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Quantitative assessment of ligand bias from bias plots: The bias coefficient "kappa"

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ABSTRACT

The current methods for quantifying ligand bias involve the construction of bias plots and the calculations of bias coefficients that can be compared using statistical methods. However, widely used bias coefficients can diverge in their abilities to identify ligand bias and can give false positives. As the empirical bias plots are considered the most reliable tools in bias identification, here we develop an analytical description of bias plot trajectories and introduce a bias coefficient, kappa, which is calculated from these trajectories. The new bias coefficient complements the tool-set in ligand bias identification in cell signaling research.

1. Introduction

"Biased agonism" or "ligand bias" describes the ability of some ligands to selectively activate a subset of signaling pathways downstream of their receptors [1,2,3]. Most studies of bias have thus far focused on G-protein coupled receptors (GPCRs), which often exhibit biased signaling in physiological contexts [4,2,5,6,7,8]. The discovery of GPCR ligand bias has revolutionized the field and has empowered the design of biased agonists and inhibitors that can target pathogenic signaling pathways [9,10–12,4,2,5,13,6,14,8]. Recently, receptor tyrosine kinases (RTKs) were also shown to engage in biased signaling [15,16,17,18,19,20,21].

The current methods for quantifying ligand bias involve the collection of experimental dose response curves for two or more ligands and two or more responses [12,2,18,22]. These responses can include recruitment of effector molecules, phosphorylation of adaptors, production of second messengers, and many other cellular behaviors associated with the pathways of interest [23,24,25]. These responses are quantified in experiments such as western blotting, fluorescence microscopy, and activity assays. A key requirement is that each response is measured over a broad range of ligand concentrations. From the dose response curves, bias plots are generated by plotting each response as a function of the second response, for the same ligand at the same concentration [1,2,26,27]. Bias plots are a method of qualitatively assessing the presence of bias, by comparing a ligand trajectory to a reference ligand trajectory [1,2,5]. There is bias if the trajectories are different, and there is no bias if the trajectories are the same. However, there is no statistical test to compare the two trajectories [2,5].

To assess if bias exists or not in a quantitative manner, several types of bias coefficients are calculated, based on best-fit parameters of the dose response curves. These different methods have been discussed extensively and compared in prior studies [9,12,2,28,5,27,29–31,32,6,33,22]. The different methods can sometimes diverge in their abilities to identify ligand bias [34,32,6]. Furthermore, these methods can produce a high level of false positive results [32]. Thus, bias plots are considered the "most important tool" in the identification of ligand bias, as they are created directly from the raw data [5]. Here we introduce a bias coefficient termed "kappa, κ", which is derived from mathematical characterization of the bias plot trajectories and allows statistical analysis to compare different ligands.

2. Methods

In the general case, an experimental dose response, R, can be fitted with the generalized Hill equation [35]:

$$R = \frac{E_{top}*[L]^n}{k_d + [L]^n} \quad (1)$$

Here E_{top} is the maximum response to the ligand, achieved at high ligand concentration (the efficacy), [L] is the ligand concentration, n is

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the Hill coefficient, and k_d is an effective dissociation constant. Eq. (1) represents a general form of an increasing saturable function.

In [6], two approaches were used to calculate bias coefficients. The first approach, called "equimolar comparison approach" is model-free, as a bias coefficient β_{lig} is calculated from the values of Etop and k_d, determined from the fit to eq. (1) when the value of the Hill coefficient is fixed at 1:

$$\beta_{lig} = log\left(\left(\frac{E_{top,A}k_{d,B}}{k_{d,A}E_{top,B}}\right)_{lig}\left(\frac{E_{top,B}k_{d,A}}{k_{d,B}E_{top,A}}\right)_{ref}\right) \quad (2)$$

where "A" and "B" denote the two responses, R_A and R_B.

The second approach models the ligand/receptor complex as a "black box" that signals to downstream transducers, and is based on the so-called "operational model" of Black and Leff [36]. This model assumes that the ligand-bound receptor, RL, activates the cellular response with an effective equilibrium dissociation constant denoted as K_{resp} according to the equation:

$$R = \frac{[RL]E_{max}}{[RL] + K_{resp}} \quad (3)$$

Here K_{resp} denotes the concentration of ligand-bound receptors that produces 50% of the largest possible response that can be achieved by the system (E_{max}).

