Quantification of receptor binding from response data obtained at different receptor levels: a simple individual sigmoid fitting and a unified SABRE approach

Peter Buchwald

Methods that allow quantification of receptor binding (occupancy) by measuring response (effect) data only are of interest as they can be used to allow characterization of binding properties (e.g., dissociation constant, $K_d$) without having to perform explicit ligand binding experiments that require different setups (e.g., use of labeled ligands). However, since response depends not just on the binding affinity-determined receptor occupancy, but also on receptor activation, which is affected by ligand efficacy (plus constitutive activity, if present), and downstream pathway amplification, this requires the acquisition and fitting of multiple concentration-response data. Here, two alternative methods, which both are straightforward to implement using nonlinear regression software, are described to fit such multiple responses measured at different receptor levels that can be obtained, for example, by partial irreversible receptor inactivation (i.e., Furchgott method) or different expression levels. One is a simple method via straightforward fitting of each response with sigmoid functions and estimation of $K_d$ from the obtained $E_{\text{max}}$ and $EC_{50}$ values as $K_d = (E_{\text{max}} - EC_{50})/EC_{50}$. This is less error-prone than the original Furchgott method of double-reciprocal fit and simpler than alternatives that require concentration interpolations, thus, should allow more widespread use of this so-far underutilized approach to estimate binding properties. Relative efficacies can then be compared using $E_{\text{max}} \cdot K_d/EC_{50}$ values. The other is a complex method that uses the SABRE receptor model to obtain a unified fit of the multiple concentration-response curves with a single set of parameters that include binding affinity $K_d$, efficacy $\varepsilon$, amplification $\gamma$, and Hill coefficient $n$. Illustrations with simulated and experimental data are presented including with activity data of three muscarinic agonists measured in rabbit myocardium.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| BCM | Benzylcholine mustard |
| BM5 | N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)acetamide |
| GPCR | G-protein coupled receptor |
| SABRE | Present model (with parameters for Signal Amplification, Binding affinity, and Receptor activation Efficacy) |
| SSE | Sum of squared errors |

Quantitative models linking ligand concentrations, $[L]$, to generated effects, $E$, are fundamental in pharmacology. Not only are they essential in establishing concentration–response relationships, which are the ultimate proof of efficacy and/or safety, but they can also provide valuable insight into the mechanisms of action of ligands and their receptors. Widely used quantitative pharmacology parameters such as the half-maximal effective or inhibitory concentration ($EC_{50}$, $IC_{50}$) are routinely derived from fitting with sigmoidal equations such as the Hill equation (Eq. 1) or its simplified form ($n = 1$, Clark equation) corresponding to a straightforward law of mass action (see Table 1 for a summary of all notations and parameters used):

Department of Molecular and Cellular Pharmacology and Diabetes Research Institute, Miller School of Medicine, University of Miami, Miami, FL 33136, USA. email: pbuchwald@med.miami.edu
Table 1. Summary of notation and parameters used.

| Symbol | Description |
|--------|-------------|
| $E$    | Effect (measured response) |
| $f^*$  | Effect (response) after partial irreversible receptor inactivation (or obtained at lower receptor expression level) (with apostrophe used to denote such values in general) |
| $E_{\text{max}}$ | Maximum effect (overall maximum response achievable) |
| $[L]$  | Ligand concentration (with square brackets […] used to denote concentration in general) |
| $[L]^*$ | Concentration that produces the same effect after partial receptor inactivation (or at lower expression level) as $[L]$ originally |
| $[R_{\text{tot}}]$ | Total (maximum) receptor concentration |
| $[R_{\text{tot}}]^*$ | Total (maximum) receptor concentration remaining after partial receptor inactivation (or achieved at lower expression level) |
| $n$    | Hill coefficient (Hill slope; including as parameter in SABRE)—characterizes the steepness of the response) |
| $f_{\text{resp}}$ | Fractional response (i.e., response as a fraction of the overall maximum: $E/E_{\text{max}}$; Eq. 1) |
| $f_{\text{occup}}$ | Fractional occupancy (i.e., fraction of ligand occupied receptors, $f_{\text{occup}} = [LR_{\text{occup}}]/[LR_{\text{max}}]$) |
| $E_{\text{C}50}$ | Half-maximal effective concentration (i.e., ligand concentration that produces 50% of the maximal possible effect of that agonist)—common measure of potency |
| $K_d$  | Equilibrium dissociation constant (including as parameter in SABRE)—common measure of binding affinity |
| $q$    | Fraction of total receptors remaining following partial receptor inactivation (or obtained at lower expression level) (i.e., $q = [R_{\text{tot}}] / [R_{\text{tot}}]$) |
| $r_{\text{A}}$ | Intrinsic relative activity (Eq. 16) |
| $g$    | Efficacy (parameter in SABRE; fraction of ligand-bound receptors that are active, Eq. (7) in ref. 1) |
| $f_{\text{RAi}}$ | Baseline receptor efficacy (parameter in SABRE; fraction of ligand-free receptors that are active, Eq. 8 in ref. 1)—characterizing constitutive activity |
| $\gamma$ | Amplification (gain) factor (parameter in SABRE; see Eq. (11) in ref. 1) |

\[
\frac{E}{E_{\text{max}}} = f_{\text{resp}} = \frac{[L]^n}{[L]^n + EC_{50}^n} \quad (1)
\]

While such functional forms often provide good fit, they may not reveal much about the signaling mechanism or even binding to the receptor because the overall response ($E$, $E_{\text{max}}$) depends not just on receptor occupancy (i.e., the number or fraction of receptors that are ligand-bound), but also on receptor activation (i.e., the number of fraction of receptors that are active either due to the presence of a ligand or to constitutive activity) and on possible downstream pathway amplification, which sometimes can be quite considerable. Thus, the parameter obtained from fitting Eq. (1) as corresponding to half-maximal effect ($EC_{50}$, $K_d$) is not necessarily closely related to the classic equilibrium dissociation constant $K_d$ that corresponds to half-maximal occupancy and determines (fractional) receptor occupancy via a similar sigmoid function:

\[
f_{\text{occup}} = \frac{[L]^n}{[L]^n + K_d^n} \quad (2)
\]

In other words, while both fractional response, $f_{\text{resp}} = E/E_{\text{max}}$, and fractional occupancy, $f_{\text{occup}} = [LR_{\text{occup}}]/[LR_{\text{max}}]$, are typically well described by such equations, they do not overlap and there could be considerable separation between them. Well-known causes of such separations include, for example, • partial agonism (where response lags behind occupancy and full occupancy does not result in full response), • signal amplification (where response runs ahead of occupancy and low occupancy can produce full or close to full response creating the appearance of “receptor reserve” or “spare receptors”), • different responses assessed at different downstream vantage points along a signaling pathway (where the same occupancy produces different responses at different readout points), and • biased agonism (where the same occupancy produces different responses along distinct divergent downstream pathways originating from the same receptor)—see reference 1 for representative illustrations.

