

MINIREVIEW

A Scale of Agonism and Allosteric Modulation for Assessment of Selectivity, Bias, and Receptor Mutation

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ABSTRACT

An index of agonism is described that can be used to quantify agonist receptor selectivity, bias, cell-based agonism, and the effects of receptor mutation on signaling. The parameter is derived from agonist concentration-response curves and comprises the maximal response to the agonist (max) and the EC_{50} in the form of $\Delta\log(\max/EC_{50})$. This parameter is derived from equations describing agonists as positive allosteric facilitators of receptor-signaling protein interaction. A similar index is also

derived to quantify the potentiating effects of positive allosteric modulators, which can be used to quantify in situ positive allosteric modulator activity in vivo. These indices lend themselves to statistical analysis and are system-independent in that the effects of the system processing of agonist response and differences in assay sensitivity and receptor expression are cancelled. The various applications of the $\Delta\log(\max/EC_{50})$ scale are described for each pharmacologic application.

Introduction

A critical component of the lead optimization process in new drug discovery for agonists and allosteric modulators is the determination of relative measures of activity that are not specifically linked only to the assays in which they are measured; that is, they are system-independent measures of activity. For full agonists, system-independent measures of activity are achieved through relative potency ratios (ratios of concentration of agonist producing half-maximal response values [EC_{50}]); however, this scale devolves into a nonlinear scale when comparing full and partial agonists, so it cannot be used for the comparison of these types of agonists. In this article, agonism is developed as a positive allosteric modulation of the natural receptor-signaling protein interaction, and the resulting scale is presented as a system-independent measure of the relative receptor activation for any set of agonists (full or partial). Separately, the same approach is applied to the assessment of the relative activity of any set of allosteric modulators, positive (PAMs) or negative (NAMs). Although the activity of NAMs can be quantified with standard methods for antagonists, the affinity of PAMs is uniquely dependent on the cobinding agonist and thus requires special methods to assess. This new scale may be particularly useful in that it can be used to furnish system-independent

measures of PAM activity in vivo for the advancement of candidate molecules.

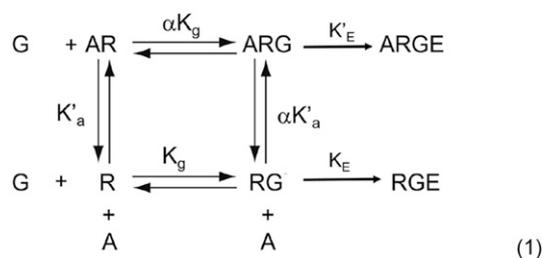
Receptor-Signaling Protein Interaction

The discovery of constitutive seven transmembrane receptor activity (Costa and Herz, 1989) and subsequent recasting of the ternary complex model for receptors to the extended ternary complex model (Samama et al., 1993) have revealed that agonists can be considered simply as PAMs of an already ongoing spontaneous association between the receptor and the signaling protein (Kenakin, 2015). In accordance with the standard functional allosteric model for receptors (Ehlert, 2005; Kenakin, 2005; Price et al., 2005), such modulators (in this case, agonists) can change the affinity of the receptor for the signaling protein (through a cooperativity term α) and the efficacy of the receptor-signaling protein complex for production of cellular response (through a cooperativity term β). In terms of binding, the relevant protein species can be described within the context of the standard Stockton-Ehlert allosteric binding model (Stockton et al., 1983; Ehlert, 1988) whereby the agonist (denoted A) and signaling protein (denoted G) bind to separate but interactive sites on the receptor. Thus, both A and G interact with the receptor with equilibrium association constants K'_a and K'_g , respectively:

This binding model (eq. 1) is then placed as the receptor species producing unit for the Black-Leff operational model

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ABBREVIATIONS: EC_{50} , concentration of agonist producing half-maximal response; NAM, negative allosteric modulator; PAM, positive allosteric modulator.



of agonism (Black and Leff, 1983) to yield the functional allosteric model. An “allosteric vector” can be described to denote the direction of modulation (Kenakin and Miller, 2010), which in this case has the binding of a ligand to the receptor directing the modification of the interaction between the receptor and a signaling protein within the cytosol; this vector constitutes agonism. It should be noted that G protein-coupled receptors have been described as allosteric proteins in the literature in early mathematical models (Karlin, 1967; Thron, 1973). The standard functional allosteric model for a cytosol-directed vector can be used to show that ratios of the maximal response (max) and EC_{50} (concentration of agonist producing half-maximal response) from agonist concentration-response curves for a set of agonists (to furnish $\Delta\log(\max/EC_{50})$ values) create a system independent scale of agonism that can be used to quantify selectivity (see Appendix 1 for derivation). This same conclusion can be reached through derivation of the term within the Black-Leff operational model as well (Kenakin, 2015) (see Appendix 2).

Historically, the relative activity of agonists has been quantified through potency ratios (e.g., differences in the negative logarithm of EC_{50} values denoted as ΔpEC_{50}). For full agonists, ΔpEC_{50} values are constant over all ranges of system sensitivity (as long as both agonists produce full system response; i.e., they are full agonists). This relationship breaks down, however, when one or both of the agonists become a partial agonist because the impact of tissue sensitivity on the potency of full and partial agonists is different. Specifically, reductions in receptor density for full agonists produce defined dextral displacement of concentration-response curves, in contrast to the effect produced on curves for partial agonists, which essentially do not change location along the concentration axis but rather show depressed maxima (Fig. 1A), producing distinctly nonlinear changes in the relative pEC_{50} values of an agonist when it expresses partial agonism

in systems of low sensitivity (see curvature in the relationship between pEC_{50} and receptor density shown in Fig. 1B). This nonlinearity makes ΔpEC_{50} values dependent on tissue sensitivity and thus not useful as a system-independent index of relative agonist activity. For dose-response curves with Hill coefficients not significantly different from unity, normalization of agonist activity through inclusion of the maximal response [in the form of $\Delta\log(\max/EC_{50})$ values] corrects this dependence on tissue sensitivity and yields a truly system-independent scale of agonism (see Fig. 1); this effect is shown with experimental data in Fig. 2 for the β -adrenoceptor partial agonist prenalterol and full agonist isoproterenol. As shown in Fig. 2, the ΔpEC_{50} values produce a distinct curvature with varying tissues, whereas $\Delta\log(\max/EC_{50})$ values remain constant through a range of tissue conditions.

Three important points to consider when discussing $\log(\max/EC_{50})$ values are the following: the fact that calculation of \max/EC_{50} reduces agonism to a single number. This is useful from the point of view of allowing statistical analyses (i.e., as in the analysis of signaling bias, Kenakin et al., 2012). The various formulae to do this are described in Appendix 3, with the key parameter to enable the calculations being an estimate of pooled variance (Kenakin et al., 2012). Thus, estimates of the agonism produced by any molecule can be made with mean $\log(\max/EC_{50})$ values \pm 95% confidence limit, comparison of the relative base agonist activity in any one system can be made with $\Delta\log(\max/EC_{50})$ values \pm 95% confidence limits; finally, comparisons of selectivity, bias, and the effects of mutation made through $\Delta\Delta\log(\max/EC_{50}) \pm$ 95% confidence limit estimates with simple adjustment of formulae based on the pooled variance (see Appendix 3).

