Evaluation of Combination Chemotherapy: Integration of Nonlinear Regression, Curve Shift, Isobologram, and Combination Index Analyses

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ABSTRACT

Isobologram and combination index (CI) analyses are the two most popular methods for evaluating drug interactions in combination cancer chemotherapy. As the commonly used CI-based software program uses linear regression, our first objective was to evaluate the effects of logarithmic data transformation on data analysis and conclusions. Monte-Carlo simulations were conducted with experimentally relevant parameter values to generate error-containing effect or concentration-effect data of single agents and combinations. The simulated data were then analyzed with linear and nonlinear regression. The results showed that data transformation reduced the accuracy and precision of the regression-derived IC_{50} curve shape parameter and CI values. Furthermore, as neither isobologram nor CI analyses provide output of concentration-effect curves for investigator evaluation, our second objective was to develop a method and the associated computer program/algorithm to (a) normalize drug concentrations in IC_{50} equivalents and thereby enable simultaneous presentation of the curves for single agents and combinations in a single plot for visual inspection of potential curve shifts, (b) analyze concentration-effect data with nonlinear regression, and (c) use the curve shift analysis simultaneously with isobologram and CI analyses. The applicability of this method was shown with experimentally obtained data for single agent doxorubicin and suramin and their combinations in cultured tumor cells. In summary, this method, by incorporating nonlinear regression and curve shift analysis, although retaining the attractive features of isobologram and CI analyses, reduced the potential errors introduced by logarithmic data transformation, enabled visual inspection of data variability and goodness of fit of regression analysis, and simultaneously provided information on the extent of drug interaction at different combination ratios/concentrations and at different effect levels.

INTRODUCTION

Evaluation of drug-drug interaction is important in all areas of medicine and, in particular, in cancer chemotherapy where combination therapy is commonly used. The nature and quantitative extent of drug interaction is usually determined in in vitro studies. Two recent reviews describe the various evaluation methods (1, 2). These methods fall in three categories, each based on a different model of drug interaction. The Bliss independence model assumes that the combined effect of two agents equals the multiplication product of the effects of individual agents. This assumption is valid only for linear drug concentration-effect relationship (i.e., drug effect increases linearly with concentration) and not for nonlinear drug concentration-effect relationship such as the commonly observed sigmoidal curve. Hence, this model has limited applicability. The additivity envelope model was developed to describe the log-linear cell survival relationship observed in radiation studies and, because this relationship is not observed for cytotoxic agents, is not widely used. The Loewe additivity model is based on the assumption that a drug cannot interact with itself. The model additionally takes into account the sigmoidal shape of the concentration-effect relationship and is, therefore, more appropriate for evaluating drugs demonstrating such a relationship.

Methods based on the Loewe additivity model include the isobologram first described in 1872 (3), the interaction index calculation (4), the median effect method (5), and several three-dimensional surface-response models (6, 7). The isobologram method evaluates the interaction at a chosen effect level and is therefore useful to inspect the drug interaction at the corresponding concentration, often the median effect concentration. The surface response methods are more complex in their calculations and have not gained wide usage. The median effect method is the most commonly used; the original publication by Chou and Talalay (5) has >900 citations, and the derived software program to calculate combination indices (CI) is widely used. The following provides an overview of the isobologram and CI analyses of drug interaction based on concentration-effect data.

The drug-induced effect, E, is described by the Hill Equation (equation A; refs. 8, 9):

$$E = E_{\text{max}} \times \frac{C^n}{IC_{50} + C^n}$$

where E is the measured effect; C is the drug concentration; \(E_{\text{max}}\) is the full range of drug effect, usually at or near 100%; \(IC_{50}\) is the drug concentration producing the median effect of
50%; and n is the curve shape parameter describing the steepness of the concentration-effect relationship. The two key parameters in the Hill Equation are IC_{50} and n.

The isobologram analysis evaluates the nature of interaction of two drugs, i.e., drug A and drug B, as follows (10). First, the concentrations of drugs A and B required to produce a defined single-agent effect (e.g., IC_{50}), when used as single agents, are placed on the x and y axes in a two-coordinate plot, corresponding to (C_{A,0}) and (0, C_{B}), respectively. The line connecting these two points is the line of additivity. Second, the concentrations of the two drugs used in combination to provide the same effect, denoted as (c_{A}, c_{B}), are placed in the same plot. Synergy, additivity, or antagonism are indicated when (c_{A}) is located below, on, or above the line, respectively.

CI analysis, similar to isobologram analysis, provides qualitative information on the nature of drug interaction, and CI, a numerical value calculated as described in equation B, also provides a quantitative measure of the extent of drug interaction.

\[
CI = \frac{C_{A,x}}{IC_{A}} + \frac{C_{B,x}}{IC_{B}}
\]  

(C)

C_{A,x} and C_{B,x} are the concentrations of drug A and drug B used in combination to achieve x% drug effect. IC_{A,x} and IC_{B,x} are the concentrations for single agents to achieve the same effect. A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively.

