Synergy between verapamil and other multidrug-resistance modulators in model membranes

Madeleine Castaing[1,2,*], Alain Loiseau[3] and Athel Cornish-Bowden[4]

[1]*Faculté de Pharmacie, 27, Boulevard Jean Moulin, 13385 Marseille, France*
[2]*Present address: CNRS Délégation Régionale Languedoc-Roussillon, 34293 Montpellier, France*
[3]*Faculté de Médecine Xavier-Bichat, 75870 Paris, France*
[4]*Institut de Biologie Structurale et Microbiologie, 13402 Marseille, France*

**Corresponding author (Email, madeleine.castaing@dr13.cnrs.fr)*

Various cationic lipophilic compounds can reverse the multidrug resistance of cancer cells. Possible interaction between these compounds, which are known as modulators, has been assessed by measuring leakage of Sulphan blue from anionic liposomes, induced both by verapamil alone and by verapamil in combination with diltiazem, quinine, thioridazine or clomipramine. An equation was derived to quantify the permeation doses and Hill coefficients of the drugs and mixtures between them by simultaneous fitting of the experimental data. The interaction was tested by two methods, the competition plot and the isobole method; both showed synergy between verapamil and each of diltiazem, quinine and thioridazine. The dose factor of potentiation for verapamil determined within membranes was 4.0 ± 0.4 with diltiazem, 3.2 ± 0.4 with quinine and 2.4 ± 0.3 with thioridazine. The results suggest that the effectiveness of reversing multidrug resistance may be increased with modulators such as verapamil and diltiazem that have a much greater effect in combination than what would be expected from their effects when considered separately.

[Castaing M, Loiseau A and Cornish-Bowden A 2007 Synergy between verapamil and other multidrug-resistance modulators in model membranes; *J. Biosci.* **32** 737–746]

1. Introduction

A major problem in the treatment of cancer is that of multidrug resistance, which remains imperfectly understood, though in some instances it certainly involves altered membrane transport in tumour cells (Pajeva *et al* 2004; Ferreira *et al* 2005). Toxicity-lowering export of drugs is mediated by the membrane protein P-glycoprotein (Higgins and Gottesmann 1992; Qu *et al* 2003), a member of the class of ABC transporters. Export of drugs by this protein is believed to result from interaction with the protein after partitioning into the membrane (Lugo and Sharom 2005). Study of this process in cancer cell lines (Ferreira *et al* 2005) has been useful, but it is made more difficult by the complicated structures of membranes *in vivo*, so for exploring interactions between drugs at the lipid bilayer it can be more informative to use of model membranes of known structure (Pajeva *et al* 2004), and we have used anionic liposomes (Castaing *et al* 2003) for this purpose. The major loss in complexity when compared with real cell membranes comes from the absence of proteins, but this should not greatly affect the interpretation, because lipophilic amphiphiles such modulators primarily interact with the lipids, and not with the proteins. We shall discuss more detail later how far one can extrapolate from model membranes to real cells (*see* Discussion).

In biomedical research, including investigation of cancer, studies of the combined action of biologically active agents, such as drugs, environmental pollutants or radiation have become increasingly important (Sühnel 1998), as

Keywords. Dose–response relationship; drug-membrane interactions; drug synergism; dye leakage; isobole method; multiple drug-resistance

combinations of agents are being increasingly used clinically for the therapeutic advantages they may provide over single agents (Berenbaum 1989). The usual approach for assessing a possible interaction in combination experiments is to calculate, from the effects of single agents, what is expected for the combination effect in the case of no interaction. An observed effect larger than expected is taken as evidence of synergism, and a smaller effect as evidence of antagonism. Five principal approaches have been used to predict the expected effect: comparison of the combined action with the most effective single constituent; multiplication of effects; summation of effects; the median-effect principle; and the isobole method (Berenbaum 1989; Sühnel 1990). Among these, the only generally applicable procedure is the isobole method, as it requires no assumptions about the shapes of the dose–response curves (Berenbaum 1989; Loewe 1928). It only requires experimental data for the agents used alone and in different dose combinations at equally effective levels, but for assessing synergy or antagonism both the expected effects and the actual effects need to be determined with high precision.

Modulators such as verapamil, quinine and others, which reverse the multidrug resistance of cancer cells (Sharom 1997), bind to membranes in the region between the head groups of the phospholipids at the membrane surface and the acyl chains of the phospholipids in the core of the membrane. The spatial positioning of these molecules in the binding site may vary according to their sizes and shapes, and according to their hydrophobicity and electric charge (Castaing *et al* 2003, 2005). We therefore set out to assess the possibility of synergy between multidrug resistance modulators in terms of drug–membrane interactions, by testing the ability of verapamil to induce dye leakage from anionic liposomes, alone (Klohs *et al* 1986) and in combination with other modulators, diltiazem (Klohs *et al* 1986), quinine (Bennis *et al* 1997), thioridazine (Ramu *et al* 1984) and clomipramine (Tsuruo 1983). We derived an equation that allows all the data to be explained simultaneously in terms of cooperative binding of the separate drugs as well as synergistic interactions between them. We show that in addition to the isobole method, widely used in pharmacological studies, the competition plot (Chevillard *et al* 1993; Cárdenas 2001) may be a useful method for assessing synergy or antagonism between the effects of drugs.

2. Materials and methods

2.1 *Materials*

L-*α*-Phosphatidylcholine prepared from fresh egg yolk, L-*α*-phosphatidic acid prepared from egg yolk lecithin, verapamil, diltiazem, quinine and clomipramine were purchased as their hydrochlorides from Sigma (St Louis, MO, USA). Thioridazine hydrochloride and the dye anhydro-4-4′-bis(diethyl-amino)triphenyl-methanol-2′′, 4′′-disulphonic acid monosodium salt (Sulphan blue) were purchased from Aldrich (Steinheim, Germany). Cholesterol was obtained from Fluka (Buchs, Switzerland). Diethyl ether, $Na_2HPO_4.2H_2O$ and $NaH_2PO_4.H_2O$ were purchased from Merck (Darmstadt, Germany), Sephadex PD–10 columns (G–25M) from Pharmacia (Uppsala, Sweden) and polycarbonate porous membranes from Nuclepore Corporation (Pleasanton, CA).

