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A Checkerboard Method to Evaluate Interactions Between Drugs

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ABSTRACT. A method to evaluate interactions between biologically active agents is presented. Synergism, zero interaction, and antagonism were easily detected with the three-dimensional approach proposed herein. This method is compatible with a checkerboard design to diagnose the interaction between agents and obviate the need to test their mixtures in a fixed concentration ratio as proposed by Chou and Talalay. Dose-response curves for individual agents were obtained, and experimental data fitted to appropriate equations by nonlinear regression. If zero interaction was present, the predicted effect could be calculated for each combination using the classical isobole equation with any spreadsheet having a command to solve mathematical equations by iteration. This allowed the selection of appropriate concentrations for the combination of two or more agents. Interaction between agents could be assessed in two ways: by comparing experimental with expected effects, if zero interaction is present; or by analyzing the reduction or increase in total dose found as a consequence of the interaction. The applicability of both approaches is discussed and, for purposes of comparison with other methods, examples based on published data are analyzed and commented upon. BIOCHEM PHARMACOL 51;5:635–644, 1996.

KEY WORDS. synergism; isobologram; interaction index; median effect equation; drug interaction; combination chemotherapy

The study of the presence, type, and degree of interaction between biologically active agents is highly relevant to many research areas, including pharmacology, immunology, toxicology, physiology, and the environmental sciences. Combination chemotherapy, for example, has proved useful in the treatment of cancer, cardiovascular diseases, and bacterial or viral infections. In addition, many biological processes are known to require the simultaneous action of two or more interacting agents to produce an effect. A variety of methods aimed at the study of the interaction of drugs in combination have been designed. These methods often give discordant results when applied to the same data, even to the point that a combination may appear as synergistic if one method is used and antagonistic if another is employed. The coexistence of several methods does not mean that they are equally valid. Some base their calculations on assumptions regarding the shape of individual dose-response curves, thereby implying the mechanism of action of the agents in question (for a review see ref. [1]).

The construction of isoboles (i.e. isoeffective-curves) is a classic procedure for the analysis of interactions between agents [2]. The isobole method has been used for the evaluation of synergism or antagonism in many fields. It requires experimental data for agents used alone and in different dose combinations at equieffective levels. These data are plotted on an isoeffective graph with axes representing the doses of each agent. If two given agents do not interact, the line joining the point corresponding to the combination with those on the axes representing doses isoeffective with the combination will be a straight line [3]. When agents in combination are more effective than what might be expected from their dose-response curves (synergy), smaller amounts will be needed to produce the effect under consideration, and a concave-up isobole results. On the other hand, when agents in combination are less effective than expected (antagonism), greater doses than expected will be needed to produce the same effect, and a concave-down isobole is generated.

The construction of isobolograms has several disadvantages. Finding a combination of drugs that would produce a given effect is often a laborious task. In addition, isobolograms can only be used to evaluate interactions at this effect level. Moreover, the type and extent of the interaction may be dose-dependent; a combination of two compounds may act synergistically within one dose range while showing antagonism within another. All these questions are indeed a reflection of the fact that the interaction between two agents is a three-dimensional problem. The effect of the combination (z axis) depends on the concentration of individual agents (x and y axes). Isobolograms are used to plot portions of the three-dimensional surface describing the effect of combination of two agents. In fact, it is theoretically possible to fit experimental data to mathematical functions describing the dose-

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† *Abbreviations:* I, interaction index; AZT, 3′-azido-3′-deoxythymidine; and HIV, human immunodeficiency virus.

response surface. Isoboles could then be calculated for any effect level [4]. This method, however, requires the use of a large number of data so that a representative surface is obtained; otherwise, artifacts may result.

Many other methods have been proposed to analyze interaction between agents. Two have become very popular in recent years: the method of Chou and Talalay [5], based on the median effect principle, and that proposed by Prichard and Shipman [6], which uses the fractional product of Webb. We report here a method to evaluate interactions between biologically active agents, which can be applied to many fields. A comparison with other methods, using practical examples based on published data, is presented.

MATERIALS AND METHODS

Synergy, zero interaction, or antagonism were evaluated in two different ways using the classical isobole equation as shown in Results. For the sake of clarity, we will follow the nomenclature proposed by Berenbaum to evaluate interactions between agents [1]: zero interaction if the effect of the combination is that expected from the dose-response curves of the agents; synergy if the effect is greater than expected; and antagonism if less than expected. The expected effect of a combination was calculated by iteration from dose-response curves of individual agents using the Microsoft® Excel spreadsheet, as explained in Results. Although this program has a specific command to solve any equation directly by iteration, a macro sheet was created to speed up calculations of the expected effects of combinations arranged in a checkerboard design.

For comparative purposes, interaction between agents was also evaluated using the fractional product of Webb [7]. In this case, the expected effect of a combination of two inhibitors was calculated as

$$E_{1,2} = E_1 + E_2 - E_1 \cdot E_2,$$

where E_1 is the fractional inhibition produced by drug 1, E_2 is the fractional inhibition produced by drug 2, and $E_{1,2}$ is the fractional inhibition produced by the combination of the two.

Curve fitting of individual dose-response curves was obtained by unweighted nonlinear regression using commercially available software [8], unless otherwise stated.

RESULTS

Expected Effect of a Combination

Although classical isobologram analysis diagnoses zero interaction, synergy, or antagonism without reference to the expected effect of a combination, this method allows these parameters to be estimated. The equation for the zero interaction line for two agents is:

$$\frac{d_1}{D_1} + \frac{d_2}{D_2} = 1, \qquad (1)$$

D_1 and D_2 being the doses of agents 1 and 2 that would individually produce the same effect as the combination (d_1 + d_2). In the case of agents that, if used alone, are without effect over a large dose-response range but may alter the effectiveness of other agents, $D_i = \infty$ and its expression can be eliminated from this equation [3, 4].