In the Chou and Talalay method, the concentration-effect curve described by equation A is linearized by logarithmic transformation as shown by equation C (5):

\[
\log(\text{fu}^{-1} - 1) = \log(\text{fa}^{-1} - 1)^{-1} = \log(\text{IC}_{50}) - \log(\text{CM}),
\]  

where fu is the fraction of cells left unaffected after drug exposure, fa is the fraction of cells affected by the exposure, C is the drug concentration used, CM is the concentration to achieve the median effect, and n is the curve shape parameter. CM and n are equivalent to IC_{50} and n, respectively, in the Hill Equation. The values of n (obtained from the slope), nlog(CM) (obtained from the absolute value of the intercept), and, therefore, CM are obtained by plotting \log(\text{fu}^{-1} - 1) versus \log(C). The effects of logarithmic data transformation on data distribution and analysis results are not known. However, because errors in low and high drug effect levels (e.g., <10% or >90%) are exaggerated because of logarithmic transformation, it is conceivable that data transformation affects the precision and accuracy of IC_{50}, n, and CI obtained with linear regression analysis. In contrast, nonlinear regression analysis does not require data transformation and presents a theoretical advantage over linear regression. The first goal of the present study was to evaluate the effects of logarithmic data transformation on data analysis and conclusions.

Although isobologram and CI analyses provide information on the nature and extent of drug interaction at different concentrations of the drugs used in combination and/or at different effect levels, neither method provides the conventional, investigator-friendly plots of drug concentration-effect curves commonly used in pharmacological studies. In isobologram analysis, a separate plot is presented for each effect level and includes only the concentrations of the drugs in combination to produce the specified effect. The typical plots provided by CI analysis as used in the Chou and Talalay method show CI as a function of effect levels and do not include the corresponding drug concentrations either as single agents or combinations. Furthermore, isobologram and CI plots, because they are based on values (e.g., CI) calculated with the IC values derived from the concentration-effect curves, do not provide information on the variability of the actual data. Accordingly, an investigator would not be able to decide with confidence that the extent of synergy or antagonism indicated by these plots is significant compared with the data variability.

On the other hand, plots of effects as a function of concentrations enable an investigator to visually inspect data variability, goodness of fit by regression analysis. Hence, the second goal of the present study was to develop a nonlinear regression-based method and the associated computer program/algorithm that enable curve shift analysis and capture the strengths of isobologram and CI analyses. An earlier version of the computer program had been published (11).

### MATERIALS AND METHODS

#### Experimental Drug Concentration-Effect Data.

The experimental data were obtained with previously described methodologies (12). Briefly, rat prostate MAT-LyLu tumor cells were cultured and treated with suramin, doxorubicin, or combinations. Drug effect was measured as inhibition of bromodeoxyuridine incorporation. We used the bromodeoxyuridine assay because the results indicate the overall drug effects, including inhibition of cell growth and induction of cell death, and, in addition, indicate the residual replication ability. The latter is not provided by other cell growth assays such as microtetrazolium reduction or sulforhodamine assays. Furthermore, we found similar results with these three assays in doxorubicin-treated rat prostate MAT-LyLu tumor cells, whereas the bromodeoxyuridine results yielded the lowest data variability and greatest data reproducibility.

The rationale for using suramin was to enhance the tumor sensitivity to doxorubicin based on our earlier observations (12–14). This study used the fixed ratio method, where the doxorubicin and suramin concentrations were present in fixed ratios of concentrations corresponding to the IC_{50} equivalents of single agents. The stock solutions contained 0, 160, 320, 640, and 1280 μmol/L suramin combined with 10,000 nmol/L doxorubicin, representing approximate suramin-to-doxorubicin IC_{50} equivalent ratios of 0, 1:400, 1:200, 1:100, and 1:50, respectively (referred to as S1D400 and so on). Cells were treated with serial dilutions (10- to 100,000-fold diluted) of the stock solutions. Controls were processed similarly but without drugs. The concentrations of single-agent suramin treatment were 0, 10, 50, 100, 500, and 1000 μmol/L. The results were analyzed with linear and nonlinear regressions to obtain the corresponding IC_{50} and n (see below).

#### General Strategy for Simulations.

We examined the effects of data transformation on regression-derived IC_{50} and n values, sensitivity of these parameters to data variability at low and high effect levels (i.e., <10% and >90%), and the calculated CI values. These studies were done with computer simulations. The parameters used to generate simulated data were...
selected based on or derived from experimental data, where appropriate. The general simulation strategy was to first select appropriate values for the parameters (i.e., IC50, n, effect variability expressed as \(/H9268\)), and CI). These values, referred to as true values, were then used together with simulations to generate sets of concentration-effect curves, which were subsequently analyzed with linear or nonlinear regression. A comparison of the analysis results with true values indicated the precision and accuracy of the two regression methods. Note that simulation of a drug concentration-effect curve requires only IC50 and n values.

**Effect of Logarithmic Data Transformation on Accuracy and Precision of IC50 and n Values Obtained from Regression Analyses.** Fig. 1 outlines the procedures. For this study, the concentration-response curves were generated with arbitrarily chosen IC50 and n values. Monte-Carlo simulations were used to generate variability or error-containing concentration-effect curves for single agents according to equations A and B, with equation D:

\[
\text{simulated effect} = \text{preselected effect} + \sigma
\]

where \(\sigma\) is the normally distributed error with a mean value of 0. The SD for \(\sigma\) ranged from 0.1 to 5%. The simulations used 10 concentrations, which cover the conventional six to eight concentrations used in concentration-response experiments (typically performed in 96-well plates).