Each of the modulators was dissolved in water.

2.2 *Liposomes loaded with Sulphan blue*

Large unilamellar vesicles composed of phosphatidylcholine, phosphatidic acid and cholesterol (8:1:1) were prepared by reverse-phase evaporation, as previously described (Castaing *et al* 2000). The external dye was removed by passage through two Sephadex G–25 columns eluted with 100 mM phosphate buffer (pH 7.4 at 37°C).

2.3 *Permeability measurements*

The absorbance of Sulphan blue at 640 nm was determined by recording the visible absorption spectra of the samples with a Uvikon 933 spectrophotometer (Milan, Italy). Permeability was measured in 1 ml suspensions of large unilamellar vesicles. The two drugs, alone or in combination, or the solvent, were mixed with 0.5 ml buffer. Following the addition of 0.5 ml liposomes, the sample was stirred for 3 min at 37°C using a Salvis SBK shaker bath (Reussbühl-Luzern, Switzerland). The external dye was removed as previously described (Castaing *et al* 2000). In these experiments, leakage was induced by 0–11 mM drug (or 0–1.7 mol drug/mol lipid), alone or in combination. It was quantified after a 3 min incubation (initial rate of leakage) at 37°C (pH 7.4).

2.4 *Analysis of dose–response curves*

The initial rate of leakage of Sulphan blue induced by verapamil was quantified, alone and at five or six constant concentrations of diltiazem, quinine, thioridazine or clomipramine, following an experimental approach similar to that of Pöch *et al* (1990). The variations in the initial rate of Sulphan blue leakage with different drug concentrations were fitted by non-linear least squares regression.

3. Theory

3.1 *Analysis of dose–response curves*

The degree of cooperativity for the binding of a single substance can be conveniently expressed in terms of the Hill

equation,

$$\text{Leakage}\,(\%) = 100 \times \frac{(a/a_{0.5})^h}{1+(a/a_{0.5})^h} \quad (1)$$

in which a is the concentration of the drug, $a_{0.5}$ is the value of a that induces 50% dye leakage, and h, the Hill coefficient, is a standard parameter to quantify the degree of cooperativity. A value of $h = 1$ would indicate no cooperativity, $h > 1$ indicates positive cooperativity, and $h < 1$ would indicate negative cooperativity. Values in the range of 3–4 are as high as one normally observes when measuring binding to a macromolecule, but higher values can be observed for binding to more elaborate structures, such as membranes. In leakage experiments $a_{0.5}$ would commonly be called the *permeation dose* and given a symbol such as PD_{50}, but we prefer to indicate that it is a concentration by using a more explicit symbol. For a full biochemical analysis of the binding it would be preferable to use the dissociation constant, consistent with the usual biochemical preference for defining inhibition behaviour in terms of inhibition constants rather than concentrations for 50% inhibition (Cortés *et al* 2001); however, this is not important here because the Hill equation is an empirical equation and the Hill coefficient has no necessary physical meaning.

Eqn (1) cannot be used as it stands to describe the behaviour of mixtures of drugs, but it can be generalized to give the following equation:

$$\text{Leakage}\,(\%) = 100 \times \frac{(a/a_{0.5})^{h_A} + (b/b_{0.5})^{h_B} + (ab/K_{AB})^{h_{A,B}}}{1+(a/a_{0.5})^{h_A} + (b/b_{0.5})^{h_B} + (ab/K_{AB})^{h_{A,B}}} \quad (2)$$

in which a, $a_{0.5}$ and h_A are the symbols for verapamil with the same meanings as in eqn (1); b, $b_{0.5}$ and h_B are the corresponding symbols for the second drug, the terms in b resulting from an obvious generalization of the equation to allow for the presence of the second drug; and K_{AB} and $h_{A,B}$ are a binding constant and a Hill coefficient that characterize interaction between the two drugs, the terms in ab allowing for the possibility that both drugs can bind simultaneously.

3.2 *Isobole analysis*

In the isobole method, the effects of drug combinations are expressed graphically in a two-dimensional plot called an 'isobologram', in which isoboles are the curves connecting equally effective dose combinations of the two drugs (Loewe 1928), isobolograms being typically drawn at a fixed effect level of 50%. The drug doses on the two axes can be expressed as the specific concentrations of the agents so that equally effective doses of the drugs, applied singly, are equal to unity (Elion *et al* 1954); in other words, the specific concentration of a modulator is its concentration in the mixture divided by the concentration that would be needed for it to produce the same effect by itself. Points below the straight line of zero interaction correspond to synergistic interaction and points above it to antagonism.

4. Results

4.1 *Drug permeation properties of mixtures of verapamil with a second multidrug-resistance modulator*

Results for verapamil with diltiazem are illustrated in figure 1, and qualitatively similar behaviour was observed for verapamil in combination with the other drugs.

It is evident from the sigmoid nature of the curves for verapamil and diltiazem in figure 1 that these drugs bind with a high degree of cooperativity, and this was true for each of the five drugs considered by itself. When the data for each experiment were fitted simultaneously to eqn (2) it was found in each case to describe the results with good accuracy ($R^2 = 0.950$–0.991; $P < 0.001$; $n = 21$–29); the permeation parameters are given in table 1.

