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Z-analysis: A new approach to analyze stimulation curves with intrinsic basal stimulation

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Abstract

In the study of receptor biology it is of considerable importance to describe the stimulatory properties of an agonist according to mathematically defined models. However, the presently used models are insufficient if the experimental preparation contains an intrinsic basal stimulation. We have developed a novel approach, tentatively named Z-analysis. In this approach, the concentration of endogenous agonist is calculated by extending the stimulation curve to zero effect. The concentration of endogenous agonist is then combined with the concentration of added agonist to estimate the true EC_{50} value. We developed a new model, the Z-model, specifically for this purpose, but in addition, we describe how Z-analysis can be applied to the traditional E_0-model. Models were applied to computer-generated curves with different Hill coefficients, using iterative curve fitting procedures. In addition to applying the models to ideal cases, we also used Monte Carlo-simulated data. Specific transformations were used to enable comparisons between parameters determined from these models. Both models were able to provide estimates of all eight parameters analyzed, both using ideal data and on Monte Carlo-simulated data. The Z-model was found to provide better estimates of the concentration of endogenous agonist, the EC_{50} values, and the Hill value, in curves with Hill coefficient deviating from one. In conclusion, Z-analysis was suitable both to determine the concentration of endogenous agonists and to determine true EC_{50} values. We found several advantages with the Z-model compared to traditional E_0-model for analysis of stimulation curves that contain basic intrinsic stimulation.

Keywords: Receptor; Curve analysis; Stimulation; Agonist concentration; EC_{50}; Hill coefficient

1. Introduction

In the study of receptor biology it is of considerable importance to describe the stimulatory properties of an agonist according to mathematically defined models. The ability to accurately do so applies to many fields of biomedical science. In general physiology it is important to determine the properties of naturally occurring receptor ligands, such as neurotransmitters and hormones. In basic pharmacological research different receptors and their subtypes are often characterized based on the properties of a combination of agonistic and antagonistic ligands [1]. Accuracy in the methods used becomes even more critical in clinical diagnosis and pharmaceutical development of new drugs intended for use by humans.

The traditional model used to analyze stimulation curves is:

$$E = \frac{E_{max}}{(EC_{50}/A)^H + 1} \quad (1)$$

where E is the effect, E_{max} is the maximal effect, EC_{50} is the agonist concentration inducing 50% of maximal effect, A is the concentration of added agonist, and H is the Hill coefficient. The Hill coefficient has a value of 1 if the sites mediating the stimulatory effect are uniform in their properties and lack cooperative interactions. If the sites are not uniform, or if there is negative cooperativety between sites, the Hill coefficient becomes <1. Conversely, the Hill coefficient becomes >1 if there is positive cooperativety between stimulatory sites.

In many cases this basic model gives sufficiently appropriate estimates of the parameters. However, the model is insufficient if the experimental preparation contains an

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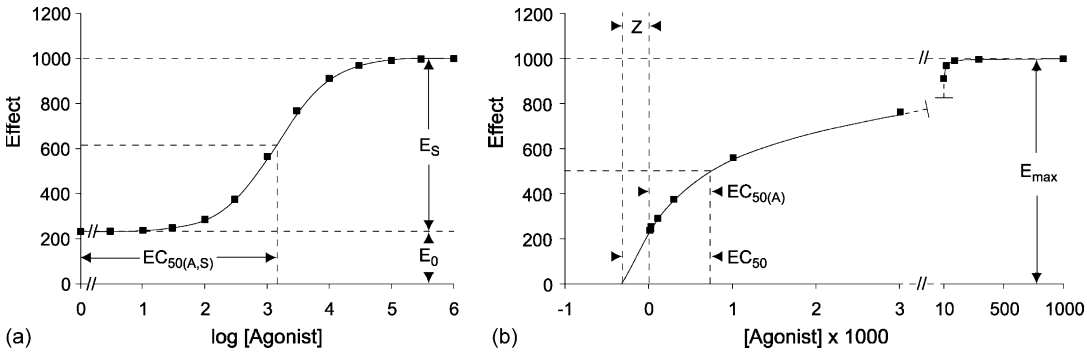

Fig. 1. Definitions of parameters used in the present study. Agonist concentrations are displayed on a logarithmic scale to show parameters determined with the E_0-model (a), or on linear scale to show parameters determined with the Z-model (b). In addition, Hill values were determined by both models. Data points were generated according to the Z-model, here at Hill = 1.