Hence, the exact relationship between $[L]$ and $E$ (even expressed as normalized fractional response $f_{\text{resp}} = E/E_{\text{max}}$) is complex, and simultaneous fitting of occupancy and response functions requires models with multiple parameters. For example, our recently introduced SABRE quantitative receptor model provides a framework to fit a variety of response data from the simplest to those of increasing complexity with a unified equation using a total of five parameters in its most general form: three characterizing the ligand–receptor interaction (binding affinity, $K_d$, efficacy, $\varepsilon$, and Hill coefficient, $n$) and two the receptor and its signaling pathway (constitutive activity, $f_{\text{RAi}}$, and amplification, $\gamma$) (Fig. 1; Eq. (17) in Methods and Models). In case of no constitutive activity ($f_{\text{RAi}} = 0$), this reduces to the form that will be used here (Eq. 18 in Methods and Models), which can also be rearranged to resemble that of Eq. (1) above:

\[
\frac{E}{E_{\text{max}}} = \frac{\varepsilon \gamma [L]^n}{(\varepsilon \gamma - \varepsilon + 1)[L]^n + K_d^n} = \frac{\varepsilon \gamma [L]^n}{(\varepsilon \gamma - \varepsilon + 1)[L]^n + \frac{K_d^n}{\varepsilon \gamma - \varepsilon + 1}} \quad (3)
\]
Thus, within the framework of SABRE, if there is no constitutive activity, half-maximal response (EC50) is observed at

\[ K_{\text{obs}} = \frac{K_d}{(\varepsilon \gamma - \varepsilon + 1)^{n-1}} \]  

(4)

highlighting that, indeed, \( K_{\text{obs}} \) depends not just on receptor binding (i.e., occupancy determined by \( K_d \)), but also on ligand efficacy (\( \varepsilon \)) and signal amplification (\( \gamma \)), not to mention cooperativity (\( n \)). It has also been shown that within the framework of SABRE (assuming \( \varepsilon = 0 \) and \( n = 1 \)), fractional response and occupancy are connected via the following function

\[ f_{\text{resp}} = \frac{\varepsilon \gamma f_{\text{occup}}}{\varepsilon (\gamma - 1) f_{\text{occup}} + 1} = \frac{\gamma}{\gamma - 1} f_{\text{occup}} + \frac{1}{\gamma (\gamma - 1)} \]  

(5)

which can even be used for direct fitting if both (fractional) response and occupancy data are available (see ref.1).

Considering these, methods that allow quantification of receptor binding (occupancy) from measurements of response (effect) data alone are of interest, as they can be used to characterize binding properties (e.g., \( K_d \)) without having to perform explicit ligand binding experiments that typically require very different setups than response assays (e.g., use of labeled ligands or displacement of known labeled ligands). One so far underutilized possibility to achieve binding quantification this way is to acquire multiple concentration–response curves at different receptor levels, which can be obtained, for example, after different partial irreversible receptor inactivation (i.e., classic Furchgott method)4,5 or, more recently, by using different expression levels, and then fitting these response curves to obtain \( K_d \) estimates. The method of irreversible receptor inactivation introduced by Furchgott4,5 is particularly useful exactly because it allows the simultaneous estimation of affinity and efficacy when concentration–response functions can be established from the same preparation before and after partial irreversible receptor inactivation. It has been used since the late 1960s, albeit in a relatively limited number of works (e.g., ~50 in PubMed). It was applied in several cases with various ligand series typically for G-protein coupled receptors (GPCRs), such as muscarinic5–8, opioid9–11, dopamine12, 5-hydroxytryptamine (5-HT)13,14, and
A₁-adenosine\textsuperscript{15,16} receptors. An interesting variation that is now possible is to obtain responses not after different levels of partial inactivation, but with cells having different levels of receptor expression. A recent example of such data has been obtained with M₁ and M₄ muscarinic receptors and three ligands (carbachol, oxotremorine, and pilocarpine) at five different levels of expression in CHO cells\textsuperscript{17}. Thus, in these experiments binding affinity (K\textsubscript{d}) estimates can be obtained from response only assays with the limitation being that it has to be confirmed that the process used to obtain different receptor levels does not significantly alter other properties (e.g., binding or downstream signaling) that might also affect the measured response.

Some type of fitting procedure is then needed to obtain the K\textsubscript{d} estimates. Here, two alternative fitting methods are described to achieve this. One is a simple method via straightforward fitting of each response with sigmoid functions and estimation of K\textsubscript{d} from the obtained E\textsubscript{max} and EC\textsubscript{50} values for each pair of control versus treated comparison. This is less error-prone than the original method of double-reciprocal fit proposed by Furchgott for his "null method" (see Eq. 7)\textsuperscript{6,7} and is simpler than alternatives that require concentration interpolations, such as those proposed by Parker and Waud (see Eq. 9)\textsuperscript{18}. As it is very straightforward to implement using nonlinear regression software, which is now widely available, it is hoped that it will allow more widespread use of this so far underutilized approach to estimate binding properties (i.e., K\textsubscript{d}) or relative efficacies. The other is a more complex method that uses the above-mentioned SABRE model\textsuperscript{1} to obtain a unified fit of the multiple concentration–response curves with a single set of parameters, one of which is K\textsubscript{d}. If sufficient data are available to allow reliable estimate of all parameters, SABRE can allow estimates obtained from all data points, which could be particularly advantageous if multiple concentration–response curves are available. For illustration, fit of simulated and experimental data are included with both models including for complex cases of multiple compounds of differing efficacies assessed at multiple receptor levels.