**Effect of Logarithmic Data Transformation on Accuracy and Precision of Calculated Combination Indices.** Fig. 2 outlines the procedures. In contrast to the study on IC50 and n determination for single agents, which was accomplished with arbitrarily chosen values, the determination of CI required using experimentally relevant concentration-response data. For this purpose, we used parameter values, including IC50, n, and CI values, which were based on the experimental results ob-

![Fig. 1 Outline of Monte-Carlo simulations to study the effects of logarithmic data transformation on the precision and accuracy of regression-derived IC50 and n values.](image)

![Fig. 2 Outline of Monte Carlo simulations to evaluate effects of logarithmic data transformation on precision and accuracy of the calculated CI values. A, simulated data without change. B, simulated data with artificially adding or subtracting 1 SD at <10% or >90% effect level.](image)
tained for the doxorubicin and suramin study described above. The SD values were varied according to the drug effect levels, as observed experimentally.

Effect of Logarithmic Data Transformation on Sensitivity of Regression-derived IC₅₀ and n Values to Data Variability. In linear regression analysis, IC₅₀ and n values are calculated based on \( \log(fa/H₁₀₀²) \) (equation 3). Because of the logarithmic transformation, the errors in fa are especially magnified at low or high fa levels or the asymptotic regions of the sigmoidal concentration-effect curve (e.g., <10% and >90%). We therefore evaluated the effects of data transformation under these conditions. For a two-drug combination, there are four potential permutations to study the effects of changes in these data points, i.e., data variability at low and high effect levels for each of the two drugs. We evaluated the effect of variability in the data for drug A at low (<10%) and high (>90%) effect levels. Note that similar studies can be done by introducing variability in the data for drug B at low and/or high effect levels. These analyses are not presented here because of space limitation.

For this purpose, the IC₅₀ and n values obtained from the experimental data for single-agent doxorubicin (70 nmol/L and 0.6, respectively) were used to simulate a concentration-effect curve and thereby identify the effects at 1 and 10,000 nmol/L doxorubicin concentration (equaling 7.2 and 95.5%, respectively). For comparison and to show the substantial effects of data variation at these high and low drug effect levels, we also calculated the concentration where the effect is near the median value, i.e., 100 nmol/L producing 55.5% effect. These effect values were then altered to include an error of up to one experimentally observed SD, which was 4% at <10% effect level, 3% at 55% effect level, and 1% at >90% effect level, and thereby generated effect levels between 3.2 and 11.2% at 1 nmol/L, between 52.3 and 58.3% at 100 nmol/L, and between 94.5 and 96.5% at 10,000 nmol/L. These error-containing effects levels and the corresponding concentrations were substituted into the original data set, and the resulting concentration-effect curves for single agents and combinations were analyzed with linear and nonlinear regressions to obtain IC₅₀ and n values.

Effect of Logarithmic Data Transformation on Sensitivity of CI Values to Data Variability. Fig. 2B outlines the procedures. Note that the methods are nearly identical to those outlined for the study of effects of logarithmic data transformation on the accuracy and precision of the calculated CI (Fig. 2A), with the exception of adding and subtracting from the effect (5%) a value (4%) equal to one experimentally observed SD. Also note that subtracting >5% SD value will result in negative drug effect, and it will not be possible to obtain the transformed effect by \( \log(fa/H₁₀₀²) \).

Development of Curve Shift Analysis and Its Incorporation with Isobologram and CI Analyses to Analyze Drug-Drug Interaction. We developed a curve shift method, in conjunction with isobologram and CI analyses, to analyze drug interaction. A computer program, written in SAS language and published elsewhere (6), was implemented to capture the strengths of all three analyses. The algorithm is outlined in Fig. 3. The applicability of this new method was shown with, as an example, experimentally obtained results of the doxorubicin/suramin combination study. Furthermore, the results of the studies outlined above indicated that logarithmic data transformation compromised data distribution and analysis, thereby introducing errors in regression-derived IC₅₀, n, and CI values, whereas these problems were avoided by using nonlinear regression analysis. Hence, we elected nonlinear regression for subsequent studies and method development.
A drug interaction experiment typically provides the concentration-effect data for single agents and their combinations. Because of the differences in the effective concentrations for the different treatments (e.g., lower drug concentrations for combinations as compared with single agents), multiple plots of concentration-effect curves would be required if the x axis is in absolute drug concentration terms (e.g., ng/mL). This limitation was overcome by normalizing the concentrations of drugs in combinations to their respective single-agent IC$_{50}$; drug concentrations were converted to fractions or multiples of the IC$_{50}$ equivalents. Equation E states the IC$_{50}$-equivalent concentration of drug A or drug B, used alone or in combination with each other, required to produce x% effect. Note that for a single agent, one of the two terms ($C_{A,x}$ or $C_{B,x}$) on the right side of the equation becomes 0.

IC-equivalent concentration = $\frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}}$  \hspace{1cm} (E)

Substituting equation E into equation A yields equation F, which describes the effects of combination therapy as a function of IC$_{50}$-equivalent concentrations. IC$_{50,\text{combo}}$ and n$_{\text{combo}}$ are the values for the combination therapy.