The operational model further considers the ligand binding constant defined as:

$$K_L = \frac{[R][L]}{[RL]} \quad (4)$$

where [R] and [L] are the concentrations of free receptor and free ligand.

Taking into account eq. 4 and the mass balance equation given by

$$[Rt] = [R] + [RL], \quad (5)$$

eq. (3) can be re-written as:

$$R = \frac{\tau[L]Emax}{[L](\tau + 1) + (K_L)} \quad (6)$$

where τ is the "transducer coefficient" defined as:

$$\tau = \frac{[Rt]}{K_{resp}} \quad (7)$$

The measured dose responses are fitted with eq. (6), with K_L known from independent binding experiments, to determine the best-fit τ.

Then, the bias coefficient is calculated as:

$$\sigma = \frac{1}{\sqrt{2}}(\sigma_A - \sigma_B) = \frac{1}{\sqrt{2}}\left(log\left(\frac{\tau_{lig}}{\tau_{ref}}\right)_A - log\left(\frac{\tau_{lig}}{\tau_{ref}}\right)_B\right) \quad (8)$$

In [6], the bias coefficients calculated using the equimolar comparison and the operational model approaches were compared to results derived from visual inspection of bias plots trajectories.

To derive an equation for the bias plot trajectories, here we first write the equations for response A and response B to a ligand as:

$$R_A = \frac{E_{top,A}*[L]^{n_A}}{k_{d,A} + [L]^{n_A}} \quad (9)$$

$$R_B = \frac{E_{top,B}*[L]^{n_B}}{k_{d,B} + [L]^{n_B}} \quad (10)$$

We rearrange eq. (9) and solve for [L]:

$$R_A*\left(k_{d,A} + [L]^{n_A}\right) = E_{top,A}*[L]^{n_A}$$

$$R_A*k_{d,A} + R_A*[L]^{n_A} = E_{top,A}*[L]^{n_A}$$

$$R_A*k_{d,A} = E_{top,A}*[L]^{n_A} - R_A*[L]^{n_A}$$

$$R_A*k_{d,A} = [L]^{n_A}*\left(E_{top,A} - R_A\right)$$

$$[L]^{n_A} = \frac{R_A*k_{d,A}}{E_{top,A} - R_A}$$

$$[L] = \left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{1}{n_A}} \quad (11)$$

We then substitute [L] into eq. (10), to obtain an expression for R_B as a function of R_A:

$$R_B = \frac{E_{top,B}*\left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{n_B}{n_A}}}{k_{d,B} + \left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{n_B}{n_A}}} \quad (12)$$

Eq. (12) is an analytical expression for a bias plot trajectory. Note that it depends on all parameters of eq. 1, namely E_{top} (the efficacy), k_d, and n. Now, any two trajectories can be compared using mathematical tools. For instance, the areas under any two bias plot trajectories can be compared over their common x-axis range, between 0 and the lower of the E_{top} values for response A (termed "m" in Fig. 1D below). The area can be calculated by either integrating the equation analytically or by using numerical methods.

Thus, the bias coefficient κ is given by the ratio of the areas under each curve as shown in eq. 13.

$$\kappa = \frac{\int_0^m R_{B,lig}\, dR_{A,lig}}{\int_0^m R_{B,ref}\, dR_{A,ref}} = \frac{\int_0^m \frac{E_{top,B}*\left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{n_B}{n_A}}}{k_{d,B} + \left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{n_B}{n_A}}}\, dR_{A,lig}}{\int_0^m \frac{E_{top,B}*\left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{n_B}{n_A}}}{k_{d,B} + \left(\frac{R_A*k_{d,A}}{E_{top,A} - R_A}\right)^{\frac{n_B}{n_A}}}\, dR_{A,ref}} \quad (13)$$

Or simply:

$$\kappa = \frac{area_{lig}}{area_{ref}} \quad (14)$$

The standard error of κ in (13) is calculated using the functional approach for multi-variable functions [37], based on the 68% confidence intervals of the fitted k_d, n, and E_{top}. Statistical significance is determined by performing a one sample *t*-test and comparing κ to 1. There is bias if $\kappa \neq 1$ and no bias if $\kappa = 1$. The magnitude of the bias scales with the deviation of κ from 1. A larger deviation of κ from 1 indicates stronger bias.

