Biochem. J. (1998) **329**, 689–698 (Printed in Great Britain)

Analysis of the combined effect of two linear inhibitors on a single enzyme

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Different methods for studying the concurrent effects of two linear inhibitors on a single enzyme have been published, including the fractional product of Webb, the Yonetani–Theorell plot or the method of Chou and Talalay. Recently the use of combination plots has also been advocated for this purpose. We have evaluated the applicability of these methods and found that most of them depend on assumptions about the mechanism of action of the inhibitors. If the mechanism of action is not completely understood, or if some assumptions about the mechanism are unfounded, the parameters obtained may be meaningless. Even if these assumptions are correct, the interaction can be advantageously measured using an alternative representation that does not require a knowledge of the inhibition constants and allows experimental data to be retrieved from the plot. In other cases it is the interpretation of the results rather than the validity of the method that is misleading. A common mistake is to take the exclusivity of the effects of two inhibitors as exclusivity of their binding. We show that this assumption is seldom justified. In any case, it is possible to decide whether the combination of two or more inhibitors is more effective than their individual use by means of isobolographic analysis, even when no information about their mechanism of action is available.

INTRODUCTION

The study of the combined effect of two inhibitors on a single enzyme is used for different purposes. Multiple inhibition analysis has been useful to determine the exclusivity of the binding of several inhibitors and to postulate the existence of distinct binding sites on an enzyme [1–6]. Other researchers have made use of this analysis to verify the catalytic mechanism of an enzyme by detecting the presence of ternary abortive complexes [7,8]. It has also proved to be valuable for the rational design of enzyme inhibitors. To maximize the affinity of an enzyme for an inhibitor, it is of interest to exploit all the potential interactions available. In proteases these interactions are numerous and intricate because of the highly extended nature of the active site [9]. The possible existence of interactions between subsites has been examined by analysing the combined effect of short peptides as competitive inhibitors of the protease, and substantial synergy has been found for some combinations [9–11]. In oncology, inhibition of farnesyl:protein transferase is an interesting approach to inhibiting Ras function. Detailed kinetic analysis revealed that the presence of phosphate or pyrophosphate increases the potency of farnesyl diphosphate competitive inhibitors [12]. These results have led to the synthesis of a derivative containing a covalently linked phosphate group, making this compound one of the most potent inhibitors against this enzyme reported to date. In anti-viral research, there is a growing interest in the combined effect of several inhibitors acting on viral DNA polymerases. For example, a key step in the HIV life cycle is the reverse transcription of genomic RNA into double-stranded DNA mediated by the virally encoded reverse transcriptase. Synergistic inhibition of HIV replication in cell cultures has been reported for mixtures of nucleoside analogues and for combinations of dideoxynucleosides with non-nucleoside inhibitors. Much effort has been devoted to finding the biochemical mechanism underlying this synergy, and analysis of the combined inhibition of these inhibitors on reverse transcriptase activity has been used for this purpose [13–21].

Several methods of analysing the effect of the combination of two or more inhibitors on a single enzyme have been published, such as those suggested by Yagi and Ozawa [22], Webb [23], Yonetani and Theorell [24], Chou and Talalay [25] and Asante-Appiah and Chan [26]. By far the most commonly used method of analysing the interaction between two inhibitors on a single enzyme is that proposed by Yonetani and Theorell [24]. In fact, most of the examples reported above have used this method. Isobolograms are sometimes found in the literature to analyse the interaction between two inhibitors [5,6,17,24], although their use has not become widespread. The method proposed by Chou and Talalay [25] is also currently employed to analyse the interaction between enzyme inhibitors [13,20,21].

These methods often give discordant results when applied to the same data [21,25,27], and, as a consequence, some authors use two or more of them on the supposition that, even if their validity is unproved, the conclusion is more likely to be correct if several methods agree. We have analysed the applicability and limitations of these methods and found that, in most cases, they depend on assumptions about the mechanism of action of the inhibitors that cannot be tested by steady-state kinetics alone. If the mechanisms of action of the inhibitors are well known, combination experiments can provide interesting information on the interaction of the inhibitors at a molecular level. But if the mechanism is not understood, the parameters obtained may be meaningless. In any case it is possible to decide whether the effects, but not the binding, of two or more inhibitors are mutually exclusive by means of isobolographic analysis.

As an illustration of the methods discussed in this paper, we have used the classical work of Yonetani and Theorell [24] on the inhibition of horse liver ethanol dehydrogenase by three fully reversible inhibitors that compete with NAD^+: ADP, ADP-ribose and *o*-phenanthroline. Experimental data were retrieved from Table V of ref. [28]. It should be noted that the units for the concentration of ADP in that Table should be mM not μM [24]. ADP and ADP-ribose are competitive mutually exclusive inhibitors, whereas ADP and *o*-phenanthroline are competitive mutually non-exclusive inhibitors. The exclusively of their binding to the enzyme was demonstrated directly by an equilibrium experiment as well as by crystallization of the complex *o*-phenanthroline–enzyme–ADP-ribose [24].

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EXPERIMENTAL

One of the main problems when dealing with analysis of combinations of inhibitors is the lack of consistency in the use of common terms between different methods, such as interaction, synergy or antagonism. In mechanism-dependent methods, two inhibitors do not interact if the observed effect of the combination corresponds to that predicted by a specific equation, and synergism and antagonism are defined as divergence between observation and expectation. Since the form of the equation depends on the mechanism assumed, the concept of no interaction will change with the method used. In isobolographic analysis, interaction is defined without reference to the mechanism of action of each inhibitor. For this reason we will use the nomenclature proposed by Berenbaum [29] to evaluate the interaction between inhibitors: 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. In this context, synergism or antagonism do not refer to the mutual influence on the binding between inhibitors but to the reduction, or increase, in the concentration needed to reach a specific effect as a consequence of the interaction. Thus two inhibitors that bind independently to an enzyme will show positive interaction, i.e. synergy, since use of the combination means that the total dose needed to obtain a specific effect can be effectively reduced.

Rate equation for two inhibitors acting on a single enzyme

No theory or equation can include all types of inhibition, even in the simple case of a single inhibitor, because the form of the equation will depend on the specific mechanism of action. A semi-generalized formulation of single-enzyme multiple inhibition by two reversible linear inhibitors has been derived (Scheme 1) [23,30]. It is assumed that I_1I_2, I_1S, I_2S and I_1I_2S are not formed and no other type of interaction, except those shown in the scheme, takes place. For binary complexes, inhibitor dissociation constants are symbolized as K with the name of the complex as subscript. Dissociation constants of ternary complexes are sometimes represented in the same way. In this case the ligand that dissociates from the complex is written in the last term. For example, $K_{EI_1I_2}$ refers to the dissociation of I_2 from the EI_1I_2 complex and is equivalent to γK_{EI_2} in Scheme 1. Factors α and β represent the change in substrate affinity induced by I_1 and I_2 respectively, or, from a different angle, the alteration in the affinity for the inhibitors due to the bound substrate. Factor γ represents the mutual influence of the two inhibitors on the binding of each other. Inhibitor binding is independent when $\gamma = 1$, whereas values of γ lower or greater than unity denote mutual facilitation or hindrance respectively. In some papers, α, β and γ are referred to as $\alpha_{1,s}$, $\alpha_{2,s}$ and $\alpha_{1,2}$ respectively [26]. If rapid-equilibrium conditions apply, the rate equation for the above mechanism is:

$$\frac{1}{v_{1,2}} = \frac{1}{V_{max}[S]}\left\{K_S\left(1+\frac{[I_1]}{K_{EI_1}}+\frac{[I_2]}{K_{EI_2}}+\frac{[I_1][I_2]}{\gamma K_{EI_1}K_{EI_2}}\right) + [S]\left(1+\frac{[I_1]}{\alpha K_{EI_1}}+\frac{[I_2]}{\beta K_{EI_2}}+\frac{[I_1][I_2]}{\alpha\beta\gamma K_{EI_1}K_{EI_2}}\right)\right\} \quad (1)$$

where V_{max} is the maximal velocity of the uninhibited reaction. Algebraic rearrangement of this equation leads to an alternative equation suitable for graphical representation:

$$\frac{1}{v_{1,2}} = \frac{1}{V_{max}}\left\{1+\frac{K_S}{[S]}+\frac{[I_1]}{K_{EI_1}}\left(\frac{K_S}{[S]}+\frac{1}{\alpha}\right) + \frac{[I_2]}{K_{EI_2}}\left(\frac{K_S}{[S]}+\frac{1}{\beta}\right)+\frac{[I_1][I_2]}{\gamma K_{EI_1}K_{EI_2}}\left(\frac{K_S}{[S]}+\frac{1}{\alpha\beta}\right)\right\} \quad (2)$$

Another useful transformation of eqn. (1) is obtained by referring the velocity of the inhibited reaction to those obtained in the absence of the inhibitor, i.e. the classical Michaelis–Menten equation:

$$\frac{v_0}{v_{1,2}} = \frac{K_S}{K_S+[S]}\left\{\left(1+\frac{[I_1]}{K_{EI_1}}+\frac{[I_2]}{K_{EI_2}}+\frac{[I_1][I_2]}{\gamma K_{EI_1}K_{EI_2}}\right) + [S]\left(1+\frac{[I_1]}{\alpha K_{EI_1}}+\frac{[I_2]}{\beta K_{EI_2}}+\frac{[I_1][I_2]}{\alpha\beta\gamma K_{EI_1}K_{EI_2}}\right)\right\} \quad (3)$$

which can be rearranged into:

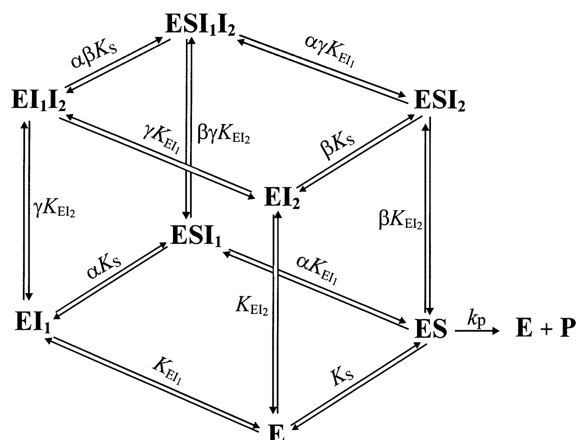
$$\frac{v_0}{v_{1,2}} = 1+\frac{[I_1]}{K_{EI_1}\left(\frac{1+[S]/K_S}{1+[S]/\alpha K_S}\right)} + \frac{[I_2]}{K_{EI_2}\left(\frac{1+[S]/K_S}{1+[S]/\beta K_S}\right)}+\frac{[I_1][I_2]}{\gamma K_{EI_1}K_{EI_2}\left(\frac{1+[S]/K_S}{1+[S]/\alpha\beta K_S}\right)} \quad (4)$$

This expression can be simplified if one realizes that, if substrate concentration is kept constant, the concentration of a linear inhibitor that reduces to half the velocity of the reaction (IC_{50} value) will be equal to:

$$(IC_{50})_1 = K_{EI_1}\left(\frac{1+[S]/K_S}{1+[S]/\alpha K_S}\right) \quad (5)$$

and by substituting in eqn. (4):

$$\frac{v_0}{v_{1,2}} = 1+\frac{[I_1]}{(IC_{50})_1}+\frac{[I_2]}{(IC_{50})_2} + \frac{[I_1][I_2]}{\gamma (IC_{50})_1(IC_{50})_2\frac{(1+[S]/\alpha K_S)(1+[S]/\beta K_S)}{(1+[S]/\alpha\beta K_S)(1+[S]/K_S)}} \quad (6)$$



Scheme 1 Equilibria among enzyme species in the presence of substrate and two linear inhibitors

In the example shown in Figure 1, IC_{50} values for ADP and *o*-phenanthroline were obtained by curve-fitting of the data by non-linear regression using Grafit 3.0 (Erithacus software) to the following equation:

$$f = \frac{1}{1 + \dfrac{IC_{50}}{[\mathrm{I}]}} \tag{7}$$

where f is the fractional inhibition exerted by [I].

RESULTS

Mechanism-dependent methods

Combination plots

It has been shown that the rate of an enzyme-catalysed reaction does not depend on the absolute concentrations of substrate and inhibitors but on the concentration relative to the specific constant for each term. This ratio is known as either relative concentration or specific concentration [23,26]. For the combination of two linear competitive inhibitors, the rate equation derived form the steady-state assumption [24] may be written as:

$$\frac{V_{\max}}{v_{1,2}} \times \frac{[\mathrm{S}]}{K_{\mathrm{m}}} = 1 + \frac{[\mathrm{S}]}{K_{\mathrm{m}}} + \frac{[\mathrm{I}_1]}{K_{\mathrm{EI}_1}} + \frac{[\mathrm{I}_2]}{K_{\mathrm{EI}_2}} + \frac{[\mathrm{I}_1][\mathrm{I}_2]}{\gamma K_{\mathrm{EI}_1} K_{\mathrm{EI}_2}} \tag{8}$$

which, in turn, using the notation proposed by Asante-Appiah and Chan [26] can be transformed into:

$$\underline{s}/\underline{v}_{1,2} = 1 + \underline{s} + \underline{i}_1 + \underline{i}_2 + \underline{i}_1\,\underline{i}_2/\gamma \tag{9}$$

The term containing the γ factor may be isolated as follows:

$$\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1 - \underline{i}_2 = \underline{i}_1\,\underline{i}_2/\gamma \tag{10}$$