If the two drugs were to bind exclusively at the same site, then the terms in ab would be negligible, but this is excluded by the fact that all the K_{AB} values in the table are finite, i.e. each $1/K_{AB}$ is significantly different from zero. One might naively expect that K_{AB}, which has the dimensions of a squared concentration, might simply be the product $a_{0.5}b_{0.5}$, but it is much smaller than this product for all of the four drug combinations tabulated, a clear indication of synergy between the effects of the different drugs. The Hill coefficients for verapamil and diltiazem are very large, beyond the range normally observed for binding to a macromolecule, and there is significant cooperativity for the other drugs also. For the verapamil–diltiazem combination it is tempting to attach significance to the fact that the Hill coefficients for the separate drugs are both about 6 and that for the combination about half that, and in fact the data fit fairly well to an equation in which the Hill coefficients are constrained to exactly these values. However, there is no corresponding suggestion from the other drug combinations.

The synergy of binding between verapamil and diltiazem is obvious in figure 1. Consider, for example, the results at 2.08 mM diltiazem. In the absence of verapamil this concentration of diltiazem produces only 7% dye leakage, the same as given by 0.95 mM verapamil in the absence of diltiazem. A concentration of verapamil of 1.90 mM, i.e. double 0.95 mM, a 'sham combination' in the terminology of Berenbaum (1989), gives 68% leakage, but a combination of 0.95 mM verapamil with 2.08 mM diltiazem gives 80%: as this is greater than 67% it indicates synergy between the two drugs.

4.2 *Competition plot for multidrug-resistance modulators*

The spatial positioning of multidrug-resistance modulators in the membrane binding site may vary depending on their structural and physicochemical characteristics (Castaing *et al* 2003; 2005). As a result, two drugs may compete exclusively for the same part of the binding site. However, the synergy evident in figure 1 indicates that this is not the case for the drugs discussed here, and the data were accordingly analysed on the basis of eqn (2) with the parameter values of table 1, by drawing a competition plot to test whether or not the binding of the modulators occurs exclusively at the same site (Chevillard *et al* 1993; Cárdenas 2001).

If a_0 and b_0 are the reference concentrations of two drugs A and B with the same leakage rate when applied singly, then any combination of these drugs has the same leakage rate if they compete for the same site and their respective concentrations a and b are varied together in such a way that

$$a = a_0(1 - p)^{1/h_A} \tag{3}$$

$$b = pb_0^{1/h_B} \tag{4}$$

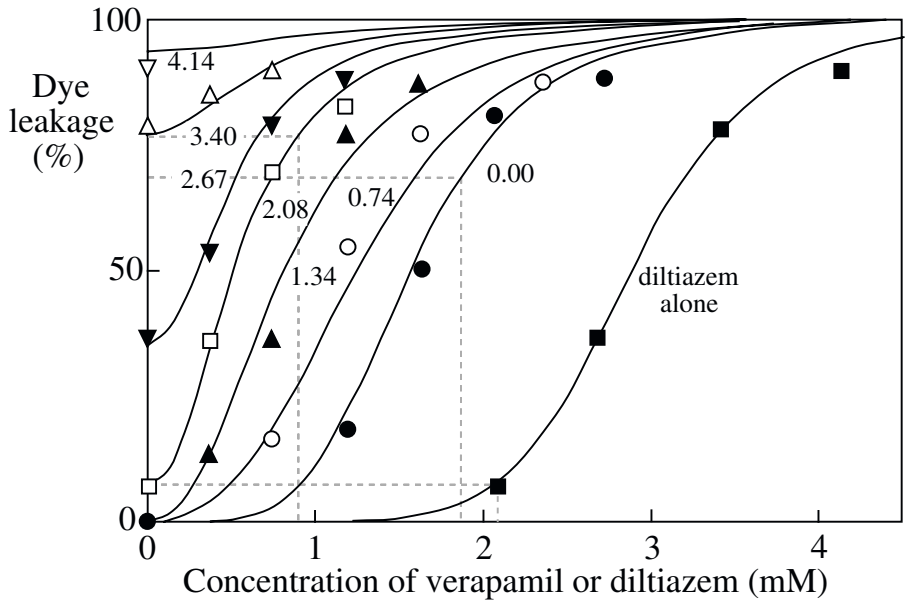

Figure 1. Dose–response curves for verapamil and diltiazem. For the curve labelled 'diltiazem alone' the abscissa scale gives the concentration of diltiazem; for all of the others the abscissa scale refers to verapamil and the curves are labelled with the diltiazem concentrations (in mM). In all cases leakage of Sulphan blue was measured and the initial rate of leakage was quantified after 3 min incubation at pH 7.4 (37°C). The curves were calculated according to eqn (2) after simultaneous fitting of the experimental data obtained on one preparation of vesicles. Each point is the result of one measurement. The broken lines illustrate an analysis of the type proposed by Pöch and Holzmann (1980), as described in the text.

Table 1. Permeation properties for combinations of drugs

Drug B	$a_{0.5}$ (mM)	h_A	$b_{0.5}$ (mM)	h_B	K_{AB} (mM^2)	$h_{A,B}$
Diltiazem	1.64 ± 0.10	5.16 ± 0.26	2.99 ± 0.14	6.49 ± 0.30	1.10 ± 0.11	2.51 ± 0.11
Quinine	1.49 ± 0.05	4.70 ± 0.14	3.44 ± 0.22	3.65 ± 0.09	1.19 ± 0.08	1.89 ± 0.08
Thioridazine	1.58 ± 0.08	4.66 ± 0.13	3.31 ± 0.23	3.86 ± 0.24	1.82 ± 0.23	1.61 ± 0.32
Clomipramine	1.41 ± 0.04	3.93 ± 0.24	5.21 ± 0.27	2.05 ± 0.22	3.60 ± 0.51	0.88 ± 0.12