Equation (1) can be easily extended to n agents:

$$\sum_{i=1}^{n} \frac{d_i}{D_i} = 1 \quad (i = 1, 2, 3, \ldots, n). \qquad (2)$$

Berenbaum [1] has shown that the expected effect of a combination, when zero interaction takes place, can be estimated graphically by finding the doses for each agent ($D_1, D_2, D_3, \ldots, D_n$) that are isoeffective with the combination ($E(D_1) = E(D_2) = E(D_3) \ldots = E(D_n) = E(d_1 + d_2 + d_3 + \ldots + d_n)$) and satisfy equation (2). Another possibility is to find these values mathematically by fitting individual dose-response curves for each agent to a monotonic function. When all dose-response curves of individual agents of a combination are simple equations (e.g. linear or simple exponential), the expected effect of the combination can be explicitly obtained from equation (2) [1, 9]. However, for more complex equations or when dose-response curves of individual agents are dissimilar, implicit equations have to be solved by iteration. For example, if the dose-response curves of two agents can be described by the median effect equation described by Chou and Talalay [5] (see equation (5) below), the dosis of each agent that produces some determined fractional effect (E) can be calculated as

$$D = D_m \cdot \left(\frac{E}{1-E}\right)^{1/m}. \qquad (3)$$

After substitution on equation (2), we have

$$\frac{d_1}{(D_m)_1 \cdot \left(\frac{E}{1-E}\right)^{1/m_1}} + \frac{d_2}{(D_m)_2 \cdot \left(\frac{E}{1-E}\right)^{1/m_2}} = 1. \qquad (4)$$

The expected effect E caused by d_1 and d_2 used in combination cannot be obtained directly from this expression. However, it could be easily calculated with any spreadsheet having a command to solve mathematical equations by iteration. Since equation (2) is valid irrespective of the shape of the agent's dose-response curves, the expected effect of a combination with any number of agents can be easily calculated by this method.

Curve Fitting of the Individual Dose-Response Curves

The isobologram technique requires the estimation of doses of each agent that produce the same effect as the combination. This estimation is usually achieved by fitting individual dose-response curves to a mathematical function and from this equation calculating the concentration of each agent that produces a given effect. As pointed out by several authors, there is no general equation that could be used to fit all dose-response curves, because the shape of the curve depends on the mechanism of action of the inhibitor [4, 10, 11]. However, in

many cases an equation can be found that fits experimental data. For example, many reversible inhibitors show dose-response curves of hyperbolic or sigmoidal form. Chou and Talalay [5] have shown that several of these curves can be fitted using the so-called "median effect equation," which states that

$$\frac{E}{1-E} = \left(\frac{D}{D_m}\right)^m \tag{5}$$

where D is the dose, E the fraction of the system affected by the dose D (fa in the original article), D_m the dose required to produce the median effect analogous to IC_{50}, ED_{50}, or LD_{50} values, and m is a coefficient indicating the sigmoidicity of the dose-effect curve [5]. This equation can be linearized by taking the logarithms on both sides,

$$\log\left(\frac{E}{1-E}\right) = m \cdot \log(D) - m \cdot \log(D_m), \tag{6}$$

showing that a graph with log (D) on the abscissa against log ($E/1 - E$) on the ordinate will be a straight line with slope m and intercept $\log(D_m)$ on the dose axis. This equation is usually presented in an equivalent form as

$$\log[(E)^{-1} - 1]^{-1} = m \cdot \log(D) - m \cdot \log(D_m). \tag{7}$$

Estimation of these parameters by the linearized version of this equation, though widely used, is not the best way to obtain these values. Figure 1 shows that simple linear regression is not applicable for the analysis of such data because this transformation distorts error distribution. It is not surprising that several authors have found that estimation of synergy by the method proposed by Chou and Talalay [5] often shows a great deal of variance, particularly on both ends of the curve [12, 13]. If linear regression is to be employed to analyze the data, it is necessary to apply appropriate weighting to compensate for the distorted error distribution. A related problem is found in the estimation of kinetic parameters of reactions following a Michaelis-Menten mechanism [14].

Nonlinear regression of the original data is an approach with a wider applicability than the use of complex weighting functions for linear regression of rearranged data. Nonlinear parameter estimation can be performed with the help of commercial easy-to-handle software without perturbing the error distribution of the data. From equation (5) we can obtain

$$E = \frac{1}{1 + \left(\frac{D_m}{D}\right)^m} \tag{8}$$

a suitable form for nonlinear regression once experimental data have been obtained.

An additional drawback of the linear transformation proposed by these authors is that linearization of experimental data precludes examination of curve fitting and analysis of the form of the curve. Sometimes low linear regression coefficients are found, whereas in other cases dose-response curves are determined within a narrow range of effects [15, 16]. For the

