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RESEARCH PAPER

An evaluation of the operational model when applied to quantify functional selectivity

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BACKGROUND AND PURPOSE

Functional selectivity describes the ability of ligands to differentially regulate multiple signalling pathways when coupled to a single receptor, and the operational model is commonly used to analyse these data. Here, we assess the mathematical properties of the operational model and evaluate the outcomes of fixing parameters on model performance.

EXPERIMENTAL APPROACH

The operational model was evaluated using both a mathematical identifiability analysis and simulation.

KEY RESULTS

Mathematical analysis revealed that the parameters R_0 and K_E were not independently identifiable which can be solved by considering their ratio, τ. The ratio parameter, τ, was often imprecisely estimated when only functional assay data were available and generally only the transduction coefficient R ($\frac{\tau}{K_A}$) could be estimated precisely. The general operational model (that includes baseline and the Hill coefficient) required either the parameters E_m or K_A to be fixed. The normalization process largely cancelled out the mean error of the calculated $\Delta log\,(R)$ caused by fixing these parameters. From this analysis, it was determined that we can avoid the need for a full agonist ligand to be included in an experiment to determine $\Delta log\,(R)$.

CONCLUSION AND IMPLICATIONS

This analysis has provided a ready-to-use understanding of current methods for quantifying functional selectivity. It showed that current methods are generally tolerant to fixing parameters. A new method was proposed that removes the need for including a high efficacy ligand in any given experiment, which allows application to large-scale screening to identify compounds with desirable features of functional selectivity.

Abbreviations

RSE, relative standard error; SSE, stochastic simulation estimation

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Introduction

Ever since the earliest description of receptor–ligand interactions (Clark, 1926), attempts to develop a reliable method for their quantification have been made. The E_{max} model provides an empirical justification for the concentration–response relationship. It makes the assumption that the effect of the ligand is directly proportional to receptor occupancy; that is to say, maximal response can only be achieved with maximal receptor occupancy (Clarke and Bond, 1998). The operational model relaxes this assumption by incorporating an (unobserved) empirical transducer function to convert occupancy into effect (Black and Leff, 1983), which allows the effect to represent a potential cascade of cellular or tissue signalling. This can explain the 'receptor reserve' phenomenon, that is, stimulation of only a fraction of the whole receptor population elicits an apparent maximal response. Furthermore, incorporation of the transducer function also allows the investigator to create a link directly from ligand concentration to response without the need to formally incorporate ligand binding as a necessary intermediary step. In this sense, the operational model is a 'middle-out' strategy in receptor theory which keeps the balance between model simplicity and mechanistic insight. Here, the equation for the operational model (Equation 1), in the same style as Equation 5 from the original paper (Black and Leff, 1983), is given:

$$\frac{E}{E_m} = \frac{R_0 A}{K_A K_E + (R_0 + K_E)A} \quad (1)$$

In the operational model described by Black and Leff, there are two key parameters: τ and K_A. This is a reparameterization (of Equation 1 here) by Black and Leff which they present in their Equation 6 (shown in this paper in Equation 5). The transducer ratio parameter, τ ($\frac{R_0}{K_E}$), encompasses receptor density (R_0) and the stimulus coupling efficiency (K_E). K_A is an implicit parameter in the model that accounts for ligand binding but is not specifically estimated in their work. In more recent developments, the operational model has been generalized into several variants, such as the conformation-based operational model (Roche *et al.*, 2013) and the operational model with constitutive activity (Slack and Hall, 2012). Among these variants, the general operational model (Equation 2) is the most widely used model that accounts for non-zero basal (*Basal*), non-unity Hill slope factor (n) and estimable maximal system response (E_m) (van der Westhuizen *et al.*, 2014). Furthermore, it is evident within the operational model framework that the classical concepts of 'full agonism' and 'partial agonism' need further examination, as such classifications are made on the combination of ligand attributes and how efficiently the system transduces the stimulus into effect (Kenakin, 2014). It is possible that one single ligand may appear to be a full agonist in an efficient transduction system but a partial agonist in a less efficient transduction system.

Recently, interest has arisen in the ability of ligands to differentially regulate different signalling pathways when coupled to a single receptor; a concept termed functional selectivity (Urban *et al.*, 2007). The need to link ligand concentration to response at (now) multiple pathways has rejuvenated interest in the operational model. In such studies, it is common to consider a single composite parameter, R, in quantifying a ligand's effect on a pathway. This parameter is defined as the ratio $\frac{\tau}{K_A}$ and is termed the transduction coefficient. It is derived from an application of the operational model to simultaneously account for both affinity and efficacy of a ligand (Kenakin and Miller, 2010). Because the system itself will complicate interpretation of this composite parameter (e.g. system bias and observation bias), it is necessary to normalize R to a reference ligand, yielding the normalized transduction coefficient $\Delta\ log\ (R)$ (Kenakin *et al.*, 2012). Finally, as the goal of the analysis is to consider how a ligand may bias the response to a particular pathway, it is convention to further normalize the transduction coefficient for one pathway to another, thereby providing a single metric to encapsulate the relative effect of a ligand on a pathway of interest compared to a reference pathway. The additional normalization leads to the now widely used metric $\Delta\Delta\ log\ (R)$ (Kenakin *et al.*, 2012).

In this work, we explore the mathematical and statistical properties of the operational and the general operational model in relation to parameter estimation, which is termed identifiability analysis. A model that contains one or more parameters that are not able to be estimated (given perfect data) is termed *not identifiable*. In other words, identifiability analysis answers the question of whether there is a unique solution for the parameters of a mathematical model based on its structure and also the experimental design (Shivva *et al.*, 2013; Lavielle and Aarons, 2016). This concept is more commonly considered in pharmacokinetic studies where, for instance, it is known that if you only administer a drug orally to an individual, then the parameter absolute bioavailability (F) cannot be estimated (as it requires both intravenous and oral administration to provide the data for this parameter). As such, this parameter is said to be *not identifiable*. When model parameters are not identifiable, this means that there are an infinite number of solutions for the model parameters that can fit the data equally well. This causes the parameters of the model to be imprecisely estimated and potentially misrepresent the data. Identifiability issues are also present in pharmacological experiments, but these properties are less well explored. The concept of identifiability is divided into two types: (i) *structural identifiability* and (ii) *deterministic identifiability* (sometimes also termed pragmatic identifiability). Structural identifiability is a formal mathematical technique to determine whether a parameter can be uniquely estimated given perfect data (data that contain no error of any type – e.g. no assay error) (Bellman and Åström, 1970). Deterministic identifiability addresses the degree of precision with which a parameter can be estimated based on typical experimental data (Guedj *et al.*, 2007). These concepts are naturally hierarchical, such that if a model is not structurally identifiable, then it cannot be applied to any data and one or more parameters will need to be either fixed or merged into a composite parameter (the latter is analogous to fixing one of the parameters to a null value; e.g. 0 or 1). Once a model is structurally identifiable, then the lower hierarchy, deterministic identifiability, yields knowledge about how well it can be estimated by a given experiment. These concepts are critical in understanding how a model can represent a given set of experimental data.

Application of the operational model to describe the responses elicited by a ligand in multiple pathways is known to result in issues with mathematical identifiability of the model (Kenakin *et al.,* 2012; van der Westhuizen *et al.,* 2014). It should be noted that application of the operational model does not explicitly include ligand binding and considers a single pathway from a single ligand at a time (hence the need for normalization). It is, in theory, possible to apply a general equilibrium model that encompasses all pathways simultaneously that would obviate some of these mathematical issues, but this is beyond the scope of this work.

