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Title page

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

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Running Title Page

Running title: Scale for Agonism and Allosteric Modulation

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Abbreviations: PAM- positive Allosteric modulator, NAM- negative allosteric modulator

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Abstract

An index of agonism is described which 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 is comprised of the maximal response to the agonist (max) and the EC_{50} (concentration of agonist producing half maximal response) 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* PAM 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 pharmacological application.

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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, i.e. they are system-independent measures of activity. For full agonists, system independent measures of activity are achieved through relative potency ratios (ratios of EC_{50} values where EC_{50} refers to the concentration of agonist producing 50% maximal response). However, this scale devolves into a non-linear scale when comparing full and partial agonists so it cannot be used for the comparison of these types of agonists. In this paper, 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). While the activity of NAMs can be quantified with standard methods for antagonists, the affinity of PAMs is uniquely dependent on the co-binding agonist and thus require special method*s* 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 advancement of candidate molecules.

Receptor-Signaling Protein Interaction

The discovery of constitutive seven transmembrane receptor (7TMR) 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) reveals that

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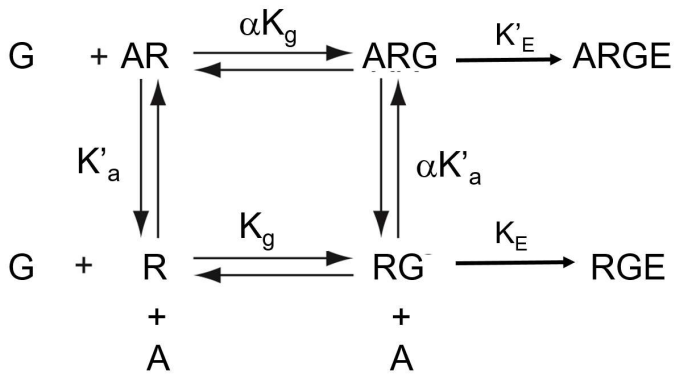
agonists can be considered simply as positive allosteric modulators of an already ongoing spontaneous association between the receptor and the signaling protein (Kenakin, 2015). In accordance with the standard functional allosteric model for receptors (Kenakin, 2005; Ehlert, 2005; Price et al, 2005) such modulators (in this case agonists) can change the affinity of the receptor for the signaling protein (through a co-operativity term α) and the efficacy of the receptor-signaling protein complex for production of cellular response (through a co-operativity 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, 1989) 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:



…[1]

This binding model is then placed as the receptor species producing unit for the Black/Leff operational model 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

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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 (see 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) creates 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- see Kenakin (2015) and Appendix 2.

Historically, the relative activity of agonists has been quantified through potency ratios (for example 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. are full agonists). However, this relationship breaks down when one or both of the agonists becomes 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 (see Fig 1A). This produces distinctly non-linear 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 non-linearity makes ΔpEC_{50}

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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 ΔLog(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 while ΔLog(max/EC_{50}) values remain constant through a range of tissue conditions.

There are three important points to consider when discussing Log(max/EC_{50}) values; the first is 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% c.l., comparison of the relative base agonist activity in any one system can be made with ΔLog(max/EC_{50}) values $\pm$ 95% confidence limits (c.l.) and finally, comparisons of selectivity, bias and the effects of mutation made through $\Delta\Delta$Log(max/EC_{50}) $\pm$ 95% c.l. estimates with simple adjustment of formulae based on the pooled variance- see Appendix 3.

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The second point is that the comparison of Log(max/EC_{50}) values between agonists in a single functional system to produce ΔLog(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 to a reference agonist and system effects are cancelled, i.e. the sensitivity of the functional system is not an issue and the ΔLog(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 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 pharmacological procedures applied to the quantitative measurement of agonist effect. As a pre-requisite 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 cyclic AMP greater than the most efficacious

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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 are 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≠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 to the true values of $\Delta Log(\tau/K_A)$; this yields an error term φ where:

$$\varphi = \Delta Log(max/EC_{50}) - \Delta Log(\tau/K_A) \quad \ldots[2]$$

Fig 3 shows a simulation surface of the dependence of φ on slope and maximal response of the agonist concentration response curves. It can be seen from this figure that slopes > 1 provide $\Delta Log(max/EC50)$ 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 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.

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

$\Delta\Delta$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 above, 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 (CHO cell GTPγS binding) from Watt et al, 2011. Selectivity can be calculated through conventional potency values (EC_{50}) or through Log(max/EC_{50}) and in the case of Talsidiidine, different outcomes illustrate the effect of ignoring differences in maximal response. In cases where partial agonism is produced, EC_{50} values over-estimate 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 to a reference compound; in this case, acetylcholine is the reference. Considering pEC_{50} values first, talsidiidine 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 talsidiidine of 2.1 for M4 receptors (talsidiidine is relatively more active on M4 than M1 receptors). Use of Log(max/EC_{50}) values leads to a different conclusion; talsidiidine is 0.022-fold as active on M1 receptors and 0.016-fold as active on M4 receptors ; this leads to an overall selectivity of 1.38-fold for M1 receptors (non-selective). This is in keeping with the lower maximal response of talsidiidine for M4 receptors (21.6%)

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indicating a lower activity for that receptor subtype. In general pEC_{50} values assume a maximal response for M4 receptors thereby over-estimating M4 activity and erroneously classifying talsididine as M4-selective. Fig 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 $\Delta\Delta Log(max/EC_{50})$ and $\Delta\Delta pEC_{50}$ increases.

