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Method to Assess Interactivity of Drugs with Nonparallel Concentration-Effect Relationships

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Abstract

Background—Commonly used methods for analyzing interactivity between drugs (*e.g.*, synergy, antagonism) such as isobologram, combination index, and curve shift are based on the Loewe Additivity principle of dose equivalence and the inherent assumption of similar concentration-effect (*C-E*) including parallel curves and equal maximum effects (E_{max}), and therefore are not suitable for drugs with dissimilar *C-E*. This study describes a new method that is without this limitation and has the additional advantage of enabling statistical analysis.

Method and Results—The method comprises two steps. First, based on the dose equivalence principle, the experimentally obtained *C-E* of one drug was used to calculate the equally effective *C-E* of the other drug at no interactivity; the resulting two *zero-interactivity C-E* formed the upper and lower boundaries of Additivity Envelope. Next, 95% confidence intervals calculated from experimental data were added to Additivity Envelope to obtain Uncertainty Envelope (UE). Experimentally observed effects of drug combinations ($C-E_{comb,observed}$) located within UE indicate additivity whereas $C-E_{comb,observed}$ located above or below UE indicate statistically significant ($p < 0.05$) synergy or antagonism, respectively. Additional *in silico* studies demonstrated the shape and size of Additivity Envelope, which determines the ability to detect drug interactivity,

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

Concepts presented in this report were previously published in the Ph.D. dissertation of L. Zhao [35]. A preliminary version of this work was presented at the 2015 annual meeting of the American Association for Cancer Research [36].

AUTHORSHIP CONTRIBUTIONS

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Conducted experiments: LZ

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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depending on the Drug A-to-B concentration ratios and the ratios of their $C-E$ curve shape parameter. Analyses of experimental results of combinations of drugs with nonparallel $C-E$ and/or unequal E_{max} indicated UE as more versatile and provided more information, compared to earlier methods.

Conclusion—UE is a broadly applicable method for analysis, including statistical significance assessment, of drug interactivity.

Keywords

Combination therapy; drug-drug interactivity; Loewe-additivity; uncertainty envelope; statistically significant synergy; antagonism

1. INTRODUCTION

Major advances in understanding the molecular defects in cancer progression and chemoresistance, together with the dramatic increase of targeted drugs, have provided unprecedented opportunities to develop new combination therapies. The scientific pursuit is further aided by the recent FDA decision to allow clinical testing of combinations involving more than one unapproved drugs [1]. Methodologies that can reliably determine the nature (synergy, additivity or antagonism) and quantify the extent of drug interactivity are valuable tools to guide the decision-making.

For drug interactivity analysis, mechanism-based models have been used where the drug actions and drug-drug interactions are well understood [2–6], whereas empirical methods are used in other situations [7–15]. Mechanism-based models are generally used to validate that drug-drug interactions involve a hypothesized mechanism, and can be useful in simulation studies to optimize the interaction. Empirical approaches provide a practical measure of benefit of a combination, and an objective criterion for selecting combinations for further studies. The empirical methods have the additional advantage of providing a quantitative measurement of the interactivity, allowing ranking of drug candidates or experimental approaches, and have played a critical role in combination therapy development [8, 9, 11, 16]. Their utility is highlighted by Faessel *et al.* who evaluated the interaction of compounds that inhibit different parts of the thymidylate synthesis machinery; the results enabled the investigators to distinguish the beneficial combinations from unfavorable combinations and to establish that inappropriate choices of drug sequencing and concentration ratios can drastically alter the outcome, *e.g.*, from synergy to antagonism [12, 17].

Drugs typically display sigmoidal concentration-effect relationship ($C-E$). The two models of interactivity analysis that accommodate sigmoidal $C-E$ are Bliss Independence and Loewe Additivity. Bliss Independence is based on probability and hence assumes an effect range between 0 and 100% [18]. Loewe Additivity is the basis for contemporary methods that include Isobologram first described in 1872 [19], interaction index in 1978 [20], median effect (also called Combination-Index) in 1984 [21], three-dimensional Response-Surface in 1990s [9, 13], and Curve-Shift in 2004 [22]. The utility and limitations of these various methods have been reviewed [9, 23]. Bliss Independence does not consider the effect of steepness of $C-E$ on interactivity. In contrast, Loewe Additivity, first described in 1926 [24],

includes the effect of steepness of $C-E$. Bliss independence evaluation is desired when Loewe additivity methods cannot be used, *e.g.*, insufficient data to define the full dose-response relationships. Recent theoretical studies using a multi-hit model show that Bliss independence is useful in cases where the actions of the combined agents are entirely independent of one another, which is extremely rare in whole cell systems [25]. In addition, Bliss independence does not agree with the expectation of no interaction with self, and may lead to erroneous conclusions of synergy for agents with steep slope, and antagonism for agents with shallow slope.

There are two unresolved issues in Loewe Additivity-based methods. First, Loewe Additivity assumes the drugs in combinations which represent dilutions of each other and have zero-interactivity (*i.e.*, yielding only additivity) [9, 18]. This in turn assumes the drugs have constant potency ratios and therefore parallel $C-E$ and equal maximal effects (E_{max}). In general, parallel $C-E$ are characterized by a constant concentration ratio for the two drugs at any effect level, which in the Hill equation is defined by equal curve shape parameter values. In practice, the motivation of combining drugs is to take advantage of their different action mechanisms, and combinations of drugs with nonparallel $C-E$ and different E_{max} are common. For example, a cytotoxic is likely to have a steeper $C-E$ compared to a cytostatic, and some drugs display incomplete cytotoxicity (*e.g.*, paclitaxel shows $E_{max} < 100\%$ [26]) whereas others produce complete cell kill. These conditions invalidate the constant potency ratio assumption, resulting in uncertainty in data interpretation (see ref. [27] for mathematical proof). This problem was first recognized in 1979 during the analysis of combination of chemotherapy and radiation that displayed nonparallel $C-E$ [28], but the problem remains largely unresolved. This is because, with the exception of Response-Surface, Loewe Additivity-based methods do not capture the curve shape parameter in their equations. Second, most of the existing methods do not include assessment of the statistical significance of drug interactivity, which is problematic since the data used to calculate interactivity are subjected to experimental variability. For example, is a calculated 30% synergy statistically significant when the data variability is 30% or higher? At present, only two published methods offer limited statistical evaluation, *i.e.*, Curve-Shift analyzes statistical significance at a single effect level (50%) but not at other levels [23], and Universal Response-Surface [9] offers an assessment on the total data but not a specific effect level.

The present report describes a new, broadly applicable method that addresses the above deficiencies of Loewe Additivity methods. In silico studies identified the factors that determine the ability to detect statistically significant drug interactivity.

2. MATERIALS AND METHOD

2.1. Computer Software Packages

All programming codes, graphical representations and calculations used Mathematica (Wolfram Research, Champaign, IL), MatLab (MathWorks, Natick, MA) and SAS (SAS, Cary, NC). Nonlinear regression was performed using the SAS/STAT PROC NLIN routine with the Marquardt iteration method.

2.2. Combination Therapy: Experimental Data and Drug Interactivity Analysis

The experiments covered several scenarios, including combinations of drugs with nonparallel C-E and equal Emax (doxorubicin+suramin) and drugs with nonparallel C-E and unequal Emax (paclitaxel+suramin), and two types of dosing methods (fixed-ratio, fixed-concentration), as follows.

Experimental data (C-E of human prostate PC3 tumor cells treated with single agents and their combinations) were obtained using the same methods as described in our earlier studies [22, 26]. Briefly, cells were cultured at 37°C in RPMI-1640 medium (supplemented with 9% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 90 µg/mL gentamicin), and treated with drugs. Drug effects were measured at predetermined times. All C-E used the customary format with % survival in the y-axis whereas the equations are expressed in E; 100% survival equals E=0% and 0% survival equals E=100%.

The doxorubicin+suramin study was to determine the benefits of overcoming the microenvironment-related chemoresistance with suramin, and therefore used the growth factors-containing conditioned medium collected from cultures of chemo-resistant tumors (rat prostate MAT-LyLu). Addition of suramin, a nonspecific growth factor inhibitor, enhanced tumor sensitivity to doxorubicin. This study used the fixed-ratio dosing, where the concentrations of the two drugs are kept at constant ratios. The stock drug solutions contained suramin:doxorubicin in their IC50-equivalent ratios of 0:1, 1:400, 1:200, 1:100, 1:50, 1:10, 1:5, and 1:0 (referred to as doxorubicin, S1D400, S1D200, S1D100, S1D50, S1D10, S1D5, and suramin). This dosing method enables the measurement of interactivity at broad concentration ranges and allows the identification of the optimal concentration ratios that give maximal synergy. Cells were treated with serially diluted solutions, and the pharmacodynamic endpoint was inhibition of DNA synthesis measured by bromodeoxyuridine incorporation.

The paclitaxel+suramin study was to determine the antagonism due to the cell cycle effect of suramin (G1 block). This study used fixed-concentration dosing, *i.e.*, cells were treated with constant concentrations of one drug plus varying concentrations of the second drug (suramin at 0, 50, 70, 100, 200, 400 and 800 µM, 48 hr before and during treatment with paclitaxel at concentrations ranging from 0 to 300 nM for 24 hr). Drug effect was measured as changes in total cell number using the sulforhodamine assay.

Drug interactivity analysis used three existing methods (Isobologram, Combination-Index, Curve-Shift) as described previously [21, 22]. Briefly, the additivity line in Isobologram was constructed by connecting the $C_{single,observed}$ of the two drugs that produced a given effect (*e.g.*, 50%); synergy, additivity, or antagonism is indicated when the $C_{comb,observed}$ producing the same effect is located below, on, or above the additivity line, respectively. Combination index (Index) was calculated using an equation with the same form as the representative Loewe Additivity equation (see next paragraph). Index values of less than, equal to, and more than 1 indicate synergy, additivity, and antagonism, respectively. Curve-Shift is a graphical method where the $C-E_{single,observed}$ of drugs are normalized to their IC50-equivalents to construct the $C-E_{additivity}$; $C-E_{comb,observed}$ locating to the left of, on, or to the right of $C-E_{additivity}$, respectively indicating synergy, additivity, and antagonism.

3. RESULTS

3.1. Existing Loewe Additivity Based Methods Are Not Suitable for Analyzing Interactivity of Drugs with Nonparallel *C-E* or Unequal *E_{max}*

In Loewe Additivity, two drugs with zero-interactivity act as different dilutions of each other (*i.e.*, as if the two drugs have different potency per mg dose) and their E_{comb} equals the effect produced by the sum of their equi-effective C_{single} . The representative Loewe Additivity equation for zero-interactivity is $(d_a/A) + (d_b/B) = 1$, and deviation from 1 indicates positive or negative interactivity. A and B are the respective concentration/amount of Drug-A and Drug-B that produce a specified effect when each is given as single agent, whereas d_a and d_b are their concentration/amount given in combination to produce the same effect. This equation is based on the assumption of constant potency ratio between Drugs A and B [9, 18, 29]. Hence, this equation is not valid for drugs with nonparallel *C-E* or unequal E_{max} . Fig. (1) provides a graphical illustration of this deficiency.

Fig. (1A) shows the situation for two drugs with parallel *C-E*. Due to the different mg potency, greater Drug-A concentrations relative to Drug-B were required to produce the same E (see left panel, data points #2 and #4 vs. #1 and #3, at $E=20\%$ and $E=80\%$). After normalizing the molar concentrations to the IC_{50} -equivalents, these data points became identical, resulting in a single, overlapping $C-E_{additivity}$ (middle panel). In contrast, Fig. (1B) shows this not to be the case for drugs with nonparallel *C-E*; the two drugs did not produce the same effects at equal IC_{50} -equivalent concentrations (#3 and #1 for Drug-A did not equal #4 and #2 for Drug-B) and it was not possible to obtain a single $C-E_{additivity}$.