The second point is that the comparison of $\log(\max/EC_{50})$ values between agonists in a single functional system to produce $\Delta\log(\max/EC_{50})$ allows the system independent scaling of agonism within a given functional system. When this is done, the agonism of a test compound is compared with a reference agonist and system effects are cancelled (i.e., the sensitivity of the functional system is not an issue), and the $\Delta\log(\max/EC_{50})$ reflects molecular efficacy and affinity in a system independent manner within a given assay. The third point involves the cross comparison of different response systems, whether they be signaling pathways in the cell or different receptors. Once the power of a test agonist is scaled to the same reference agonist in two systems, then $\Delta\Delta\log(\max/EC_{50})$ values cancel cross-system effects (including differences in assay sensitivity) and become independent

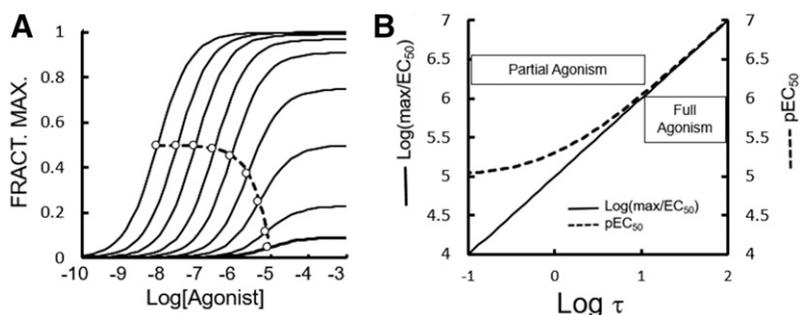


Fig. 1. Effect of changing receptor density (and/or tissue sensitivity) on concentration response to an agonist. (A) Note how less sinistral displacement of EC_{50} values is observed for the agonist when it produces partial agonism compared to full agonism. (B) $\Delta\log(\max/EC_{50})$ values (solid line) and ΔpEC_{50} values (dotted line) with changing tissue sensitivity for two agonists, one of which produces partial agonism at low tissue sensitivities. Note how ΔpEC_{50} values vary with tissue sensitivity, whereas $\Delta\log(\max/EC_{50})$ values remain stable.

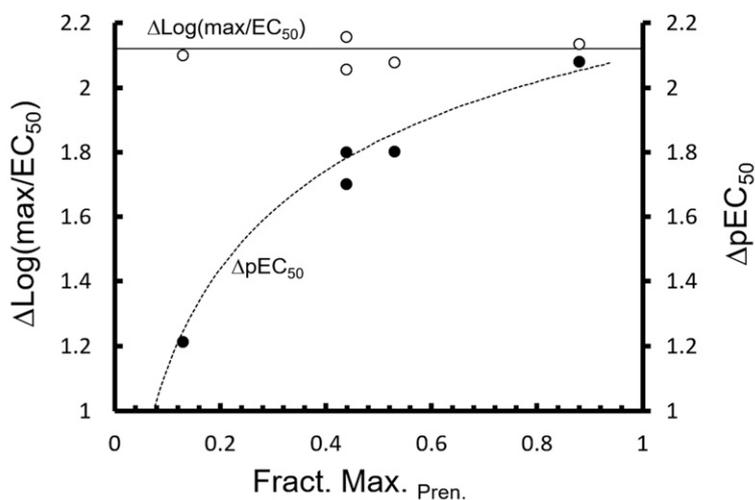


Fig. 2. Ratios of $\log(\max/EC_{50})$ values for the β -adrenoceptor full-agonist isoproterenol and the partial agonist prenalterol (left ordinate axis; data as open circles) as a function of the maximal response to prenalterol in range of isolated tissues (abscissae). Dotted line shows the ratio of pEC_{50} values (right ordinate axis; data in filled circles). Data from Kenakin and Beek (1982).

	Isoproterenol			Prenalterol				
	max	pEC_{50}	$\log(\max/EC_{50})$	max	pEC_{50}	$\log(\max/EC_{50})$	$\Delta\log(\max/EC_{50})$	ΔpEC_{50}
Left atria	1.0	8.21	8.21	0.13	7.00	6.11	2.10	1.21
Right atria	1.0	8.70	8.70	0.44	6.90	6.54	2.16	1.80
Control-hyperthyroid	1.0	8.50	8.50	0.44	6.80	6.44	2.06	1.70
Control-desensitized	1.0	8.80	8.80	0.53	7.00	6.72	2.08	1.80
R. atria-hyperthyroid	1.0	9.60	9.60	0.88	7.52	7.47	2.13	2.08

measures of the power of the agonist activation across the two systems. This can be in terms of different receptors (receptor selectivity), different signaling pathways (biased signaling), cell-based agonist selectivity, or measures of the impact of receptor mutation on a given agonist activity. It is worth considering these settings as they are fundamental pharmacologic procedures applied to the quantitative measurement of agonist effect. As a prerequisite, it is useful to consider some operational features of the analysis.

Practical Use of the $\log(\max/EC_{50})$

It is important to note that the maximal response for agonism must be expressed as a fraction of the maximal window available in the assay to express agonism and not simply as the maximal response to the most efficacious agonist in the assay. For example, if direct activation of adenylate cyclase with forskolin in a given functional assay produces a maximal elevation of cAMP greater than the most efficacious agonist in the assay, then the maximal response to the agonist must be expressed as a fraction of the maximal effect of forskolin. In addition, the derivation given in Appendix 1 assumes that the Hill coefficient of the agonist concentration response curves is not significantly different from unity. In the comparison of the Black-Leff operational model scale of $\Delta\log(\tau/K_A)$, to $\Delta\log(\max/EC_{50})$ values, this is an explicit requirement to equate the two values (Appendix 2). In some instances in experimental pharmacology, this is not the case; therefore, it is useful to explore the effects of slopes differing from unity on the immutability of the $\Delta\log(\max/EC_{50})$ scale with receptor density and tissue sensitivity. Specifically, the comparison is made between a curve fit to the Hill equation of response = $[A]^n \max / ([A]^n + EC_{50}^n)$ where $n = 1$ and $n \neq 1$. Then comparisons of different $\log(\max/EC_{50})$ values can be made through simulation whereby the actual values of

$\Delta\log(\max/EC_{50})$ values for concentration-response curves of different slopes can be compared with the true values of $\Delta\log(\tau/K_A)$; as shown in eq. 2, this yields an error term φ , where

$$\varphi = \Delta\log(\max/EC_{50}) - \Delta\log(\tau/K_A). \quad (2)$$

Figure 3 shows a simulation surface of the dependence of φ on slope and the maximal response of the agonist concentration-response curves. It can be seen from this figure that slopes > 1 provide $\Delta\log(\max/EC_{50})$ values that depend only slightly on slope (as indicated by the small deviation with agonist maxima). In fact, the main region of deviation occurs with agonist concentration-response curves of low maximal response and slopes significantly less than unity. This should be

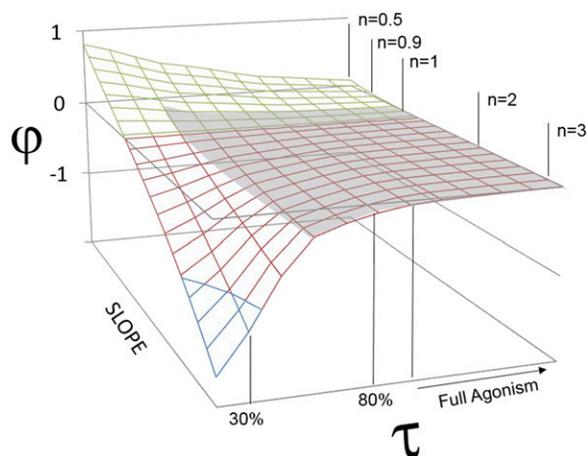


Fig. 3. Effects of efficacy (range of receptor densities) τ and slope of the agonist concentration-response curve (slope) on the differences between indices of agonism as calculated by $\log(\tau/K_A)$ versus $\log(\max/EC_{50})$ values.

considered as a caution to the use of $\Delta\log(\max/EC_{50})$ values for low-efficacy agonists (maximal responses <35%) demonstrating concentration-response curves with slopes significantly lower than 0.5.