The leakage of Sulphan blue through large unilamellar vesicle membranes was induced by 0–2.7 mM verapamil (drug A), alone or in combination with 0–9 mM diltiazem, quinine, thioridazine or clomipramine, i.e. the second multidrug-resistance modulator, drug B. The initial rate of leakage was quantified following incubation for 3 min at 37°C (pH 7.4). The permeation parameters of A, B and mixtures between them were determined according to eqn (2) by simultaneous fitting of the experimental data obtained on one vesicle preparation. Data are given ± SEM (n = 4), each error estimate being based on four separate determinations of the parameters from experiments with four different vesicle preparations. The surprisingly low value of h_A in the bottom line can probably be explained by aggregation of clomipramine in the aqueous phase (Atherton and Barry 1985), which reduced its thermodynamic activity.

where p varies from 0 to 1 and defines the concentrations of the two drugs in each mixture, i.e. $p = (b/b_0)^{h_B}$ and $1-p = (a/a_0)^{h_A}$ (Chevillard *et al* 1993). Application of eqn (2) with the parameter values of table 1 allowed a_0, b_0, a and b to be calculated as functions of p, this being done for leakage rates of 20% for the pure drugs. The leakage rates for the mixtures should not vary with p if the drugs compete exclusively for the same site. However, as illustrated in figure 2, the leakage rates of combinations of verapamil with other drugs varied greatly with p, being 4.4 ± 0.1 times higher with diltiazem at p = 0.5, 3.6 ± 0.1 times higher with quinine, 2.9 ± 0.1 times higher with thioridazine, and 2.2 ± 0.2 times higher with clomipramine. These very large deviations from the behaviour expected for simple competition, well outside experimental error, indicate that although multidrug-resistance modulators bind to the same region of the membrane the binding is not exclusive (especially for the verapamil–diltiazem combination), and this allows synergy between these drugs.

4.3 *Isoboles for multidrug-resistance modulators*

Figure 3 shows isoboles obtained with mixtures of multidrug-resistance modulators at an effect level of 50% (initial leakage rate). In this figure, the drug doses on the abscissa and ordinate are respectively expressed as $A = (a/a_{0.5})^{h_A}$ and $B = (b/b_{0.5})^{h_B}$, and because of the experimental design (Figure 1), the B values corresponding to any given A level had to be calculated from eqn (2) with the data of table 1. The variations of B with A were fitted to the following equation (R^2 = 0.993–1.000; $P < 0.001$; n =11):

$$B = (1 - A) / (1 + \alpha A), \tag{5}$$

where $B = (b/b_{0.5})^{h_B}$ and $A = (a/a_{0.5})^{h_A}$, so that equally effective doses of A and B, applied singly, are equal to unity, and α is a parameter related to the interaction between them.

The dashed line connecting A = 1 to B = 1 represents all dose combinations with zero interaction, i.e. for the simpler equation $B = 1 - A$. This line is also called the dose-additivity line of Loewe (1928). The curves for the mixtures of drugs all pass well below the zero-interaction straight line, i.e. the sum of the two normalized drug concentrations in each mixture is less than 1.0, even for the mixtures with the highest level of experimental error, those with clomipramine and verapamil, as indicated by the shaded region in the figure. Shading is not used in the other cases, as it would be barely visible at the scale of the figure for mixtures of thioridazine and verapamil, and not visible at all for the other two. The results therefore clearly indicate synergistic interactions between verapamil and each of the other four modulators.

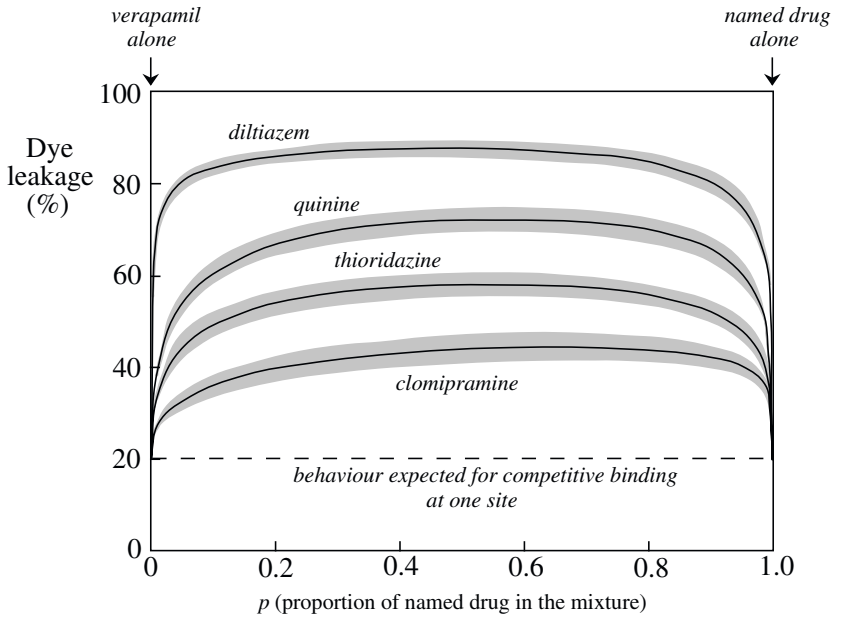

Figure 2. Competition plot for multidrug-resistance modulators. The reference concentrations a_0 (verapamil) and b_0 (drug named on the graph) were chosen so that the effect of verapamil and the second drug, applied singly, was equal to 20%. The total leakage rate for mixtures was calculated according to eqn (2) with the data in table 1, at concentrations $a = a_0 (1 - p)^{1/h_A}$ and $b = b_0 p^{1/h_B}$, where p specifies the proportion of the second drug in the mixture. If the modulators competed exclusively for the same binding site of the membrane, the amount of leakage would not vary with p (Chevillard *et al* 1993). The curves drawn in this figure were calculated from eqns (2–4), and the shaded region around each curve shows the calculated standard errors of the mean.