The overarching aim of this paper is to provide a ready-to-use understanding of the application of the operational model for quantifying functional selectivity. This encompasses three specific objectives: (i) to systematically assess the identifiability (structural and deterministic) of both the operational model and the general operational model; (ii) to evaluate the effects of parameter mis-specification on the quantification of ligand bias; and (iii) to assess whether we can relax the need for the presence of a full agonist to quantify ligand bias.

Methods

The methods are described in two parts. In the first part, the technical details of the methodology are provided as they relate to both structural and deterministic identifiability as well as simulation estimation methods. In the second part, methods are provided for each of the three aims.

Part 1

Structural identifiability analysis. This type of identifiability determines whether a unique set of parameter values exist within the model, given perfect data. If this is not true, then the model is said to be *not identifiable*. This means that the model parameters cannot be estimated in its current form and a parameter (or parameters) will need to be fixed. The structural identifiability analysis followed the processes developed in previous work (Shivva *et al.,* 2013). The Population OPTimal design for Identifiability (POPT_I), a MATLAB-based software (available at http://www.otago.ac.nz/pharmacometrics/downloads/index.html), was used for the assessment of structural identifiability. Brief details of the application of this method are provided here for completeness. In this work, two necessary criteria for claiming a structurally identifiable, fixed-effect model were adopted: (i) there was a continuous linear log–log relationship between the log of the determinant of Fisher information matrix (M_F) and the log of the random noise; (ii) the determinant should approach infinity as residual variance approaches zero (Shivva *et al.,* 2013). The values for the random noise $log(\sigma^2)$ were the same for all analyses, −7, −6, −5, −4, −3, −2, −1, 0, 1 and 2.

Deterministic identifiability analysis. Deterministic identifiability is concerned with the precision of parameter estimation. The relative magnitude of the SE is an indication of the precision of the parameter estimates and is amenable to the experimental design. The SE value can be obtained either theoretically, *via* the Fisher information matrix (M_F), or they can be obtained after a data set has been analysed using standard software (such as GraphPad Prism®). Technically, these are termed the *expected* SE or the *observed* SE values respectively. Both methods to calculate the SE values give very similar results. Irrespective of the method used to calculate the SE values, large values are a measure of uncertainty in the parameter values that (if large enough) may yield a claim that the model is *not deterministically identifiable*. In this work, in order to alleviate the need to generate and analyse data, we use the information matrix (M_F) and calculate the expected SE values. Hence, it is straightforward (with appropriate software) to calculate the expected SE values for any model given any potential experimental design without having to do the experiment. For convenience, we express the SE value for a parameter as its value relative to the parameter value (in order to normalize to scale). This is termed relative SE (RSE) which we show as per cent. Values greater than 50% are suggestive of potential problems with deterministic identifiability.

Briefly, and for completeness, the SE values are calculated as the square root of the diagonal elements of the inverse of the M_F. The M_F can be calculated as the product of the sensitivity matrices weighted by the residual error. In this work, optimal design software PFIM (Bazzoli *et al.,* 2010), available at http://www.pfim.biostat.fr/, was used to calculate the M_F and provide the expected SE values.

Stochastic simulation and estimation (SSE)

Outline of stochastic simulation estimation (SSE) unit. In practice, the slope factor n is commonly added to improve curve fitting. Hence, we intend to explore a range of values of n to assess their influence on the results of this analysis. We tried an exact numerical evaluation, but it became unfeasible (this failure is shown in the Supporting Information). A more general method involving simulating data and estimating the model parameters was therefore used. This is termed stochastic simulation and estimation (SSE). Practically, this method requires the data to be simulated with random error (aka 'experimental noise'), termed stochastic simulation, and then estimated using an appropriate software application. It is common to use SSE methods when the model is too complicated to perform exact mathematical analyses. Both the simulation (SSE) method and exact calculation (when possible) provide equivalent answers and interpretations.

The SSE methods in this work were used to assess the influence of mis-specification of parameter values (i.e. when the parameters were fixed to something other than their true value). Each SSE unit consists of three steps: dataset simulation, parameter estimation and analysis of the results. In the simulation step, based on the given parameter values and study design, pseudo-experimental data were generated from the general operational model (Equation 2) using R version 3.0.2 (http://www.r-project.org/) within Rstudio IDE Version 0.98.490 (http://www.rstudio.com/). In total, there were 1000 replicates. In each replicate, two concentration–response curves corresponding to a pair of reference and test ligands were generated with random error. Then, in the estimation step, each replicate was analysed in GraphPad Prism (v7.0; GraphPad Software, La Jolla, CA,

USA) following usual estimation methods (van der Westhuizen *et al.,* 2014). R code for stochastic simulation and GraphPad Prism code for batch analysis are included in the Supporting Information. In the post-estimation analysis step, the normalized transduction coefficient (Δ *log* (*R*)) value was calculated for each replicate by taking the difference of *log*(*R*) between reference and test ligand. For the final outcome of each SSE unit, only the results from the replicates with successful estimation were included and contributed to calculation of the median and 95% confidence intervals (CI) for *log*(*R*) and Δ *log* (*R*).

Criteria for successful estimation. A successful estimation was defined as not *ambiguous* estimates of the parameter *log* (*R*) for the pair of ligands in a replicate. The term *ambiguous* is a term coined by Prism to describe the case that the parameters cannot be precisely estimated. This is akin to defining the parameters as *not deterministically identifiable*. Such parameters are marked in Prism with a '~' before the best fit values. Ambiguous values were omitted from further computation. The successful estimation rate was defined as the fraction of successful estimation runs within 1000 replicates. In general, the estimation method was considered to be robust if its successful estimation rate was higher than 90%.

Part 2

Systematic assessment of the identifiability of both the operational model and the general operational model

Structural identifiability analysis. The original form of the operational model (Equation 1) has been extended into the general operational model (Equation 2, the same as Equation 5 in van der Westhuizen *et al.,* 2014) to account for non-zero basal (*Basal*), non-unity slope factor (*n*) and an estimable maximal system response (E_m) (van der Westhuizen *et al.,* 2014).

$$E = Basal + \frac{E_m - Basal}{\left(\frac{1+A/10^{\log(K_A)}}{A\cdot 10^{\log(R)}}\right)^n + 1} \quad (2)$$

Here, E_m is the system maximal response, *Basal* is the baseline response in the absence of ligand and *n* is the slope factor. These three parameters are system parameters. Note, in this expression, it is assumed that the basal activity of the system is constant over both time and applied ligand concentrations. Further generalization is possible but not considered here. Both K_A and *R* are drug-specific parameters. Conventionally, the parameters K_A and *R* are transformed into logarithms (i.e. $10^{\log(K_A)}, 10^{\log(R)}$). Note here that this re-parameterization does not affect the structural identifiability of the model.

The structural identifiability analysis (a formal assessment of the model to determine if the parameters could be uniquely estimated) was performed on both the Black and Leff operational model (Equation 1) and the general operational model (Equation 2) in POPT_I. A generic study design with sampling concentrations *log*(*A*) from −13 to −4, increment of 1, was assumed for both models. An arbitrary set of parameter values was used (Table 1). [Since this work is dependent on the particular set of parameter values, then the structural identifiability analysis is said to be *local* – meaning the model is identifiable (or not) based on a specific set of parameter values. This does not affect interpretation of this work.] Structural identifiability was assessed for the Black and Leff operational model based on four scenarios: (i) all the model parameters (R_0, K_E, K_A) were considered to be unknown and estimable; (ii) R_0 was assumed to be known and fixed; (iii) K_E was assumed to be known and fixed; and (iv) R_0 and K_E were reduced into one single parameter τ, and both τ and K_A were considered to be unknown and estimable. For the general operational model, four scenarios were evaluated for the identifiability analysis: (i) all the model parameters (E_m, *log*(K_A), *log*(*R*)) were considered to be unknown and estimable; (ii) E_m was assumed to be known and fixed; (iii) *log*(K_A) was assumed to be known and fixed; and (iv) *log*(*R*) was assumed to be known and fixed.

