Analysis of the interactions between an enzyme and multiple inhibitors using combination plots

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The concurrent effects of two enzyme inhibitors have been analysed previously with the Yonetani–Theorell plot to obtain insight into the interactions between bound inhibitors. This procedure, like many other traditional graphical methods in enzymology, is based on the estimation of intersecting tendencies in a family of lines. In a recent paper from this laboratory [Chan (1995) *Biochem. J.* **311**, 981–985] it was shown that a plot of this nature may sometimes be replaced, with advantage, by a ‘combination plot’ in which all data points are accommodated in a single line. We have now extended this approach to analyse the

effects of multiple inhibitors and have developed combination plots which illustrate the interaction behaviour in an optimal manner. Thus, in these plots, the synergistic or antagonistic nature of the interactions is clearly evident from the slope, which also provides a direct estimate of the interaction coefficient. The analysis is more efficient and consequently requires fewer enzyme assays. This approach is applicable to various special cases, including that in which three inhibitors bind simultaneously to the enzyme.

INTRODUCTION

The study of inhibitors can contribute significantly to the understanding of enzyme catalysis as well as the development of useful drugs. Since many enzyme reactions involve two or more molecules as substrates and cofactors, it is reasonable to expect that the active site of an enzyme can frequently accommodate two small inhibitors simultaneously. Even for a reaction which, formally speaking, has only one substrate (e.g. the hydrolysis of a peptide), the enzyme may contain an extended active site capable of many interactions [1]. The concurrent effects of multiple inhibitors have been investigated in the past by various workers [2–4], and some interesting results (e.g. synergism) have been obtained. These results can complement other studies because they provide quantitative information concerning binding affinity which is not available from structural work. However, this type of study has not been conducted extensively, perhaps in part because current methods for the analysis of data tend to be cumbersome.

In a recent paper from this laboratory [5], one of us (W.W.-C.C.) advocated the use of combination plots as graphical tools for the study of enzyme inhibition. By combining a family of lines (e.g. from a Dixon plot [6]) into a single linear plot, this method improves the efficiency of analysis and allows greater freedom in experimental design. In the present work we have extended the approach to analyse the concurrent effects of two inhibitors. We describe below graphical methods which simplify the analysis and enhance the presentation of data substantially. A major benefit of the procedure is that fewer enzyme assays are required for a comparable degree of accuracy. This approach is readily adaptable to even more complex systems. As an illustration, we include in the present paper the unusual case of three inhibitors interacting simultaneously with one enzyme.

MATERIALS AND METHODS

Enzymes and reagents

Pig pancreatic carboxypeptidase B (EC 3.4.17.2) and its chromogenic substrate *N*-(3-[2-furyl]acryloyl)-Ala-Lys [7] were obtained

from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Mercaptoethanol was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Other inhibitors and reagents were also products of Sigma Chemical Co.

The activity of carboxypeptidase B was determined in 100 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl at 25 °C with an enzyme concentration of 1.2×10^{-8} M. The reaction was monitored continuously by measuring the decrease in A_{345} for 2 min in a Gilford Response spectrophotometer.

Rate equations in relative values

The rate equations for an enzyme with two or three inhibitors are sufficiently complex as to warrant the use of special abbreviations. It is also desirable to adopt a standard formulation which is generally applicable and brings out clearly the differences among the various systems. With this aim in mind, we have rearranged the kinetic equations so that all terms consist of dimensionless quantities. This can be illustrated with the equation for a single, purely competitive inhibitor which may be written as:

$$\frac{[S]}{K_m} \times \frac{V_{\max}}{v_i} = 1 + \frac{[S]}{K_m} + \frac{[I]}{K_i} \quad (1)$$

(For convenience, we have collected together in Table 1 all the symbols used in this paper.) The prominent feature of the above formulation is that all variables are expressed as ratios to the corresponding kinetic constants. The terms can therefore be referred to as ‘relative values’ and given a standard abbreviation. Thus we can denote the quantity $[S]/K_m$ as the relative value of substrate concentration and give it the symbol $\underline{s}$. Similarly, $[I]/K_i$ and $v_i/V_{\max}$ may be called the relative values of inhibitor concentration and of reaction velocity respectively and given the symbols $\underline{i}$ and $\underline{v}_i$. With these notations (see Table 1), we can reduce eqn. (1) to a very simple form:

$$\underline{s}/\underline{v}_i = 1 + \underline{s} + \underline{i} \quad (2)$$

The advantage of this formulation becomes apparent when we

Abbreviations used: the algebraic symbols are defined in Table 1.

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Table 1 List of algebraic symbols

Symbol	Description	Mathematical function
[S]	Substrate concentration	
K_m	Michaelis constant	
$\underline{s}$	Relative value of [S]	$\equiv [S]/K_m$
v_0	Velocity with no inhibitor	
V_{max}	Maximum velocity	
$\underline{v}_0$	Relative value of v_0	$\equiv v_0/V_{max}$
v_i	Velocity with one inhibitor	
$\underline{v}_i$	Relative value of v_i	$\equiv v_i/V_{max}$
[I]	Inhibitor concentration	
[E]	Concn. of free enzyme	
[EI]	Concn. of complex between E and I	
K_i	Inhibition constant	$\equiv [E][I]/[EI]$
$[E]_t$	Total concn. of all enzyme species	
i	Relative value of [I]	$\equiv [I]/K_i$
$[I_n]$	Concn. of inhibitor n ($n = 1, 2, 3$ etc.)	
$[EI_n]$	Concn. of complex between E and I_n	
K_n	Inhibition constant for I_n	$\equiv [E][I_n]/[EI_n]$
i_n	Relative value of $[I_n]$	$\equiv [I_n]/K_n$
$v_{1,2}$	Velocity with inhibitors 1 and 2	
$\underline{v}_{1,2}$	Relative value of $v_{1,2}$	$\equiv v_{1,2}/V_{max}$
$[EI_1I_2]$	Concn. of complex containing E, I_1 , and I_2	
$\alpha_{1,2}$	Interaction constant between inhibitors 1 and 2	$\equiv [EI_1][I_2]/[EI_1I_2]K_2$ (or $[EI_2][I_1]/[EI_1I_2]K_1$)
$\alpha_{1,3}$	Interaction constant between 1 and 3	$\equiv [EI][S]/[EIS]K_m$
$v_{1,2,3}$	Velocity with inhibitors 1, 2, and 3	
$\underline{v}_{1,2,3}$	Relative value of $v_{1,2,3}$	$\equiv v_{1,2,3}/V_{max}$
$[EI_1I_2I_3]$	Concn. of complex containing E, I_1 , I_2 and I_3	
β_1	Interaction constant for inhibitor 1 within the quaternary complex	$\equiv [I_1][EI_2I_3]/[EI_1I_2I_3]K_1$
τ	Tripartite interaction constant	$= \beta_1/\alpha_{1,2}\alpha_{1,3}$ etc.