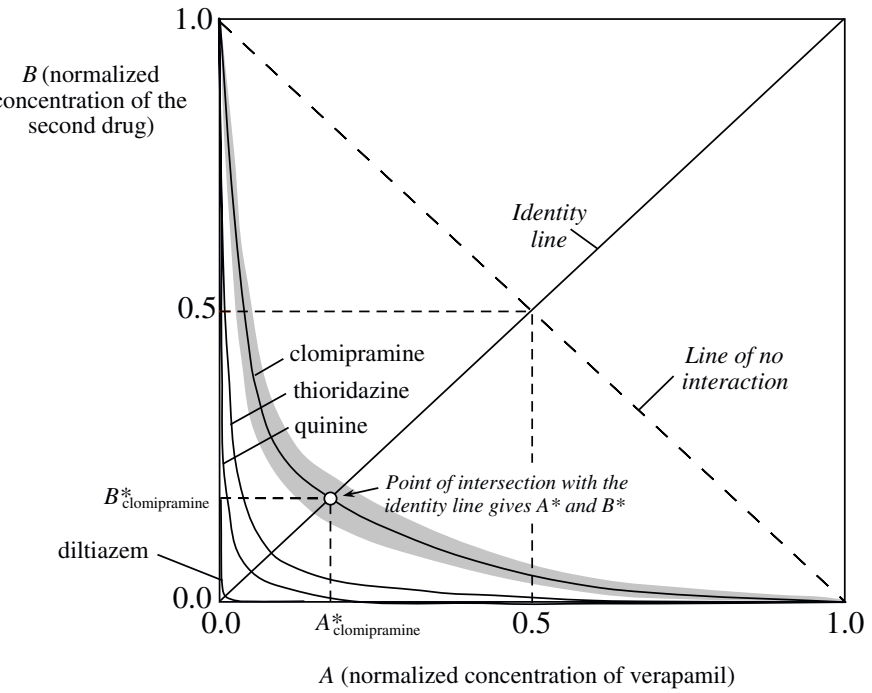

Figure 3. Isoboles for mixtures of multidrug-resistance modulators**.** The isoboles were drawn at an effect level of 50% (initial leakage rate). The abscissa and ordinate axes were respectively normalized to $A = (a/a_{0.5})^{h_A}$ and $B = (b/b_{0.5})^{h_B}$, so that equally effective doses of drugs A and B, applied singly, are equal to unity. The dotted line connecting $B = 1$ to $A = 1$ characterizes mixtures with no interaction. The identity line intersects the isoboles of the modulators at $A = A^*$ and $B = B^*$, and the line for zero interaction at $A = 0.5$ and $B = 0.5$. The isoboles were calculated from eqn (5). The triangle below the line of no interaction represents the case of synergy: note that all the curves are well within this triangle. The shaded region around the curve for verapamil with clomipramine shows the calculated standard errors of the mean.

4.4 *Dose ratio and dose factor of potentiation*

Pöch's method (Pöch 1980) for quantifying the dose factor of potentiation of synergistic drugs from isobolograms was first applied to drugs showing non-cooperative binding, i.e. $h_A = h_B = 1$. The dose ratios were defined as $DR_A = 0.5/A^*$ and $DR_B = 0.5/B^*$, the equal dose ($A = B$) inducing a given effect level being $A = 0.5$ and $B = 0.5$ for zero interacting drugs, and $A = A^*$ and $B = B^*$ for synergistic drugs, the doses A^* and B^* being determined graphically by the method illustrated for clomipramine in figure 3, and the dose factor DF value calculated as $DF = DR_A \times DR_B$. In the present study, the cooperative dose–response curves precluded this simple approach, and to allow for cooperativity the dose ratios were expressed as $DR_A = (0.5/A^*)^{1/h_A}$ and $DR_B = (0.5/B^*)^{1/h_B}$, and the A^* and B^* values were quantified mathematically. As shown in figure 3, the coordinates of the intersection point of the identity line ($A = B$) with the isoboles of the modulators correspond to $A^* + B^*$ mixtures (specified by a star for the mixture of verapamil with clomipramine), and to a 0.5 + 0.5 mixture in the case of zero interaction. The value of A^* was taken as the positive solution of the quadratic equation $\alpha A^2 + 2A - 1 = 0$ that results from substituting the identity-line condition $B = A$ into eqn (5), and then B^* could be calculated from eqn (5). The calculated values of DR_A and DR_B led to the dose factors shown in table 2: with the exception of clomipramine, the dose factors differ significantly from unity, clearly indicating synergistic interactions of verapamil with three of the other drugs within membranes, the highest synergy being with diltiazem.

5. Discussion

The object of this study was to assess interaction between multidrug-resistance modulators (Klohs *et al* 1986; Bennis *et al* 1997; Ramu *et al* 1984; Tsuruo 1983) within the membrane of anionic liposomes. Apart from the importance of these molecules in cancer therapy, they have an additional theoretical interest by reason of the high cooperativity of their dose–response curves. Their action in combination was assessed by establishing the dose–response curves of verapamil alone and in combination with several concentrations of a second modulator, diltiazem, quinine, thioridazine or clomipramine (Pöch *et al* 1990). The equation used to relate the variations in the dye leakage rate with different concentrations of verapamil and other

drugs, eqn (2), described the experimental results well over the entire range of effect levels (figure 1). The permeation parameters ($a_{0.5}$ and h) of the drugs and mixtures between them were thus quantified accurately (table 1).