In this expression, the left-hand side, termed the 'residual function of the rate', would be known if the K_{m}, $V_{\max}$, K_{EI_1} and K_{EI_2} parameters were obtained in separate experiments. To obtain these parameters, Asante-Appiah and Chan [26] proposed four sets of experiments containing no inhibitor, one of the inhibitors in turn or both inhibitors. Within each set, inhibitor and/or substrate concentrations are varied and the four parameters calculated. When the 'residual function' is plotted against $\underline{i}_1\,\underline{i}_2$, a straight line with a slope of $1/\gamma$ is obtained. However, if one or both of the inhibitors deviated from competitive behaviour, the above plot will no longer be linear. Alternative plots have been proposed for these combinations making use of modified rate equations. In fact, a more general expression can be obtained transforming eqn. (2) into:

$$\frac{V_{\max}}{v_{1,2}} \times \frac{[\mathrm{S}]}{K_{\mathrm{S}}} = 1 + \frac{[\mathrm{S}]}{K_{\mathrm{S}}} + \frac{[\mathrm{I}_1]}{K_{\mathrm{EI}_1}} + \frac{[\mathrm{I}_2]}{K_{\mathrm{EI}_2}} + \frac{[\mathrm{S}]}{K_{\mathrm{S}}} \times \frac{[\mathrm{I}_1]}{\alpha K_{\mathrm{EI}_1}} + \frac{[\mathrm{S}]}{K_{\mathrm{S}}} \times \frac{[I_2]}{\beta K_{\mathrm{EI}_2}} + \frac{[\mathrm{I}_1][\mathrm{I}_2]}{\gamma K_{\mathrm{EI}_1} K_{\mathrm{EI}_2}} + \frac{[\mathrm{S}]}{K_{\mathrm{S}}} \times \frac{[\mathrm{I}_1][\mathrm{I}_2]}{\alpha\beta\gamma K_{\mathrm{EI}_1} K_{\mathrm{EI}_2}} \tag{11}$$

which reduces to:

$$\underline{s}/\underline{v}_{1,2} = 1 + \underline{s} + \underline{i}_1 + \underline{i}_2 + \underline{i}_1\,\underline{s}/\alpha + \underline{i}_2\,\underline{s}/\beta + \underline{i}_1\,\underline{i}_2/\gamma + \underline{i}_1\,\underline{i}_2\,\underline{s}/\alpha\beta\gamma \tag{12}$$

In addition to γ, the mutual influence on the binding between substrate and each inhibitor must be considered. Although six parameters ($V_{\max}$, K_{m}, K_{EI_1}, K_{EI_2}, α and β), rather than four, must be obtained to calculate γ, no additional experiments, other than those proposed by Asante-Appiah and Chan [26], are needed. From experiments with a single inhibitor, where both substrate and inhibitor concentrations are varied, both competitive and uncompetitive constants may be calculated. These constants can be obtained by non-linear regression, or by means of a $1/v_{\mathrm{i}}$ versus [I] plot for the competitive constant, and an $[\mathrm{S}]/v_{\mathrm{i}}$ versus [I] plot for the uncompetitive constant [31]. If rapid-equilibrium conditions hold, these parameters correspond to the dissociation constants of inhibitor from the EI (K_{EI}) and from ESI complexes ($K_{\mathrm{ESI}} = \alpha K_{\mathrm{EI}}$) respectively (Scheme 1). We can then substitute $\underline{i}_{1\mathrm{s}}$ for $\underline{i}_1/\alpha$, where $\underline{i}_{1s} = [\mathrm{I}_1]/K_{\mathrm{ESI}_1}$. Making the same substitution for I_2, eqn. (12) can be transformed into:

$$\underline{s}/\underline{v}_{1,2} = 1 + \underline{s} + \underline{i}_1 + \underline{i}_2 + \underline{i}_{1\mathrm{s}}\,\underline{s} + \underline{i}_{2\mathrm{s}}\,\underline{s} + \underline{i}_1\,\underline{i}_2/\gamma + \underline{i}_{1\mathrm{s}}\,\underline{i}_{2\mathrm{s}}\,\underline{s}/\gamma \tag{13}$$

where the last two terms refer to the formation of the $\mathrm{EI}_1\mathrm{I}_2$ and $\mathrm{ESI}_1\mathrm{I}_2$ complexes respectively. Making use of the concept of 'residual function', the terms containing the γ factor may be isolated as follows:

$$\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1 - \underline{i}_2 - \underline{i}_{1\mathrm{s}}\,\underline{s} - \underline{i}_{2\mathrm{s}}\,\underline{s} = \underline{i}_1\,\underline{i}_2/\gamma + \underline{i}_{1\mathrm{s}}\,\underline{i}_{2\mathrm{s}}\,\underline{s}/\gamma = \underline{i}_1\,\underline{i}_2/\gamma + \underline{i}_1\,\underline{i}_2\,\underline{s}/\alpha\beta\gamma \tag{14}$$

If, as considered by Asante-Appiah and Chan [26], $\mathrm{ESI}_1\mathrm{I}_2$ is not formed when one of the inhibitors is competitive, the last term can be eliminated from the equation. If both complexes are present, an interaction plot can still be obtained by rearranging the equation as follows:

$$\frac{\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1 - \underline{i}_2 - \underline{i}_{1\mathrm{s}}\,\underline{s} - \underline{i}_{2\mathrm{s}}\,\underline{s}}{1 + \underline{s}/\alpha\beta} = \underline{i}_1\,\underline{i}_2/\gamma \tag{15}$$

This approach allows the use of a single combination plot to show the interaction between two or more linear inhibitors. Combination plots have the merit of providing a visual insight into the mutual influence on the binding of the two inhibitors to the enzyme, and a means of obtaining γ. In addition, this method has the property that all the terms are dimensionless quantities, making it possible to place separate experiments together in the same diagram for comparison [26]. However, it also has some drawbacks. The main objection involves the validity of mechanism-dependent methods to evaluate the interaction between two inhibitors. From the above considerations it is clear that the form of the equation will depend on whether the $\mathrm{EI}_1\mathrm{I}_2$ and/or $\mathrm{ESI}_1\mathrm{I}_2$ complexes are formed, and this information cannot be gathered from steady-state kinetics alone. This point will be discussed below. Moreover, transformation of data does not allow experimental data to be retrieved from the graph. Another limitation is that, to use this methodology, up to six parameters and constants must be accurately obtained in previous experiments.

Yonetani–Theorell plot

The Yonetani–Theorell plot has been the most popular way of evaluating the interaction between two enzyme inhibitors. From eqn. (2) it is obvious that, if the binding of two linear inhibitors to the enzyme is mutually exclusive ($\gamma = \infty$), plotting $1/v_{1,2}$ against $[\mathrm{I}_1]$ at fixed $[\mathrm{I}_2]$ would result in parallel straight lines. If they can bind simultaneously to the enzyme ($\gamma \neq \infty$), the slope will depend on the concentration of I_2 and the lines will intersect. For any pair of mutually non-exclusive bound linear inhibitors, the intersection of the lines, provided that I_1 is the variable inhibitor, will occur at an abscissa value of:

$$[I_1] = -\gamma K_{\mathrm{EI}_1} \frac{\left(1 + \dfrac{[\mathrm{S}]}{\beta K_{\mathrm{S}}}\right)}{\left(1 + \dfrac{[\mathrm{S}]}{\alpha\beta K_{\mathrm{S}}}\right)} \tag{16}$$

Table 1 Abscissa value of the intersection point of the lines generated in a Yonetani–Theorell plot depending on the inhibitors combined and the probable enzyme complexes formed

Inhibition dissociation constants K_{ESI_1} and K_{ESI_2} refer to αK_{EI_1} and βK_{EI_2} constants shown in Scheme 1. δ is equal to $K_{EI_1I_2}/K_{ESI_2} = \gamma/\beta$ if I_2 is the uncompetitive inhibitor or to $K_{EI_2I_1}/K_{ESI_1} = \gamma/\alpha$ if I_1 is the uncompetitive inhibitor (see the text).