Table 1 shows the sequential procedures required to apply the $\Delta\log(\max/EC_{50})$ scale for quantification of selectivity for different receptors, signaling pathways, cell types, and receptor sequence.

$\Delta\log(\max/EC_{50})$ Quantification of Receptor Selectivity

Historically, receptor selectivity has been expressed as the ratio of agonist potencies, and for full agonists, this yields useful and system independent measures; however, as discussed, the use of maximal responses extends this scale to all agonists, partial and full, and provides a more inclusive scale. Table 2 shows data describing concentration-response curves for four muscarinic agonists on M1 and M4 receptors (Chinese hamster ovary cell GTP γ S binding) from Watt et al. (2011). Selectivity can be calculated through conventional potency values (EC_{50}) or through $\log(\max/EC_{50})$; in the case of talsaclidine, different outcomes illustrate the effect of ignoring differences in maximal response. In cases where partial agonism is produced, EC_{50} values overestimate the agonism of the ligand because full agonism is assumed in the calculation. The first step is to cancel the effect of the sensitivity of each assay by comparing agonism with a reference compound; in this case, acetylcholine is the reference. Considering pEC_{50} values first, talsaclidine is 0.032-fold as active as acetylcholine on M1 receptors and 0.066-fold as active as acetylcholine on M4 receptors; this leads to an overall selectivity of talsaclidine of 2.1 for M4 receptors (talsaclidine is relatively more active on M4 than M1 receptors). Use of $\log(\max/EC_{50})$ values leads to a different conclusion; talsaclidine is 0.022-fold as active on M1 receptors and 0.016-fold as active on M4 receptors, which leads to an overall selectivity of 1.38-fold for M1 receptors (nonselective). This is in keeping with the lower maximal response of talsaclidine for M4 receptors (21.6%), indicating a lower activity for that receptor subtype. In general, pEC_{50} values assume a maximal response for M4 receptors, thereby overestimating M4 activity and erroneously classifying talsaclidine as M4-selective. Figure 4 shows the selectivity of the four agonists in these recombinant functional systems, where it can be seen that as the maximal responses to the agonists diminish, the disparity between $\Delta\log(\max/EC_{50})$ and $\Delta\Delta pEC_{50}$ increases.

TABLE 1
Practical application of the $\Delta\log(\max/EC_{50})$ scale to quantify selectivity

Procedure	Rationale
1) Fits dose-response data to function to yield \max (maximal response) and EC_{50}	Furnishes individual values for $\log(\max/EC_{50})$, a single index of agonism
2) Choose a reference agonist for comparison of all test agonists; use the same reference agonist for all systems (receptors, pathways, cell types)	Ratios to the index for the reference agonist cancels differences in receptor density, cell sensitivity, assay sensitivity, etc.
3) Calculate $\Delta\log(\max/EC_{50})$ values for each test agonist ($\Delta\log(\max/EC_{50}) = \log(\max/EC_{50})_{ref} - \log(\max/EC_{50})_{test}$)	Scales agonist activity of the test agonist to that of the reference agonist within a given system (receptor type, signaling pathway, cell type, etc.)
4) Calculate $\Delta\Delta\log(\max/EC_{50})$ values across the two systems being compared (different receptors, signaling pathways, cell types, protein sequence, etc.) ($\Delta\Delta\log(\max/EC_{50}) = \Delta\log(\max/EC_{50})_{ref} - \Delta\log(\max/EC_{50})_{test}$)	With the individual differences in sensitivity between the two systems cancelled, $\Delta\Delta\log(\max/EC_{50})$ values provide a system-independent measure of the relative agonism of each test agonist in both systems

The general tissue selectivity of agonists also can be quantified; Fig. 5 shows the relative activity of eight muscarinic agonists, compared with that of acetylcholine, in guinea pig bladder and ileum. Relative selectivity is calculated through $\Delta\log(\max/EC_{50})$ values and relative agonist potency ratios as $\Delta\Delta pEC_{50}$ values. In this calculation, the value for each agonist within a given tissue is compared with that of acetylcholine through $\log(\max/EC_{50})$ or pEC_{50} values, and then the selectivity between the tissues is assessed through differences in these values in these two tissues to yield $\Delta\log(\max/EC_{50})$ or $\Delta\Delta pEC_{50}$ values for tissue selectivity (to cancel differences in tissue sensitivity between the assays). The result is a measure of how well the agonists activate the muscarinic receptors of guinea pig bladder and ileum. Tissue-selective differences might be seen with varying mixtures of receptor type in each tissue- or in a cell-type effect on biased agonism (Kenakin, 2016); the analysis makes no assumptions as to the nature of the differences in potency and functions only as an operational measure of observed selectivity. It can be seen that, as expected, estimates are identical when both agonists produce full agonism. In drug discovery programs, however, where new test molecules of low intrinsic activity are compared with powerful standard agonists, this scale becomes important, illustrated by the deviations in values in Fig. 5, in which one of the agonists is a partial agonist in either tissue (see far right column of table showing relative maxima and compare differences between $\Delta\log(\max/EC_{50})$ and $\Delta\Delta pEC_{50}$).

$\Delta\log(\max/EC_{50})$ Quantification of Signaling Bias

Just as extracellular agonist selectivity can be quantified using this scale, so too can intracellular selectivity (agonist bias). This occurs when a given agonist that interacts with a pleiotropically coupled receptor selectively activates one or more of the signaling cascades at the expense of others (i.e., it biases the stimulus and does not distribute activation evenly among the available pathways). This is predicted to be the result of the stabilization of different receptor active states by different agonists (Kenakin and Morgan, 1989; Kenakin, 1995) and, irrespective of mechanism, is a widespread phenomenon in pharmacology studied by numerous research groups and given a variety of names [i.e., *stimulus trafficking* (Kenakin, 1995), *biased signaling* (Jarpe et al., 1998), *functional selectivity* (Lawler et al., 1999); collateral efficacy (Kenakin, 2005), *functional dissociation* (Whistler and van Zastrow, 1999), *biased inhibition* (Kudlacek et al., 2002), and *differential engagement* (Manning, 2002)]. Insofar as signaling

TABLE 2
M1/M4 receptor selectivity for agonists

	Max.	EC ₅₀ (nM)	log(max/EC ₅₀)	pEC ₅₀	Δlog(max/EC ₅₀)	Rel. Ag ^a	ΔpEC ₅₀	Rel. Ag. _b
Relative agonism at M1 receptors								
Acetylcholine	1	25.7	7.59	7.59	0	1	0	1
Sabcom.	0.389	56.2	6.84	7.25	-0.75	0.178	-0.34	0.457
Talsaclidine ^c	0.693	812.8	5.93	6.09	-1.66	0.022	-1.5	0.032
Xanol	0.637	43.7	7.16	7.36	-0.43	0.375	-0.23	0.589
Relative agonism at M4 receptors								
Acetylcholine	0.87	52.5	7.22	7.28	0	1	0	1
Sabcom	0.2	67.6	6.47	7.17	-0.75	0.178	-0.11	0.776
Talsaclidine	0.216	794.3	5.43	6.1	-1.79	0.016	-1.18	0.066
Xanol	0.46	63.1	6.86	7.2	-0.36	0.44	-0.08	0.832

^aRelative agonism based on log(max/EC₅₀) values.

^bRelative agonism based on EC₅₀ values.

^cTalsaclidine selectivity was calculated as ΔΔlog(max/EC₅₀). ΔΔlog(max/EC₅₀) = 0.13: Talsaclidine is 10^{0.13} = 1.38 selective for M1 receptors. Talsaclidine selectivity calculated as ΔΔpEC₅₀. ΔΔpEC₅₀ = -0.32: Talsaclidine is 10^{-0.32} = 0.48 selective for M1 receptors (i.e., 2.1 × selective for M4 receptors).