The isobole method, used here to quantify the interaction between multidrug-resistance modulators (Loewe 1928), normally requires experimental data for various dose combinations at equally effective levels and, according to Sühnel (1990), obtaining these may be a laborious task. Here, however, interaction between modulators was investigated within the membrane of liposomes, a physicochemical system of limited complexity, and the dose combinations could be calculated simply at any given effect level with eqn (2) and the data of table 1. The isobologram was drawn at an effect level of 50%, with axes normalized to $A = (a/a_{0.5})^{h_A}$ and $B = (b/b_{0.5})^{h_B}$ so that equally effective doses of the drugs alone were equal to unity (Elion *et al* 1954). This normalization and the high values of h_A and h_B ($2.05 \leq h \leq 6.49$) explains the very high curvature of the isoboles seen in figure 3. This figure may be perceived as misleading with respect to both the strength of the synergy and the rank order of the synergistic mixtures (table 2), but this is inherent in the properties of the compounds studied, and a line for non-interacting drugs can only be obtained if this normalization is made. The variations of B with A are well explained by eqn (5), an equation derived here to describe the isoboles of the modulators. Note that if $\alpha = 1$ then eqn (5) describes the isobole for synergistic mutually non-exclusive drugs, according to the concept of combination index applied to these agents by Chou and Talalay (1984). In figure 3, the high error of the B values with the mixture between verapamil and clomipramine is likely to be due to the unusual shape of clomipramine, a tricyclic and nearly planar aromatic ring with an aliphatic side chain, as this structure favours its aggregation in the aqueous phase at low concentrations (Atherton and Barry 1985).

As the competition plots and isobolograms were based on the same experimental data, they did not differ in the experimental effort that they required. However, the greater computational effort needed to calculate the isobolograms reflects what would have been a difference in experimental effort if they had been from different experiments. In simple terms, the competition plot demands measurement of the actual behaviour in conditions that the simplest model predicts should produce no variation, whereas the isobole approach reverses this, as it requires experimental determination of the conditions that do in fact produce no variation. This difference exactly parallels the kinetic case: the competition plot (Chevillard *et al* 1993) looks for experimental deviations from a null expectation, whereas the constant-velocity plot (Whitehead 1984) looks for experimental conditions that give a null result.

Table 2. Dose ratios and dose factors of potentiation

Drug B	DR_A	DR_B	Dose factor
Diltiazem	2.17 ± 0.08	1.85 ± 0.06	4.02 ± 0.41
Quinine	1.66 ± 0.02	1.92 ± 0.12	3.18 ± 0.44
Thioridazine	1.49 ± 0.02	1.62 ± 0.09	2.40 ± 0.28
Clomipramine	1.35 ± 0.02	1.77 ± 0.27	2.38 ± 0.84

The dose ratios $DR_A = (0.5/A^*)^{1/h_A}$ and $DR_B = (0.5/B^*)^{1/h_B}$, and the dose factor of potentiation, calculated as $DR_A \times DR_B$, were determined from the isoboles of the modulator mixtures at an effect level of 50% (figure 3). Data are given ± SEM ($n = 4$).

The dose ratios indicate substantial potentiation of verapamil by the second modulator, the order of effectiveness being diltiazem > quinine > thioridazine > clomipramine. The rigid tricyclic rings in the chemical structures of thioridazine and clomipramine probably interfered with their insertion into the core of the membrane, decreasing their ability to interact with verapamil. The dose ratios of the second drugs, apart from clomipramine, were similar and significantly different from unity: this suggests that the ability to potentiate the effect of verapamil does not vary with the identity of the second drug unless it can aggregate in the aqueous phase (Atherton and Barry 1985).

The dose factors of potentiation within liposomal membranes prove that there is synergy between verapamil and diltiazem, quinine and thioridazine (table 2), the order of effectiveness being diltiazem > quinine > thioridazine. The effectiveness of the modulators studied, with dose factors in the range of 2.4–4.0, is similar to that of anti-tumour drugs combinations applied to various cancer cell lines, with dose factors in the range of 2.1–10.5 (Müller *et al* 1976; De Vincenzo *et al* 1996; Photiou *et al* 1997). Verapamil has already been found to increase the effects of papaverine (dose factor of 2.3) and isoprenaline (7.5) on smooth muscle (Pöch and Holzmann 1980). In addition, combinations of antimalarial drugs such as quinine are highly synergistic, with dose factors in the range of 15–36 (Rollo 1955). However, such high potentiation levels, leading to dose factors as high as 15–600, usually result from sequential blockade of steps along the same biochemical pathway (Pöch and Holzmann 1980; Rollo 1955).

It follows that there is synergy between verapamil and other multidrug-resistance modulators within model membranes. If this synergy also occurs in the membranes of cancer cells — and in view of the physical chemistry involved, and the fact that the proteins present in cell membranes but not in our models are unlikely to interact strongly with multidrug-resistance molecules, which are highly lipophilic, there is little reason to expect otherwise— then a promising approach to combat multidrug resistance will be the clinical use of combinations of modulators such as verapamil and diltiazem that exhibit a greater effect together than that expected from study of the single agents.

We now examine the extent to which results with model membranes shed light on the problem of drug efflux from cancer cells. The ideal would, of course, be to carry out exact measurements on resistant cancer cells under reproducible conditions, but biological systems are too complex for such an approach to yield all the answers. Model membranes provide reproducibility and greater precision at the cost of moving further from pathological conditions. Their use cannot prove what happens in cancer treament, but it can suggest hypotheses that ought to be testable in clinical studies. To justify this point of view it may be helpful to describe briefly the principle of the dye-leakage method: an impermeable dye is trapped inside the liposomes, and dye efflux follows the partitioning of a multidrug-resistance modulator into the lipids if the strength of the modulator-membrane interaction is sufficient. The dye efflux does not, in any case, mimic the efflux of the modulator tested or of an anticancer agent. Our aim was to show that there may be synergy between verapamil and other modulators within model membranes, and the synergy that we have shown to exist in this simple system suggests that it may also exist in the lipid phase of multidrug-resistant cells.