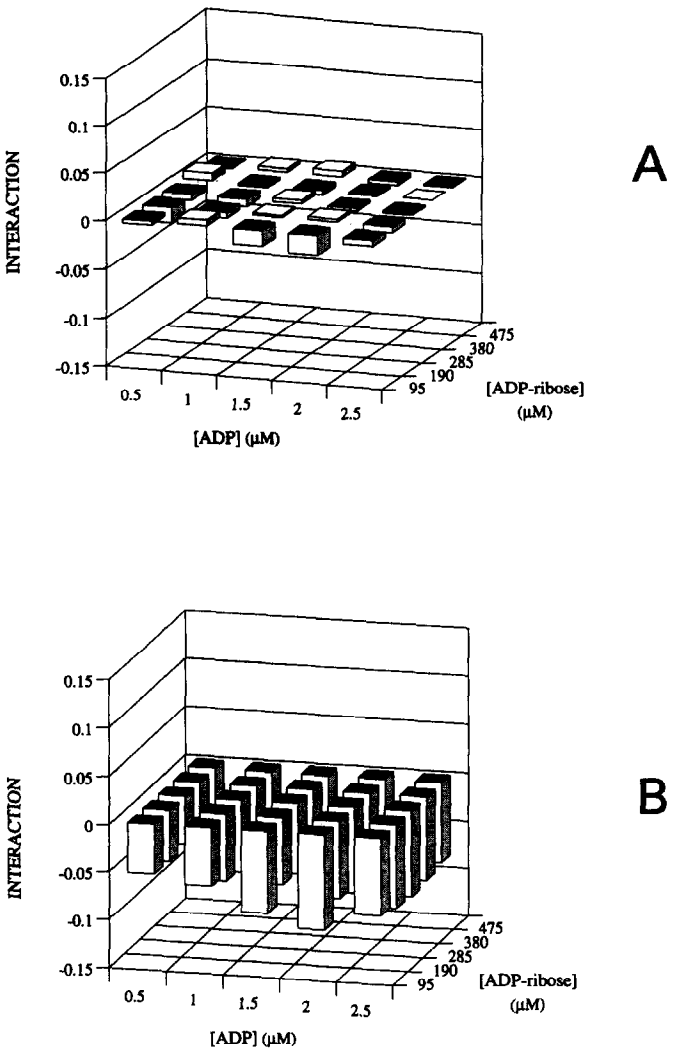

FIG. 1. Interaction between ADP and ADP-ribose on the inhibition of horse liver alcohol dehydrogenase. Interaction was assessed as the difference between the measured and expected fractional effect for each combination obtained (A) by iteration on equation (4) or (B) by the fractional product of Webb. Inhibition data were retrieved from Table V of reference [22]. Positive values indicate synergy, zero or near zero values indicate zero interaction, and negative values (columns having black bases) indicate antagonism.

use of isobolographic analysis, it is not sufficient that experimental data fit equation (7) with a high regression coefficient, as suggested by Chou and Talalay. There is no guarantee that outside this range real data should follow the median effect equation. In our experience, some reversible enzyme inhibitors do not behave as expected from equation (8). This equation predicts that if $D \gg D_m$ the whole system is affected ($E = 1$), whereas if $D \ll D_m$ the system would not be affected at all ($E = 0$). In some cases, complete inhibition is not achieved by any dose of the inhibitor. Although most enzyme inhibitors studied have been interpreted as full inhibitors, it is likely that in many of these cases only partial inhibition could be operating, since common plots used to represent kinetic data do not allow a clearcut discrimination between them [17]. It should be pointed out that this type of inhibition is not detected unless

a complete dose-response curve within a broad range of concentrations is obtained.

Other possible causes for this behavior, such as solubility problems, antimicrobial or cellular resistance and tolerance development, might be elucidated in many research areas; in any event, though, curves cannot be described by equation (8). For example, as judged by the data obtained by Eron *et al.* [15], the maximal inhibition achievable on HIV+ growth by AZT in an AZT-resistant clinical isolate is approximately 30%. Although these data can hardly be fitted into equation (7), the authors show that in the range of concentrations they have used, the linear regression coefficient is as high as 0.9. Taking into account that the inhibition caused by the combinations of AZT and ddC are within the range of 66%–99.8%, doses of AZT that individually would produce such an effect obviously cannot be obtained from this equation. Many of these curves can be fitted into the more general expression

$$E = \frac{a}{1 + \left(\frac{D_m}{D}\right)^m} \tag{9}$$

where a is the maximum effect achieved with an infinite amount of inhibitor [18]. This equation cannot be solved by any linear transformation, because three parameters (a, D_m, and m) have to be estimated; however, these values can be easily obtained by nonlinear regression.

Although we have centered our discussion on inhibitors having dose-response curves of sigmoidal shape, some conclusions can be derived irrespective of the form of the curve. The use of nonlinear fitting for other kinds of inhibitors is also strongly recommended. Equations related to expression (9) are included in the software we have used [8], and other equations suitable for nonlinear fitting of experimental data can easily be added. It should be stressed that dose-response curves must be obtained for the whole possible range. This will lead to the correct estimation of doses for any effect level.

Design of the Combination Experiment and Analysis of Data

To determine the interaction between two or more drugs, five steps should be followed: (1) A complete dose-response curve for each drug alone within a wide range of concentrations will have to be obtained. (2) An appropriate equation to fit inhibition data of each agent by nonlinear regression should be chosen. As pointed out above, there is no universal equation applicable to all inhibitors. In the examples discussed below, equation (8) has been used to fit the data, but in other cases different equations should be used. Some authors have expressed an overt preference for mechanistic over empirical models. However, one should always bear in mind that if the model and the data are in conflict, it is the model that must be changed. (3) The combination experiment should be designed in a checkerboard manner. To select the appropriate combinations of inhibitors, the expected effect of each combination can be calculated. As explained above, individual dose-response equations combined with the isobole equation (equation 2) make it possible to obtain the expected effect of each combination. For example, with a mix of two inhibitors that follow the median effect principle (equation (8)), the expected effect of each combination can be obtained by iteration from equation (4). Calculations can be done easily with any spreadsheet having a command to solve equations by iteration. The goal is to determine the expected effect (E) that makes the first member of the equation equal to unity. When designing the experiment, it should be remembered that some concentrations selected to obtain individual dose-response curves might not be the most adequate for combination analysis; it is clear that mixing inhibitors at very high concentrations will not help in diagnosing synergy. (4) The experiment should be performed. (5) The interaction between drugs should be analyzed.

