol does lead to weak β-arrestin recruitment and signaling in the absence of G protein activation (Wisler et al., 2007). Other studies at this receptor have also identified potentially β-arrestin-biased agonists using direct comparisons of pharmacologic or biophysical parameters (Drake et al., 2008; Galandrin and Bouvier, 2006; Reiner et al., 2010).

We collected concentration-response data for β-arrestin recruitment, using an assay based on release of a transcription factor upon β-arrestin recruitment to a modified receptor (Barnea et al., 2008), and cAMP generation, using a luminescence-based cAMP biosensor (Fan et al., 2008), for a panel of clinically used β2AR ligands (Fig. 3A-F). There is a suggestion of bias in this data as formoterol is more potent than isoproterenol in the β-arrestin recruitment assay while the two drugs are equipotent in the cAMP assay (Fig. S1). However, an equimolar comparison does not demonstrate any significantly biased compounds (Fig. 4A and Fig. S2), due to the large difference in amplification between the cAMP and β-arrestin recruitment assays that results in strongly hyperbolic equimolar comparison curves for all compounds. The concentration-response data were fit well by logistic equations (Supp. Table S1) and bias factors were calculated using an equiactive comparison (Fig. 4B, Table 1). While the equimolar

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comparison was unable to identify any biased ligands, the equiactive analysis identifies a number of potentially β-arrestin biased compounds: pindolol (Pin), dichloroisoproterenol (DCI), salmeterol (Slm) and formoterol (For) ($p < 0.05$ by unpaired t-test). However, for the weak partial agonists pindolol and DCI, the change in bias factor is driven by differences in the EC_{50} between the cAMP and β-arrestin assays derived from poor fits (Fig. S3). The fits for formoterol and salmeterol do not suffer from this problem and their calculated bias factors likely represent a true difference in efficacies between the G protein- and β-arrestin-mediated pathways.

The operational model was then used to fit this data and calculate relative signaling efficacies compared to epinephrine, which was chosen as the reference compound because it is an endogenous agonist that activates the receptor physiologically. A comparison of the effective signaling in each pathway ($\sigma_{pathway}$) of the panel of ligands to epinephrine is shown in Fig. 4C. Balanced compounds, with similar bias to epinephrine, would be expected to lie on a line of unity in this analysis (red line, Fig. 4C). Epinephrine, considered a full agonist in most studies, is actually less effective in stabilization of the G protein- and β-arrestin signaling states than the synthetic agents fenoterol and isoproterenol, neither of which appear biased. Bias factors analogous to those calculated from the equactive comparison were then calculated (Fig. 4D). Here, formoterol and salmeterol are again identified as having bias towards β-arrestin recruitment ($p < 0.05$ by unpaired t-test). Pindolol and DCI, identified as biased compounds in the equiactive comparison, are not significantly biased in this analysis.

Notably, the three different approaches for quantifying bias yielded different results. A major limitation in the equimolar comparison is its inability to identify weakly biased agonists when assays have significantly different levels of amplification. The equiactive comparison performed poorly with data from suboptimal fits of weak partial agonists (Pin and DCI), which display little signaling activity. This problem is less of an issue with the operational model, where the additional information provided by the dissociation constant improves the quality of these fits and yields a better estimation of the bias

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factors. Therefore, we conclude that formoterol and salmeterol, two long acting beta agonists (LABAs) in our panel of ligands, are β-arrestin-biased agonists of the β2AR. These compounds were not identified as biased in a previous analysis of β2AR ligands (Drake et al., 2008), a finding that is likely due to differences in the assays used for assessment of G protein and β-arrestin signaling and the methodology for quantifying bias. In that earlier study, both signaling parameters had significant kinetic components, with β-arrestin signaling quantified by the rate of β-arrestin recruitment to the receptor as measured by fluorescence resonance energy transfer and G protein signaling quantified by the integrated signal of a cAMP-binding fluorescent biosensor over time. In this study, the assays used have significantly higher levels of amplification and are measured at a single late time point. Also of note, norepinephrine, which was identified as a biased agonist in a recent publication based on biophysical and signaling experiments performed at saturating doses of ligands (Reiner et al., 2010), does not display any significant signaling bias compared to epinephrine. Carvedilol, a weakly β-arrestin-biased agonist, was not tested in these assays as it is an inverse agonist of G protein signaling (Wisler et al., 2007) and, therefore, by definition is biased.

Some rather counter-intuitive findings arise from this type of analysis compared to one based on the classic pharmacologic parameters of maximal responses and potencies. In a comparison of maximal responses, it would appear that dobutamine would be a strongly cAMP-biased agonist, reaching a maximal response in the cAMP assay (Emax ~ 96%) but only very limited activity (E_{max} < 5%) in the β-arrestin recruitment assay. This finding, however, is wholly due to the weak partial agonism of dobutamine, which can still lead to a maximal response in the assay with significant receptor reserve and amplification (cAMP formation) but results in a very weak response in an assay with little receptor reserve (β-arrestin recruitment). Thus, within the errors of this experiment, the response of dobutamine is no different than a low dose (~ 10 nM) of the reference agonist epinephrine. However, no

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concentration of epinephrine could result in the pattern of cAMP formation and β-arrestin recruitment of a truly biased agonist, such as formoterol.

Identification of Biased Ligands at the $AT_{1A}R$

The $AT_{1A}R$ is notable among 7TMRs in that a number of well-characterized β-arrestin-biased agonists have been described at this receptor. These include $Sar^1Gly^4Gly^8$ (SGG) and $Sar^1Ile^4Ile^8$ angiotensin II (SII) (Holloway et al., 2002). SII recruits β-arrestin and leads to β-arrestin-mediated ERK phosphorylation in the absence of significant G protein activation. SII is also capable of enhancing the contraction of isolated cardiac myocytes (Rajagopal et al., 2006), as does a more potent β-arrestin-biased agonist, TRV120027, which has been found to reduce blood pressure and increase cardiac performance in rats (Violin et al., 2010). We chose a panel of ten derivatives of angiotensin II (AngII) to test whether those compounds had more bias than the index compound SGG. We used an assay for β-arrestin recruitment based on enzyme complementation (Fig. 5A-C) and an assay for G_q signaling based on inositol 1-phosphate (IP_1) formation (Fig. 5D-F). Notably, from a comparison of representative concentration-response curves, a number of compounds appear to be biased, with partial activity with respect to β-arrestin recruitment and little IP_1 formation.

The equimolar comparison clearly identifies such compounds (TRV120026, TRV120034, TRV120045, TRV120044 and SGG) as β-arrestin-biased ligands while the other compounds appear to be balanced (TRV120055, TRV120056, A1 and S1C4) (Fig. 6A and Supp. Fig. S4). For example, the SGG and TRV120044 compounds are shifted to the left portion of the plot while the balanced agonists AngII and TRV120055 both have similar hyperbolic shapes consistent with increased amplification in the IP_1 assay compared to the β-arrestin recruitment assay (Fig. 6A). The plots for these two β-arrestin-biased compounds suggest that TRV120044 (red) has more β-arrestin bias than SGG (green), although it is difficult to ascertain in such a qualitative analysis. Bias factors for all of the compounds using the

Molecular Pharmacology Fast Forward. Published on May 24, 2011 as DOI: 10.1124/mol.111.072801
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equiactive approach were then calculated (Fig. 6B). Consistent with the equimolar comparison, the TRV120026, TRV120034, TRV120044, TRV120045 and SGG compounds all had bias factors consistent with β-arrestin bias, although the large errors for a number of these compounds led to the differences being statistically insignificant. This was due to the poor fits of the IP_1 concentration-response data, where many of the compounds displayed little to no signaling activity.

We then compared effective signaling for the G protein and β-arrestin-mediated pathways as calculated by the operational model (Fig. 6C) using experimentally determined dissociation constants from radioligand competition binding (Table S3). Again the compounds separate into two groups, with the β-arrestin-biased compounds displaying preserved β-arrestin signaling in the absence of G protein signaling and a number of balanced compounds that signal through both pathways. This was confirmed by a calculation of bias factors derived from the operational model (Fig. 6D), which have an excellent correlation with the bias factors calculated using the equiactive approach (Table 2). While some of the synthetic compounds do not appear to display any significant bias, such as TRV120055 and TRV120056, other compounds have nearly an order of magnitude more bias than the initially described β-arrestin-biased agonist SGG (Holloway et al., 2002). In this case, all three approaches yielded similar results.

Discussion

In this work, we develop a general methodology for the quantification of ligand bias by using three different approaches, each with its own strengths and weaknesses. Both the equimolar and equiactive approaches are free of the assumptions inherent in pharmacologic models, e.g., that signaling is mediated by a 1:1 receptor:agonist complex and that the effects are due to a receptor:ligand complex at equilibrium. Therefore they can be used more generally, e.g., for analyzing bias in systems with receptor dimers or allosteric modulators. While the equimolar comparison is intuitively appealing and graphically displays different levels of bias, it is unable to identify weakly biased ligands when assays

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with markedly different levels of amplification are compared, and, more generally, it is unable to quantify bias. The equiactive comparison allows for a quantification of bias, however the resulting bias factors are prone to error with partial agonists or strongly biased compounds due to the poor fits of the concentration-response data with weak signal-to-noise levels. In contrast, these large errors are not as problematic in the operational model, where the additional information from a separate ligand binding experiment constrains the fits and yields a better estimate of bias. This model not only allows for quantification of bias, but also yields an estimate of efficacy, the effective signaling (σ). Therefore, we conclude that the best approach to quantifying bias is given by the operational model, although a good estimate of bias can be obtained using the equiactive comparison if the dissociation constant for a ligand is not known.