Methods and models

Implementation and data fitting. All data fittings were performed in GraphPad Prism (GraphPad, La Jolla, CA, USA, RRID:SCR_002798) including those with the SABRE model as described before\textsuperscript{1,3}. Data used for fitting were normalized and considered as having no baseline (i.e., in the 0–100% range); parameters constrained to specific values are indicated for each fitting separately. Simulated data were generated with the same model in Prism using the "Simulate XY data" algorithm with 5% random error). Experimental data used for illustrations of model fit in Fig. 3 were reproduced from reference 18; those used for Fig. 5 were obtained from Ehlert\textsuperscript{19}.

Models for data fitting. Fit using double reciprocals and linear regression (Furchgott’s “null method”). Following Furchgott, this is a method relying on comparing equal responses before and after treatment based on the hypothesis that equal stimuli should produce equal responses irrespective of the actual shape of the transduction function linking the stimulus S to the generated response (effect) \( \tilde{E} \). If one assumes that inactivation reduces the number of total receptors to a \( q \) fraction of the original, \( [R_{\text{tot}}]' = q[R_{\text{tot}}] \), while response continues to be generated via the same transduction function, the concentrations [L] and [L]' that produce the same effect pre- and post-inhibition must create the same stimulus S (using the original terminology of Furchgott). Hence, if \( S \) is proportional with the concentration of occupied receptors [LR], \( S = \epsilon[L][LR] \), and the concentration of occupied receptors [LR] is connected to that of the ligand [L] via the standard hyperbolic function (law of mass action), equal pre- and post-inactivation responses imply that

\[
\frac{[R_{\text{tot}}]}{[L] + K_d} = q[R_{\text{tot}}] \frac{[L]'}{[L] + K_d}
\]

This can be rearranged into the linear relationship between the reciprocals of equiactive concentrations that is the basis of the original Furchgott method introduced in the late 1960s\textsuperscript{6,20}:

\[
\frac{1}{[L]} = \frac{1 - q}{qK_d} + \frac{1}{q[L]}
\]

The slope and intercept of this line allow the simultaneous determination of \( q \) (fraction of receptor inactivated) and K\textsubscript{d} (receptor affinity). It was considered an important advantage that this Furchgott analysis is a null method approach that makes no assumption regarding the nature of the transduction function other than it stays the same after partial inactivation of R\textsubscript{tot}. It has, however, several implicit assumptions\textsuperscript{19} including that response is produced via only one transduction system (i.e., no poly-pharmacology) and that receptor occupation follows a classic law of mass action (i.e., hyperbolic function as shown in Eq. 2 with no cooperativity, Hill slope \( n = 1 \)). One of its important drawbacks is that it relies on linear regression using a double-reciprocal plot, and, hence, just as the Lineweaver-Burke plot, it is prone to errors typical for such plots: it tends to amplify the errors of the measurements at low concentrations (where 1/[L] is large), as those points will weigh more heavily in the corresponding linear regression, so that the very worst data are emphasized the most, as the smallest measured numbers have the largest influence on the regression due to the use of reciprocal values\textsuperscript{21}. Another important drawback is that, except for rare lucky coincidences, actual measurements at equiactive concentrations are not available, so that some interpolation method has to be used to estimate at least one of the concentration values producing the same response.

Fit using hyperbolic functions and interpolated concentrations (Parker-Waud). Following the suggestion of Parker and Waud in the early 1970s, an often used approach to overcome the problems of this double-reciprocal method was to (1) fit the control (untreated) data with a standard hyperbolic function, (2) use this to obtain
the needed interpolated \([L]\) concentrations, (3) fit those using another nonlinear regression for the equation connecting \([L]\) and \([L']\), and then (4) use the parameters of this fitting to estimate \(q\) and \(K_d\). Specifically, first a nonlinear regression is used to fit the pre-inactivation control data with a classic sigmoid (Hill) function similar to Eq. (1)

\[
E = E_{\text{max}} \frac{[L]^n}{[L]^n + EC_{50}^n}
\]  

(8)

Next, this is used to interpolate the \([L]\) concentrations that would produce the same responses pre-inactivation as those measured post-inactivation at the \([L']\) values tested, and then another nonlinear regression is used to fit the corresponding \([L]\) and \([L']\) pairs to the hyperbolic equation connecting them from which \(q\) and \(K_d\) can be obtained (essentially, the reverse of Eq. 7):

\[
[L] = qK_d \frac{[L']}{1 - q} \frac{[L']}{[L] + \frac{1}{K_d}}
\]  

(9)

These make fitting a cumbersome and multi-step process—quite possibly a main reason behind the relatively rare use of this otherwise very informative approach to estimate \(K_d\) from response data alone. A nested version of this, where Eq. (9) is used to replace \([L]\) in Eq. (8) and fit \(E'\) as a function of \(L'\) in parallel with fitting \(E\) to \([L]\) using Eq. (8), was proposed by James and co-workers in the late 1980s.

Fit using individual sigmoid functions (present simple method). To overcome these cumbersome fittings, here, first a simple alternative is proposed with fitting that can be easily done with software tools for nonlinear regression that are now widely available and used to directly obtain estimates for \(K_d\) and \(q\). One possible alternative to avoid the use of double-reciprocal regression and the need for concentration interpolations is to simply fit both sets of activity data with standard hyperbolic curves (i.e., sigmoid on semi-log scale) and use the parameters from this to obtain \(q\) and \(K_d\). This was avoided in the original approach to not make any assumptions on the shape of the transducer function. However, hyperbolic functions are good representations of typical concentration–response functions, and because of the extrapolations needed to obtain concentrations producing equipo-

\[
\text{Vol.:(0123456789)}
\]

...
compounds were assayed in the same system, relative efficacies can also be estimated once \( K_c \) estimates are available by comparing fractional occupancies \( f_{\text{occup}} \) (calculated from these \( K_c \) values) that cause the same (fractional) effect. A simple formula for estimating relative efficacies using the \( E_{\text{max}} \), \( EC_{50} \), and \( K_c \) values obtained via the sigmoid fitting is (see derivation assuming sigmoid response and occupancy functions in Supporting Information, Appendix 1 and with SABRE’s formalism below using Eq. 26):

\[
\frac{\varepsilon_2}{\varepsilon_1} = \frac{E_{\text{max},2}/EC_{50,2}}{E_{\text{max},1}/EC_{50,1}}
\]  
\( (15) \)