3.2. Study Overview

The current study comprises three parts. First, we applied the Loewe Additivity principle's null hypothesis of zero-interactivity for Drug-A and Drug-B, developed the equations to construct Additivity Envelope that depicts the expected $C-E_{additivity}$, and added the 95% confidence intervals (CI) to obtain Uncertainty Envelope (UE). UE represents the boundaries of $C-E_{additivity}$ beyond which there is statistically significant interactivity. Comparison of experimental $C-E_{comb, observed}$ to UE indicates nature of interactivity; $C-E_{comb, observed}$ falling within UE indicates additivity, whereas $C-E_{comb, observed}$ that is greater or inferior relative to UE indicates significant synergy and antagonism ($p < 0.05$). In addition, we developed equations to quantify the degree of interactivity. Next, we used *in silico* studies to demonstrate that three parameters (*i.e.*, $C-E_{single}$, ratios of concentrations ($R_{da:db}$) and C-E curve shape parameters ($R_{na:nb}$) of Drug-A:Drug-B) affected the Envelope shape and size and thereby determined the sensitivity for detecting, and the statistical significance of, drug interactivity. Lastly, we demonstrated the application of this method to analyze the experimental data of combinations of drugs with nonparallel *C-E* and/or unequal E_{max} , and compared the UE results to those of three currently used methods.

The current method differs from the existing Loewe Additivity- based methods in two ways. The earlier methods use equations derived from the representative Loewe Additivity equation and similarly conclude on interactivity based on the relative drug concentration/ amount used as single agents or in combination, and therefore also are not valid for drugs

with nonparallel C - E or unequal E_{max} . In contrast, the present method analyzes interactivity by comparing $E_{comb,observed}$ to $E_{additivity}$ and uses equations that express $E_{additivity}$ in the sigmoidal C - E format (*i.e.*, Hill equation) and thereby capture the variations in n and E_{max} . Hence, the current method is free of the constant potency ratio assumption and applies to all situations. In addition, only two of the earlier methods provide limited statistical analysis at selected E levels, whereas the present method enables statistical analysis at all E levels.

3.3. Development of Additivity/Uncertainty Envelope

Additivity Envelope is circumscribed by the two zero-interactivity C - $E_{additivity}$ of Drugs A and B that were calculated using the experimental C - $E_{single,observed}$ of these drugs and Eq. 1–4 described below. Adding the 95% CI calculated using experimental data variations yielded UE. Additional equations and their derivations are shown in Appendix. In graphical presentations, drug concentrations are represented in their IC_{50} -equivalents when appropriate. This allows description of the C-E of different agents on a single concentration scale, so that C - E , Additivity Envelope, and UE can be presented on a two-dimensional plot. Additivity Envelope for a two-agent combination is defined as the area between the pair of C - $E_{additivity}$. UE is defined as the Additivity Envelope plus its 95% CI.

3.4. Obtaining Boundaries of Additivity Envelope: General Equations

We applied the Loewe additivity principles of dose equivalence (*i.e.*, concentration d_a of Drug-A is equally effective to concentration d_b' of Drug-B) and sham combination (*i.e.*, adding d_b' to any d_b gives the expected additive effect of the summed concentration (d_a+d_b) as if only Drug-B was given), to calculate the zero-interactivity $E_{additivity,a}$ for Drug-A and $E_{additivity,b}$ for Drug-B, as functions (f_a , f_b) of their concentrations (d_a , d_b) or equi-effective concentrations (d_b' , d_a') (Eq. 1).

$$E_{additivity,a}=f_a(d_a+d_a'); E_{additivity,b}=f_b(d_b+d_b') \quad (1)$$

Therefore, $E_{additivity,a}$ is the expected additive effect of the A/B combination, calculated by converting all concentrations to A-equivalents. $E_{additivity,b}$ similarly expresses concentrations in terms of B. As mathematical functions, $E_{additivity}$ changes with d_a and d_b . Operationally, these functions were calculated one point at a time, at a selected d_a'/d_b combination (using the SAS program or Excel spreadsheet in Supplementary Materials). This is illustrated below using the example shown in Fig. (2B) (for a combination using a ratio of A to B in IC_{50} units ($R_{da:db}$) of 1:1). On a 2-dimensional plot of C vs. E , this function for Drug-B corresponds to $C_{additivity,b}=(d_a+d_b)$ and $E_{additivity,b}=f_b(d_b+d_b')$. We first transformed d_a to its equi-effective d_b' at one E value, *e.g.*, at $E_{single,a}=20\%$, d_a equaled 0.14 IC_{50} units of Drug-A and d_b' equaled 0.57 IC_{50} units of Drug-B. Because $R_{da:db}$ was 1:1 (hence $d_b=d_a=0.14$), the sum, d_b+d_b' , equaled 0.71 units of Drug-B. We then found the corresponding $E_{additivity,b}$ from C - $E_{single,b}$ at 0.71 units of Drug-B; the value was 30%. $C_{additivity,b}$, which equaled d_a+d_b , was 0.28 IC_{50} units of total drug. Plotting $C_{additivity,b}$ vs. $E_{additivity,b}$ provided one data point on the C - $E_{additivity,b}$ (denoted by the star symbol in Fig. 2B). Repeating the same procedures at all d_a'/d_b combinations produced the complete C - $E_{additivity,b}$. The

complete $C-E_{additivity,a}$ was similarly obtained. These two functions are boundaries of Additivity Envelope within which there is zero interactivity.

There are three noteworthy aspects. First, the calculation of Additivity Envelope required only $C-E_{single,observed}$ and did not require $C-E_{comb,observed}$. Second, the conversion of d_a to d_b' or d_b to d_a' can only be performed up to E_{max} (see below for proposed solution). Third, $R_{da:db}$, as it determines $C_{additivity}$, will affect the shape and size of Additivity Envelope, and different values of $R_{da:db}$ (e.g., 1:1 vs. 10:1) will result in different Envelopes (see *in silico* results).

While Eq. 1 does not assume the type or shape of $C-E$ (e.g., sigmoidal Hill equation, log-linear equation, Weibull distribution), the present study focused on drugs exhibiting sigmoidal $C-E$. Substituting the Hill equation form for f_a or f_b in Eq. 1 yielded Eq. 2–4.

3.5. Obtaining Boundaries of Additivity Envelope: Combinations of Agents with Parallel C-E and Equal E_{max}

$E_{additivity,a}$ and $E_{additivity,b}$ at equal n and equal E_{max} ($n_a=n_b=n$ and $E_{max,a}=E_{max,b}=E_{max}$) are calculated using Eq. 2, and the resulting overlapping $C-E_{additivity}$ are shown in Fig. (2A).

$$E_{additivity,a}=E_{additivity,b}=E_{max} \frac{\left(\frac{d_a}{IC_{50,a}} + \frac{d_b}{IC_{50,b}}\right)^n}{1 + \left(\frac{d_a}{IC_{50,a}} + \frac{d_b}{IC_{50,b}}\right)^n} \quad (2)$$

3.6. Obtaining Boundaries of Additivity Envelope: Combinations of Agents with Nonparallel C-E and Equal E_{max}

Eq. 3 shows the calculation of $E_{additivity,a}$ and $E_{additivity,b}$ at equal E_{max} ($E_{max,a}=E_{max,b}$) and unequal n ($n_a \neq n_b$). Fig. (2B) shows an example of $C-E_{additivity}$ of Drug-A and Drug-B at 1:1 $R_{da:db}$.

$$E_{additivity,a}=E_{max} \frac{\left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}}\right)^{\frac{n_b}{n_a}}\right)^{n_a}}{1 + \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}}\right)^{\frac{n_b}{n_a}}\right)^{n_a}};$$

$$E_{additivity,b}=E_{max} \frac{\left(\left(\frac{d_a}{IC_{50,a}}\right)^{\frac{n_a}{n_b}} + \frac{d_b}{IC_{50,b}}\right)^{n_b}}{1 + \left(\left(\frac{d_a}{IC_{50,a}}\right)^{\frac{n_a}{n_b}} + \frac{d_b}{IC_{50,b}}\right)^{n_b}} \quad (3)$$

3.7. Obtaining Boundaries of Additivity Envelope: Combinations of Agents with Nonparallel C-E and Unequal E_{max}

Eq. 4 is for unequal E_{max} ($E_{max,a} \neq E_{max,b}$) and nonparallel $C-E$ ($n_a \neq n_b$). A comparable equation for unequal E_{max} and parallel $C-E$ is obtained by substituting with $n_a = n_b$.

$$E_{\text{additivity},a} = E_{\text{max},a} \frac{\left(\frac{d_a + d'_a}{\text{IC}_{50,a}}\right)^{n_a}}{1 + \left(\frac{d_a + d'_a}{\text{IC}_{50,a}}\right)^{n_a}};$$

$$E_{\text{additivity},b} = E_{\text{max},b} \frac{\left(\frac{d_b + d'_b}{\text{IC}_{50,b}}\right)^{n_b}}{1 + \left(\frac{d_a + d'_b}{\text{IC}_{50,b}}\right)^{n_b}} \quad (4)$$

Unequal E_{max} prohibits the transformation of d_b to its equi-effective d'_a at E exceeding $E_{\text{max},a}$, i.e., it would not be possible to transform d_b at 80% E of Drug-B to d'_a if the E of Drug-A did not reach 80%. For example, Fig. (2C) shows the two C - $E_{\text{additivity}}$ for the combinations at 1:1 $R_{da:db}$ with different n and a higher E_{max} for Drug-B (i.e., $E_{\text{max},a}=70\%$ and $n_a=0.7$ for Drug-A; $E_{\text{max},b}=100\%$ and $n_b=2.5$ for Drug-B). Because Drug-B had the higher E_{max} , the d_a values could be converted to d'_b values and $E_{\text{additivity},b}$ could be obtained. On the other hand, at E equaling or exceeding $E_{\text{max},a}$ it was not possible to convert d_b to d'_a , and consequently this portion of $E_{\text{additivity},a}$ could not be determined, resulting in an incomplete C - $E_{\text{additivity},a}$. To address this limitation, we proposed the portion of $E_{\text{additivity},a}$ beyond $E_{\text{max},a}$ would at least equal the effect of single agent Drug-B (i.e., $f_b(d_b)$); this is represented by the broken line starting at the star symbol in Fig. (2C).

3.8. Uncertainty Envelope: Statistical Analysis of Drug Interactivity

The null hypothesis is no interactivity between Drugs A and B. In UE analysis; this is indicated if $E_{\text{comb,observed}}$ falls between the UE (i.e., between $E_{\text{additivity},a}$ and $E_{\text{additivity},b}$). Conversely, the null hypothesis is rejected if $E_{\text{comb,observed}}$ falls outside the UE. The experimental data contained variability. $E_{\text{additivity},a}$ and $E_{\text{additivity},b}$ as they were determined based on parameters derived from experimental data (n , IC_{50} , E_{max}), also contained variability; their standard errors (SEM) were calculated using the error propagation method [22, 30]. The errors from these two sources were used to calculate the distance from the Additivity Envelope that would show significance by the Student's t-test, referred to as the 95% CI and equaling the multiplication product of (critical Student's t value) and (SEM of the difference between the Additivity Envelope and an experimental data point; see Appendix). Hence any $E_{\text{comb,observed}}$ located beyond UE would be different from $E_{\text{additivity}}$ at a 5% level of significance.

We also considered the conventional approach of analysis of variance (ANOVA) as it is a widely accepted method to evaluate differences between groups of experimental values. However, the underlying equations for ANOVA assume the combined effect to be the linear addition of the effects of the single agents. This is not the case in the Loewe principle where the expected combined effect follows the Hill equation and is sigmoidal rather than linear in nature. In addition, ANOVA gives the overall results for all data, wherein the synergy in one region (e.g., at a selected effect level) can be canceled out by antagonism at a different effect

level, leading to an overall outcome of no significant interactivity. In contrast, the current UE method enables the distinction of different interactions at different effect levels, *e.g.*, synergy at high effect levels vs. antagonism at low effect levels.

We further note that the error propagation technique we used to calculate confidence intervals is a broadly applicable technique and could likely be applied to other methods assessing drug-drug interactivity. However, such calculations are not widely used in the field nor included in the commercially available program to perform Combination Index calculations.