Several approaches have been previously proposed to quantify ligand bias in an effort to overcome the limitations associated with an analysis of classic pharmacologic parameters. Some methods are qualitative, such as 'bias plots' (Gregory et al., 2010) or a comparison of rank order of potencies (Kenakin, 1995), while others are quantitative, such as comparisons of transduction ratios (Evans et al., 2010; Gregory et al., 2010; Kenakin and Miller, 2010; Koole et al., 2010) or intrinsic relative activities (Ehlert, 2008; Figueroa et al., 2009). The qualitative approaches to identify biased ligands are inherently limited in their scope, while the current quantitative approaches have theoretical or practical limitations. Recently, "transduction ratios" (Evans et al., 2010; Figueroa et al., 2009; Gregory et al., 2010; Kenakin and Miller, 2010; Koole et al., 2010), defined as τ/K_A derived from the operational model (where K_A denotes the dissociation constant), have been used to estimate ligand bias. We chose not to use this approach for a number of reasons. First, the parameters of interest in assessing bias are the ligand's different efficacies through different signaling pathways, which is quantified by the ligand's coupling efficiency (τ) for the different pathways (Black and Leff, 1983; McPherson et al., 2010) and not by its dissociation constant, K_A (see Suppl. Materials). Second, in these studies, the effective dissociation

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constant is derived directly from the concentration-response data itself (Evans et al., 2010; Gregory et al., 2010; Koole et al., 2010), which may differ for the same ligand in different signaling assays due to the formation of different receptor ternary complexes with G proteins or β-arrestins in each assay (Colquhoun, 1985; De Lean et al., 1980). Also, the detailed method for fitting data using the transduction ratio approach has yet to be published (Evans et al., 2010). In fitting our data, we chose to use a dissociation constant determined from competition radioligand binding experiments under conditions that would limit formation of a receptor ternary complex, which should allow a separation of affinity and efficacy (Kenakin, 1999) in our analysis. Even with these differences, the bias factors calculated from transduction ratios (Evans et al., 2010) should be similar to those from our operational analysis as the dissociation constant terms would largely cancel out.

Over the past few years there has been an explosion in publications describing the identification of biased agonists at a wide variety of 7TMRs (recently reviewed by Whalen et al. (Whalen et al., 2010)). In such studies, it is important to optimize experimental conditions to avoid the false identification of biased ligands due to differences in compound stability or variations in cell types and other conditions used for different assays. Many biased ligands have been identified in screening, while for other well-known drug targets such as the β2AR, strongly biased agonists have yet to be identified. However, most presumably biased compounds have been identified based on comparisons of classic pharmacological parameters such as EC_{50} and E_{max}, which are prone to errors in interpretation in the setting of receptor reserve. Therefore, it is still unclear the extent to which these ligands are biased. Conversely, it is likely that there are a number of weakly biased ligands that have yet to be identified due to the inability to properly quantify ligand bias. Here we have demonstrated that weakly biased ligands, which could serve as tool compounds to dissect receptor biology or as lead compounds in the drug development process, can be identified using these approaches. Notably, the weakly biased ligands identified at the β2AR, formoterol and salmeterol, are used clinically, suggesting that a number of drugs that are used in the

clinic today may also be similarly biased. The ability to quantify such signaling bias may facilitate the mechanistic understanding of both desirable and undesirable properties of such therapeutics.

Acknowledgements

We thank Tommaso Costa, Terry Kenakin, Arthur Christopoulos and Ryan Strachan for valuable discussions. We thank Gilad Barnea and Richard Axel for providing the β2AR Tango construct. We thank Donna Addison and Quivetta Lennon for secretarial assistance.

Authorship Contributions

Participated in Research Design: Rajagopal, Ahn, Rominger, Gowen-McDonald, DeWire, Violin, Lefkowitz

Conducted Experiments: Rajagopal, Ahn, Rominger, Gowen-McDonald, Lam, DeWire

Performed Data Analysis: Rajagopal, Ahn, Rominger, Gowen-McDonald

Wrote or contributed to the writing of the manuscript: Rajagopal, Ahn, DeWire, Violin, Lefkowitz

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Molecular Pharmacology Fast Forward. Published on May 24, 2011 as DOI: 10.1124/mol.111.072801
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Footnotes

This work was supported in part by National Institutes of Health Grants [HL16037 and HL70631] to RJL. RJL is an Investigator of the Howard Hughes Medical Institute. SR is supported by a National Institutes of Health T32 training grant [HL07101-34].

Molecular Pharmacology Fast Forward. Published on May 24, 2011 as DOI: 10.1124/mol.111.072801
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Figure Legends

Figure 1. Limitations of Classic Pharmacologic Parameters in Quantifying Bias. (A) In an assay with significant receptor reserve, such as that for second messengers, both full (red, $\tau = 100$) and partial (blue, $\tau = 10$) agonists reach close to a maximal response. (B) In assays with little to no receptor reserve, such as those based on translocation or recruitment, full agonists (red, $\tau = 10$) reach higher maximal responses than partial agonists (blue, $\tau = 1$). Potencies are similarly affected by receptor reserve, as (C) in assays with high levels of receptor reserve, a full agonist ($\tau = 100$) would have a greater left shift compared to a partial agonist ($\tau = 10$) from its dissociation constant. However, in assays with lower levels of receptor reserve (D), these shifts are do not correlate in a linear fashion (full agonist, $\tau = 10$; partial agonist, $\tau = 1$). Simulated data was generated using the operational model (see text) with the dissociation constant set to 100 nM.

Figure 2. **Approaches to Quantifying Ligand Bias**. (A) In the equimolar comparison, data for a single ligand is collected in 2 different assays (left panel). The responses of these 2 different assays at the same concentration of ligand (middle panel) are then plotted against each other (right panel). (B) In the equiactive comparison, concentration-response data is fit to a logistic equation, yielding EC_{50} and E_{max} (middle panel). This then allows the calculation of a bias factor (right panel). (C) In the operational model, the data is fit to the equation proposed by Black and Leff(Black and Leff, 1983) (left panel). From the coupling coefficient, τ, the effective signaling of each ligand in each assay can be calculated (middle panel), which then allows a calculation of a bias factor (right panel).

Figure 3. **Concentration-response for β-arrestin recruitment and cAMP generation at the β2AR**. Normalized β-arrestin recruitment(A-C) and cAMP generation (D-F) for (A, D) Isoproterenol (Iso), epinephrine (Epi), dobutamine (Dob) and dichloroisoproterenol (DCI); (B,E) Fenoterol (Fen), salbutamol (Salb) and norepinephrine (Norepi); (C,F) Formoterol (Form), clenbuterol (Clen), salmeterol (Salm) and

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pindolol (Pind). (β-arrestin and cAMP signals normalized to formoterol, n=3, error bars denote standard errors of the mean)

Figure 4. **Identification of weakly β-arrestin-biased ligands at the β2AR**. (A) An equimolar comparison between the G protein and β-arrestin-mediated assays does not demonstrate any significant bias. Fenoterol (black), salbutamol (red), norepinephrine (blue), formoterol (green). (B) Bias factors from an equiactive comparison demonstrate bias for dichloroisoproterenol (DCI), pindolol (Pin), salmeterol (Slm) and formoterol (For) ($p < 0.05$ by t-test). (C) Comparison of effective signaling (σ) in β-arrestin recruitment and cAMP generation for a panel of ligands compared to the reference agonist epinephrine. The red line is the theoretical line of balanced signaling. (D) Bias factors calculated from the operational model. Only formoterol (For) and salmeterol (Slm) are significantly biased ($p < 0.05$ by t-test).

Figure 5. **Concentration-response for β-arrestin recruitment and IP_1 formation at the $AT_{1A}R$**. Normalized β-arrestin recruitment (A-C) and IP_1 generation (D-F) for (A, D) Angiotensin II (AngII), TRV120026 (red), TRV120055 (blue) and TRV120056 (green); (B,E) TRV120044 (black), TRV120045 (red) and TRV120034 (blue); (C,F) S1C4 (black), S1G4G8 (red), and A1 (blue). (β-arrestin and IP_1 signals normalized to AngII, n=3, error bars denote standard errors of the mean)

Figure 6. **A group of angiotensin II analogs at the $AT_{1A}R$ are significantly β-arrestin-biased.** (A) The equimolar comparison identifies the ligands SGG (green) and TRV120044 (red) as β-arrestin-biased compared to the reference agonist angiotensin II (black) or TRV120055 (blue). A complete equimolar analysis for all compounds is shown in Figure S4 (B) Bias factors calculated using the equiactive model for the set of $AT_{1A}R$ ligands. Due to large errors, only SGG is identified as a biased ligand. (C) Effective signaling via G proteins and β-arrestins compared to the endogenous agonist AngII. TRV120026, TRV120034, TRV120044, TRV120045 and SGG are significantly β-arrestin-biased while all the other compounds appear balanced (red line). (D) Bias factors calculated using the operational model.

Molecular Pharmacology Fast Forward. Published on May 24, 2011 as DOI: 10.1124/mol.111.072801
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TRV120044 and 45 have nearly an order of magnitude more bias compared to one of the initially described ligands, SGG (*, $p < 0.05$ by t test).

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Table 1. Bias factors for a panel of ligands at the β2AR. The column "Bias" denotes whether a statistically significant difference in bias compared to the reference balanced agonist epinephrine is present (either towards G protein or β-arrestin) while 'Non' denotes an insignificant difference from the balanced agonist. β, bias factor. σ, effective signaling. SEM denotes standard error of the mean.