intrinsic basal stimulation. Traditionally, the solution has been to adjust for the basal effect by simply subtracting it [2]. The modified model, here called the E_0-model, then becomes:

$$E = \frac{E_S}{(EC_{50(A,S)}/A)^H + 1} + E_0 \quad (2)$$

where E is the effect, A is the concentration of added agonist, E_S is the effect induced by A, E_0 is the basal effect, i.e., effect without added agonist, and $EC_{50(A,S)}$ is the concentration of A that causes a 50% stimulation above E_0, i.e. A at $E_0 + E_S/2$ (Fig. 1). This model is adequate in many cases when the intrinsic basal effect is negligible and constant. However, in many preparations both in vivo and in vitro the intrinsic basal stimulation is large and/or variable. In vivo, the added agonist may inevitably compete with endogenous agonists, the concentration of which may differ between subjects or between different target regions within each subject. In vitro, there may be residual endogenous agonist that interferes with subsequent agonist stimulation. The addition of competitive antagonists can be used to demonstrate the degree of a basal effect that is due to intrinsic agonist stimulation.

There are ways to attempt to reduce the impact of endogenous stimulation, such as for example increased washing of membrane preparations. This may, however, not always be feasible. In certain cases, extensive washing may damage the preparation, and in other cases, low amounts of endogenous agonists are constantly leaking from tissue stores. In vivo, it is usually very difficult to reduce the concentrations of endogenous agonist.

During conditions where there is a clear intrinsic basal stimulation, the parameter $EC_{50(A,S)}$ may deviate substantially from the true EC_{50} value, which often is a primary value of interest. Furthermore, such deviations will result in problems when comparing stimulation curves where the basal stimulation varies, e.g. between different experimental subjects (e.g. whole animals, tissues, cell populations, or subcellular preparations) or between different regions within a subject.

One way to eliminate this problem is to directly measure the concentration of the endogenous agonist and to add this concentration to that of the added agonist [3]. However, in many cases this approach is not possible.

To overcome these problems we have developed a novel approach that we have tentatively named Z-analysis. In this approach, the concentration of endogenous agonist is calculated by extending the stimulation curve to zero effect. The concentration of endogenous agonist is then combined with the concentration of added agonist to estimate the true EC_{50} value. Hence, Z-analysis can be used both to determine the concentration of endogenous agonists and to determine true EC_{50} values. The analysis is useful when the added and endogenous agonists are identical, or when their relative potencies are known. We have developed a new model, the Z-model, specifically for this purpose, but in addition, we describe how Z-analysis can be applied to the traditional E_0-model. We found several advantages with the Z-model compared to traditional E_0-model for analysis of stimulation curves that contain basic intrinsic stimulation.

2. Methods

2.1. Generation of ideal curve data

We generated a set of stimulation curve data points that included a basal effect in the absence of added agonist. The data was generated with the assumptions that the basal effect was entirely due to stimulation by endogenous agonist, and that the properties of the endogenous and exogenous agonists were identical. Ideal data were generated according to the Z-model, a novel formula based on (1):

$$E = \frac{E_{max}}{(EC_{50}/(A+Z))^H + 1} \quad (3)$$

where E is the effect (rounded to four significant figures), E_{max} is the maximal effect, EC_{50} is the concentration of $(A + Z)$ that cause a 50% stimulation of E_{max}, A is the

concentration of added agonist, Z is the concentration of endogenous agonist, and H is the Hill coefficient (see Fig. 1). Hence, Z is equivalent to $-$A at $E = 0$). We chose the following parameter values: $E_{max} = 1000$, $A = 1, 3, 10 \ldots -1{,}000{,}000$ (13 data points), $Z = 300$, $EC_{50} = 1000$, and $H = 0.5$, 1 (Fig. 1), or 2.

2.2. Generation of pseudo-experimental data

Using the parameters and values for A described above, pseudo-experimental data were generated using Monte Carlo-simulation where a random error is added to each data point using an inverse normal distribution [4]. A standard deviation of 5 was selected based on observed variability in experimental situations [3]. In total, data for 500 curves at each of the three Hill values were generated.

2.3. Stimulation curve models

Two models were used to produce stimulation curve parameters: the novel Z-analysis model, which is identical to the formula used to derive ideal data (3), and the traditional E_0-analysis model (2). To be able to calculate the parameters Z and EC_{50} with the E_0-model, the H value was required to be fixed at a value of 1.