Table 1

The parameter values for structural identifiability analysis of the operational model

Parameters	Operational model	General operational model
n	–	1
E_m	–	1
Basal	–	0.1
R_0	10	–
K_E	10	–
τ		1
log(K_A)		−8
log(*R*)		8
Sampling concentrations: *log* (*A*)	From −13 to −4, increased by 1	
Random noise levels: *log*(σ^2)	−7, −6, −5, −4, −3, −2, −1, 0, 1, and 2	

Deterministic identifiability analysis. A deterministic identifiability analysis (assessment of SEs of the parameter estimates) was performed on both the operational model and the general operational model. A similar parameter set-up was used as in the structural identifiability analysis. Details are provided in Table 2. A proportional measurement error with 10% coefficient of variation was assumed for data arising under both models. The deterministic identifiability was assessed for each value of τ. In terms of the general operational model, three arbitrary sets of the parameter values were used and each stood for one of the following circumstances: (i) a pair of highly efficacious ligands; (ii) one highly efficacious ligand and one less efficacious ligand; and (iii) a pair of low-efficacy ligands (Table 3). Efficacy is defined based on τ values. Under each circumstance, two scenarios were evaluated for the deterministic identifiability: (i) E_m was assumed to be known and fixed; and (ii) K_A values of both ligands were assumed to be known and fixed.

Table 2

The parameter values for deterministic identifiability analysis of the operational model

Design	Value
log(A)	From −13 to −4, increased by 1
Random noise	–
prop. err	0.1
Parameters	–
τ	From 0.1 to 100, increased by 0.1
K_A	1.0E − 8
R	$\frac{\tau}{K_A}$

Evaluation of the effects of parameter mis-specification on the quantification of ligand bias

Current estimation methods for the quantification of ligand bias. In current practice, three methods have been adopted for the estimation of the general operational model. In method I, the value of system maximal response (E_m) is set to the empirically measured response of a known maximal stimulant (e.g., forskolin for cyclic AMP and phorbol 12-myristate 13-acetate for phosphorylated ERK) or the common maximal response among multiple ligands (Kenakin *et al.,* 2012). In method II, the equilibrium dissociation constant (K_A) is fixed to a previously estimated value from a separate binding assay (Rajagopal *et al.,* 2011). In method III, a slight modification is made into the operational model framework (van der Westhuizen *et al.,* 2014). In this method, ligands are subjectively classified into two categories: full and partial agonists, based on the value of observed maximal response of each ligand ($E_{max,\ i}$). For partial agonists, the concentration–response curve is directly fitted to the general operational model, whereas for full agonists, one more constraint is applied with K_A arbitrarily assigned to 1 mol·L^{-1} (the identity for most mathematical operations), a value typically 6 to 8 orders of magnitude from expected.

SSE study I: evaluation of the effects of mis-specified E_m. SSE study I was performed to evaluate the effects of mis-specified E_m on the quantification of ligand bias. For the simulation step, a generic study design for the sampling concentrations (Table 4) was assumed for all the SSE units and three arbitrary sets of the parameter values (Table 4) were used with the only difference in slope factor (n=[0.7, 1, 1.5]). Then, in the estimation step, for these three simulated datasets, each was used to estimate the parameters using estimation method I with E_m fixed to three different values 0.8, 1(true) or 1.2. Therefore, there were nine SSE units in this SSE study.

SSE study II: Evaluation of the effects of mis-specified K_A. SSE study II was conducted to evaluate the effects of mis-specified K_A on the quantification of ligand bias. In the simulation step, a generic study design for the sampling concentrations (Table 4) was assumed for all the SSE units and 10 arbitrary sets of the parameter values (Table 4) were used with the only difference in slope factor (n=[0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.25, 1.5, 1.75, 2]). The value of τ for the reference ligand was assigned to a large number (Table 4) to represent full agonist (i.e. highly efficacious ligand). In the following, each simulated data were estimated by two different estimation methods: method II with K_A values fixed to true or method III with K_A of full agonist arbitrarily assigned to 1 mol·L^{-1}. In the final step, the post-estimation analysis was applied to get summarized outcomes from these 20 SSE units.

Assessment of the relaxation of the need for a full agonist in quantifying functional selectivity

Method IV for the quantification of ligand bias. The current estimation method for the general operational model requires a full agonist to have been identified within the study. We propose a generalization to this estimation method (termed method IV here) to account for the situation when only low-efficacy ligands are present. The key steps were listed:

Table 3

The parameter values for deterministic identifiability analysis of the general operational model

		Ligand pair study					
		High + high[a]		High + low		Low + low	
Setting		Ligand I	Ligand II	Ligand I	Ligand II	Ligand I	Ligand II
Individual parameters	τ	15	25	15	0.8	0.8	0.4
	log(K_A)	-8	−7	−8	−7	−7	−8
	log(R)	9.18	8.40	9.18	6.90	6.90	7.60
Design	*log(A)*	From −13 to −4, increased by 1					
System parameters	E_m	1					
	Basal	0.1					
	n	1.0					
	prop. err	0.1					

[a]High = high efficiency and low = low efficiency.

Table 4

The parameter values for the SSE studies

Design	Impact of E_m mis-specification		Impact of K_A mis-specification		Evaluation of method IV	
log(A)	From −13 to −4, increased by 1		From −13 to −4, increased by 0.5		From −13 to −4, increased by 0.5	
System parameters	–		–		–	
n	0.7, 1, 1.5		Ranging from 0.5 to 2		Ranging from 0.5 to 2	
E_m	1		500		500	
Basal	0.1		10		10	
prop. err	0.1		0.1		0.1	
Individual parameters	**Ligand I**	**Ligand II**	**Ligand I**	**Ligand II**	**Ligand I**	**Ligand II**
τ	1.5	0.8	6	2	0.6	0.2
$log(K_A)$	−8	−7	−8	−7	−8	−7
log(R)	8.176	6.903	8.778	7.301	7.778	6.301
Δ *log (R)*	1.273		1.477		1.477	

1) Fit the classical E_{max} model (Equation 3) to the data from every ligand. For each pathway, the value of *n* and *Basal* are shared for all the ligands and there is no constraint on the values of E_{max} and EC_{50}.

$$E = Basal + \frac{(E_{max} - Basal)}{1 + 10^{(logEC_{50} - logA)\cdot n}} \quad (3)$$

Here, *Basal* and *n* are system parameters shared among different ligands. E_{max} and $logEC_{50}$ are ligand-specific parameters

2) Select the ligand with maximal $E_{max,i}$ from the current study and set as if it were a full agonist (termed pseudo full agonist). Classify all the remaining ligands as partial agonists.
3) For partial agonists, the concentration–response curve is directly fitted with the general operational model, whereas for the pseudo full agonist, the value of K_A is set to 1 mol·L^{-1}. For each pathway, the values of *n*, *Basal* and E_m are shared for all the ligands and there is no constraint on the values of $log(R)$ and $log(K_A)$.

An illustrating example with step-by-step analysis processes and results is included in the Supporting Information.

SSE study III: Evaluation of the performance of method IV. SSE study III was performed to evaluate the performance of method IV by comparing to methods I and II. The same set-up was used for the simulation as for SSE study II, except that the values of τ were smaller in SSE study III (Table 4) to represent low-efficacy ligands. Then, for the parameter estimation, each simulated data were analysed by three different estimation methods: method I with E_m set to true; method II with K_A values set to true; and method IV. Finally, the results of a total of 30 SSE units were obtained from the post-estimation analysis.

