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.

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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):

$$E = E_{max} \times \frac{C^n}{IC_{50}^n + C^n}, \quad (A)$$

where E is the measured effect; C is the drug concentration; E_{max} is the full range of drug effect, usually at or near 100%;

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, c_B) 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.

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}} \qquad (B)$$

$C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A and drug B used in combination to achieve x% drug effect. $IC_{x,A}$ and $IC_{x,B}$ 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):

$$\log(fu^{-1} - 1) = \log(fa^{-1} - 1)^{-1} = n\log(C) - n\log(Cm), \qquad (C)$$

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 $\log(fu^{-1} - 1)$ *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 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 simu-

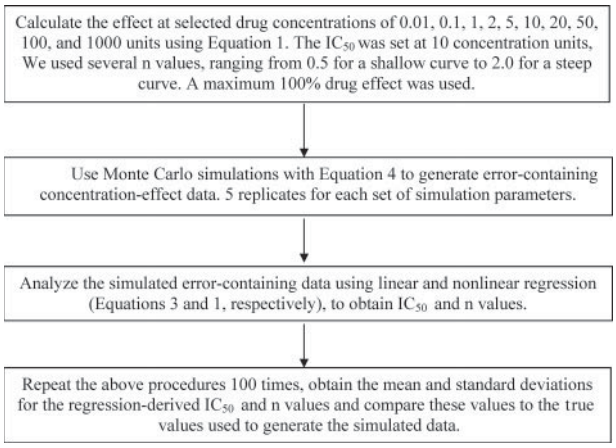

Fig. 1 Outline of Monte-Carlo simulations to study the effects of logarithmic data transformation on the precision and accuracy of regression-derived IC_{50} and n values.

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.,* IC_{50}, n, effect data variability expressed as σ, 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 IC_{50} and n values.

Effect of Logarithmic Data Transformation on Accuracy and Precision of IC_{50} and n Values Obtained from Regression Analyses. Fig. 1 outlines the procedures. For this study, the concentration-response curves were generated with arbitrarily chosen IC_{50} 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:

$$\text{simulated effect} = \text{preselected effect} + \sigma \qquad \text{(D)}$$

where σ is the normally distributed error with a mean value of 0. The SD for σ 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*A* outlines the procedures. In contrast to the study on IC_{50} 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 IC_{50}, n, and CI values, which were based on the experimental results ob-

