A Complementary Scale of Biased Agonism for Agonists with Differing Maximal Responses

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Compelling data in the literature from the recent years leave no doubt about the pluridimensional nature of G protein-coupled receptor function and the fact that some ligands can couple with different efficacies to the multiple pathways that a receptor can signal through, a phenomenon most commonly known as functional selectivity or biased agonism. Nowadays, transduction coefficients (log(τ/KA)), based on the Black and Leff operational model of agonism, are widely used to calculate bias. Nevertheless, combining both affinity and efficacy in a single parameter can result in compounds showing a defined calculated bias of one pathway over other though displaying varying experimental bias preferences. In this paper, we present a novel scale (log(τ)), that attempts to give extra substance to different compound profiles in order to better classify compounds and quantify their bias. The efficacy-driven log(τ) scale is not proposed as an alternative to the affinity&efficacy-driven log(τ/KA) scale but as a complement in those situations where partial agonism is present. Both theoretical and practical approaches using μ-opioid receptor agonists are presented.

G protein-coupled receptors (GPCRs) are membrane receptors responsible for numerous physiological responses in living systems by transducing the signals embodied in the chemical structure of hormones, neurotransmitters and synthetic ligands from outside to inside the cells¹. Many diseases are associated with an abnormal functioning of these receptors, which makes them one of the most important target families in drug discovery programs².

Plasticity is a property inherent to the flexible nature of proteins which GPCRs make proficient use of for signaling purposes³. It is now well established that GPCRs signal not only through G protein-dependent pathways but also through β-arrestin and other accessory proteins⁴. This multiplicity of signaling pathways has led to a collection of pharmacological concepts (pluridimensional efficacy⁵,⁶, stimulus trafficking⁷, functional selectivity⁸,⁹, and biased agonism¹⁰) which all share a common link: the differential modulation that ligands may exert on receptor signaling.

Given that ligands may differentially drive receptor signaling, either potentiating or inhibiting one pathway over others, the issue arises of how to measure functional responses in order to establish reproducible scales for drug comparison¹¹. The approach that is currently most commonly used (the log(τ/KA) scale) is based on a combination of estimates of efficacy (τ) and affinity (KA) to cancel the confounding interacting effects between both parameters¹². In an attempt to provide new insights on the measurement of receptor signaling bias, this article presents a new scale (the log(τ) scale) that follows from the one described by Kenakin and coworkers to complement it and give extra substance to the classification of compounds’ bias. We use both scales to study a group of μ-opioid receptor ligands as this receptor’s bias behavior has been thoroughly described¹³,¹⁴. We show that two ligands’ properties, efficacy and concentration range, play a role in the results yielded by these two scales, allowing for a better classification of compounds and therefore aiding in the initial stages of drug discovery and hopefully in the development of new therapeutic drugs.

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The herein described efficacy-driven log(τ) scale is not proposed as an alternative to the widely used affinity
& efficacy-driven log(τ/Kₐ) scale but as a complement in those situations where partial agonism is present. We argue that using only the log(τ/Kₐ) scale is not sufficient for the analysis of biased agonism for compounds with differing maximal responses. In these cases the expression of the maximum response of a given agonist is $E_{\text{max}} = E_m^\tau/(1 + \tau)$, which does not include affinity, a note of caution seems opportune. As biased agonism is becoming a reality in routine screening studies, increasingly accurate protocols for biased signal detection are now necessary.

Materials and Methods

Substances. Buprenorphine and fentanyl were supplied by Johnson Matthey (310030 and 310100). Morphine was acquired at Alcaliber. TRV130 was synthesized by Esteve. Finally, endomorphine-2 and Damgo were acquired from Sigma-Aldrich (SCP0133 and E7384).

**G protein-independent pathway: β-arrestin-2 recruitment assay.** Chinese hamster ovary (CHO)-K1 cells engineered to co-express the ProLink™ (PK) tagged human µ-opioid receptor and the Enzyme Acceptor (EA) tagged β-Arrestin-2 from DiscoverX were used (93-0213C2). 5000 cells/well were seeded in 20 µL of PathHunter Cell Plating Reagent in 384 well plates. Twenty-four hours later, 5 µl ligands (dissolved in Hanks’ balanced salt solution (HBSS) containing 20 mM Hepes) were added to the plate. Cells were incubated for 90 min at 37°C. 6 µL of detection reagent (PathHunter Detection Reagent) were then added and the incubation continued at room temperature for 60 min. Luminescence was recorded (integration time of 1 s) in a Tecan Infinite M1000 Pro reader.