Combination therapy effect

\begin{align*}
E_{\text{max}} & = \frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}}^{n_{\text{combo}}} + (IC_{50,\text{combo}})^{n_{\text{combo}}} \hspace{1cm} (F)
\end{align*}

Plotting the effects of single agents and combinations against IC$_{50}$-equivalent drug concentrations enabled the simultaneous presentation of these concentration-effect curves in a single plot.

**Computer Software Packages and Procedures.** All programming codes, graphical representations and calculations used SAS language and procedures (SAS, Cary, NC). Linear and nonlinear regressions were done with the SAS/STAT Proc REG routine and the SAS/STAT Proc NLIN routine with the Marquardt iteration method, respectively.

**Fig. 4** Concentration-effect curves of single-agent doxorubicin and suramin and their combinations in rat prostate tumor cells. Effects of single agent doxorubicin/suramin and their combinations were measured. The four combinations of suramin and doxorubicin, S1D400, S1D200, S1D100, and S1D50, correspond to the suramin-to-doxorubicin concentration ratios. The experimental data were fitted with equation 1 or 5. **Solid line**, fitting results with nonlinear regression. **Dotted line**, fitting results with linear regression. Note the inverse relationship between survival and drug effect, i.e., 10% survival is equivalent to 90% effect level.
The output of the drug-drug interaction results obtained from the 96-well microplate reader was used as input to simultaneously perform curve shift, isobologram, and CI analyses, as outlined in Fig. 3.

A pilot study evaluated whether the quality of data fitting could be improved by weighing the data with 1/concentration as the weight. The results showed no significant improvement. Accordingly, simple regression was used.

RESULTS

Experimental Data. Fig. 4 shows the concentration-effect curves for single-agent doxorubicin and suramin and their combinations, analyzed with linear and nonlinear regressions. In all cases, nonlinear regression provided better-fitted curves, as indicated by the higher R² values (range, 0.9959 to 0.9998; mean, 0.9977), compared with linear regression (range, 0.8892 to 0.9883; mean, 0.9499). Similar results were obtained with Akaike Information Criterion analysis (data not shown). Single-agent suramin and doxorubicin showed different curve shapes, with a steeper curve for suramin.

The nonlinear regression analysis yielded, for single-agent doxorubicin and suramin, IC₅₀ of 70 nmol/L and 450 µmol/L and n values of 0.5 and 1.6, respectively. The CI values were between 0.1 and 0.9.

Effect of Logarithmic Data Transformation on Accuracy and Precision of Regression Results: IC₅₀ and n Values for a Single Agent. Table 1 summarizes the results. The nonlinear regression analysis yielded, for single-agent doxorubicin and suramin, IC₅₀ of 70 nmol/L and 450 µmol/L and n values of 0.5 and 1.6, respectively. The CI values were between 0.1 and 0.9.

| Experiment no. | True values | IC₅₀ | N | SD in effect, % | Mean ± SD (CV, %) | Dev, % | Mean ± SD (CV, %) | Dev, % | Mean ± SD (CV, %) | Dev, % | Mean ± SD (CV, %) | Dev, % | DEV, % |
|---------------|-------------|------|---|----------------|-------------------|-------|-------------------|-------|-------------------|-------|-------------------|-------|
| 1             | 10 0.5      | 2    | 10.07 ± 0.57 (5.7) | +0.7              | 10.12 ± 0.57 (5.7) | +1.2  | 0.50 ± 0.01 (2.0) | 0.0   | 0.51 ± 0.02 (4.0) | +2.0  |
| 2             | 10 0.5      | 5    | 10.84 ± 1.39 (13.9) | +8.4             | 9.71 ± 1.14 (11.4) | −2.9  | 0.51 ± 0.03 (6.0) | +2.0  | 0.50 ± 0.04 (4.0) | 0.0   |
| 3             | 10 1        | 2    | 10.25 ± 0.23 (2.3) | +2.5             | 9.23 ± 0.83 (8.3)  | −7.7  | 1.01 ± 0.02 (2.0) | +1.0  | 0.86 ± 0.07 (7.0) | −14.0 |
| 4             | 10 1        | 5    | 10.74 ± 0.61 (6.1) | +7.4             | 9.01 ± 0.23 (2.5)  | −9.9  | 1.03 ± 0.06 (6.0) | +3.0  | 0.75 ± 0.08 (8.0) | −23.0 |
| 5             | 10 1, 5     | 2    | 10.17 ± 0.17 (1.7) | +1.7             | 8.87 ± 0.98 (9.8)  | −11.3 | 1.52 ± 0.04 (2.7) | +1.3  | 1.03 ± 0.11 (7.3) | −31.3 |
| 6             | 10 1, 5     | 5    | 10.44 ± 1.14 (11.4) | +4.4             | 8.64 ± 1.41 (14.1) | −13.6 | 1.59 ± 0.50 (33.3) | +6.0  | 0.87 ± 0.11 (7.3) | −42.0 |
| 7             | 10 2        | 2    | 9.76 ± 1.91 (19.1) | −2.4             | 8.55 ± 1.23 (12.3) | −14.0 | 2.00 ± 0.38 (19.0) | 0.0   | 1.11 ± 0.13 (6.5) | −44.5 |
| 8             | 10 2        | 5    | 10.24 ± 1.51 (15.1) | +2.4             | 8.66 ± 1.98 (19.8) | −13.4 | 2.09 ± 0.65 (32.5) | +4.5  | 0.90 ± 0.12 (6.0) | −55.0 |
| Average SD or Dev | (9.4)      | +3.1 | (10.5) | −9.0  | (12.9) | +2.2  | (6.3) | −26.2 |

NOTE. Simulated data were analyzed using linear and nonlinear regressions to obtain IC₅₀ and n values, which were compared with true values used to generate the simulations.