3.9. Shape and Size of Additivity/Uncertainty Envelope: *In silico* Results

Simulations were performed to identify the determinants of the shape and size of Additivity Envelope and UE. The $R_{da:db}$ values used in simulations were varied between 0.1 to 10, in order to cover a wide range of combinations, *e.g.*, $R_{da:db}$ of 0.1 comprises 10% Drug A and 90% Drug B, with Drug concentrations expressed in IC_{50} units. As UE is derived from Additivity Envelope, the following findings on Additivity Envelope apply to both. The main difference is that the size of UE, since it equals Additivity Envelope plus 95% CI, is additionally affected by the variance of the experimental results.

With respect to shape, Envelope is bound by two sigmoidal $C-E_{additivity}$ with varying distance between data points; Figs. (2A–2C) show Envelopes for combinations at 1:1 $R_{da:db}$. Envelope shape was affected by $R_{da:db}$, and typically followed $C-E_{single}$ of the drug that contributed the most to $C-E_{additivity}$ at the selected E levels. For example, the Envelope shape for the combination of Drug-A ($E_{max,a}=100\%$, $n_a=0.7$) and Drug-B ($E_{max,b}=100\%$, $n_b=2.5$) at 1:1 $R_{da:db}$ aligned closely with $C-E_{single,a}$ at $E<50\%$ and $C-E_{single,b}$ at higher E (Fig. 2B).

Envelope size determines the sensitivity of detecting interactivity and is defined by the height and the width, where height is the vertical distance between the upper and lower envelope boundaries (h , expressed in unit of E), and width is the horizontal distance between the left and right envelope boundaries (w , expressed in unit of C). Both h and w were dependent on C and E . For example, at 1:1 $R_{da:db}$ and 2.5:0.7 $R_{na:nb}$, h at one IC_{50} unit was the highest and stretched from 67% to 41%, indicating that at C_{comb} of one IC_{50} -equivalent (*e.g.*, 0.5 IC_{50} -equivalents each of Drug-A and Drug-B), the $E_{comb,observed}$ had to fall below 67% or exceed 41% to demonstrate synergy or antagonism, respectively. In contrast, at C_{comb} of 0.2 IC_{50} -equivalent, Envelope narrowed considerably and the demonstration of synergy or antagonism required $E_{comb,observed}$ of $<17\%$ or $>24\%$, respectively. Similarly, w was at the highest value at about $E=50\%$, with lower values at higher or lower E .

The second determinant of h and w was $R_{da:db}$. $R_{da:db}$ of 10:1 and 1:10 (Fig. 3A) yielded narrower Envelopes (smaller maximal h and w values) compared to 1:1 (Fig. 2B). Based on the *in silico* results (simulated using varying $R_{da:db}$ from 1:10 to 10:1, at $n_a=0.7$, $n_b=2.5$ and equal E_{max} of 100%), h and w showed the highest values at 0.4 and 0.8 $R_{da:db}$, respectively, and lower values at all other $R_{da:db}$ (Figs. 3C and 3D). The extent of decline of h was determined by $C-E_{single}$ of the agent that contributed the most to $C-E_{comb}$. For example, a higher h was found for 1:10 $R_{da:db}$ compared to 10:1 $R_{da:db}$ (24.3% vs. 5.5%) due to the steeper $C-E_{single,b}$ that resulted in a larger Envelope (Figs. 3A and 3C).

The third determinant of h and w was $R_{na:nb}$ and n values (Fig. 3B). Envelope collapsed to a single sigmoidal $C-E$ (i.e., $h=0$) at equal n values ($n_a = n_b=2.5$). For two combinations with 1:5 $R_{na:nb}$ (0.5:2.5 and 2.5:12.5), the combination with higher n values yielded several times greater h (Fig. 3B). Based on the *in silico* results (simulated using constant $n_a=1$ and varying n_b from 0.1 to 10, at 1:1 $R_{da:db}$ and equal E_{max} of 100%), h and w showed the lowest values of 0, and higher values at all other $R_{na:nb}$ (Figs. 3C and 3D).

The w value highlights a noteworthy difference in the results of UE and Combination-Index analyses. A w of 0.48 IC_{50} units (from 0.77 to 1.25) within the Envelope indicates additivity within this range, whereas the corresponding Index values would identify either synergy or antagonism ($1/0.77=1.30$ -fold synergy and $1.25/1=1.25$ -fold antagonism).

3.10. Quantification of Extent of Interactivity

In a fixed ratio dosing study, the difference between (d_a+d_b) and the lower boundary $C_{additivity}$ at a given $E_{comb,observed}$ provides a measure of the extent of synergy whereas the difference between (d_a+d_b) and the upper boundary $C_{additivity}$ provides the extent of antagonism. For the calculation of extent of interactivity (EI), we used the ratio of (d_a+d_b) and (logarithmic mean of $C_{additivity,a}$ and $C_{additivity,b}$). The equations and the numerical solutions are shown in the Appendix.

3.11. Drug Interactivity Analysis of Experimental Data Using Uncertainty Envelope: Comparison with Existing Methods

Figs. (4A and 4B) show the experimental data for two drugs with nonparallel $C-E$ and equal E_{max} , doxorubicin and suramin, as single agents and in combination, and Figs. (5A and 5B) show the data for two drugs with nonparallel $C-E$ and unequal E_{max} , paclitaxel and suramin. The former combination used fixed-ratios of drug concentrations, whereas the latter used fixed concentrations of suramin plus varying paclitaxel concentrations. The same Hill equation parameters of these drugs (n , E_{max} and IC_{50} ; Table 1) were used in analyses by UE and three commonly used methods (Isobologram, Combination-Index, Curve-Shift). Hence, differences in the interactivity results were solely due to the analysis methods.

For doxorubicin and suramin, both drugs showed equal E_{max} of 100% and were applied at broader dose range including the 100% cytotoxic doses. The resulting UE was fully enclosed extending over the full E range of 0–100% (Fig. 4C). The S1D400 combination is used as an example for discussion; the analysis results of UE and the other three methods are shown in Figs. (4C–4F and Table 2A). All four methods showed synergy at mid-range $E_{comb,observed}$ values (e.g., ~50%), with additivity, slight antagonism or synergy at the lowest and highest E (<10% and >95%). Second, method-related differences depended on $E_{comb,observed}$. Full agreement was found at the mid-range E of 50–60% and for conditions with substantial synergy of more than 2.5-fold (EI and Combination Index <0.4). In contrast, different conclusions were found for situations showing minor interactivity (<2-fold synergy or antagonism) and at lowest and highest $E_{comb,observed}$ (<10% and >90%). Hence, Isobologram, where analysis was limited to one E of 50%, showed full agreement with other methods. Combination-Index yielded disagreements in 17 of 30 occasions with either Curve-Shift or UE. For example, Combination-Index identified antagonism at $E_{comb,observed}$ of

1.9% and 16% whereas the other two methods identified additivity. In comparison, Curve-Shift and UE were more often in agreement, with different conclusions in only 4 of 30 occasions; this is likely because both analyses were based on $C-E_{single,observed}$. Note that because UE accounted for the experimental data variations, its results provided statistical assessment of interactivity at 5% level of significance. In comparison, the other three methods did not have this capability and would have concluded interactivity where in fact there was none; *e.g.*, UE concluded no statistically significant interactivity at $E_{comb,observed}$ of 97% and 80.3%, whereas Curve-Shift and/or Combination-Index concluded antagonism or synergy.

The paclitaxel+suramin combination, due to their unequal E_{max} (58.5% for paclitaxel *vs.* 100% for suramin) and the fixed-concentration dosing, yielded very different UE. In addition, the $C_{suramin}$ used was below 100% cytotoxic level and yielded <100% E . These properties resulted in UE that was not enclosed, did not extend over 0–100% E , and had boundaries that were dependent on $C_{suramin}$ and whether $E_{single,suramin}$ exceeded or was below $E_{max,paclitaxel}$. Figs. (5C and 5G) show the UE for two combinations containing 200 and 800 μ M suramin, where $E_{single,suramin}$ was below and exceeded $E_{max,paclitaxel}$ respectively. For the combination containing 200 μ M suramin, the upper envelope boundary started at $E_{single,suramin}$ of 36%, and changed with increasing paclitaxel concentrations to eventually plateau at $E_{max,paclitaxel}$. In comparison, the combination containing 800 μ M suramin showed an upper boundary that started and remained constant at $E_{single,suramin}$ of 76%, *i.e.*, no further increase by increasing $C_{paclitaxel}$ since $E_{single,suramin}$ exceeded $E_{max,paclitaxel}$. In both cases, the lower envelope boundaries started at $E_{single,suramin}$ and increased with increasing paclitaxel concentrations to reach a plateau level that was in-between $E_{max,single}$ of the two drugs (see Appendix for calculation). Figs. (5C–5H) compare the results by different analysis methods (Table 2). Overall, UE results indicated statistically significant interactivity that was highly dependent on the concentrations of both drugs, with synergy for combinations containing lower suramin concentrations (100 μ M or 30% of its IC_{50} of 317 μ M) plus higher paclitaxel concentration (30 nM, or ~3-times its IC_{50} of 11 nM) and antagonism for combinations containing higher suramin concentrations (800 μ M) or lower paclitaxel concentrations (10 nM). Of the remaining methods, only Curve-Shift provided results at all $E_{comb,observed}$, whereas Isobologram and Combination-Index, which require knowing the concentrations of Drug-A and Drug-B that produce the same specified effect when given alone, were not applicable to situations where $E_{comb,observed}$ exceeded $E_{max,paclitaxel}$ (*e.g.*, combinations containing 800 μ M suramin). There is general agreement of antagonism for combinations containing low paclitaxel (10 nM) (Table 2). The only exception is in the 70 μ M suramin dataset, UE showed additivity while Curve-Shift and Combination-Index yielded antagonism. In view of the nearly identical values of EI and Combination Index, this difference appears to be due to the requirement of statistical significance for UE. Comparison of UE with Curve-Shift, with respect to shape and size, indicates that unequal E_{max} resulted in a wider gap at the right ends of $C-E$ in Curve-Shift, yielding a greater likelihood of concluding additivity for $C-E_{comb,observed}$ data points within this region in Curve-Shift, *e.g.*, UE identified significant antagonism whereas Curve-Shift identified only additivity for the combination containing 800 μ M suramin. A comparison of EI values with Combination Index values in the cases presented in Table 2 shows differences

of less than 10% when Combination Index values could be obtained. Note that Combination Index values could not be calculated for $E_{comb,observed}$ exceeding $E_{max,paclitaxel}$ while EI could be calculated in all cases.

4. DISCUSSION

Analysis of drug interactivity is gaining importance due to the increasing clinical use of combination therapy and the need to guide decision-making in drug development. The search of sound methodologies to analyze drug interactivity has been ongoing since the first description of Isobologram in 1872. While a number of methods have been developed and adopted by the biomedical community, it is increasingly recognized that several popular methods, because they use the general Loewe Additivity equation, are not suitable for drugs with nonparallel $C-E$ or unequal E_{max} [9, 18, 27]. However, the mathematics are such that this issue remains underappreciated in the biology and pharmacology fields. We provided in Fig. (1) a graphical explanation why this assumption, when wrongly applied, will lead to incorrect analysis results and conclusions. We further developed UE to resolve two longstanding issues in drug interactivity analysis and to enable the preselection or prioritization of candidate combinations. The major features of this new method are as follows.

First, UE retains the dose-effect consideration in the Loewe Additivity model and hence is suitable for drugs with sigmoidal $C-E$ [18].

Second, this method does not use the constant potency ratio assumption. Our results show that it is broadly applicable to drugs with parallel or nonparallel $C-E$ and equal or unequal E_{max} . The ability to handle unequal E_{max} values also enables the use of the variable E_{max} model where E_{max} is an estimated parameter preferred for unbiased data analysis [31]. In contrast, Isobologram, Combination-Index and Curve-Shift require a constant potency ratio assumption and are not applicable to agents with nonparallel $C-E$ or unequal E_{max} . The governing equations of Response-Surface account for unequal curve shape parameters [9]. A modified isobologram-like method was recently described for agents with nonparallel $C-E$; this method, as in the current method, uses A-to-B and B-to-A dose transformation, to obtain two curved isoboles that circumscribe the area of additivity. It also uses the delta method (same as error propagation) to enable statistical analysis [27, 32]. The notable differences are (a) UE covers all E values whereas the earlier isobologram-like method, due to the one-effect-at-a-time nature of Isobologram, is limited to a single E , and (b) UE is the only method that covers the unequal E_{max} situation (*i.e.*, provides a complete envelope).