**G protein-dependent pathway: Measurement of cAMP responses by Homogeneous Time Resolved Fluorescence.** cAMP measurements on CHO-K1 cells that stably express the human µ-opioid receptor (Perkin Elmer ES-542-C) were performed by using a system based on Homogeneous Time Resolved Fluorescence (HTRF). The HTRF cAMP kit from CisBio (62AM4PEJ) was used according to the manufacturer’s recommendation. 2500 cells/well were seeded the day before the experiment in 10 µl of Opti-Mem (Gibco, 11058-021). On the following day, β-funaltrexamine (β-FNX, Sigma Aldrich, O003) was prepared in OptiMem and cells were treated with 5 µl of either concentration of β-FNX (0, 1, 3, 10, 30, 100, 300 nM) for 2 hours. After that time, cells were washed twice with 40 µl of OptiMem. 10 µl of OptiMem were finally added and cells were left for one hour at 37°C. Opioid agonists were prepared in OptiMem with 3-isobutyl-1-methyl-xanthine (Sigma-Aldrich, 15879-5G) and forskolin (Tocris, 1099) at 0.5 mM and 7.5 µM respectively and 10 µl added to the cells. After 45 min at 37°C the reaction was stopped by lysing the cells with a mixture of 10 µl of each of HTRF detection reagents. Plates were incubated for an additional hour at room temperature and read at 665 nm/620 nm using a RubyStar Plate reader (BMG LabTech). The conditions were followed as described in ref.16.

Parameter estimation. Curve fitting was performed by using nonlinear least squares regression. The NLIN procedure of SAS statistical package was applied (SAS/STAT 9.2; SAS Institute, Cary, NC, USA). The Gauss iterative method was employed in solving the nonlinear least square problem. Equation 1 (this article) of the operational model of agonism15 was used for affinity and efficacy parameter estimation. It is known that the operational method cannot be applied to fit a single effect/agonist concentration (E/ [A]) curve because there is not a single solution for the estimated parameters15. In this regard, two different fitting procedures, namely, the receptor inactivation and the comparative method, were followed depending on the experimental assay performed. For the G protein-dependent cAMP assay, the receptor inactivation method15 was used: seven curves for each tested ligand were obtained by varying the concentration (0, 1, 3, 10, 30, 100 and 300 nM, respectively) of the irreversible antagonist β-FNX. Common operational $E_m$, $n$ and $K_A$ parameters were shared between curves whereas a $\tau$ parameter was defined for each β-FNX concentration-dependent curve15. The $\tau$ parameter corresponding to the curve yielded in the absence of β-FNX was used for the biased agonism analysis. For the G protein-independent β-arrestin-2 recruitment assay the same compounds as in the cAMP assay were used. However, because of the absence of an appropriate irreversible antagonist for the β-arrestin-2 assay, an alternative method was necessary. The comparative method18 was considered suitable because the tested compounds behave as partial agonists in the assay. In the comparative method, it is assumed that the maximal response ($E_{\text{max}}$) and the slope parameter (m) yielded by a full agonist through the Hill equation ($E = E_{\text{max}}[A]^m / (A^m + [A]^m$)) match, respectively, the operational parameters maximum response of the system ($E_{\text{max}}$) and slope parameter (n). Once determined, $E_{\text{max}}$ and m can be used as fixed values in Equation 1 (this article) of the operational model for the estimation of $K_A$ and $\tau$ parameters of partial agonists. Damgo was used as the full agonist in the present study and its curve data were fitted through the Hill equation. The $E_{\text{max}}$ and m parameters of Damgo curve were then used as fixed $E_{\text{max}}$ and m values in the fitting of the selected compounds under the operational model (Equation 1, this article). In all fitting procedures, $K_A$ and $\tau$ were estimated as logarithms to approximate the assumption of normality distribution18.

The two scales compared in the present study are based on log(τ) and log(τ/Kₐ) estimates. Parameter estimates for log(τ) and log(Kₐ) and their corresponding standard errors were obtained from the nonlinear-regression curve fitting described above. Log(τ/Kₐ) was estimated as log(τ) – log(Kₐ). Standard errors for log(τ/Kₐ) were calculated from the standard errors of log(τ) and log(Kₐ) by including the correlation (r) between both parameters because they are not independent properties. Thus, representing log(τ/Kₐ) as x and log(Kₐ) as y, the standard error (se) of log(τ/Kₐ) was calculated as $se_{\Delta \text{y}} = \sqrt{se_x^2 + se_y^2 - 2rse_xse_y}$. The 95% confidence intervals of log(τ/Kₐ) were calculated as $IC95\%_{\text{y}} = x - y \pm 1.96 se_{\Delta \text{y}}$, where the value of r for the degrees of freedom of the Student’s t-function depends on whether the variances of x and y are statistically equal or different (F-Fisher test).