Abbreviations: Dev, deviation from true values. +, overestimation; −, underestimation; CV, coefficient of variation.
Table 2  Effect of logarithmic data transformation on accuracy, precision, and sensitivity of CI values to data variability

<table>
<thead>
<tr>
<th>Combination group</th>
<th>True CI</th>
<th>Regression Method</th>
<th>IC50 for Drug A MeanSD (CV, %)</th>
<th>n for Drug A MeanSD (CV, %)</th>
<th>10</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeanSD (CV, %)</td>
<td>Dev, %</td>
<td>MeanSD (CV, %)</td>
<td>Dev, %</td>
<td>MeanSD (CV, %)</td>
<td>Dev, %</td>
<td>MeanSD (CV, %)</td>
</tr>
<tr>
<td>A. Perfect data, 5% variation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1D400 0.8</td>
<td>Nonlinear</td>
<td>71.7±3.1 (4)</td>
<td>0.394±0.008</td>
<td>0.0</td>
<td>0.808±0.08 (10.5)</td>
<td>0.4</td>
<td>0.816±0.07 (8.5)</td>
<td>1.7</td>
<td>0.824±0.07 (8.3)</td>
</tr>
<tr>
<td>S1D200 0.5</td>
<td>Nonlinear</td>
<td>71.9±3.1 (4)</td>
<td>0.394±0.008</td>
<td>0.0</td>
<td>0.808±0.08 (10.5)</td>
<td>0.4</td>
<td>0.816±0.07 (8.5)</td>
<td>1.7</td>
<td>0.824±0.07 (8.3)</td>
</tr>
<tr>
<td>S1D100 0.2</td>
<td>Nonlinear</td>
<td>71.9±3.1 (4)</td>
<td>0.394±0.008</td>
<td>0.0</td>
<td>0.808±0.08 (10.5)</td>
<td>0.4</td>
<td>0.816±0.07 (8.5)</td>
<td>1.7</td>
<td>0.824±0.07 (8.3)</td>
</tr>
<tr>
<td>S1D50 0.2</td>
<td>Nonlinear</td>
<td>71.9±3.1 (4)</td>
<td>0.394±0.008</td>
<td>0.0</td>
<td>0.808±0.08 (10.5)</td>
<td>0.4</td>
<td>0.816±0.07 (8.5)</td>
<td>1.7</td>
<td>0.824±0.07 (8.3)</td>
</tr>
</tbody>
</table>

NOTE. Simulated concentration-effect data were generated with IC50 and n values of 70 nmol/L and 0.6 for drug A (doxorubicin), values of 450 nmol/L and 1.4 for drug B (suramin), and analyzed as described in Materials and Methods. S1D50, S1D100, S1D200, and S1D400 refer to the combinations containing suramin-to-doxorubicin ratios (in IC50 units) of 1:50, 1:100, 1:200, and 1:400, respectively. Results for 20, 30, 70, and 80% effect levels are in line with the results at the other effect levels and are not shown because of space limitation. For simulated data, Dev represents deviation from true CI values. The effect of data transformation on the accuracy and precision of regression-derived CI values as a function of data variability at 5% effect level was studied with simulated data generated with error-free effect data (0%, top panel) or error-containing data (generated by increasing or decreasing the effect of drug A by 1 SD or 4%).

Table 3  Effect of data transformation on sensitivity of IC50 and n values of a single agent to data variability