Third, UE takes into account the experimental data variability and requires a 95% likelihood that observations are synergistic or antagonistic before the conclusion of additivity is rejected. This is demonstrated by its more conservative conclusions on interactivity compared to three existing Loewe Additivity-based methods. For example, the UE analysis results did not show synergy in cases where other methods did not find synergy, and only showed synergy in some of the cases found to be synergistic by other methods. On the other hand, the ability of UE to account for data variations enhances the confidence of data interpretation. Despite its more conservative nature, UE did determine synergy in cases that

could not be analyzed by other methods because $E_{comb,observed}$ exceeded the lower E_{max} of a different E_{max} combination. In comparison, many of the existing methods do not include an assessment of statistical probability, and are therefore more likely to reach a conclusion of interactivity without providing the statistical significance. For example, the Combination-Index results do not include additivity in the example of Table 2, as all findings are either synergistic or antagonistic. Note that Combination-Index is frequently applied using the commercial software CalcuSyn, which, according to the provider's website, uses Monte Carlo simulations to estimate the accuracy of CI estimates [33]; recent publications using this software indicate that the range of CI values of 0.8 to 1.2 are considered additive [34]. The current finding that different analysis methods yielded different conclusions on the nature of interactivity highlights the need of statistical analysis to reach sound data interpretation.

Fourth, UE facilitates identification of synergistic combinations and aids in the evaluation of the experimental design. The UE method, when used in combination with the curve shift analysis we described earlier, will facilitate the identification of promising combinations while at the same time highlighting where the noise in the experiment data would prevent detection of possible synergy. This is because the critical factors for accurately identifying synergy are a good description of the single agent C-E curves (*e.g.*, at least 3 concentration-effect (C-E) observations between 20 and 80% effect, including one near the 50% effect level) and high accuracy and precision of the experimental C-E data. The curve shift analysis shows the experimental C-E curves for single agents, and thereby enables an investigator to judge the quality of the experimental C-E data. The UE method enables an investigator to determine if the combination C-E data is located within the data variability (if it equals the 95% confidence interval used to construct the UE), in which case the investigator will reject synergy. Conversely, if the combination C-E data is located outside the UE, an investigator will accept interactivity (either antagonism or synergy) with $p < 0.05$. In addition, the statistical analysis provided by the UE method enables distinguishing statistically valid findings from chance observations.

Fifth, UE is more versatile compared to Combination- Index, in two ways. The calculation of Additivity Envelope requires only $C-E_{single,observed}$ and does not require $C-E_{comb,observed}$ whereas Combination-Index requires both. In addition, UE can be applied to experiments using either fixed-concentration or fixed-ratio dosing methods, whereas Combination-Index is limited to the latter.

Sixth, UE is more sensitive for detecting interactivity among drugs with unequal E_{max} compared to, *e.g.*, Curve-Shift. UE further provides a quantitative assessment of interaction in cases where the Combination Index cannot be calculated.

Seventh, the representation of the upper and lower boundaries of Additivity Envelope in equation format makes it amenable to computerized analysis, with potential advantages over graphic methods (see Supplementary Materials for software code). For example, Isobologram requires drawing an isobole plot for each E . In addition, the Additivity Envelope equations can be extended to include sigmoidal, log-linear, logistic and other functions, and similarly can be extended to evaluate combinations of more than two agents

(*e.g.*, construct three Envelopes to evaluate combination of three agents). More importantly, it can be used, together with *in silico* studies, to prioritize the candidate combinations. For example, our *in silico* results indicate that $R_{da:db}$ and $R_{na:nb}$ determine the Envelope size and shape and therefore the sensitivity for detecting positive or negative interactivity as well as the extent of interactivity. These findings can be extended to screen or select combinations, including the type of drugs (*e.g.*, higher n for cytotoxics and lower n for cytostatic) and the treatment conditions, *e.g.*, drugs with similar n values will yield a narrower Envelope and the $R_{da:db}$ can be preselected to maximize the detectability of interactivity. In comparison, other Loewe Additivity-based methods use $C-E_{single, observed}$ for the sole purpose of analyzing $C-E_{comb, observed}$ for interactivity, do not include the n term in their equations and do not support *in silico* studies using varying $R_{da:db}$ and $R_{na:nb}$.

In summary, the major benefits of the UE method over the most commonly applied current methods are that the UE method (a) does not rely on mathematics that is theoretically incorrect when analyzing combinations of agents with nonparallel $C-E$ and/or unequal E_{max} ; (b) includes statistical evaluation of significance at all effect levels and therefore takes data variability into account in the assessment of interaction; (c) handles evaluation of interactivity of agents with unequal E_{max} best; (d) is suitable for analysis of studies with fixed-ratio dosing design and fixed-concentration dosing design; and (e) provides quantitative scores of drug interaction to facilitate rank-ordering of compounds or experimental variables. Note that a limitation of UE is that the inclusion of 95% CI, which typically equals or exceeds 1%, renders it difficult to conclude antagonism or synergy at the extremes of $C-E$ (close to 0% and 100%). Another requirement, as in all Loewe additivity-based methods, is sigmoidal and well-defined $C-E$ curves of the single agents. Because variability in $E_{additivity}$ is estimated from the variability in the parameter estimates, good precision in these estimates is required.

We previously compared the analysis results by four existing Loewe Additivity-based methods (the above three plus Universal Response-Surface), and concluded these methods as offering complementary information [23]. Universal Response-Surface provides a single value summarizing the nature of interactivity for the overall data with statistical analysis, but is technically challenging. We recommend using a combination of methods, in the following sequence. First, use Curve-Shift to visually inspect the parallelism of $C-E$ and E_{max} of single agents and draw a preliminary conclusion on drug interactivity. Second, use UE to identify the combinations that result in statistically significant synergy and antagonism, or uncertain outcomes, and to quantify the extent of interactivity. For additional verification, Combination-Index may be used to quantify the extent of interactivity. Note that the Combination-Index results are valid only for drugs with parallel $C-E_{single}$. Finally, we recommend using high quality experimental data (*i.e.* low variability) to maximize the confidence of analysis results and conclusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
<i>C</i>	Concentration
<i>C-E</i>	Concentration-effect relationship
CI	Confidence interval
<i>d_a</i> or <i>d_b</i>	Concentration/dose of Drug-A or Drug-B used in combination to generate a specified effect
<i>E</i>	Effect
<i>E_{max}</i>	Maximal effect in Hill equation
EI	Extent of interactivity
Index	Combination index
<i>h</i> and <i>w</i>	Vertical height and horizontal width of Additivity Envelope
<i>IC_{x,y}</i>	Concentration of Drug y needed to generate x% of its <i>E_{max}</i>
<i>n</i>	<i>C-E</i> curve shape parameter in Hill equation
<i>SE</i>	Standard error
<i>R_{da:db}</i> and <i>R_{na:nb}</i>	Ratio of Drug-A: Drug-B concentration (in <i>IC₅₀</i> units) and their <i>C-E</i> curve shape parameter (<i>n</i> in Hill equation) UE, Uncertainty Envelope. Subscripts denote single agent or combination (<i>e.g.</i> , <i>C_{single}</i>), Drug-A or Drug-B (<i>e.g.</i> , <i>E_{single,a}</i>), experimentally observed data (<i>e.g.</i> , <i>E_{comb, observed}</i>) or calculated zero-interactivity data (<i>e.g.</i> , <i>E_{additivity}</i>).

APPENDIX

Hill Equation

The Hill equation is broadly used in pharmacology to describe the sigmoidal *C-E* of drug actions. It was used to obtain Eq. 2–4. In oncology, the typical *C-E* is a survival curve where a drug causes inhibition of survival, where the concentration of drug x is expressed as *d_x*, the maximal drug effect as *E_{max,x}*, the *d_x* resulting in 50% of *E_{max,x}* is *IC_{50,x}*, and the curve shape parameter of *C-E* is *n*. The Hill equation for single agent Drug-A describes its effect

($E_{single,a}$) as a function of d_a or, in the standard notation, $E_{single,a} = f_a(d_a)$. The two equivalent forms of the Hill equation are described in Eq. A1. The same equations are used for Drug-B by replacing the subscripts a with b .

$$E_{single,a} = f_a(d_a) = E_{\max,a} \frac{\left(\frac{d_a}{IC_{50,a}}\right)^{n_a}}{1 + \left(\frac{d_a}{IC_{50,a}}\right)^{n_a}} = \frac{E_{\max,a} \cdot d_a^{n_a}}{IC_{50,a}^{n_a} + d_a^{n_a}} \quad (A1)$$

Inverse Function

An inverse function converts the value of a function to the value of its argument. For example, the natural logarithm is the inverse of an exponential function: $y = e^x$, as $\ln(y) = \ln(e^x) = x$. The standard notation for the inverse function of y is $f^{-1}(y)$. Inverse functions can only be used for continuously increasing or continuously decreasing functions, so that there is only a single argument for each value of the function. The Hill equation fulfills this requirement. In the derivations below, we used the inverse function $f^{-1}(y)$ to convert the value of $E_{additivity,a}$ to the value of its argument d_a . Expressing d_a as a function of $E_{single,a}$ and rearranging Eq. A1 gives Eq. A2.

$$d_a = f_a^{-1}(E_a) = \frac{(E_{single,a})^{1/n_a} IC_{50,a}}{(E_{\max,a} - E_{single,a})^{1/n_a}} \quad (A2)$$

Substituting $E_{single,a} = f_a(d_a)$ in Eq. A2 yielded Eq. A3.

$$d_a = f_a^{-1}(E_{single,a}) = f_a^{-1}(f_a(d_a)) \quad (A3)$$

Equations for Calculating d_a' and d_b'

The amount of Drug-A that produces the same effect E as the amount d_b of Drug-B is d_a' , so that

$$E_{single,a} = f_a(d_a') = f_b(d_b) \quad (A4)$$

Using the properties of inverse functions, Eq. A4 is rewritten to Eq. A5.

$$d_a' = f_a^{-1}(E) = f_a^{-1}(f_b(d_b)) \quad (A5)$$

Note that Eq. A5 applies generally, *i.e.*, without assuming a particular function to describe the C - E data. Applying the Hill equation and the d_a' per Eq. A2, and substituting E for $E_{single,a}$ yielded Eq. A6. The equation for d_b' (A7) was similarly obtained.

$$d'_a = f_a^{-1}(E) = f_a^{-1}(f_b(d_b)) = \frac{E^{1/n_a} \cdot IC_{50,a}}{(E_{\max,a} - E)^{1/n_a}} \quad (A6)$$

$$d'_b = f_b^{-1}(E) = f_b^{-1}(f_a(d_a)) = \frac{E^{1/n_b} \cdot IC_{50,b}}{(E_{\max,b} - E)^{1/n_b}} \quad (A7)$$

Derivation of Eq. 2 for Combination of Drugs with Parallel C-E and Equal

$E_{\max}$

The following shows that the equality of $E_{\text{additivity},a}$ and $E_{\text{additivity},b}$ is a general property for parallel C-E and does not depend on use of the Hill equation. Drugs with parallel C-E follow the relationship described in Eq. A8, where k is the fold-shift between the C-E of the two drugs.

$$f_a(d_a) = f_b(k d_a) \text{ or } f_b(d_b) = f_a\left(\frac{1}{k} d_a\right) \quad (A8)$$

For example, if the C-E for Drug-B is shifted 2-fold to the right relative to Drug-A (*i.e.*, Drug-B is 2-times less potent on a molar basis), then $f_a(d_a) = f_b(2 d_a)$. Similarly,

$$f_b(d_b) = f_a\left(\frac{1}{2} d_b\right).$$

Substituting d'_a from Eq. A5 into Eq. 1 and applying Eq. A8 yielded Eq. A9.

$$E_{\text{additivity},a} = f_a\left(d_a + f_a^{-1}(f_b(d_b))\right) = f_b\left(k \cdot d_a + k \cdot f_a^{-1}(f_b(d_b))\right) \quad (A9)$$