2.4. Curve fitting algorithms

Data were analyzed with iterative non-linear regression procedures using an enhanced in-house version of the program EasyBound, a custom-developed program which was designed to handle automated analysis of large amounts of data [5]. The curve fitting utilized the Gauss–Newton algorithm as modified by Levenberg [6] and Marquardt [7].

2.5. Initial estimates

Initial estimates for the iterative curve fitting were derived from linear regression of transformed data [8] using the standard formula:

$$E_{linear} = mA + b$$

where

$$m = \frac{n(\sum xy) - \sum x \sum y}{n(\sum x^2) - (\sum x)^2}$$

$$b = \frac{\sum x^2 \sum y - \sum x \sum xy}{n \sum x^2 - (\sum x)^2}$$

n: number of data points

$$x = \log_{10}(A + 0.001)$$

$$y = \frac{\log_{10}((E - 0.999 \times \min(E)/(1.001 \times \max(E) - E))}{\log_{10}(A + 0.001)}$$

For the Z-model:

$$Z = \frac{\min(E)}{\max(E)/2} \times 10^{b/m}$$

$$E_{max} = \max(E)$$

$$EC_{50} = 10^{b/m}$$

$$H = m$$

For the E_0-model:

$$E_0 = \min(E)$$

$$E_S = \max(E)$$

$$EC_{50(A,S)} = 10^{b/m}$$

$$H = m$$

Analyses were performed with or without a weighting factor of $1/E^2$ [9,10].

2.6. Derivations used to allow comparisons between models

To be able be perform direct comparisons between the parameters determined by Z-analysis and E_0-analysis additional parameters were defined and derived. For Z-analysis:

$$E_0 = \frac{E_{max}}{(EC_{50}/Z)^H + 1} \quad (4)$$

Note that Eq. (4) only is valid for $Z > 0$. At $Z = 0$, $E_0 = 0$.

$$E_S = E_{max} - E_0 \quad (5)$$

$$E_0 = \frac{EC_{50}}{H\sqrt{(E_{max}/(E_S/2 + E_0))^H - 1}} - Z \quad (6)$$

$$EC_{50(A)} = EC_{50} - Z \quad (7)$$

($EC_{50(A)}$ is defined as A at $E = E_{max}/2$)

For E_0-analysis, the following parameters were derived:

$$E_{max} = E_S + E_0 \quad (8)$$

$$Z = -A = \frac{EC_{50(A,S)}}{E_S/E_0 + 1} \quad (9)$$

Note that Eq. (9) only is valid if $H = 1$, since transformation of the Hill exponent will result in a negative root expression that cannot be solved. Hence, Z and EC_{50} can only be determined when H is fixed at the value of 1.

$$EC_{50(A,S)} = \frac{EC_{50(A,S)}}{H\sqrt{(E_S/(E_{max}/2 - E_0))^H - 1}} \quad (10)$$

Note that Eq. (10) is valid only for $E_0 < E_{max}/2$. At $E_0 = E_{max}/2$, $EC_{50(A)} = 0$. If H is fixed to 1, Eq. (10) is valid also at $E_0 > E_{max}/2$.

$$EC_{50} = Z + EC_{50(A)} \quad (11)$$

Table 1
Parameters derived from curve analyses of defined data with Hill values of 0.5, 1, and 2 (top, middle, and bottom panels, respectively), using the *Z*-model and the E_0-model

	H	*Z*	$EC_{50(A)}$	EC_{50}	$EC_{50(A,S)}$	E_0	E_S	E_{max}
Defined data	0.500	300	700	1000	4091	354	646	1000
Z-model	0.500	300	700	1000	4091	354	646	1000
E_0-model	0.668[a]	*1373*[a]	660	*2033*[a]	3743	337	644	981
Defined data	1.000	300	700	1000	1300	231	769	1000
Z-model	1.000	300	700	1000	1300	231	769	1000
E_0-model	1.000	*326*	700	*1026*	1300	231	769	1000
Defined data	2.000	300	700	1000	786	83	917	1000
Z-model	2.000	300	700	1000	786	83	917	1000
E_0-model	1.355[a]	*58*[a]	734	*792*[a]	839	85	935	1019

Values in italics are calculated with Hill fixed at a value of 1.
[a] Deviations >10% from the defined original curves.