Data from Watt et al., 2011.

bias may be a therapeutically exploitable favorable agonist property, it is useful to have a quantitative scale to guide medicinal chemists in efforts to optimize this effect.

A theoretically optimal scale for this uses ΔΔlog(τ/K_A) values (Kenakin et al., 2012; Kenakin and Christopoulos, 2013), and just as ΔΔlog(max/EC₅₀) values can be useful surrogates for agonist selectivity, they can also function as the same for signaling bias. Thus, when the slopes of the concentration response curves to the agonists are >0.5 or maximal responses >35%, ΔΔlog(max/EC₅₀) values can provide convenient and rapid assessment of signaling bias. As for receptor selectivity, log(max/EC₅₀) values are calculated for each agonist for two signaling pathways and then compared through Δlog(max/EC₅₀) values using a reference agonist in each (the reference agonist must be the same for both pathways); this cancels the relative effects of assay sensitivity in each assay. This is extremely important because signaling assays, such as effects on second messengers (e.g., cAMP) are highly coupled and much more sensitive than assays quantifying β-arrestin complementation. After this, cross pathway

comparison can be done through comparison of Δlog(max/EC₅₀) values to yield values of ΔΔlog(max/EC₅₀). The bias is then calculated as the antilog of ΔΔlog(max/EC₅₀) values. It should be noted that the bias is a vector that can be expressed in two directions. For example, a bias for two agonists A and B showing that agonist A favors the cAMP system (over β-arrestin) by a factor of 5 can also be expressed as agonist A having a bias away from β-arrestin of 0.2. In general, when bias values are reported, this vector orientation must always be denoted.

Figure 6 shows the bias of five opioid receptor agonists compared with that of salvinorin A for κ-opioid receptor inhibition of cAMP production and β-arrestin signaling pathways (White et al., 2014). As with receptor selectivity, it can be seen that bias estimates differ when one of the agonists produces partial maximal response and simple EC₅₀ values (in the form of ΔΔpEC₅₀) versus consideration of maxima (in the form of ΔΔlog(max/EC₅₀) estimates are used. In general, bias is underestimated if only EC₅₀ values are used.

Assessment of Cell-Type Specificity

A well known observation in pharmacology is the imposition of cell-type effects on receptor selectivity. For example, expression of the same receptor in different host cell types can produce differences in the relative potency ratios of agonists (e.g., calcitonin) (Christmanson et al., 1994; Watson et al., 2000). Although this is incompatible with a monotonic receptor coupling scheme for agonists in cells, it can occur if agonists produce biased signaling at the receptor and the difference host cell types emphasize the heterogenous signals in different ways (Kenakin, 2016); in these cases, ΔΔlog(max/EC₅₀) values can be used to identify cell-type specificity. Specifically, bias plots, where the response to an agonist in one cell type is expressed as a function of the response in another cell type, can furnish visual data to indicate where an agonist produces a unique response in a given cell type over other agonists. For example, Fig. 7 shows label-free responses to muscarinic agonists in HT-29 and SF268 cells (Deng et al., 2013). A linear relationship would not necessarily be expected as different cell types may have differing receptor expression levels and efficiency of receptor coupling, but if the agonists

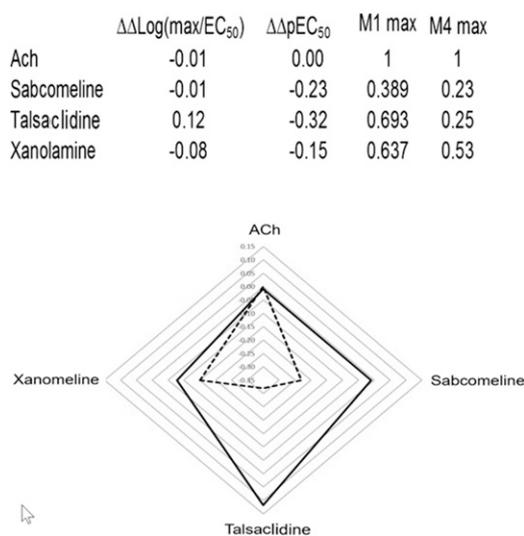


Fig. 4. Radar plot showing muscarinic receptor selectivity for four agonists activating M1 compared with M4 receptors. Selectivity expressed as ΔΔlog(max/EC₅₀) values (solid line) and ΔΔpEC₅₀ values (dotted line). Data recalculated from Watt et al., 2011.

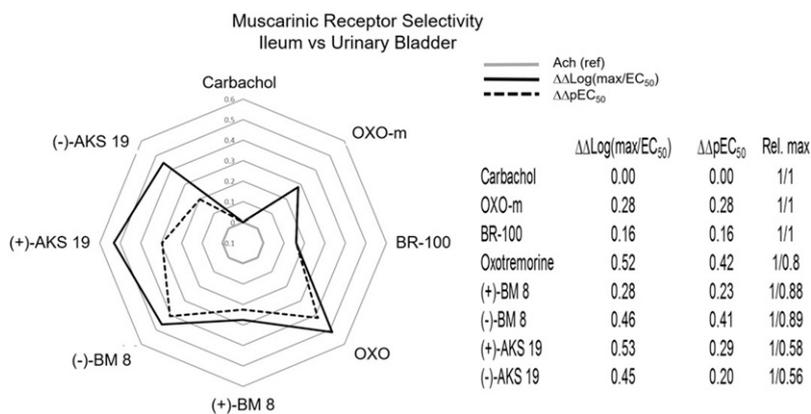


Fig. 5. Radar plot of receptor selectivity, compared with acetylcholine as a reference agonist, expressed as $\Delta\Delta\log(\max/EC_{50})$ values (solid line) and $\Delta\Delta pEC_{50}$ values (dotted line) for eight muscarinic agonists for agonism in guinea pig ileum and urinary bladder. Data from Ringdahl (1987). Right-most column indicates the maxima of agonists relative to that of acetylcholine. Note how $\Delta\Delta pEC_{50}$ values deviation from $\Delta\Delta\log(\max/EC_{50})$ increases with partial agonism.

produce a uniform receptor-active state, then a concordance (i.e., no deviations in the relationship for any one agonist) in this bias plot would be expected for all agonists tested. As seen in Fig. 7, however, although most of the agonists followed a fairly uniform pattern, bethanechol shows a distinctly different bias, being uniquely more active in SF268 cells than in HT-29 cells (compared with the other agonists). This difference can be quantified and statistically estimated through $\Delta\Delta\log(\max/EC_{50})$ values. For example, the data shown in the table with Fig. 7 shows that bethanechol is 7.86-fold biased toward producing responses in SF268 cells versus HT-29 cells. If, in the example shown in Fig. 7, the mean cell bias toward response in SF268 cells for acetylcholine, methacholine, carbachol, and Oxo-M is 1.7, bethanechol produces a $7.86/1.7 = 4.5$ -fold selective bias toward SF268 cells compared with these other agonists. This type of analysis might be applied to the testing of ligands in healthy cells those from disease models (or tumor versus normal cells) to identify unique cell-based activity for therapeutic applications.