The basic assumption of a relationship between partitioning of drugs in model membranes and similar effects *in vivo* is well accepted. Most modulators are highly lipophilic, and Seydel *et al* (1994) have speculated that interactions between these compounds and the membrane phospholipids might contribute to the mechanism underlying multidrug-resistance modulation. Pajeva *et al* (1996) suggest that drug-membrane interactions may lead to the reversal of multidrug resistance, either directly by changing the membrane fluidity and permeability, or indirectly by modifying the structural organization of the lipids surrounding embedded proteins, thus changing their functional modes. Moreover, Drori *et al* (1995) have found a correlation between the ability of modulators (including verapamil and quinine) to induce dye leakage through the membrane of liposomes and their capacity to potentiate trimetrexate cytotoxicity in multidrug-resistant T19 cells, suggesting that cytotoxicity potentiation by various modulators may involve an increase in membrane permeability to a variety of anticancer cytotoxic agents. Like various mild detergents, modulators induced a dose-dependent dye leakage of carboxyfluorescein encapsulated in liposomes. In addition, like specialized membrane fluidizers, various modulators induced a dose-dependent membrane fluidization, regardless of the lipid-bilayer source used (artificial liposomes, canalicular membrane vesicles or various wild-type and multidrug-resistant animal and human cells). The increased permeability induced by modulators favoured the passive entry of anticancer agents into sensitive or resistant cancer cells, the active efflux of these compounds (modulators and anticancer agents) being mediated by the membrane protein P-glycoprotein.

Although the model membranes used here differ from cancer cells in their lipid composition, they offer a reasonable model in terms of physical chemistry. Biological membranes are essentially composed of phospholipids, cholesterol and embedded proteins, and can be considered as hydrophobic barriers separating two aqueous phases. The presence of proteins does not greatly affect the interpretation, because lipophilic amphiphiles such as modulators primarily interact with the lipid phase (head groups of the phospholipids and their acyl chains, cholesterol, and so on).

Pajeva *et al* (1996) have likewise stressed that the ability of modulators to reverse resistance in tumour cells is likely to be mediated through their ability to interact with the membrane phospholipids. They concluded from their results that modulators within artificial membranes composed of neutral and negatively charged phospholipids provided a convenient model for screening compounds for their potential ability to reverse multidrug-resistance in tumour cells.

The relatively high drug:lipid molar ratios used in the study suggest weak interactions between the drug and lipid, and indeed, Pajeva *et al* (1996) stressed the moderate nature of the membrane interaction with verapamil; they showed that membrane interactions with verapamil occurred at drug:lipid molar ratios varying from 0.05:1 to 0.40:1. In the present study, this ratio amounted to 0.21–0.25:1 at the a_{50} of verapamil, a result in agreement with the data of these authors. At the a_{50} of the modulators, the drug:lipid ratio ranged here from 0.21:1 to 0.78:1. The highest value of 1.7:1 used in our experiments was not typical, being used only for clomipramine when inducing 90% dye leakage in 3 min, which was made necessary by the aggregation of clomipramine in the aqueous phase. It may also be noted that some drug:lipid ratios used here had high values because of the requirement for all the rates measured to correspond to initial rates, regardless of the modulator and of the leakage level (0–90%).

Drug-membrane interactions are controlled by numerous physico-chemical parameters related to the properties (i) of the modulator (partition coefficient or distribution coefficient, electric charge, size and shape, etc.), (ii) of the membrane (lipid composition, chain length and degree of saturation of the phospholipids, presence of cholesterol, which modifies the dipolar potential of the membrane, membrane surface potential, etc.), and (iii) of the aqueous phases surrounding the membrane (ionic strength, pH, temperature, etc.). We believe that measurements of the initial rates of dye efflux from liposomes offer the simplest way of quantifying the combined effects of all of these parameters. As we have shown, this method is quite capable of demonstrating the existence of synergistic interactions between modulators.

Experiments with lipid vesicles do not of course prove beyond any doubt that similar behaviour occurs in cancer

cells, but they provide a useful indication. Given that all biological membranes (including cancer cells and multidrug resistant cancer cells) contain phospholipids, it seems justifiable to hypothesize that synergy between verapamil and other modulators may also occur in resistant tumour cells.

Acknowledgements

We thank Professors J A Rodriguez and J Barbe for their encouragement during this work, and we thank Dr M. L. Cárdenas for many helpful comments on the manuscript.

References

Atherton A D and Barry B W 1985 Photon correlation spectroscopy of surface active cationic drugs; *J. Pharm. Pharmacol.* **37** 854–862

Bennis S, Faure P, Chapey C, Hu Y P, Fourche J, El Yamani J and Robert J 1997 Cellular pharmacology of lipophilic anthracyclines in human tumor cells in culture selected for resistance to doxorubicin; *Anti-Cancer Drugs* **8** 610–617

Berenbaum M C 1989 What is synergy? *Pharmacol. Rev.* **41** 93–141

Cárdenas M L 2001 The competition plot: a kinetic method to assess whether an enzyme that catalyzes multiple reactions does so at a unique site; *Methods* **24** 175–180

Castaing M, Brouant P, Loiseau A, Santelli-Rouvier C, Santelli M, Alibert-Franco S, Abdallah A and Barbe J 2000 Membrane permeation by MDR-modulators and non-modulators: effects of hydrophobicity and electric charge; *J. Pharm. Pharmacol.* **52** 289–296

Castaing M Loiseau A and Djoudi L 2003 Effects of cholesterol on dye leakage induced by multidrug-resistance modulators from anionic liposomes; *Eur. J. Pharm. Sci.* **18** 81–88

Castaing M, Loiseau A and Mulliert G 2005 Multidrug resistance modulator interactions with neutral and anionic liposomes: membrane binding affinity and membrane perturbing activity; *J. Pharm. Pharmacol.* **57** 547–554