General tissue selectivity of agonists also can be quantified; Fig 5 shows the relative activity of 8 muscarinic agonists, compared to that of acetylcholine, in guinea pig bladder and ileum. Relative selectivity is calculated through $\Delta\Delta Log(max/EC_{50})$ 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 to that of acetylcholine through $\Delta Log(max/EC_{50})$ or ΔpEC_{50} values and then the selectivity between the tissues assessed through differences of these values in these two tissues to yield $\Delta\Delta Log(max/EC5_0)$ 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 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. However, in drug discovery programs where new test molecules of low intrinsic activity are compared to powerful standard agonists, this scale becomes important. This is illustrated by the deviations in values in Fig 5 when

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one of the agonists is a partial agonist in either tissue (see far right column of table showing relative maxima and compare differences between $\Delta\Delta$Log(max/EC_{50}) and $\Delta\Delta$pEC_{50}).

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

Just as extracellular agonist selectivity can be quantified with 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 amongst 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); 'differential engagement' (Manning, 2002)). Insofar as signaling 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 utilizes $\Delta\Delta$Log(τ/K_A) values (Kenakin et al, 2012; Kenakin and Christopoulos, 2013) and just as $\Delta\Delta$Log(max/EC_{50}) 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

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>0.5 or maximal responses > 35%, $\Delta\Delta$Log(max/EC_{50}) values can provide convenient and rapid assessment of signaling bias. Just as for receptor selectivity, Log(max/EC_{50}) values are calculated for each agonist for two signaling pathways and then compared through ΔLog(max/EC_{50}) 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 as signaling assays such as effects on second messengers (i.e. cyclic AMP) 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/EC_{50}) values to yield values of $\Delta\Delta$Log(max/EC_{50}). The bias is then calculated as the antilog of $\Delta\Delta$Log(max/EC_{50}) 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.

Fig 6 shows the bias of 5 opioid receptor agonists, compared to that of salvinorin A, for κ-opioid receptor inhibition of cAMP production and β-arrestin signaling pathways (White et al, 2013). Just as with receptor selectivity, it can be seen that bias estimates differ when one of the agonists produces partial maximal response and simple EC_{50} values (in the form of $\Delta\Delta$pEC_{50}) vs consideration of maxima (in the form of $\Delta\Delta$Log(max/EC_{50}) estimates are used. In general, bias is under-estimated if only EC_{50} values are utilized.

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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 (i.e. calcitonin, Christmansson et al, 1994; Watson et al, 2000). While 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 $\Delta\Delta$Log(max/EC_{50}) 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 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. However, as seen in Fig 7, while most of the agonists followed a fairly uniform pattern, bethanechol shows a distinctly different bias being uniquely more active in SF268 cells than HT-29 cells (as compared to 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 theTable with Fig 7, shows that bethanechol is 7.86-fold biased toward producing responses in SF268 cells vs HT-29 cells. If, in the

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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 to these other agonists. This type of analysis might be applied to the testing of ligands in healthy cells those from disease models (or tumor vs 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 due to variations in receptor expression. The application of $\Delta\Delta\text{Log}(\text{max}/EC_{50})$ 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 vs mutation), then comparisons between them can be made that will be corrected for efficiency of transduction and expression with $\Delta\Delta\text{Log}(\text{max}/EC_{50})$ 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 to the natural agonist to yield a measure of predicted differences in signaling with the synthetic agonist (as opposed to natural signaling). While natural signaling is sometimes referred to as ‘unbiased signaling’ this is a misnomer since the natural agonist will be biased according to the

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physiological 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). Fig 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 under-estimated if $\Delta\Delta pEC_{50}$ values are utilized (as opposed to $\Delta\Delta Log(max/EC_{50})$).

Quantifying PAM Effects

An important distinction between negative allosteric modulators (NAMs) and positive allosteric modulators (PAMs) is that the effective affinity of the latter species (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:

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

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

An 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 R50 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

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response- see Appendix 4 for derivation. Specifically, differences between $Log(max/R_{50})$ values of R_{50} curves yield differences between the molecular system-independent parameters describing PAM, namely α,β and K_B:

$$\Delta Log(max/R_{50}) = \Delta Log(\alpha\beta/K_B) \quad \ldots[4]$$

This has the potential to be an extremely useful parameter 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 of this is relatively unknown *in vivo*. However, through standard pharmacological null experimentation, 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. Fig 10 shows two $Log(max/R_{50})$ curves for in vitro potentiation of muscarinic receptor activity of acetylcholine by two experimental PAMs (Mistry et al,2016). In this particular case, the $\Delta Log(max/R_{50})$ values indicate comparable PAM effects; this 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 $\Delta Log(max/R_{50})$ shows a comparable value ($\Delta Log(max/R_{50})$=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

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This paper 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 DR 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, $Log(max/EC_{50})$, 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, $\Delta Log(max/EC_{50})$ values become system-independent measures of the power of the test agonist(s) (compared to a reference agonist) to induce response in the defined system. These indices then can be used to compare different systems; thus, $\Delta\Delta Log(max/EC_{50})$ 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 upon the presence of the co-binding ligand (in this case, the endogenous agonist) and this may be variable in vivo. However, if different PAMs are compared under similar conditions in an in vivo system, relative measures of

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PAM activity based on molecular parameters of ligand-receptor interaction, may be derived.

These approaches are clearly applicable to the advancement of candidate molecules in drug discovery programs (quantifying selectivity and bias). However, they also can 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.

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Authors contributions:

TPK wrote the manuscript

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Legends for Figures

Fig 1. Effect of changing receptor density (and/or tissue sensitivity) on concentration response to a high efficacy agonist ($Agonist_1$) and low Efficacy Agonist ($Agonist_2$). A. Concentration response curves to $Agonist_1$ (Solid line curves) and $Agonist_2$ (dotted line curves) with increasing sensitivity of tissue. Note how less sinistral displacement of EC_{50} values for $Agonist_2$ are observed vs that for $Agonist_1$. B. $\Delta Log(max/EC_{50})$ values (solid line) and ΔpEC_{50} values (dotted line) with changing tissue sensitivity for Agonists 1 and 2. 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).