<table>
<thead>
<tr>
<th>Concentration, nmol/L</th>
<th>Mean effect, % (no. of SD)</th>
<th>Nonlinear regression Estim</th>
<th>Linear regression Dev, %</th>
<th>Nonlinear regression Estim</th>
<th>Linear regression Dev, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2 (1)</td>
<td>72.58 +3.69</td>
<td>95.88 +36.98</td>
<td>0.63 +4.90</td>
<td>0.68 +13.42</td>
</tr>
<tr>
<td>5.2 (0.5)</td>
<td>71.42 +2.03</td>
<td>80.84 +15.48</td>
<td>0.62 +2.78</td>
<td>0.63 +5.72</td>
<td></td>
</tr>
<tr>
<td>7.0 (1)</td>
<td>70.00 +0.00</td>
<td>70.00 +0.00</td>
<td>0.60 +0.00</td>
<td>0.60 +0.00</td>
<td></td>
</tr>
<tr>
<td>9.2 (0.5)</td>
<td>67.31 -3.85</td>
<td>57.94 +17.22</td>
<td>0.57 +4.52</td>
<td>0.56 +6.63</td>
<td></td>
</tr>
<tr>
<td>11.2 (1)</td>
<td>64.84 -7.37</td>
<td>51.03 +27.10</td>
<td>0.55 +8.23</td>
<td>0.54 +10.62</td>
<td></td>
</tr>
<tr>
<td>50.8 (0.5)</td>
<td>72.53 +10.38</td>
<td>72.88 +4.11</td>
<td>0.60 +0.82</td>
<td>0.58 +2.87</td>
<td></td>
</tr>
<tr>
<td>5.38 (0.5)</td>
<td>73.69 +5.27</td>
<td>71.42 +2.03</td>
<td>0.60 +0.47</td>
<td>0.60 +0.00</td>
<td></td>
</tr>
<tr>
<td>55.3 (0)</td>
<td>70.00 +0.00</td>
<td>70.00 +0.00</td>
<td>0.60 +0.00</td>
<td>0.60 +0.00</td>
<td></td>
</tr>
<tr>
<td>66.5 (0.5)</td>
<td>65.55 -5.00</td>
<td>68.59 +2.03</td>
<td>0.60 +0.47</td>
<td>0.60 +0.00</td>
<td></td>
</tr>
<tr>
<td>58.3 (1)</td>
<td>63.18 -9.74</td>
<td>67.20 +4.00</td>
<td>0.61 +1.30</td>
<td>0.60 +0.00</td>
<td></td>
</tr>
<tr>
<td>10,000</td>
<td>94.7 (0.5)</td>
<td>74.14 +5.91</td>
<td>0.59 -0.95</td>
<td>0.58 +2.87</td>
<td></td>
</tr>
<tr>
<td>94.7 (0.5)</td>
<td>74.14 +5.91</td>
<td>74.14 +5.91</td>
<td>0.59 -0.95</td>
<td>0.58 +2.87</td>
<td></td>
</tr>
<tr>
<td>95.2 (0)</td>
<td>70.00 +0.00</td>
<td>70.00 +0.00</td>
<td>0.60 +0.00</td>
<td>0.60 +0.00</td>
<td></td>
</tr>
<tr>
<td>95.7 (0.5)</td>
<td>67.82 -3.11</td>
<td>67.82 +1.47</td>
<td>0.60 +0.78</td>
<td>0.61 +1.65</td>
<td></td>
</tr>
<tr>
<td>96.2 (1)</td>
<td>69.57 -0.61</td>
<td>65.55 -6.36</td>
<td>0.61 +0.93</td>
<td>0.62 +3.50</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. A concentration-effect curve for doxorubicin was generated with true values of 70 nmol/L and 0.6 for drug A (doxorubicin), values of 450 nmol/L and 1.4 for drug B (suramin), and analyzed as described in Materials and Methods. S1D50, S1D100, S1D200, and S1D400 refer to the combinations containing suramin-to-doxorubicin ratios (in IC50 units) of 1:50, 1:100, 1:200, and 1:400, respectively. Results for 20, 30, 70, and 80% effect levels are in line with the results at the other effect levels and are not shown because of space limitation. For simulated data, Dev represents deviation from true CI values. The effect of data transformation on the accuracy and precision of regression-derived CI values as a function of data variability at 5% effect level was studied with simulated data generated with error-free effect data (0%, top panel) or error-containing data (generated by increasing or decreasing the effect of drug A by 1 SD or 4%).

Abbreviations: +, overestimation; -, underestimation.
n) at higher SDs (e.g., compare the deviations at 0.5× and 1× the SD). The average deviations for the nonlinear regression results at low and high effect levels (i.e., 7.2 and 95.2%) remained negligible at an average of 3.4% for IC_{50} and 4.1% for n. In contrast, the deviations for the linear regression results were −5.7-fold higher at 19.4% for IC_{50} and 1.8-fold higher at 7.3% for n. In comparison, the parameter values derived from linear regression were less sensitive to the change at the mid effect level of 55% (average deviations of 2.4% for IC_{50} and 0.57% for n).

**Effect of Logarithmic Data Transformation on Sensitivity of Combination Indices to Data Variability.** Fig. 5B and Table 2B show the results when the 5% effect level was reduced by 4% (1 SD), and Fig. 5C and Table 2C shows the results when the 5% effect was increased by 4%. A comparison of these results to the results generated with the perfect data (i.e., 0% variation; Fig. 5A and Table 2A) showed two major differences. First, for perfect data, the two regression methods yielded regression-derived CI values that were closely aligned with true CI values and with similar accuracy. On the other hand, data variation at the 5% effect level in either direction yielded larger deviations between the regression results and true CI values for either regression methods. Second, the linear regression results showed substantially lower accuracy and precision compared with the nonlinear regression results. These results are additionally described below.

With respect to accuracy, irrespective of true CI values and regression methods, a 4% reduction at the 5% effect level led to overestimated CI values at 10 to 70% effect levels and slight underestimation at 90% effect level. In general, the magnitude of overestimation increased with decreasing effect level and was −3-fold greater for linear regression. On the contrary, a 4% increase in the 5% effect level resulted in underestimated CI values.

**Fig. 5** Effect of logarithmic data transformation on regression-derived CI values. The CI values were obtained as outlined in Fig. 2. True CI values were 0.2, 0.5, or 0.8 (indicated by dotted lines). The four combinations of suramin and doxorubicin, S1D400 (□), S1D200 (▼), S1D100 (▼), and S1D50 (■), correspond to the suramin-to-doxorubicin concentration ratios. All drug concentrations were normalized to IC_{50} equivalents of single agents (see Materials and Methods). Simulated data were generated with error-free effect data of single agent treatment (0%, □) or with effect data with −4% error (B) or +4% error (C) at 5% effect level (i.e., by subtracting or adding 4%).
values at lower effect levels (≤ 30% for nonlinear regression and ≤70% for linear regression) and overestimated CI values at higher effect levels; the magnitude of over- or underestimation increased with the departure of the effect level away from the median effect level.