compare the above equation with the Michaelis–Menten equation written in the same way:

$$\underline{s}/\underline{v}_0 = 1 + \underline{s} \quad (3)$$

or with the analogous rate equation for a non-competitive inhibitor:

$$\underline{s}/\underline{v}_i = 1 + \underline{s} + \underline{i} + \underline{i}\underline{s} \quad (4)$$

It can be seen that a more complex system differs from its simpler counterpart only by having extra term(s) in the rate equation. This characteristic is particularly useful because it applies to systems of even greater complexity. The favourable properties of the above formulation have a relatively simple physical basis. As pointed out in [5], eqn. (2) can be expressed in terms of the concentration of various enzymic species as follows (for a description of the symbols see Table 1):

$$[E]_t/[E] = ([E] + [ES] + [EI])/[E] \quad (5)$$

Therefore the Michaelis–Menten equation has one term less because in that case EI does not exist and the expression for a non-competitive inhibitor has an extra term because in the latter system EIS can also form. For convenience, we have collected in Table 2 all the equations expressed in relative values and also listed the physical basis of the extra terms in them. The above formulation can be readily applied to the rate equations for two and three concurrent inhibitors, and these are discussed fully in the Results section because they constitute the main focus of this paper.

Concept of residual functions

In the selection of mathematical functions for combination plots, we have generally found it useful to group together all terms in the rate equation which have known values so that the terms containing unknown quantities are isolated. For the above case of a competitive inhibitor (eqn. 2), we therefore obtain:

$$\underline{s}/\underline{v}_i - 1 - \underline{s} = \underline{i} \quad (6)$$

In order to understand fully the underlying principles of this approach, we may consider the following concept of residual functions. As explained earlier, the rate equations describing systems of various complexities differ from each other by the presence of extra terms. For any system under consideration, we may choose a reference system which is one level lower in complexity to represent the standard state. Thus in the case of a single competitive inhibitor, the uninhibited enzyme may be selected as the ‘reference system’. We may therefore view the above approach of grouping together the known terms in the equation as a method to account for the effects attributable to

Table 2 Rate equations for inhibition studies in abbreviated notation

Behaviour	Equation	Physical basis of each term
No inhibitor		
Michael–Menten	$\underline{s}/\underline{v}_0 = 1 + \underline{s}$	$\underline{s}/\underline{v}_0 = [E]_t/[E]$ $1 + \underline{s} = ([E] + [ES])/[E]$
Single inhibitor, I		
Competitive	$\underline{s}/\underline{v}_i = 1 + \underline{s} + \underline{i}$	$\underline{i} = [EI]/[E]$
Uncompetitive	$\underline{s}/\underline{v}_i = 1 + \underline{s} + \underline{i}\underline{s}$	$\underline{i}\underline{s} = [EIS]/[E]$
Linear mixed	$\underline{s}/\underline{v}_i = 1 + \underline{s} + \underline{i} + \underline{i}\underline{s}/\alpha_{1,3}$	$\underline{i}\underline{s}/\alpha_{1,3} = [EIS]/[E]$ (for non-competitive inhibition $\alpha_{1,3} = 1$)
Two inhibitors, I_1, I_2		
Both competitive		
I_1 competitive	$\underline{s}/\underline{v}_{1,2} = 1 + \underline{s} + \underline{i}_1 + \underline{i}_2 + \underline{i}_1\underline{i}_2/\alpha_{1,2}$ $\underline{s}/\underline{v}_{1,2} = 1 + \underline{s} + \underline{i}_1 + \underline{i}_2 + \underline{i}_1\underline{i}_2/\alpha_{1,2} + \underline{i}_2\underline{s}$	$\underline{i}_1\underline{i}_2/\alpha_{1,2} = [EI_1I_2]/[E]$
I_2 non-competitive		$\underline{i}_2\underline{s} = [E_2S]/[E]$
Three inhibitors, I_1, I_2, I_3		
All competitive	$\underline{s}/\underline{v}_{1,2,3} = 1 + \underline{s} + \sum_{n=1}^{n=3} \underline{i}_n + \sum_{m=1}^{m=2} \sum_{n=m+1}^{n=3} \underline{i}_m\underline{i}_n/\alpha_{m,n} + \underline{i}_1\underline{i}_2\underline{i}_3/\tau\alpha_{1,2}\alpha_{2,3}\alpha_{1,3}$	$\underline{i}_1\underline{i}_2\underline{i}_3/\tau\alpha_{1,2}\alpha_{1,3}\alpha_{2,3} = [EI_1I_2I_3]/[E]$

Table 3 Residual functions for different number of inhibitors

Number of inhibitors	Symbol	Terms contained in the function
1	$f_r(v_i)$	$\equiv \frac{s}{v_i} - 1 - \underline{s}$
2	$f_r(v_{1,2})$	$\equiv \frac{s}{v_{1,2}} - 1 - \underline{s} - \frac{i_1}{i_2} - \frac{i_2}{i_1}$ $n=3 \quad m=2n=3$
3	$f_r(v_{1,2,3})$	$\equiv \frac{s}{v_{1,2,3}} - 1 - \underline{s} - \sum_{n=1} i_n - \sum_{m=1} \sum_{n=2} \frac{i_n i_m}{\alpha_{m,n}}$ $m=1n=2$