Ligand	Equiactive			Operational Model						
	β	SEM_{β}	Bias	σ_{cAMP}	$SEM_{\sigma cAMP}$	$\sigma_{\beta arr}$	$SEM_{\sigma\beta arr}$	β	SEM_{β}	Bias
Isoproterenol	-0.310	0.152	Non	1.196	0.129	1.500	0.052	-0.215	0.137	Non
Epinephrine	0.0	0.153	Non	0.0	0.128	0.000	0.044	0.0	0.136	Non
Dobutamine	-0.585	0.260	Non	-1.285	0.125	-1.603	0.247	0.225	0.218	Non
Dichloroisoproterenol	-1.976	0.454	**βarr**	-2.714	0.114	-2.071	0.360	-0.454	0.284	Non
Fenoterol	-0.267	0.196	Non	0.287	0.129	0.445	0.048	-0.112	0.137	Non
Salbutamol	-0.145	0.203	Non	-0.557	0.123	-0.610	0.045	0.038	0.133	Non
Norepinephrine	-0.490	0.227	Non	-0.993	0.127	-0.730	0.064	-0.186	0.139	Non
Formoterol	-0.982	0.153	**βarr**	-0.475	0.129	0.812	0.051	-0.910	0.137	**βarr**
Clenbuterol	-0.725	0.220	Non	-0.944	0.126	-0.899	0.046	-0.32	0.135	Non
Salmeterol	-1.380	0.186	**βarr**	-1.891	0.107	-0.970	0.045	-0.651	0.126	**βarr**
Pindolol	-1.757	0.559	**βarr**	-3.258	0.134	-2.571	0.721	-0.486	0.528	Non

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Table 2. Bias factors for a panel of ligands at the $AT_{1A}R$. The column "Bias" denotes whether a statistically significant difference in bias compared to the reference balanced agonist angiotensin II is present (either towards G protein or β-arrestin) while 'Non' denotes an insignificant difference from the balanced agonist. β, bias factor. σ, effective signaling. SEM denotes standard error of the mean.

Ligand	Equiactive			Operational Model						
	β	SEM_β	Bias	σ_{IP1}	$SEM_{\sigma IP1}$	$\sigma_{\beta arr}$	$SEM_{\sigma\beta arr}$	β	SEM_β	Bias
AngII	0.000	0.093	Non	0.000	0.126	0.000	0.121	0.000	0.174	Non
TRV120026	-2.342	0.938	Non	-1.983	0.188	-0.290	0.107	-1.197	0.217	**βarr**
TRV120055	-0.029	0.119	Non	1.582	0.111	1.010	0.120	0.404	0.163	Non
TRV120056	-0.007	0.119	Non	1.119	0.111	0.510	0.119	0.431	0.163	Non
TRV120044	-2.122	2.308	Non	-2.343	0.376	-0.223	0.106	-1.500	0.390	**βarr**
TRV120045	-1.812	1.189	Non	-2.202	0.273	-0.118	0.111	-1.474	0.295	**βarr**
TRV120034	-1.349	0.582	Non	-1.892	0.166	-0.105	0.112	-1.264	0.201	**βarr**
S1C4	0.195	0.133	Non	0.594	0.113	-0.306	0.105	0.636	0.154	Non
SGG	-1.237	0.282	**βarr**	-1.634	0.141	-0.271	0.106	-0.964	0.176	**βarr**
A1	-0.089	0.120	Non	-0.312	0.126	-0.126	0.118	-0.131	0.173	Non

FIGURE 1

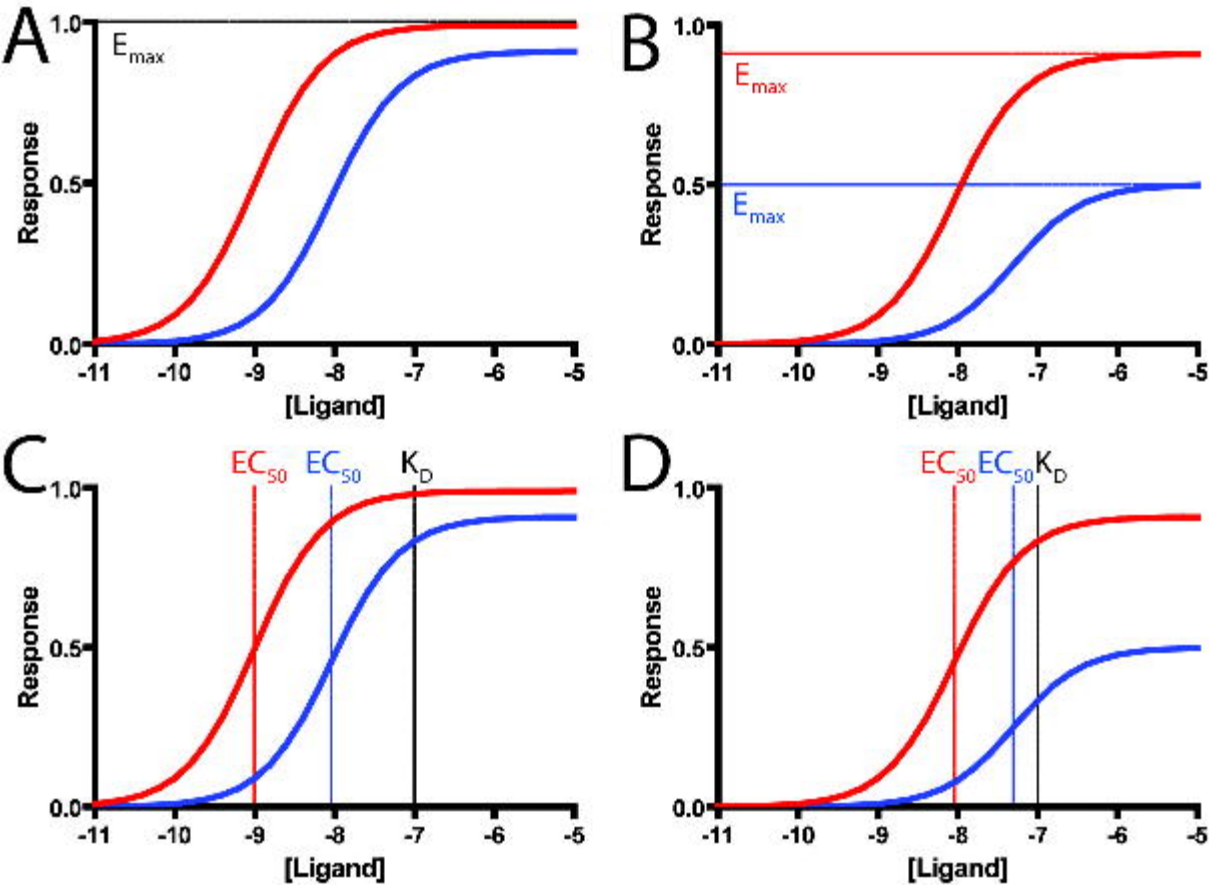

A
E_{max}
Response
1.0
0.5
0.0
-11
-10
-9
-8
-7
-6
-5
[Ligand]
B
E_{max}
E_{max}
Response
1.0
0.5
0.0
-11
-10
-9
-8
-7
-6
-5
[Ligand]
C
EC_{50}
EC_{50}
K_D
Response
1.0
0.5
0.0
-11
-10
-9
-8
-7
-6
-5
[Ligand]
D
EC_{50}
EC_{50}
K_D
Response
1.0
0.5
0.0
-11
-10
-9
-8
-7
-6
-5
[Ligand]

FIGURE 2

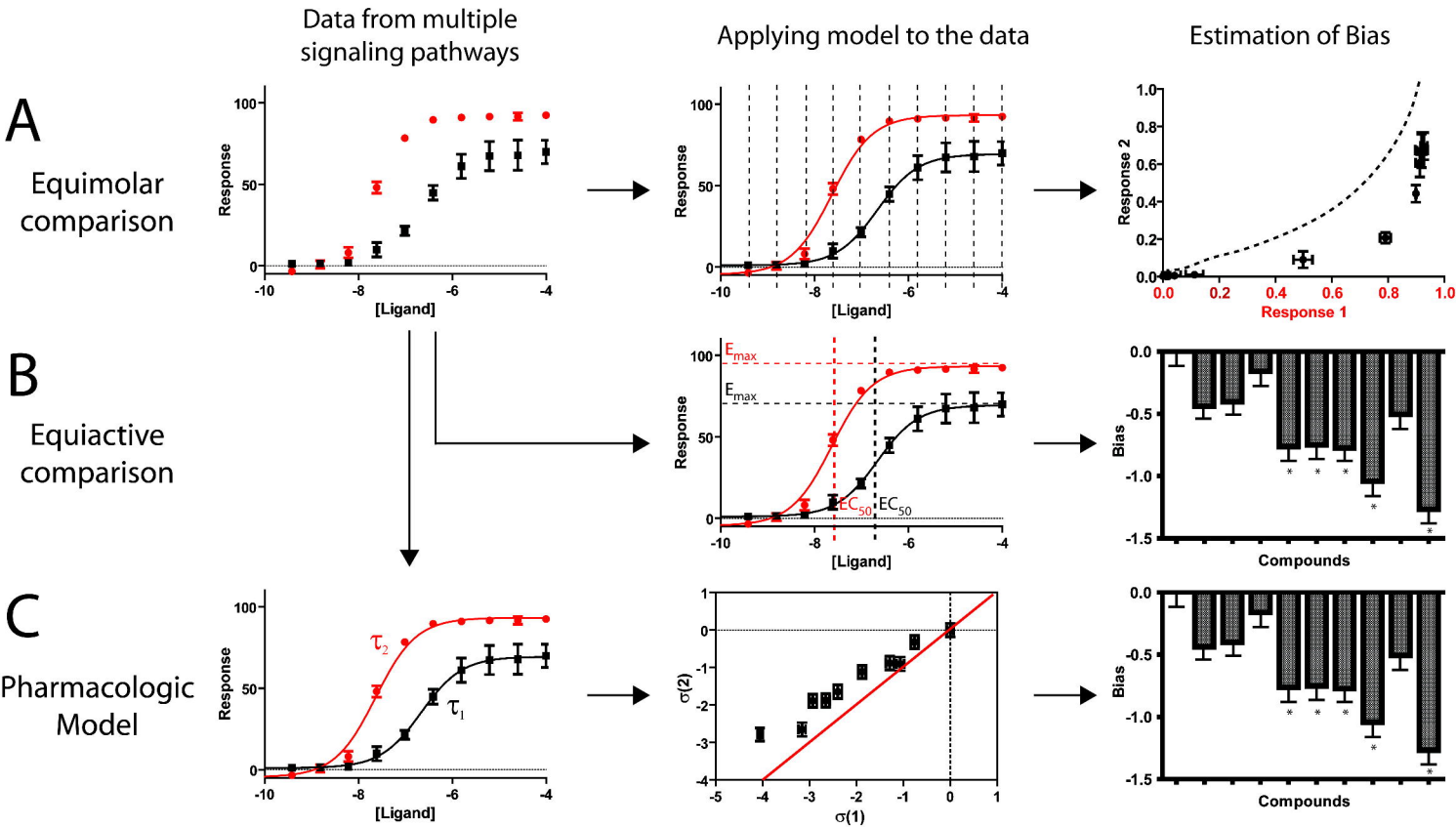

Data from multiple signaling pathways
Applying model to the data
Estimation of Bias
A
Equimolar comparison
B
Equiactive comparison
C
Pharmacologic Model
Response
[Ligand]
Emax
EC50
τ2
τ1
σ(2)
σ(1)
Response 2
Response 1
Bias
Compounds