In the calculation of bias through both $\Delta \log(\tau)$ G-protein, β-arrestin and $\Delta \log(\tau/K_A)$ G-protein, β-arrestin scales we conclude that there is no bias in one or the other when the confidence interval includes zero. However, inasmuch
as a collection of compounds is evaluated, the issue of multiple testing appears and a corresponding correction
is necessary. The issue of multiple testing was considered by adjusting the significance level through the Holm's
method. To do that we first transformed the IC95% of each of the compounds in a p-value for a t-test with a null
hypothesis of μ = 0. Then the p-values for the selected compounds were adjusted according to the Holm's method.
Afterwards, the IC95% were recalculated by adjusting the α value according to the relative position of the previ-
souly calculated p-value. This resulted in adjusted IC95% more prone to include the zero value, which parallels
the conventional conservative process of multiple testing involving p-values (a lesser propensity to reject the null
hypothesis). Due to the statistical consistency of both inference methods, confidence intervals and hypothesis
testing produced the same conclusion (biased agonism or not) for each of the compounds.

Results and Discussion

The log(τ/Kₐ) and log(τ) scales. The two scales for biased agonism we discuss herein are based on the
operational model of agonism, presented in a seminal work by Black and Left13. Equation 1 is the general E/[A]
equation of the operational model of agonism.

\[
E = \frac{E_m \tau^\mu [A]^\nu}{(K_\alpha + [A])^\beta + \tau^\mu [A]^\nu}
\]

Where E is the pharmacological effect; Eₘ, the maximum effect of the system; [A], the concentration of the ago-
nist A; τ, the operational efficacy of A in the receptor system; K_α, the dissociation constant of A for the receptor;
and n, a parameter related with the slope of the E/[A] curves. A value of n equal to 1 yields rectangular hyperbolic
E/[A] curves whereas n values greater and lower than 1 allow for steeper and flatter curves than rectangular
hyperbola, respectively15,20.

Under the operational model of agonism15, τ is defined as \( \tau = \tau_0 \exp(ΔE/RT) \) where \( \tau_0 \) represents the
total receptor concentration and K_α the value of the concentration of agonist-receptor complex, [AR], for half the maximum
possible effect, Eₘ in other words, the inverse of K_α reflects the intrinsic efficacy of the AR complex. Thus, τ
contains both tissue and ligand-receptor efficacy parameters. Moreover, K_α is not a thermodynamic equilibrium
dissociation constant but a conditional or functional constant. This is because K_α does not correspond to an indi-
vidual equilibrium step, the binding of the agonist to the inactive receptor conformation, but it incorporates, in
addition, the receptor conformational change associated with receptor activation. From a molecular perspective,
the concept of receptor activation is present in both τ and K_α parameters. As it has been shown15, τ may reflect
the binding of the transducer G protein to the receptor. More precisely, in the presence of GTP and GDP, receptor
activation is proportional to the active state of the quaternary complex (AR*G-GDP), with R* indicating the active
conformation of the receptor15. In addition, K_α is a combined expression of the parameter values for the
agonist binding to the bare receptor and the receptor conformational change from the inactive (R) to the active
(R*) state15,20.

The asymptotic maximum (E_{max} in Equation 2) and location (logEC_{50} in Equation 3) parameters allow for the
quantification of E/[A] curve shape20. LogEC_{50} provides information about the potency of the agonist and E_{max}
reflects agonist efficacy. We see that logEC_{50}, or its commonly used negative value, pEC_{50}, includes operational K_α
and τ parameters whereas K_α is not present in the definition of E_{max}.

\[
E_{max} = \lim_{x \to \infty} E = \frac{E_m}{1 + \tau^\mu}; \text{ with } x = \log[A]
\]

\[
\logEC_{50} = \log \frac{1}{\left(\frac{2}{K_\alpha} + \left(\frac{\tau}{K_\alpha}\right)^n\right)^{\frac{1}{\tau}}}; \text{ with } \logEC_{50} = \log[A] \text{ for } E = E_{max}/2
\]

Equation 2 shows that high values of τ are associated with high values of E_{max} which at the limit (full agonists)
reach E_m. On the contrary, low values of τ determine partial agonism: A value of τ as low as one unit makes E_{max}
equal to half E_m. With respect to potency, Equation 3 shows that higher potency values (lower logEC_{50}) result
from lower K_α and higher τ values. Moreover, high values of τ (full agonists) lead to logEC_{50} = \log \left(\frac{1}{K_\alpha}\right).

Kenakin et al12, combining the K_α and τ parameters of the operational model15, defined a parameter designated
the transduction coefficient, \( \log(\tau/K_\alpha) \), which provides a one-parameter scale able to classify agonists acting
through one receptor. They demonstrated that this scale can be transferred between systems with differing recep-
tor densities and, what is more, a ratio of this parameter relative to a reference ligand provides normalization by
taking into account the natural bias of the system, and so is useful for comparing experimental and physiological
tissues. Precedents of the transduction coefficient can be found in some publications by Ehlert25–27, who used
either the ε/K_α ratio, with ε being intrinsic efficacy, or the τ/K_α ratio.

