

Evaluating Response to Antineoplastic Drug Combinations in Tissue Culture Models

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Summary

The mainstay of clinical antineoplastic chemotherapy is multiagent combinations, most of which were developed empirically. Because of the desire to speed research and decrease costs, there is increasing interest in moving new drugs into clinical trials in potentially active combinations based on preclinical testing data. Different mathematical models have been proposed for evaluating drug interactions, which can be classified as synergistic (combinations demonstrating greater than the additive activity expected from each agent alone), additive, or antagonistic (drugs showing less activity in combination than expected from the sum of each agent alone). Here, we briefly review some of the principles for testing cytotoxic drug interactions. We focus this review on application of the Combination Index method (as developed by Chou and colleagues) in the evaluation of drug interactions in cell culture assays.

Key Words

Drug synergism; drug antagonism; chemotherapy; combination index; isobologram.

1. Introduction

Virtually all curative chemotherapy regimens for cancer employ multiagent drug combinations (1). Although ideal drug combinations would be those that are synergistically active against malignant cells without increased systemic toxicity, additive antitumor activity with a favorable toxicity profile can also be clinically beneficial. The large majority of currently employed combination chemotherapy regimens have been developed empirically. While patterns of cross-resistance, degree of normal organ toxicity overlap, and mechanisms of drug mechanisms are often considered in designing combination regimens, formal preclinical testing of the combinations has played a minor role in clinical trials of combination chemotherapy (2). Thus, whether clinically active multiagent com-

binations are effective due to increased cytotoxicity for tumor cells that is additive or synergistic remains undefined for most combination chemotherapies. As the number of drugs available for clinical trials continues to increase, the number of possible combinations of agents increases almost exponentially. This has enhanced interest in developing robust preclinical models to assess drug combinations, which may identify clinically useful combinations, enabling reduced development costs and more rapid clinical delivery (3). Of particular importance is the ability to identify and preclude the testing of drug combinations that are likely to be antagonistic when tested in clinical trials.

Combination testing of drugs can be done directly (i.e., delivered concurrently), or sequentially, in time. Although the principles of defining interactions can be applied to either case, in general, most experimental studies exploring synergy employ direct testing of drug combinations. Comparison of the dose-response curves of two agents in combination (in fixed-ratio concentrations) to either agent alone may demonstrate apparent antagonism or apparent synergy by inspection alone when the skilled eye is employed. However, formal quantitative analysis of experimental data is always preferred over descriptive presentation, to preclude unintended observer bias, and also because such analysis is generally required to distinguish additive from truly synergistic effects. Formal methods that have been employed to evaluate drug interactions include isobologram methodology (4–6), the Nonlinear Mixture (surface response) model (5,7–9), and the combination index (CI) (6,10–13). We focus here on the adaptation of the CI method (based on the multiple drug-effect equation of Chou-Talalay, originally derived for use in enzyme kinetic models) to *in vitro* anticancer drug testing. CI analysis has been widely used for this purpose, and its application is simplified by the availability of user-friendly microcomputer software (14).

Drug interactions can also be studied *in vivo*, and elegant demonstrations of synergy in mouse xenografts have been reported (6,15–18). However, the numbers of variables that impact synergy studies in xenografts are substantial, requiring large numbers of animals to achieve statistically valid results, with consequent investment of time and cost. Formal models used for *in vitro* modeling of synergy are applicable to xenograft studies, but relatively few laboratories have the resources to conduct experiments of the size needed to truly define synergy using either the isobologram or CI approaches. A model more suitable for xenograft studies employing the *F*-test has been proposed, and successfully applied, to the testing of drug combinations, but it still requires a significant investment of resources (19). One alternative approach to large xenograft experiments for defining synergistic drug interactions would be first to define synergy in robust tissue culture models, and then to confirm a

beneficial interaction between the two drugs (i.e., increased antitumor response) in more limited xenograft studies.