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

Fig 4. Radar plot showing muscarinic receptor selectivity for four agonists activating M1 vs M4 receptors. Selectivity expressed as $\Delta\Delta Log(max/EC_{50})$ values (solid line) and $\Delta\Delta pEC_{50}$ values (dotted line). Data recalculated from Watt et al, 2011.

Fig 5. Radar plot of receptor selectivity, compared to acetylcholine as a reference agonist, expressed as $\Delta\Delta Log(max/EC_{50})$ values (solid line) and $\Delta\Delta pEC_{50}$ values (dotted

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line) for 8 muscarinic agonists for agonism in guinea pig ileum and urinary bladder. Data from Ringdahl (1987). Column furthest to the right in the table indicates the maxima of agonists relative to that of acetylcholine- note how $\Delta\Delta$LogpEC$_{50}$ values deviation from $\Delta\Delta$Log(max/EC$_{50}$) increases with partial agonism.

Fig 6 Radar plot showing biased signaling of κ-opioid agonists (G proteins vs β-arrestin) either through $\Delta\Delta$Log(max/EC$_{50}$) values (solid line) or $\Delta\Delta$pEC$_{50}$ values (dotted line); reference agonist is salvinorin A. Data from White et al, 2012.

Fig 7 Cell-based agonism: Bias plots showing relative responses to muscarinic agonists in HT-29 and SF268 cells. While the system bias for four of the agonists are relatively uniform, 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.

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 sub-maximal agonist effect with 2 PAMs. Panel A shows the effects of PAM$_1$ with α = 120 / β= 0.8 / K$_B$= 1 μM; curves shown for control ([PAM$_1$]=0) and 5 nM, 20 nM , 0.1 μM, 0.5 μM, 2 μM and 10 μM. Panel C shows the effects of PAM$_2$ α=50 / β= 3 / K$_B$= 10 μM; curves shown for control ([PAM$_2$]=0) and 50 nM, 0.2

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μM, 1 μM, 5 μM, 20 μM and 100 μM. Panel B shows the dose-response curves for the PAMs (R_{50} curves) as potentiation of the agonist response. Log(max/EC_{50}) value for PAM_1 = 6.58 and Log(max/EC_{50}) for PAM_2 = 5.76 providing a ΔLog(max/EC_{50}) value of 0.82. From separate estimates of α, β, and K_B used to construct the curves the value for ΔLog($\alpha\beta/K_B$) is 0.81.

Fig 10. R_{50} curves for 2 PAMs for muscarinic M1 receptors (filled circles = CMPD 10d / open circles CMPD 1). Table on right shows calculation of ΔLog(max/R_{50}) values (0.12); Log($\alpha\beta/K_B$) = 0.05 from separate estimation of α,β, and K_B (Mistry et al, 2016).

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Table 1
Practical Application of the ΔΔLog(max/EC_{50}) Scale to Quantify Selectivity

Procedure	Rationale
1. Fit DR date to a function to yield max (maximal response) and EC_{50} (concentration of agonist producing 50% maximal response to the agonist).	This 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 for all systems (receptors, pathways, cell types)	Ratios to the index for the reference aognist will allow cancellation of receptor denstiy, cell sensitivity, assay sensitivity differences
3. Calculate Δlog(max/EC_{50}) values for each test agonist (ΛLog(max/EC_{50}) = Log(max/EC_{50})$_{ref}$ - Log(max/EC_{50})$_{test}$)	This will scale the agonist activity of the test agonist to the reference agonist within a given system (receptor type, signaling pathway, cell type etc)
4. Calculate ΔΔLog(max/EC_{50}) values across the 2 systems being compared (different receptors, signaling pathways, cell types, receptor protein sequence) (ΔΔLog(max/EC_{50}) = ΔΛog(max/EC_{50})$_{ref}$ - ΔΛog(max/EC_{50})$_{test}$)	With the individual differences in sensitivity between the two systems cancelled, ΔΔLog(max/EC_{50}) values provide a system independent measure of the relative agonism of each test agonist in both systems

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Table 2

M1/M4 Receptor Selectivity for Agonists

	max	EC_{50} (nM)	Log(max/EC_{50})	pEC_{50}	Δlog(max/EC_{50})	Rel. Ag.[1]	ΔpEC_{50}	Rel Ag.[2]
Relative Agonism at M1 Receptors								
Ach	1	25.7	7.59	7.59	0.00	1.000	0	1.000
Sabcomeline	0.389	56.2	6.84	7.25	-0.75	0.178	-0.34	0.457
Talsadidine	0.693	812.8	5.93	6.09	-1.66	0.022	-1.5	0.032
Xanolamine	0.637	43.7	7.16	7.36	-0.43	0.375	-0.23	0.589
Relative Agonism at M4 Receptors								
Ach	0.87	52.5	7.22	7.28	0.00	1.000	0	1.000
Sabcomeline	0.2	67.6	6.47	7.17	-0.75	0.178	-0.11	0.776
Talsadidine	0.216	794.3	5.43	6.1	-1.79	0.016	-1.18	0.066
Xanolamine	0.46	63.1	6.86	7.2	-0.36	0.440	-0.08	0.832

Talsadidine selectivity calculated as $\Delta\Delta$Log(max/EC_{50})

$\Delta\Delta$Log(max/EC_{50})= 0.13 : Talsadidine is $10^{0.13}$ = 1.38 selective for M1 receptors

Talsadidine selectivity calculated as $\Delta\Delta pEC_{50}$

$\Delta\Delta pEC_{50}$= -0.32: Talsadidine is $10^{-0.32}$= 0.48 selective for M1 receptors

(2.1x selective for M4 receptors)

[1] Relative Agonism based on Log(max/EC_{50}) values

[2] Relative Agonism based on EC_{50} values

Data from Watt et al, 2011.