Using the equality of Eq. A3, and the property of parallel shift of Eq. A11 below, Eq. A9 becomes Eq. A10. From Eq. 1, the right hand side of Eq. A10 equals $E_{\text{additivity},b}$

$$\begin{aligned} E_{\text{additivity},a} &= f_b\left(f_b^{-1}(f_b(k \cdot d_a)) + k \cdot f_a^{-1}\left(f_a\left(\frac{1}{k} \cdot d_b\right)\right)\right) = \\ &f_b\left(f_b^{-1}\left(f_a\left(\frac{1}{k} \cdot (k \cdot d_a)\right)\right) + k \cdot \left(\frac{1}{k} \cdot d_b\right)\right) = f_b\left(f_b^{-1}(f_a(d_a)) + d_b\right) \end{aligned} \quad (A10)$$

For drugs that follow the Hill equation and show parallel C- E_{single} (hence $n_a = n_b = n$) and equal $E_{\max}$ ($E_{\max,a} = E_{\max,b} = E_{\max}$), the condition of constant potency ratios is met and the ratios of equi-effective concentrations of Drugs A and B, d'_a and d'_b , equal the ratio of their respective IC_{50} (Eq. A11).

$$d'_a = \frac{IC_{50,a}}{IC_{50,b}} \cdot d_b \quad (A11)$$

Substituting Eq. A1, A5 and A11 into Eq. 1 yields Eq. A12.

$$E_{\text{additivity},a} = f_a(d_a + d'_a) = E_{\text{max},a} \frac{\left(\frac{d_a + \frac{IC_{50,a}}{IC_{50,b}} d_b}{IC_{50,a}} \right)^{n_a}}{1 + \left(\frac{d_a + \frac{IC_{50,a}}{IC_{50,b}} d_b}{IC_{50,a}} \right)^{n_a}} \quad (A12)$$

Replacing $E_{\text{max},a}$ with E_{max} and n_a with n , and simplifying the bracketed terms yielded Eq. 2. Repeating these steps for Drug-B yielded $E_{\text{additivity},b}$. Due to the identical values of E_{max} and n for the two drugs, $E_{\text{additivity},b}$ is identical to $E_{\text{additivity},a}$. Hence, the two functions collapsed into a single sigmoidal C - $E_{\text{additivity}}$.

Derivation of Eq. 3 for Combination of Drugs with Nonparallel C - E and Equal E_{max}

Under these situations, $n_a = n_b$ and $E_{\text{max},a} = E_{\text{max},b} = E_{\text{max}}$. Combining Eq. A5 and A6, and substituting E_{max} for $E_{\text{max},a}$ yielded Eq. A13.

$$d'_a = f_a^{-1}(f_b(d_b)) = f_a^{-1}(E) = \frac{E^{1/n_a} \cdot IC_{50,a}}{(E_{\text{max}} - E)^{1/n_a}} \quad (A13)$$

Substitution E with the Hill equation term of Eq. A1 and using the equation for Drug-B, plus substituting E_{max} for $E_{\text{max},b}$ yielded Eq. A14.

$$E = f_b(d_b) = \frac{E_{\text{max}} \cdot d_b^{n_b}}{IC_{50,b}^{n_b} + d_b^{n_b}} \quad (A14)$$

Substituting E in Eq. A14 into Eq. A13 yielded Eq. A15.

$$d'_a = f_a^{-1}(f_b(d_b)) = \frac{\left(\frac{E_{\text{max}} \cdot d_b^{n_b}}{IC_{50,b}^{n_b} + d_b^{n_b}} \right)^{1/n_a} \cdot IC_{50,a}}{\left(E_{\text{max}} - \frac{E_{\text{max}} \cdot d_b^{n_b}}{IC_{50,b}^{n_b} + d_b^{n_b}} \right)^{1/n_a}} \quad (A15)$$

Rearranging Eq. A15 yielded Eq. A16.

$$d'_a = \text{IC}_{50,a} \left(\frac{d_b}{\text{IC}_{50,b}} \right)^{\frac{n_b}{n_a}} \quad (\text{A16})$$

Substituting Eq. A1 into Eq. 1 yielded Eq. A17.

$$E_{\text{additivity},a} = f_a(d_a + d'_a) = E_{\text{max},a} \frac{\left(\frac{d_a + d'_a}{\text{IC}_{50,a}} \right)^{n_a}}{1 + \left(\frac{d_a + d'_a}{\text{IC}_{50,a}} \right)^{n_a}} \quad (\text{A17})$$

Substituting Eq. A16 into Eq. A17 followed by simplification yielded Eq. 3 for $E_{\text{additivity},a}$. The equation for $E_{\text{additivity},b}$ was obtained similarly, using d_b and d'_b instead of d_a and d'_a .

Derivation of Eq. 4 for Combination of Drugs with Nonparallel C-E and Unequal E_{max}

Under these situations, $n_a = n_b$, and $E_{\text{max},a} \neq E_{\text{max},b}$. Eq. 4 for $E_{\text{additivity},a}$ was obtained by substituting Eq. A1 into Eq. 1, and replacing the argument (d_a) with $(d_a + d'_a)$. d'_a was first calculated using Eq. A5 and the resulting numerical value was used in Eq. 4. Similar procedures were used to obtain Eq. 4 for $E_{\text{additivity},b}$.

Calculation of Additivity Envelope for Combinations of Drugs with Unequal E_{max} and Studied Using Fixed-concentration Dosing

The fixed-concentration dosing design keeps the concentration of one drug constant while changing the concentration for the other drug. The resulting shape and size of Additivity Envelope for combinations of drugs with unequal E_{max} depend on which agent is given at the fixed concentration. The following calculation is for the situation where Drug-A has a lower E_{max} and is given at varying concentrations, and where Drug-B has a higher E_{max} and is given at a fixed concentration. From Eq. 1, $E_{\text{additivity},a}$ equals $f_a(d_a + d'_a)$, and $E_{\text{additivity},b}$ equals $f_b(d_b + d'_b)$. In the plot of survival vs. concentration, the former is the upper boundary of Additivity Envelope and the latter is the lower boundary (*i.e.*, lower survival or higher E). We consider the two situations where Drug-B, given at a fixed concentration, produces a fixed E_{single} that is below $E_{\text{max},b}$ but is either below or above $E_{\text{max},a}$.

For the situation where $E_{\text{single},b}$ is below $E_{\text{max},a}$, d_b can be converted to d'_a and because d_b is fixed, $f_a(d_a + d'_a)$ initially changes with d_a until it reaches $E_{\text{max},a}$. Hence, the upper envelope boundary plateaus at $E_{\text{max},a}$. In comparison, $f_b(d_b + d'_b)$, because d_b is fixed, changes with d'_b until it reaches the level that produces $E_{\text{max},a}$ in which case the lower envelope boundary plateaus at an E that exceeds $E_{\text{max},a}$ but lies below $E_{\text{max},b}$. This was the case for the example of paclitaxel+suramin in Fig. (5C) (200 μM suramin produced $E_{\text{single},b}$ of about 36%, which was below the $E_{\text{max},a}$ of 58.5% for paclitaxel). $E_{\text{additivity},a}$ (upper boundary) changed with d_a

to reach the plateau level of $E_{max,a}$, whereas $E_{additivity,b}$ (lower boundary) plateaued at 70% E , which was between the E_{max} of paclitaxel and suramin.

For the situation where $E_{single,b}$ exceeds $E_{max,a}$, d_b cannot be converted to d'_a . Under our proposed solution of substituting the portion of $E_{additivity,a}$ beyond $E_{max,a}$ with $f_b(d_b)$ (see Results), $E_{additivity,a}$ remains at a plateau level that equals $E_{single,b}$ at the fixed d_b . In comparison, $f_b(d_b + d'_b)$, because d_b was constant, changes with d'_b until d'_b reaches the level producing $E_{max,a}$ at which point $f_b(d_b + d'_b)$ reaches a plateau level, e.g., 84% E in (Fig. 5G), which is between $E_{single,b}$ at the fixed d_b and $E_{max,b}$.

The above discussion is for situations where the agent with a higher E_{max} was given in fixed concentrations, in which case the two boundaries of Additivity Envelope do not converge because the upper envelope boundary, which plateaus at the lower of $E_{max,a}$ or $E_{single,b}$ levels, does not meet or cross the $E_{max,b}$ or $E_{single,a}$. In situations where the agent with a lower E_{max} is given in fixed concentrations, the envelope boundaries will converge at E_{max} of the agent with the higher E_{max} . Another noteworthy feature for experiments that use the fixed-concentration dosing is that in the absence of Drug-A (i.e., $d_a = d'_b = 0$), $E_{additivity,a}$ equals $f_a(d'_a)$ and $E_{additivity,b}$ equals $f_b(d_b)$, which both equal to $E_{single,b}$ at d_b . Hence, Additivity Envelope for these combinations will originate from $E_{single,b}$ instead of from 100% survival as for the combinations that use fixed-ratio dosing (e.g., Fig. 4).

Calculation of Standard Errors of $E_{additivity,a}$ and $E_{additivity,b}$

$E_{additivity,a}$ and $E_{additivity,b}$ as described by Eq. 2–4 are functions of the Hill equation parameters (IC_{50} , n , E_{max}) of the individual drugs. These parameters and the associated standard errors (SE) were obtained by nonlinear regression of the $C-E_{single,observed}$. For this step, we recommend including all raw data to obtain the best estimates of Hill equation parameters and corresponding SE, as well as the associated degrees of freedom. The SE in the Hill equation parameters were used together with the error propagation method [22, 30] to calculate the SE in $E_{additivity,a}$ and $E_{additivity,b}$; Eq. A18 shows the equation for $E_{additivity,a}$. The partial derivatives in Eq. A18 are shown in closed form below.

$$SE_{E_a(d_a, d_b)} = \sqrt{\left(\frac{\partial E_a(d_a, d_b)}{\partial n_a}\right)^2 \cdot SE_{n_a}^2 + \left(\frac{\partial E_a(d_a, d_b)}{\partial IC_{50,a}}\right)^2 \cdot SE_{IC_{50,a}}^2 + \left(\frac{\partial E_a(d_a, d_b)}{\partial n_a}\right) \cdot \left(\frac{\partial E_a(d_a, d_b)}{\partial IC_{50,a}}\right) \cdot SE_{n_a IC_{50,a}}^2 + \left(\frac{\partial E_a(d_a, d_b)}{\partial n_b}\right)^2 \cdot SE_{n_b}^2 + \left(\frac{\partial E_a(d_a, d_b)}{\partial IC_{50,b}}\right)^2 \cdot SE_{IC_{50,b}}^2 + \left(\frac{\partial E_a(d_a, d_b)}{\partial n_b}\right) \cdot \left(\frac{\partial E_a(d_a, d_b)}{\partial IC_{50,b}}\right) \cdot SE_{n_b IC_{50,b}}^2} \quad (A18)$$

Covariance terms are calculated as a product of correlation coefficient and error terms. As an example, $SE_{n_a IC_{50,a}}^2$ was calculated from the nonlinear regression output as follows, using the results for suramin (Table 1) as shown in Eq. A19.

$$SE_{n_a IC_{50,a}}^2 = \text{Correlation}(IC_{50,a}, n_a) \cdot SE_{IC_{50,a}} \cdot SE_{n_a} = 0.303 \cdot 22.8 \cdot 0.152 = 1.050 \quad (A19)$$

Calculation of Standard Error of the Difference

The 95% CI of UE equaled t^* times the SE of the difference between $E_{observed}$ and $E_{additivity,a}$ or $E_{additivity,b}$, where t^* is the critical value in the Student t-test. The SE of the difference is calculated using Eq. A20.

$$SE_{\text{difference}} = SE_{\text{pool}} \sqrt{\frac{1}{df_{\text{observed}} + 1} + \frac{1}{df_{\text{additivity}} + 1}}$$

$$\text{Where: } S_{\text{pool}} = \sqrt{\frac{SD_{\text{observed}}^2 \cdot df_{\text{observed}} + SD_{\text{additivity}}^2 \cdot df_{\text{additivity}}}{df_{\text{observed}} + df_{\text{additivity}}}}$$

SD_{observed} is a function of d_a and d_b that approximates the standard deviation of the observations. We have used zero order (constant value) and first order functions. df is degrees of freedom.