	I_2			
I_1	Competitive	Non-competitive	Uncompetitive	Mixed
Competitive	$-\gamma K_{EI_1}$*	$-\gamma K_{EI_1}\left(1+\frac{[S]}{K_S}\right)$*	$-\delta K_{EI_1}\frac{[S]}{K_S}$*	$-\gamma K_{EI_1}\left(1+\frac{[S]}{\beta K_S}\right)$*
Non-competitive	$-\gamma K_{EI_1}$	$-\gamma K_{EI_1}$	$-\gamma K_{EI_1} = -\gamma K_{ESI_1}$	$-\gamma K_{EI_1}$
Uncompetitive	$-\gamma K_{EI_1}$* $= -\delta K_{ESI_1}$	$-\gamma K_{ESI_1}\left(1+\frac{K_S}{[S]}\right)$†	$-\gamma K_{ESI_1}$†	$-\gamma K_{ESI_1}\left(1+\frac{\beta K_S}{[S]}\right)$†
Mixed	$-\gamma K_{EI_1}$*	$-\gamma K_{EI_1}\frac{\left(1+\frac{[S]}{K_S}\right)}{\left(1+\frac{[S]}{\alpha K_S}\right)}$	$-\gamma K_{ESI_1}$†	$-\gamma K_{EI_1}\frac{\left(1+\frac{[S]}{\beta K_S}\right)}{\left(1+\frac{[S]}{\alpha\beta K_S}\right)}$

* Provided that the ESI_1I_2 complex is not formed.
† Provided that the EI_1I_2 complex is not formed.

Table 1 shows the value of the abscissa intercept point obtained in this plot depending on the inhibitors combined and the probable complexes formed. For example, any combination having a competitive inhibitor will intersect at $-\gamma K_{EI_1}$, provided that the competitive inhibitor is the constant inhibitor (I_2) and the ESI_1I_2 complex is not formed. An interesting case, analysed by Asante-Appiah and Chan [26], is the interaction between a competitive and an uncompetitive inhibitor. It is conceivable that the binding of a competitive inhibitor to the enzyme might in some cases allow the binding of an uncompetitive inhibitor that otherwise does not bind to the free enzyme. In this case the ternary EI_2I_1 complex would be formed. In a Yonetani–Theorell plot, if I_1 is an uncompetitive inhibitor and I_2 is the competitive one, the value of the abscissa intersection point will be $-\gamma K_{EI_1}$. Since for an uncompetitive inhibitor $K_{EI_1} = \infty$, if this inhibitor has some affinity for the EI_2 complex, the γ value will be close to 0. This can be misinterpreted as a very strong interaction being present when, in fact, it only reflects the uncompetitive nature of one of the inhibitors. For this combination it is preferable to express the interaction in terms of K_{ESI_1}. Replacing K_{EI_1} with K_{ESI_1}/α, the intersection point will be $-(\gamma/\alpha)K_{ESI_1} = -\delta K_{ESI_1}$, where $\delta = \gamma/\alpha$. Since $K_{ESI_1} = \alpha K_{EI_1}$ and $K_{EI_2I_1} = \gamma K_{EI_1}$, $\delta = \gamma/\alpha = K_{EI_2I_1}/K_{ESI_1}$, i.e. δ does not represent the mutual influence of the two inhibitors on the binding of each other, as γ does, but it rather compares the dissociation constant of I_1, the uncompetitive inhibitor, from the EI_2I_1 and ESI_1 complexes. If $\delta = 1$, I_1 has the same affinity for ES as for EI_2, whereas if $\delta = \infty$, the EI_2I_1 complex is not formed and parallel lines will result. It should be noted that in the derivation of Asante-Appiah and Chan [26] for the combination of a competitive with an uncompetitive inhibitor, although not explicitly stated in the text, $\alpha_{1,2}$ refers to δ and not to γ, and for the other combinations $\alpha_{1,2}$ has the same meaning as γ.

Modified plots

It has been argued that the Yonetani–Theorell plot offers no visual insight into the interaction between two inhibitors, because the point of intersection depends on the K_{EI_1} value [26]. Since most of the combinations usually tested have at least a competitive inhibitor, we have found that a clear picture of the interaction present can be obtained if $1/v_{1,2}$ is plotted against $[I_1]/K_{EI_1}$, provided that I_2 is the competitive inhibitor, because the abscissa intersection point directly represents the interaction factor ($-\gamma$). For combinations having a competitive (I_2) and an uncompetitive (I_1) inhibitor, a plot $1/v_{1,2}$ versus $[I_1]/K_{ESI_1}$ would be preferable, the intersection point giving the δ value. In contrast with combination plots, only one parameter (K_{EI_1} or K_{ESI_1}), rather than six, is needed to obtain this factor, and original data can be easily retrieved from the graph. It should be noted that if $v_0/v_{1,2}$ instead of $1/v_{1,2}$ is used, the abscissa intersection point will be the same and separate experiments can be placed together in the same diagram.

In fact, γ, or δ, values can be obtained for most combinations by means of eqn. (6) without any previous knowledge of inhibition constants. If $v_0/v_{1,2}$ versus $[I_1]/(IC_{50})_1$ is plotted, lines will intersect at an abscissa point of:

$$\frac{[I_1]}{(IC_{50})_1} = -\gamma\frac{\left(1+\frac{[S]}{\alpha K_S}\right)\left(1+\frac{[S]}{\beta K_S}\right)}{\left(1+\frac{[S]}{\alpha\beta K_S}\right)\left(1+\frac{[S]}{K_S}\right)} \quad (17)$$

Table 2 shows the abscissa value of the intercepting lines on this graph for the combination presented in Table 1. For most of the combinations, a knowledge of inhibitor constants is not needed to obtain the γ value. For example, for the mixture of two competitive inhibitors, if $[S] = K_S$ the intersecting point will be $-\gamma/2$, irrespective of the dissociation constants of the inhibitors tested. In addition to K_S, the only parameter needed is the IC_{50} value of the inhibitors, which can be obtained at the same time as the combinations are tested. Another advantage of this plot is that it clearly shows the effect of the substrate concentration on the interaction between two inhibitors. It has been pointed out that two inhibitors that bind at independent sites ($\gamma = 1$) usually do not produce independent inhibitory effects, since inhibitors

Table 2 Abscissa value of the intersection point of the lines generated in a plot of v_0/v_i against $[I]_1/(IC_{50})_1$ at different fixed concentrations of I_2 depending on the inhibitors combined and the probable enzyme complexes formed

Inhibition dissociation constants K_{ESI_1} and K_{ESI_2} refers to αK_{EI_1} and βK_{EI_2} constants shown in Scheme 1. δ is equal to $K_{EI_1I_2}/K_{ESI_2} = \gamma/\beta$ if I_2 is the uncompetitive inhibitor or to $K_{EI_2I_1}/K_{ESI_1} = \gamma/\alpha$ if I_1 is the uncompetitive inhibitor (see the text).