Table 4 Summary of combination plots for various purposes

Functions plotted	Coefficient displayed*	Reference
One competitive inhibitor $K_m\{1 + f_r(v_i)\}$ versus $[I]$	K_m (ver.), $-K_i$ (hor.)	[5]
One competitive inhibitor of any simple behaviour $f_r(v_i)/[I]$ versus $\underline{s}$	$1/K_i$ (ver.), $-\alpha_{i,s}$ (hor.)	[5]
Two inhibitors, both competitive $f_r(v_{1,2})$ versus $i_1 i_2$	$1/\alpha_{1,2}$ (slp.)	The present paper
$f_r(v_{1,2}) - i_1 i_2$ versus $i_1 i_2$	$1/\alpha_{1,2} - 1$ (slp.)	The present paper
Two inhibitors, i_2 may deviate from competitive behaviour $f_r(v_{1,2})/i_2$ versus i_1	$1/\alpha_{1,2}$ (slp.), $\underline{s}/\alpha_{2,s}$ (ver.)	Appendix 1
$f_r(v_{1,2})/i_1 i_2$ versus $1/i_1$	$1/\alpha_{1,2}$ (ver.), $\underline{s}/\alpha_{2,s}$ (slp.)	Appendix 1
Two inhibitors, K_2 unknown $(\underline{s}/v_i - 1 - \underline{s})/[I_2]$ versus i_1	$1/\alpha_{1,2} K_2$ (slp.)	Appendix 2
$\{f_r(v_{1,2}) + i_2 - i_1 i_2 / \alpha_{1,2}\} / [I_2]$ versus $\underline{s}$	$1/K_2$ (ver.), $-\alpha_{2,s}$ (hor.)	Appendix 2
Three inhibitors, all competitive $f_r(v_{1,2,3})$ versus $i_1 i_2 i_3 / \alpha_{1,2} \alpha_{1,3} \alpha_{2,3}$	$1/\tau$ (slp.)	The present paper

* Intercepts are indicated as vertical (ver.) or horizontal (hor.) and slopes are labelled as (slp.).

the reference system by subtracting away all terms corresponding to it (in this case $1 + \underline{s}$ or $\underline{s}/v_0$; see eqn. 3). The resulting expression may be called a 'residual function', because it represents the quantity which remains after correcting for the reference system. The concept of residual function has a concrete physical basis, since the terms in the rate equations are directly related to the concentration of various enzymic species (see eqn. 5 and Table 2). Thus starting from the total enzyme concentration (as represented by $\underline{s}/v_i$ in eqn. 6), we can take into account all the species of the reference system (with known concentrations) to arrive at the 'residue' which represents the species unique to the system under consideration (whose concentration has yet to be determined). We suggest the symbol $f_r(v_i)$ to represent the 'residual function of the rate in the presence of a single inhibitor'. Thus, by definition:

$$f_r(v_i) \equiv (\underline{s}/v_i) - 1 - \underline{s} \equiv (\underline{s}/v_i) - (\underline{s}/v_0)$$

(for all types of inhibitory behaviour).

Eqn. (6) for a competitive inhibitor then becomes simply:

$$f_r(v_i) = i \quad (7)$$

The residual functions for two and three inhibitors have been similarly defined in Table 3. The use of these functions achieves a more concise description of the various plots and shows more clearly the relationship between them. For the purposes of comparison, we have collected the descriptions of all such plots in Table 4, including those published previously [5].

Scheme 1 Equilibria between enzyme-inhibitor complexes in a three-inhibitor system

For the sake of clarity, two diagrams are used to represent alternate views of the cube which contains all the possible equilibria. The back of the cube is out of sight in the top diagram, but is shown in the bottom diagram by removing the front corner.

The tripartite interaction coefficient (τ) for three inhibitors

The interaction of an enzyme with three concurrent inhibitors (Scheme 1) is in principle similar to that occurring between two inhibitors (see Scheme 2 in the Results section), except that additional coefficients are necessary to describe the increased number of equilibria. A useful representation of the equilibria governing the formation of all possible enzyme-inhibitor complexes is to arrange them in the form of a cube (assuming that all three inhibitors are competitive towards the substrate and that their binding to the enzyme is reversible). Each corner of the cube denotes a distinct enzymic species which can be converted to other species via a one-, two- or three-step process (see Scheme 1). As in the simpler situation with only two inhibitors, the formation of any ternary complex containing two inhibitors involves an interaction coefficient, but, in this case, three separate coefficients (α with distinct subscripts) must be provided for. In addition, other coefficients (β with various subscripts) must be introduced to describe the formation of the quaternary complex $EI_1I_2I_3$. From the inherent properties of such interconnected equilibria, the following relationship must hold:

$$\alpha_{1,2}\beta_3 = \alpha_{1,3}\beta_2 = \alpha_{2,3}\beta_1$$

The description of the system can be simplified by replacing the three β coefficients with a single coefficient τ (the tripartite interaction coefficient) such that:

$$\tau = \beta_1/\alpha_{1,2}\alpha_{1,3} = \beta_2/\alpha_{1,2}\alpha_{2,3} = \beta_3/\alpha_{1,3}\alpha_{2,3}$$

The rate equation for the above system can be formulated in terms of relative values (Table 2) and contains an extra term with this new coefficient τ . This term, $i_1 i_2 i_3 / \tau \alpha_{1,2} \alpha_{1,3} \alpha_{2,3}$, corresponds to the quaternary complex (being equal to $[EI_1 I_2 I_3]/[E]$) and τ (like α) expresses the extent to which the formation of that particular complex is unfavourable thermodynamically. It should be pointed out that the above formulation can be applied generally to any three-inhibitor system. By giving the value of infinity to the appropriate coefficient (α or τ), we can describe cases where, for some reason (e.g. steric hindrance), the formation of a certain complex is made impossible.

Practical considerations

The various rate equations in the present paper can be formulated in an alternative manner [9] using values of v_0 (the corresponding rate in the absence of any inhibitor) instead of $V_{\max}$. For example, eqn. (6) may be written as:

$$(1 + \underline{s})(v_0 - v_i)/v_i = i$$

and in fact the combination plot published earlier for a single inhibitor [5] was presented in this form. Similarly, the plot of Hunter and Downs [10] (which might be regarded as the first combination plot) was also expressed in terms of v_0 . However, there is nothing to be gained by this formulation, since the K_m value is still required ($\underline{s} = [S]/K_m$) and therefore v_0 and $V_{\max}$ are interconvertible. As pointed out previously [5], the use of $V_{\max}$ allows for greater flexibility and avoids statistical fluctuations in v_0 values.

In our experience, the most efficient procedure for determining the concurrent effects of two inhibitors involves four sets of experiments each containing five assays. The different sets contain either no inhibitor, or one of the inhibitors in turn or both inhibitors. Within each set, substrate concentration is varied in a linear manner and the inhibitor concentrations (where applicable) are also varied to obtain well-spaced data points. The first set (with no inhibitor) is used to obtain K_m and $V_{\max}$ (preferably by plotting v_0 versus $v_0/[S]$). The assays with a single inhibitor are then analysed by plotting $f_i(v_i)/[I]$ versus $\underline{s}$ (see [5]) using the above K_m and $V_{\max}$ values. Finally, the results with both inhibitors are plotted as $f_i(v_{1,2})$ versus $i_1 i_2$ using the K_1 , K_2 values obtained above. If greater accuracy is required, the number of assays in each set can simply be increased from the five recommended here.