Assessment of Receptor Mutation

In the study of the effects of receptor mutations on agonist function and functional signaling, important considerations are differences owing to variations in receptor expression. The application of $\Delta\Delta\log(\max/EC_{50})$ values negates this problem through comparison of effects to a common standard for both the wild type and mutated receptor. Just as with the assessment of signaling bias, the internal comparison of agonist function to a common reference agonist for both the

wild-type and mutated receptor cancels any effective differences in the disposition of the two types of receptor protein by the cell. Once the relative agonism of two agonists is quantified for each receptor species (wild-type versus mutation), then comparisons between them can be made that will be corrected for efficiency of transduction and expression with $\Delta\Delta\log(\max/EC_{50})$ values. One possible difference from the process used to assess signaling bias is in the choice of reference agonist. Specifically, when quantifying signaling bias, usually the test agonist is compared with the natural agonist to yield a measure of predicted differences in signaling with the synthetic agonist (as opposed to natural signaling). Whereas natural signaling is sometimes referred to as “unbiased signaling,” this is a misnomer since the natural agonist will be biased according to the physiologic needs of the organ; therefore, what is measured as “bias” for the synthetic ligand is simply a difference from the bias of the natural ligand. In contrast, when exploring the effects of mutation on receptor function, the aim often is to assess the effects of the mutation on the natural wild-type receptor interacting with the natural agonist. Under these circumstances, a synthetic ligand is chosen as the reference agonist (to cancel systems effects), and the induced bias on the natural ligand is thus measured as an assessment of the effects of the mutation (Tschammer et al., 2011). Figure 8 shows the comparison of the wild-type dopamine D_{2L} receptor with a D_{2L} H393^{6,35}A receptor mutant through $\Delta\Delta\log(\max/EC_{50})$ and $\Delta\Delta pEC_{50}$ values; it can be seen from this figure that, as with receptor selectivity and agonist bias, the effects of mutation are underestimated if $\Delta\Delta pEC_{50}$ values are used (as opposed to $\Delta\Delta\log(\max/EC_{50})$).

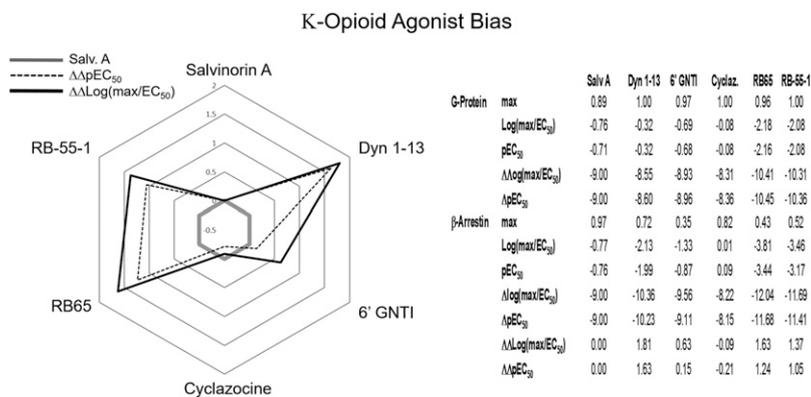


Fig. 6. Radar plot showing biased signaling of κ -opioid agonists (G proteins versus β -arrestin) either through $\Delta\Delta\log(\max/EC_{50})$ values (solid line) or $\Delta\Delta pEC_{50}$ values (dotted line); reference agonist is salvininorin A. Data from White et al., 2014.

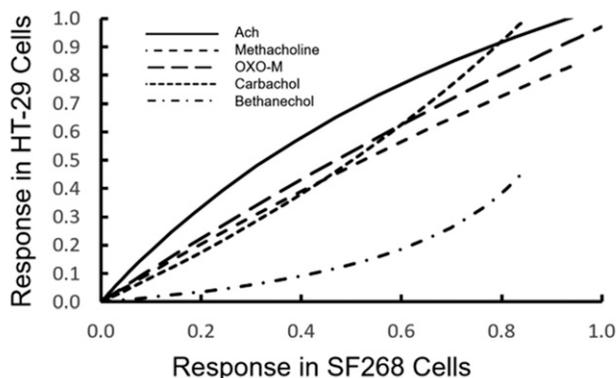


Fig. 7. Cell-based agonism: Bias plots showing relative responses to muscarinic agonists in HT-29 and SF268 cells. The system bias for four of the agonists is relatively uniform, but bethanechol stands out as being more active in SF268 cells than HT-29 cells. This effect can be quantified through a bias calculation as shown in the table below the figure. If the mean bias toward SF268 cells for acetylcholine, methacholine, Oxo-M, and carbachol is 1.75, then bethanechol is $7.86/1.75 = 4.5$ -fold selective for SF268 cells. Data from Deng et al., 2013.

	HT-29 Cells						SF268 Cells				
	pEC ₅₀	max	Log(max/EC ₅₀)	Δlog(max/EC ₅₀)	ΔΔlog(max/EC ₅₀)	BIAS	Δlog(max/EC ₅₀)	Log(max/EC ₅₀)	pEC ₅₀	max	
Ach	6.28	1.00	6.28	0	0	1	0	6.00	6	1	
OXO-M	6	0.97	5.99	-0.31	0.23	1.69	-0.08	5.92	5.92	1	
Meta	6.08	0.84	6.00	-0.29	0.27	1.86	-0.02	5.98	6	0.95	
CARB	5.36	0.99	5.36	-0.94	0.39	2.43	-0.55	5.45	5.52	0.84	
BETH	4.72	0.46	4.38	-1.92	0.90	7.86	-1.02	4.98	5.28	0.50	

Quantifying PAM Effects

An important distinction between NAMs and PAMs is that the effective affinity of the latter species (i.e., PAMs) depends much more on the co-binding ligand than does the former (NAMs). The reason for this comes from the expression for effective affinity of allosteric ligands in the Stockton-Ehlert allosteric binding model (Stockton et al., 1983; Ehlert, 1988). This predicts that the effective observed affinity of the allosteric modulator (expressed as K_{obs}) is given by eq. 3:

$$K_{obs} = \frac{K_B ([A]/K_A + 1)}{\alpha [A]/K_A + 1}, \quad (3)$$

where K_B is the equilibrium dissociation constant of the modulator-receptor complex with no cobinding ligand present and α is the effect of the modulator on the affinity of the cobinding ligand. It can be seen that for NAMs (where $\alpha \ll 1$), there will be a negative effect of cobinding ligand commensurate with standard antagonist experiments (i.e., basically a modified Cheng-Prusoff (Cheng and Prusoff, 1973) relationship between observed and microaffinity); however, in vivo, ambient agonist concentrations are probably not high, and this modification of NAM potency will not be extensive. In

contrast, for PAMs where $\alpha \gg 1$, the cobinding ligand will have a profound effect on the effective affinity of the modulator, even for low concentrations of agonist. For a NAM with $\alpha = 0.01$, and assuming a concentration of agonist = K_A , the correction will be a factor of 1.1, whereas for a PAM with $\alpha = 100$, the correction will be a 50-fold increase in observed affinity. This effect means that a useful estimation of the effective affinity of the PAM cannot be obtained in the absence of the cobinding ligand, a fact implicitly considered in the standard screening assay for PAMs. In these assays the PAM is added to an assay already partially activated by the endogenous agonist. When this is done, the resulting potentiation of the endogenous agonist effect produces a sigmoidal concentration-response curve to the PAM referred to as an R_{50} curve (see Fig. 9).