3. Results

To demonstrate how to calculate κ, we utilized published dose response curves [6] for a GPCR, angiotensin type II receptor (AT1R) and their published fit parameters (Tables S1A, S1B, S2A, and S2B Supplemental Data in [6]). These results were later independently confirmed using orthogonal approaches [38]. Rajagopal and colleagues characterized how AT1R responds to its ligand, angiotensin II (chosen as reference ligand), as well as to a series of angiotensin derivatives [6]. They measured (A) β-arrestin recruitment by the GPCR and (B) the concentration of inositol 1-phosphate (IP), a downstream signaling molecule. The dose response curves are shown in Fig. 1A&B. Based on these responses, the bias plots are created by plotting one response versus the other (shown in Fig. 1C with the symbols).

We see that the trajectories in the bias plot fall into two major groups. Four of the derivative trajectories are very similar to the reference ligand trajectory, while the other five appear distinctly different. Thus, by comparing the bias plots, we reason that TRV0120026, TRV0120044, TRV0120045, TRV0120034, S1G4G8 are biased ligands when compared to angiotensin, while TRV0120055, TRV0120056, S1C4, and A1 are not.

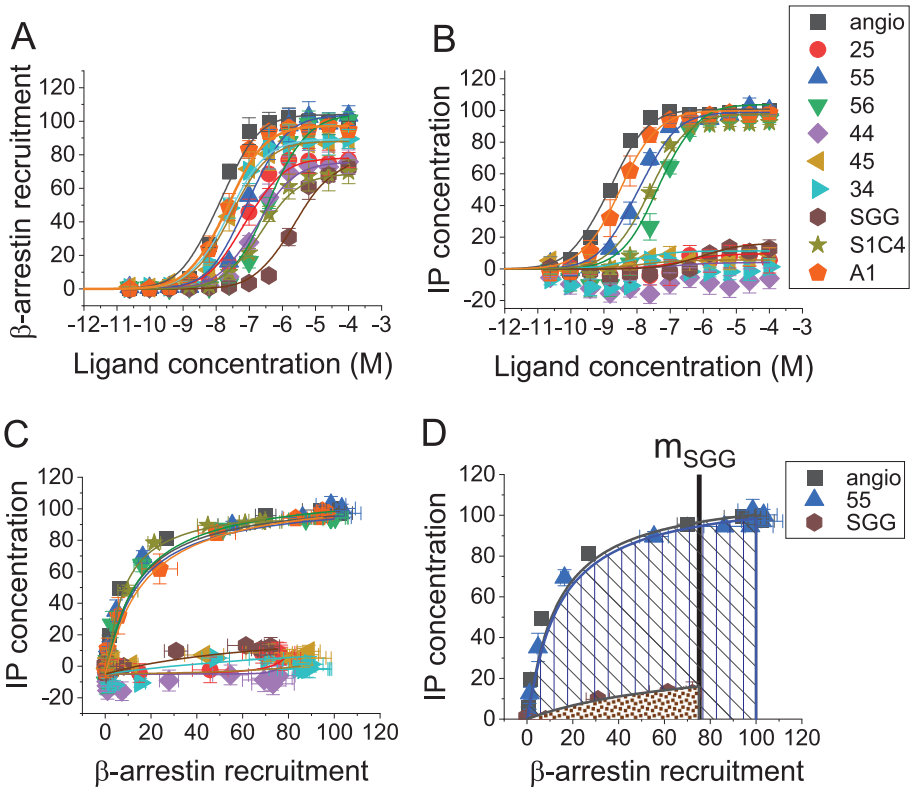

Fig. 1. (A) and (B) Dose response curves for $AT_{1A}R$ β-arrestin recruitment (A) and concentration of inositol 1-phosphate (IP), a downstream signaling molecule (B). Shown are raw data and fits to eq. 1 with $n = 1$, as reported in Tables S1A and S1B in [6]. (C) Bias plots showing the raw data and the analytical curves created with eq. 12. (D) Illustration of comparison of areas under the bias plot trajectories (hatched areas), used to calculate κ. m is the lower of the E_{top} values for response A.