	I_2			
I_1	Competitive	Non-competitive	Uncompetitive	Mixed
Competitive	$\dfrac{-\gamma}{\left(1+\dfrac{[S]}{K_S}\right)}$ *	$-\gamma$	$\dfrac{-\delta}{\left(1+\dfrac{K_S}{[S]}\right)}$ *	$-\gamma\dfrac{\left(1+\dfrac{[S]}{\beta K_S}\right)}{\left(1+\dfrac{[S]}{K_S}\right)}$ *
Non-competitive	$-\gamma$	$-\gamma$	$-\gamma$	$-\gamma$
Uncompetitive	$\dfrac{-\delta}{\left(1+\dfrac{K_S}{[S]}\right)}$ *	$-\gamma$	$\dfrac{-\gamma}{\left(1+\dfrac{K_S}{[S]}\right)}$ †	$-\gamma\dfrac{\left(1+\dfrac{\beta K_S}{[S]}\right)}{\left(1+\dfrac{K_S}{[S]}\right)}$ †
Mixed	$-\gamma\dfrac{\left(1+\dfrac{[S]}{\alpha K_S}\right)}{\left(1+\dfrac{[S]}{K_S}\right)}$ *	$-\gamma$	$-\gamma\dfrac{\left(1+\dfrac{\alpha K_S}{[S]}\right)}{\left(1+\dfrac{K_S}{[S]}\right)}$ †	$-\gamma\dfrac{\left(1+\dfrac{[S]}{\alpha K_S}\right)\left(1+\dfrac{[S]}{\beta K_S}\right)}{\left(1+\dfrac{[S]}{\alpha\beta K_S}\right)\left(1+\dfrac{[S]}{K_S}\right)}$

* Provided that the ESI_1I_2 complex is not formed.
† Provided that the EI_1I_2 complex is not formed.

can influence indirectly the binding of each other by altering the substrate affinity [32]. Two independent bound linear inhibitors that compete with the substrate will facilitate the binding of each other, since the binding of one of them will preclude the binding of the substrate. Conversely, two inhibitors binding independently of each other, but exerting opposite effects on substrate affinity, will make their simultaneous binding to the enzyme more difficult. Such an indirect substrate-mediated mutual influence cannot be exerted when one or both of the two independently bound inhibitors is non-competitive (α and/or $\beta = 1$). In this case eqn. (6) is transformed into:

$$\frac{v_0}{v_{1,2}} = 1+\frac{[I_1]}{(IC_{50})_1}+\frac{[I_2]}{(IC_{50})_2}+\frac{[I_1][I_2]}{\gamma(IC_{50})_1(IC_{50})_2} \quad (18)$$

If these inhibitors are combined at their respective IC_{50} values, a fractional inhibition of 0.75 will be obtained. For the combination of two linear competitive inhibitors, eqn. (6) becomes:

$$\frac{v_0}{v_{1,2}} = 1+\frac{[I_1]}{(IC_{50})_1}+\frac{[I_2]}{(IC_{50})_2}+\frac{[I_1][I_2]}{\dfrac{\gamma}{\left(1+\dfrac{[S]}{K_S}\right)}(IC_{50})_1(IC_{50})_2} \quad (19)$$

In this case, if $[S] = K_S$, the combination of two independently bound competitive inhibitors mixed at their respective IC_{50} values will result in a fractional inhibition of 0.8. However, if substrate concentration was equal to $10 \times K_S$ or $0.1 \times K_S$ and both inhibitors were tested in each case at their IC_{50} value, fractional inhibition would be 0.93 and 0.76 respectively. If we are interested in the effect of the combination with respect to the individual effect of each inhibitor, the parameter of interest is not the γ value but the composite factor $\gamma/(1+[S]/K_S)$. This is exactly the abscissa intersection point that gives this plot.

Fractional product of Webb

Webb [23], on probabilistic grounds, assumed that if two inhibitors bind independently to an enzyme, the expected effect of their combination can be expressed by the product of their fractional activities, i.e.

$$\frac{v_{1,2}}{v_0} = \frac{v_1}{v_0}\times\frac{v_2}{v_0} = (1-f_1)(1-f_2) \quad (20)$$

where f_1, f_2 and $f_{1,2}$ are the fractional inhibitions measured in the presence of I_1, I_2 and their combination respectively. However, it can be easily demonstrated that this statement is unfounded. When the concentration of the substrate is kept constant, linear enzyme inhibitors have dose–response curves of hyperbolic shape that can be fitted to [25,30]:

$$\frac{v_i}{v_0} = \frac{1}{1+\dfrac{[I]}{(IC_{50})_i}} \quad (21)$$

and substitution in eqn. (20) gives:

$$\frac{v_0}{v_{1,2}} = \frac{v_0}{v_1}\times\frac{v_0}{v_2} = \left(1+\frac{[I_1]}{(IC_{50})_1}\right)\left(1+\frac{[I_2]}{(IC_{50})_2}\right)$$
$$= 1+\frac{[I_1]}{(IC_{50})_1}+\frac{[I_2]}{(IC_{50})_2}+\frac{[I_1][I_2]}{(IC_{50})_1(IC_{50})_2} \quad (22)$$

By comparing eqn. (22) with eqn. (6), it appears that the fractional product of Webb correctly predicts the effect of the combination between two independently bound ($\gamma = 1$) linear inhibitors only when at least one of the inhibitors is non-

competitive (α and/or $\beta = 1$). For the other combinations, this method does not take into account that, as stated in the preceding point, inhibitors can influence indirectly the binding of each other by altering substrate affinity. In particular cases, it is possible that the effect of the combination matches those predicted by the fractional product of Webb even when neither of two independently bound inhibitors is non-competitive [32]. For example, for the combination of two independent competitive inhibitors, the effect matches those calculated by the fractional product of Webb if $[S] \ll K_S$ [see eqn. (19)]. For the combination of two uncompetitive inhibitors this happens when $[S] \gg K_S$.

Limitations of mechanism-dependent methods

In the preceding methods, two inhibitors do not interact if the observed effect of the combination fits to what is predicted by a specific equation. Since the form of the equation will depend on the mechanism assumed, the concept of no interaction will change with the method used. If the mechanism of action is not well understood, or if the model is inappropriate, the analysis will be meaningless. As conclusions on mechanism often vary in the light of new investigations, whereas experimental observations do not, some representation reflecting the real experimental data must be used to facilitate future discussions. It should be noted that eqn. (2) is based on the rapid-equilibrium assumption. Steady-state treatment would give rise to complex equations containing higher-degree terms both in substrate and inhibitor concentrations. This could result in curved Yonetani–Theorell plots in the presence of the inhibitor. However, the predicted deviations from simple kinetics are difficult to detect experimentally, and adherence to simple kinetics is not adequate evidence that inhibition constants are true dissociation constants [31]. Moreover, as shown below, many combinations of inhibitors, even irreversible inhibitors, may result in linear Yonetani–Theorell plots provided that they have individual dose–response curves of hyperbolic shape. If indeed rapid equilibrium is not appropriate or if the model is unfounded, α, β and γ will not reflect the change in affinity for a ligand when the other ligand is bound to the enzyme. For the combination of two competitive inhibitors, this may not to be a problem if enzyme–inhibitor complexes are dead-end complexes. For this combination, steady-state treatment results in an equation similar to eqn. (2) (K_m substitutes for K_S and α and β are equal to ∞ [see eqn. (8)] [24]. However, even in this case, it should be kept in mind that the interaction between two inhibitors may be substantially more complex than what is represented in Scheme 1, and the form of the equation velocity would consequently change. Only knowing in detail the mechanism of action of the inhibitors is it possible to obtain these parameters from kinetic data.