FIGURE 3

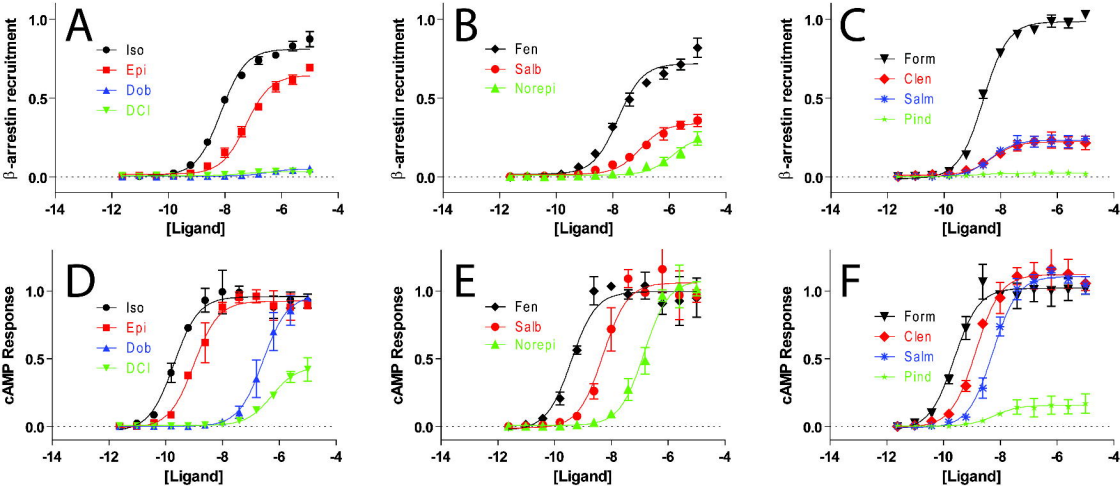
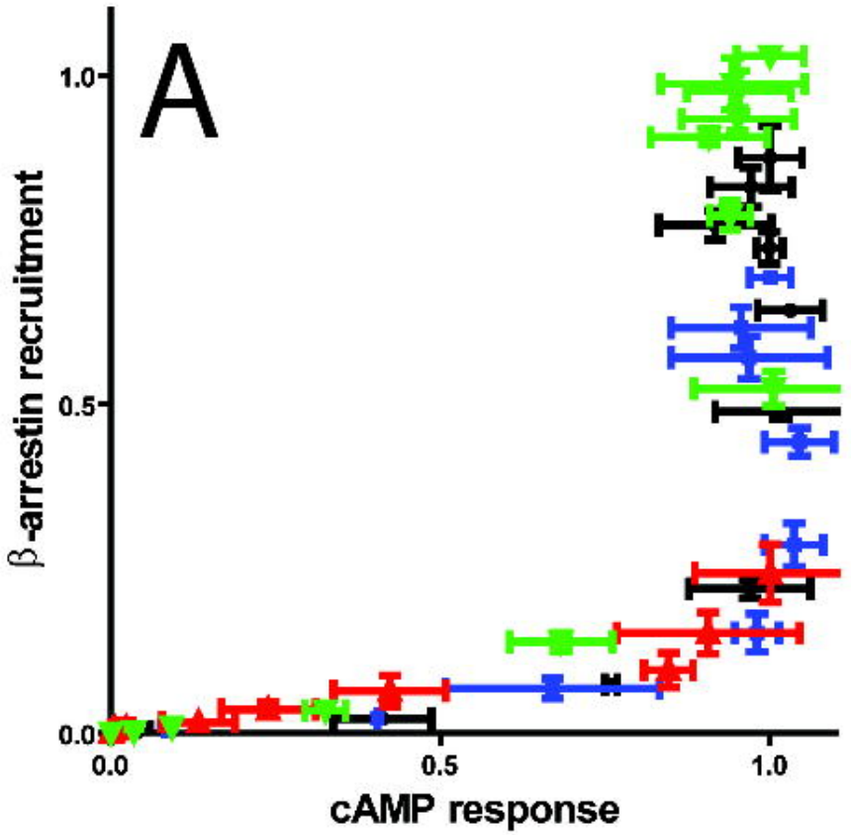
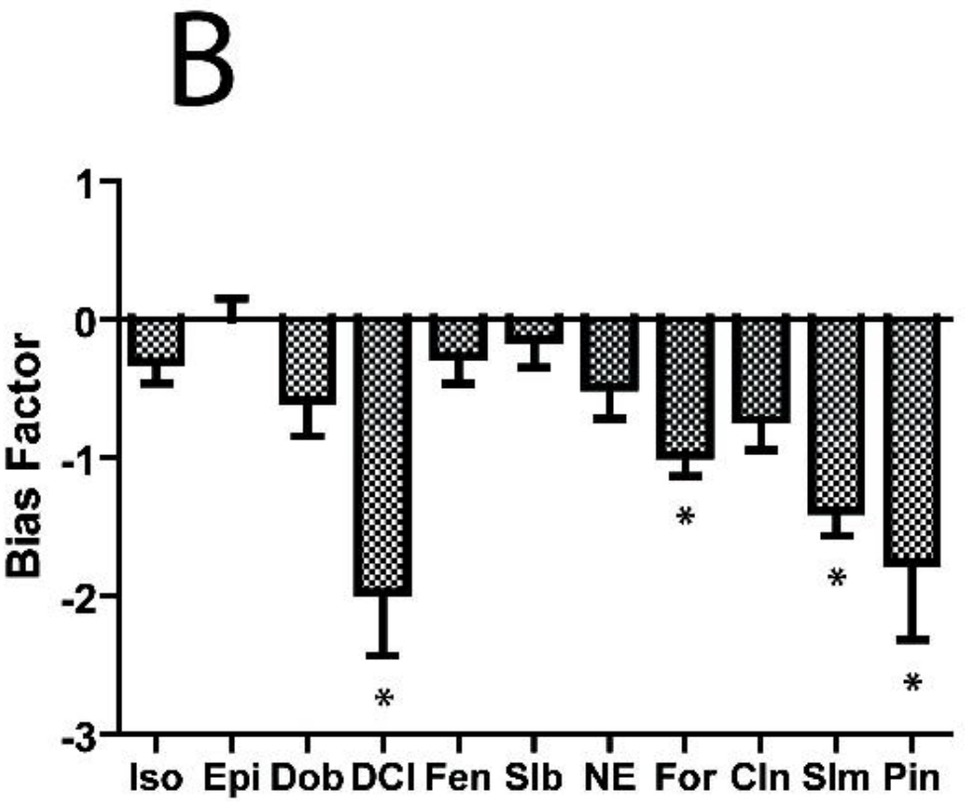
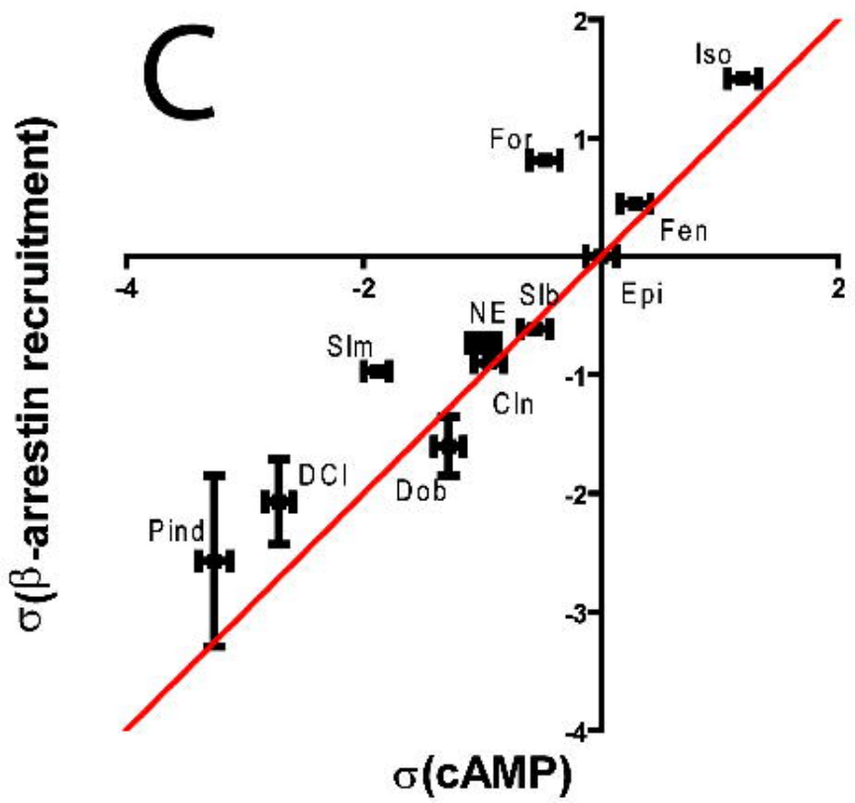