There are at least two ways of analyzing interactions between two agents. The first is to compare the experimental results with the expected effects obtained by iteration. Differences can be displayed in a three-dimensional plot where x and y axes represent concentrations of each agent, and the z axis is the difference between actual and expected effects. Positive differences indicate synergy; no interaction is represented by zero or near-zero values; and negative values indicate antagonism. Graphical representation can be done in a three-dimensional plot that allows direct inspection of the results (see Figs. 1A, 2A, and 3A). If combinations with three or more agents are tested, graphical representation will not be possible and the results can be tabulated.

However, synergy may be viewed from another angle more related to isobolographic principles. In isobolographic analysis, substances do not interact if the effect of the combination remains unaltered when a part of one of the constituents is replaced by the effect-equivalent dose of the other substance [19]. Interaction index (I) is defined as

$$I = \sum_{i=1}^{n} \frac{d_i}{D_i} \quad (i = 1, 2, 3, \ldots, n), \tag{10}$$

where d_i corresponds to the doses of the individual agents in a combination, and D_i to the doses of the agents that individually would produce the same effect as that caused by the combination [3, 4]. When $I = 1$, agents in the combination do not interact; if $I > 1$ the combination is antagonistic; and if $I < 1$ the combination is synergistic. This parameter is equivalent to the combination index proposed by Chou and Talalay for the quantification of interactions between mutually exclusive drugs [5]. Once individual dose-response curves for each inhibitor are fitted to appropriate equations, the interaction index can be easily obtained from equation (10) by determining the concentration of each separate agent ($D_1, D_2, \ldots, D_n$) that gives the same effect as that of the combination ($d_1 + d_2 + \ldots + d_n$). This index can be plotted in a three-dimensional graph as in the preceding method. A drawback to direct plotting of this parameter is that the resulting graph is highly

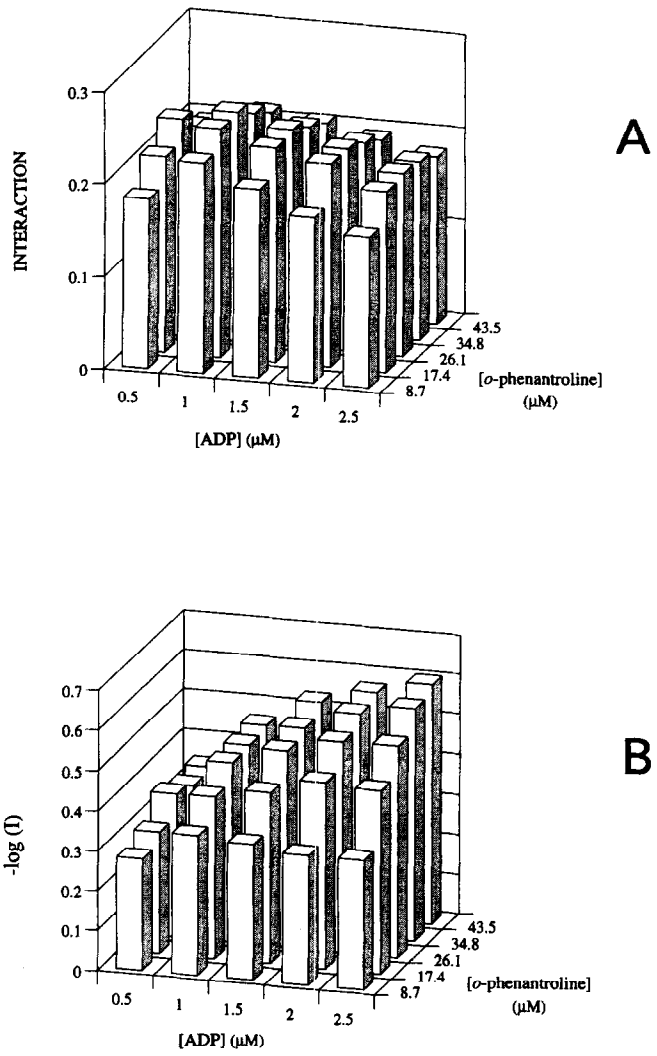

FIG. 2. Interaction between ADP and *o*-phenantroline on the inhibition of horse liver alcohol dehydrogenase. Interaction was assessed (A) as the difference between the measured and expected fractional effect obtained by iteration on equation (4), or (B) by plotting the logarithm of the interaction index with its sign changed. The interaction index (*I*) was obtained from each combination using equation (10). Inhibition data were retrieved from Table V of Ref. [22]. In both cases positive values indicate synergy, zero or near zero values indicate zero interaction, and negative values indicate antagonism.