Is the Δlog(τ/K_α) scale sufficient for biased agonism description? Combining the log(τ/K_α) and log(τ) scales: A
theoretical example. As explained in the Appendix (Supplementary Material), both Δlog(τ) and Δlog(τ/K_α)
represent useful scales to classify ligands independently of receptor density. An initial look at both scales reveals
the fact that while the former only takes into account ligand operational efficacy, the latter also balances this
efficacy in respect to ligand affinity, and so naturally these two scales classify ligands differently and the bias cal-
culated from those scales differs as well. Because the Δlog(τ/K_α) scale is currently being used in a routine way,
the proposal of a complementary scale invites justification. At this point it is worth comparing both scales with a theoretical example:

Let us suppose a drug screening study consisting of two pathways that is aimed at identifying ligands with a positive bias effect of Pathway 2 with respect to Pathway 1. Figure 1 shows the concentration-response curves for three ligands with agonistic properties acting through a given receptor in the two pathways, where the values of $\tau$ and $K_a$ for each ligand at each pathway are displayed in Table 1. We have assumed that the ligands have the same operational affinities and efficacies in Pathway 1 and different operational affinities and efficacies in Pathway 2. A normalized value of 100 has been assumed for $E_{\text{m}}$ in both pathways.

For a proper comparison between a collection of ligands within a single pathway and in various pathways, a reference ligand must be defined. This allows for the cancellation of system effects. Assuming Ligand 1 as the reference ligand, calculated parameters for both scales, $\Delta \log(\tau/K_a)$ and $\Delta \log(\tau)$, at each pathway and the bias of Pathway 2 relative to Pathway 1, $\Delta \Delta \log(\tau/K_a)$ and $\Delta \Delta \log(\tau)$, are shown in Table 1. It can be seen that both scales classify ligands in a different order. For the $\Delta \log(\tau/K_a)$ scale the order is Ligand 2 > Ligand 1 > Ligand 3 while for the $\Delta \log(\tau)$ scale the order is Ligand 3 > Ligand 2 > Ligand 1. Thus, taking Ligand 1 as the reference ligand whose bias is to be optimized, the second scale, $\Delta \log(\tau/K_a)$, would provide that both Ligand 3 and Ligand 2 are optimized to a greater degree and Ligand 3 to a larger extent than Ligand 2, while with the first scale, $\Delta \log(\tau)$, only Ligand 2 is optimized with respect to Ligand 1.

Another practical output from the combination of the two scales comes from the comparison of the results obtained for Ligand 2 and Ligand 3 in both scales. While for Ligand 2 the two parameters $\Delta \Delta \log(\tau/K_a)$ and $\Delta \Delta \log(\tau)$ result in a positive number, suggesting an improvement of the bias with respect to Ligand 1, a different situation is found for Ligand 3. For this last agonist the two parameters show opposing results due to an improvement in affinity but a worsening in efficacy versus Ligand 1.

Although at first sight these results seem contradictory, we understand that the two scales are complementary, each offering information not found in the other. Parameter derivations from the operational model (Equation 1)
show that while $\Delta \Delta \log(\tau/K_A)$ is driven by both potency ($EC_{50}$) and affinity ($K_A$) (Equation 3) $\Delta \Delta \log(\tau)$ is driven by efficacy ($E_{\text{max}}$) (Equation 2). A closer look at the concentration-response curves of Pathway 2 in Fig. 1 shows that the concentration at which Ligand 2 and Ligand 3 cross separates the relative activity of both ligands, as explained below.

**Concentration is the key.** Following the example above, the agonist concentration at which the $E/[A]$ curves of two agonists at a given pathway cross can be determined. Let us consider the $E/[A]$ curves of two agonists: $A_1$ and $A_2$.

For Agonist 1: $E_1 = \frac{E_{\text{max}}n_1[A_1]^n}{(K_{A1} + [A_1])^n + \frac{1}{\tau_1}[A_1]^n}$ (4)

For Agonist 2: $E_2 = \frac{E_{\text{max}}n_2[A_2]^n}{(K_{A2} + [A_2])^n + \frac{1}{\tau_2}[A_2]^n}$ (5)

where it is assumed that $E_{\text{max}}$ and $n$ are system-dependent parameters.