A
Iso
Epi
Dob
DCI
β-arrestin recruitment
[Ligand]
B
Fen
Salb
Norepi
C
Form
Clen
Salm
Pind
D
cAMP Response
E
F

FIGURE 4


A
β-arrestin recruitment
cAMP response
0.0
0.5
1.0


B
Bias Factor
1
0
-1
-2
-3
*
Iso Epi Dob DCI Fen Slb NE For Cln Slm Pin


C
σ(β-arrestin recruitment)
σ(cAMP)
Iso
For
Fen
Epi
Slb
NE
Slm
Cln
DCl
Dob
Pind
2
1
-1
-2
-3
-4
-4
-2
2

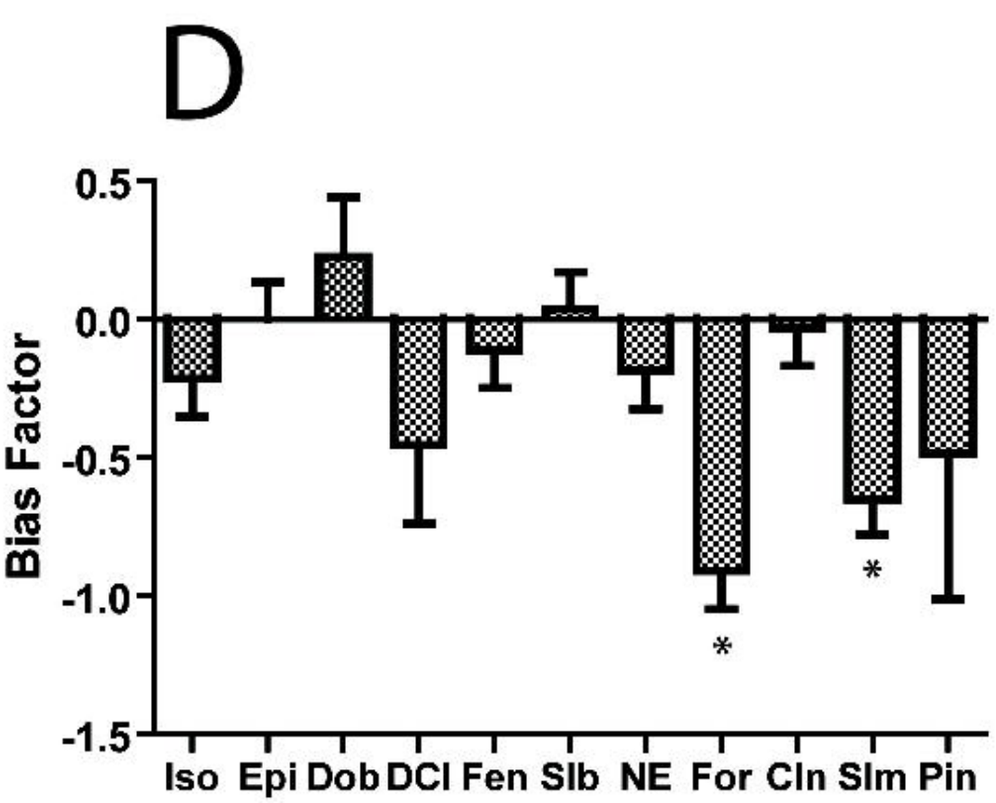

D
Bias Factor
0.5
0.0
-0.5
-1.0
-1.5
*
Iso Epi Dob DCI Fen Slb NE For Cln Slm Pin

FIGURE 5

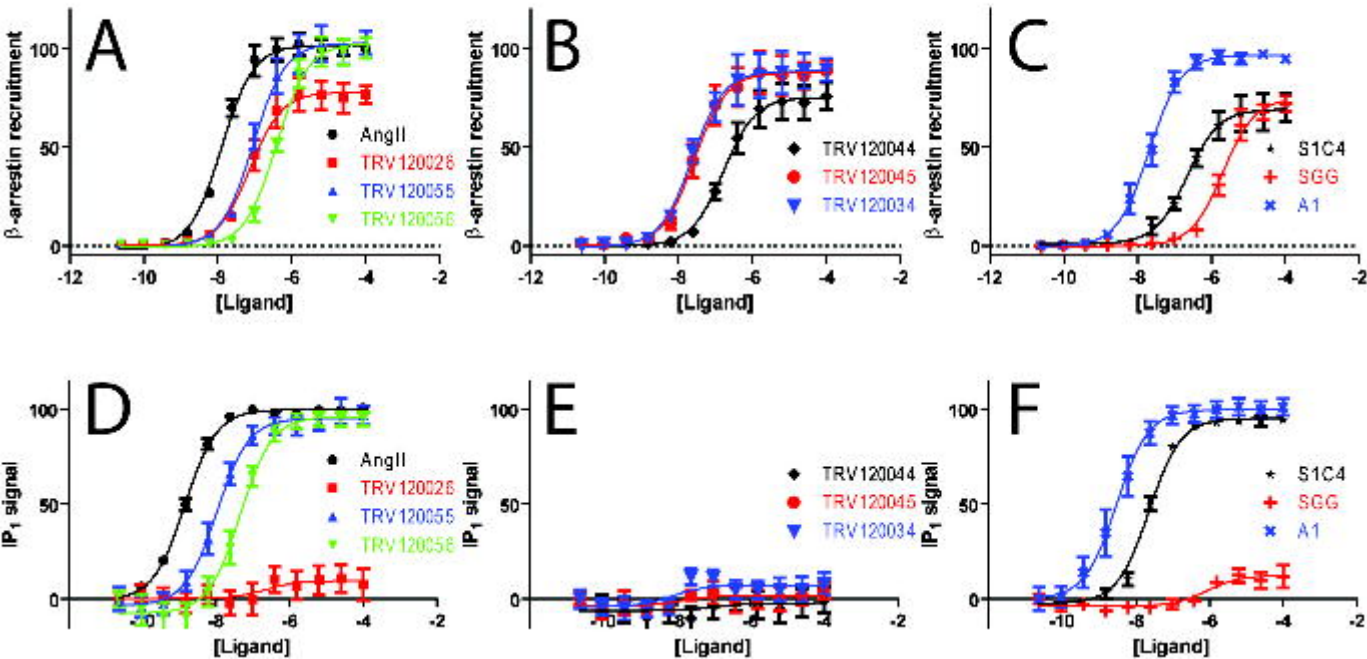
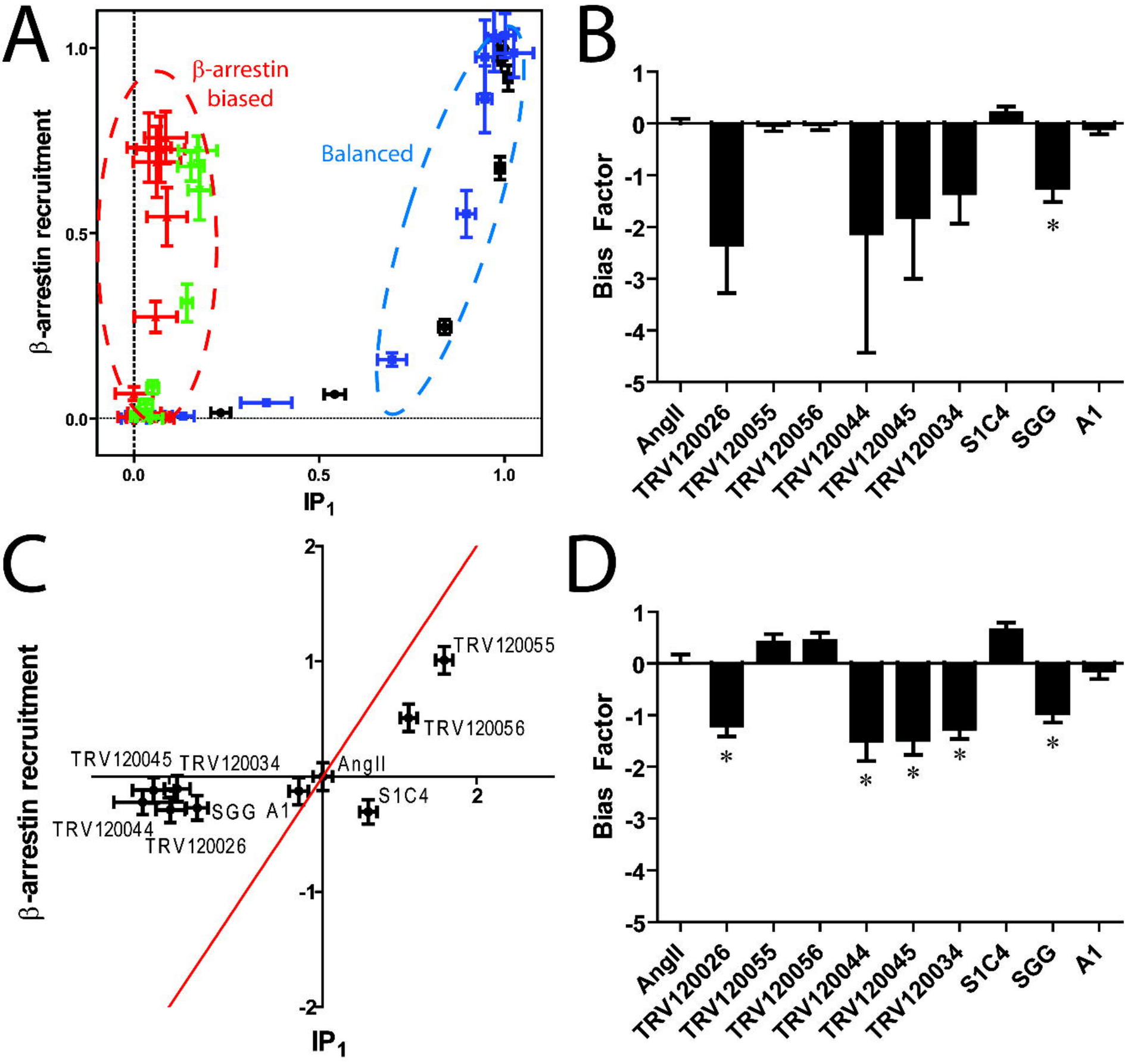