Results

Systematic assessment of the identifiability of both the operational model and the general operational model

Structural identifiability of the operational model. Given pharmacological response data only, the original form of the operational model (Equation 1) was confirmed as not structurally identifiable. As conceptually demonstrated in Equation 4 (a re-expression of Equation 1 by dividing K_E), there were infinite possible combinations of R_0 and K_E that rendered the same result. For example, the two parameter sets, (R_0 = 100, K_E = 20, K_A = 10^{-8}) and (R_0 = 10, K_E = 2, K_A = 10^{-8}), produced exactly identical curves.

$$\frac{E}{E_m} = \frac{\frac{R_0}{K_E}\cdot A}{K_A + \left(\frac{R_0}{K_E} + 1\right)A} \quad (4)$$

Structural identifiability analysis was performed in Equation 1. As shown in Figure S1A, the result indicated that this equation was unidentifiable when all parameters were considered to be estimable. Fixing either R_0 or K_E rendered the model structurally identifiable. In the absence of measuring (or estimating) R_0 or K_E from an additional experiment, it is not possible to distinguish their values. In order to solve this problem and as indicated by the work of Black and Leff, R_0 and K_E are therefore reduced into a single identifiable quantity ($\frac{R_0}{K_E}$), defined as the transducer ratio τ. This yielded the reduced form of the operational model, Equation 5, which was structurally identifiable (Figure S1B).

$$\frac{E}{E_m} = \frac{\tau\cdot A}{K_A + (\tau + 1)\cdot A} \quad (5)$$

Deterministic identifiability of the operational model. It was seen from the results of a deterministic identifiability analysis (Figure 1) that the operational model (Equation 5) generally

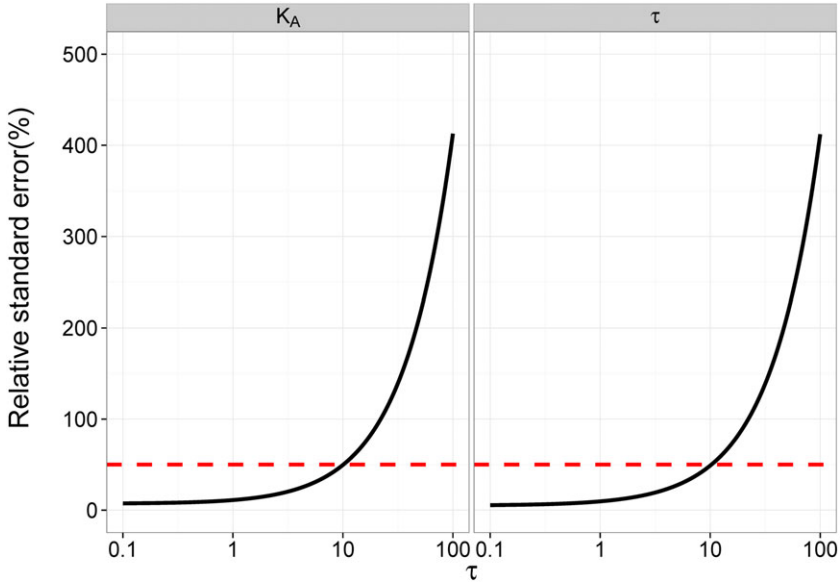

Figure 1

The RSE of estimated parameters versus τ for Equation 5. In this study, a generic study design with sampling concentrations $log(A)$ from −13 to −4, increment of 1, was adopted. A proportional measurement error with 10% coefficient of variation were assumed. K_A was set to an arbitrary value, 10^{-8} mol·L^{-1}. The deterministic identifiability was assessed for each value of τ, ranging from 0.1 to 100. The left panel is for K_A and the right panel is for τ. The red dashed line indicates the 50% RSE, considered as the threshold for precise estimation.

yielded appropriately low RSE% values (<50%) for τ and K_A for standard study designs. However, it was also evident that the precision of the estimate of both K_A and τ decreased with increasing values of τ, indicating that the operational model would not be deterministically identifiable for the large values of τ, even though this model is structurally identifiable (Figure 1). This is conceptually demonstrated in Equations 6 and 7: when values of τ greatly exceed 10 (i.e. highly efficacious ligand), Equation 5 is well approximated by Equation 6, which is then unidentifiable. Equation 6 is re-expressed as Equation 7 by dividing K_A. We now see that τ and K_A only appear as the ratio when τ exceeds 10 and hence there will be infinite possible combinations of τ and K_A that provide indistinguishable results (i.e. the model is structurally unidentifiable).

$$\frac{E}{E_m} = \frac{\tau \cdot A}{K_A + (\tau + 1) \cdot A} \xrightarrow{\tau > 10} \frac{\tau \cdot A}{K_A + \tau \cdot A} \tag{6}$$

$$\frac{E}{E_m} = \frac{\frac{\tau}{K_A} \cdot A}{1 + \frac{\tau}{K_A} \cdot A} \tag{7}$$

In order to circumvent this problem, the transduction coefficient (R) was introduced, defined as the ratio between transducer ratio (τ) and equilibrium dissociation constant (K_A). Substituting R into Equation 5 yielding Equation 8:

$$\frac{E}{E_m} = \frac{R \cdot A}{1 + (R + 1/K_A) \cdot A} \tag{8}$$

A deterministic identifiability analysis was also performed on Equation 8. Contrary to the behaviour of K_A, the RSE of R is now independent of the value of τ (Figure S2), indicating that the transduction coefficient (R) could be precisely estimated, even for highly efficacious ligands. In this sense, R was not only a combination of efficacy and affinity, as originally described, but also the minimal robust element that could be directly derived from the operational model analysis for quantifying agonism.

Structurally identifiability of the general operational model. Structural identifiability analysis was performed on Equation 2. As shown in Figure S3A, the result indicated that this equation was unidentifiable when all parameters were considered to be estimable. Furthermore, case deletion assessment (Figure S3B–D) identified that the general operational model had one unidentifiable parameter and one of E_m, $log(K_A)$, or $log(R)$ would be required to be fixed to yield a structurally identifiable model. However, since $log(R)$ is regarded as the target from these analyses, only E_m or $log(K_A)$ could be considered to be fixed. Note here that neither n nor *Basal* affects the structural identifiability of the model.

Deterministic identifiability of the general operational model. A deterministic identifiability analysis was performed on Equation 2 for a pair of ligands. As shown in Table 5, in general, with fixed E_m or K_A, the target parameter R could be precisely estimated with low RSE% values (<50%), when at least one highly efficacious ligand existed. For a pair of low-efficacy ligands, estimation of R became less precise and fixing E_m gave more precise parameter estimation of R than fixing K_A. For all highly efficacious ligands, given only

Table 5

The results of deterministic identifiability analysis of the general operational model

Scenario		Fixed parameters	E_m RSE(%)	K_A RSE(%)	R RSE(%)
Ligand pair	High + high[a]	E_m	–	109.6[b], 172.3	24.6, 23.7
		K_{A1}, K_{A2}	5.2	–	27.6, 27.5
	High + low	E_m	–	120.9, 47.3	27.9, 44.5
		K_{A1}, K_{A2}	6.6	–	29.3, 20.3
	Low + low	E_m	–	75.8, 67.4	76.1, 88.4
		K_{A1}, K_{A2}	80.9	–	159.8, 147.9

[a]High = high efficacy and low = low efficacy.
[b]If the RSE value is less than 50%, the parameter it describes is interpreted as precisely estimable.

pharmacological response data, K_A could not be precisely estimated (RSE% values >100%), consistent with previous findings in the operational model.