While this method of fitting sigmoid functions appears to abandon the apparent elegance of the “null method” of making no assumptions regarding the actual shape of the transduction function, in practice, it makes little difference as sigmoid (or some other similar) functions had to be used anyway for the needed data interpolations. Note also that \( q \) as estimated with this method is the same as the ratio of equiactive molar ratios (EAMR; i.e., ratio of molar concentrations that produce the same response at low enough concentrations), later termed intrinsic relative activity \((RA_i)\) introduced by Ehrlert and co-workers, which is the ratio of \( E_{\text{max}}/EC_{50} \) values for a Hill slope of 1.2,29.

\[
RA_i = \frac{E_{\text{max},1}/EC_{50,1}}{E_{\text{max},ref}/EC_{50,ref}}
\]  
\( (16) \)

This became the basis of bias quantification methods that commonly rely on calculating the shift \( A_\Delta \text{log}(E_{\text{max}}/EC_{50}) \) which is estimated as \( A_\Delta \text{log}(\tau/K_c) \) if the operational model is used, versus a selected reference compound, e.g., a logarithmic bias factor is obtained as \((\log(E_{\text{max},\text{P1,1}}/EC_{50,\text{P1,1}})) − \log(E_{\text{max},\text{P2,1}}/EC_{50,\text{P2,1}}))/(\log(E_{\text{max},\text{P1,ref}}/EC_{50,\text{P1,ref}})) − \log(E_{\text{max},\text{P2,ref}}/EC_{50,\text{P2,ref}}))\). In fact, using \( E_{\text{max}}, K_c/EC_{50} \) from Eq. (15) derived here as a measure to compare relative activities or efficacies makes more sense than using \( E_{\text{max}}/EC_{50} \) from Eq. (16), which are being used as intrinsic relative activities \((RA_i \text{ or EAMR}) \), as the former is a dimensionless quantity (%), while the latter is not (mol\(^{-1}\)). When used for the same compound (e.g., comparing activities at different receptor levels, i.e., Furchgott method, or for different diverging downstream pathways, i.e., biased agonism), the comparison in Eq. (15) reduces to the same as Eq. (16), as \( K_c \) are the same and are eliminated from the ratio.

Fit using SABRE (present, unified method). A more complex approach is also described that uses the recently introduced multiparametric SABRE receptor model to achieve a single unified fit of the multiple concentration–response curves and, hence, a \( K_c \) estimate based on all data available. The SABRE quantitative receptor model in its most general form uses a total of five parameters: three characterizing the interaction of the ligand with the receptor (binding affinity, \( K_c \), efficacy, \( \varepsilon \), and Hill coefficient, \( n \)) and two characterizing the receptor signaling pathway (amplification, \( y \), and constitutive activity, \( \varepsilon_{\text{const}} \)), (Fig. 1):3–5

\[
E_i/E_{\text{max}} = \frac{\varepsilon y[L]^n + \varepsilon_{\text{const}} y K_d^n}{\varepsilon y y^{-1} + (\varepsilon - \varepsilon_{\text{const}})[L]^n + (\varepsilon_{\text{const}} y - \varepsilon_{\text{const}} + 1)K_d^n}
\]  
\( (17) \)

With the incorporation of separate parameters for signal amplification and (intrinsic) efficacy, SABRE can fit concentration–response functions even for complex connections between fractional response \( f_{\text{occup}} \) and occupancy \( f_{\text{occup}} \). An important advantage compared to other complex receptor models, such as those based on the operational (Black and Leff) model,30 is that SABRE’s equations can be collapsed into functional simplifications by fixing the parameters as special values (Fig. 1), and these can and should be used on their own when adequate. Furthermore, SABRE can accommodate experimental \( K_c \) data and, hence, connect experimentally determined fractional occupancy and fractional response data, which is not possible within the framework of the original operational model. Note also that the operational model is not well suited to fit Furchgott type data; it could not provide independent estimates of agonist affinity and efficacy.31

As discussed before, the simplest form of SABRE that still provides adequate fit should be used to avoid over-parametrization, and selection of the model to be used should also be guided by the consideration that reliable fitting and well-defined parameter values can only be achieved if there are at least 5–10 (well-distributed) data points for each adjustable parameter.32–34. As data here do not involve constitutive activity \((\varepsilon_{\text{const}} = 0) \) but still allows a Hill-type extension was used for all fittings (Fig. 1, second from top; also Eq. 3):

\[
E_i/E_{\text{max}} = \frac{\varepsilon y y^{-1} + (\varepsilon - \varepsilon_{\text{const}})[L]^n + K_d^n}{\varepsilon y y^{-1} + (\varepsilon_{\text{const}} y - \varepsilon_{\text{const}} + 1)K_d^n}
\]  
\( (18) \)

As shown before, SABRE can be used for the quantitative modeling of responses measured after partial irreversible receptor inactivation.1 The loss of total receptors due to irreversible inhibition, \([R_i] = q[R_{\text{tot}}] \), leads to a corresponding loss in the concentration of active receptors \([LR^*] \) responsible for generating the response and, hence, a loss in response. Assuming that the inactivation does not affect the post-receptor signal amplification function, the fractional response after inactivation in the formalism of SABRE becomes:

\[
E_i/E_{\text{max}} = \frac{q y y^{-1} + q \varepsilon_{\text{const}} y [L] + K_d}{[q y y^{-1} + q \varepsilon_{\text{const}} + 1]y [L] + K_d}
\]  
\( (19) \)
Thus, a q-fold decrease in $R_{\text{at}}$ translates into an apparent q-fold reduction of efficacy as used in SABRE: $\varepsilon' = q\varepsilon$. Two illustrations for the use of SABRE with this formalism of "fractional efficacy" $\varepsilon'$ and unified amplification ($\gamma$) and $K_d$ to fit data from Furchgott type experiments have been provided in ref.2: one with a series of compounds acting at the dopamine receptor, which was inactivated by the irreversible antagonist N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)12, and one with the muscarinic agonists carbachol and oxotremorine and receptor inactivation with phenoxbenzamine (PHB)35. Further examples with simulated and experimental data are presented below including with muscarinic agonist activities measured in rabbit myocardium after different levels of inactivation caused by an irreversible muscarinic antagonist (BCM).