Using the dose response fit parameters of Rajagopal (given in Tables S1A and S1B in Supplemental Data in [6], we plot the analytical expressions for the bias plots, as given by eq. 12, in Fig. 1C. We then calculate κ according to eq. 13 using a Matlab code, which calculates the areas under the curves as shown in Fig. 1D, the ratio of the areas, κ, as well as the κ standard error (the 68% confidence interval). The results are shown in Table 1, and are in complete agreement with the bias plots. In addition, we obtain *p*-values which report on the statistical significance of differences observed between two ligands. A low *p*-value suggests that a ligand is strongly biased as compared to the reference ligand, while a *p*-value >0.05 indicates no bias.

Table 1
Values of κ, calculated for the data set in Fig. 1.

	κ[a]	*p*-value[b]	bias plot[c]	β	σ	m
	0.02 ±			−2.34 ±	−1.20 ±	
TRV0120026	0.03	<0.001	O	0.94	0.22	5.73
	0.98 ±			−0.03 ±	0.40 ±	
TRV0120055	0.04	>0.05	X	0.12	0.16	96.43
	1.02 ±			−0.01 ±	0.43 ±	
TRV0120056	0.04	>0.05	X	0.12	0.16	99.35
	0.01 ±			−2.12 ±	−1.50 ±	
TRV0120044	0.02	<0.001	O	2.31	0.39	0.13
	0.02 ±			−1.81 ±	−1.47 ±	
TRV0120045	0.02	<0.001	O	1.19	0.30	2.78
	0.08 ±			−1.35 ±	−1.26 ±	
TRV0120034	0.03	<0.001	O	0.13	0.20	8.53
	0.13 ±			−1.24 ±	−0.96 ±	
S1G4G8	0.02	<0.001	O	0.28	0.18	14.13
	1.09 ±			0.20 ±	0.64 ±	
S1C4	0.05	>0.05	X	0.13	0.15	96.27
	0.97 ±			−0.089 ±	−0.13 ±	
A1	0.04	>0.05	X	0.12	0.17	96.30

[a] Calculated using eq. 13. Standard error is calculated using the functional approach for multi-variable functions.
[b] Calculated using a Wilcoxon *t*-test.
[c] Based on visual inspection of bias plots. O indicates bias. X indicates no bias.

Two widely used biased coefficients, σ and β_{lig}, based on the operational model (σ) or the equiactive comparison (β_{lig}), respectively, are calculated from best-fit parameters of the dose response curves (see Methods). Results from our κ calculations are compared to the values of β_{lig} and σ, calculated by Rajagopal et al., in Table 1. We see that σ and κ calculations lead to identical conclusions, in agreement with the bias plots. However, β_{lig} fails to identify the biased ligands (with the exception of S1G4G8) [6].

We further used the data for the β-2 Adrenergic Receptor (β2AR) and its ligands, reported in the same paper [6]. In this data set, two responses were measured following stimulation with increasing concentrations of ligand: (A) β-arrestin recruitment and (B) cAMP concentration. The dose response curves are shown as symbols in Fig. 2A and B and the fits are shown as solid lines. Fig. 2C shows the bias plots for the raw data (symbols) and their fits (solid line). As discussed in [6], the bias plots suggest that dichloroisoproterenol, norepinephrine, salmeterol, and pindolol are biased when epinephrine is used as the reference ligand. The bias plots further suggest that salbutamol and formoterol may be also biased when compared to epinephrine, but the difference is small and a conclusion cannot be drawn.

We calculate κ according to eq. 13, using fit values in Tables S2A and S2B in [6], and we display the results in Table 2. The calculated *p* values suggest that dichloroisoproterenol, norepinephrine, salmeterol, and pindolol are biased with high statistical significance. The *p*-values for salbutamol and formoterol are 0.04 and 0.05, respectively. These values are close to the cut-off for significance and thus a definitive conclusion cannot be drawn, similar to the conclusion from the visual inspection of the bias plot. However, the κ analysis produces a distinct *p*-value, unlike the bias plot. Interestingly, in this example β_{lig} identifies bias in the cases of dichloroisoproterenol, norepinephrine, salmeterol, and pindolol, just