With respect to precision, either increasing or decreasing the 5% effect by 4% resulted in greater SDs in the linear regression results, at all effect levels, as compared with nonlinear regression results. A comparison of the SD values in the linear regression results, under the scenarios of perfect data or error-containing data, additionally showed highest SD values or lowest precision in the regression-derived CI values when the 5% effect contained a 4% upward error.

The data in Table 2 also show the relationship between data variation and the regression-derived IC₅₀, n, and CI values. As discussed above, the nonlinear regression method is less sensitive to data variability at <10% and >90% effect levels and thereby yielded IC₅₀ values that were closely aligned with true IC₅₀ values. In contrast, the linear regression method is highly sensitive to these data variations; when the 5% effect was reduced erroneously by 4 to 1%, the concentration-effect curve was shifted downward and thereby resulted in underestimated IC₅₀ value. As stated in equation B, an underestimated IC₅₀ value results in overestimated CI value. Conversely, when the 5% was increased erroneously to 9%, the concentration-effect curve was shifted upward, thereby overestimating the IC₅₀ value and understating the CI value. This explains the higher accuracy and precision associated with the nonlinear regression results.

Fig. 6 A nonlinear regression-based drug interaction evaluation method. The experimental results described in Fig. 4 were normalized to IC₅₀ equivalents of single agents. Data were analyzed with nonlinear and linear regressions, and the results are shown in left and right panels, respectively. A, curve shift analysis. The dots and bars are mean values and 1 SD. The lines are best-fitted regressed lines. A leftward shift of concentration-effect curves for combinations when compared with single-agent curves indicates synergism and a rightward shift indicates antagonism. At 75% survival level, curves from left to right represent S1D100, S1D50, S1D200, S1D400, doxorubicin, and suramin. B, results of isobologram analysis. The diagonal line is the line of additivity. Experimental data points, represented by dots, located below, on, or above the line indicates synergy, additivity, or antagonism, respectively. C, combination index analysis. CI values less than, equal to, or greater than 1 indicates synergy, additivity, or antagonism, respectively. ●, doxorubicin. ○, S1D400. ▼, S1D200. ▽, S1D100. ■, S1D50. ○, suramin.
Integration of Nonlinear Regression-Based Curve Shift Analysis, Isobologram, and Combination Analyses to Evaluate Drug-Drug Interactions.

Fig. 6 shows the output of data analysis with the algorithm outlined in Fig. 3. The results of linear and nonlinear regression analyses are also shown for comparison.

Fig. 6A shows the curve shift analysis results. Note that the plots were generated from the procedures outlined in Fig. 3, with equations 4 and 5. The conversion of actual drug concentrations to IC_{50} equivalents enabled the simultaneous representation of all concentration-effects curves for single agents and combinations on a single plot. In line with Loewe’s principle of additivity, a leftward shift or a rightward shift of the concentration-effect curve for a combination, when compared with concentration-effect curves for both single agents, indicates synergy or antagonism, respectively. When the concentration-effect curve for a combination falls between the concentration-effect curves for single agents, combination index values are needed to decide the nature of drug interaction. Note that concentration-effect curves for single agents will overlap each other at the one IC_{50}-equivalent concentration level.

Fig. 6B shows the isobolograms for the 50% effect level. Both linear and nonlinear regression results show that the IC_{50} equivalent concentrations for various suramin/doxorubicin combinations were located below the line of additivity, indicating synergistic interaction at all combinations. The separation of the points in the isobologram was consistent with that in the CI-effect plot. For example, both isobologram and CI analyses showed a wider separation between the combinations S1D200 and S1D400 analyzed by nonlinear versus linear regression.

Fig. 6C shows the CI values. Both regression methods showed CI values < 1 in nearly all cases and thereby identified synergy between suramin and doxorubicin at various combination ratios. However, the CI values obtained with linear regression were lower than the values obtained with nonlinear regression at 10 to 80% effect levels and were higher only at 90% effect level.

Note that the three analysis methods, although each provided different types of information on drug interaction, consistently identified the two combinations, S1D50 and S1D100, corresponding to respective suramin-to-doxorubicin concentration ratios of 1:50 and 1:100, as the most synergistic combinations (i.e., the greatest leftward shift in the curve shift analysis, the greatest distance from the line of additivity in the isobologram analysis, and the lowest CI values).

To additionally illustrate the effects of logarithmic data transformation on the CI values, we compared the linear regression results to the nonlinear regression results with the latter as the reference values. The comparison showed that linear regression resulted in under- or overestimation of the CI values, depending on the effect levels and the combination ratios of the two drugs. In general, the linear regression results showed lower CI values at 10 to 70% effect levels, with greater underestimation at lower effect levels; CI was underestimated, on average, by ~45% at 50% effect levels and by ~75% at 10% effect level. This trend was reversed at the 90% effect level, where the linear regression results overestimated the average CI by ~45%.