Recently the use of combination plots has been proposed to display graphically the interaction between two inhibitors [26]. This analysis has been suggested to be more efficient and to require fewer assays than conventional methods. In fact, the reduction of the number of experimental data is more theoretical than real. In the first place, previous experiments must be performed in order to obtain the kinetic parameters needed to construct the combination plot. In addition, by plotting the product of the concentrations of the two inhibitors, very different combinations fall into a single point. In this model it is assumed that α, β and γ factors remain constant over the whole range of concentrations, whereas it has been shown that for several combinations the type and extent of interaction is dose-dependent [29]. Whether or not experimental data agree with a model can only be determined if multiple combinations are tested. A checkerboard design, such as those proposed by Yonetani and Theorell, seems to be preferable for this purpose [24,27].

Mechanism-independent methods: isobolographic analysis

It is indeed possible to decide whether or not the effects of two or more inhibitors are mutually exclusive, even when no information is available about their mechanism of action, by means of isobolographic analysis. Two inhibitors that do not interact are no more or less effective in combination than they are separately; in other words, there is no advantage in combining them. There is one sort of combination that must always behave in this way; the mock combination of an agent with itself [33]. Consider that we introduce an inhibitor into two containers, labelled 1 and 2, and conduct an experiment to find out what combinations of 1 and 2 will produce a given effect. We know, because 1 and 2 are in fact the same inhibitor, that the same effect is produced, for example, by 10 mg of 'sample 1', or 10 mg of 'sample 2', or the combination of 5 mg of sample 1+5 mg of sample 2, or 1 mg of sample 1+9 mg of sample 2, and so on. Let d_1 and d_2 be the concentrations of 1 and 2 when used in combination and D_1 and D_2 the concentrations of each inhibitor that individually produce the same effect as the combination (d_1+d_2). Then, for all the combinations of 1 and 2, the following equation is fulfilled [33]:

$$\frac{d_1}{D_1}+\frac{d_2}{D_2}=1 \tag{23}$$

Let us suppose now that sample 2 is mixed with an equal part of an inert material that has no effect on the inhibition. Now, D_1 is 10 mg and D_2 20 mg, and the same effect is produced by 10 mg of sample 1, 20 mg of sample 2, or by such combinations as 5 mg of sample 1+10 mg of sample 2, 7 mg of sample 1+6 mg of sample 2, 1 mg of sample 1+18 mg of sample 2, and so on. Obviously, eqn. (23) still holds, irrespective of the effect specified or the shape of the dose–response curve of the inhibitor. Eqn. (23) is therefore a useful criterion of no interaction, zero interaction, between inhibitors. To apply this methodology, experimental data for agents used alone and in different dose combinations at equi-effective levels are required. These data are plotted on an iso-effective graph with axes representing the doses of each agent. From eqn. (23) we have:

$$d_2 = D_2-\frac{D_2}{D_1}d_1 \tag{24}$$

i.e. if two given inhibitors show zero interaction, the line joining the point corresponding to the combination with those on the axes representing doses iso-effective with the combination will be a straight line [33]. All the combinations (d_1+d_2) iso-effective with D_1 or D_2 will fall on a straight line with slope D_2/D_1 which intercepts both axes at D_1 and D_2 respectively. When agents in combination are more effective than what might be expected from their individual dose–response curves, smaller amounts will be needed and a concave-up isobole results. When agents in combination are less effective than expected, larger doses will be needed to produce the same effect and a concave-down isobole is generated. It has been pointed out that the result obtained does not depend on any assumption about the mechanism of action of the inhibitor and, in fact, this method is widely used to analyse the interaction between biologically active agents in many fields [29].

An interesting result is obtained when the isobole method is used to analyse the combination of two exclusive linear inhibitors that follows Michaelis–Menten kinetics. When the concentration of the substrate is kept constant, these inhibitors show dose–response curves of hyperbolic shape that can be fitted to eqn. (7). If this equation is combined with the isobole equation [eqn. (23)], the following equation is obtained:

$$\frac{d_1}{(IC_{50})_1\left(\frac{f}{1-f}\right)}+\frac{d_2}{(IC_{50})_2\left(\frac{f}{1-f}\right)}=1 \quad (25)$$

and from this:

$$\frac{1}{1-f}=\frac{v_0}{v_{1,2}}=1+\frac{d_1}{(IC_{50})_1}+\frac{d_2}{(IC_{50})_2} \quad (26)$$

which has the same form as eqn. (6) in the case of two mutually exclusive enzyme inhibitors ($\gamma = \infty$). From eqn. (26) it can be concluded that, if two inhibitors having dose–response curves of hyperbolic shape show zero interaction, the family of lines generated by plotting $1/(1-f)$ versus the concentration of one inhibitor, keeping the other at constant concentration, are parallel. This is the same conclusion as that reached by independent means by Yonetani and Theorell [24] for the combination of two linear competitive inhibitors. It is important to appreciate that this result does not depend on any consideration about the reversibility of the inhibitors or on their mechanism of action, but only on the shape of their individual dose–response curves. An interesting example is the inhibition of HIV reverse transcriptase by chain-terminating nucleotides, such as 3′-azido-3′-deoxythymidine 5′-triphosphate or 2′,3′-dideoxycytidine 5′-triphosphate. These inhibitors exert their function not by direct binding to the enzyme, but by acting as chain terminators of DNA polymerization. In fact, competitive inhibition with natural deoxynucleotides in the absence of chain termination has been found to be irrelevant in the action of these nucleotide analogues [19]. Since HIV reverse transcriptase does not have the 3′–5′ proof-reading function, once incorporated into the primer these compounds preclude DNA synthesis. Owing to the processivity of the reaction, the polymerase remains bound to the template–primer until it dissociates and binds to another primer. This results in a reduction in global rate of polymerization when compared with uninhibited reactions. Under some conditions, inhibition of reverse transcriptase by chain-terminator nucleotides results in Michaelis–Menten kinetics. What is more, combinations of these inhibitors, with either other chain terminators or non-competitive inhibitors, usually give parallel Yonetani–Theorell plots, indicating mutually exclusive effects [15,16]. Obviously, in this case, Scheme 1, and all the parameters derived from it, are meaningless. However, the result obtained can be safely interpreted in the sense that, under these conditions, the combination has no advantage over the individual use of each inhibitor.