RESULTS AND DISCUSSION

Current methods of analysis

Among the various methods proposed for the analysis of multiple inhibitors, that of Yonetani and Theorell [2] is probably the most suitable for obtaining insight into the underlying molecular processes. The superiority of this method for the above purpose lies in its ability to provide a quantitative measure (in the form of the coefficient α) describing the interaction between the two inhibitors. The physical significance of this coefficient is indicated in Scheme 2, where α represents the factor by which the first inhibitor bound to the enzyme influences the binding of the second inhibitor. Since K_i denotes the dissociation constant, a small value of α (< 1) indicates a synergistic interaction between the inhibitors, while a large value of α (> 1) signifies mutual

Scheme 2

antagonism. Two special cases are of particular interest. When $\alpha = 1$, the two inhibitors bind to the enzyme in a completely independent manner. At the other extreme, if the binding of the two inhibitors are mutually exclusive, then α would approach infinity. It is therefore clear that α is the key parameter which describes the behaviour of the system and has a direct physical significance in terms of the molecular processes involved.

In the analysis according to Yonetani and Theorell [2], the rate equation (for the case of two inhibitors, each of which is by itself competitive towards the substrate) is transformed as follows:

$$\frac{1}{v_{1,2}} = \frac{1}{V_{\max}} + \frac{K_m}{[S]} \times \frac{1}{V_{\max}} \left(1 + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha_{1,2} K_1 K_2} \right) \quad (8)$$

where $v_{1,2}$ denotes the initial rate in the presence of inhibitors 1 and 2, and $\alpha_{1,2}$ (the subscripts are used to distinguish it from similar coefficients described below) represents the coefficient of interaction between these inhibitors (see Table 1).

The recommended experimental design consists of a series of assays using the same concentrations of enzyme and substrate and varying the concentration of each inhibitor in turn while keeping the concentration of the other constant. The results may be plotted as $1/v_{1,2}$ versus $[I_1]$ (or $[I_2]$) to generate a family of lines (as illustrated in Figure 1(a)). As originally pointed out by Yonetani and Theorell [2], the intersection of these lines provides the value of $-\alpha_{1,2} K_1$ (if plotted against $[I_1]$) from which the value of $\alpha_{1,2}$ can be calculated (using the value of K_1 determined in a separate experiment).

In our experience with the above method [11–14], there are a number of important deficiencies. First, as a means of determining the value of $\alpha_{1,2}$, the method suffers from the fact that the family of lines do not generally intersect at a single point owing to the scatter in the data associated with experimental error (as shown in Figure 1a). In order to avoid subjective judgement, we have previously used a secondary plot (as recommended by Segel [8]) consisting of the slope from the above plot versus the concentration of the variable inhibitor (Figure 1b). Alternatively, the value of $\alpha_{1,2}$ can be obtained by computer-fitting of the data to the original equation. In either case a considerable number of assays are routinely required (the amount of data shown in Figure 1(a) may be regarded as the minimum). Secondly, as a graphical tool for illustrative purposes, the method offers no visual insight into the behaviour of the system. This deficiency is due to the fact that the point of intersection gives the composite term $-\alpha_{1,2} K_1$ from which the value of $\alpha_{1,2}$ is not immediately obvious (except where it is equal to infinity).

Combination plots for two inhibitors

As previously shown in the case of a single inhibitor [5], the use of combination plots can overcome the above deficiencies in-

Figure 1 Concurrent effects of methyl guanidine and succinate on carboxypeptidase B

(a) The Yonetani–Theorell [2] plot. The concentration of I_1 (succinate) is shown on the abscissa, and those of I_2 (methylguanidine, in mM) are: $\triangle$, 0.1; $\blacktriangle$, 0.3; $\square$, 0.4; and $\blacksquare$, 0.5. The substrate concentration was 0.4 mM in all cases. The lines are drawn according to linear regression of the data and $\alpha_{1,2}$ is found to be about 0.05 as judged from the intersection point and calculated using a dissociation constant of 28 mM (for succinate). (b) Secondary plot of the slope of each line in (a) above against the concentration of methylguanidine. From the horizontal intercept a value of about 0.06 for $\alpha_{1,2}$ is obtained on the basis of a dissociation constant of 1.3 mM for methylguanidine. (c) Combination plot using 14 of the same data points in (a) above (the remaining two have been omitted because they have $i_1 i_2$ values which duplicate those of other data points). Two other assays without any inhibitor were conducted to obtain a value for $V_{\max}$ which is required in this plot. (A previously determined K_m value of 1.25 mM

is inherent in plots involving a family of lines. These plots differ from traditional graphical methods in that the mathematical functions used are not restricted to simple derivatives of a single variable, but may include composite functions containing several variables. Thus this approach allows the use of a considerable range of functions, and the choice of a particular function can be made on the basis of the graphical characteristics deemed to be most desirable. In the case of two inhibitors described in Scheme 2, only one constant ($\alpha_{1,2}$) needs to be determined and this can be displayed graphically to the best advantage.

To arrive at the most suitable function, the rate equation for two competitive inhibitors (eqn. 8) may be transformed as follows:

$$\frac{V_{\max}}{v_{1,2}} \times \frac{[S]}{K_m} = 1 + \frac{[S]}{K_m} + \frac{[I_1]}{K_1} + \frac{[I_2]}{K_2} + \frac{[I_1][I_2]}{\alpha_{1,2}K_1K_2} \quad (9)$$

A special property of this transformation is that the terms are all dimensionless quantities which consist of ratios between the variable and the corresponding kinetic constants. Consequently, we may use the idea of relative values (for details, see the Materials and methods section), the abbreviated notations ($\underline{s} = [S]/K_m$; $\underline{v}_{1,2} = v_{1,2}/V_{\max}$; $\underline{i}_1 = [I_1]/K_1$ and $\underline{i}_2 = [I_2]/K_2$) and reduce the above equation (eqn. 9) to a very simple form:

$$\underline{s}/\underline{v}_{1,2} = 1 + \underline{s} + \underline{i}_1 + \underline{i}_2 + \underline{i}_1 \underline{i}_2 / \alpha_{1,2} \quad (10)$$

The only unknown quantity in this equation is $\alpha_{1,2}$, since all other constants (K_m , $V_{\max}$, K_1 and K_2) can be obtained in separate experiments. Therefore the term containing $\alpha_{1,2}$ may be isolated as follows:

$$\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1 - \underline{i}_2 = \underline{i}_1 \underline{i}_2 / \alpha_{1,2} \quad (11)$$