The curves for Agonists 1 and 2 cross at the $([A], E)$ point ($[A_1] = [A_2] = [A], E_1 = E_2 = E$). Making Equations 4 and 5 equal and rearranging terms leads to Equation 6.

$$[A] = \frac{\tau_2 K_{A1} - \tau_1 K_{A2}}{\tau_1 - \tau_2}$$ (6)

where $[A]$ is the ligand concentration, $\tau_1$ and $\tau_2$ the operational agonist efficacies of Ligand 1 and Ligand 2, respectively, and $K_{A1}$ and $K_{A2}$ the operational agonist equilibrium dissociation constants.

By substituting the value of $\tau$ in Equation 6, we obtain Equation 7.

$$[A] = \frac{[R_1] K_{A1} - [R_2] K_{A2}}{[E_1] K_{E1} - [E_2] K_{E2}} = \frac{K_{A1} K_{E2} - K_{A2} K_{E1}}{K_{E2} - K_{E1}}$$ (7)

The ligand concentration ([A]) at which both concentration-response curves cross does not depend upon total receptor concentration ([R]) and it is constant over the whole range of receptor densities. Looking at the $E/[A]$ curves for Pathway 2 in Fig. 1 we see that Ligand 3 shows greater effect than Ligand 2 at concentrations below that at which both concentration-response curves cross, in agreement with the $\Delta \Delta \log(\tau)$ scale; while at concentrations above that of curve crossing, Ligand 2 shows the greater effect, in agreement with the $\Delta \log(\tau)$ scale.

Figure 2. Schematic diagram for analysis of agonist bias using log($\tau$) and log($\tau/K_A$) scales. Properly designed functional data for the pathways to be studied are fitted to Black & Leff operational model to yield log($\tau$) and log($\tau/K_A$) values. If all agonists behave as full agonists in all pathways then log($\tau/K_A$) scale alone can be used to classify compounds. If not, then both scales should be used. As a result, four different scenarios may result.
clear bias throughout the full concentration range in the tissue with a high receptor density. However, in the tissue with low receptor density, the agonist exhibits a change of the preference of one pathway over the other at the ligand concentration at which the E/\[A\] curves for the two pathways cross. Therefore, for this particular ligand in these particular \textit{in vivo} conditions, the \(\Delta \log(\tau/K_A)\) scale does not reflect correctly the experimental results.

The approximation presented here, a joint consideration of the \(\Delta \log(\tau)\) and \(\Delta \log(\tau/K_A)\) scales aids in identifying experimentally-found differences, provides better fine tuning of the classification of compounds and allows for the calculation of a concentration value that determines the relationship between the scales.

Figure 2 shows a diagram for the analysis of agonist bias using the \(\Delta \log(\tau)\) and \(\Delta \log(\tau/K_A)\) scales calculated by fitting functional data to the Black and Leff operational model\(^1\). If all agonists studied behave as full agonists in all the pathways analyzed, then \(\Delta \log(\tau/K_A)\) scale alone can be used. But, if those agonists are not all full agonists, then both scales should be used, ending up with four different situations. The first situation, where both scales

|                   | \(E_m\)   | \(n\)     | Log(\(K_A\)) | Log(\(\tau\)) | Log(\(\tau/K_A\)) |
|-------------------|-----------|-----------|--------------|---------------|-------------------|
| Morphine          | 98.33 ± 1.41 | 1.04 ± 0.05 | −5.96 ± 0.11 | 2.12 ± 0.12   | 8.08 ± 0.04       |
| Fentanyl          | 93.99 ± 1.01 | 1.22 ± 0.08 | −7.74 ± 0.10 | 2.23 ± 0.11   | 9.97 ± 0.05       |
| TRV130            | 98.15 ± 1.44 | 0.90 ± 0.04 | −7.63 ± 0.07 | 1.82 ± 0.09   | 9.45 ± 0.05       |
| Endomorphine-2    | 96.64 ± 2.12 | 1.02 ± 0.07 | −7.38 ± 0.17 | 1.97 ± 0.20   | 9.35 ± 0.07       |
| Buprenorphine     | 96.75 ± 0.81 | 0.98 ± 0.21 | −9.35 ± 0.23 | 0.85 ± 0.20   | 10.20 ± 0.10      |