$$SD_{\text{additivity}} = SE_{E_a(d_a, d_b)} \cdot \sqrt{df_{\text{additivity}} + 1} \text{ or}$$

$$SD_{\text{additivity}} = SE_{E_b(d_a, d_b)} \cdot \sqrt{df_{\text{additivity}} + 1} \quad (A20)$$

Closed Forms of Partial Derivatives of Eq. A18

The partial derivatives for Eq. A18 were obtained using Mathematica, and are provided in Eq. A21.

$$\frac{\partial E_a}{\partial n_a} = \frac{E_{\text{max},a} \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{n_a} \left((d_a + IC_{50,a} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}}) n_a \log \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right) - IC_{50,a} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} n_b \log \left(\frac{d_b}{IC_{50,b}} \right) \right)}{\left(1 + \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{n_a} \right)^2 (d_a + IC_{50,a} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}}) n_a}$$

$$\frac{\partial E_a}{\partial IC_{50,b}} = \frac{d_a E_{\text{max},a} \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{n_a} n_a}{IC_{50,a} \left(1 + \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{n_a} \right)^2 (d_a + IC_{50,a} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}})}$$

$$\frac{\partial E_a}{\partial IC_{50,b}} = \frac{E_{\max,a} IC_{50,a} \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{\frac{n_b}{n_a}} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} n_b}{IC_{50,b} \left(1 + \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^2 \right) (d_a + IC_{50,a} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}})}$$

$$\frac{\partial E_a}{\partial n_b} = \frac{E_{\max,a} IC_{50,a} \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{\frac{n_b}{n_a}} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \log \left(\frac{d_b}{IC_{50,b}} \right)}{\left(1 + \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^2 \right) (d_a + IC_{50,a} \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}})}$$

$$\frac{\partial E_a}{\partial E_{\max,a}} = \frac{\left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^{\frac{n_b}{n_a}}}{1 + \left(\frac{d_a}{IC_{50,a}} + \left(\frac{d_b}{IC_{50,b}} \right)^{\frac{n_b}{n_a}} \right)^2} \quad (A21)$$

Quantification of extent of interactivity

The extent of interactivity (EI) is calculated as the ratio of the experimental ($d_a + d_b$) and (logarithmic mean of $C_{\text{additivity},a}$ and $C_{\text{additivity},b}$ on the two boundaries of Additivity Envelope) found at the same effect level $E_{\text{comb,observed}}$ in a fixed-ratio dosing experiment. Hence, EI can be calculated from Additivity Envelope generated for individual (d_a/d_b) combinations, using the methods outlined below. To determine $C_{\text{additivity},a}$ we need to back-calculate the d_a and d_b combination that produces $E_{\text{comb,observed}}$ under the assumption of no interactivity; these terms are defined as d_{abacka} and d_{bbacka} . For convenience, the concentrations d_{abacka} and d_{bbacka} are expressed in IC_{50} -equivalents. When d_{abacka} and d_{bbacka} are known, $C_{\text{additivity},a}$ can be calculated according to Eq. A22.

$$C_{\text{additivity},a} = d_{\text{abacka}} + d_{\text{bbacka}} \quad (A22)$$

Solving for d_{abacka} and d_{bbacka} requires a set of equations, as follows. We first used Eq. A23 to describe their ratio (R) since Additivity Envelope is constructed based on R of d_a to d_b . Substituting d_{abacka} and d_{bbacka} into the general equation for $E_{\text{additivity},a}$ (Eq. 4) yielded Eq. A24, where d'_{bbacka} is the amount of Drug B that is equi-effective with d_{abacka} of Drug A, and d'_{abacka} of Drug A is equi-effective with d_{bbacka} of Drug B.

$$\frac{d_{\text{abacka}}}{d_{\text{bbacka}}} = R \quad (A23)$$

$$E_{\text{additivity},a} = E_{\max,a} \frac{(d_{\text{abacka}} + d'_{\text{abacka}})^{\frac{n_a}{n_a}}}{1 + (d_{\text{abacka}} + d'_{\text{abacka}})^{\frac{n_a}{n_a}}} \quad (A24)$$

In view of the analogous form of Eq. A24 and Eq. A1, $E_{additivity,a}$ can be written as $f_a(d_{abacka} + d'_{abacka})$. In analogy with Eq. A6, we then obtained Eq. A25.

$$d_{abacka} + d'_{abacka} = f_a^{-1}(E_{additivity,a}) = \frac{E_{additivity,a}^{1/n_a}}{(E_{\max,a} - E_{additivity,a})^{1/n_a}} \quad (\text{A25})$$

Let E_b be the effect caused by d_{bbacka} . In analogy with Eq. A6, d'_{abacka} is described by Eq. A26, and E_b by Eq. A27.

$$d'_{abacka} = f_a^{-1}(f_b(d_{bbacka})) = f_a^{-1}(E_b) = \frac{E_b^{1/n_a}}{(E_{\max,a} - E_b)^{1/n_a}} \quad (\text{A26})$$

$$E_b = E_{\max,b} \frac{d_{bbacka}^{n_b}}{1 + (d_{bbacka})^{n_b}} \quad (\text{A27})$$

Substituting Eq. A23 sequentially into Eq. A27, A26, A25, and A24, followed by simplification yielded Eq. A28. Eq. A28 shows d_{abacka} as the only unknown. The root for d_{abacka} can be determined numerically. Next, Eq. A22 and A23 were combined to yield Eq. A29. Eq. A30 and A31 are the analogous equations for $C_{additivity,b}$.

$$\frac{E_{additivity,a}^{1/n_a}}{(E_{\max,a} - E_{additivity,a})^{1/n_a}} - d_{abacka} = \left(\frac{E_{\max,b} \cdot (d_{abacka}/R)^{n_b}}{E_{\max,a} + (E_{\max,a} - E_{\max,b}) \cdot (d_{abacka}/R)^{n_b}} \right)^{1/n_a} \quad (\text{A28})$$

$$C_{additivity,a} = d_{abacka} \cdot (1 + 1/R) \quad (\text{A29})$$

$$\frac{E_{additivity,b}^{1/n_b}}{(E_{\max,b} - E_{additivity,b})^{1/n_b}} - d_{abacka} = \left(\frac{E_{\max,a} \cdot (d_{bbackb} \cdot R)^{n_a}}{E_{\max,b} + (E_{\max,b} - E_{\max,a}) \cdot (d_{bbackb} \cdot R)^{n_a}} \right)^{1/n_b} \quad (\text{A30})$$

$$C_{\text{additivity},b} = d_{\text{bbackb}} \cdot (1+R) \quad (\text{A31})$$

For cases with unequal E_{max} , when $E_{\text{comb,observed}}$ exceeds the lower E_{max} , $E_{\text{additivity}}$ is replaced by E_{single} . In these cases, $C_{\text{additivity}}$ has an algebraic solution. For $E_{\text{max},a} < E_{\text{max},b}$ and $E_{\text{comb,observed}} > E_{\text{max},a}$ we obtained Eq. A32, which when combined with Eq. A23 yielded Eq. A33. The reverse situation where $E_{\text{max},a} > E_{\text{max},b}$ and $E_{\text{comb,observed}} > E_{\text{max},b}$ is shown in Eq. A34. EI is calculated using Eq. A35.

$$d_{\text{bbacka}} = f_b^{-1}(E_{\text{comb,observed}}) = \frac{E_{\text{comb,observed}}^{1/n_b}}{(E_{\text{max},b} - E_{\text{comb,observed}})^{1/n_b}} \quad (\text{A32})$$

$$C_{\text{additivity},a} = d_{\text{abacka}} + d_{\text{bbacka}} = \frac{E_{\text{comb,observed}}^{1/n_b}}{(E_{\text{max},b} - E_{\text{comb,observed}})^{1/n_b}} \cdot (1+R) \quad (\text{A33})$$

$$C_{\text{additivity},b} = d_{\text{abackb}} + d_{\text{bbackb}} = \frac{E_{\text{comb,observed}}^{1/n_a}}{(E_{\text{max},b} - E_{\text{comb,observed}})^{1/n_a}} \cdot (1 + 1/R) \quad (\text{A34})$$

$$\text{EI} = \frac{d_a + d_b}{\sqrt{C_{\text{additivity},a} \cdot C_{\text{additivity},b}}} \quad (\text{A35})$$

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Fig. (1). Existing Lowe Additivity-based methods are not suitable to analyze interactivity of drugs with nonparallel *C-E*

(A) Combinations of two drugs with parallel *C-E*. After normalization, for example with respect to IC_{50} , the curves overlap and the two drugs act the same. The combined effect of the two drugs in any combination ratio is then expected to follow the same *C-E*. If an experimental observation provides a certain level of effect at a combined concentration that is lower than expected (*i.e.*, the observation falls left of the zero-interactivity *C-E*), then the combination is synergistic. An observation to the right of the zero-interactivity *C-E* indicates antagonism. This is captured by the Loewe Additivity equation (see text). (B) Nonparallel *C-E*. The curves do not overlap after normalization, and the zero-interactivity *C-E* cannot be simply concluded. The Loewe Additivity equation cannot be applied and methods based on the Loewe Additivity equation are not theoretically correct.

Fig. (2). Uncertainty Envelopes for drugs with parallel or nonparallel C-E and with equal or unequal E_{max}

Thin solid line: $C-E_{single,a}$. Dotted line: $C-E_{single,b}$. (A) UE for two drugs with equal E_{max} (100%) and equal n (0.7) was calculated using Eq. 2. The two $C-E_{additivity}$ for Drugs A and B overlapped with each other, yielding a single zero-interactivity $C-E_{additivity}$. (B) UE (thick solid lines) for two drugs with equal E_{max} (100%) and unequal n ($n_a=0.7$, $n_b=2.5$), at 1:1 $R_{da:db}$, was calculated according to Eq. 3. The horizontal line denotes the conversion of an amount d_a of Drug-A to an equi-effective amount d_b' of Drug-B. See text for the calculation of a single point of $f_b(d_b+d_b')$ denoted by the star. Repeating this for other d_a values yielded the full $C-E_{additivity,b}$. Similar steps were used to obtain $C-E_{additivity,a}$. The two curves circumscribe the Additivity Envelope (light shade). The vertical distance between the upper and lower envelope boundaries is the envelope height (h) and the horizontal distance is the envelope width (w). (C) UE for two drugs with unequal E_{max} ($E_{max,a}=70\%$, $E_{max,b}=100\%$) and unequal n ($n_a=0.7$, $n_b=2.5$) was calculated according to Eq. 4. For $E=70\%$, d_b could not be converted to d_a' . Hence, the Envelope was “broken” at $E_{max,a}$ (point denoted by star). We extended the Envelope by substituting with $f_b(d_b)$ (see text), resulting in a closed Additivity Envelope (light shade).

Fig. (3). Shape and size of Additivity Envelope

In silico studies were conducted to identify the determinants of the shape and size of Additivity Envelope. **(A)** Effect of $R_{da:db}$ on the Envelope shape and size. For two drugs with equal E_{max} and unequal n ($E_{max,a}=100\%$, $n_a=0.7$; $E_{max,b}=100\%$, $n_b=2.5$). Two sets of heavy broken lines: One envelope for 1:10 $R_{da:db}$ (darker shade) and one for 10:1 $R_{da:db}$ (lighter shade) $C-E_{single,a}$ and $C-E_{single,b}$ are shown in Fig. (2B). **(B)** Effect of $R_{na:nb}$ on the envelope shape and size. For two drugs with equal E_{max} of 100% at 1:1 $R_{da:db}$. Three envelopes are shown. For equal n values ($n_a = n_b = 2.5$), the envelope collapsed to a single curve ($h=0$). The other two Envelopes compare two drugs with a 5-fold difference in the n values, either with shallow or steep $C-E$ curve shape, *i.e.*, $n_a=0.5$ and $n_b=2.5$ (shallow) *vs.* $n_a=2.5$ and $n_b=12.5$ (steeper), the combination with the steeper curve shapes showed greater h at one IC_{50} unit but similar w . **(C and D)** Effects of $R_{da:db}$ and $R_{na:nb}$ on the envelope height h and width w . Simulations with varying $R_{da:db}$ values used $n_a=2.5$, $n_b=0.7$. Simulations with varying $R_{na:nb}$ values used $n_a=1$, $d_a:d_b=1$.