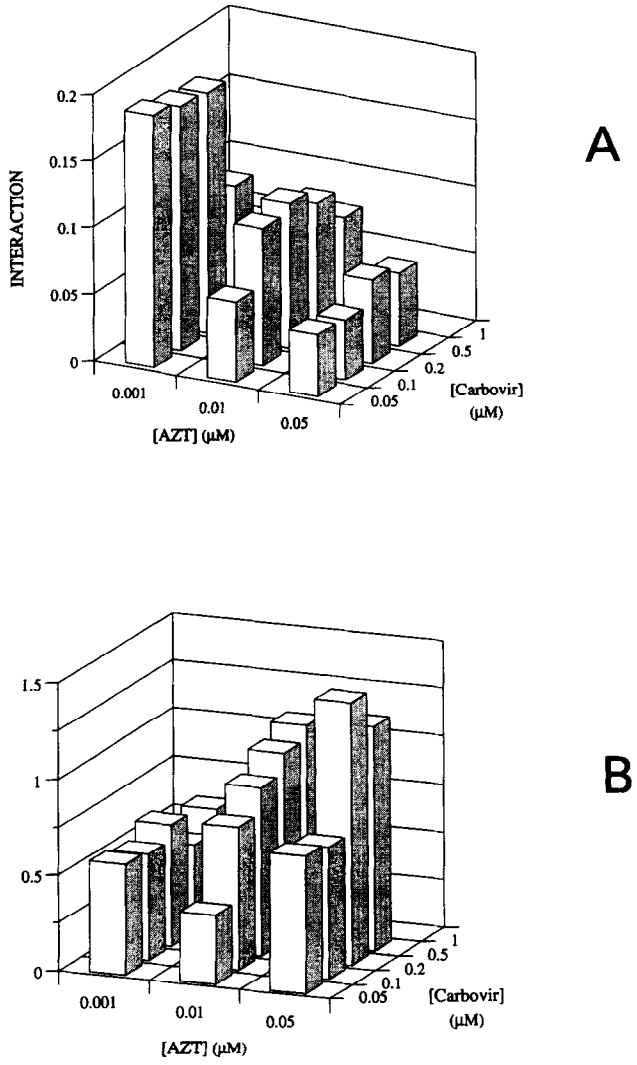

FIG. 3. Interaction between AZT and carbovir on the inhibition of HIV virus replication. Interaction was assessed (A) as the difference between measured and expected fractional inhibition for each combination obtained by iteration on equation (4), or (B) by plotting the logarithm of the interaction index for each combination with its sign changed. The interaction index (*I*) was obtained from each combination using equation (10). In both graphs, positive values indicate synergy, zero or near zero values indicate zero interaction, and negative values indicate antagonism. Inhibition data were retrieved from Table 2 of Ref. [23]. The combination of 0.05 μM of AZT with 1 μM of carbovir was not tested by these authors.

asymmetric. Synergistic combinations can take values between 0 and 1, while antagonistic interactions are represented by any number between 1 and infinity. This asymmetry is clearly perceived in the presentation proposed by Chou and Talalay [5], one used by many authors. This problem can be minimized by plotting not the interaction index itself, but its logarithm, as discussed when analyzing specific examples (Figs. 2B and 3B). To maintain consistency with the preceding method (i.e. synergy above the plane and antagonism below it), it is preferable to plot the logarithm of the combination index with its sign changed.

Although both methods give similar results for each combination (synergy, zero interaction, or antagonism), they are conceptually different. Whereas the first method shows the variation of effects found as a consequence of the interaction, the second is a reflection of the reduction or increase in total dose, taking into account the potency of each agent, as a result of this interaction. Unfortunately, most authors claiming a synergism or antagonism for the combination of two agents show the outcome of the analysis according to a model, precluding examination of experimental data. In the next section, we will present two examples obtained from articles where original data were available and combinations of agents at several concentration ratios were tested.

Example 1: Inhibition of Horse Liver Alcohol Dehydrogenase

Yonetani and Theorell [20] analyzed the inhibition of horse liver alcohol dehydrogenase by three inhibitors that compete with NAD^+: ADP, ADP-ribose, and *o*-phenantroline. ADP and ADP-ribose are competitive, mutually exclusive inhibitors, while ADP and *o*-phenantroline are competitive, mutually nonexclusive inhibitors. Individual dose-response curves obtained with ADP, ADP-ribose, and *o*-phenantroline on this enzyme are presented in Fig. 4. These three inhibitors closely follow the median effect equation in the range of concentrations analyzed [21]. However, there is no experimental evidence that data fit the curve outside this range. A close inspection of the original data [22] reveals that several combinations of ADP and *o*-phenantroline resulted in inhibitions of enzyme activity greater than 90%. Estimating the concentration of ADP that when taken alone would give this inhibition, by extrapolation on the dose-response curve shown in Fig. 4, can be misleading.

Combinations of ADP and ADP-ribose or ADP and *o*-phenantroline were tested in a 5 × 5 checkerboard arrangement allowing synergy to be estimated. Using the method proposed by Chou and Talalay [5], only combinations of two inhibitors in a fixed concentration ratio can be used to evaluate interactions between them. For this reason, only five of the twenty-five combinations tested are useful if this method is followed. However, as explained above, the expected effect for each combination can be obtained by iteration by applying equation (4). Differences between experimentally measured and calculated inhibitions for the combination of ADP and ADP-ribose are presented in Fig. 1A. As expected from their mechanism of action, when two competitive exclusive inhibitors, ADP and ADP-ribose, are combined, zero interaction is reflected. A very similar plot was obtained when the interaction index was calculated and plotted as explained above (data not shown).

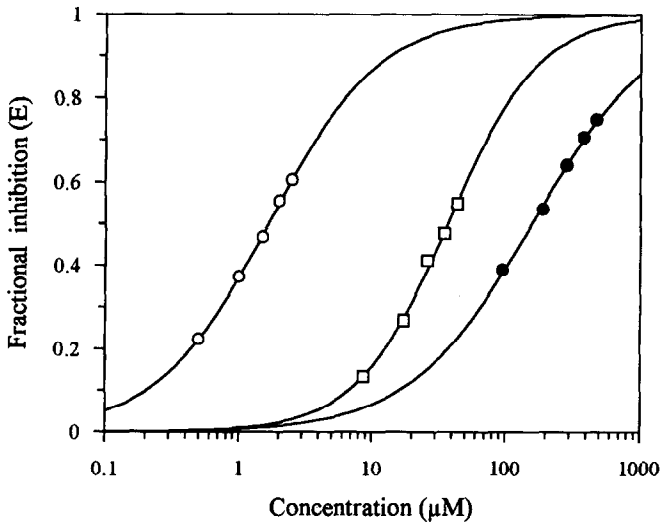

FIG. 4. Dose-response curves for the inhibition of horse liver alcohol dehydrogenase by ADP, ADP-ribose, and *o*-phenantroline. Curves were obtained by fitting experimental data to equation (8) by unweighted nonlinear regression as explained in Materials and Methods. Data for ADP (○) and ADP-ribose (●) were retrieved from Table IV of Ref. [22], and inhibition caused by *o*-phenantroline (□) was obtained from Table V of Ref. [22]. For ADP D_m = 1.657 μM, m = 1.045; for ADP-ribose D_m = 156.4 μM, m = 0.9650; and for *o*-phenantroline D_m = 36.84 μM, m = 1.288.