The framework provided by SABRE can also be used to confirm Eqs. (13–15) obtained for the fitting of individual sigmoid functions above. Using the rearranged form of SABRE (shown in Eq. 3)

$$E/E_{\text{max}} = \frac{\varepsilon \gamma}{(\varepsilon \gamma - \varepsilon + 1)} \frac{[L]^n}{[L]^n + K_d^n} e^{ \frac{\varepsilon \gamma - 1}{\varepsilon \gamma - \varepsilon + 1} }$$  \hspace{1cm} (20)

it is clear that half-maximal response (EC50, $K_{\text{obs}}$) is observed at

$$K_{\text{obs}} = \frac{K_d}{(\varepsilon \gamma - \varepsilon + 1)^n}$$  \hspace{1cm} (21)

and the maximum fractional effect is

$$f_{\text{resp,max}} = \frac{\varepsilon \gamma}{(\varepsilon \gamma - \varepsilon + 1)}$$  \hspace{1cm} (22)

Thus, the term of interest for ratio as intrinsic relative activity (equiactive molar ratio; Eq. 16), which also corresponds to the "transduction coefficient" $\delta/K_d$ of the operational model, is $\varepsilon \gamma/K_d$ in SABRE assuming $n = 1$ (see Appendix 2 of ref.1):  \hspace{1cm} (23)

$$\frac{E_{\text{max}}}{E_{\text{max}}^{50}} = \frac{\varepsilon \gamma}{(\varepsilon \gamma - \varepsilon + 1)} \frac{K_d}{K_d} = \frac{\varepsilon \gamma}{K_d}$$

Thus, fitting of two individual sigmoid curves and using Eq. (13) indeed reproduces q in the formalism of SABRE too with the modified efficacy, $\varepsilon' = q\varepsilon$:

$$\frac{E_{\text{max}}}{E_{\text{max}}^{50}} = \frac{\varepsilon \gamma}{K_d}$$  \hspace{1cm} (24)

Similarly, Eq. (14) reproduces $K_d$:

\begin{equation}
\frac{E_{\text{max}} - E_{\text{max}}^{50}}{E_{\text{max}}^{50} - E_{\text{max}}^{50}} = \frac{\varepsilon \gamma}{(\varepsilon \gamma - \varepsilon + 1)} \left( \frac{K_d}{K_d} \right) - \frac{q \varepsilon \gamma}{(q \varepsilon \gamma - q \varepsilon + 1)} = \frac{q \varepsilon \gamma}{(q \varepsilon \gamma - q \varepsilon + 1)}
\end{equation}

$$= \varepsilon \gamma K_d - K_d$$

If multiple compounds are assayed in the same system, Eq. (23) can be used to obtain an estimate of relative efficacies for two compounds ($\varepsilon_1/\varepsilon_2$) even with the sigmoid fitting approach once $K_d$ have been calculated from Eq. (14). Since pathways and hence $y$s are the same, the ratio of two efficacies (i.e., relative efficacy versus a reference compound) as expressed from Eq. (23)

$$\varepsilon \gamma = \frac{K_d}{E_{\text{max}}^{50}}$$

reproduces exactly the one shown in Eq. (15) earlier.

Results and discussion

Illustration 0: fit of response data with sigmoid function and SABRE. First, fitting of response only data including partial agonists is shown to illustrate the parallel between use of classic sigmoid functions (i.e., Eq. 1) and that of SABRE (Eq. 18) with its simplified form that also assumes no amplification ($\gamma = 1$), hence, has only three adjustable parameters, $K_d$, $\varepsilon$, and $n$:

$$E/E_{\text{max}} = \frac{\varepsilon [L]^n}{[L]^n + K_d^n}$$  \hspace{1cm} (27)

Simulated data generated for three different hypothetical agonists (with a 5% random error) was used as shown in Fig. 2. Fitting with SABRE produced the very same parameters as fitting with classic sigmoid functions (i.e., "log(agonist) versus response—Variable slope (four parameters)" in GraphPad Prism) with the same constrains (Bottom = 0, HillSlope shared values for all datasets) and nicely reproduced the log $K_d$ and maximum fractional response $f_{\text{resp,max}}$ values used to generate the data: $-7.998 \pm 0.017$, $-6.211 \pm 0.023$, and $-7.564 \pm 0.056$ for the log $K_d$ values used of $-8.000$, $-6.200$, and $-7.500$, and $0.989 \pm 0.007$, $0.765 \pm 0.008$, $0.308 \pm 0.007$ for the
maximum responses used of 100%, 75%, and 30% (Supplementary information, Table S1). For further generalizability, data corresponding to a Hill slope of $n = 2$ were used here, and fitting was done by constraining $n$ to the same value for all compounds resulting in a fitted value of 1.828 ± 0.090.

Hence, if adequately constrained ($\varepsilon_R0 = 0$, $\gamma = 1$), SABRE can perform the same role as the widely used Hill or Clark ($n = 1$) equation and can be used to derive straightforward EC50 ($K_{\text{obs}}$) values. However, it can do much more than that if its other parameters are released and can be adequately fitted. For example, it can fit the same type of response data, but with integration of occupancy ($K_d$) data obtained from a different, independent assay in the same system. In other words, it can connect fractional response, $f_{\text{resp}}$, and fractional occupancy, $f_{\text{occup}}$, data even in complex cases where the fractional response can be either ahead or behind the fractional receptor occupancy.