2. Materials

1. Data from a suitable cytotoxicity assay such as DIMSCAN (*see Note 1*).
2. Microsoft Excel (Microsoft Office). Data can be collected easily in the Excel program and manipulated, e.g., to calculate averages or log transformations, as required.
3. SigmaPlot (Jandell, San Rafael, CA). Analyzed data can be copied from Excel to SigmaPlot to create publication-quality graphics.
4. CalcuSyn (Biosoft, Cambridge, UK; www.biosoft.com). Data from both fixed-ratio and non-fixed-ratio drug combination experiments can be analyzed using this Microsoft Windows–based program. CalcuSyn analyzes drug interactions and provides tables and graphics of a variety of values from application of median-effect equations to the data. In addition to calculating the various tables, graphs, and values for assessing drug interactions described below, CalcuSyn can determine the effective concentrations needed for each drug to produce a given dose effect. In such calculations, the concentration of a drug needed to affect 90% of cells is termed an EC_{90} (effective concentration for 90%). For cytostatic assessment, this is generally referred to as an IC_{90} (inhibitory concentration) and for cytotoxic agents as an LC_{90} (lethal concentration).

3. Methods

3.1. CI Method

The CI method is based on the median-effect principle derived by Chou (10,20,21). The median-effect equation correlates the drug dose and cytotoxicity or cytostatic effect in the following form:

$$f_a / f_u = (D/D_m)^m \text{ or its alternative form, } D = Dm[f_a / (1 - f_a)]^{1/m}$$

in which D is the dose of the drug; D_m is the median-effect dose signifying the potency, determined from the x -intercept of the median-effect plot; f_a is the fraction affected by the dose; f_u is the fraction unaffected ($f_u = 1 - f_a$); and m is an exponent that signifies the sigmoidicity (shape) of the dose-effect curve, which is determined by the slope of the median-effect plot.

The median-effect equation is utilized to calculate D_x , which is the dose of a drug that inhibits (or kills) “ x ” percent of cells. The CI is then calculated as

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

The preceding equation is employed when “mutually exclusive” drugs are used that have the same or similar modes of action (i.e., those drugs that are, by

analogy to enzyme kinetics, competitive inhibitors of each other; e.g., two agents that both inhibit the same active site of a kinase, or perhaps, two microtubule inhibitors) (**10,21**). For drugs with “mutually nonexclusive” mechanisms of action (i.e., those drugs that are, by analogy, noncompetitive inhibitors of each other; e.g., a DNA alkylator and a microtubule inhibitor, or a tyrosine kinase inhibitor and a topoisomerase inhibitor), the following equation applies:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1(D)_2}{(D)_1(D)_2}$$

Owing to the complexity of whole-cell biological systems, the CalcuSyn program automatically analyzes a data set using both the mutually exclusive and mutually nonexclusive assumptions. The CI equation determines the additive effect of drug combinations, such that synergism is defined as a greater-than-the-expected-additive effect, and antagonism is defined as less-than-an-expected-additive effect. Thus, $CI = 1$ indicates an additive effect, $CI < 1$ indicates a synergistic effect, and $CI > 1$ indicates antagonism. The precise biological significance of various degrees of synergism or antagonism remains to be defined, but it has been proposed that CI values be interpreted as follows:

| | |
|----------|----------------------------------|
| <0.1 | very strong synergism |
| 0.1–0.3 | strong synergism |
| 0.3–0.7 | synergism |
| 0.7–0.9 | moderate to slight synergism |
| 0.9–1.1 | nearly additive |
| 1.1–1.45 | slight to moderate antagonism |
| 1.45–3.3 | antagonism |
| >3.3 | strong to very strong antagonism |

Because CI values may change with the fraction affected (F_a) in a nonlinear manner, the CI should optimally be presented for each effective concentration (EC) tested, or an overall CI value presented that is generally reflective of the CI values calculated at the various ECs tested (e.g., EC_{50} , EC_{90} , and EC_{99} or EC_{99}).