Fig. (4). Comparison of interactivity analysis methods for combinations of agents with nonparallel $C-E$ and equal E_{max}

Combinations of doxorubicin and suramin were studied using the fixed-ratio dosing design.

Symbols are means and standard deviations of $C-E_{comb,observed}$. (A and B) $C-E$ of single agent doxorubicin and suramin, and their combinations at six $R_{da:db}$ (S1D400, S1D200, S1D100, S1D50, S1D10, and S1D5). For example, S1D400 contains IC_{50} -equivalent concentrations of suramin:doxorubicin in a 1:400 ratio, in serial dilutions. Relative Units are relative to the lowest concentration of the dilution series. Figs. (4C–4F) are for the S1D400

combination analyzed by four methods. In UE and Curve-Shift analysis, drug concentrations are normalized to the concentration that produces 50% E and are expressed as IC_{50} equivalents. (C) UE analysis. Dotted and solid lines are the upper and lower boundaries of Additivity Envelope (note the two $C-E_{additivity}$ for doxorubicin and suramin overlap each other). The shaded area indicates UE (which equals Additivity Envelope plus 95% CI). Symbols are experimental data (mean $\pm$ SD) of $C-E_{comb,observed}$. Comparison of experimental $C-E_{comb,observed}$ to UE indicates nature of interactivity; $C-E_{comb,observed}$ falling within UE indicates additivity, whereas $C-E_{comb,observed}$ that is greater or inferior relative to UE indicate significant synergy and antagonism ($p<0.05$). (D) Curve-Shift analysis. Curves are the best-fitting $C-E_{single}$ to experimental data by nonlinear regression. Symbols are experimental data (mean $\pm$ SD) of $C-E_{comb,observed}$. Experimental $C-E_{comb,observed}$ data points located within the two $C-E_{single}$ curves indicate additivity, whereas data points located to the left indicate synergy and data points to the right indicate antagonism. (E) Isobologram analysis. Diagonal line: line of additivity at $E=50\%$. Dot: $E_{comb,observed}$. Because there were no experimental data points that yielded precisely 50% effect, we used the interpolated value from the best-fit $C-E_{comb}$ curve. The experimental $C-E_{comb,observed}$ data point located below the line of additivity, indicating synergy. (F) Combination-Index analysis. Calculated Combination Index values (dots) are shown; values below 1 indicate synergy and values above 1 indicate antagonism.

Fig. (5). Comparison of interactivity analysis methods for combinations of agents with nonparallel $C-E$ and unequal E_{max}

Combinations of suramin and paclitaxel were studied using the fixed-concentration dosing (*i.e.*, fixed suramin concentrations plus varying paclitaxel concentrations). (**A** and **B**) $C-E$ of single agent paclitaxel and suramin, and their combinations at seven suramin concentrations. (**C–F**) Analysis of results obtained from combinations containing 200 μM suramin (equaled 62% of its IC_{50}), analyzed by four methods, as described in Fig. (4). (**G** and **H**) Analysis of results obtained from combinations containing 800 μM suramin (equaled 252% of its IC_{50})

using UE and Curve-Shift. The other two methods were not applicable (see text). In C and G, curves are upper and lower boundaries of Additivity Envelope, and Shaded area represents the Uncertainty Envelope.

Parameter estimates for $C-E$ of single agents and combinations. Parameter estimates, approximate standard error of the estimate, and correlation between parameters (Correlation Coefficients) were obtained as output of nonlinear regression fitting of the Hill equation to experimentally obtained $C-E_{single}$. The correlation coefficients were used in the error propagation calculations (see Appendix).

Table 1

Treatment	Parameter	Estimate	Standard Error	Correlation Coefficients	
				IC_{50}	n
Doxorubicin and suramin (nonparallel $C-E$, equal E_{max} of 100%)					
Doxorubicin	IC_{50} (nM)	55.4	9.0	1.000	-0.003
	n	0.709	0.074	-0.003	1.000
Suramin	IC_{50} (μM)	310.3	22.8	1.000	0.303
	n	1.693	0.152	0.303	1.000
Paclitaxel and suramin (nonparallel $C-E$, unequal E_{max})					
Paclitaxel	IC_{50} (nM)	11.0	0.28	1.000	-0.276
	n	2.23	0.131	-0.276	1.000
	E_{max} %	58.5	0.55	0.497	-0.491
Suramin	IC_{50} (μM)	317.3	6.48	1.000	0.044
	n	1.25	0.06	0.044	1.000
	E_{max} %	100	0	0	0

Table 2

Drug interactivity analysis by Uncertainty Envelope and three existing Loewe Additivity-based methods. Experiments used PC3 cells. E_{Dox} (or E_{Pac}) and E_{Sur} are analogous to the zero-interactivity $E_{additivity,a}$ and $E_{additivity,b}$ described in Eq. 1. $E_{comb,observed}$ is the experimentally measured effect for the combination. (A) Combinations of doxorubicin (Dox) and suramin (Sur) using the fixed-ratio dosing method. SID400 through SID5 correspond to the ratios of suramin-to-doxorubicin concentrations in IC_{50} equivalents, in serial dilutions. (B) Combinations of paclitaxel (Pac) and suramin using the fixed-ratio dosing method (S50 through S800 correspond to 7 fixed suramin concentrations between 50 and 800 μ M, with paclitaxel concentrations changing from 0.1 to 300 nM). Isobologram analysis was limited to $E=50\%$ (see text; ND or not determined because E was above or below 50%). Combination-Index analysis was not applicable (NA) to analyze paclitaxel+suramin combinations that yielded E exceeding E_{max} of single agent paclitaxel (see text). EI and CI are values of extent of interactivity and Combination Index as calculated by Uncertainty Envelope and Combination-Index methods, respectively.

A. Combinations of doxorubicin and suramin using the fixed-ratio method													
Combination	Drug Concentration		$E_{comb,observed}$ % $\pm$ SD	Additivity-Envelope		Uncertainty-Envelope		Interactivity by method of analysis					
				E_{Dox} or E_{Pac} % $\pm$ SEM	E_{Sur} % $\pm$ SEM	Lower boundary	Higher boundary	Uncertainty Envelope		Isobologram	Curve-Shift	Combination-Index	
	Nature	EI						Nature	Nature			Nature	Nature
	SID400	6250		90	97.0 $\pm$ 1.0	96.6 $\pm$ 1.2	96.8 $\pm$ 1.1	94.2	99.0	Additivity	0.88	ND	Additivity
625		9	80.3 $\pm$ 3.1	84.8 $\pm$ 2.7	85.0 $\pm$ 2.7	79.3	90.4	Additivity	1.57	ND	Antagonism	Antagonism	1.57
62.5		0.9	59.3 $\pm$ 4.4	52.1 $\pm$ 2.9	52.3 $\pm$ 2.9	46.4	58.0	Synergy	0.67	Synergy	Synergy	Synergy	0.67
6.25		0.09	16.0 $\pm$ 3.9	17.6 $\pm$ 2.9	17.6 $\pm$ 2.9	11.8	23.3	Additivity	1.17	ND	Additivity	Antagonism	1.17
0.625		0.009	1.9 $\pm$ 3.8	4.0 $\pm$ 1.3	4.0 $\pm$ 1.3	1.30	6.69	Additivity	2.94	ND	Additivity	Antagonism	2.94
SID200	6250	180	96.6 $\pm$ 0.9	96.6 $\pm$ 1.2	97.0 $\pm$ 1.0	94.2	99.0	Additivity	1.09	ND	Additivity	Antagonism	1.09
	625	18	89.3 $\pm$ 1.7	84.8 $\pm$ 2.7	85.2 $\pm$ 2.6	79.3	90.5	Additivity	0.58	ND	Synergy	Synergy	0.58
	62.5	1.8	74.6 $\pm$ 3.3	52.1 $\pm$ 2.9	52.4 $\pm$ 2.9	46.4	58.1	Synergy	0.25	Synergy	Synergy	Synergy	0.25
	6.25	0.18	20.9 $\pm$ 5.3	17.6 $\pm$ 2.9	17.6 $\pm$ 2.9	11.8	23.3	Additivity	0.74	ND	Additivity	Synergy	0.74
	0.625	0.018	2.9 $\pm$ 7.6	4.0 $\pm$ 1.3	4.0 $\pm$ 1.3	1.30	6.69	Additivity	1.60	ND	Additivity	Antagonism	1.60
SID100	6250	360	97.1 $\pm$ 1.3	96.6 $\pm$ 1.2	97.3 $\pm$ 0.8	94.3	99.0	Additivity	0.95	ND	Additivity	Synergy	0.94
	625	36	94.7 $\pm$ 0.9	84.8 $\pm$ 2.7	85.7 $\pm$ 2.5	79.3	90.7	Synergy	0.22	ND	Synergy	Synergy	0.21
	62.5	3.6	76.1 $\pm$ 2.2	52.1 $\pm$ 2.9	52.6 $\pm$ 2.8	46.4	58.3	Synergy	0.23	Synergy	Synergy	Synergy	0.23
	6.25	0.36	35.0 $\pm$ 4.6	17.6 $\pm$ 2.9	17.6 $\pm$ 2.9	11.8	23.4	Synergy	0.27	ND	Synergy	Synergy	0.27

A. Combinations of doxorubicin and suramin using the fixed-ratio method														
Combination	Drug Concentration		$E_{\text{comb, observed}} \%$ $\pm$ SD	Additivity-Envelope			Uncertainty-Envelope		Interactivity by method of analysis					
				$E_{\text{Dox or } E_{\text{Pac}}}$	E_{Sur}	% $\pm$ SEM	Lower boundary	Higher boundary	Uncertainty Envelope		Isobologram	Curve-Shift	Combination-Index	
	Nature	EI							Nature	Nature			Nature	CI
SID50	0.625	0.036	0.0 $\pm$ 4.9	4.0 $\pm$ 1.3	4.0 $\pm$ 1.3	4.0 $\pm$ 1.3	1.30	6.70	Additivity	>10	ND	Additivity	Antagonism	>10
	6250	720	98.0 $\pm$ 1.1	96.6 $\pm$ 1.3	97.9 $\pm$ 0.6	94.5	99.1	Additivity	0.71	ND	Additivity	Synergy	0.70	
	625	72	96.8 $\pm$ 0.6	84.8 $\pm$ 2.8	86.5 $\pm$ 2.3	79.3	91.1	Synergy	0.12	ND	Synergy	Synergy	0.12	
	62.5	7.2	80.9 $\pm$ 1.7	52.1 $\pm$ 2.9	53.1 $\pm$ 2.8	46.4	58.7	Synergy	0.16	Synergy	Synergy	Synergy	0.16	
	6.25	0.72	39.7 $\pm$ 4.6	17.6 $\pm$ 2.9	17.7 $\pm$ 2.9	11.8	23.4	Synergy	0.21	ND	Synergy	Synergy	0.21	
	0.625	0.072	1.6 $\pm$ 4.8	4.0 $\pm$ 1.3	4.0 $\pm$ 1.3	1.30	6.70	Additivity	3.77	ND	Additivity	Antagonism	3.77	
SID10	6250	3600	97.2 $\pm$ 0.9	98.7 $\pm$ 0.4	99.3 $\pm$ 0.3	97.9	99.8	Antagonism	2.39	ND	Additivity	Antagonism	2.19	
	625	360	95.9 $\pm$ 1.4	85.8 $\pm$ 2.6	91.0 $\pm$ 1.5	80.7	94.0	Synergy	0.33	ND	Synergy	Synergy	0.31	
	62.5	36	82.6 $\pm$ 1.3	52.2 $\pm$ 2.9	56.5 $\pm$ 2.6	46.5	61.8	Synergy	0.17	Synergy	Synergy	Synergy	0.17	
	6.25	3.6	40.3 $\pm$ 4.8	17.6 $\pm$ 2.9	18.3 $\pm$ 2.9	11.8	24.0	Synergy	0.21	ND	Synergy	Synergy	0.21	
	0.625	0.36	0.9 $\pm$ 5.4	4.0 $\pm$ 1.3	4.0 $\pm$ 1.4	1.30	6.75	Additivity	8.57	ND	Additivity	Antagonism	8.57	
	6250	7200	97.4 $\pm$ 0.5	99.5 $\pm$ 0.2	99.7 $\pm$ 0.1	99.1	100	Antagonism	3.84	ND	Additivity	Antagonism	3.41	
SID5	625	720	95.4 $\pm$ 0.9	88.9 $\pm$ 1.9	94.0 $\pm$ 1.2	85.2	96.4	Additivity	0.60	ND	Additivity	Synergy	0.54	
	62.5	72	81.8 $\pm$ 1.8	52.6 $\pm$ 2.8	60.4 $\pm$ 2.5	46.9	65.4	Synergy	0.24	Synergy	Synergy	Synergy	0.23	
	6.25	7.2	38.4 $\pm$ 6.9	17.6 $\pm$ 2.9	19.0 $\pm$ 2.9	11.8	24.7	Synergy	0.25	ND	Synergy	Synergy	0.25	
	0.625	0.72	5.1 $\pm$ 3.9	4.0 $\pm$ 1.3	4.1 $\pm$ 1.4	1.30	6.81	Additivity	0.71	ND	Additivity	Synergy	0.71	
B. Combinations of paclitaxel and suramin using the fixed-concentration method														
S50	300	50	66.4 $\pm$ 3.0	58.5 $\pm$ 0.5	61.8 $\pm$ 0.9	55.1	64.1	Synergy	0.19	ND	Additivity	NA	NA	
	100	50	68.5 $\pm$ 2.5	58.1 $\pm$ 0.5	61.5 $\pm$ 0.9	54.8	63.7	Synergy	0.16	ND	Additivity	NA	NA	
	30	50	62.5 $\pm$ 2.1	54.4 $\pm$ 0.7	57.0 $\pm$ 1.1	50.1	62.1	Synergy	0.25	ND	Synergy	NA	NA	
	10	50	23.1 $\pm$ 4	39.2 $\pm$ 1.1	34.3 $\pm$ 1.2	28.3	45.8	Antagonism	1.60	Antagonism	Antagonism	Antagonism	1.52	
	3	50	0	19.8 $\pm$ 0.8	13.2 $\pm$ 1.0	8.2	24.7	Antagonism	>10	ND	Antagonism	Antagonism	>10	
	1	50	0	12.5 $\pm$ 0.7	9.6 $\pm$ 0.8	5.5	16.6	Antagonism	>10	ND	Antagonism	Antagonism	>10	
S70	0.1	50	0	9.4 $\pm$ 0.6	9.1 $\pm$ 0.9	4.6	13.6	Antagonism	>10	ND	Antagonism	Antagonism	>10	
	300	70	76.9 $\pm$ 3.2	58.5 $\pm$ 0.5	63.1 $\pm$ 0.4	55.1	65.2	Synergy	0.12	ND	Additivity	NA	NA	