Chevillard C Cárdenas M L and Cornish-Bowden A 1993 The competition plot: a simple test of whether two reactions occur at the same active site; *Biochem. J.* **289** 599–604

Chou T-C and Talalay P 1984 Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors; *Adv. Enzyme Regul.* **22** 27–55

Cortés A, Cascante M, Cárdenas M L and Cornish-Bowden A 2001 Relationships between inhibition constants, inhibitor concentrations for 50% inhibition and types of inhibition: new ways of analysing data; *Biochem. J.* **357** 263–268

De Vincenzo R, Scambia G, Benedetti Panici P, Fattorossi A, Bonanno G, Ferlini C, Isola G, Pernisco S and Mancuso S 1996 Modulatory effect of tamoxifen and ICI 182,780 on adriamycin resistance in MCF-7 human breast-cancer cells; *Int. J. Cancer* **68** 340–348

Drori S, Eytan G D and Assaraf Y G 1995 Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability; *Eur. J. Biochem.* **228** 1020–1029

Elion G B, Singer S and Hitchings G H 1954 Antagonists of nucleic acid derivatives VIII. Synergism in combinations of biochemically related antimetabolites; *J. Biol. Chem.* **208** 477–488

Ferreira M J, Gyemant N, Madureira A M, Tanaka M, Koos K, Didziapetris R and Molnar J 2005 The effects of jatrophane derivatives on the reversion of MDR1- and MRP-mediated multidrug resistance in the MDA-MB-231 (HTB-26) cell line; *Anticancer Res.* **25** 4173–4178

Higgins C F and Gottesmann M M 1992 Is the multidrug transporter a flippase?; *Trends Biochem. Sci.* **17** 18–21

Klohs W D, Steinkampf R W, Havlick M J and Jackson R C 1986 Resistance to anthrapyrazoles and anthracyclines in multi-resistant P388 murine leukemia cells: reversal by calcium blockers and calmodulin antagonists; *Cancer Res.* **46** 4352–4356

Loewe S 1928 Die quantitativen Probleme der Pharmakologie; *Ergebn. Physiol.* **27** 47–187

Lugo M R and Sharom F J 2005 Interaction of LDS-751 and rhodamine 123 with P-glycoprotein: evidence for simultaneous binding of both drugs; *Biochemistry* **44** 14020–14029

Müller W E G, Rohde H J, Steffen R, Maidhof A and Zahn R K 1976 Potentiation of the effectiveness of bleomycin by AT-specific DNA ligands *in vitro* as well as *in vivo*; *Cancer Lett.* **1** 127–132

Pajeva I K, Wiese M, Cordes H P and Seydel J K 1996 Membrane interactions of some catamphiphilic drugs and relation to their multidrug-resistance-reversing ability; *J. Cancer Res. Clin. Oncol.* **122** 27–40

Pajeva I, Todorov D K and Seydel J 2004 Membrane effects of the antitumor drugs doxorubicin and thaliblastine: comparison to multidrug resistance modulators verapamil and *trans*-flupentixol; *Eur. J. Pharm. Sci.* **21** 243–250

Photiou A, Shah P, Leong L K, Moss J and Retsas S 1997 *In vitro* synergy of paclitaxel (taxol) and vinorelbine (navelbine) against human melanoma cell lines; *Eur. J. Cancer* **33** 463–470

Pöch G 1980 Dose factor of potentiation derived from isoboles; *Arzneim-Forsch/Drug Res.* **30** 2195–2196

Pöch G, Dittrich P, Reiffenstein R J, Lenk W and Schuster A 1990 Evaluation of experimental combined toxicity by use of dose–frequency curves: comparison with theoretical additivity as well as independence; *Can. J. Physiol. Pharmacol.* **68** 1338–1345

Pöch G and Holzmann S 1980 Quantitative estimation of overadditive and underadditive drug effects by means of theoretical, additive dose–response curves; *J. Pharmacol. Methods* **4** 179–188

Qu Q, Chu J W and Sharom F J 2003 Transition state P-glycoprotein binds drugs and modulators with unchanged affinity, suggesting a concerted transport mechanism; *Biochemistry* **42** 1345–1353

Ramu A, Spanier R, Rahamimoff H and Fuks Z 1984 Restoration of doxorubicin responsiveness in doxorubicin-resistant P388 murine leukaemia cells; *Br. J. Cancer* **50** 501–507

Rollo I M 1955 The mode of action of sulphonamides, proguanil and pyrimethamine on *Plasmodium gallinaceum; Brit. J. Pharmacol.* **10** 208–214

Seydel J K, Coats E A, Cordes H P and Wiese M 1994 Drug membrane interaction and the importance for drug transport, distribution, accumulation, efficacy and resistance; *Arch. Pharm. (Weinheim)* **327** 601–610

Sharom F J 1997 The P-glycoprotein efflux pump: how does it transport drugs? *J. Membrane Biol.* **160** 161–175

Sühnel J 1998 Parallel dose–response curves in combination experiments; *Bull. Math. Biol.* **60** 197–213

Sühnel J 1990 Evaluation of synergism and antagonism for the combined action of antiviral agents; *Antivir. Res.* **13** 23–40

Tsuruo T 1983 Reversal of acquired resistance to Vinca alkaloids and anthracycline antibiotics; *Cancer Treat. Rep.* **67** 889–894

Whitehead E P 1984 Subunit interactions and enzyme kinetics: the linkage approach; in *Dynamics of Biochemical Systems* (ed) J Ricard and A Cornish-Bowden (New York and London: Plenum Press) pp 45–64

MS received 7 October 2006; accepted 31 January 2007

*e*Publication: 17 April 2007

Corresponding editor: Amit Chattopadhyay