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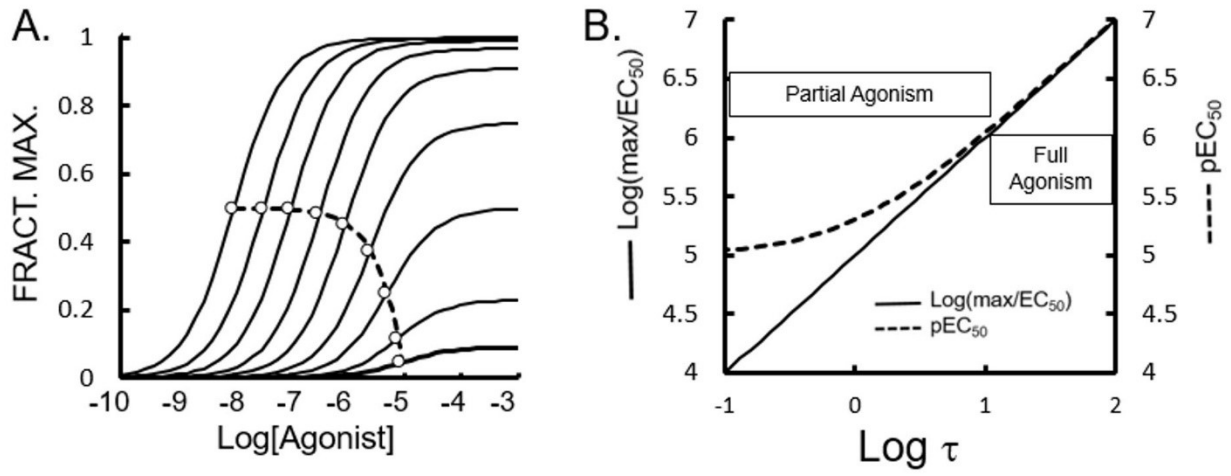

Figure 1

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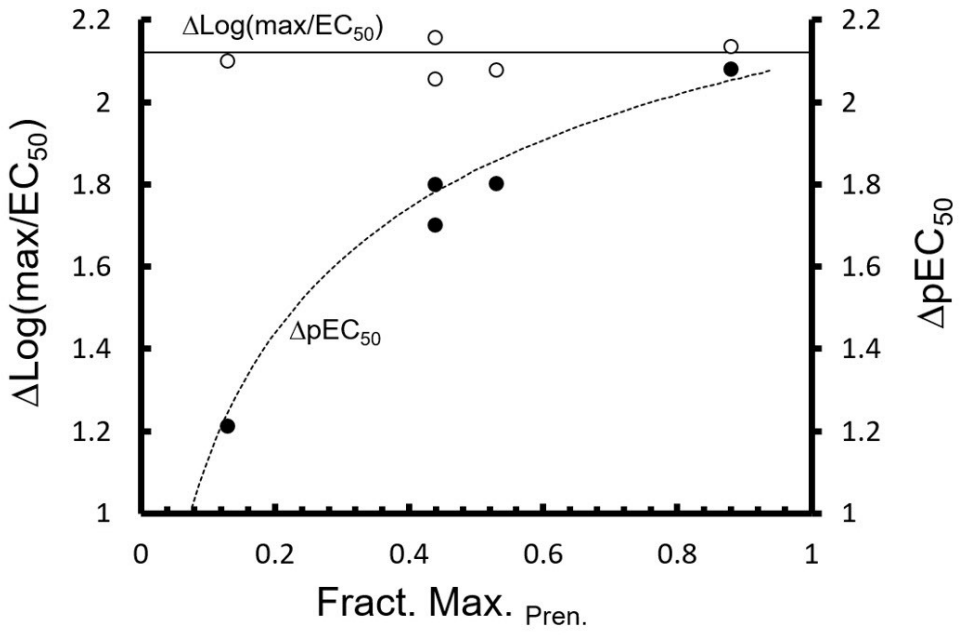

	Isoproterenol			Prenalterol				
	max	pEC_{50}	$Log(max/EC_{50})$	max	pEC_{50}	$Log(max/EC_{50})$	$\Delta log(max/EC_{50})$	ΔpEC_{50}
Left atria	1.0	8.21	8.21	0.13	7.00	6.11	2.10	1.21
Right atria	1.0	8.70	8.70	0.44	6.90	6.54	2.16	1.80
Control-hyperthyroid	1.0	8.50	8.50	0.44	6.80	6.44	2.06	1.70
Control-desensitized	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