To quantify the interaction between two inhibitors, an interaction index (I) is defined as [27,33]:

$$\frac{d_1}{D_1}+\frac{d_2}{D_2}=I \quad (27)$$

When $I = 1$ inhibitors do not interact, if $I > 1$ the combination is antagonistic, and if $I < 1$ the combination is synergistic. Eqn. (27) can be easily extended to the combination of three or more inhibitors [33]. The interaction index is equivalent to the combination index (CI) proposed by Chou and Talalay [25] for mutually exclusive drugs. The interaction between two inhibitors can be graphically displayed in a three-dimensional plot where x and y axes represent concentrations of each agent, and the z axis is the logarithm of the interaction index [27]. If two inhibitors show zero interaction, the value of the interaction index is equal to 1 at any concentration. For combinations showing synergistic interaction, this index usually decreases as concentrations of the inhibitors are raised, although more complex patterns can be obtained [25,27,29]. This decrease has a physical meaning. At low concentrations of an inhibitor the effect obtained is usually proportional to the amount of inhibitor present. As the concentration of the inhibitor is raised, saturation is more manifest. If we mix two non-exclusive inhibitors at low concentrations, their combined effect is the same as if we were using an equivalent concentration of one of them. Only when saturation approaches does the combination have an advantage over the individual use of a single inhibitor. Chou and Talalay [25] have proposed that, although eqn. (27) is valid for two mutually exclusive inhibitors, zero interaction for the combination of two mutually non-exclusive inhibitors must be defined as:

$$\frac{d_1}{D_1}+\frac{d_2}{D_2}+\frac{d_1 d_2}{D_1 D_2}=1 \quad (28)$$

It was assumed that, for two non-exclusive independently bound enzyme inhibitors, this combination index would be equal to 1, whereas it would be greater or less than unity if favourable interaction (synergism) or mutual hindering (antagonism) was present. However, this assumption is unfounded. Isobolographic analysis is useful for predicting the expected effect of a combination showing zero interaction, but it is not possible to predict the combined effect of two interacting inhibitors, since the form of the equation depends on their mechanism of action. As shown previously, two independently bound inhibitors can affect indirectly the binding of each other by affecting the substrate affinity. Even if one, or both, of the two independently bound inhibitors is non-competitive, and consequently this indirect effect cannot be exerted, eqn. (28) does not hold. If eqn. (7) is combined with eqn. (28), we obtain:

$$\frac{d_1}{(IC_{50})_1\left(\frac{f}{1-f}\right)}+\frac{d_2}{(IC_{50})_1\left(\frac{f}{1-f}\right)}+\frac{d_1 d_2}{(IC_{50})_1(IC_{50})_2\left(\frac{f}{1-f}\right)\left(\frac{f}{1-f}\right)}=1 \quad (29)$$

d_1 and d_2 being the concentrations of I_1 and I_2 in the combination.

As $1/(1-f) = v_0/v_i$, there is no way to convert this equation into eqn. (18). The right solution would be:

$$\frac{d_1}{(IC_{50})_1\left(\frac{f}{1-f}\right)}+\frac{d_2}{(IC_{50})_2\left(\frac{f}{1-f}\right)}+\frac{d_1 d_2}{(IC_{50})_1(IC_{50})_2\left(\frac{f}{1-f}\right)}=1 \quad (30)$$

and therefore:

$$\frac{d_1}{D_1}+\frac{d_2}{D_2}+\frac{d_1 d_2}{D_1(IC_{50})_2}=\frac{d_1}{D_1}+\frac{d_2}{D_2}+\frac{d_1 d_2}{(IC_{50})_1 D_2}=1 \quad (31)$$

It is obvious that eqns. (28) and (31) are different. Only when the fractional effect of the combination is equal to 0.5 do the two equations give the same result. It should be noted that, whereas eqn. (31) and eqn. (22) agree, eqns. (28) and (22) are incompatible. It can be concluded that, if isobolographic analysis is employed, eqn. (27) must be used for all types of combination. With this method, we can conclude whether or not agents in combination are dose-additive.

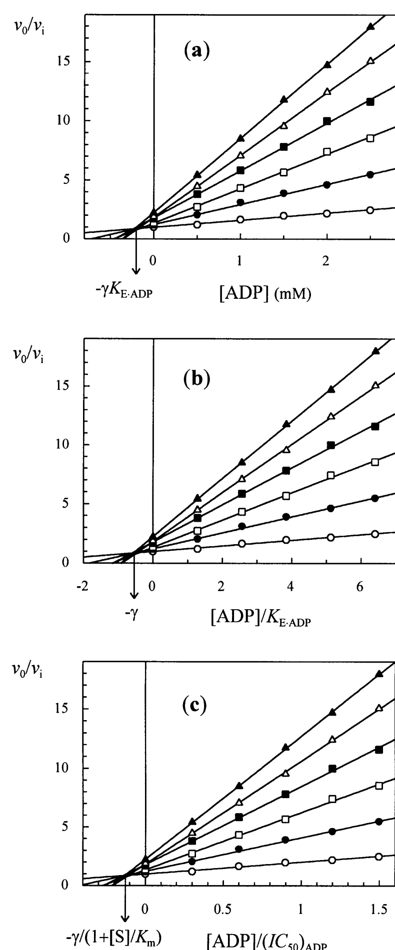


Figure 1 Effect of the combination of ADP and *o*-phenanthroline on horse liver ethanol dehydrogenase

Experimental data were from Yonetani and Theorell [24] as retrieved by Chou and Talalay [28]. Concentrations of *o*-phenanthroline are 0 (○), 8.7 (●), 17.4 (□), 26.1 (■), 34.8 (△) and 43.5 (▲) μM. (**a**) Yonetani–Theorell plot, ADP being the variable inhibitor (I_1). (**b**) Plot of v_0/v_i versus $[I_1]/K_{EI_1}$ at different fixed concentrations of *o*-phenanthroline. A previously reported K_{EI} value of 0.390 mM for ADP was used for calculations [24]. (**c**) Plot of v_0/v_i versus $[I_1]/(IC_{50})_1$ at different fixed concentrations of *o*-phenanthroline. [S] and K_m were 50 and 14 μM respectively [24], and IC_{50} values for ADP and *o*-phenanthroline, 1.67 mM and 40.2 μM respectively, were obtained by fitting the dose–response curve for each inhibitor alone to eqn. (7). A value of 0.51, similar to the value of 0.5 reported previously using other methods [24,26], was obtained for γ by fitting experimental data to eqn. (19) by unweighted non-linear regression.

Example: inhibition of horse liver ethanol dehydrogenase by ADP, ADP-ribose and *o*-phenanthroline

It may be of interest to analyse how the different methods perform with a practical example. The combination of ADP with ADP-ribose, two mutually exclusive competitive inhibitors, was originally analysed by means of the Yonetani–Theorell plot [24]. As expected from their mechanism of action, the combination of ADP and ADP-ribose gave parallel lines, showing that the effects of both inhibitors were mutually exclusive. The same conclusion is reached by inspecting the isobolograms presented by Yonetani and Theorell [24], or if interaction index is used to measure the interaction [27]. However, by using the fractional product method, antagonism is diagnosed for all concentrations [27]. The method of Chou and Talalay [25] gives consistent results if the equation for mutually exclusive inhibitors, i.e. eqn. (23), is employed, but not if eqn. (28), proposed for mutually non-exclusive inhibitors, is used instead.