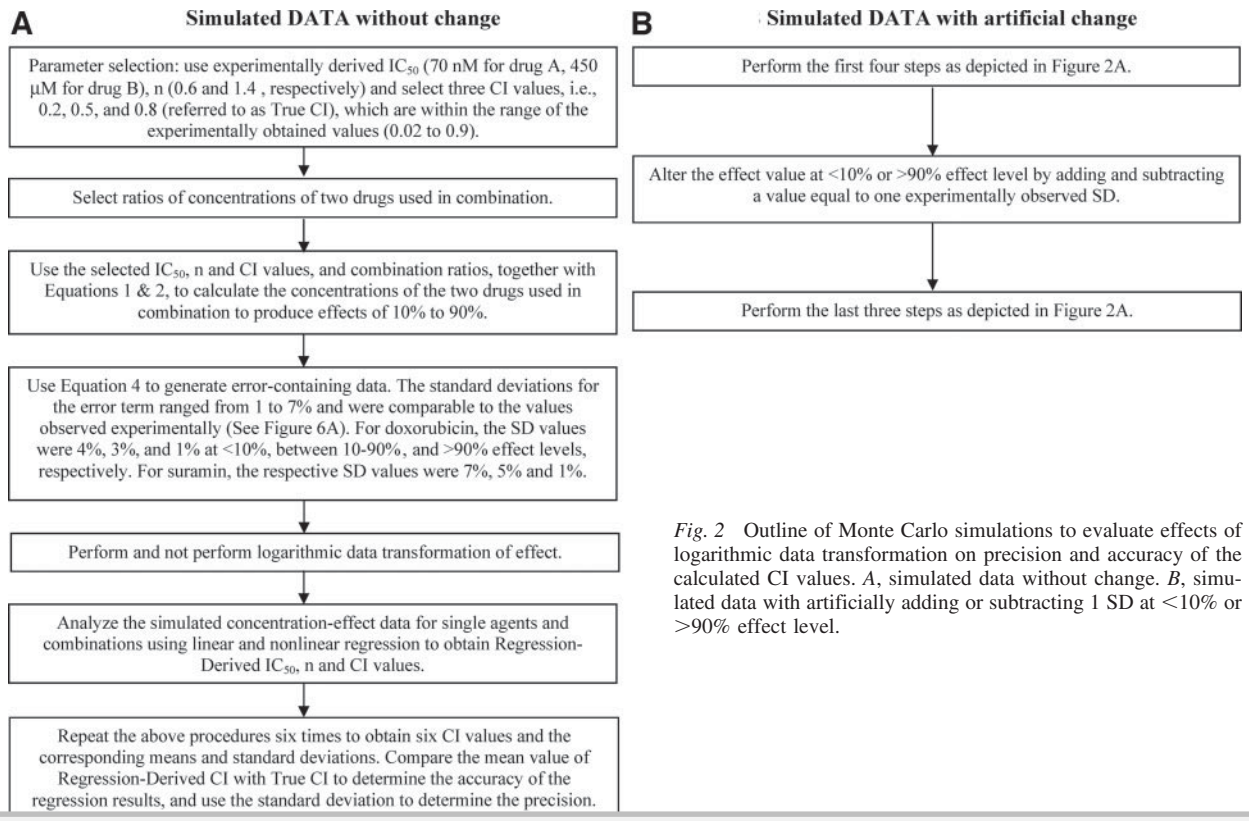

Fig. 2 Outline of Monte Carlo simulations to evaluate effects of logarithmic data transformation on precision and accuracy of the calculated CI values. *A*, simulated data without change. *B*, simulated data with artificially adding or subtracting 1 SD at <10% or >90% effect level.

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 IC_{50} and n Values to Data Variability. In linear regression analysis, IC_{50} and n values are calculated based on $\log(fa^{-1} - 1)^{-1}$ (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 IC_{50} 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 IC_{50} and n values.

Effect of Logarithmic Data Transformation on Sensitivity of CI Values to Data Variability. Fig. 2*B* 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. 2*A*), 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 $\log(fa^{-1} - 1)^{-1}$.

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 IC_{50}, 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.

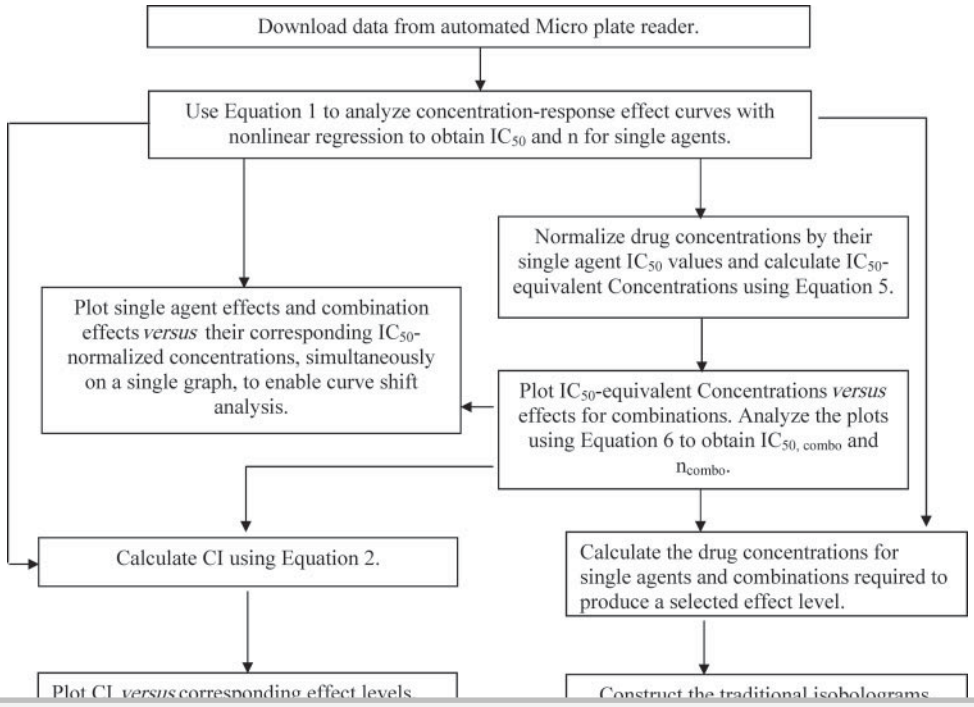

Fig. 3 Algorithm for developing a nonlinear regression-based method to integrate curve shift, isobologram, and CI analyses of drug interaction data.