Table 2. Operational parameters (estimates ± standard errors) for the \(\mu\)-opioid-dependent pathway. Data obtained from concentration-response curves of \(\mu\)-opioid agonists in presence of various concentrations of the irreversible antagonist \(\beta\)-FNX analyzed with the operational model of agonism (Fig. 3). Parameter estimates and standard errors of operational parameters \(E_m\), \(n\), Log(\(K_A\)) and Log(\(\tau\)) were produced by global fitting. Common \(E_m\), \(n\) and \(K_A\) parameters were shared between curves whereas a \(\tau\) parameter was defined for each \(\beta\)-FNX concentration-dependent curve. In the Table, Log(\(\tau/K_A\)) for \(\beta\)-FNX concentration equal to 0 is shown. For buprenorphine, the fitting did not converge when \(E_m\) was included as a free parameter; thus, we set \(E_m\) equal to the mean of the values obtained for the other ligands (96.75) and kept it fixed as such in the fitting process. Log(\(\tau/K_A\)) values and their standard errors were calculated from estimated \(\tau\) and \(K_A\) parameters (see Parameter estimation in Methods).
agonists for the μ-opioid receptor13,31. Buprenorphine, a classically classified partial agonist; and finally endomorphine-2 and TRV130, which are biased μ-receptor antagonists for the β-arrestin pathway. To determine its ability to recruit and signal through the β-receptor on the G protein signaling pathway, while an enzyme complementation assay (DiscoverX) was used to recruit β-arrestin, is already in Phase II clinical trials30. Bearing this in mind, we used the pathway over that of a review). What is more, TRV130, a ligand described as a biased μ-opioid agonist favoring the G protein signaling pathway (Fig. 4) we used a different experimental approach: the comparative method18 with Damgo as full agonist (Fig. 3). The Hill equation was used for fitting to Damgo data. The values obtained for Damgo for maximal response (95.14) and slope parameter (1.48) were used for all ligands in the Table as Em and n parameters in the operational model and kept fixed as such in the fitting process. Parameter estimates and standard errors of operational parameters log(KA) and log(τ) were produced by global fitting. Log (τ/KA) values and their standard errors were calculated from estimated τ and KA parameters (see Parameter estimation in Methods).

Table 3. Operational parameters (estimates ± standard errors) for β-arrestin pathway. Data obtained from concentration-response curves of μ-opioid agonists using the comparative method18 with Damgo as full agonist (Fig. 4). The Hill equation was used for fitting to Damgo data. The values obtained for Damgo for maximal response (95.14) and slope parameter (1.48) were used for all ligands in the Table as Em and n parameters in the operational model and kept fixed as such in the fitting process. Parameter estimates and standard errors of operational parameters log(KA) and log(τ) were produced by global fitting. Log (τ/KA) values and their standard errors were calculated from estimated τ and KA parameters (see Parameter estimation in Methods).

show an improvement in the bias they calculate (ΔΔτlog(τ/KA) > 0) meaning that bias has been optimized. A second one where ΔΔτlog(τ/KA) > 0 but Δlog(τ) < 0 would represent a situation where only the first scale would point to an improvement in the bias pursued. As the difference between both scales resides in the KA value of the first one, we identify this bias improvement as affinity-driven, due to an increase in affinity (KA) of the ligand studied compared to the reference ligand. A third scenario, where ΔΔτlog(τ/KA) < 0 but Δlog(τ) > 0 would represent a situation where only the second scale points to an improvement in bias. In this case we identify this bias improvement as efficacy-driven due to an increase in efficacy (τ) of the ligand studied versus the reference. Finally, in the last situation both scales point out that bias improvement has not been achieved (ΔΔτlog(τ) and ΔΔτlog(τ/KA) < 0).

A practical example. Biased signaling has already been analyzed for the μ-opioid receptor13,28 (see also29 as a review). What is more, TRV130, a ligand described as a biased μ-opioid agonist favoring the G protein signaling pathway over that of β-arrestin, is already in Phase II clinical trials29. Bearing this in mind, we used the μ-opioid receptor to apply our proposal for bias calculation.

When μ-opioid receptors couple to Gi/o subtypes they inhibit the production of cAMP and they can also recruit β-arrestins. An HTRF (Cisbio) cAMP determination assay was used to determine the activity of this receptor on the G protein signaling pathway, while an enzyme complementation assay (DiscoverX) was used to determine its ability to recruit and signal through the β-arrestin pathway. The ligands used in this study were: morphine and fentanyl, two opioids commonly used for pain-relief; buprenorphine, a classically classified partial agonist; and finally endomorphine-2 and TRV130, which are biased agonists for the μ-opioid receptor13,31.

Figure 3 shows the results of the five μ-opioid receptor agonists in the cAMP determination assay. It is known22 that the operational model cannot satisfactorily fit a single experimental E/[A] curve. A solution to the problem can be reached by using the irreversible inactivation method17 (see Parameter estimation in Methods), which produces a collection of experimental curves with lower maximal effect by decreasing receptor density. With this procedure, a single solution for each of the curves, with particular τ and common Em, n and KA parameters, is obtained. We followed this approach for parameter determination in the G protein pathway. Results are shown in Table 2.