DISCUSSION

Results of the present study show the significant effects that the choice of the regression method has on the drug interaction results. Our findings indicate that logarithmic data transformation resulted in underestimation of the steepness of concentration-effect curves with increasing underestimation at increasing steepness. The erroneous representation of the experimental data by shallower-than-actual curves, in turn, led to underestimation of the effective drug concentrations at nearly all effect levels (i.e., 10–80% effect levels) and reduced the accuracy but not the precision of the regression-derived IC_{50} and n values. The factors important for the accuracy and precision in the determination of IC_{50}, n, and CI values are discussed below.

Data variability at <10% and >90% effect levels introduced significant errors in the IC_{50} and n values. This problem was especially critical for data analyzed with the linear regression method. At 2 to 4% variability at these effect levels, the deviations of the regression-derived IC_{50} and n values from their true values were ~6- and ~2-fold greater, respectively, compared with the nonlinear regression results. In contrast, the IC_{50} and n values were less sensitive to variability in the data at about median effect level. This is because small changes in fa results in large changes in log(fa^{-1} − 1)^{-1} when fa approaches 0 or 1 during logarithmic transformation. On the other hand, according to the D-optimality design method, these data points are important for reducing the variance associated with the parameter estimates and must be included in the analysis (15). Accordingly, we have three recommendations: (a) use nonlinear regression for data analysis, (b) increase the number of replicates to reduce the variability of data at the two ends of the concentration-effect curve, and (c) exercise care to incorporate the variability of the data at the low and high effect levels in deciding on the quality of the regression results (e.g., higher variability indicates less reliable results).

As shown in Table 2, at n values of between 1 to 1.5, which represent the curve shape parameter for most drugs, the linear regression typically and significantly underestimated the IC_{50} values by ~11%, whereas the nonlinear regression underestimated the values by ~4%. As IC_{50} values are used to calculate the CI, the lower accuracy of the linear regression results also affected the accuracy and precision of the calculated CI values. Our results generated with concentration-effect data simulated with true CI values of 0.2 to 0.8 indicate the following. With perfect data, both linear and nonlinear regression methods were comparably accurate in the CI determination, although the linear regression results were less precise. Second, data variation at <10% and >90% effect levels reduced the accuracy and precision of the regression-derived CI values by both regression methods, with much lower accuracy and lower precision for linear regression. The extent of underestimation or overestimation in the CI values was relatively high and materially important; the deviation for the nonlinear regression results (11.0 ± 11.3) was significantly lower compared with the deviation for the linear regression results (34.4 ± 27.0, P < 0.0002). As CI values are indicative of the nature of drug interactions, we conclude that the linear regression method is associated with greater errors in determining synergy or antagonism between...
drugs and recommend using the nonlinear regression method to analyze drug interaction.

Evaluation of synergy or antagonism of agents used in combination is an integral part of cancer chemotherapy development. Various methods contributing complementary aspects to the data analysis are available. Simultaneous use of multiple methods enhances the confidence the investigator can have in her/his conclusions. We describe here a curve shift analysis method, where the concentration-effect curves of single agents and combinations are simultaneously presented on a single plot and that thereby enables visual inspection on data variability, goodness of fit by regression analysis, and parallelism of the curves. According to Loewe’s principle of additivity, a leftward shift of the concentration-effect curve for a combination indicates synergy and a rightward shift indicates antagonism. The shape of the curve may additionally suggest the nature of interaction; a parallel curve shift suggests equal interactions at all concentrations or effect levels, whereas a nonparallel shift suggests concentration-dependent or effect-dependent interactions. We postulate that this may have pharmacological relevance because an anticancer drug may produce antiproliferation at low concentrations and cell kill at higher concentrations. A change in the curve shape at the different parts of the concentration-effect curve may hint at the mechanisms of drug interaction; a more shallow shape at low concentrations or effect levels for the combination, whereas the rest of the curve shows a parallel shift, when compared with single agents, would suggest a greater interaction on the antiproliferation effect. It is noted that the curve shift analysis does not provide statistical estimates of drug interaction. Quantitative statistical approaches represent an area that requires additional attention (2, 6, 7).

The present study additionally described an algorithm for a nonlinear regression-based method that integrates curve shift, isobologram, and CI analyses. The incorporation of isobologram and CI analyses provides additional qualitative and quantitative information on drug interaction at different combination ratios/concentrations and different effect levels.

Finally, it is of interest to note the synergy between suramin and doxorubicin. For example, the CI values from 10 to 70% drug effect levels were between 0.1 and 0.2 (equivalent to 10- to 5-fold synergy) for the S1D50 and S1D100 combinations. The corresponding suramin concentrations under these conditions are between 30 nmol/L and 7 μmol/L. This finding is consistent with our earlier observation that low concentrations of suramin (e.g., 10 μmol/L), acting as a nonspecific inhibitor of acidic and basic fibroblast growth factors, enhance the activity of chemotherapeutic agents with diverse chemical structures and action mechanisms both in vitro and in vivo (12–14).

In conclusion, the analysis of drug-drug interaction benefits from an evaluation by several methods, including direct visualization of the data by the curve shift analysis presented here. Furthermore, the use of nonlinear regression analysis rather than the conventional linear regression of logarithmically transformed data is essential to obtain precise results of drug-drug interaction studies.

REFERENCES