Analysis of the midpoint and maximal asymptote of this curve yields an interesting parameter of PAM activity. Specifically, it can be seen that the parameter \max/R_{50} (where R_{50} is EC_{50} of the R_{50} curve) of this curve (see Fig. 9) furnishes a parameter of agonist potentiation that, when used as a ratio, provides a system-independent measure of the power of the PAMs involved to potentiate agonist response (see Appendix 4 for derivation). Specifically, different between $\log(\max/R_{50})$ values of R_{50} curves yield differences between the molecular

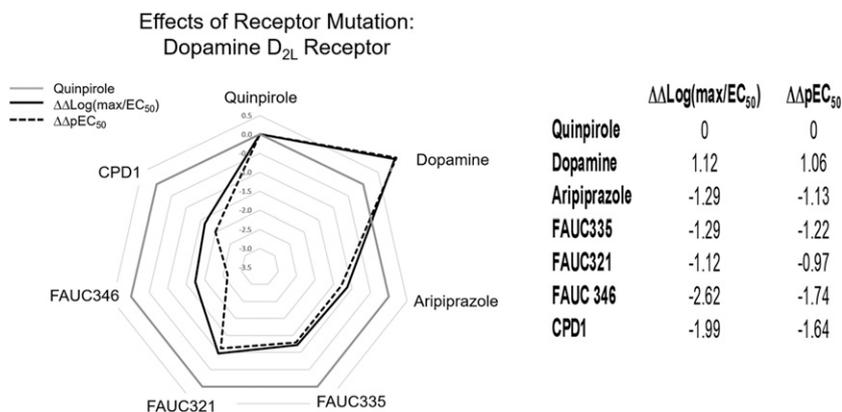


Fig. 8. Effects of mutation on dopamine D_{2L} receptor (comparison of wild-type to D_{2L} H393^{6.35}A receptor) through a radar plot showing $\Delta\Delta\log(\max/EC_{50})$ values (solid line) or $\Delta\Delta pEC_{50}$ values (broken line). Reference agonist is quinpirole. Data from Tschammer et al., 2011.

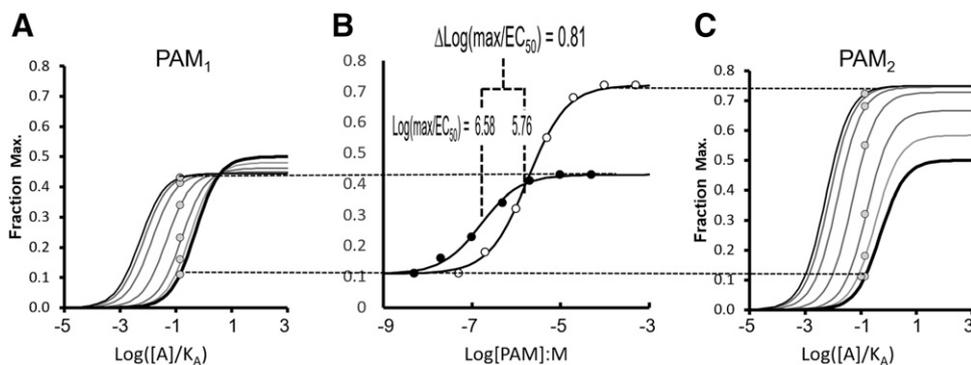


Fig. 9. Potentiation of a submaximal agonist effect with 2 PAMs. (A) Effects of PAM₁ with $\alpha = 120/\beta = 0.8/K_B = 1 \mu\text{M}$; curves shown for control ($[\text{PAM}_1] = 0$) and 5 nM, 20 nM, 0.1 μM , 0.5 μM , 2 μM , and 10 μM . (C) Effects of PAM₂ $\alpha = 50/\beta = 3/K_B = 10 \mu\text{M}$; curves are shown for control ($[\text{PAM}_2] = 0$) and 50 nM, 0.2 μM , 1 μM , 5 μM , 20 μM , and 100 μM . (B) Dose-response curves for the PAMs (R_{50} curves) as potentiation of the agonist response. $\log(\text{max}/\text{EC}_{50})$ value for PAM₁ = 6.58 and $\log(\text{max}/\text{EC}_{50})$ for PAM₂ = 5.76, providing a $\Delta\log(\text{max}/\text{EC}_{50})$ value of 0.82. From separate estimates of α , β , and K_B used to construct the curves the value for $\Delta\log(\alpha\beta/K_B)$ is 0.81.

system-independent parameters describing PAM (eq. 4), namely α , β , and K_B :

$$\Delta\log(\text{max}/R_{50}) = \Delta\log(\alpha\beta/K_B). \quad (4)$$

This parameter has the potential to be extremely useful since, in theory, it can be used to measure the relative effects of PAMs in vivo. This is important since the effective activity of PAMs is expressed only in the presence of the natural agonist, and the effect is relatively unknown in vivo. Through standard pharmacologic null experimentation, however, R_{50} curves obtained in vivo can be used to compare PAMs in a system-independent manner by simply comparing the effects of the PAMs on natural ambient agonist activity in the in vivo system. Figure 10 shows two $\log(\text{max}/R_{50})$ curves for in vitro potentiation of muscarinic receptor activity of acetylcholine by two experimental PAMs (Mistry et al., 2016). In this particular case, the $\Delta\log(\text{max}/R_{50})$ values indicate comparable PAM effects, which is confirmed by individual estimation and calculation of $\Delta\log(\alpha\beta/K_B)$ values measured from separate experiments fitting data to the functional allosteric model. Specifically, the $\Delta\log(\alpha\beta/K_B)$ estimate for the compounds shown is 0.05 and the $\Delta\log(\text{max}/R_{50})$ shows a comparable value ($\Delta\log(\text{max}/R_{50}) = 0.12$). This method is based on the null cancellation of the basal activity level of the system and the isolation of the effect of a PAM on that basal level of response.

Conclusions

This article proposes that two descriptive parameters for dose-response curves, namely, the EC_{50} and maximal response, can be used to furnish system-independent ratios of

agonist activity in a variety of settings. The inclusion of maximal response into the index for agonism takes into account the heterogeneous effects of varying system sensitivity on dose-response curves for partial and full agonists. This, in turn, allows seamless comparisons to be made between full and partial agonists in functional systems. The index, $\log(\text{max}/\text{EC}_{50})$, embodies agonism into a single number, which then lends itself to statistical analysis and allows null methods to cancel tissue effects such as receptor number, receptor coupling efficiency, and amplification within functional assays between agonists for any given system. Once this cancellation has been done, $\Delta\log(\text{max}/\text{EC}_{50})$ values become system-independent measures of the power of the test agonist(s) (compared with a reference agonist) to induce response in the defined system. These indices then can be used to compare different systems; thus, $\Delta\Delta\log(\text{max}/\text{EC}_{50})$ values can be used to quantify extracellular receptor selectivity, intracellular receptor selectivity (biased signaling), cell-specific agonism, and the effects of receptor mutation on natural signaling.

In addition, the same parameters from a different type of dose-response curve, namely, the potentiation of an ambient agonist response by a PAM, can be used to quantify allosteric modulation both in vitro and in vivo. This may be especially useful for the in vivo comparison of PAM effects since the affinity and potentiating activity of these types of molecules are dependent on the presence of the cobinding ligand (in this case, the endogenous agonist), and this may be variable in vivo. If different PAMs are compared under similar conditions in an in vivo system, relative measures of PAM activity based on molecular parameters of ligand-receptor interaction may be derived.

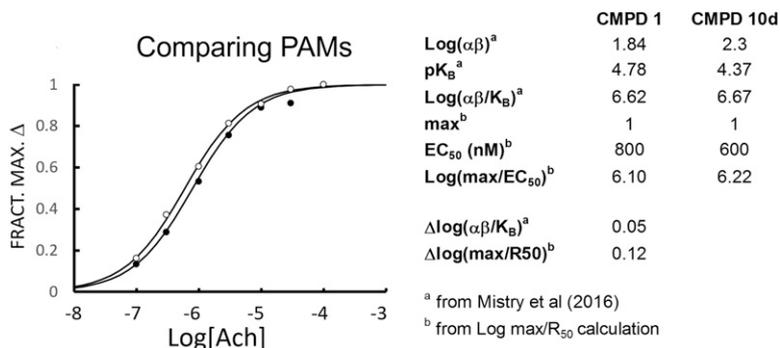


Fig. 10. R_{50} curves for two PAMs for muscarinic M1 receptors (filled circles = CMPD 10 d/open circles CMPD 1). Table on right shows calculation of $\Delta\log(\text{max}/R_{50})$ values (0.12); $\log(\alpha\beta/K_B) = 0.05$ from separate estimations of α , β , and K_B (Mistry et al., 2016).