2.7. Data analysis

For the analysis of ideal curves, determined parameters deviating >10% from the ideal curve parameters were defined as significant deviations.

For the analysis of parameters determined from Monte Carlo-simulated data, geometric mean and ±1 S.D. range of the geometric mean values were calculated on logarithmically transformed data, since this may lead to a more normal distribution [4]. Parameters where the ±1 S.D. range of the geometric mean value were outside the predefined curve parameters were defined as significant deviations.

3. Results

3.1. General observations

Analysis of curves with $H = 0.5$, 1, or 2 showed that the *Z*-analysis approach was feasible using both models. This

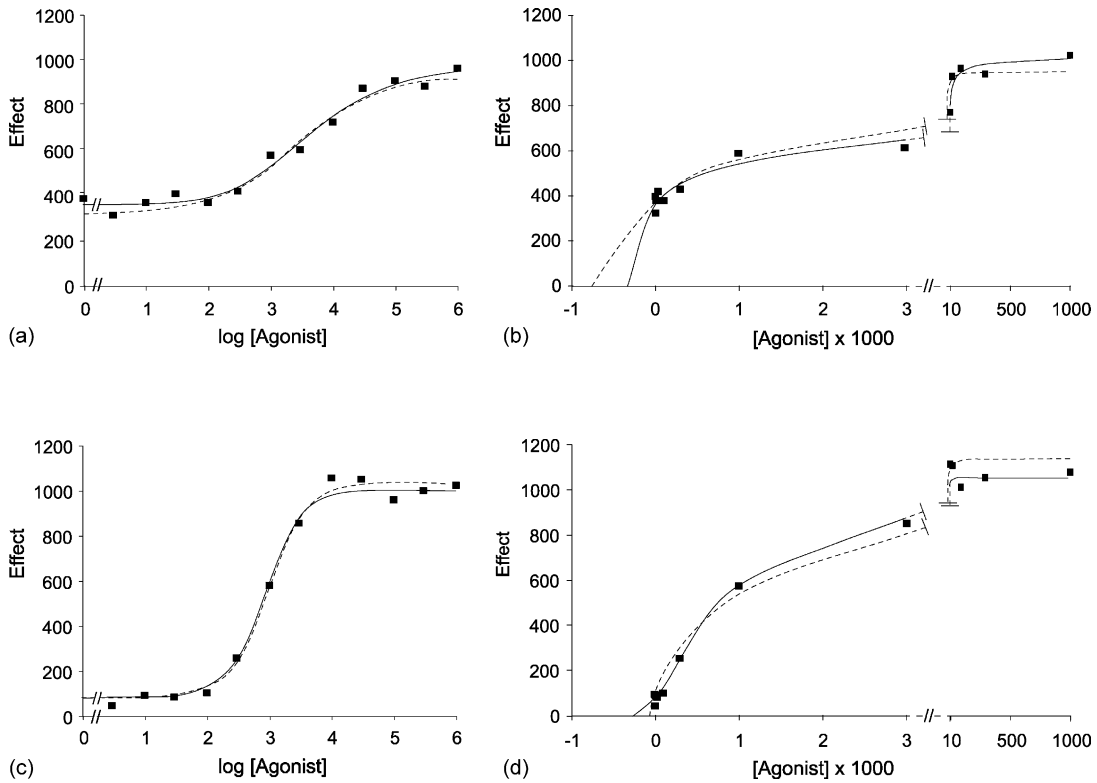

Fig. 2. Graphs show representative Monte Carlo-simulated data points and curves fitted with the *Z*-model (solid line) and the E_0-model (hatched line). Data points (see Table 2 for defined parameter values) were generated according to the *Z*-model using Hill values of 0.5 (a, b) and 2 (c, d), displayed on a logarithmic scale (a, c) or on linear scale (b, d). Note that the E-model in (b, d) uses a fixed Hill value, which is required to enable determination of *Z*-values and hence true EC_{50} values. The Hill values determined by the *Z*-model and E_0-model were 0.50 and 0.65 (a), and 1.78 and 1.39 (c). The *Z* and EC_{50} values as determined by the *Z*-model were 327 and 949 (b), and 260 and 1046 (d). The corresponding values as determined by E_0-model with Hill fixed at a value of 1 were 740 and 1166 (b) and 73 and 890 (d).