Evaluation of the effects of parameter mis-specification on the quantification of ligand bias

The source of parameter mis-specification addressed here was restricted to some unavoidable cases, that is, theoretically inevitable mis-specification or simply fixing the parameter to an (arbitrary) value for convenience. Note that the effects of mis-specified K_A arising from binding assay experimental error, while potentially important, was not considered on the performance of method II and was beyond the scope of current evaluation.

In method I, E_m was prone to mis-specification. In real cases, the true value of E_m is rarely known and often empirically approximated from the maximal observed response among a number of ligands or the response to a known maximal stimulant (e.g. forskolin for cyclic AMP) (Evans *et al.*, 2011; Kenakin *et al.*, 2012). In so doing, E_m is set to the lower bound of its true value. On the other hand, K_A is simply fixed to an (arbitrary) value for convenience in method III. Instead of plugging in estimated K_A values from independent binding assays (method II), the K_A values of full agonists were arbitrarily fixed to 1 mol·L^{-1}. This value greatly exceeds the biologically reasonable range of possible values of K_A (10^{-4}~10^{-12} mol·L^{-1}). In the following, we evaluate the effects of parameter mis-specification on quantification of ligand bias under mis-specification of E_m and K_A.

The influence of the mis-specified E_m. In the case where the slope factor (n) is equal to one, an exact numerical evaluation was performed to explore the effects of the mis-specification of E_m (see Appendix A). It is clearly shown that the mis-specified value of E_m leads to an inaccurate estimation of R. Here, we are referring to the mean error in the estimate of R, which we specify as the true value minus the estimated value [we use *mean error* to avoid the use of bias in two different settings (statistical and pharmacological)]. The mis-specification of R has a similar effect on different ligands. Therefore, the normalization process would cancel out this estimation mean error, and the estimation of $\Delta\ log\ (R)$ would remain accurate. In other words, the estimation of $\Delta\ log\ (R)$ is tolerant to the mis-specification of E_m.

Table 6

The results from the evaluation of the effects of the mis-specified E_m on the estimation of $log(R)$ and $\Delta\ log\ (R)$

n	E_m assigned value	$log(R)$ Ligand I median [95% CI]	$log(R)$ Ligand II median [95% CI]	$\Delta\ log\ (R)$ Median [95% CI]	Successful estimation rate (%)
0.7	0.8	8.40 [7.92–8.84]	7.15 [6.59–7.70]	1.25 [0.86–1.70]	98.4
	1(true)	8.18 [7.52–8.79]	6.93 [6.24–7.63]	1.25 [0.86–1.70]	98.1
	1.2	8.02 [7.22–8.73]	6.77 [5.95–7.56]	1.25 [0.85–1.70]	97.9
1	0.8	8.28 [7.82–8.81]	7.03 [6.43–7.62]	1.27 [0.92–1.65]	98.2
	1(true)	8.18 [7.55–8.89]	6.93 [6.17–7.66]	1.26 [0.91–1.65]	97.5
	1.2	8.09 [7.34–8.80]	6.83 [5.98–7.60]	1.27 [0.91–1.65]	95.3
1.5	0.8	8.19 [7.73–8.66]	6.94 [6.34–7.40]	1.26 [0.94–1.63]	93.4
	1(true)	8.20 [7.58–8.72]	6.94 [6.25–7.45]	1.27 [0.96–1.62]	94.0
	1.2	8.17 [7.49–8.71]	6.91 [6.13–7.45]	1.27 [0.97–1.62]	91.2

For the case where the slope factor is different from unity, a stochastic simulation-estimation study was performed to explore the effect of the mis-specification of E_m. In Table 6, it is noted that estimation of $log(R)$ is inaccurate with the mis-specified E_m. For instance, in the case that n is equal to 0.7 and E_m is underestimated by 20%, the estimated $log(R)$ values for ligands I and II were 3–5% greater than their true values (i.e. negligibly affected). The estimation mean error of ligand bias caused by the mis-specification of E_m appears therefore to be largely cancelled out *via* the normalization process.

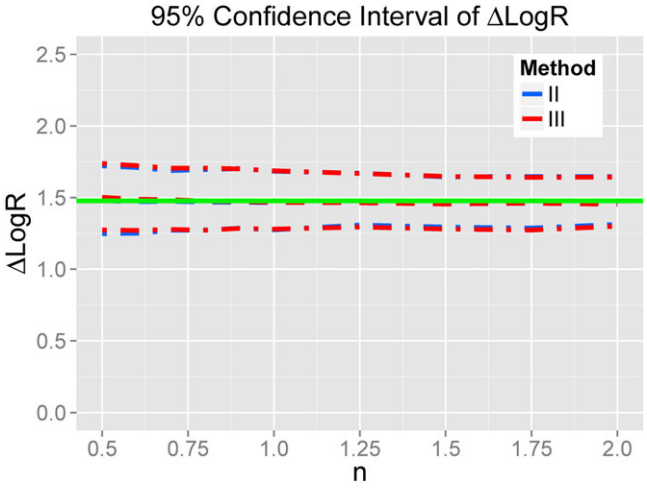

Figure 2

Values of Δ *log* (*R*), with its 95% CI, over different values of *n*. The green line shows the true value. Dashed line indicates the median estimated value and the dash-dot line the 95% CI of estimated value. Blue indicates the method of assigning K_A to measured values from binding assay (method II); red indicates the method of arbitrarily fixing K_A of full agonists to 1 (method III). The successful estimation rates of three methods were all higher than 95%.

The influence of mis-specified K_A. As shown for the mis-specification of E_m, we can exactly determine the effect of the mis-specification of K_A on estimation mean error (detailed in Appendix B). Through the derivation, it is evident that the mis-specification of K_A would lead to inaccurate estimation of R. However, again, this affects all ligands in question equally, and hence, estimation of Δ *log* (*R*) remains accurate.

When the slope factor was different from unity, we performed a stochastic simulation-estimation study to explore the influence of the mis-specification of K_A. In Figure 2, it is demonstrated that Δ *log* (*R*) calculated from method III (red) is essentially accurate, even when the slope factor n departs from unity. It is also seen that arbitrarily assigning K_A to 1-mol·L^{-1} (method III, red line) is indistinguishable from the result produced from plugging in the true values of K_A (method II, blue line), indicating that the influence of this mis-specification of K_A on the quantification of ligand bias is trivial.

Assessment of the relaxation of the need for a full agonist in quantifying functional selectivity

It was shown in the deterministic identifiability analysis that estimation of $log(R)$ after fixing E_m (method I) or fixing K_A (method II) became less precise in the absence of highly efficacious ligands (Table 5). Moreover, the application of method III was restricted, by definition, to cases wherein a full agonist (highly efficacious ligand) was used in the experiment. Method IV was proposed to relax the need for a full agonist in method III. In this case, E_m was taken to be the highest efficacy level observed within the range of ligands considered.

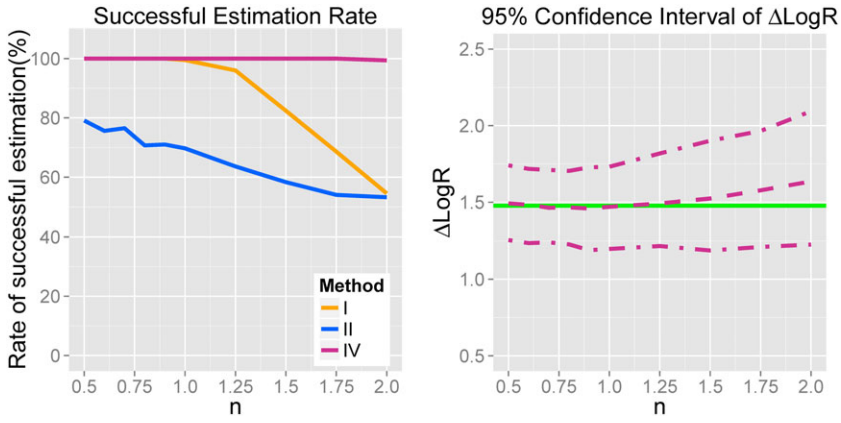

Figure 3

Performance comparison of method IV and other estimation methods. (A) The successful estimation rates for three estimation methods over the range of *n*. Method I involves assigning E_m to known system maximal response; Method II involves assigning K_A to measured values from binding assay; Method IV involves arbitrarily fixing K_A of pseudo full agonists to 1. (B) Values of Δ *log* (*R*), with its 95 % CI, over the normal range of *n* for Method IV. Green line indicates the true value. Dashed line indicates the median value of estimated value. Dash-dot line indicates the 95% CI of estimated value.