A
β-arrestin recruitment
100
50
0
-12
-10
-8
-6
-4
-2
[Ligand]
AngII
TRV120026
TRV120055
TRV120056
B
β-arrestin recruitment
[Ligand]
TRV120044
TRV120045
TRV120034
C
β-arrestin recruitment
[Ligand]
S1C4
SGG
A1
D
IP1 signal
[Ligand]
AngII
TRV120026
TRV120055
TRV120056
E
IP1 signal
[Ligand]
TRV120044
TRV120045
TRV120034
F
IP1 signal
[Ligand]
S1C4
SGG
A1

FIGURE 6


A
β-arrestin biased
Balanced
β-arrestin recruitment
IP1
1.0
0.5
0.0
B
Bias Factor
AngII
TRV120026
TRV120055
TRV120056
TRV120044
TRV120045
TRV120034
S1C4
SGG
A1
C
β-arrestin recruitment
IP1
TRV120055
TRV120056
TRV120045
TRV120034
AngII
S1C4
SGG
A1
TRV120044
TRV120026
D
Bias Factor

Title: Quantifying Ligand Bias at Seven-Transmembrane Receptors

Authors: Sudarshan Rajagopal, Seungkirl Ahn, David H. Rominger, William Gowen-McDonald,

Christopher M. Lam, Scott M. DeWire, Jonathan D. Violin and Robert J. Lefkowitz

Journal: Molecular Pharmacology

Supplementary Materials

Equiactive Comparison

This method is based on Furchgott's approach to estimate agonist dissociation constants(Furchgott, 1966). In Furchgott's approach, the stimulus for downstream signaling is equal to the intrinsic efficacy multiplied by the agonist:receptor complex concentration (s = ε[AR]). Then, by plotting the ligand concentrations required to obtain equiactive responses before and after partial receptor inactivation ([A] and [A']) allows an estimation of K_D (where q the fraction of receptors active in the irreversibly blocked sample).

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{(1-q)}{q\,K_D}$$

Rather than comparing signaling between different conditions of receptor blockade (denoted by q), we can compare signaling between different signaling pathways downstream of the receptor at equiactive concentrations. This model can be modified to account for allostery by implementing the Katz model for receptor activity(del Castillo and Katz, 1957), the simplest model that accounts for receptor allostery:

$$A + R \overset{K_D}{\rightarrow} AR \overset{\varepsilon}{\rightarrow} AR^*$$

where K_D is the dissociation constant for agonist to the receptor (affinity) and ε, which is equal to ([AR*]/[AR]), is the ability of the agonist to form the signaling-competent receptor conformation R*, i.e., efficacy(Kenakin, 1999). To account for different levels of amplification inherent to each response downstream of the stimulus of AR*, we multiply each stimulus by an amplification factor a_{path}, resulting in s = a_{path} * [AR*]. We can express the fraction of receptors in a signaling-competent conformation in the Katz model as:

$$\frac{[AR^*]}{[R_0]} = \frac{\varepsilon[A]}{K_D + (1+\varepsilon)[A]}$$

When the responses of the 2 pathways are equal, the underlying stimuli are equal:

$$a_1 * \frac{\varepsilon_1\ [A_1]}{K_D + (1+\varepsilon_1\ )[A_1]} = a_2 * \frac{\varepsilon_2\ [A_2]}{K_D + (1+\varepsilon_2\ )[A_2]}$$

After some straightforward manipulations, 1/[A_2] can be expressed in terms of 1/[A_1] as:

$$\frac{1}{[A_2]} = \frac{a_2}{a_1}\frac{\varepsilon_2}{\varepsilon_1} * \frac{1}{[A_1]} + \frac{1}{K_D}\left(\frac{a_2}{a_1}\frac{\varepsilon_2}{\varepsilon_1}(1+\varepsilon_1\ ) - (1+\varepsilon_2\ )\right)$$

In this linear relationship, $m = \frac{a_2}{a_1}\frac{\varepsilon_2}{\varepsilon_1}$ is the slope of the line. If we then compare the slopes from one ligand (m_{lig}) to the reference balanced agonist (m_{ref}), the amplification terms cancel out and:

$$\frac{m_{lig}}{m_{ref}} = \frac{\varepsilon_{2,lig}}{\varepsilon_{2,ref}}\frac{\varepsilon_{1,ref}}{\varepsilon_{1,lig}}\ and \log\left(\frac{m_{lig}}{m_{ref}}\right) = \log\left(\frac{\varepsilon_{2,lig}}{\varepsilon_{2,ref}}\right) - \log\left(\frac{\varepsilon_{1,lig}}{\varepsilon_{1,ref}}\right) = \beta$$

where β is the bias factor and estimates the molecular efficacy of pathway 1 versus pathway 2 on a logarithmic scale, e.g., a bias factor of 1 for G_i/arrestin means that the ligand is 10 times better at generating the active receptor conformation for G_i signaling compared to arrestin signaling (compared to the reference balanced agonist).

If we derive this same relationship in terms of a pharmacologic fit with Hill coefficient set to 1 and no basal activity, we obtain a similar expression after some straightforward manipulations as shown below:

$$R_1 = \frac{E_{max,1}[A_1]}{[A_1] + EC_{50,1}} = \frac{E_{max,1}}{1 + \frac{EC_{50,1}}{[A_1]}} = \frac{E_{max,2}}{1 + \frac{EC_{50,2}}{[A_2]}} = R_2$$

$$E_{max,1}(1 + \frac{EC_{50,2}}{[A_2]}) = E_{max,2}(1 + \frac{EC_{50,1}}{[A_1]})$$

$$\frac{1}{[A_1]} = \frac{1}{E_{max,2}EC_{50,1}}\left(E_{max,1}\left(1 + \frac{EC_{50,2}}{[A_2]}\right) - E_{max,2}\right)$$

$$\frac{1}{[A_1]} = \left(\frac{E_{max,1}}{EC_{50,1}}\frac{EC_{50,2}}{E_{max,2}}\right)\frac{1}{[A_2]} + \frac{(E_{max,1} - E_{max,2})}{E_{max,2}EC_{50,1}} = RA_{12}\frac{1}{[A_2]} + \frac{(E_{max,1} - E_{max,2})}{E_{max,2}EC_{50,1}}$$

where RA_{12} is the intrinsic relative activity proposed by Ehlert and coworkers(Ehlert, 2008). Therefore, if the data can be fit well by a logistic expression with Hill coefficient equal to 1, the intrinsic relative activity is equal to the ratio of equilibrium constants for receptor activation. Then:

$$\beta = \log\left(\frac{RA_{12,lig}}{RA_{12,ref}}\right) = \log\left(\left(\frac{E_{max,1}}{EC_{50,1}}\frac{EC_{50,2}}{E_{max,2}}\right)_{lig} * \left(\frac{E_{max,2}}{EC_{50,2}}\frac{EC_{50,1}}{E_{max,1}}\right)_{ref}\right)$$

where the 1 and 2 subscripts refer to signaling through pathways 1 and 2, *lig* refers to the ligand and *ref* to the reference compound. Thus, using this approach, signaling bias can be quantified using concentration-response data alone.

Operational Model

The operational model of Black and Leff(Black and Leff, 1983) yields essentially identical mathematical terms to other pharmacologic models that are based on the original work of Stephenson(Stephenson, 1956). In the operational model, the response to a stimulus is related in a hyperbolic fashion to the concentration of the agonist:receptor complex [AR] by a factor K_E. By introducing a coupling coefficient τ, which is equal to the total receptor concentration divided by K_E, the response of the system can be described in terms of agonist concentration, the coupling coefficient τ and the agonist:receptor dissociation constant K_D:

$$\frac{E}{E_m} = \frac{[AR]}{[AR] + K_E} = \frac{\tau\,[A]}{\tau\,[A] + ([A] + K_D)}$$

where E_m is the maximal response of the system to a full agonist, K_D is the agonist dissociation constant and τ is "coupling efficiency" between the agonist:receptor complex and its downstream signaling partners. This coupling efficiency τ can be considered to be composed of two components ($\tau=\tau^*\varepsilon$), where the τ^* term accounts for the amplification inherent to the downstream signaling pathway that is the same for all ligands in the same assay, and the other component (ε) accounts for a ligand's efficacy at generating a signaling-competent agonist:receptor conformation. The ability of an agonist to signal to downstream pathways can then be compared to a reference agonist by the effective signaling (σ_{lig}):

$$\sigma_{lig} = \log\left(\frac{\tau_{lig}}{\tau_{ref}}\right) = \log\left(\frac{\varepsilon_{lig}}{\varepsilon_{ref}}\right)$$

It would be expected that for balanced agonists, the effective signaling would be equal in both pathways assayed would be equal ($\sigma_{P1} = \sigma_{P2}$), and on a graph comparing these two values balanced agonists would lie on a line of unity. Therefore, a bias factor, β, can then be calculated as the distance between the effective signaling factors for the test compound compared to the line of unity for balanced agonists:

$$\beta_{lig} = \frac{\sigma_{lig}^{path1} - \sigma_{lig}^{path2}}{\sqrt{2}}$$

Supplementary Figure Legends

Figure S1. A near-reversal in rank order of potencies for isoproterenol (black) and formoterol (red) comparing assays of (A) cAMP formation and (B) β-arrestin recruitment. (β-arrestin and cAMP signals normalized to formoterol, n=3, error bars denote standard errors of the mean)

Figure S2. Equimolar comparison of β-arrestin recruitment and cAMP response at the β2AR. Shown are the means from three independent experiments with error bars denoting standard errors of the mean.