Figure 2

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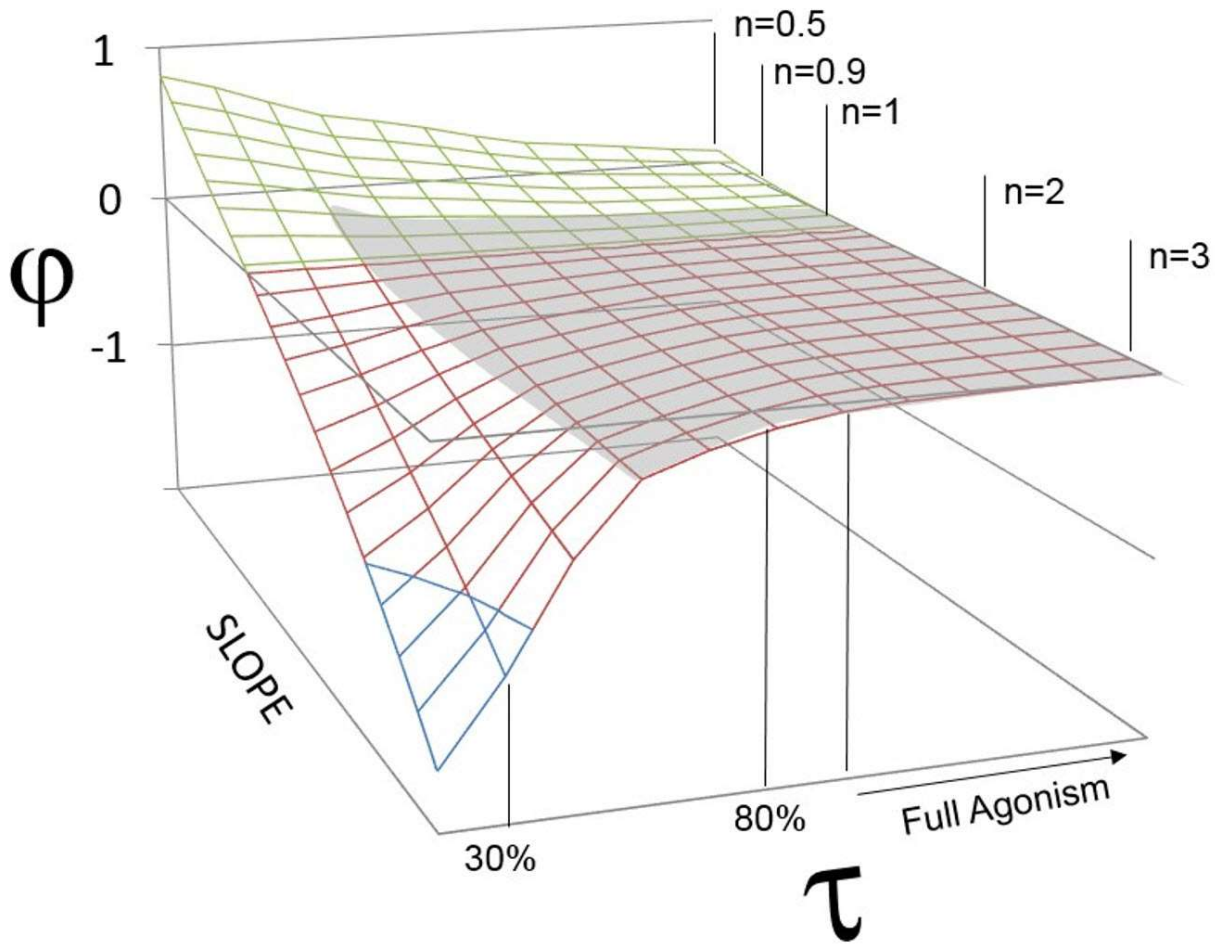

Figure 3

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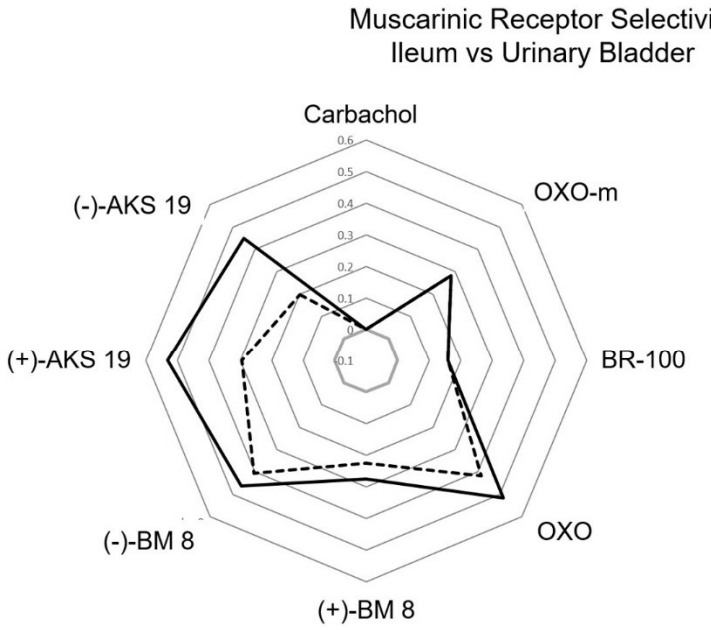

	ΔΔLog(max/EC50)	ΔΔpEC50	Rel. max
Carbachol	0.00	0.00	1/1
OXO-m	0.28	0.28	1/1
BR-100	0.16	0.16	1/1
Oxotremorine	0.52	0.42	1/0.8
(+)-BM 8	0.28	0.23	1/0.88
(-)-BM 8	0.46	0.41	1/0.89
(+)-AKS 19	0.53	0.29	1/0.58
(-)-AKS 19	0.45	0.20	1/0.56

Figure 4

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	$\Delta\Delta Log(max/EC_{50})$	$\Delta\Delta pEC_{50}$	M1 max	M4 max
Ach	-0.01	0.00	1	1
Sabcomeline	-0.01	-0.23	0.389	0.23
Talsadidine	0.12	-0.32	0.693	0.25
Xanolamine	-0.08	-0.15	0.637	0.53