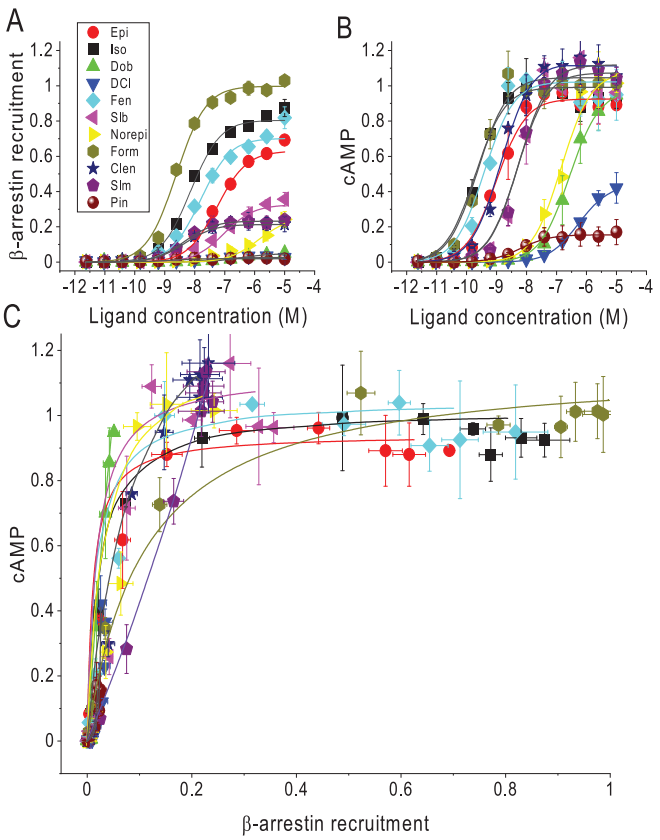

Fig. 2. (A) and (B) Dose response curves for β2AR β-arrestin recruitment (A) and cAMP concentration (B). Shown are raw data and fits to eq. (1) with $n = 1$, as reported in Tables S1A and S1B in [6]. (C) Bias plots showing the raw data and the analytical curves corresponding to eq. 12.

as κ, but σ fails to do so, an intriguing reversal from the example in Fig. 1.

4. Discussion

Here we propose a new method to calculate a bias coefficient called κ, to be used alongside other widely used bias coefficients such as β_{lig}, σ, and ΔΔLog [36,1,27,6]. This calculation is meant to recapitulate the insights gained from bias plots while allowing statistical comparisons of the data. It involves fitting dose response curves with the Hill equation and then using the fit parameters to describe the bias plot trajectory according to eq. 12. With the analytical expressions for the bias plots known, we compare the areas under the curves for a ligand of interest and a reference ligand.

To demonstrate the utility of this method, we used published data sets for AT1R and β2AR. In these examples, σ and β_{lig} are able to detect bias in one or the other data set, in a way that is consistent with the bias plots, but neither is able to detect bias in both cases. κ is able to consistently detect bias, in agreement with the bias plots in both data sets. The approach outlined here relies on a calculation of the areas underneath each trajectory in the bias plots. This results in increased sensitivity to weakly biased agonists and improved specificity when there are poor fits to the dose response curves. The unique utility of κ over the visual inspection of the bias plot is that κ can be analyzed for statistical significance to obtain a *p*-value. Here the analysis of κ suggests the presence of slight bias for salbutamol as compared to epinephrine ($p = 0.04$), which neither the equiactive or operational model-derived coefficients are able to detect. Of note, the *p*-values generated from κ agree with the bias plots, showing borderline significance when the plots are similar and high statistical significance when the plots are very different.

Table 2
Values of κ, calculated for the data set in Fig. 2.