Fit with SABRE can be considered consistent if adequate fit can be obtained with well-defined log $K_d$ values (that are consistent with their experimental values if those are available) and a single amplification parameter $\gamma$ characterizing the system (pathway) as a whole. Examples for this have been shown before for simulated1 as well as experimental data including among others imidazoline type $\alpha$-adrenoceptor agonists data36 often used as textbook illustration of mismatch between $f_{\text{resp}}$ and $f_{\text{occup}}$. Contrary to SABRE, the classic two-parameter ($\tau$, $K_D$) operational model29,30 can neither be simplified back to the Hill or Clark equation by constraining its $\tau$ parameter nor used to connect the response to independently determined binding data ($K_D$)1,3. A “special edition” extension of the operational model “with given $K_D$ values” introduced by Rajagopal and coworkers for bias quantification can use experimental $K_D$ values as its $K_D$s (instead of fitted $K_D$s) to constrain the regression25,27, but this required introduction of an additional scaling factor $\alpha$ making it a three- and not a two-parameter model (see ref.1 for details and correspondence between the parameters of these models).

**Illustration 1: guinea pig ileum treated with dibenamine.** A first illustration of fit of receptor inactivation data (Fig. 3) is provided with the relatively simple experimental data obtained with guinea pig ileum and used by Parker and Waud to introduce their fitting method for concentration-responses following partial irreversible receptor inactivation18. Because of the limited number of data points available, fits were done with the Hill slopes fixed as unity ($n = 1$), especially since even if released, the fitted values for $n$ were not significantly different from 1. The two fits here (individual sigmoid curves and SABRE) produced very similar results, i.e., completely overlapping curves in Fig. 3 and predicted fraction of receptor inactivated $q = 0.40$ and receptor affinity log $K_d = −4.11$ for both, which are in good agreement with the original estimates obtained by Parker and Waud (Supplementary information, Table S2).

**Illustration 2: complex simulated receptor inactivation data.** An example with simulated data generated to illustrate that full agonists can become partial ones following receptor inactivation with significant change in apparent and even relative efficacies is shown in Fig. 4. The three different responses (corresponding to no treatment, Trt_0, and two inactivation treatments, Trt_1 and Trt_2) obtained for each of the three compounds (color coded as blue, red, and green for compounds 1, 2, and 3, respectively) are shown as a function of log concentration individually and in

![Figure 2. Fit of response only data for full and partial agonists with SABRE using its form shown in Eq. (20) that allows non-unity Hill slope. Simulated data (symbols) for three different compounds were generated in GraphPad Prism as described in the Methods (“Simulate XY data” algorithm with 5% random error) and then fitted with SABRE (lines) using the following constrains: $\varepsilon_R0 = 0$, $\gamma = 1$, $n$ shared. Fit with classic sigmoid functions, “log(agonist) versus response—Variable slope (four parameters)” with Bottom = 0 and HillSlope shared as constrains, produces the exact same fit (Supplementary information, Table S1) with overlapping lines. Dashed lines show fit if only unity Hill slopes are allowed; data here correspond to a Hill slope of $n = 2$ (note the wide range of data included that covers nine orders of magnitude).](https://www.nature.com/scientificreports/)

\[ E_i / E_{\text{max}} = \frac{\varepsilon_i}{(L^n + \varepsilon_{R0}K_{d,i})^{1 / n}} \]

parameters: $n$, $\varepsilon_{R0}$, $K_{d,i}$ $i = 1..3$
combination in Fig. 4. Unified fit with SABRE nicely reproduced the amplification ($\gamma = 2000$), receptor inactivation ($q_1 = 0.030$, $q_2 = 0.001$), binding affinity ($\log K_d$: $-5.0$, $-7.0$, and $-5.0$), and ligand efficacy ($\epsilon$: $1.00$, $0.02$, and $0.30$) values used to generate the data (Table S3B). For example, the estimates obtained from fitting were $-5.031 \pm 0.043$, $-6.949 \pm 0.031$, and $-4.955 \pm 0.044$ for the log $K_d$ values used to generate the data of $-5.000$, $-7.000$, and $-5.000$. Fit with individual sigmoids also produced acceptable estimates (one for each inactivation treatment, which can be averaged for overall estimates; Table S3A). Note, however, that on such multiple inactivation data, SABRE provides more reliable estimates overall because of the unified fit of all data, if it can provide one. Here, individual fit with sigmoids could not obtain sufficiently reliable estimates for $K_{d,Cpd1}$ from treatment 1 data (Cpd1Trt_1) as the two maxima were very similar, i.e., log $K_d$ estimate of $-4.589$ versus the actual $-5.000$, and for $K_{d,Cpd2}$ from treatment 2 data (Cpd2Trt_2) where the responses were too small for reliable fitting, i.e., log $K_d$ estimate of $-7.578$ versus the actual $-7.000$. These values that correspond to $K_d$ values that are three-fourfold off, as well as the $q_j$ value derived from the data of Cpd2 that is also about fourfold off, are shown in italic in Table S3A. For the same reason, the relative efficacy estimates calculated using Eq. (15) are also almost two-fold.
off compared to the values used to generate the simulated data, unless they are calculated omitting these values (Table S3A; italic versus non-italic values in row denoted with asterisk).