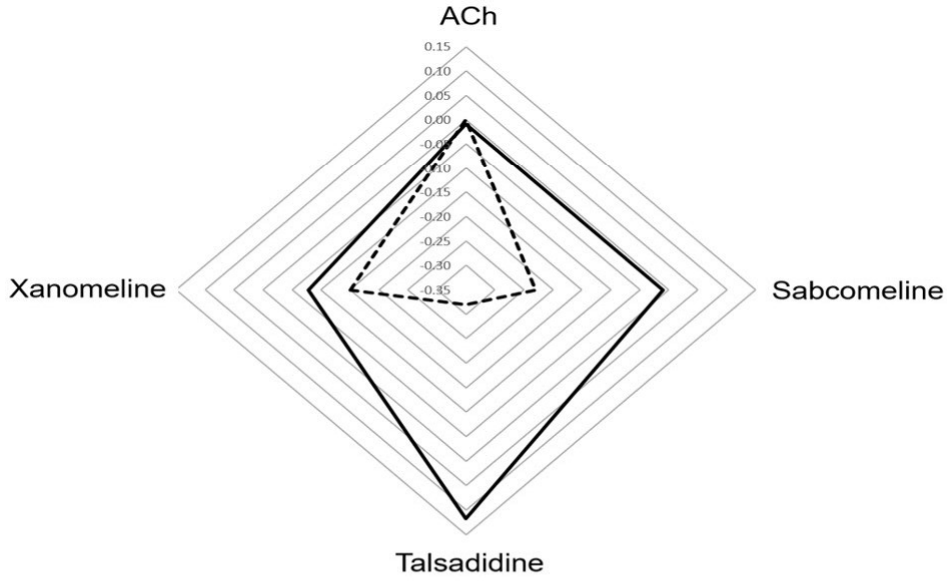

Figure 5

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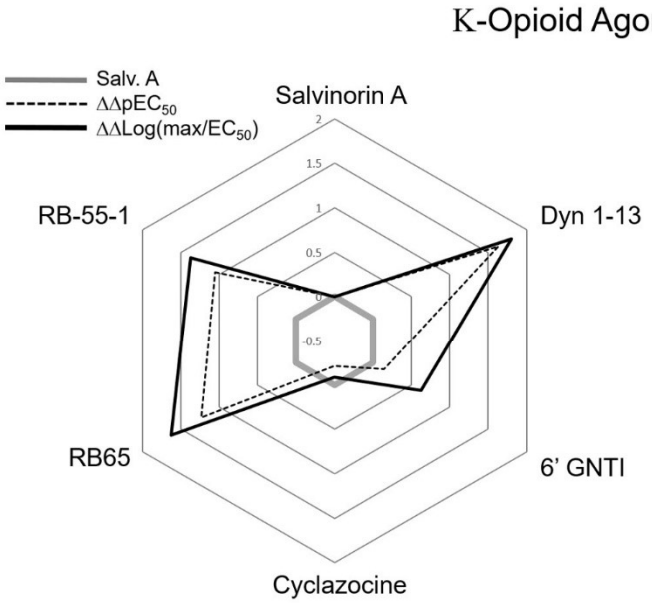

		Salv A	Dyn 1-13	6' GNTI	Cyclaz.	RB65	RB-55-1
G-Protein	max	0.89	1.00	0.97	1.00	0.96	1.00
	$Log(max/EC_{50})$	-0.76	-0.32	-0.69	-0.08	-2.18	-2.08
	pEC_{50}	-0.71	-0.32	-0.68	-0.08	-2.16	-2.08
	$\Delta Log(max/EC_{50})$	-9.00	-8.55	-8.93	-8.31	-10.41	-10.31
	ΔpEC_{50}	-9.00	-8.60	-8.96	-8.36	-10.45	-10.36
β-Arrestin	max	0.97	0.72	0.35	0.82	0.43	0.52
	$Log(max/EC_{50})$	-0.77	-2.13	-1.33	0.01	-3.81	-3.46
	pEC_{50}	-0.76	-1.99	-0.87	0.09	-3.44	-3.17
	$\Delta log(max/EC_{50})$	-9.00	-10.36	-9.56	-8.22	-12.04	-11.69
	ΔpEC_{50}	-9.00	-10.23	-9.11	-8.15	-11.68	-11.41
	$\Delta\Delta Log(max/EC_{50})$	0.00	1.81	0.63	-0.09	1.63	1.37
	$\Delta\Delta pEC_{50}$	0.00	1.63	0.15	-0.21	1.24	1.05

Figure 6

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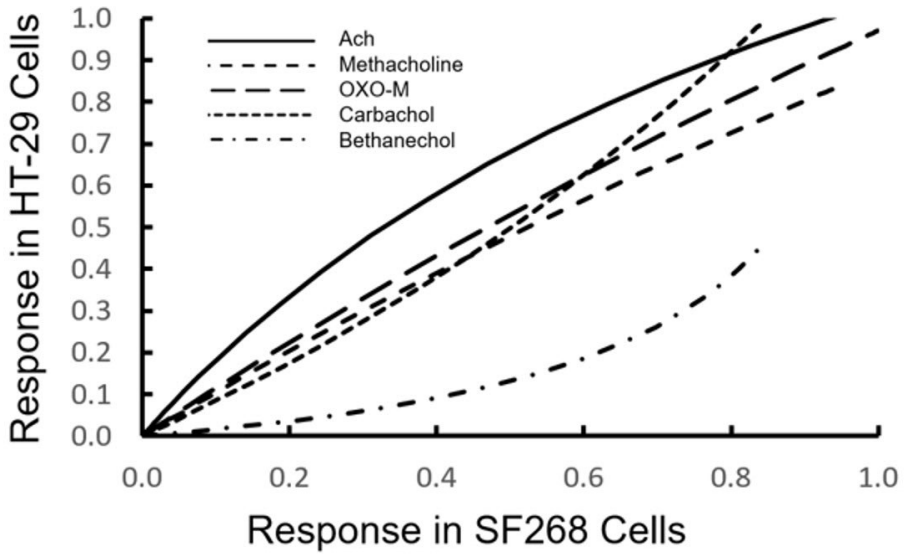

	HT-29 Cells						SF268 Cells			
	pEC_{50}	max	$Log(max/EC_{50})$	$\Delta log(max/EC_{50})$	$\Delta\Delta Log(max/EC_{50})$	BIAS	$\Delta log(max/EC_{50})$	$Log(max/EC_{50})$	pEC_{50}	max
Ach	6.28	1.00	6.28	0	0	1	0	6.00	6	1
OXO-M	6	0.97	5.99	-0.31	0.23	1.69	-0.08	5.92	5.92	1
Meta	6.08	0.84	6.00	-0.29	0.27	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