A stochastic simulation-estimation study was performed to compare the performance of method IV with other estimation methods, that is, methods I (E_m set to the system maximal response) and II (K_A set to the value from a previous binding experiment). Method III was excluded from this comparison because it, by definition, could only be applied to the case that a full agonist was present.

The method IV estimation of $log(R)$ was found to be robust (using GraphPad Prism v7.0), with a successful estimation rate close to 100%. Methods I and II, however, had quite poor rates of successful estimation (less than 60%), especially for large values of n (Figure 3A). This suggests that method IV is more robust than typical analysis methods. Furthermore, in Figure 3B, it was clear that the $\Delta\ log\ (R)$ calculated using method IV was either accurate or minimally inaccurate within the normal range of n (from 0.5 to 2).

An exact numerical evaluation (detailed in Appendix C) supports these findings, showing that method IV yields an accurate $\Delta\ log\ (R)$. As demonstrated before, the influence of mis-specified K_A can be cancelled out through the normalization process. Hence, the estimation of $\Delta\ log\ (R)$ is accurate using method IV. As demonstrated in Appendix C, fixing K_A for a given partial agonist to an arbitrarily large value (1 mol·L^{-1}) forces the apparent value of τ (τ') to be sufficiently large for estimation of R to be precise, despite its lower efficacy. Furthermore, it is noted in the deterministic identifiability analysis that a large value of τ contributes to the precise estimation of transduction coefficient (R). Hence, R and therefore $\Delta\ log\ (R)$ can be precisely estimated in method IV.

Discussion and conclusions

Functional selectivity describes the ability of ligands to differentially regulate several different signalling pathways when coupled to a single receptor (Clarke and Berg, 2010). This is a complicated pharmacological process involving multiple biological steps (e.g. receptor binding, cellular and intracellular signalling), and how these multiple signalling pathways interact with the receptor is still unclear (Kenakin and Christopoulos, 2013b). Due to the complexity of functional selectivity, the implementation of a full model that can accommodate multiple receptor states where each state links to a signalling pathway remains impractical (Weiss *et al.*, 1996; Ehlert, 2008; Stein and Ehlert, 2015). Therefore, it has been necessary to use a simplified model. Several simplified models have been proposed, such as the three-state model (Leff *et al.*, 1997), the operational model (Black and Leff, 1983), the operational model with non-zero constitutive activity (Slack and Hall, 2012) and the conformation-based operational model (Roche *et al.*, 2013). Among them, the simplest version is the operational model (Black and Leff, 1983), which has been widely used to quantify functional selectivity (Kenakin *et al.*, 2012).

The purpose of the current study is to gain a more in-depth understanding of the application of the operational model for quantification of functional selectivity. To do this, we first systematically assessed identifiability of the operational model and the general operational model (where basal and system maximum are also considered as parameters). In so doing, additional rationale has been provided for utilization of current metrics for the quantification of functional selectivity. Second, current estimation methods for the quantification of ligand bias were evaluated *via* both exact (algebraic) and numerical (stochastic simulation-estimation) approaches, with the goal of providing a better understanding of the effect of parameter mis-specification. Finally, based on the insights from these evaluations, the current method was further generalized into a more robust and objective procedure that has the potential to simplify current analyses.

In many cases, metrics arise from a need to solve the mathematical issue of quantification. For instance, the parameter τ was introduced to reduce two entangled parameters, R_0 and K_E, into one single parameter (Black and Leff, 1983). While this parameter may not have biological meaning, its form is a necessary part of the application of the operational model. Likewise, the transduction coefficient R, the ratio of τ to K_A, was initially constructed to simultaneously account for both affinity and efficacy of a ligand (Kenakin and Miller, 2010). However, we see that this was also required mathematically to ensure that the model parameters were able to be estimated with reasonable precision. The issue of parameter estimation is only evident for highly efficacious ligands, where the value of τ is sufficiently large (say, 10) such that the model collapses to the ratio of τ to K_A (namely, R). In other words, with only functional response assay data (and no binding data), R is the minimal element that can be directly derived from the operational model.

The normalized transduction coefficient, $\Delta\ log\ (R)$, was initially designed to cancel out the system bias and observation bias in the *in vitro* assays (Kenakin *et al.*, 2012). Through our analysis, we have found that this metric could also cancel out the possible estimation mean error introduced by the mis-specification of fixed parameters. In other words, the value of $\Delta\ log\ (R)$ would be accurate even when E_m and K_A were unknown and their values fixed (hence mis-specified). This makes $\Delta\ log\ (R)$ an important, robust quantity in current practice. Therefore, there is no need to run a separate assay to determine an accurate estimate of E_m or K_A, when the aim of study is to quantify ligand bias based on $\Delta\Delta\ log\ (R)$. In so doing, our work helps alleviate the concern that may arise on fixing K_A to a non-physiological value (1 mol·L^{-1}), instead of plugging in realistic value estimated from binding assay (Rajagopal, 2013; Onaran *et al.*, 2014).

In this work, we propose a generalization of the accepted method III, whereby each experiment must include a full agonist which is used as an approximation to the system maximum (E_m). Compared to the accepted method, the only modification made in the proposed method (method IV) is to relax the requirement of a full agonist and select the ligand with the highest efficacy level among the tested ligands as if it were a full agonist (termed a 'pseudo full agonist'). Hence, method IV inherits most of the advantages and limitations of method III; only functional assay data are required, and the estimation of operational model parameters is inaccurate but tolerated due to the subsequent normalization. We also found that method IV provides more precise estimation results even in the absence of highly efficacious ligands. As efficacy profiling of ligands is not required, method IV (using the maximum effect from the entire range of ligands as an approximation to the system E_m)

therefore has the potential to be applied to large-scale screening to accelerate the discovery of novel lead compounds with desirable features of functional selectivity.

The choice of the various methods (I–IV) for estimating the parameters from the operational model largely depends on the aim of the work and the available data. If the aim is solely to calculate, $\Delta\ log\ (R)$ and/or $\Delta\Delta\ log\ (R)$ for the quantification of ligand bias, it is our recommendation that method IV be chosen. However, if an accurate estimate of E_m, K_A, R and τ is the target, then methods I or II would be more appropriate. In addition, with more information at hand (e.g. binding assay data and separate E_m information), it is possible to gain insights into the system *via* methods I or II, which is not available from methods III or IV where these values are compressed (Buchwald, 2017).

A limitation of the current study is that we did not consider biological constraints associated with different pathways. In this work, we consider a range of values of n to encompass values that are seen in empirical applications of the operational model. We do not attempt to provide a mechanistic interpretation of the value. Method II, in its original form (Rajagopal *et al.,* 2011), fixes the K_A from different pathways to the same empirical dissociation constant, determined from a binding assay under conditions that would limit the formation of a receptor ternary complex. However, the feasibility of this biological constraint is still under debate (Rajagopal, 2013; Kenakin and Christopoulos, 2013a). There is emerging evidence that a single value of K_A cannot describe data from a ligand that acts as the weak agonists in two pathways with very different EC_{50} values (Kenakin and Christopoulos, 2013a). Through the theoretical analysis from (cubic) ternary complex model, the binding affinity of agonist can be affected by allosteric modulation of effector (G protein) and different receptor states (Weiss *et al.,* 1996; Ehlert, 2008; Stein and Ehlert, 2015). This may explain the reason why a single binding affinity may be insufficient to describe a biological system. Therefore, in the evaluation of the methods in this work, we allowed the K_A values to be different in different pathways. In so doing, the performance of the current method II (without the potentially necessary biological constraint on K_A) should always be superior to its original version (which included the biological constraint).