However, when these data are analyzed using the fractional method of Webb, antagonism for all concentrations is diagnosed (Fig. 1B). This approach has been employed by some authors to estimate interaction between agents [6]. Although this method gives correct estimates of the inhibition caused by a mixture of two mutually nonexclusive inhibitors that follow Michaelis-Menten-type hyperbola, it cannot be applied to other cases [22]. In fact, analyzing interactions between agents with this method can give paradoxical results. A combination of an agent with itself could result in synergy, no interaction, or antagonism, depending on the doses chosen and the shape of the agent's dose-response curve [1, 4].

For the combination of ADP and *o*-phenantroline, two competitive, mutually non-exclusive inhibitors, differences between experimental and expected effects and interaction index (I) are plotted in Fig. 2A and B. Both representations indicate marked synergy at any of the concentrations tested, but the "optimum" combination (i.e. that producing the maximum synergy) does not coincide. According to the interaction index, synergy increases as the concentration of ADP and *o*-phenantroline increases, whereas in the other graph maximum synergy appears at moderate concentrations of the inhibitors. The reason for this discrepancy is that the information given in the graphs differs. When several agents are mixed at high concentrations, the effect for each agent alone is usually high, so that the combination of these agents cannot result in an inhibition noticeably higher than that observed with agents assayed alone. However, the concentrations needed to reach a specific inhibition level can be substantially decreased. Plotting differences between actual and expected effects underestimates synergy at high inhibitor concentrations. This can lead to the false impression that synergy is larger at moderate concentrations of the agents than that actually present when they are mixed at high concentrations. When the fractional product method is used and differences between actual and expected effects are plotted, a more marked decrease in synergy with increasing concentrations of inhibitors is found (data not shown).

Using the same data, Chou and Talalay [5] reached different conclusions. As the expected effect for the combination of two nonexclusive inhibitors is greater than for two mutually exclusive inhibitors, they reasoned that equation (10) cannot be used. Instead they proposed that the combination index (CI) for the mixture of two nonexclusive inhibitors may be calculated as follows:

$$CI = \frac{d_1}{D_1} + \frac{d_2}{D_2} + \frac{d_1 \cdot d_2}{D_1 \cdot D_2}. \qquad (11)$$

Using equation (11), they found that the combination of ADP and *o*-phenantroline results in moderate antagonism at

low inhibition values and marked synergism at high inhibition values [5]. This conclusion is misleading, since equation (11) predicts zero interaction for the combination of first-order mutually nonexclusive inhibitors, whereas the interaction of these inhibitors, as expected from their mechanism of action, is necessarily synergistic [1].

Example 2: Inhibition of Human Immunodeficiency Virus Type 1 by Carbovir and AZT

To evaluate the interaction between carbovir and AZT in the inhibition of HIV-1 virus replication in primary human peripheral blood mononuclear cells, Smith *et al.* [23] employed combinations of these inhibitors at several ratios of drugs and analyzed their interaction with the method proposed by Chou and Talalay [5]. Dose-response curves for AZT and carbovir were calculated according to the median effect equation and are presented in Fig. 5. The presumption that AZT follows the median effect equation is not justified, because only four concentrations of this drug were tested, and significant deviations from predicted values were detected at low concentrations of the drug. These deviations are more clearly seen in the original dose-response curve (Fig. 5) than in the linearized form, where a high regression coefficient ($r = 0.95$) is found. Whether these deviations are relevant or not cannot be deduced from these data, because standard deviations were not presented in the original article.

Assuming that AZT and carbovir follow the median effect equation, differences between experimental and expected effects obtained by iteration applying equation (4) and interaction index (I) were calculated and represented as before (Fig. 3A and B). In both cases marked synergism was found for all combinations tested. It should be noted that in several combinations the fractional inhibition achieved combining both agents exceeded 0.91, the maximal inhibition measured with each agent alone. Smith *et al.* [23] did not calculate combination indices when the fraction affected was greater than 0.98. In fact, combination indices calculated for effects greater than 0.91 should be considered provisional until complete individual dose-response curves for AZT and carbovir are obtained.

As in the preceding case, estimation of synergy by these two methods gives different quantitative results. In Fig. 3A, maximum synergy is observed when agents are combined at moderate concentrations, whereas synergy decreases as concentrations increase. The opposite is true if the interaction index is used to measure synergy (Fig. 3B). In fact, the expected inhibition for six of the combinations of AZT and carbovir, if zero interaction holds, are greater than 90%. As discussed above, estimation of synergy as the difference between real and expected inhibition underestimates synergy at high concentrations of inhibitors. Moreover, for combinations having 0.001 μM of AZT, synergy is overestimated in both cases because inhibition obtained from the curve is smaller than the experimentally measured effect (Fig. 5).

If the fractional product method is employed to analyze these data, modest synergy (<3%) or no interaction is diagnosed for all combinations but two, where moderate antagonism is found (data not shown). As in the preceding case, synergy decreases as the concentration of inhibitors increases.