These approaches are clearly applicable to the advancement of candidate molecules in drug discovery programs (quantifying selectivity and bias); however, they can also be used to quantify molecular properties of receptors (differences in receptor signaling seen with receptor mutation) and even operational effects of different cell types on receptor signaling. This latter process could be especially useful in the optimization of cell type (i.e., pathology-related) agonism through medicinal chemistry.

Appendix 1: Agonism as a Positive Allosteric Modulation of Receptor-Signaling Protein Interaction

The functional allosteric model (Scheme 1) yields two receptor species that produce cellular response, namely [RG] and [ARG]. These interact with the cell stimulus response mechanisms: [RG] with an equilibrium dissociation constant K_E to a signaling species [RGE] and [ARG] producing response with an equilibrium dissociation constant K'_E to a signaling species [ARGE].

From scheme 1, the system defines the following:

$$[RG] = [ARG]/\alpha[A]K'_A \quad (5)$$

$$[AR] = [ARG]/\alpha[G]K_g \quad (6)$$

$$[R] = [ARG]/\alpha[A]K'_A[G]K_g \quad (7)$$

The receptor conservation equation ($[R_{tot}] = [R] + [AR] + [RG] + [ARG]$) can be rewritten using eq. 5–7 as:

$$[R_{tot}] = [G]/K_G \left(1 + \alpha[A]/K'_A \right) + [A]/K'_A + 1, \quad (8)$$

where K_G and K'_A are equilibrium dissociation constants ($K'_A = 1/K'_a$ and $K_G = 1/K_g$).

Substituting the term in eq. 8 for $[R_{tot}]$ and defining the fraction of receptors RG as ρ_G and ARG as ρ_{AG} respectively yields:

$$\rho_G = \frac{[RG]}{[R_{tot}]} = \frac{[G]/K_G}{[G]/K_G \left(1 + \alpha[A]/K'_A \right) + [A]/K'_A + 1} \quad (9)$$

$$\rho_{AG} = \frac{[ARG]}{[R_{tot}]} = \frac{\alpha[A]/K'_A[G]/K_G}{[G]/K_G \left(1 + \alpha[A]/K'_A \right) + [A]/K'_A + 1} \quad (10)$$

The subsequent interaction of the receptor-signaling protein complex (either agonist bound or not) is processed through the Black-Leff operational model (Black and Leff, 1983) as a forcing function to generate a response from the agonist. Specifically, these fractional receptor species can be entered into the Black-Leff operational model form for response:

$$\text{Response} = \frac{\left([RG]/K_E + [ARG]/K'_E \right) E_m}{\left([RG]/K_E + [ARG]/K'_E \right) + 1} \quad (11)$$

The spontaneous active state receptor has a natural efficacy (denoted τ_G) for the production of response through coupling to the signaling protein. Defining the efficacy of the active state receptor as $\tau_G = [R_{tot}]/K_E$ and the efficacy of the agonist-bound active state receptor as $\tau_A = [R_{tot}]/K'_E$ further defines the factor β as the ratio of the efficacy of the nonagonist-bound

receptor (τ_G) and agonist-bound receptor. The efficacy of the agonist in terms of the Black-Leff operational model (τ_A) therefore yields the term β as τ_A/τ_G , and the operational model equation can be rewritten:

$$\text{Response} = \frac{(\rho_G \tau_G + \rho_{AG} \beta \tau_G)}{\rho_G \tau_G + \rho_{AG} \beta \tau_G + 1} \quad (12)$$

Substituting for ρ_G and ρ_{AG} from eq. 9 and eq. 10 yields:

$$\text{Response} = \frac{\left(\alpha \beta \tau_G [A]/K'_A [G]/K_G + \tau_G [G]/K_G \right) E_m}{[A]/K'_A \left(1 + \alpha [G]/K_G (1 + \beta \tau_G) \right) + \tau_G [G]/K_G + 1} \quad (13)$$

Equation 13 defines a sigmoidal curve for the agonist results from which values of maximal response (denoted *max*) can be derived:

$$\text{max} = \frac{\alpha \beta \tau_G [G]/K_G E_m}{1 + \alpha [G]/K_G (1 + \beta \tau_G)} \quad (14)$$

It should be noted that for all calculations utilizing the Black-Leff operational model and these indices of agonist activity, the maximal response to the agonist must be expressed as a fraction of the maximal window of response available in the assay. Thus, no agonist can produce a maximal response greater than unity (the maximal response window for the assay). Similarly, the EC_{50} is given as:

$$EC_{50} = \frac{K'_A (\tau_G [G]/K_G + 1)}{1 + \alpha [G]/K_G (1 + \beta \tau_G)} \quad (15)$$

Combining eq. 14 and eq. 15 yields:

$$\frac{\text{max}}{EC_{50}} = \frac{\alpha \beta \tau_G [G]/K_G E_m}{K'_A (\tau_G [G]/K_G + 1)} \quad (16)$$

It can be shown that a ratio of the quotients max/EC_{50} (where *max* refers to the maximal response to the agonist and the EC_{50} (the concentration of agonist producing 50% of the agonist maximal response) results in a system independent parameter quantifying agonism. Used as $\Delta \log(\text{max}/EC_{50})$ values for two agonists (denoted agonist_1 and agonist_2), this can be shown to be:

$$\Delta \log(\text{max}/EC_{50})_{1-2} = \log(\alpha_1 \beta_1 / K'_{A-1}) - \log(\alpha_2 \beta_2 / K'_{A-2}). \quad (17)$$

Specifically, eq. 17 reveals that $\log(\text{max}/EC_{50})$ is a combination of an assay and tissue term and a strictly agonist term (specifically $\alpha \beta / K'_A$):

$$\log \left[\frac{\text{max}}{EC_{50}} \right] = \log \left[\frac{\tau_G [G]/K_G E_m}{\tau_G [G]/K_G + 1} \right] + \log \left[\frac{\alpha \beta}{K'_A} \right] \quad (18)$$

Therefore, the ratio of max/EC_{50} values, which subtracts and thus cancels the two $\log((\tau_G [G]/K_G E_m)/(\tau_G [G]/K_G + 1))$ terms is independent of the assay and tissue effects and becomes a unique identifier of for the two agonists; for agonist_1 and agonist_2 the $\Delta \log(\text{max}/EC_{50})$ is $\Delta \log(\alpha \beta / K'_A)$ which is a system independent ratio of agonism.

The value $\alpha \beta / K'_A$ comprises only drug parameters (α is the change in the affinity of the receptor for the signaling protein produced by the binding of the agonist and reciprocally the affinity of the agonist when the signaling protein interacts with the receptor), K'_A is the equilibrium dissociation of the

receptor agonist complex when the receptor does not interact with the signaling protein, and β is the change in the efficacy of the receptor for production of response produced by the agonist.