Illustration 3: muscarinic activity in rabbit myocardium. A further detailed illustration with experimental data is included using the muscarinic receptor-mediated inhibition of adenylate cyclase activity measured in rabbit hearts (Fig. 5). Activity of three muscarinic agonists, namely oxotremorine-M, oxotremorine, and BM5 (N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)acetamide), was determined in perfused rabbit myocardium homogenates in the absence and presence of the irreversible muscarinic antagonist benzilcholine mustard (BCM; 0, 1.0, and 10.0 nM, 15 min)\textsuperscript{19}. Unified fit with SABRE of 81 data points from all 9 response data sets (3 compounds assessed at 9 concentrations each at 3 different pretreatments; 3 × 9 × 3 = 81) using only 10 parameters (3 × $K_d$, 3 × $\varepsilon$, $\gamma$, $n$, $q_1$, $q_2$; Table S4B) resulted in good overall fit (Fig. 5) accounting for 99.3% of the variability in the data ($r^2 = 0.993$) with an amplification of $\gamma = 7.3 \pm 1.6$ (Table S4B). Here, individual fit with sigmoids (Table S4A) also produces good fit, in fact, slightly better one ($r^2 = 0.994$; SSE 474.1 vs. 550.0 with SABRE); however, it requires a total of 19 parameters (2, $K_d$ and $E_{\text{max}}$, for each of the 3 compounds in 3 treatments plus $n$; i.e., $2 \times 3 \times 3 + 1 = 19$). According to the fit with SABRE, BCM caused fractional inhibitions ($q$) of 0.406 and 0.065 for the 1.0 and 10.0 nM pre-treatments, respectively (to be compared with estimates of 0.380 and 0.020 obtained in the original publication using analysis of equiactive agonist concentrations\textsuperscript{19}). Fit with sigmoids produced averaged $q$ estimates of 0.406 and 0.065, similar to those from SABRE; however, the three different estimates from the three compounds for $q_2$ were a bit different covering an approximate two-fold range from 0.051 to 0.092 (Table S4A). For the three compounds, log $K_d$ estimates from SABRE came out as $-5.27$, $-5.85$, and $-6.31$ for oxotremorine-M, oxotremorine, and BM5, respectively (corresponding to $K_d$ of 5.3, 1.4, and 0.5 μM) in nice agreement with the values measured by competition assays (4.0, 1.1, and 0.2 μM) as well as those estimated from fitting (7.5, 2.2, and 0.2 μM) in the original work\textsuperscript{19}. Efficacy ($\varepsilon$) estimates from SABRE were 1.0, 0.75, and 0.13—in reasonable agreement with the original estimates of 1.0, 0.52, and 0.04 obtained by Ehlert using equiactive concentrations\textsuperscript{19}. Relative efficacies estimated using the sigmoid approach for fitting and then Eq. (15) were also similar to those from SABRE being 0.64 for oxotremorine and 0.12 for BM5 compared to oxotremorine-M as reference (Supplementary information, Table S4A).

Conclusion
In conclusion, multiple response only data measured at different receptor levels can be used to quantify receptor binding without having to perform ligand binding/displacement experiments; however, careful fitting is required to separate binding affinity from efficacy (i.e., partial agonism) and signal amplification (“receptor reserve”). Here, two fitting methods were described that are both straightforward to use with widely available nonlinear regression software. One is a simple method that relies on fitting of individual sigmoid functions to provide $K_d$ estimates from the obtained $E_{\text{max}}$ and $E_{\text{50}}$ values. Since it requires no interpolation and needs only easily obtainable parameter estimates, e.g., $K_d = (E_{\text{max}} - E_{\text{50}} - E_{\text{max}} - E_{\text{50}})/E_{\text{max}} - E_{\text{50}}$, it is hoped that it will allow more widespread use of this so far underutilized approach to estimate binding affinities. The other method uses the SABRE model to obtain a unified fit of the multiple concentration–response curves with a single set

Figure 5. Fit of experimental responses obtained for three muscarinic agonists (oxotremorine-M, oxotremorine, and BM5) measured before and after partial irreversible receptor inactivation with BCM (0, 1.0, and 10.0 nM, 15 min)\textsuperscript{19}. Unified fit of all 81 data points done with SABRE with 10 parameters (3 × $K_d$, 3 × $\varepsilon$, $\gamma$, $n$, $q_1$, $q_2$; Table S4B) is shown in color-coded thick lines. For comparison, fit with individual sigmoids is also included (thin lines; 19 parameters: 3 × 3 × $K_d$, 3 × 3 × $E_{\text{max}}$; n; Table S4A).
of parameters. This could be particularly advantageous if multiple concentration–response curves are available and a consistent fit can be obtained, as it provides binding affinity, $K_a$, as well as efficacy, $e$, and amplification $γ$, parameters derived based on the entire set of data.

Data availability
Data used for illustrations of model fit are either simulated data generated as described or reproduced from previous publications as indicated in the corresponding figures. The datasets generated and/or analyzed are available from the corresponding author upon reasonable requests.

Received: 23 August 2022; Accepted: 2 November 2022
Published online: 06 November 2022