For the β-arrestin pathway (Fig. 4) we used a different experimental approach: the comparative method18 (see Parameter estimation in Methods). In this method, it is assumed that the maximal response (Emax) and the slope parameter (m) yielded from a full agonist by fitting curve data with the Hill equation match, respectively, the operational Em and n parameters, and, once determined, can be used as fixed values for the estimation of the
efficacy and affinity of partial agonists within the operational model. In this assay, we used Damgo as the full agonist. As all the other opioids in the assay behaved as partial agonists, their $K_A$ and $\tau$ values could be directly calculated using the operational model by substituting $E_{\text{m}}$ and $n$ parameters with Damgo $E_{\text{max}}$ and $m$ and keeping them fixed as such in the fitting process. Results are shown in Table 3. It is worth noting the discrepancies in $K_A$ between the two pathways for each of the agonists (Tables 2 and 3). This is an acceptable result under the operational model of agonism because $K_A$ is a functional affinity of the agonist which includes the interaction of the activated receptor with the signaling protein either the G protein or $\beta$-arrestin.$^{26}$

At this point it is worth mentioning, for the sake of correct data interpretation, that it is convenient to analyze all the studied pathways in the same cell line to minimize any functional influence of receptor tagging or modification that needs to be performed. Unfortunately, this is not always possible because, depending on the signaling pathway, different receptor or signaling protein constructs must be used.$^{35}$ This is particularly evident when more than two pathways are analyzed, as in the study by Thompson and colleagues$^{29}$, where bias was calculated for cAMP, GTP-$\gamma$S and pERK1/2 determinations using the wild-type $\mu$-opioid receptor, but for other signaling pathways such as $\beta$-arrestin-1, $\beta$-arrestin-2 or receptor internalization an Rluc-tagged receptor was used.$^{25}$ It is worth noting that concerns about receptor tagging have been extensively addressed in the literature.$^{28}$ It is worth noting that concerns about receptor tagging have been extensively addressed in the literature.$^{28}$ DeWire and colleagues (2013)$^{14}$ reported a bias favoring the G protein pathway for TRV130 using relative intrinsic activities ($R_A$) and because $R_A$ values can reduce to $\tau/K_A$ (when $n = 1$)$^{15}$, their data resembles the transduction coefficient scale ($\Delta \Delta \log (\tau/K_A)$) that we present here. Regarding endomorphine-2, some studies$^{13,37}$ have reported a bias favoring the $\beta$-arrestin pathway for this ligand. In these studies$^{15,37}$, $\tau$ values were estimated
Table 4. Calculation of \((G \text{ protein} - \beta \text{-arrestin})\ \Delta \Delta \log (\tau)\) and \(\Delta \log (\tau/K_A)\) bias. Raw data for \(\log (\tau)\) and \(\log (\tau/K_A)\) were taken from Tables 2 and 3. Morphine was taken as the reference compound. Parameter estimates ± standard errors are shown. The confidence intervals of 95% (CI95%) for \(\Delta \Delta\) estimates are shown in parentheses. Multiple testing was considered in the calculation of confidence intervals through Holm’s method (see Parameter estimation in Methods). A * has been added to those CI95% for \(\Delta \Delta\) estimates which do not include zero and show thus statistical significance for bias signaling.

| Compound | \(\Delta \Delta \log (\tau) G\) protein pathway | \(\Delta \Delta \log (\tau) G\) protein \(-\beta\) arrestin pathway | \(\Delta \Delta \log (\tau/K_A) G\) protein pathway | \(\Delta \Delta \log (\tau/K_A) G\) protein \(-\beta\) arrestin pathway |
|----------|-----------------------------------------------|---------------------------------------------------------------|-----------------------------------------------|---------------------------------------------------------------|
| Morphine | 0                                             | 0                                                             | 0                                             | 0                                                             |
| Fentanyl | \(0.11 \pm 0.16\)                             | \(0.54 \pm 0.08\)                                            | \(-0.43 \pm 0.18\) (\(-0.84, -0.02)\)*      | \(1.89 \pm 0.06\)                                             |
| TRV130   | \(-0.3 \pm 0.15\)                            | \(-0.92 \pm 0.15\)                                          | \(0.62 \pm 0.21\) (0.06, 1.18) *             | \(1.37 \pm 0.06\)                                             |
| Endomorphine-2 | \(-0.15 \pm 0.23\)             | \(0.48 \pm 0.04\)                                           | \(-0.63 \pm 0.23\) (\(-1.19, -0.07)\) *     | \(1.27 \pm 0.08\)                                             |
| Buprenorphine | \(-1.27 \pm 0.23\)       | \(-0.9 \pm 0.04\)                                           | \(-0.37 \pm 0.23\) (\(-0.83, 0.09)\)         | \(2.12 \pm 0.11\)                                             |

Figure 6. \(\mu\)-opioid agonist inhibition of forskolin-stimulated cAMP production assay. The concentration-response curves for morphine and buprenorphine determined in both the absence and presence (1, 3, 10, 30, 100, 300 nM) of the irreversible antagonist \(\beta\)-funaltrexamine are represented jointly in the same graph. The concentrations at which the curves for each agonist cross with each other are marked. Results were obtained in at least three independent experiments. In each experiment, data points were obtained in quadruplicates.

from the operational model using \(K_A\) values obtained from independent binding experiments. These results for endomorphine-2 favoring the \(\beta\)-arrestin pathway are in agreement with our results in the \(\Delta \Delta \log (\tau)\) scale, though in our fitting procedure \(\tau\) and \(K_A\) are both estimated from the operational model.