Figure S3. Concentration-response data for pindolol and DCI. Shown are the means from three independent experiments with error bars denoting standard errors of the mean.

Figure S4. Equimolar comparison of β-arrestin recruitment and IP_1 formation at the $AT_{1A}R$. Shown are the means from three independent experiments with error bars denoting standard errors of the mean.

Figure S5. β-arrestin recruitment for selected compounds using the DiscoveRx PathHunter assay. In this assay, the human β2AR was modified by addition of a Prolink tag that allows enzyme complementation to a modified β-arrestin. The same pattern in the concentration-response data is seen here as in the Tango assay, with formoterol displaying higher potency than isoproterenol (A), and clenbuterol and salmeterol displaying nearly identical potencies and maximal responses (B). (RLU= Relative luminescence units, n=3, error bars denote standard errors of the mean)

Figure S6. cAMP responses to β2AR ligands in HEK293 cells stably transfected with the β2AR Tango construct for β-arrestin recruitment and transiently transfected with the Glosensor construct for detection of cAMP. As compared to Figure 3D-F, the compounds display essentially the same activities. (β-arrestin and cAMP signals normalized to formoterol, experiment performed once in triplicate, error bars denote standard errors of the mean)

Figure S1

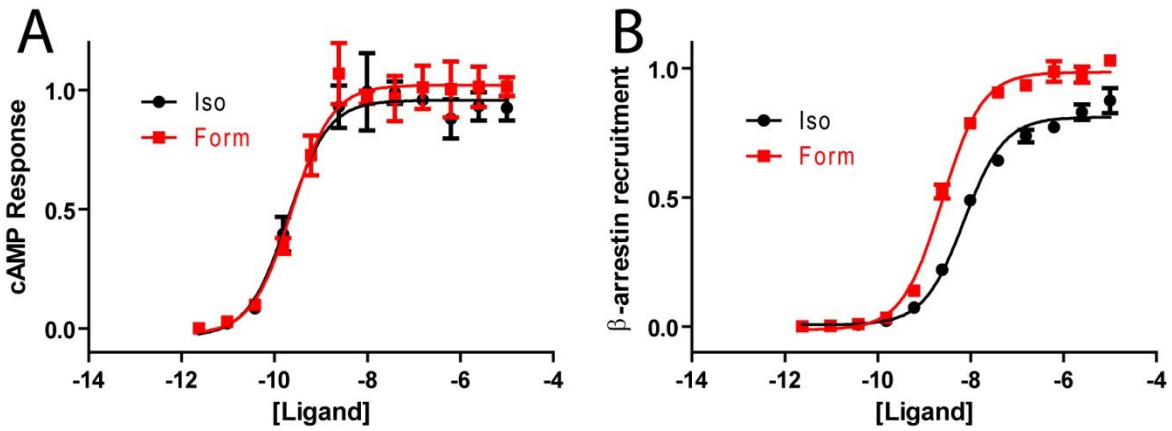


Figure S2

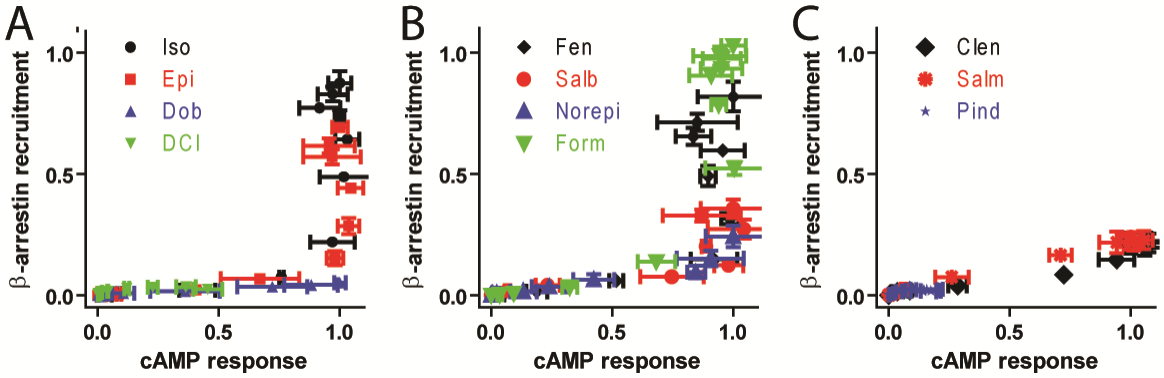


Figure S3

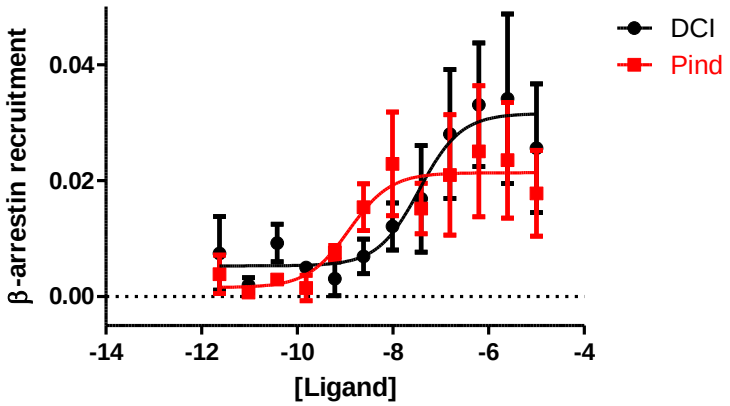


Figure S4

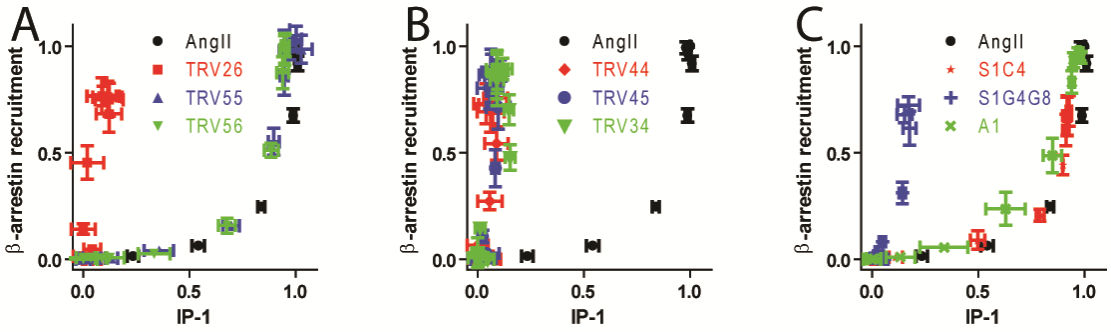


Figure S5

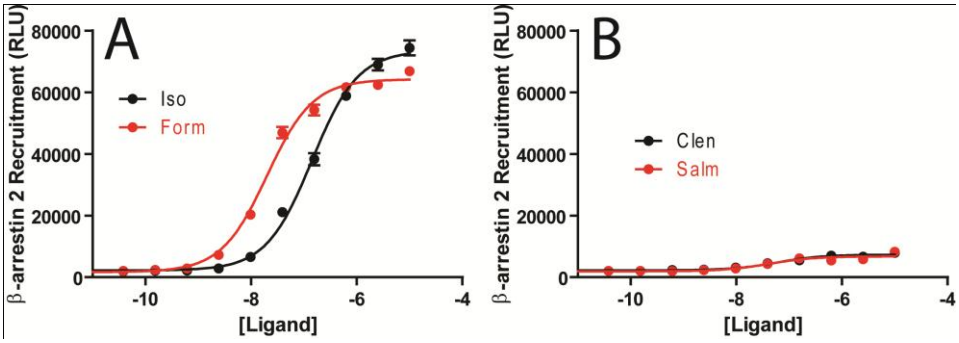


Figure S6

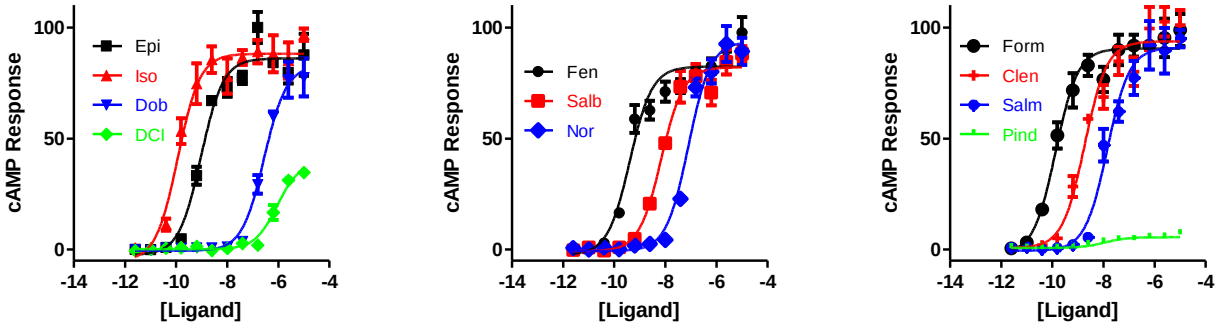


Table S1. Fitting parameters for concentration-response data from the β2AR.

A. Application of a simple logistic equation to cAMP generation as assayed by the Glosensor assay. E_{max}: Maximal response; SEM_{Emax}: Standard error of the mean for E_{max}; $LogEC_{50}$: The log base 10 of the concentration that elicits half-maximal response; $SEMl_{ogEC50}$: Standard error of the mean for $LogEC_{50}$.