Table 2
Parameters derived from curve analyses of Monte Carlo-simulated data points based on defined data (shown) with Hill values of 0.5, 1, and 2 (top, middle, and bottom panels, respectively), using the E_0-model and the *Z*-model

	H	*Z*	$EC_{50\ (A)}$	EC_{50}	$EC_{50\ (A,S)}$	E_0	E_S	E_{max}
Defined data	0.500	300	700	1000	4091	354	646	1000
Z-model	0.528	273	818	1327	4949	350	666	1021
	(0.319–0.875)	(70–1066)	(381–1755)	(637–2761)	(2038–12017)	(321–380)	(562–789)	(919–1134)
E_0-model	0.685	*767*	367	*1170*	2125	318	616	941
	(0.527–0.891)*	(*506–1162*)*	(200–675)*	(*782–1749*)	(1405–3214)*	(273–371)	(547–694)	(895–988)*
Defined data	1.000	300	700	1000	1300	231	769	1000
Z-model	1.107	313	704	1132	1322	229	772	1004
	(0.642–1.909)	(101–974)	(564–879)	(684–1873)	(1049–1666)	(203–260)	(723–827)	(967–1043)
E_0-model	0.999	*305*	673	*987*	1261	230	771	1004
	(0.753–1.325)	(*208–447*)	(461–981)	(*694–1404*)	(847–1877)	(199–264)	(704–844)	(954–1058)
Defined data	2.000	300	700	1000	786	83	917	1000
Z-model	1.656	181	726	1025	842	83	919	1008
	(0.943–2.907)	(47–695)	(579–910)	(690–1524)	(659–1076)	(56–123)	(870–971)	(970–1046)
E_0-model	1.316	*95*	822	*928*	959	89	941	1037
	(0.988–1.753)*	(*62–145*)*	(616–1098)	(*716–1203*)	(709–1297)	(60–132)	(882–1004)	(990–1086)

Values in italics are calculated with Hill fixed at a value of 1. Data show geometric mean with the range of ±1 standard deviation of logarithmically transformed values. $N = 500$ simulations. Asterisks indicate that the range does not encompass the corresponding ideal parameter value.

was found both for ideal curves (Table 1) and for pseudo-experimental curves (Fig. 2, Table 2). Hence, it was in most cases possible to provide estimates of the concentration of intrinsic agonist (Z), and to use this concentration to aid in the determination of the true EC_{50} value.

$EC_{50(A,S)}$, as determined on ideal curves by both models, was strongly dependent of the Hill value, in spite of constant true EC_{50} values (Table 1). At $H < 1$, $EC_{50(A,S)}$ was substantially higher than the true EC_{50}, whereas at $H > 1$, $EC_{50(A,S)}$ was somewhat lower than the true EC_{50}. Thus, $EC_{50(A,S)}$ is a poor estimate of the true EC_{50} value if endogenous agonists are present.

It was generally found that analyses using weighting with $1/E^2$ gave a better goodness of fit than those without weighting (data not shown). The *H* values and the parameters expressed as concentrations (i.e. *Z* values and the three EC_{50} parameters) became more normally distributed after logarithmic transformation, whereas the three *E* parameters (E_0, E_s and E_{max}) were close to normally distributed both with and without any transformation (data not shown). Hence, all data presented are from the calculations of logarithmically transformed data where weighting with $1/E^2$ was applied.

3.2. Analysis of ideal curves

The nonlinear iterative curve fitting procedures utilizing the *Z*-analysis model produced curves with parameter estimates that were close to identical to the defined parameters (Fig. 2, Table 1). Minute differences in the decimal portions were observed, which could be attributed to the initial rounding of the generated *E* values (not shown). This validation shows that the curve fitting algorithm is sufficiently powerful to recreate ideal data, and that the *Z*-model, in theory, is the perfect model to analyze this type of data.

In contrast, curve-fitting utilizing the traditional E_0-model produced estimates that in many cases deviated markedly from the true parameters. These discrepancies were most prominent at $H \neq 1$, and the most erroneous determinations were obtained for *H*, *Z*, and EC_{50} (Fig. 2, Table 1). *H* values determined by the E_0-model were higher than the defined *H* when $H < 1$, and lower than the defined *H* when $H > 1$.