As explained more fully in the Materials and methods section, this approach of subtracting away terms of known values leads to ‘residual functions’ which are graphically useful quantities. In this case, the expression on the left-hand side may be termed the ‘residual function of the rate in the presence of two inhibitors’ and abbreviated as $f_r(v_{1,2})$ so that:

$$f_r(v_{1,2}) = \underline{i}_1 \underline{i}_2 / \alpha_{1,2} \quad (12)$$

From this equation it is obvious that a plot of $f_r(v_{1,2})$ versus $\underline{i}_1 \underline{i}_2$ will give a straight line through the origin with a slope of $1/\alpha_{1,2}$ (Figure 1c). Using the same data from the above Yonetani–Theorell plot, it can be seen that all points fall on one line regardless of the different values for the two inhibitor concentrations. The most striking result from this ‘combination’ plot is that the number of assays in the above experiment is rather excessive and may be reduced by about half without significant sacrifice in accuracy. As already pointed out in the case for one inhibitor [5], the approach of combination plots is more efficient because all the data are compared directly. By contrast, in the Yonetani–Theorell plot, the comparison is a two-stage process which is conducted first within different subsets of data (each with one inhibitor concentration held constant) and then among the subsets (either by intersection or by a secondary plot).

For the purpose of graphical display, the above combination plot is virtually ideal in providing visual insight into the molecular interactions. Thus the slope ($1/\alpha_{1,2}$) represents directly the extent to which the two bound inhibitors interact favourably. This aspect of the combination plot is well-illustrated using the original

was used for calculation.) In this way, the plots (a) and (c) may be regarded as comparable, because they both involve 16 assays. The slope of this plot yields a value of 0.040 ± 0.006 for $\alpha_{1,2}$, which is identical with that obtained by non-linear regression using the program Enzfitter [23].

Figure 2 Concurrent effects of two inhibitors on horse liver alcohol dehydrogenase [2]

The data had been retrieved in numerical form previously by Chou and Talalay [16] and these are presented here in combination plots. The kinetic constants used for calculation are those reported previously [2]: K_m for NAD^+ = 14 μM , K_i for ADP = 390 μM , K_2 for α -phenanthroline = 9 μM and K_2' for ADP-ribose = 26 μM . The slope (which is equivalent to $1/\alpha_{1,2}$) has the value of 2.0 and 0 for the case of ADP/ α -phenanthroline (□) and ADP/ADP-ribose (△) respectively.

data of Yonetani and Theorell [2] for alcohol dehydrogenase (Figure 2). Once again, it is quite clear that much of the data is redundant, especially when we consider that 11 of the 25 data points now become duplicates because they have the same values of $i_1 i_2$. Furthermore, it can be readily appreciated that the greater efficiency of the combination plot allows data from two separate experiments (involving different pairs of inhibitors) to be placed together in the same diagram for comparison. In the original Yonetani–Theorell plot, the juxtaposition of two data sets in this way would have been quite impossible. In the above combination plot, the different behaviours of the two pairs of inhibitors are shown in a highly contrasted fashion. Thus the significantly synergistic interaction between α -phenanthroline and ADP is indicated by a substantial slope, while the mutually exclusive binding of ADP and ADP-ribose is represented as a completely horizontal line. It should be pointed out that this plot is highly sensitive to any deviation from a completely exclusive binding behaviour, because even a small slope can be readily detected.

Alternative plots and special cases

As listed in Table 4 of the Materials and methods section, there are several alternative combination plots based on the above equation. One of these plots (Figure 3) provides a particularly useful visual representation in which synergism and antagonism are clearly distinguished. The choice of function in this plot is based on an extension of the residual function approach (see the Materials and methods section). In this case, the chosen reference system involves the independent binding of the two inhibitors ($\alpha_{1,2} = 1$) rather than their mutually exclusive binding ($\alpha_{1,2} = \infty$) which constituted the reference system in the above plot (Figures 1c and 2). We must therefore subtract from the residual function the term $i_1 i_2$, which corresponds to the expected formation of the

Figure 3 An alternative combination plot for two concurrent inhibitors of carboxypeptidase B

In each series of experiments, the concentrations of the two inhibitors were varied over a five-fold range (as indicated below) and similarly for the substrate (0.4–2 mM in all cases). The three pairs of inhibitors are: ▲, succinate (2–10 mM) and methylguanidine (0.1–0.5 mM), $\alpha_{1,2} = 0.04$; ■, 2-mercaptoethanol (0.8–4 mM) and methylguanidine (0.25–1.25 mM), $\alpha_{1,2} = 0.83$; ●, 2-mercaptoethanol (0.8–4 mM) and succinate (5–25 mM), $\alpha_{1,2} = \infty$. The above values of α have been calculated in each case from the slope, which is equivalent to $(1/\alpha_{1,2}) - 1$.

EI_1I_2 complex in the new reference system used. In other words, eqn. (12) can be transformed into:

$$f_r(v_{1,2}) - i_1 i_2 = i_1 i_2 [(1/\alpha_{1,2}) - 1] \quad (13)$$

Thus, by plotting $f_r(v_{1,2}) - i_1 i_2$ against $i_1 i_2$, we are examining how the actual behaviour (synergism or antagonism) differs from that of the reference system. The resulting linear plot has a positive or negative slope depending on whether the behaviour is synergistic ($\alpha < 1$) or antagonistic ($\alpha > 1$). This is arguably the most natural method for illustrating the two types of behaviour, since antagonism is shown as the reverse of synergism by being cast in a negative quadrant of the diagram. At the same time, independent binding has a neutral representation as a horizontal line.

To illustrate the above plot, we have chosen some data from experiments designed to take advantage of these combination plots (with five equally spaced points). It is evident from Figure 3 that very few enzyme assays are required to determine the behaviour of each system and obtain a reasonable estimate of the interaction coefficient ($\alpha_{1,2}$). It is also clear that a wide range of behaviour (with $\alpha_{1,2}$ values from 0.04 to ∞) can be displayed

effectively in one diagram. Because the plots are based on dimensionless quantities, even quite different experiments may be compared graphically. Thus comparisons may involve different enzymes or variations in pH or temperature.

In the majority of cases that are likely to be of interest, each of the inhibitors will be competitive towards the substrate. However, in the unusual event that one of the inhibitors deviates from competitive behaviour the above plots will no longer be linear. Thus any departure from linearity will serve as a diagnostic for unusual inhibition behaviour. For the purpose of estimating $\alpha_{1,2}$, the above situation can normally be anticipated because the inhibition behaviour becomes apparent during the determination of K_i undertaken earlier for each inhibitor. It is then a simple matter to modify the combination plot by subtracting extra terms from the 'residual function'. However, certain universal plots (see Appendix 1) can be devised which are applicable even when one of the inhibitors deviates from competitive behaviour. There is another unusual situation (see Appendix 2) in which the solubility of one inhibitor is too low for its K_i to be determined directly, but owing to its synergistic interaction with another inhibitor, the concurrent effects of the two inhibitors can be studied. Since these cases will not be encountered frequently in routine work, their analysis are given in Appendices at the end of the present paper.