References
1. Buchwald, P. A single unified model for fitting simple to complex receptor response data. Sci. Rep. 10, 13386. https://doi.org/10.1038/s41598-020-70220-w (2020).
2. Buchwald, P. A three-parameter two-state model of receptor function that incorporates affinity, efficacy, and signal amplification. Pharmacol. Res. Perspect. 5, e00311. https://doi.org/10.1002/prp2.311 (2017).
3. Buchwald, J. P. A receptor model with binding affinity, activation efficacy, and signal amplification parameters for complex fractional response versus occupancy data. Front. Pharmacol. 10, 605. https://doi.org/10.3389/fphar.2019.00605 (2019).
4. Furchgott, R. F. The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. Adv. Drug Res. 3, 21–55 (1966).
5. Furchgott, R. F. & Bursztyn, P. Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann. N. Y. Acad. Sci. 144, 882–889. https://doi.org/10.1111/j.1749-6632.1967.tb5817x.1 (1967).
6. Harden, T. K., Heng, M. M. & Brown, J. H. Receptor reserve in the calcium-dependent cyclic AMP response of astrocytoma cells to muscarinic receptor stimulation: Demonstration by agonist-induced desensitization, receptor inactivation, and phorbol ester treatment. Mol. Pharmacol. 30, 200–206 (1986).
7. Eglen, R. M. & Whiting, R. L. Estimation of apparent agonist affinity constants using desensitization of the ileal muscarinic receptor. J. Pharmacol. Exp. Ther. 240, 404–409 (1987).
8. Porter, A. C. et al. M1 muscarinic receptor signaling in mouse hippocampus and cortex. Brain Res. 944, 82–89. https://doi.org/10.1016/0006-8993(02)02721-x (2002).
9. Chavkin, C. & Goldstein, A. Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. Proc. Natl. Acad. Sci. U.S.A. 81, 7253–7257. https://doi.org/10.1073/pnas.81.22.7253 (1984).
10. Adams, J. U., Paronis, C. A. & Holtzman, S. G. Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using parameters derived based on the entire set of data.
11. Fox, P. D. & Hentges, S. T. Differential desensitization observed at multiple effectors of somatic mu opioid receptors underlies the postinactivation concentration-response curve to a nested hyperbolic equation. J. Pharmacol. Exp. Ther. 255, 1027–1032 (1990).
12. Fox, P. D. & Hentges, S. T. Differential desensitization observed at multiple effectors of somatic mu opioid receptors underlies sustained agonist-mediated inhibition of proopiomelanocortin neuron activity. J. Neurosci. 37, 8667–8677. https://doi.org/10.1523/JNEUROSCI.1030-17.2017 (2017).
13. Meller, E., Bohmker, K., Namba, Y., Friedhoff, A. J. & Goldstein, M. Relationship between receptor occupancy and response at striatal dopamine autoreceptors. Mol. Pharmacol. 31, 592–598 (1987).
14. Meller, E., Goldstein, M. & Bohmker, K. Receptor reserve for 5-hydroxytryptamine1A-mediated inhibition of serotonin synthesis: Possible relationship to anxiolytic properties of 5-hydroxytryptamine1A agonists. Mol. Pharmacol. 37, 231–237 (1990).
15. Dennis, D., Jacobson, K. & Belardinelli, L. Evidence of spare A1-adenosine receptors in guinea pig atrioventricular node. Am. J. Physiol. 262, H661–H671. https://doi.org/10.1152/ajpheart.1992.262.3.H661 (1992).
16. Morey, T. E., Paronis, C. A. & Holtzman, S. G. Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using parameters derived based on the entire set of data.
17. Jakub, J. et al. Applications and limitations of fitting of the operational model to determine relative efficacies of agonists. Sci. Rep. 9, 4637. https://doi.org/10.1038/s41598-019-40993-w (2019).
18. Parker, R. B. & Waud, D. R. Pharmacological estimation of drug-receptor dissociation constants. Statistical evaluation. I. Agonists. J. Pharmacol. Exp. Ther. 177, 1–12 (1971).
19. Ehler, F. J. Coupling of muscarinic receptors to adenylate cyclase in the rabbit myocardium: Effects of receptor inactivation. J. Pharmacol. Exp. Ther. 240, 23–30 (1987).
20. Jenkinson, D. H. Textbook of Receptor Pharmacology 3–78 (CRC Press, Florida, 2010).
21. Ochs, R. S. The problem with double reciprocal plots. Curr. Enz. Inhib. 6, 164–169. https://doi.org/10.2174/157340810793384098 (2010).
22. James, M. K., Morgan, P. H. & Leighton, H. J. A new method for estimation of agonist dissociation constants (KA): Directly fitting the postinactivation concentration-response curve to a nested hyperbolic equation. J. Pharmacol. Exp. Ther. 249, 61–69 (1989).
23. Ehler, F. J., Griffin, M. T. & Gildzen, P. F. The interaction of the enantiomers of acodrine with subtypes of the muscarinic receptor. J. Pharmacol. Exp. Ther. 279, 1335–1344 (1996).
24. Griffin, M. T., Figueroa, K. W., Lilier, S. & Ehler, F. J. Estimation of agonist activity at G protein-coupled receptors: Analysis of M2 muscarinic receptor signaling through Gi/o, Gs, and G13. J. Pharmacol. Exp. Ther. 321, 1193–1207. https://doi.org/10.1124/jpet.107.120857 (2007).
25. Önaran, H. O. et al. Systematic errors in detecting biased agonism: Analysis of current methods and development of a new-model-free approach. Sci. Rep. 7, 44247. https://doi.org/10.1038/srep44247 (2017).
26. Michel, M. C. & Charlton, S. J. Biased agonism in drug discovery - is it too soon to choose a path?. Mol. Pharmacol. 93, 259–265. https://doi.org/10.1124/mol.117.110890 (2018).
27. Raigopal, S. et al. Quantifying ligand bias at seven-transmembrane receptors. Mol. Pharmacol. 80, 367–377. https://doi.org/10.1124/mol.111.072801 (2011).
28. Kolb, P. et al. Community guidelines for GPCR ligand bias: IUPHAR review 32. Br. J. Pharmacol. 179, 3651–3674. https://doi.org/10.1111/bph.15811 (2022).
29. Black, J. W. & Leff, J. Operational models of pharmacological agonism. Proc. R. Soc. Lond. B Biol. Sci. 220, 141–162. https://doi.org/10.1098/rspb.1983.0093 (1983).
30. Kenakin, T. Receptor theory. Curr. Protoc. Pharmacol. 41, 1.2.1–1.2.28. https://doi.org/10.1002/0471141755.ph0102s41 (2008).
31. Van der Graaf, P. H. & Stam, W. B. Analysis of receptor inactivation experiments with the operational model of agonism yields correlated estimates of agonist affinity and efficacy. J. Pharmacol. Toxicol. Methods 41, 117–125. https://doi.org/10.1016/S1056-8719(99)00029-5 (1999).
32. Knofczynski, G. T. & Mundfrom, D. Sample sizes when using multiple linear regression for prediction. *Ed. Psychol. Measur.* **68**, 431–442. https://doi.org/10.1177/0013164407310131 (2008).
33. Austin, P. C. & Steyerberg, E. W. The number of subjects per variable required in linear regression analyses. *J. Clin. Epidemiol.* **68**, 627–636. https://doi.org/10.1016/j.jclinepi.2014.12.014 (2015).
34. Kenakin, T. P. *A Pharmacology Primer: Techniques for More Effective and Strategic Drug Discovery* 5th edn. (Academic Press, New York, 2018).
35. Kenakin, T. P. *Pharmacological Analysis of Drug-Receptor Interactions* 2nd edn. (Raven Press, New York, 1993).
36. Ruffolo, R. R. Jr., Rosing, E. L. & Waddell, J. E. Receptor interactions of imidazolines. I. Affinity and efficacy for alpha adrenergic receptors in rat aorta. *J. Pharmacol. Exp. Ther.* **209**, 429–436 (1979).
37. Ritter, J. M. *et al.* *Rang and Dale’s Pharmacology* 9th edn. (Elsevier, Amsterdam, 2020).

**Acknowledgements**
The author would like to thank Fred Ehlert for providing the original experimental data obtained for the muscarinic agonists used for illustrations here and for his insightful comments and input on an early version of this manuscript.

**Author contributions**
P.B. is the sole author; he originated the project, performed the calculations and data fittings, and wrote the manuscript.

**Competing interest**
The author declares no competing interests.

**Additional information**
*Supplementary Information* The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-23588-w.

**Correspondence** and requests for materials should be addressed to P.B.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022