In the E_0-model, *Z* values could only be determined if using a fixed *H* value of 1. The resulting *Z*-values were higher than the defined *Z* when $H < 1$, and lower than the defined *H* when $H > 1$. This led to corresponding errors of the EC_{50} values, which thus were higher than the defined values when $H < 1$, and lower than the defined values when $H > 1$.

3.3. Analysis of pseudo-experimental curves

Z-model analysis of the pseudo-experimental data produced geometric means that were similar to the defined parameters in all cases (Table 2). In all cases the range of ± 1 S.D. was encompassing the defined base parameter. In contrast, as in the analysis of ideal data, the E_0-model produced estimates that in many cases deviated markedly from the defined parameters. These discrepancies were most prominent at $H \neq 1$, and the most erroneously determined parameters were *H* and *Z*.

As for the analysis of ideal data, *H* values determined by the E_0-model were higher than the defined *H* when $H < 1$, and lower than the defined *H* when $H > 1$. Hence, *H* values determined by the E_0-model show artificially small deviations of the *H* values. Similarly, the *Z*-values determined by the E_0-model were higher than the defined *Z* values when $H < 1$, and lower than the defined *Z* when $H > 1$. However, since opposite deviations were observed for $EC_{50(A)}$ determinations (at least at $H = 0.5$), the resulting estimates of

EC_{50} were reasonably good at all H values. The $EC_{50(A,S)}$ and E_{max} values determined with the E_0-model at $H = 0.5$ were somewhat lower than the defined data.

4. Discussion

In the present study, we describe the novel approach Z-analysis to investigate stimulation curves that contain an intrinsic basal stimulation. The analysis is valid for preparations where the stimulation level without agonist (intrinsic and exogenous) is zero (as established, e.g. by antagonists), or at some other specific level as defined by some other means. In such cases, the curve fitting models will need modifications, e.g. by adding a fixed baseline factor. Hence, we found that it was possible to use and extend curve-fitting parameters to provide estimates of the concentration of endogenous agonist, and use this parameter to estimate the true EC_{50} value. In some investigations, the determination of intrinsic concentration may be the primary aim. Thus, this method may complement or in certain cases replace direct biochemical analysis of the endogenous agonist [3], which may be difficult or even impossible in some cases.

Z-analysis is valid if the endogenous agonist is identical with the added agonist. However, it may also be appropriate if their EC_{50} values are different, provided that they otherwise share the same properties. In such a situation Z will not reflect the absolute concentration but a value expressed in agonist equivalents. If the intrinsic and added agonists are further deviating in their properties, other assumptions and determinations are required, requiring further mathematical extensions of the models.

We found that Z-analysis can be applied to both the traditional E_0-model and to the novel Z-model. The latter seems to be preferable in most cases, and showed no systematic deviations from the defined parameters. In contrast, many parameters determined with the E_0-model showed marked deviations, especially at true Hill values deviating from unity. This was the case both for analysis of ideal data and pseudo-experimental data. This bias appears to be related directly to the H values rather than to differences in the magnitude of the E_0 values, since the bias was clearly observed also at $H = 2$, where the E_0 only was about 8% of E_{max}.

H values determined by the E_0-model showed artificially small deviations from unity. This was the case both for analysis of ideal data and pseudo-experimental data. Hence, the use of the E_0-model will not only bias the estimates of the absolute Hill values, but may also allow true deviations from unity to escape detection, and to lure experimenters to conclude that the H value is close to unity also when this is not the case.

The Z values determined by the E_0-model were overestimated at low Hill values and underestimated at high Hill values. Hence, the E_0-model provides poor estimates of the concentrations of endogenous agonist. This bias results in erroneous determinations of true EC_{50} values, although this error disappears as the experimental variation increases.

The parameter $EC_{50(A,S)}$, as determined by both models, was found to fluctuate greatly under the influence of the Hill value, in spite of the EC_{50} value being constant. Hence, $EC_{50(A,S)}$ is clearly not very useful as an estimate of the true EC_{50} value, when endogenous agonists are present.

In conclusion, we found that Z-analysis is a workable approach to analyze stimulation curves with an intrinsic basal stimulation, providing the possibility to estimate the concentration of the intrinsic agonist as well as the true EC_{50} value. The Z-model was found to provide better estimates than the E_0-model, especially for H, Z, and EC_{50} values when $H \neq 1$. Hence, Z-analysis should be a valuable tool in biological and pharmacological research and development.

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