It is noted that in Figure 2 that the 95% CI becomes narrower along the range of evaluated n values (i.e. yields more precise parameter estimation). Yet, in Figure 3A, there is a decrease in the successful estimation fraction with an increase in n, and Figure 3B shows wider 95% CI with the increment of n (i.e. less precise estimation). These directly opposite behaviours may be due to the difference of the ligand efficacy in two scenarios presented in the two figures. Figure 2 is a case of a highly efficacious ligand (large value of τ, τ is greater than 1) whereas Figure 3 considers low efficacious ligands (low value of τ, τ is less than 1). From the equation $\frac{E_{max}}{E_m} = \frac{\tau^n}{\tau^n+1}$, when τ is greater than 1, the increment of n would increase the value of $\frac{\tau^n}{\tau^n+1}$ (more efficacious), while, when τ is less than 1, the increment of n would decrease the value (less efficacious). Hence, it is speculated that for the same method, more efficacious ligands will have a narrower 95% CIs (i.e. more precise estimation).

Our work provides a formal assessment of the issues pertaining to identifiability and other mathematical observations that have been raised by others (Ehlert, 2005; Kenakin *et al.,* 2012; Rajagopal, 2013; Kenakin and Christopoulos, 2013a; van der Westhuizen *et al.,* 2014). For instance, it was noted that two different parameter sets were able to produce nearly identical curves in the general operational model (Kenakin *et al.,* 2012), which is a function of observability (rather than identifiability, *per se*). It has also been reported that separate estimation of τ or K_A values for full agonists from direct fitting of the operational model to a concentration–response curve was usually not possible (Rajagopal, 2013; van der Westhuizen *et al.,* 2014), which was explored here with deterministic identifiability. To our knowledge, the current study provides the first formal assessment of the identifiability of commonly used models in functional selectivity, illustrating the utility of identifiability analysis tools developed in the field of pharmacometrics (Bazzoli *et al.,* 2010; Shivva *et al.,* 2013). This recent trend in quantitative pharmacology sees the development of more mechanism-based models, which are inevitably more complicated in their interrelationships but are built from the building blocks provided by standard receptor theory (Wajima *et al.,* 2009; Peterson and Riggs, 2012; Benson *et al.,* 2014). It is therefore likely that the models explored here, based on the operational model, will form the basis of many other models within quantitative systems pharmacology, which require appropriate mathematical underpinning.

In conclusion, we have systematically assessed the identifiability of the operational model and its variants and demonstrated that current methods for quantifying ligand bias is tolerant to mis-specification of those parameters that are set to fixed constants. Furthermore, an objective method is proposed that relaxes the need for specific choice of ligands in any given experiment and provides accurate estimates of biased ligand profiles.

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Author contributions

X.Z. designed and performed experiments, analysed the data and wrote the paper. D.B.F. analysed the data and wrote the paper. M.G. designed experiments, analysed the data and wrote the paper. S.B.D. designed experiments, analysed the data and wrote the paper.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Appendix A

Theoretical assessment of the influence of the mis-specified E_m on the quantification of ligand bias

From Equation A1

$$E = Basal + \frac{(E_m - Basal)\cdot\tau\cdot A}{K_A + (\tau + 1)\cdot A} \quad \text{(A1)}$$

the location and asymptote are defined as follows:

$$Basal_{i_{obs}} = Basal \tag{A2}$$

$$E_{\max.i_{obs}} = Basal + \frac{(E_m - Basal)\cdot\tau_i}{\tau_i + 1} \tag{A3}$$

$$EC_{50.i_{obs}} = \frac{K_{Ai}}{\tau_i + 1} \tag{A4}$$

Here, *Basal* and E_m were the true system parameter values shared by all ligands, and τ_i and K_{Ai} were the true parameter values for ith ligand.

Since these constraints (i.e. Equations A2–A4) need to be met by all the workable parameter sets, the links between true parameter values and mis-specified parameter values were set-up as follows:

$$Basal^{'} = Basal \tag{A5}$$

$$Basal^{'} + \frac{\left(E_m^{'} - Basal^{'}\right)\cdot\tau_i^{'}}{\tau_i^{'} + 1} = Basal + \frac{(E_m - Basal)\cdot\tau_i}{\tau_i + 1} \tag{A6}$$

$$\frac{K_{A_i}^{'}}{\tau_i^{'} + 1} = \frac{K_{Ai}}{\tau_i + 1} \tag{A7}$$

Here, the prime symbol denotes the corresponding mis-specified parameter values.

The general form for the mis-specified value of E_m was defined as follows:

$$E_m^{'} = \alpha\cdot(E_m - Basal) + Basal \tag{A8}$$

Here, α is a scalar that quantifies the difference between true parameter value (E_m) and mis-specified parameter value ($E_m^{'}$). The value of α should be larger than the maximal value of $\frac{\tau_i}{\tau_i+1}$. Doing so ensures that $E_m^{'}$ is larger or equal to the maximal observed response among all the ligands.

Substituting Equations A5 and A8 into Equations A6–A7 for ligand one yielded the expressions for $\tau_1^{'}$ and $K_{A1}^{'}$:

$$\tau_1^{'} = \frac{\tau_1}{\alpha(\tau_1 + 1) - \tau_1} \tag{A9}$$

$$K_{A1}^{'} = \frac{\alpha K_{A1}}{\alpha(\tau_1 + 1) - \tau_1} \tag{A10}$$

By definition, R (transduction coefficient) is the ratio between τ and K_A. So $R_1^{'}$ was calculated as follows:

$$R_1^{'} = \frac{\tau_1^{'}}{K_{A1}^{'}} = \frac{\frac{\tau_1}{\alpha(\tau_1+1)-\tau_1}}{\frac{\alpha K_{A1}}{\alpha(\tau_1+1)-\tau_1}} = \frac{\tau_1}{\alpha K_{A1}} = \frac{R_1}{\alpha} \tag{A11}$$

Similarly, $R_2^{'}$ was derived as follows:

$$R_2^{'} = \frac{R_2}{\alpha} \tag{A12}$$

Finally, $\Delta\ log\ (R^{'})$ was derived by taking the difference between two ligands:

$$\begin{aligned}\Delta\ \log\left(R^{'}\right) &= \log\left(R_1^{'}\right) - \log\left(R_2^{'}\right) = \log\left(\frac{R_1}{\alpha}\right) - \log\left(\frac{R_2}{\alpha}\right)\\ &= \log(R_1) - \log(R_2) = \Delta\ \log(R)\end{aligned} \tag{A13}$$

Through the derivation above, mis-specified value of E_m would lead to inaccurate estimation of R, while the estimation of $\Delta\ log\ (R)$ could still be accurate when the slope factor was equal to unity. The estimation mean error caused by the mis-specification of E_m has been fully cancelled out in this situation. In other words, the estimation of $\Delta\ log\ (R)$ was robust to the mis-specification of E_m.