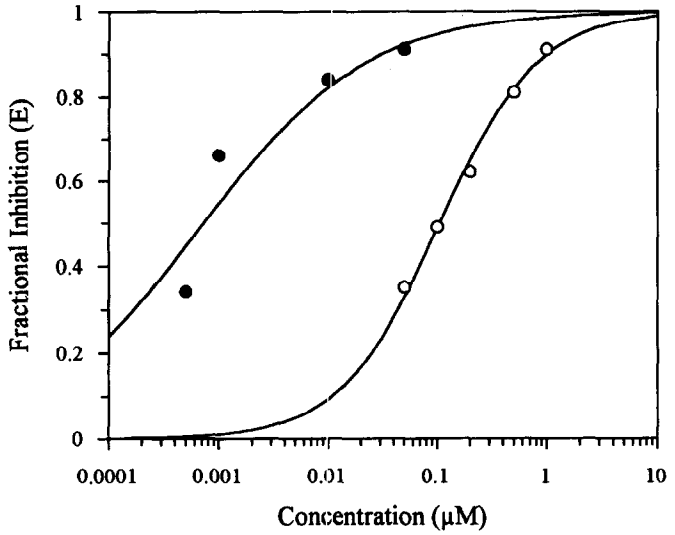

FIG. 5. Dose-response curves for the inhibition of HIV-1 virus replication in primary human peripheral blood mononuclear cells by AZT and carbovir. Although these curves are presented in the nonlinearized form, for comparative purposes their parameters were obtained as in the original article by linear transformation according to equation (6), using the method proposed by Chou and Talalay [5]. For AZT (○) D_m = 0.000723 μM, m = 0.583; and for carbovir (●) D_m = 0.104 μM, m = 0.970. Inhibition data were retrieved from Table 2 of Ref. [23].

DISCUSSION

Since its publication in 1984, the method proposed by Chou and Talalay [5] to estimate interaction between agents has become popular in many research areas. The method requires a relatively small number of data to evaluate interactions between agents, and a computer program facilitating the calculations is available. However, it has several disadvantages. Only agents that alone and in combination have dose-response curves fitting the median effect equation can be used, and many inhibitors show dose-response curves that cannot be fitted by equation (5). An unfortunate consequence is that some authors use this method when data do not follow the median effect principle or when data are obtained in a narrow range of effects [15, 16]. Moreover, although experimental data follow equation (5), the linear transformation proposed by Chou and Talalay is not the best way to estimate m and D_m parameters (Fig. 6). Nonlinear regression is a superior means of obtaining these values, and allows direct inspection of data as depicted in Figs. 4 and 5. Moreover, it permits the use of many other equations for fitting experimental data. Another problem with the method proposed by Chou and Talalay [5] is that inhibitors must be tested in a fixed, arbitrary concentration ratio. Some authors, aware of this limitation, test their compounds at several ratios to obtain representative results. A more logical design is to diagnose interaction in a checker-

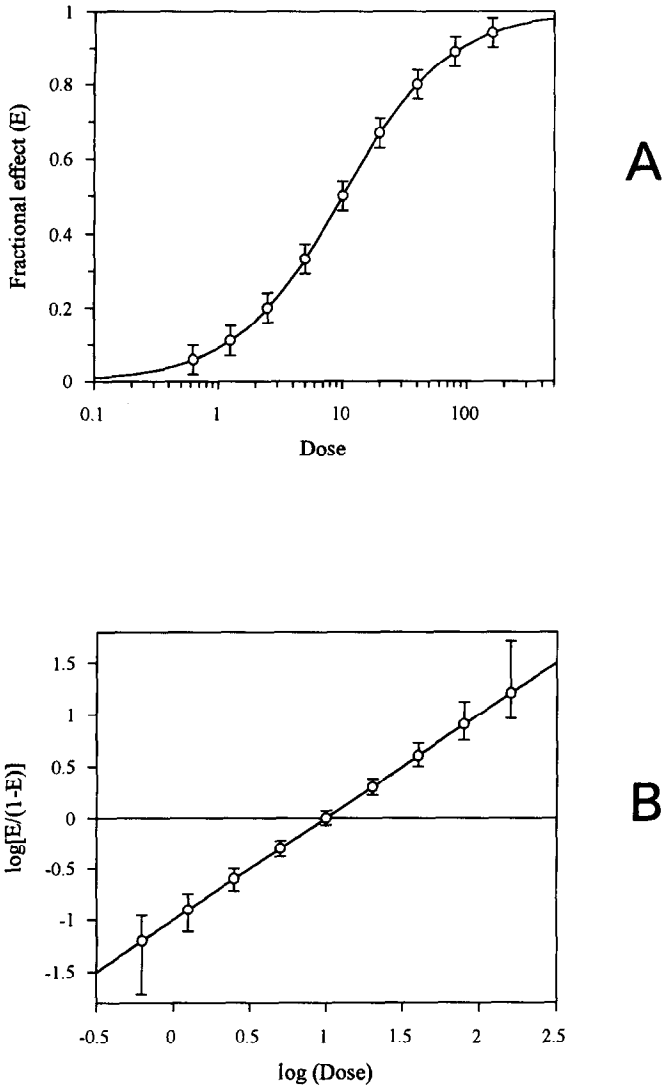

FIG. 6. Distorted error distribution as a consequence of linearization of experimental data. A hypothetical dose-response curve for an inhibitor that follows the median effect equation is shown in (A) original or (B) linearized form. For this inhibitor $D_m = 10$, $m = 1$, and a constant y error is assumed for all data. It is clearly shown that linearization asymmetrically distorts error distribution, precluding accurate estimation of these parameters.

board manner at several concentrations of both inhibitors, as set out in Results.