3.2. Conducting Fixed-Ratio Analysis of Drug Interactions

A prerequisite for fixed-ratio calculations is the generation of accurate dose-response curves for the agents tested, both alone and in combination. The accuracy of assessing drug interactions will depend directly on the accuracy of the method used to assess their cytotoxicity or growth inhibition. In testing the antitumor properties of antineoplastic drugs, the effect can be an inhibition of growth or cytotoxicity (most assays measure a combination of both). The

dynamic range of the assay is important, because effective combinations should achieve an inhibition of growth over time, or direct cytotoxicity, of >2 to 3 logs (the “two-log” rule) for an expectation of clinical response (based on current response definitions and calculated disease burdens in leukemia patients and solid tumor masses) (22). Accuracy of the calculations is also impacted by the number of different points taken along the dose-response curve.

To determine potency and shape in a dose-response relationship, three data points are a minimum value, although CI can be calculated using any number of data points. The linear correlation coefficient (r) of the median-effect plot should be reasonably good ($r > 0.9$ is common for cell culture experiments).

To assess synergy, each drug should have some effect as a single agent. Both the potency (D_m) and shape (m) parameters that are derived from dose-response data are required for assessing synergism or antagonism. A CI can be derived for a combination of a noneffective agent and an effective agent, and although these can be referred to in terms of synergism or antagonism, such interactions are more properly termed *modulation* (potentiation, augmentation, or inhibition) of the active agent by the inactive agent.

CI values for each data point of a nonconstant ratio experiment can also be calculated, as long as m and D_m parameters are available for each single drug. An example of such an experiment is the use of one drug at a fixed concentration while varying the concentration of the second drug. However, such an experimental design does not allow calculation of an F_a -CI plot simulation (though the points can still be placed on the F_a -CI plot), nor does it allow plotting of a classic isobologram.

The preferred experimental design is to employ each drug alone, and in combination, at a fixed ratio of concentrations (e.g., 2:1 or 1:3). Drugs with two different units (micromolar vs micrograms/milliliter) can be analyzed directly in fixed-ratio combinations. One approach to selecting values for the fixed ratio combinations is to combine each drug at its equipotent ratio (i.e., the ratio of the EC_{50} concentrations), and then to create a combination that is four- to eight-fold higher than the EC_{50} , and use serial dilutions of the highest concentration combination to generate the dose-response curve (each single agent in the combination is tested alone in the same manner) (see **Note 2**).

3.3. Examples of Assessing Drug Interaction Data

The first example (**Fig. 1**) provided is from a published study that assessed the synergistic interaction of fenretinide (a cytotoxic retinoid known to stimulate generation of ceramides in tumor cells *in vitro*) and safinolol (a stereoisomer of sphinganine, a precursor of ceramide) (23). First, the data for **Fig. 1** were obtained from the DIMSCAN assay of fixed-ratio exposures to each drug