Appendix B

Theoretical assessment of the influence of the mis-specified K_A on the quantification of ligand bias

In a two ligand system, choosing the ligand with higher efficacy as the reference ligand and fixing its equilibrium dissociation constant to an arbitrary large value yielded Equation A14:

$$K_{A1}^{'} = \beta\cdot K_{A1} \tag{A14}$$

Here, β was the scaling factor quantifying the difference between true parameter value (K_{A1}) and mis-specified parameter value ($K_{A1}^{'}$). The value of β should be larger than 1.

Substituting Equation A5 and A14 into Equations A6–A7 and solving the equation set for ligand one yields the expressions for other parameters:

$$\tau_1^{'} = \beta\cdot(\tau_1 + 1) - 1 \tag{A15}$$

$$E_m^{'} = \gamma\cdot(E_m - Basal) + Basal \tag{A16}$$

Here, γ was equal to $\frac{\beta\tau_1}{\beta\cdot(\tau_1+1)-1}$.

By definition, R (transduction coefficient) was the ratio between τ and K_A. So $R_1^{'}$ was calculated as follows:

$$R_1^{'} = \frac{\tau_1^{'}}{K_{A1}^{'}} = \frac{\beta\cdot(\tau_1 + 1) - 1}{\beta\cdot K_{A1}} = \frac{\beta\cdot(\tau_1 + 1) - 1}{\beta\cdot\tau_1}\cdot\frac{\tau_1}{K_{A1}} = \frac{R_1}{\gamma} \tag{A17}$$

At the same time, $Basal^{'}$ and $E_m^{'}$ were system parameters shared between two ligands. Substituting them into

Equations A6 and A7 for ligand two yielded the expressions for $\tau_2^{'}$ and $K_{A2}^{'}$:

$$K_{A2}^{'} = \frac{\gamma \cdot K_{A2}}{\gamma \cdot (\tau_2 + 1) - \tau_2} \tag{A18}$$

$$\tau_2^{'} = \frac{\tau_2}{\gamma \cdot (\tau_2 + 1) - \tau_2} \tag{A19}$$

Similarly, $R_2^{'}$ was derived:

$$R_2^{'} = \frac{\tau_2^{'}}{K_{A2}^{'}} = \frac{\frac{\tau_2}{\gamma(\tau_2+1)-\tau_2}}{\frac{\gamma K_{A2}}{\gamma(\tau_2+1)-\tau_2}} = \frac{\tau_2}{\gamma K_{A2}} = \frac{R_2}{\gamma} \tag{A20}$$

Combining Equations A17 and A20 yielded the expression for $\Delta\ log\ (R^{'})$:

$$\begin{aligned} \Delta\ \log\left(R^{'}\right) &= \log\left(R_1^{'}\right) - \log\left(R_2^{'}\right) = \log\left(\frac{R_1}{\gamma}\right) - \log\left(\frac{R_2}{\gamma}\right) \\ &= \log(R_1) - \log(R_2) = \Delta\ \log(R) \end{aligned} \tag{A21}$$

Through the derivation above, it was clearly shown that a mis-specified value of K_A could lead to inaccurate estimation of R, while the estimation of $\Delta\ log\ (R)$ was still accurate when the slope factor was equal to unity. The estimation mean error caused by the mis-specification of K_A has been completely cancelled out *via* normalization process.

Appendix C

Theoretical assessment of the feasibility of method IV

Method IV could be considered a natural generalization of method III. The requirement of full agonist was relaxed into the ligand with highest efficacy among all the ligands in the study.

The general operational model has only one unidentifiable parameter, that is, fixing either E_m or K_A will render the model structurally identifiable. Therefore, with mis-specified K_A, the estimated values of other parameters have to change accordingly in line with the constraints from observed features of the concentration response curve (e.g. E_{max} and EC_{50}).

The location parameter EC_{50} of the general operational model was derived as follows:

$$EC_{50} = \frac{K_A}{(2 + \tau^n)^{\frac{1}{n}} - 1} \tag{A22}$$

Similarly, this constraint should also be applied to the case that K_A was fixed to 1 mol·L^{-1}, as shown in Equation A23.

$$EC_{50} = \frac{1}{(2 + \tau^{'n})^{\frac{1}{n}} - 1} \tag{A23}$$

Transforming Equation A23 yielded the expression for the apparent value of τ (denoted here as $\tau^{'}$) when K_A was mis-specified to 1 mol·L^{-1}:

$$\tau^{'} = \sqrt[n]{\left(\frac{1}{EC_{50}} + 1\right)^n - 2} \tag{A24}$$

As the normal value of EC_{50} was much smaller than 1 mol·L^{-1} (10^{-4}~10^{-12} mol·L^{-1}), the apparent value ($\tau^{'}$) appeared to be much larger than 1. In other words, setting K_A to 1 ensured the apparently high efficacy of the ligand. In method IV, fixing K_A to 1 mol·L^{-1} was not the result from a full agonist, but rather a 'pseudo full agonist' was created by fixing K_A to 1 mol·L^{-1} for the highest efficacy agonist in the set of compared agonists. Furthermore, as noted in the previous deterministic identifiability analysis, the large apparent value of τ (i.e. highly efficacious ligand) could also substantially improve the estimation precision of parameter R.

Method IV could also be considered as the generalization of method I. The asymptote parameter E_{max} of the general operational model was derived as follows:

$$E_{max} = \frac{E_m \cdot \tau^n}{1 + \tau^n} \tag{A25}$$

Similarly, this constraint should also be applied to the pseudo full agonist, as shown in Equation A26.

$$E_{max} = \frac{E_m^{'} \cdot \tau^{'n}}{1 + \tau^{'n}} \tag{A26}$$

Reorganizing Equations A26 yields A27:

$$E_m^{'} = \frac{E_{max} \cdot \left(1 + \tau^{'n}\right)}{\tau^{'n}} \tag{A27}$$

As demonstrated before, when K_A was assigned to 1-mol·L^{-1}, the apparent value of τ ($\tau^{'}$) was much larger than 1. Therefore, $\frac{E_{max} \cdot \left(1+\tau^{'n}\right)}{\tau^{'n}}$ could be reduced to E_{max}, that is, E_m was implicitly fixed to the E_{max} value of the pseudo full agonist in method IV, rather than the system maximal response.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1 log|M$_F$| *vs*. log residual variance for operational model. A) all parameters estimated from operational model (Eq. 1) with data from functional assay; B) reduced operational model (Eq. 5) with data from functional assay. |M$_F$|: the determinant of the Fisher Information Matrix. The criteria for claiming identifiability of a model: (i) log|M$_F$|

should have a continuous linear log–log relationship with the log of the random noise and (ii) $|M_F|$ should approach infinity as residual variance approaches zero.

Figure S2 The relative standard error of estimated parameters *vs*. τ for Eq. 8. In this study, a generic study design with sampling concentrations *log* (*A*) from −13 to −4, increment of 1, was adopted. A proportional measurement error with 10% coefficient of variation were assumed. K_A was set to an arbitrary value, 10^{-8} mol L^{-1}. *R* was defined as the ratio of τ and K_A. The deterministic identifiability was assessed for each value of, ranging from 0.1 to 100. The left panel is for K_A and the right panel is for *R*. The red dashed line indicates the 50% relative standard error, considered as the threshold for precise estimation.

Figure S3 $\log|M_F|$ *vs*. log residual variance for general operational model (Eq. 2). A) all parameters estimated (E_m, *Basal*, *n*, *log* (K_A), *log* (*R*)); B) E_m fixed; C) *log* (K_A) fixed; D) *log* (*R*) fixed.

Figure S4 Concentration–response curves for cAMP formation showing pplss-3HA-hCB1 HEK signalling, on stimulation with 2.5 μM FSK and a panel of six agonists, following >16 h pretreatment in the presence of PTX. The data is fitted by Method IV.

Table S1 The estimation of transduction coefficient (log (*R*)) *via* Method IV.