Combination plot for three inhibitors

The Yonetani–Theorell plot is not applicable to the case of three inhibitors interacting simultaneously with the enzyme because three separately variable concentrations of inhibitors cannot be represented by a family of lines in a two-dimensional graph. In fact, no graphical method appears to be available for such a system. With combination plots, on the other hand, there is no limit to the number of variables which may be included in the chosen function. This approach is therefore ideally suited to a highly complex situation of this nature. As in the case for two inhibitors, the rate equation is conveniently expressed in terms of relative values (for details see Materials and methods section). Thus for three inhibitors (each of which is in itself competitive with respect to the substrate) we obtain the analogous equation:

$$\frac{s}{v_{1,2,3}} = 1 + \frac{s}{\sum_{n=1}^{n=3} i_n} + \sum_{m=1}^{m=2} \sum_{n=2}^{n=3} \frac{i_m i_n}{\alpha_{m,n}} + \frac{i_1 i_2 i_3}{\tau \alpha_{1,2} \alpha_{2,3} \alpha_{1,3}} \quad (14)$$

Although this equation appears complex, it can be readily understood by considering the enzymic species whose concentrations correspond to the various terms in it. Thus $s/v_{1,2,3}$ represents the total concentration of all enzymic species (being equal to $[E]/[E]$). The terms 1 and s correspond to the species $[E]$ and $[ES]$ and there are three terms of the type i_n (under the summation sign) which represent *binary* enzyme–inhibitor complexes (EI_1 , EI_2 and EI_3). The concentrations of these complexes are simply governed by their dissociation constants (e.g. $i_1 = [I_1]/K_1$). Similarly, there are three terms of the type $i_m i_n / \alpha_{m,n}$ (under the double summation signs) which are contributed by *ternary* complexes (i.e. $EI_m I_n$). The formation of these complexes are controlled by a set of equilibria illustrated in Scheme 1 (see the Materials and methods section) and their concentrations are also affected by the interaction coefficient (α with the appropriate subscripts) applicable in each case. Finally, the possible existence of the *quaternary* complex $EI_1 I_2 I_3$ is represented by the last term in the equation. The extent to which this complex is formed is dependent on the mutual interaction occurring within a given pair of bound inhibitors which is accounted for by the various α coefficients (measured in three separate experiments). In addition, another coefficient, τ (the tripartite interaction coefficient; for

details, see the Materials and methods section), represents the factor by which the presence of two bound inhibitors influences the binding of the third inhibitor beyond what each bound inhibitor would exert singly. This coefficient has a physical significance closely similar to that of α , except that it applies to the quaternary complex $EI_1 I_2 I_3$. Thus antagonism or synergism will be characterized by τ values greater or less than 1 respectively. As in the case of two inhibitors described earlier, all the parameters except one (τ in this case) can be obtained in separate experiments, so that we can rearrange the above equation (eqn. 14) as follows:

$$\frac{s}{v_{1,2,3}} - 1 - \frac{s}{\sum_{n=1}^{n=3} i_n} - \sum_{m=1}^{m=2} \sum_{n=2}^{n=3} \frac{i_m i_n}{\alpha_{m,n}} = \frac{i_1 i_2 i_3}{\tau \alpha_{1,2} \alpha_{2,3} \alpha_{1,3}} \quad (15)$$

As explained in greater detail in the the Materials and methods section, the expression on the left-hand side of the above equation may be denoted the 'residual function of the rate in the presence of three inhibitors' and abbreviated $f_r(v_{1,2,3})$ so that:

$$f_r(v_{1,2,3}) = \frac{i_1 i_2 i_3}{\tau \alpha_{1,2} \alpha_{2,3} \alpha_{1,3}} \quad (16)$$

Thus, $f_r(v_{1,2,3})$ may be plotted against $i_1 i_2 i_3 / \alpha_{1,2} \alpha_{1,3} \alpha_{2,3}$ to give $1/\tau$ as the slope.

Example of a three-inhibitor system

Carboxypeptidase B is well-suited for demonstrating the validity of the above analysis because the active site has three or more distinct features which contribute to substrate binding [15]. To begin with, the enzyme contains a recognition site for the side chain of basic amino acids as well as the terminal carboxy group. In addition, the Zn^{2+} ion essential for catalysis can be probed with a variety of metal-specific ligands. There is also a relatively hydrophobic binding region corresponding to the extended carbon chain in the arginine or lysine moiety of the substrate. Thus methylguanidine is a relatively strong inhibitor of this enzyme [13]. In preliminary experiments we also noticed that the binding of 2-mercaptoethanol and methyl succinate were not only mutually compatible but in fact moderately synergistic ($\alpha = 0.38$). Presumably the thiol group of 2-mercaptoethanol interacts with the zinc atom, while one of the carboxy groups of methyl succinate binds to the C-terminal recognition site. (The interactions of the other functional groups in these inhibitors with the enzyme remain unclear.)

Having first determined the interaction coefficients for the three pairwise combinations of the above compounds (Table 4), we proceeded to measure the effect on the enzyme when all three inhibitors were present. Analysis of the data using the above combination plot (Figure 4) indicates that the three inhibitors were able to bind simultaneously to form the quaternary complex ($EI_1 I_2 I_3$). If this complex were unable to form, the resulting plot would have been a horizontal line. From the slope, a value of 1.1 ± 0.3 for the tripartite interaction constant (τ) was obtained. Since this value is not significantly different from unity, we may conclude that no additional synergistic or antagonistic effects were produced beyond what was expected from pairwise interactions.

Comparison with other methods

The study of multiple enzyme inhibitors may be undertaken for a variety of purposes. This diversity may explain the difference in approach which has been used to analyse the results. Thus, in advocating the method of median effects, Chou and Talalay [16] emphasized that their analysis required no prior knowledge of kinetic constants. This special property might well be an ad-

Figure 4 Combination plot for three concurrent inhibitors

The concentrations of all three inhibitors were varied over a five-fold range (as indicated below) and similarly for the substrate (0.4–2 mM). The three inhibitors were methylguanidine (0.04–0.2 mM), methylsuccinate (0.1–0.5 mM) and 2-mercaptoethanol (0.1–0.5 mM). Their kinetic constants are listed below. The slope, which represents $1/\tau$, has a value of 0.92 ± 0.15 .