It is a matter of considerable controversy whether equation (2) fulfills general and valid criteria to estimate synergy between biologically active agents. Based on kinetic principles, Chou and Talalay [22] have demonstrated that the expected effect of combining two nonexclusive enzyme inhibitors is greater than that obtained combining two exclusive inhibitors at the same concentrations. For this reason they argued that although equation (2) is valid for exclusive inhibitors, interaction between two nonexclusive inhibitors must be estimated by equation (11). In fact, what they demonstrated is that synergy is expected from the interaction between nonexclusive inhibitors, but this does not argue against the validity of equation (2). As shown by Berenbaum [1], equation (11) predicts synergy and not zero interaction. Other authors maintain that equation (2) is only valid when dose-response curves of individual agents have a linear shape. This interpretation is misleading in the sense that additivity in isobolographic analysis is referred to as additivity of doses and not as additivity of effects. In an isobologram we can determine, for a determined effect level, if agents in combination are dose-additive, or if total doses required, taking into account the relative potency of each agent, are smaller (synergy) or greater (antagonism) than expected. The effect obtained by iteration in equation (2) is the expected one if agents were dose-additive, not effect-additive. In fact, no equation can be found that predicts the combined effect of two drugs irrespective of the shape of dose-response curves of individual agents. Only with a profound knowledge of the mechanism of action of the drugs is it sometimes possible to predict their combined effect.

In spite of this limitation, many models used to evaluate interactions between agents require estimation on the expected inhibition of two drugs as if they were effect-additive. Experimental data are compared with expected results and the interaction diagnosed. Several of these methods are based on the fractional product of Webb [7]. Some investigators have assumed that if two inhibitors act independently, the expected effect of the combination can be calculated by the product of their fractional activities. Using this principle, Prichard and Shipman [6] have proposed a three-dimensional model that allows interactions between agents to be assessed in a checkerboard manner. For the combination of a competitive and a noncompetitive inhibitor obeying the Michaelis-Menten equation, this model predicts zero interaction, while synergy is expected from their mechanism of action. Moreover, application of these criteria to the combination of two doses of the same compound can give synergy, zero interaction, or antagonism, depending on the shape of its dose-response curve and the concentrations chosen [1, 4, 24]. This can lead one to diagnose antagonism when zero interaction is present, as seen above for ADP and ADP-ribose (Fig. 1). Prichard and Shipman [6] suggest that this is a more restrictive criterion to evaluate synergy than the utilization of equation (2). Even if the fractional product method is a more restrictive criterion, which is not always the case, its use is not justified. In some cases it would be sufficient if the combination of agents were not antagonistic as long as other benefits (e.g. reduction in toxicity or better bioavailability) were obtained.

Another drawback of the method proposed by Prichard and Shipman [6] is that interaction is assessed as the difference between actual and expected effects. This method was originally developed to evaluate interactions in the combination of antiviral agents suppressing viral replication. In this case the interaction index is a more useful parameter because plotting differences between experimental and expected effects underestimates synergy at high inhibitor concentrations. For example, in the fight against HIV infection, several cocktails of inhibitors have been assayed to knock out virus replication, preventing the development of drug resistance [25]. Some of these compounds can, if they are used at sufficiently high (sometimes toxic) concentrations, individually effectively

block viral replication *in vitro*. The aim of combining several agents is the possibility of reducing the doses of individual agents lowering cellular toxicity and increasing the therapeutic index, rather than increasing total inhibition [25, 26]. In other cases, differences between experimental and expected effects may be more informative. If this criterion is used to evaluate interaction between agents, it should be limited to those cases where expected inhibition for the combinations is not very high; otherwise, synergy could be underestimated.

Some methods propose a direct three-dimensional approach to the combination of two drugs, fitting experimental data to a dose-response surface obtained with the aid of complex mathematical functions and sophisticated graphic programs [4, 6, 9, 27]. The dose-response surface experimentally obtained is then compared with the expected dose-response surface, obtained according to a model, and the interaction is then evaluated. It is important to keep in mind that the validity of a method depends on the validity of the model used to define zero interaction for the combination of the agents. In the method of Prichard and Shipman [6], zero interaction is defined by the fractional product of Webb, which, as discussed above, could lead to incorrect results. In the method of Greco *et al.* [27], dose-response curves for all agents are supposed to follow some strictly predetermined equation, such as the median effect equation. As pointed out above, there is no general equation that would fit all dose-response curves, because the shape of the curve depends on the mechanism of action of the inhibitor. In addition, the evaluation of synergy by this approach has been found to be in disagreement with that obtained with the isobole method [10, 11].

Even in the event that the method employed is well founded, the surface fitting of experimental data has several disadvantages. Mathematical equations employed for curve fitting may have up to seven or eight parameters, and the number of combinations that must be examined in order to fit a dose-response surface rises exponentially with the number of parameters [1, 24]. If piecewise fitting is used, arbitrary mathematical smoothing has to be applied to the experimental dose-response surface, modifying experimental data [4]. In addition, extension to three or more agents would be very complex. Rather than use complicated formulae to describe three-dimensional surfaces, it is simpler and more flexible to use curve fitting for each agent alone and then combine them with the isobole equation, as explained above. In this way, many different equations can be used to fit individual dose-response curves of individual agents.

Some conclusions can be derived concerning experimental design aimed at evaluating synergy between agents. Dose-response curves for individual agents must be obtained for the broadest possible range of effects to obtain a complete dose-response curve for each agent. In this article we have centered our attention on dose-response curves of sigmoidal shape, but many other types exist that can be fitted by nonlinear regression using appropriate equations. Once individual dose-response curves are obtained, several concentrations of agents can be tested in a checkerboard design. The predicted effect for each combination can be calculated by iteration using the classical isobole equation. A macro has been written in a spreadsheet that allows calculation of the expected effects of combinations arranged in a checkerboard design if zero interaction is present. Using this macro, appropriate concentrations of individual agents are selected for each combination, allowing interaction to be diagnosed. If synergy is expected for the combination of two or more inhibitors, mixing them at very high concentrations would not be useful, since the expected effect of the combination would, in fact, be very high. Interaction between agents can be assessed in two ways: by comparing experimental with expected effects if zero interaction is present, or by analyzing the reduction or increase in total dose found as a consequence of the interaction. Which information is more relevant depends on what advantage is expected as a result of the interaction between agents.

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