Appendix 2: Relationship between $\Delta\log(\max/EC_{50})$ and $\Delta\log(\tau/K_A)$ Through the Black-Leff Operational Model

Agonist response is modeled by the Black-Leff operational model for systems yielding response with a variable Hill coefficient slope as (Black et al, 1985):

$$\text{Response} = \frac{[A]^n \tau_A^n E_m}{[A]^n \tau_A^n + ([A] + K_A)^n}, \quad (19)$$

where τ_A is the efficacy of the agonist, n is the Hill coefficient of the agonist concentration-response curve, and E_m the maximal response window of the functional assay. It should be noted that the K'_A in eq. 18 in terms of the Black-Leff model is the equilibrium dissociation constant of the agonist-response complex for agonism, with the receptor interacting with the signaling protein. Therefore, the K_A term is the operational equilibrium dissociation constant of the agonist-receptor complex (i.e., agonist binding to the receptor as it interacts with the signaling protein). If the agonist is viewed as a modulator of signaling protein interaction, then the operational K_A is equal to α/K'_A . Black et al. (1985) provided expressions for the maximal response (max) as:

$$\max = \frac{\tau_A^n E_m}{(1 + \tau_A^n)} \quad (20)$$

and for the EC_{50} for half maximal response as:

$$EC_{50} = \frac{K_A}{(2 + \tau_A^n)^{1/n} - 1} \quad (21)$$

This leads to an expression for \max/EC_{50} of:

$$\frac{\max}{EC_{50}} = \frac{\tau_A^n ((2 + \tau_A^n)^{1/n} - 1) E_m}{K_A (1 + \tau_A^n)} \quad (22)$$

For $n = 1$, $\max/EC_{50} = \tau E_m/K_A$; ratios of (\max/EC_{50}) values cancel the tissue E_m term and yield a strictly agonist-dependent term τ/K_A . Therefore, ratios of \max/EC_{50} values (in the form of $\Delta\log(\max/EC_{50})$ values for systems where the slope of the agonist concentration response curves is not significantly different from unity) yield strictly agonist dependent (and system-independent) values for relative agonism:

$$\Delta\log(\max/EC_{50}) = \Delta\log(\tau_A/K_A) \quad (23)$$

Appendix 3: Statistical Assessment of Difference Using $\Delta\Delta\log(\max/EC_{50})$ Values

If individual estimates of $\log(\max/EC_{50})$ are available, then a statistical estimate of mean $\log(\max/EC_{50})$ values, $\Delta\log(\max/EC_{50})$ values, and $\Delta\Delta\log(\max/EC_{50})$ values can be calculated in the form of 95% confidence limits of the estimated values. For a set of k to n values for agonist y activating signaling system j , s_{ij}^2 is defined as:

$$S_{ij}^2 = \frac{1}{n_{ij} - 1} \sum_{k=1}^{n_j} (y_{ijk} - y_{\text{mean}})^2 \quad (24)$$

Values for s_{ij}^2 are calculated for sets of K agonists and all signaling pathways to yield a pooled variance defined by:

$$s_{\text{pooled}} = \sqrt{\frac{\sum_{i=1}^K \sum_{j=1}^2 s_{ij}^2}{df_{\text{error}}}}, \quad (25)$$

where df_{error} is given as:

$$df_{\text{error}} = \sum_{i=1}^K \sum_{j=1}^2 (n_{ij} - 1). \quad (26)$$

From these values, a 95% confidence limit with two-tailed T values ($T_{97.5}$) can be calculated. For a mean $\log(\max/EC_{50})$ estimate:

$$95\% \text{ c.I.} = T_{97.5} \times s_{\text{pooled}} \sqrt{\frac{1}{n_{ij}}}. \quad (27)$$

Within any one assay, agonist comparison with a standard yields a ratio of $\log(\max/EC_{50})$ values denoted as $\Delta\log(\max/EC_{50})$. The 95% c.l. of this ratio is defined as

$$95\% \text{ c.I.} = T_{97.5} \times s_{\text{pooled}} \sqrt{\frac{1}{n_{ij}} + \frac{1}{n_{i'j}}}. \quad (28)$$

Once values have been normalized to a reference standard agonist within each group (receptor type, signaling pathway, cell type), then a 95% c.l. can be calculated for selectivity or bias for the $\Delta\Delta\log(\max/EC_{50})$ value as:

$$95\% \text{ c.I.} = T_{97.5} \times s_{\text{pooled}} \sqrt{\frac{1}{n_{i1}} + \frac{1}{n_{i'1}} + \frac{1}{n_{i2}} + \frac{1}{n_{i'2}}}. \quad (29)$$

See Figure 11 for the application of equations (24) through (29).

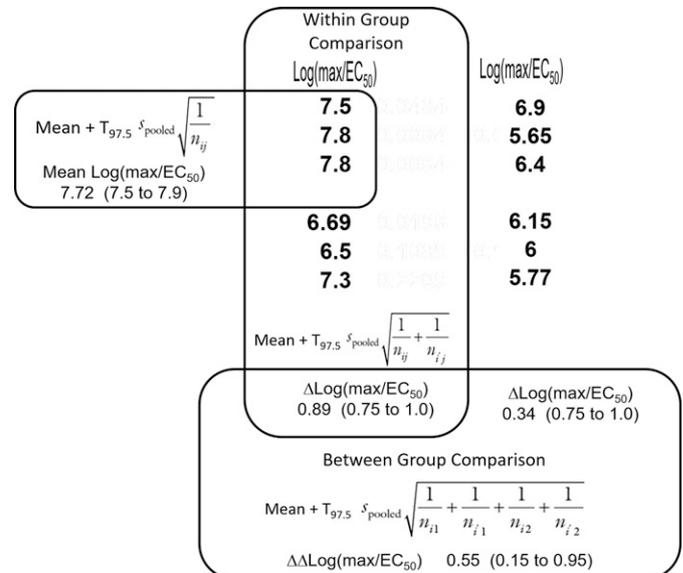


Fig. 11. The application of equations (24) through (29).

Appendix 4: Application of log(Max/R₅₀) Values from R₅₀ Curves to Quantify the Effects of PAMs

The model for allosteric effects in functional systems defines agonist response as (Ehlert, 2005; Kenakin, 2005; Price et al, 2005):

$$\text{Response} = \frac{\tau_A [A]/K_A (1 + \alpha \beta [B]/K_B)}{[A]/K_A (1 + \alpha [B]/K_B + \tau_A (1 + \alpha \beta [B]/K_B)) + [B]/K_B + 1}, \quad (30)$$

where α is the effect of the modulator ($[B]$) on the affinity of the agonist for the receptor, and β is the effect of the modulator on the efficacy of the agonist. This equation can be rewritten in terms of the modulator as the active species to:

$$\text{Response} = \frac{\alpha \beta \tau_A [B]/K_B ([A]/K_A + \tau_A [A]/K_A)}{[B]/K_B (1 + \alpha [A]/K_A (1 + \beta \tau_A)) + [A]/K_A (1 + \tau_A) + 1}, \quad (31)$$

This defines the R_{50} curve for a potentiating modulator (PAM) increasing the effect of an ambient agonist response due to a presence of agonist acting on the receptor (in the form of $[A]/K_A$).

The maximal response of the R_{50} curve is thus given as:

$$\max = \frac{\alpha \beta \tau_A [A]/K_A}{(1 + \alpha [A]/K_A (1 + \beta \tau_A))}, \quad (32)$$

and the hal-maximal effect of the R_{50} curve (defined as the R_{50}) is given as:

$$R_{50} = \frac{K_B ([A]/K_A (1 + \tau_A) + 1)}{(1 + \alpha [A]/K_A (1 + \beta \tau_A))}. \quad (33)$$

This leads to the ratio of \max/R_{50} as:

$$\frac{\max}{R_{50}} = \frac{\alpha \beta \tau_A [A]/K_A}{K_B ([A]/K_A (1 + \beta \tau_A)) + 1}. \quad (34)$$

It can be seen that this expression is a mixture of tissue specific and agonist specific factors:

$$\frac{\max}{R_{50}} = \frac{\alpha \beta}{K_B} \times \frac{\tau_A [A]/K_A}{([A]/K_A (1 + \beta \tau_A)) + 1}. \quad (35)$$

Therefore, the ratios of \max/R_{50} values can provide system independent estimates of the relative activity of PAMs in potentiating agonist response:

$$\Delta \log(\max/R_{50})_{A-B} = \log(\alpha \beta / K_B)_A - \log(\alpha \beta / K_B)_B. \quad (36)$$

Authorship Contributions

Performed data analysis: Kenakin

Wrote or contributed to the writing of the manuscript: Kenakin

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