Figure 7

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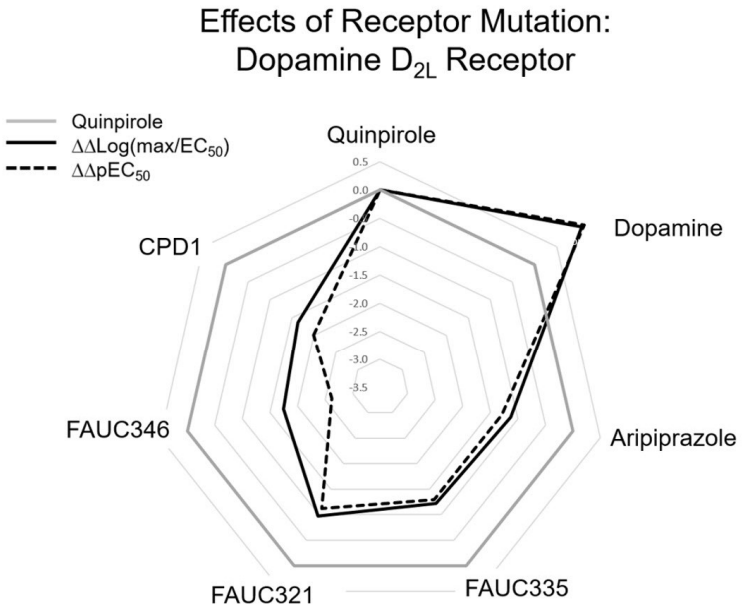

	ΔΔLog(max/EC50)	ΔΔpEC5(
Quinpirole	0	0
Dopamine	1.12	1.06
Aripiprazole	-1.29	-1.13
FAUC335	-1.29	-1.22
FAUC321	-1.12	-0.97
FAUC 346	-2.62	-1.74
CPD1	-1.99	-1.64

Figure 8

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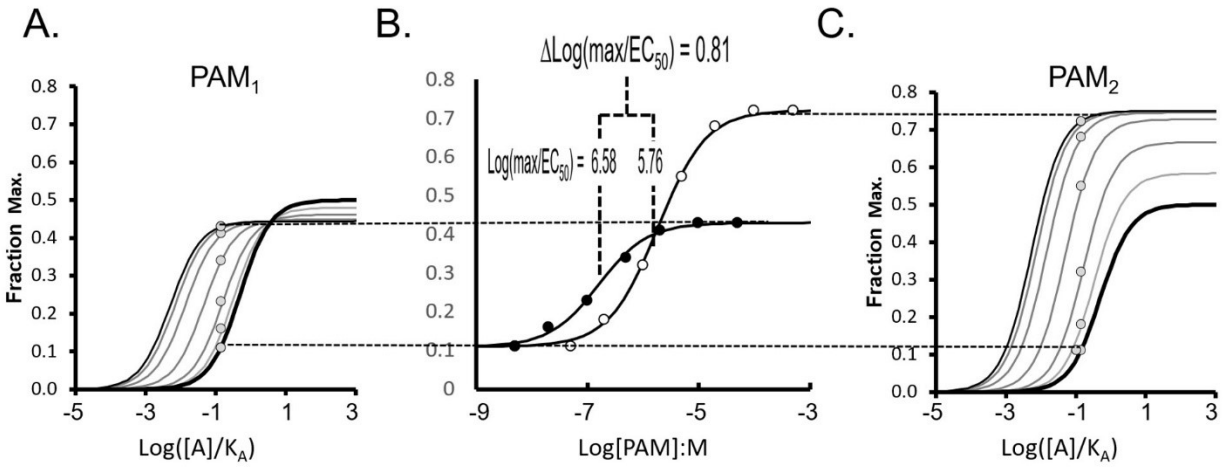

Figure 9

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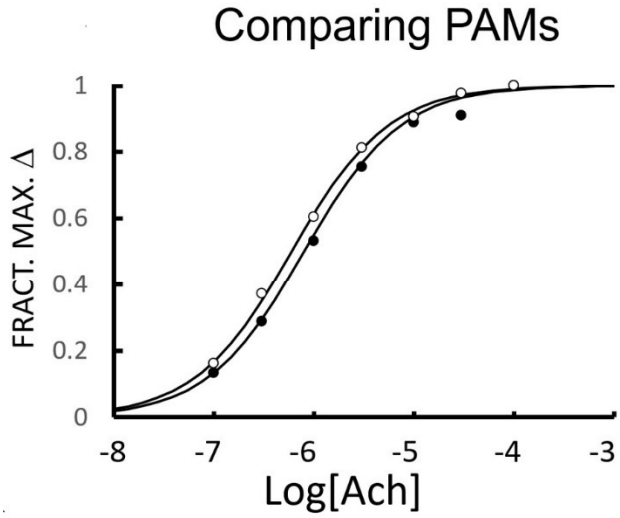

	CMPD 1	CMPD 10d
Log(αβ)[a]	1.84	2.3
pK_B[a]	4.78	4.37
Log(αβ/K_B)[a]	6.62	6.67
max[b]	1	1
EC_{50} (nM)[b]	800	600
Log(max/EC_{50})[b]	6.10	6.22
Δlog(αβ/K_B)[a]	0.05	
Δlog(max/R50)[b]	0.12	

[a] from Mistry et al (2016)
[b] from Log max/R_{50} calculation

Figure 10

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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:

$$[RG] = [ARG]/\alpha[A]K'_a \quad \ldots\ldots[5]$$

$$[AR] = [ARG]/\alpha[G]K_g \quad \ldots..[6]$$

$$[R] = [ARG]/\alpha[A]K'_a[G]K_g \quad \ldots..[7]$$

The receptor conservation equation ($[R_{tot}] = [R] + [AR] + [RG] + [ARG]$) can be re-written using equations 5 to 7 as:

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

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

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

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

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:

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

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 non agonist-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:

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

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

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

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

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

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 midpoint sensitivity of effect (denoted EC_{50}) is given as:

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

Combining equations 14 and 15 yields:

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

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. Utilized as $\Delta\text{Log}(\text{max}/EC_{50})$ values for two agonists (denoted agonist_1 and agonist_2), this can be shown to be:

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

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

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

Therefore, the ratio of max/EC_{50} values, which subtracts and thus cancels the two Log((τ_G[G]/$K_G E_m$)/(τ_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 ΔLog(max/EC_{50}) is ΔLog($\alpha\beta/K'_A$) which is a system independent ratio of agonism.