A. Combinations of doxorubicin and suramin using the fixed-ratio method														
Combination	Drug Concentration		$E_{\text{comb, observed}} \pm \text{SD}$	Additivity-Envelope			Uncertainty-Envelope		Interactivity by method of analysis					
	Dox or Pac, nM	Sur, μM		$E_{\text{Dox or } E_{\text{Pac}}}$	E_{Sur}	% $\pm$ SEM	Lower boundary	Higher boundary	Uncertainty Envelope		Isobologram	Curve-Shift	Combination-Index	
									Nature	EI			Nature	Nature
S100	100	70	76.4 $\pm$ 4.3	58.1 $\pm$ 0.5	62.8 $\pm$ 0.4	54.8	64.9	Synergy	0.12	ND	Additivity	Nature	NA	NA
	30	70	71.1 $\pm$ 5.3	54.7 $\pm$ 0.7	58.5 $\pm$ 1.0	50.4	63.5	Synergy	0.18	ND	Synergy	Nature	NA	NA
	10	70	33.1 $\pm$ 4.3	41.3 $\pm$ 1.0	37.2 $\pm$ 1.2	31.5	47.8	Additivity	1.24	Antagonism	Antagonism	Antagonism	Antagonism	1.20
	3	70	13.8 $\pm$ 3.1	23.9 $\pm$ 0.8	17.2 $\pm$ 1.0	12.2	28.9	Additivity	1.40	ND	Antagonism	Antagonism	Antagonism	1.42
	1	70	10 $\pm$ 3.5	16.8 $\pm$ 0.7	13.7 $\pm$ 1.0	9.1	21.2	Additivity	1.44	ND	Antagonism	Antagonism	Antagonism	1.47
	0.1	70	8.6 $\pm$ 5.1	13.5 $\pm$ 0.6	13.2 $\pm$ 1.1	7.9	18.4	Additivity	1.48	ND	Antagonism	Antagonism	Antagonism	1.48
	300	100	70.7 $\pm$ 2.6	58.5 $\pm$ 0.5	64.8 $\pm$ 0.4	55.1	66.8	Synergy	0.26	ND	Additivity	Nature	NA	NA
	100	100	71.0 $\pm$ 5.0	58.1 $\pm$ 0.5	64.5 $\pm$ 0.4	54.9	66.5	Synergy	0.26	ND	Additivity	Nature	NA	NA
	30	100	70.1 $\pm$ 3.1	55.0 $\pm$ 0.7	60.6 $\pm$ 1.0	50.9	65.4	Synergy	0.27	ND	Synergy	Nature	NA	NA
	10	100	34.3 $\pm$ 4.4	43.8 $\pm$ 1.0	41.2 $\pm$ 1.1	35.7	50.1	Antagonism	1.34	Antagonism	Antagonism	Antagonism	Antagonism	1.31
S200	3	100	18.1 $\pm$ 3.4	29.1 $\pm$ 0.8	22.9 $\pm$ 1.0	18.1	33.9	Antagonism	1.42	ND	Antagonism	Antagonism	Antagonism	1.45
	1	100	14.1 $\pm$ 3.9	22.7 $\pm$ 0.7	19.7 $\pm$ 1.0	14.8	27.1	Antagonism	1.47	ND	Antagonism	Antagonism	Antagonism	1.49
	0.1	100	12.1 $\pm$ 2.5	19.5 $\pm$ 0.7	19.1 $\pm$ 1.1	13.6	24.7	Antagonism	1.55	ND	Antagonism	Antagonism	Antagonism	1.56
	300	200	66.6 $\pm$ 1.6	58.5 $\pm$ 0.5	69.7 $\pm$ 0.3	55.1	71.2	Additivity	0.73	ND	Additivity	Nature	NA	NA
	100	200	68.5 $\pm$ 1.1	58.2 $\pm$ 0.5	69.4 $\pm$ 0.3	54.9	71.0	Additivity	0.61	ND	Additivity	Nature	NA	NA
	30	200	65.5 $\pm$ 2.0	55.9 $\pm$ 0.6	66.4 $\pm$ 0.9	52.0	70.8	Additivity	0.63	ND	Additivity	Nature	NA	NA
	10	200	48.1 $\pm$ 3.0	47.4 $\pm$ 0.9	52.0 $\pm$ 1.1	44.0	57.2	Additivity	1.10	Antagonism	Antagonism	Antagonism	Antagonism	1.13
	3	200	33.1 $\pm$ 1.5	41.7 $\pm$ 0.6	38.8 $\pm$ 0.8	34.9	45.5	Antagonism	1.30	ND	Antagonism	Antagonism	Antagonism	1.35
	1	200	31.7 $\pm$ 2.9	38.1 $\pm$ 0.5	36.4 $\pm$ 0.8	32.6	41.3	Antagonism	1.22	ND	Antagonism	Antagonism	Antagonism	1.25
	0.1	200	30.7 $\pm$ 3.1	36.2 $\pm$ 0.5	36.0 $\pm$ 0.9	31.8	40.2	Antagonism	1.21	ND	Antagonism	Antagonism	Antagonism	1.22
S300	300	300	66.7 $\pm$ 1.7	58.5 $\pm$ 0.5	73.5 $\pm$ 0.3	55.1	74.7	Additivity	1.08	ND	Additivity	Nature	NA	NA
	100	300	65.5 $\pm$ 3.0	58.2 $\pm$ 0.5	73.3 $\pm$ 0.3	54.9	74.6	Additivity	1.10	ND	Additivity	Nature	NA	NA
	30	300	64.7 $\pm$ 3.4	56.7 $\pm$ 0.6	70.9 $\pm$ 0.8	53.1	75.0	Additivity	0.90	ND	Additivity	Nature	NA	NA
	10	300	54.8 $\pm$ 3.4	53.6 $\pm$ 0.8	60.0 $\pm$ 1.1	48.8	65.3	Additivity	1.03	Antagonism	Additivity	Antagonism	Antagonism	1.08
	3	300	44.7 $\pm$ 2.9	50.4 $\pm$ 0.5	50.3 $\pm$ 0.8	46.3	54.2	Antagonism	1.22	ND	Antagonism	Antagonism	Antagonism	1.28

A. Combinations of doxorubicin and suramin using the fixed-ratio method													
Combination	Drug Concentration		$E_{\text{comb, observed}} \pm \text{SD}$	Additivity-Envelope		Uncertainty-Envelope		Interactivity by method of analysis					
				E_{Dox} or E_{Pac}	E_{Sur}	Lower boundary	Higher boundary	Uncertainty Envelope		Isobologram	Curve-Shift	Combination-Index	
								Nature	El			Nature	CI
S400	1	300	44.3 ± 3.5	49.1 ± 0.4	48.5 ± 0.6	45.5	51.6	Antagonism	1.16	ND	Antagonism	Antagonism	1.19
	0.1	300	42.3 ± 3.2	48.3 ± 0.4	48.3 ± 0.6	45.1	51.4	Antagonism	1.21	ND	Antagonism	Antagonism	1.22
	300	400	72.2 ± 1.5	58.5 ± 0.5	76.5 ± 0.2	55.1	77.5	Additivity	0.94	ND	Additivity	NA	NA
	100	400	71.3 ± 1.6	58.3 ± 0.5	76.4 ± 0.2	55.2	77.5	Additivity	0.98	ND	Additivity	NA	NA
	30	400	69.1 ± 3.0	58.0 ± 0.5	74.5 ± 0.8	54.8	78.3	Additivity	0.94	ND	Additivity	NA	NA
	10	400	67.9 ± 1.5	57.6 ± 0.7	65.9 ± 1.1	53.4	71.4	Additivity	0.81	ND	Synergy	NA	NA
	3	400	59.1 ± 3.4	57.3 ± 0.5	58.7 ± 0.9	54.1	63.2	Additivity	0.96	ND	Additivity	NA	NA
	1	400	58.2 ± 1.9	57.2 ± 0.4	57.4 ± 0.7	53.8	61.0	Additivity	0.97	ND	Additivity	Synergy	0.98
	0.1	400	58.4 ± 2.0	57.2 ± 0.4	57.2 ± 0.7	53.8	60.6	Additivity	0.96	ND	Additivity	Synergy	0.96
	300	800	71.8 ± 1.2	76.0 ± 0.0	84.3 ± 0.1	76.0	84.8	Antagonism	1.92	ND	Additivity	NA	NA
S800	100	800	72.1 ± 1.3	76.0 ± 0.1	84.2 ± 0.2	75.5	85.1	Antagonism	1.76	ND	Additivity	NA	NA
	30	800	70.9 ± 1.0	76.0 ± 0.3	83.3 ± 0.6	73.9	86.4	Antagonism	1.54	ND	Additivity	NA	NA
	10	800	71.8 ± 1.6	76.0 ± 0.5	79.5 ± 1.2	72.9	85.1	Antagonism	1.28	ND	Additivity	NA	NA
	3	800	69.4 ± 2.4	76.0 ± 0.5	76.6 ± 1.2	70.8	82.4	Antagonism	1.32	ND	Additivity	NA	NA
	1	800	66.9 ± 1.5	76.0 ± 0.6	76.1 ± 1.1	70.7	81.6	Antagonism	1.44	ND	Additivity	NA	NA
	0.1	800	68.1 ± 1.8	76.0 ± 0.6	76.0 ± 1.1	70.7	81.4	Antagonism	1.38	ND	Additivity	NA	NA