Inhibitor	K_i	α
Methylguanidine (i_1)	1.5 ± 0.2	$\alpha_{1,2} = 0.040 \pm 0.005$
Methyl succinate (i_2)	0.30 ± 0.05	$\alpha_{2,3} = 0.38 \pm 0.05$
2-Mercaptoethanol (i_3)	5.0 ± 1.0	$\alpha_{1,3} = 0.83 \pm 0.15$

vantage in determining dosage effects of drugs in complex systems such as cell cultures or the whole body. However, for studies of molecular interactions in isolated enzymes, it is rather inconceivable that one would actually avoid determining kinetic constants. Instead, the rational procedure in such cases should be to analyse first the simplest system (i.e. with no inhibitor) and then to progress in a stepwise fashion to more complex ones (i.e. from single to multiple inhibitors). By using combination plots, the entire exercise of determining K_m , V_{max} , K_1 , K_2 (and inhibition pattern or $\alpha_{1,2}$, $\alpha_{2,3}$) and the interaction coefficient $\alpha_{1,2}$ for a two-inhibitor system could be accomplished with as little as 20 assays (as explained in the Materials and methods section).

It is perhaps significant that, in the above-mentioned work of Chou and Talalay [16], the main focus concerned mutually exclusive inhibitors. The fact that two inhibitors of sufficiently large size could not be accommodated simultaneously in one active site, however, provides little information in terms of molecular interactions. When the above authors considered non-exclusive inhibitors, they assumed (often without stating explicitly) that the interaction coefficient ($\alpha_{1,2}$) was equal to 1. Our

extensive work (covering seven different enzymes) [4,11–14] indicates, in contrast, that this condition is rarely fulfilled. Instead α may range from less than 0.01 all the way to infinity. Thus equations based on the above assumption are of very restricted applicability. Although an alternative criterion such as the combination index suggested by Chou and Talalay [17] may be useful for comparing effectiveness of drugs taken concurrently, it does not provide insight into the molecular basis of the phenomenon. In fact, by using their procedure to analyse the previous data of Yonetani and Theorell [2], the above authors came to the apparent conclusion [16] that a higher-order (i.e. co-operative and non-Michael–Menten) kinetic system was involved. Our analysis, however, clearly demonstrates (Figure 2) that the same data can be fully explained in terms of a simple system with moderately synergistic interaction ($\alpha = 0.5$) between the inhibitors.

In any method of analysis, there are normally certain implications on the experimental design of the study. In this respect, some approach to the analysis of multiple inhibitors can be highly restrictive. For example in the median-effect method [16], only data with constant molar ratio of the two inhibitors may be used, and similar restrictions apply to other methods [18,19]. Even in the Yonetani–Theorell analysis [2], there is the requirement that the concentration of one inhibitor be held constant in each subset of data. In contrast, the approach of combination plots described in the present paper allows virtually complete freedom in the choice of the experimental parameters. Even substrate and enzyme concentrations may be varied, because the effects are automatically taken into account by the equation. Therefore the only criterion to bear in mind in the experimental design is the accuracy of the measurements. In special cases, the power of the method can be extended by restricting some variable in order to probe into particular aspects of the systems (as shown in Appendices 1 and 2).

With the general availability of microcomputers, curve-fitting of experimental data based on non-linear regression has become the method of choice for determining kinetic constants [20]. However, it has been emphasized [21] that graphical display is still indispensable for assessing the goodness-of-fit. Although this can be accomplished with a non-linear graph, the linear combination plots described here have the advantage that any deviation in the data can be readily assessed in terms of the value of $\alpha_{1,2}$ because this is represented directly as the slope. The ability to inspect data visually is a feature of graphical analysis which continues to appeal widely to biochemists and has been strongly endorsed by some experts ([22] and references therein). Thus we hope that the convenience of combination plots and their favourable illustrative properties may stimulate further interest in the study of multiple inhibitors.

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APPENDIX 1

Universal plots for two concurrent inhibitors of which one may deviate from competitive behaviour

The combination plots described in the main section are designed for the most common case where both inhibitors are fully competitive towards the substrate. When one of the inhibitors (say I_2) deviates from competitive behaviour, we may include a new coefficient, $\alpha_{2,s}$, to take into account the interaction between this inhibitor and the substrate. The rate equation can be written in terms of relative values as follows:

$$\underline{s}/v_{1,2} = 1 + \underline{s} + i_1 + i_2 + i_1 i_2 / \alpha_{1,2} + i_2 \underline{s} / \alpha_{2,s} \quad (A1)$$

where the last term represents the formation of the EI_2S complex (being equal to $[EI_2S]/[E]$). This equation is valid for non-competitive inhibition (where $\alpha_{2,s} = 1$) or linear mixed inhibition ($1 < \alpha_{2,s} < \infty$) and reduces to that for competitive inhibition (eqn. 10 of the main paper) by giving $\alpha_{2,s}$ the value of ∞ . In the case of an uncompetitive inhibitor (again, I_2) the term i_2 disappears from the above equation and the last term is reduced to $i_2 \underline{s}$:

$$\underline{s}/v_{1,2} = 1 + \underline{s} + i_1 + i_1 i_2 / \alpha_{1,2} + i_2 \underline{s} \quad (A2)$$

One approach in such cases would be to customize the combination plot by adding appropriate terms to, or subtract them from, the residual function used in the plot. For example, if one inhibitor is uncompetitive (eqn. A2), we may plot the function $\underline{s}/v_{1,2} - 1 - \underline{s} - i_1 - i_2 \underline{s}$ against $i_1 i_2$. However, in most situations, it would be beneficial to have a single plot to cover all simple inhibition behaviour. Two ‘universal’ combination plots of this kind are presented below.