Quantitative pharmacology of signaling bias may offer a structure-function framework which can be useful for drug discovery purposes. An elegant study on the \(M_3\) muscarinic acetylcholine receptor combining various approaches including mutation and molecular modeling identified orthosteric and allosteric site mutations that contribute to ligand-selective signaling bias\(^{38}\). The authors suggested that the functional selectivity of some of the compound might arise from a bitopic mechanism\(^{38}\). Other examples with similar methodological approaches can be cited as, for example, studies focusing on the glucagon-like peptide-1 receptor\(^{40}\) or the \(M_1\) muscarinic acetylcholine receptor\(^{40}\). Moreover, a detailed review on the functional analysis of receptor states can be found in ref.\(^{21}\).

In the theoretical example used, we determined the concentration at which the concentration-response curves of two agonists for a given receptor cross at each other in a given signaling pathway. We showed that this concentration does not depend on the total amount of receptor present. In Fig. 6 we have represented the concentration-response curves for morphine and buprenorphine in the cAMP inhibition assay in the presence or absence of \(\beta\)-FNX to illustrate this point. Visual inspection of Fig. 6 shows that the concentration-response curves of morphine and buprenorphine at various \(\beta\)-FNX concentrations cross at similar concentration values (shown by blue dots), in agreement with theoretical predictions (Equation 7). Applying Equation 6 to the data generated from these two agonists gives the following concentration values (critical concentrations) at which the curves cross: 80 nM, 102 nM, 124 nM, 90 nM, 82 nM, 71 nM and 88 nM, respectively, for each of the \(\beta\)-FNX pretreatment conditions. We see that the effect elicited by buprenorphine is always greater than that produced by morphine at concentrations lower than \(~100\) nM whereas the opposite is true at concentrations above \(~100\) nM.
Conclusions

Biased agonism is a hot topic in current pharmacologic research with known therapeutic implications. Accurate and standardized measurement of this property is fundamental to drug discovery and development. Currently, the most widely used scale is one based on \(\log(\tau/K_A)\). It has the advantage of combining efficacy and affinity properties in a single parameter thus providing simplicity. However, in those situations in which different maximal responses are found, the \(\log(\tau/K_A)\) scale appears to be insufficient. In this regard, because the efficacy parameter \(\tau\) is directly related with the maximal response achieved by an agonist, the \(\log(\tau)\) scale can complement the \(\log(\tau/K_A)\) scale in those cases which include ligands with different maximal responses. Of note, we have shown that the \(\log(\tau)\) scale accomplishes the same requirement as that of the \(\log(\tau/K_A)\) scale, namely the ratio of \(\tau\) values for two ligands across receptor systems with varying receptor density remains constant. We have also shown that concentration plays a role in these cases and how the decision of whether to use a biased agonism approach based on either pure efficacy (the \(\Delta\log(\tau)\) scale) or a combination of efficacy and affinity parameters (the \(\Delta\log(\tau/K_A)\) scale) depends on the experimental concentration window used. In this regard, the signs of the \((\Delta\log(\tau/K_A), \Delta\log(\tau))\) pairs provide an indication on whether there is \((+, +)\) or not \((-,-)\) an optimization of the bias in one pathway relative to the other also whether it is mainly affinity- or efficacy-driven, \((+, +)\) and \((-,-)\), respectively.

Finally, we have illustrated the application of the proposed methodology to the \(\mu\)-opioid receptor scenario by considering the G protein and the \(\beta\)-arrestin pathways and selected full and partial agonists.

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**Acknowledgements**

This study was supported in part by Ministerio de Economía y Competitividad (ERA-NET NEURON PCIN-2013-018-C03-02 and SAF2014-58396-R) and Xunta de Galicia (IN853A2014-08). We thank José Brea and Mabel Loza for carefully reading of the manuscript and helpful comments.

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Contributed to the conception and design of the study: Burgueño and Giraldo. Performed the mathematical modeling: Burgueño and Giraldo. Wrote the manuscript: Burgueño and Giraldo. Carried out the statistical analyses: Roche and Giraldo. Conducted and analyzed the pharmacological assays: Pujol, Varela, and Monroy. Contributed to the design, analysis and interpretation of experiments: Merlos.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-15258-z.

**Competing Interests:** The authors declare that they have no competing interests.

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