For the combination of ADP and *o*-phenantroline, two competitive mutually non-exclusive inhibitors, intersecting lines were found in the Yonetani–Theorell plot (Figure 1a). It should be noted that, for the combination of two dead-end competitive inhibitors, steady-state treatment results in an equation similar to eqn. (19), except in one thing, that K_m replaces K_S, and consequently the result obtained does not depend on the validity of a rapid-equilibrium assumption. Moreover, since the mechanisms of action of these inhibitors are well known, the value of γ effectively represents the mutual influence on their binding. As pointed out above, a clear picture of the interaction present can be obtained if $v_0/v_{1,2}$ is plotted against $[I]/K_{EI}$ (Figure 1b). It is also possible to obtain the value of γ even when no information exists about the numerical value of the inhibition constants (Figure 1c). The value of γ may also be obtained from combination plots [26], although four kinetic constants are needed and experimental data cannot be retrieved from this graph. The evaluation of the interaction by isobolographic analysis leads to the conclusion that this combination is clearly synergistic, as expected from the mechanism of action of both inhibitors [24,27]. When the method of Chou and Talalay [25] is applied to these data under the assumption of mutual non-exclusivity, a moderate antagonism is found at low inhibition values, whereas a marked synergism exists at high inhibition values. If the fractional product method is used as a criterion for no interaction and differences between actual and expected effects are plotted, a marked decrease in the synergy with increasing concentrations of inhibitors is found [27].

DISCUSSION

Although several plots to represent the interaction between enzyme inhibitors have been proposed, that of Yonetani and Theorell [24] has been the most generally used for this purpose. As pointed out above, this plot gives straight lines for many combinations that do not follow the mechanism proposed by these authors, and this probably has contributed to its popularity. With hindsight, this has been fortunate. Although conclusions reached by some researchers were misinterpreted, original data can be easily retrieved from the graph, facilitating further analysis by the reader. A common mistake in the interpretation of combination experiments is to take the exclusivity of the effects of two inhibitors as exclusivity of their binding. Two linear mutually exclusive enzyme inhibitors that follow Scheme 1 will give rise to parallel lines in a Yonetani–Theorell plot. However, parallel lines are sometimes found with inhibitors that follow a completely different mechanism, as occurs in the inhibition of HIV reverse transcriptase by chain-terminating nucleotides. In this case the exclusivity of the effects cannot be taken as exclusivity of their binding, as is usually done [14,16–19,21]. Moreover, two inhibitors that bind independently to an enzyme ($\gamma = 1$) usually do not produce independent effects. As indicated above, two inhibitors can affect indirectly the binding of each other by altering substrate affinity. Furthermore, independent inhibitor binding, if deduced from binding studies in the absence of the substrate, cannot be taken as independence of the effects of their combination [32]. In addition, two inhibitors that are not able to bind simultaneously to the enzyme may give rise to synergistic inhibition. It has been shown that a combination of two structurally related non-nucleoside inhibitors results in synergistic inhibition of HIV reverse transcriptase. Although both

inhibitors bind to the same site on the enzyme, their binding is directed to different mechanistic forms [20].

Some researchers seem to be not fully aware of the limitations of each method and its consistency with other methods developed to analyse interactions between inhibitors. For example, Zhang et al. [21] have reported that two inhibitors do not seem to interact according to the Yonetani–Theorell plot, but that substantial synergy was found by calculating the combination index under the assumption of mutually exclusive conditions, i.e. by applying eqns. (23) or (27). This statement is self-contradictory since, as indicated, if two inhibitors give parallel lines in a Yonetani–Theorell plot, zero interaction must be obtained in isobolographic analysis, irrespective of the mechanism of action of the inhibitors [see eqn. (26)]. Some authors also encountered problems when applying the method of Chou and Talalay [25], since this method requires previous knowledge of whether or not the binding of two inhibitors is mutually exclusive. As information about exclusivity of the binding of inhibitors is not always available, some authors use both criteria [13], or apply the assumption of non-exclusivity, in the belief that the conclusion is more likely to be correct if a more restrictive criterion is used. In fact, there is no need to know whether or not two inhibitors bind simultaneously to the enzyme, since eqn. (23) can be used in both cases.

It is interesting to point out that several of the methods reviewed here, such as those proposed by Chou and Talalay [25], are often used in other research areas, such as pharmacology, toxicology and cancer research, to analyse the interaction between biologically active compounds. From analysis of the strengths and weaknesses of each method for the inhibition obtained with a single enzyme, interesting conclusions can be reached that may of great value in other fields. For example, Prichard and Shipman [34] have developed a method to evaluate the synergy between anti-microbial agents that has become popular in this area. The theoretical basis of the method is the fractional product of Webb [23]. As seen before, if both inhibitors act on the same enzyme, this criterion is seldom met. Even if their targets are different, the validity of this approach depends on the shape of the dose–response curve of each inhibitor [29]. More importantly, one of the main drawbacks of this method is that interaction is assessed as the difference between the actual effect and that predicted by the fractional product of Webb [23]. Accordingly, the 'optimum combination', i.e. that producing maximum synergy, is found at rather moderate concentrations of both inhibitors [27,35]. The reason is that when agents are mixed at high concentrations, each agent alone produces strong inhibition, so that the combination of two inhibitors cannot result in inhibition that is noticeably greater than that observed with each inhibitor alone. However, the concentration needed to reach a specific effect can be substantially decreased, as is evident from isobolographic analysis. This should be taken into account especially when interactions are studied in order to guide clinical trials [36].

It has been a matter of controversy of whether the isobole method is a generally valid procedure for analysing interactions between inhibitors, irrespective of their mechanism of action or the similarity or dissimilarity of their dose–response curves. Some authors maintain that eqn. (23) 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 additivity of effects. By definition, eqn. (23) holds if the combination is dose-additive, i.e. 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. On the other hand, if the dose–response curves of the inhibitors have different shapes, there is no agreement between the exclusivity of the binding of inhibitors to the enzyme and the interaction assessed by means of isobolographic analysis. This fact does not argue against the validity of eqn. (23) to evaluate the interaction between inhibitors. By using eqn. (23) [and eqn. (27) derived from it] we can ascertain for each combination whether the doses needed to reach a specific level of effect, taking into account the relative potency of each agent, are lower (synergy) or higher (antagonism) than those required when each agent is used alone. This information is interesting in itself, and therefore this equation is a useful criterion for evaluating the interaction between these inhibitors. However, caution must be exercised when these results are interpreted from a mechanistic point of view, since there is no direct correlation between the exclusivity of the binding of two inhibitors and the interaction measured by means of isobolographic analysis. In any case, it may be prudent to establish that a significant interaction is taking place before investigating the hypothetical mechanism of this putative interaction.

In conclusion, the applicability of the methods usually employed to analyse the interaction between two linear enzyme inhibitors has been revised. If the mechanisms of action of the inhibitors are well known, it would be of interest to derive an equation that predicts their combined effect. This approach would provide interesting information on the interaction at a molecular level. Interaction can then be graphically displayed by using some of the plots discussed in this paper. However, caution must be exercised when the mechanism of the inhibitors is not well understood or when some assumptions might not be valid. In this case, the values of the parameters obtained may be meaningless. It should be remembered that the validity of these assumptions cannot be settled by steady-state kinetic data. The Yonetani–Theorell plot, commonly used to present graphically the effect of combinations, gives straight lines for many combinations that do not follow the mechanism presented in Scheme 1. It should also be clearly stated that exclusivity of the effects usually does not correlate with exclusivity of the binding. If the mechanisms of action of the inhibitors are unknown, it is safer to apply isobolographic principles to the analysis of data. By using this methodology it is possible to know whether the combination of two or more inhibitors is more effective than their individual use.

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Received 25 June 1997/2 September 1997; accepted 19 September 1997