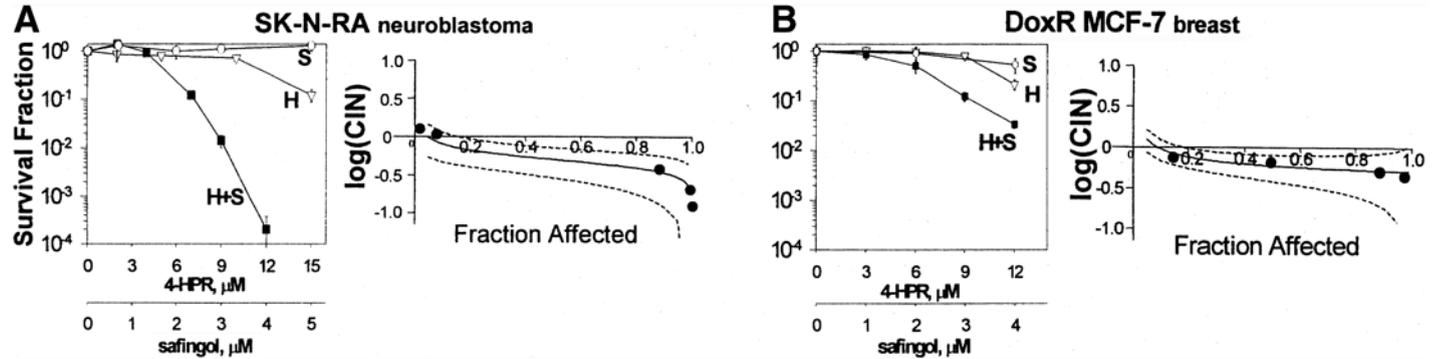


Fig. 1. Effects of *L-threo*-dihydrosphingosine (safingol) on fenretinide (4-HPR) cytotoxicity in a neuroblastoma and a breast cancer cell line (23). The cytotoxicity dose response of (A) the SK-N-RA neuroblastoma cell line and (B) the DoxR MCF-7 breast cancer cell line to 4-HPR (H), safingol (S), and 4-HPR/safingol (3:1 ratio) (H+S) using a fluorescence-based assay employing digital imaging microscopy (DIMSCAN) is shown (24). Cell lines were exposed to drug(s) and responses were assayed at 4 d. (∇) 4-HPR; (\circ) safingol; (\blacksquare) 4-HPR/safingol (3:1 ratio). Synergy was quantified by Combination Index (CIN) analysis and expressed as $\log_{10}(\text{CIN})$ vs fraction affected. By this method, $\log_{10}(\text{CIN}) < 0$ indicates synergy; $\log_{10}(\text{CIN}) = 0$ indicates an additive effect; and $\log_{10}(\text{CIN}) > 0$ indicates antagonism. Ninety-five percent confidence intervals are shown on CIN plots where calculable. Bars indicate 95% confidence intervals. Note that CIN is used as an abbreviation for Combination Index because the journal in which the data were originally published used CI as an abbreviation for confidence interval.

alone and in combination, and the data were analyzed in CalcuSyn. Second, values from the CI tables were then plotted using SigmaPlot to generate publication-quality graphics demonstrating the synergistic interaction between fenretinide and safingol.