The value $\alpha\beta/K'_A$ is comprised of 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 the change in the efficacy of the receptor for production of response produced by the agonist.

Appendix 2. Relationship Between ΔLog(max/EC_{50}) and ΔLog(τ/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):

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

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where τ_A is the efficacy of the agonist, n 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 equation 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:

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

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

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

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

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

For n=1, max/EC_{50}= τ 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 ΔLog(max/EC_{50}) values for systems where the slope of the agonist concentration response curves is not significantly different form unity) yield strictly agonist dependent (and system-independent) values for relative agonism:

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$$\Delta\text{Log}(\text{max}/EC_{50}) = \Delta\text{Log}(\tau_A/K_A) \quad \ldots[23]$$

Appendix 3. Statistical Assessment of Difference Using ΔΔLog(max/EC50) Values

If individual estimates of Log(max/EC50) are available, then a statistical estimate of mean Log(max/EC50) values, ΔLog(max/EC50) values and ΔΔLog(max/EC50) 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:

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

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

$$s_{pooled} = \sqrt{\frac{\sum_{i=1}^{K} \sum_{j=1}^{2} s_{ij}^2}{df_{error}}} \quad \ldots[25]$$

Where df_{error} is given as:

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

From these values, a 95% confidence limit with two-tailed T values ($T_{97.5}$) can be calculated. For a mean Log(max/EC50) estimate:

$$95\%\ \text{c.l.} = T_{97.5} \times s_{pooled} \sqrt{\frac{1}{n_{ij}}} \quad \ldots[27]$$

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Within any one assay, agonist comparison to a standard yields a ratio of Log(max/EC_{50}) values denoted as ΔLog(max/EC_{50}). The 95% c.l. of this ratio is defined as:

$$95\%\ \text{c.l.} = T_{97.5} \times s_{\text{pooled}} \sqrt{\frac{1}{n_{ij}} + \frac{1}{n_{i'j}}} \quad \ldots[28]$$

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:

$$95\%\ \text{c.l.} = T_{97.5} \times s_{\text{pooled}} \sqrt{\frac{1}{n_{i1}} + \frac{1}{n_{i'1}} + \frac{1}{n_{i2}} + \frac{1}{n_{i'2}}} \quad \ldots.[29]$$

The application of these formulae are depicted in the figure below.

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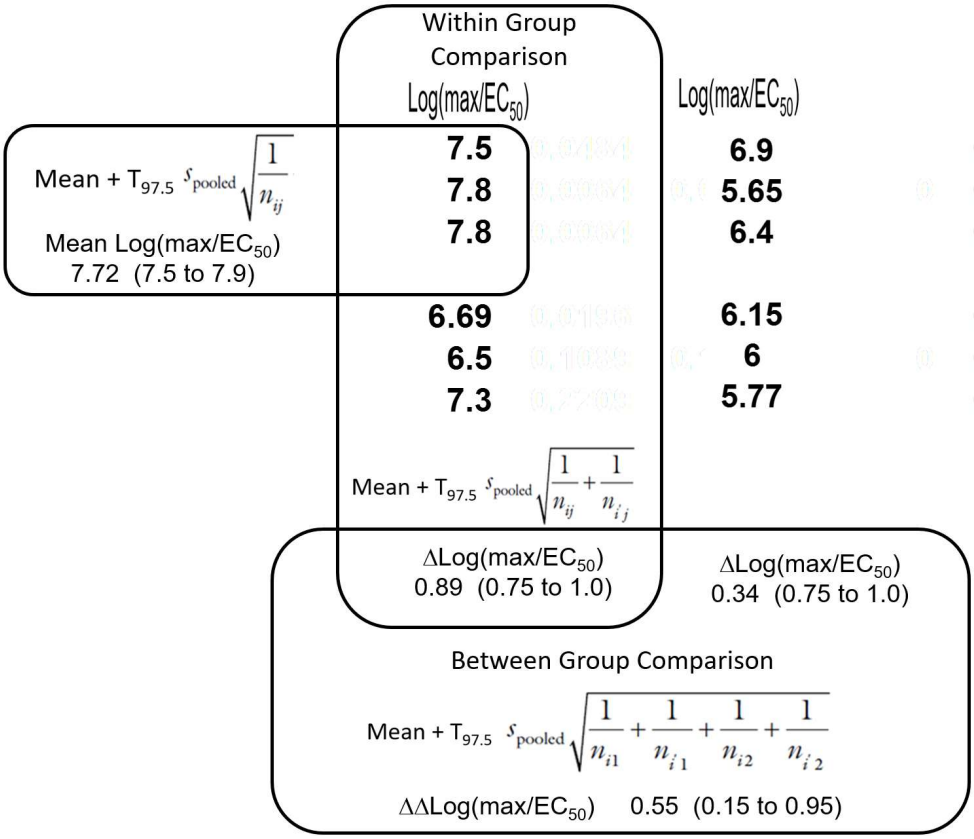

Appendix 4. Application of Log(max/R_{50}) values from R_{50} Curves to Quantify the Effects of PAMs

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

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

where α is the effect of the modulator ([B]) on the affinity of the agonist for the receptor and β 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:

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

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:

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

And the half maximal effect of the R50 curve (defined as the R50) is given as:

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

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

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

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

$$\frac{\text{max}}{R_{50}} = \underbrace{\frac{\alpha\beta}{K_B}}_{\text{PAM specific}} \times \underbrace{\frac{\tau_A[A]/K_A}{([A]/K_A(1 + \beta\tau_A)) + 1}}_{\text{System specific}} \quad \ldots[35]$$

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

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$$\Delta \text{Log}(\text{max}/R_{50})_{A\text{-}B} = \text{Log}(\alpha\beta/K_B)_A - \text{Log}(\alpha\beta/K_B)_B \quad \ldots[36]$$