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.

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

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

Combination therapy effect

$$= \frac{E_{max}\left(\frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}}\right)^{n}_{combo}}{\left(\frac{C_{A,x}}{IC_{50,A}} + \frac{C_{B,x}}{IC_{50,B}}\right)^{n}_{combo} + (IC_{50,combo})^{n}_{combo}} \qquad \text{(F)}$$

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.

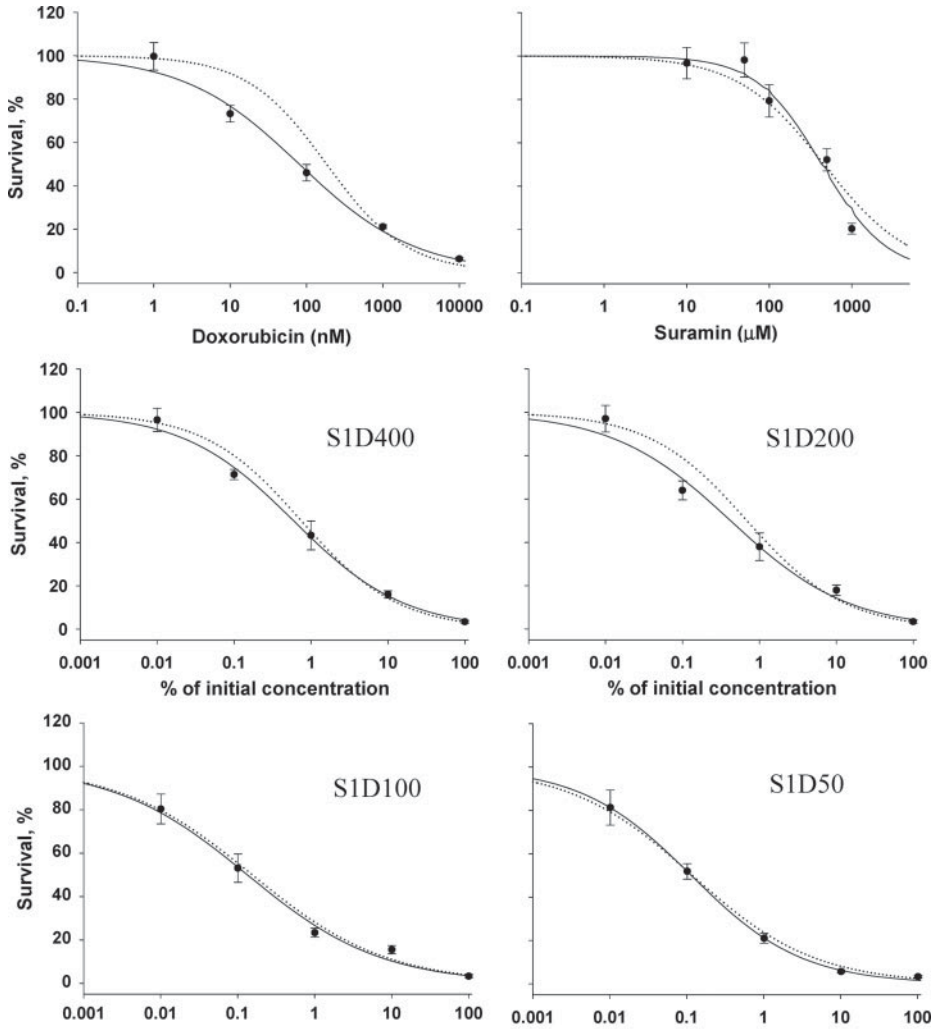

Fig. 4 Concentration-effect curves of single-agent doxorubicin and suramin and their combinations in rat prostate tumor cells. Effects of single agent doxorubicin/suramin and their combinations were measured. The four combinations of suramin and doxorubicin, S1D400, S1D200, S1D100, and S1D50, correspond 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.

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