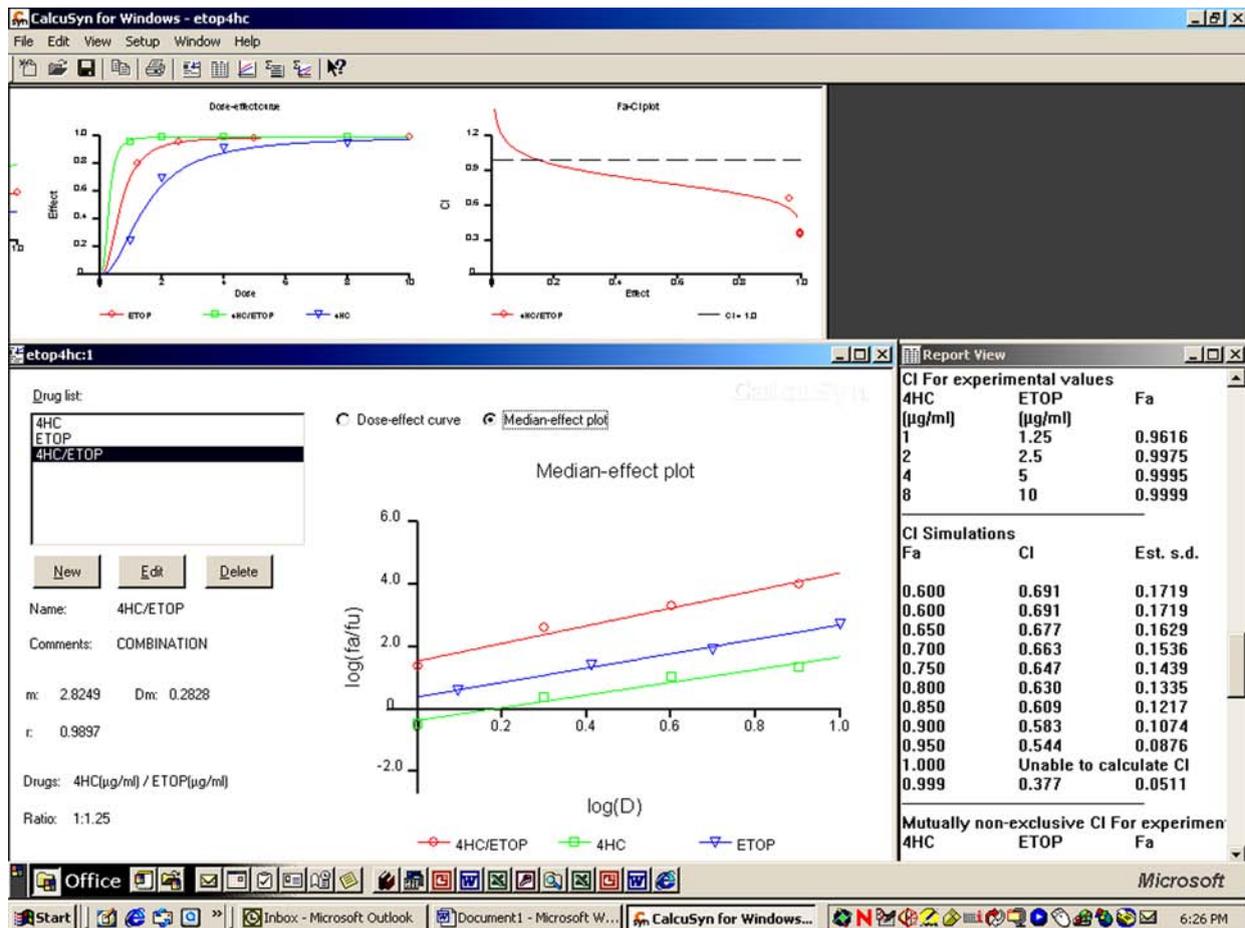
The second example is a computer screen showing CalcuSyn after it has generated the median-effect and CI plots that are used to analyze drug interactions for the combination of cyclophosphamide (as the active metabolite 4-hydroperoxycyclophosphamide [4-HC]) and etoposide (**Fig. 2**). The data, data analysis, and methods of obtaining and analyzing the data associated with **Fig. 2** are presented elsewhere in this volume (**24**) (*see Note 3*). A number of examples of the use of the CI approach to assess interactions of antineoplastic drugs can be found in the literature (**12,23,25–29**).

4. Notes

1. Determination of drug interactions for antineoplastic agents is ideally done using an assay with a ≥ 3 log dynamic range, especially if each individual agent is capable of 1 to 2 logs of cytotoxicity. Some commonly used cytotoxicity assays (such as 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) cannot achieve >2 logs of dynamic range with many cell types and, thus, should not be employed for assessing synergy and antagonism. We find that the DIMSCAN assay, owing to its 4 log dynamic range, is particularly suitable for drug combination testing (**23,25**). DIMSCAN and other in vitro cytotoxicity assays are reviewed by Keshelava et al. in Chapter 12 (**24**).
2. If the actual clinically achievable plasma level for each drug is known or suspected, then the ratio between the drugs should reflect this. The drug concentration ratios should also reflect the lower than maximal drug concentrations likely to be achieved when using the combination owing to the additive systemic toxicities of each agent, and the possible lower drug levels achieved in tumor tissue relative to plasma. Otherwise, in vitro overmodeling may occur, which can diminish the predictive value of the preclinical studies when the drug combination is tested in clinical trials.
3. Details on the use of CalcuSyn are found in the software user's manual (**14**).

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Fig. 2. (see opposite page) Screen captured from a computer analyzing data from a fixed-ratio analysis of the combination of 4-HC + etoposide using a human neuroblastoma cell line. Dose-response curves and data tables from the same experiment as shown in **Fig. 2** can be found elsewhere in this volume (**24**). Shown are windows containing the dose-effect curves (upper left), the F_a -CI plot (upper right), the median-effect plot (lower left), and a small portion of CI tables (lower right).

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