	κ[a]	*p*-value[b]	Bias Plot[c]	β	σ	m
Isoproterenol	1.02 ± 0.05	>0.05	X	−0.31 ± 0.15	−0.22 ± 0.14	0.61
Dobutamine	0.88 ± 0.08	>0.05	X	−0.59 ± 0.26	0.23 ± 0.22	0.04
Dichloroisoproterenol	0.12 ± 0.03	<0.001	O	−1.98 ± 0.45	−0.45 ± 0.28	0.02
Fenoterol	1.08 ± 0.06	>0.05	X	−0.27 ± 0.20	−0.11 ± 0.14	0.61
Salbutamol	1.14 ± 0.06	0.04	–	−0.15 ± 0.20	0.04 ± 0.13	0.31
Norepinephrine	1.07 ± 0.07	>0.05	X	−0.49 ± 0.23	−0.19 ± 0.14	0.22
Formoterol	0.89 ± 0.05	0.05	–	−0.98 ± 0.15	−0.91 ± 0.14	0.61
Clenbuterol	0.97 ± 0.06	>0.05	X	−0.73 ± 0.22	−0.32 ± 0.14	0.20
Salmeterol	0.63 ± 0.04	<0.001	O	−1.38 ± 0.19	−0.65 ± 0.13	0.22
Pindolol	0.10 ± 0.03	<0.001	O	−1.76 ± 0.56	−0.49 ± 0.53	0.02

[a] Calculated using eq. 13. Standard error is calculated using the functional approach for multi-variable functions.
[b] Calculated using a Wilcoxon t-test.
[c] Based on visual inspection of bias plots. O indicates bias. X indicates no bias. – indicates inconclusive.

A unique feature of this method, as compared to all others, is that it can assess ligand bias over specific ligand concentration ranges. This can be done simply by adjusting the limits of integration for the area under the bias plot trajectories in eq. 13. This can be useful in cases such as the formoterol, as compared to the epinephrine, case shown in Fig. 2C, where the bias plots clearly diverge over a certain concentration range, and then cross. If data are truncated before the lines cross, κ will predict more significant bias over the specified concentration range. For example, if the cutoff is set to 0.45, then κ becomes 0.82 ± 0.04 (compared to 0.89 ± 0.05) and the *p*-value becomes 0.001 (compared to 0.05). Thus, conclusions about ligand bias can be drawn in the context of biological processes that occur at a specific range of ligand concentrations, or in the context of specific drug dosages.

As in all methods that calculate bias coefficients, the calculations of κ are largely dependent on the quality of the dose response curve fits [12,34,6]. Neither of the quantitative approaches used to identify biased agonists can overcome limitations in the underlying signaling data, such as artifacts from non-receptor-mediated signaling events, although they can address amplification that is generated downstream of a receptor-initiated event. Most dose response curves are well fit by the generalized Hill equation, which includes a variable Hill slope to account for both steep and shallow dose responses. In rare cases when satisfactory fits cannot be obtained, numerical integration could be applied to the raw bias plot data points to calculate the area under the curve, even without fitting. In theory, this is a model-free approach for ligand bias assessment in cases when the dose response curve fits are not robust. This makes the approach receptor-agnostic, and it could also be used to assess other systems that display functional selectivity in the absence of true ligand bias (e.g., receptor or system bias). However, the numerical approach also has weaknesses. For example, integration requires

interpolation between points. Without a theoretical model, the interpolation function is arbitrary, which can lead to large errors. Further, numerical integration can be strongly influenced by the uncertainties in individual points, especially when combined with large interpolations.

None of the methods to identify bias, discussed here, explicitly consider the physical chemistry of ligand binding to the receptor, i.e., the number of binding sites and the cooperativity of binding. The effective dissociation constant K_L in the Black and Leff operational model is assumed to describe simple 1:1 ligand to receptor binding. This simplification is justified, as the mode of ligand binding is just one of many factors that affect the response. The value of K_{resp} in the operational model (eq. 3), which describes the coupling between the receptors and downstream signaling molecules, and therefore the value of τ in eq. 6, also strongly influences the shape of the dose response curve. On the phenomenological level, complex modes of ligand binding will manifest themselves in a binding curve with a Hill slope that is different from 1. Importantly, the new bias coefficient κ does not require that the Hill slope is fixed at 1 and can be used for any n. Thus, κ is well suited for comparisons of ligands that exhibit different modes of binding, to determine if they are biased or not.

In conclusion, here we present a new method to calculate bias coefficients from bias plots. A Matlab script which calculates kappa using numerical methods can be found at https://engineering.jhu.edu/hristova/.

Authorship contributions

KK: participated in research design, performed data analysis, contributed to the writing of the manuscript; SR: contributed to the writing of the manuscript. KH: participated in research design, contributed to the writing of the manuscript.

Declaration of Competing Interest

No author has an actual or perceived conflict of interest with the contents of this article.

Data availability

Data will be made available on request.

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