Using the concept of residual functions (see the Materials and methods section of the the main paper) and the corresponding abbreviations we may rearrange eqn. (A1) as follows:

$$f_r(v_{1,2})/i_2 = i_1/\alpha_{1,2} + \underline{s}/\alpha_{2,s} \quad (A3)$$

and similarly, for uncompetitive inhibition, eqn. (A2) can be written as:

$$f_r(v_{1,2})/i_2 = (i_1/\alpha_{1,2}) + \underline{s} - 1 \quad (A4)$$

By keeping $[S]$ constant, it is then possible to plot $f_r(v_{1,2})/i_2$ against i_1 for both the above cases (Table 4 of the main paper). If the inhibitor in question (I_2) is in fact competitive (in which case $\alpha_{2,s} = \infty$), then the plot is virtually identical with that recommended above in the main paper (Figure 1c) and goes through the origin. However, deviation from competitive behaviour will result in a vertical intercept which is usually equal to $\underline{s}/\alpha_{2,s}$, thus allowing this additional coefficient to be easily determined. In the case of uncompetitive behaviour, the intercept is equal to $\underline{s} - 1$, and this could have a negative value if $[S]$ is chosen to be less than K_m (since $\underline{s} = [S]/K_m$). A useful feature of this plot is that, in all cases, the slope represents the extent of favourable interaction ($1/\alpha_{1,2}$) between the two inhibitors.

A variation of the above method is to plot $f_r(v_{1,2})/i_1 i_2$ against $1/i_1$ (Table 4 of the main paper). The difference in behaviour is now displayed as a slope rather than an intercept. Again, if $\underline{s}$ is chosen to be appreciably less than unity, the distinctive appearance of a negative slope ($\underline{s} - 1$) will indicate uncompetitive behaviour. This plot may be inferior to the above because the reciprocal ($1/i_1$) is used. However, if $\alpha_{2,s}$ is large (but distinguishable from ∞) then it may be easier to detect $\underline{s}/\alpha_{2,s}$ as an appreciable slope in this plot rather than as an intercept with a small value. For this particular situation it would certainly be advisable to use high $[S]$ rather than low $[S]$, as recommended above for uncompetitive inhibition.

Normally the nature of inhibition will already have been determined for each inhibitor before their concurrent effects are examined. However, one may occasionally wish to ascertain that the behaviour is maintained in the presence of the second inhibitor. This can be done with the above plots and the same data may be plotted against i_1 and i_2 in turn. In fact the same diagram should accommodate both graphs, since the functions used contain only dimensionless quantities.

APPENDIX 2

Synergistic interaction between two inhibitors of which one has low solubility

In the study of enzyme inhibition, the situation may occasionally arise where the compound of interest has a solubility limit substantially lower than its K_i , so that the latter cannot be reliably measured. However, we have found several instances [1,2] in which the presence of a second inhibitor generates sufficient synergism that their concurrent effects can be measured at much lower concentrations. In other words, a very low value of α (0.1 or less) may well bring αK_i within the range of solubility of the compound under study.

We have previously used the Yonetani-Theorell plot [3] to analyse both $\alpha_{1,2}$ and the K_i of the sparingly soluble inhibitor [1]. This analysis was possible provided that the K_i for the other inhibitor was accurately known (and α is small). Our previous method was, however, not completely rigorous, because it

assumed that the compound with a low solubility was a competitive inhibitor. In addition, it shared the same cumbersome properties with other plots based on a family of lines. In the present Appendix, we present a rigorous and highly efficient approach for this special situation.

Let us assume that I_1 is a competitive inhibitor with known K_1 and I_2 is the sparingly soluble inhibitor. Since K_2 is unknown, the variable i_2 in the relevant equations must be expressed as $[I_2]/K_2$. Eqn. (A1) of Appendix 1 above may therefore be arranged as:

$$(\underline{s}/v_{1,2} - 1 - \underline{s} - i_1)/[I_2] = (1 + i_1/\alpha_{1,2} + \underline{s}/\alpha_{2,s})/K_2 \quad (A5)$$

Similarly eqn. (A2) of Appendix 1 for uncompetitive inhibition may be written as:

$$(\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1)/[\underline{I}_2] = (\underline{i}_1/\alpha_{1,2} + \underline{s})/K_2 \quad (\text{A6})$$

If the function on the left-hand side of these equations is plotted against $\underline{i}_1$, it is clear that a straight line will result with a slope of $1/\alpha_{1,2}K_2$ in both cases, provided that $[\underline{S}]$ is held constant. By conducting a series of assays (say five) in which $\underline{i}_1$ is varied, we can therefore determine $1/\alpha_{1,2}K_2$ regardless of the inhibition behaviour. This value can then be used to calculate $\underline{i}_1\underline{i}_2/\alpha_{1,2}$ (since $\underline{i}_2 = [\underline{I}_2]/K_2$) so that we can now rearrange eqn. (A5) as follows:

$$(\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1 - \underline{i}_1\underline{i}_2/\alpha_{1,2})/[\underline{I}_2] = 1/K_2 + \underline{s}/\alpha_{2,s}K_2 \quad (\text{A7})$$

Similarly, eqn. (A6) becomes:

$$(\underline{s}/\underline{v}_{1,2} - 1 - \underline{s} - \underline{i}_1 - \underline{i}_1\underline{i}_2/\alpha_{1,2})/[\underline{I}_2] = \underline{s}/K_2 \quad (\text{A8})$$

If a second series of assays (say four) are performed in which $\underline{s}$ is varied ($[\underline{I}_1]$ and $[\underline{I}_2]$ may also be varied), then a plot of the left-hand side of eqns. (A7) and (A8) above against $\underline{s}$ will generate a straight line with a vertical intercept of $1/K_2$ and a horizontal intercept of $-\alpha_{2,s}$ (or, for uncompetitive inhibition, a line through the origin with a slope of $1/K_2$). It should be mentioned that the data in the first series of assays may also be used in this second

plot, but they will provide only a single additional point because the value of $\underline{s}$ was held constant. This plot is completely equivalent to the 'optimized' combination plot previously published [4] for a single inhibitor {namely $(\underline{s}/\underline{v}_{1,2} - 1 - \underline{s})/[\underline{I}]$ versus $\underline{s}$ }, and the equivalence is achieved here by taking fully into account the effects of $\underline{I}_1$ (through subtraction of the terms $\underline{i}_1$ and $\underline{i}_1\underline{i}_2/\alpha_{1,2}$). As in the case of the previous plot [4], each type of inhibition behaviour is indicated by a distinctive appearance of the resulting plot. Furthermore, the horizontal intercept of $\alpha_{2,s}$ serves as a quantitative criterion for the inhibition pattern (e.g. $\alpha_{2,s} = 1$ for non-competitive behaviour).

Having determined both $1/K_2$ and $1/\alpha_{1,2}K_2$, we can obviously calculate $\alpha_{1,2}$. A more accurate estimate may consist of plotting together the data from both series of assays in a combination plot such as $f_r(v_{1,2})$ versus $\underline{i}_1\underline{i}_2$. If the values of $[\underline{I}_1]$ and $[\underline{I}_2]$ have been suitably chosen, a line with nine well-spaced points will be obtained from the above recommended procedure.

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