Ligand	E_{max}	SEM_{Emax}	$LogEC_{50}$	$SEM_{logEC50}$	R^2
Isoproterenol	99.14	4.631	-9.709	0.09880	0.9617
Epinephrine	93.52	3.762	-9.006	0.09277	0.9680
Dobutamine	96.77	4.544	-6.572	0.09236	0.9653
Dichloroisoproterenol	43.35	2.327	-6.274	0.09655	0.9613
Fenoterol	102.2	5.879	-9.412	0.1274	0.9397
Salbutamol	107.3	5.746	-8.298	0.1267	0.9432
Norepinephrine	107.4	4.501	-6.842	0.08748	0.9695
Formoterol	104.6	5.066	-9.623	0.1040	0.9582
Clenbuterol	112.8	4.124	-8.854	0.08521	0.9731
Salmeterol	111.8	2.832	-8.257	0.05992	0.9867
Pindolol	15.48	2.087	-8.200	0.3185	0.7240

B. Application of a simple logistic equation to β-arrestin recruitment by the Tango assay. E_{max}: Maximal response; SEM_{Emax}: Standard error of the mean for E_{max}; $LogEC_{50}$: The log base 10 of the concentration that elicits half-maximal response; $SEMl_{ogEC50}$: Standard error of the mean for $LogEC_{50}$

Ligand	E_{max}	SEM_{Emax}	$LogEC_{50}$	$SEM_{logEC50}$	R^2
Isoproterenol	80.47	1.593	-8.140	0.04674	0.9872
Epinephrine	62.84	1.690	-7.254	0.05985	0.9783
Dobutamine	4.803	0.5361	-6.491	0.2150	0.7645
Dichloroisoproterenol	2.626	0.4890	-7.445	0.4228	0.4779
Fenoterol	70.17	2.314	-7.812	0.07703	0.9658
Salbutamol	32.41	1.506	-7.024	0.1002	0.9403
Norepinephrine	24.89	2.629	-5.892	0.1617	0.8518
Formoterol	99.75	1.511	-8.612	0.03563	0.9925
Clenbuterol	21.35	1.540	-8.332	0.1706	0.8535
Salmeterol	23.26	1.361	-8.352	0.1384	0.8985
Pindolol	1.979	.4001	-8.916	0.4692	0.4311

C. Application of the operational model to cAMP generation as assayed by the Glosensor assay. The experimentally determined dissociation constant from radioligand binding is listed as Log K_D, the maximal efficacy for the system is Emax, log τ is the log base 10 of the coupling coefficient τ, and $SEM_{\log \tau}$ is the standard error of the mean for log τ.

Ligand	E_{max}	Log K_D	log τ	SEM $_{\log \tau}$	R^2
Isoproterenol	110.6	-6.00	3.819	0.09463	0.9512
Epinephrine		-6.54	2.518	0.09481	0.9478
Dobutamine		-5.46	1.028	0.09152	0.9735
Dichloroisoproterenol		-6.83	-0.3114	0.08296	0.8750
Fenoterol		-6.53	2.846	0.09587	0.9334
Salbutamol		-6.42	1.908	0.09708	0.9222
Norepinephrine		-5.36	1.641	0.09502	0..9278
Formoterol		-7.55	2.080	0.09762	0.9643
Clenbuterol		-7.48	1.422	0.09991	0.9774
Salmeterol		-8.51	0.5235	0.08528	0.8368
Pindolol		-9.32	-0.8291	0.1489	0.6565

D. Application of the operational model to β-arrestin recruitment by the Tango assay. The experimentally determined dissociation constant from radioligand binding is listed as Log K_D, the maximal efficacy for the system is Emax, log τ is the log base 10 of the coupling coefficient τ, and $SEM_{\log \tau}$ is the standard error of the mean for log τ.

Ligand	E_{max}	Log K_D	log τ	SEM $_{\log \tau}$	R^2
Isoproterenol	89.87	-6.00	1.947	0.04167	0.9671
Epinephrine		-6.54	0.4472	0.03078	0.9741
Dobutamine		-5.46	-1.156	0.2449	0.5720
Dichloroisoproterenol		-6.83	-1.624	0.3588	0.3728
Fenoterol		-6.53	0.8919	0.03702	0.9379
Salbutamol		-6.42	-0.1632	0.03304	0.9144
Norepinephrine		-5.36	-0.2828	0.05583	0.8382
Formoterol		-7.55	1.259	0.04105	0.9572
Clenbuterol		-7.48	-0.4518	0.03460	0.7638
Salmeterol		-8.51	-0.5224	0.03236	0.8707
Pindolol		-9.32	-2.124	0.7208	0.1540

Table S2. Fitting parameters for concentration-response data from the $AT_{1A}R$.

A. Application of a simple logistic equation to IP_1 generation. E_{max}: Maximal response; SEM_{Emax}: Standard error of the mean for E_{max}; $LogEC_{50}$: The log base 10 of the concentration that elicits half-maximal response; $SEMl_{ogEC50}$: Standard error of the mean for $LogEC_{50}$.

Ligand	E_{max}	SEM_{Emax}	$LogEC_{50}$	$SEM_{logEC50}$	R^2
Angiotensin II	100.8	1.448	-8.843	0.02962	0.9923
TRV0120026	9.478	3.752	-6.637	0.9144	0.1272
TRV0120055	98.88	2.450	-7.971	0.05729	0.9738
TRV0120056	103.8	3.377	-7.341	0.07696	0.9545
TRV0120044	3.958	3.832	-6.880	2.267	0.02340
TRV0120045	5.550	2.773	-7.889	1.162	0.08322
TRV0120034	11.28	2.751	-8.118	0.5571	0.2798
S1C4	97.81	1.54	-7.634	0.03700	0.9890
S1G4G8	15.98	1.864	-6.057	0.2598	0.6510
A1	99.88	3.582	-8.516	0.07830	0.9502

B. Application of a simple logistic equation to β-arrestin recruitment by the PathHunter™ assay. E_{max}: Maximal response; SEM_{Emax}: Standard error of the mean for E_{max}; $LogEC_{50}$: The log base 10 of the concentration that elicits half-maximal response; $SEMl_{ogEC50}$: Standard error of the mean for $LogEC_{50}$.

Ligand	E_{max}	SEM_{Emax}	$LogEC_{50}$	$SEM_{logEC50}$	R^2
Angiotensin II	103.9	2.563	-7.895	0.05735	0.9738
TRV0120026	77.91	3.266	-7.130	0.09892	0.9268
TRV0120055	103.4	3.443	-7.046	0.07838	0.9527
TRV0120056	103.4	2.782	-6.415	0.06088	0.9700
TRV0120044	75.38	3.467	-6.788	0.1072	0.9144
TRV0120045	87.95	4.157	-7.566	0.1113	0.9090
TRV0120034	89.17	4.332	-7.634	0.1142	0.9045
S1C4	68.31	3.169	-6.660	0.1073	0.9137
S1G4G8	75.20	2.650	-5.686	0.07119	0.9566
A1	97.18	2.499	-7.682	0.06034	0.9713

C. Application of the operational model to IP_1 generation. The experimentally determined dissociation constant from radioligand binding is listed as Log K_D, the maximal efficacy for the system is Emax, log τ is the log base 10 of the coupling coefficient τ, and $SEM_{\log \tau}$ is the standard error of the mean for log τ.

Ligand	E_{max}	Log K_D	log τ	SEM $_{\log \tau}$	R^2
Angiotensin II	107.1	-7.9	0.9152	0.089	0.989
TRV0120026		-7.3	-1.068	0.166	0.108
TRV0120055		-5.5	2.497	0.066	0.968
TRV0120056		-5.3	2.034	0.067	0.954
TRV0120044		-6.3	-1.428	0.365	0.020
TRV0120045		-7.4	-1.287	0.259	0.077
TRV0120034		-7.5	-0.977	0.140	0.238
S1C4		-6.1	1.509	0.070	0.985
S1G4G8		-5.4	-0.719	0.109	0.599
A1		-8.2	0.603	0.089	0.894

D. Application of the operational model to β-arrestin recruitment by the PathHunter™ assay. The experimentally determined dissociation constant from radioligand binding is listed as Log K_D, the maximal efficacy for the system is Emax, log τ is the log base 10 of the coupling coefficient τ, and $SEM_{\log \tau}$ is the standard error of the mean for log τ.

Ligand	E_{max}	Log K_D	log τ	SEM $_{\log \tau}$	R^2
Angiotensin II	127.6	-7.9	0.399	0.085	0.897
TRV0120026		-7.3	0.109	0.064	0.874
TRV0120055		-5.5	1.409	0.084	0.918
TRV0120056		-5.3	0.908	0.083	0.958
TRV0120044		-6.3	0.176	0.062	0.913
TRV0120045		-7.4	0.281	0.071	0.891
TRV0120034		-7.5	0.293	0.073	0.882
S1C4		-6.1	0.092	0.061	0.906
S1G4G8		-5.4	0.128	0.063	0.955
A1		-8.2	0.273	0.082	0.804

Table S3. Radioligand binding data for the rat AT1AR. Data was collected as described in Materials and Methods.

	rat AT1R ^{125}I *[S*^{1}*I*^{8}*]Ang II apparent affinity (pKi)*		
Peptide	***mean pKi***	***SEM***	***n***
human Angiotensin II	***7.9***	***0.1***	***4***
TRV0120045	***7.4***	***0.1***	***3***
TRV0120026	***7.3***	***0.1***	***5***
TRV0120034	***7.5***	***0.2***	***3***
TRV0120055	***5.6***	***0.1***	***3***
TRV0120056	***5.2***	***0.0***	***3***
TRV0120044	***6.3***	***0.1***	***3***
[Sar^{1}Gly^{4}Gly^{8}]AngII	***5.4***	***0.2***	***3***
[Ala^{1}]AngII	***8.2***	***0.1***	***5***
[Sar^{1}Cha^{4}]AngII	***6.1***	***0.1***	***3***

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