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Interaction of Combinations of Drugs, Chemosensitizers, and Peptides with the P-Glycoprotein Multidrug Transporter

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ABSTRACT. P-Glycoprotein functions as an ATP-driven efflux pump for hydrophobic natural products and peptides, and gives rise to resistance to multiple chemotherapeutic drugs. The inhibition of colchicine transport via P-glycoprotein by various compounds was determined in a plasma membrane vesicle model system. A chemotherapeutic drug (vinblastine) and several chemosensitizers (verapamil, reserpine, cyclosporin A) and hydrophobic peptides (*N*-acetyl-leucyl-leucyl-methioninal, leupeptin, pepstatin A, valinomycin) were examined, both as individual species and as combinations of compounds. The median effect analysis was used to determine the concentration of each combination required to produce a median effect, D_m, as well as the sigmoidicity of the concentration–effect plot, m. The combination of cyclosporin A and verapamil was the only one established to be mutually nonexclusive, whereas several mutually exclusive pairs of compounds were identified. The combination index, CI, was calculated for several combinations of drugs, chemosensitizers, and peptides, and used to ascertain whether effects were synergistic, antagonistic, or additive. Some combinations (vinblastine/verapamil; verapamil/valinomycin) showed antagonism over the entire concentration range. Other combinations (valinomycin/*N*-acetyl-leucyl-leucyl-methioninal; cyclosporin A/verapamil) displayed both synergism and antagonism over different regions of the CI plot. Many combinations of compounds displayed additive interactions over most of the CI plot. The median effect analysis may be helpful in identifying potentially useful additive or synergistic combinations of compounds for reversal of Pgp-mediated drug resistance. BIOCHEM PHARMACOL 53;12:1789–1797, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance; plasma membrane vesicles; drug transport; median effect analysis; synergism; antagonism

Many human tumours are either intrinsically resistant to a number of chemotherapeutic drugs or develop such resistance after one or more rounds of treatment. Clinically, resistance to multiple drugs presents a serious barrier to curing cancers. A major cause of MDR† is the overexpression of a 170-kDa integral membrane glycoprotein, known as Pgp. Pgp is a member of the ABC (ATP-binding cassette) superfamily of membrane proteins [1, 2] and, based on its close resemblance to other members of this group of proteins, is proposed to operate as an energy-dependent drug exporter, powered by ATP hydrolysis (for reviews, see Refs. 3–5). Our understanding of MDR at the molecular level has been greatly improved by the development of *in vitro* models displaying this phenotype. MDR cells expressing high levels of Pgp have provided the starting material for purification and subsequent biochemical study of the transporter. Our laboratory has made extensive use of plasma membrane vesicles from MDR cells, and reconstituted proteoliposomes containing purified Pgp, to demonstrate that Pgp is indeed an active drug transporter, energized by ATP hydrolysis, which generates a drug gradient across the membrane bilayer [6–8]. In addition, Pgp substrates have been shown to compete with each other for transport, and either stimulate or inhibit Pgp ATPase activity in vesicle model systems. Various peptides and ionophores also interact with Pgp to inhibit drug transport and increase ATPase activity [9–11]. Recent work in our laboratory has shown that, like chemotherapeutic drugs, linear hydrophobic peptides are actively transported by Pgp [12].

The action of Pgp may be blocked by certain compounds known as MDR chemosensitizers, reversers, or modulators [13–15]. There is much interest in the use of chemosensitizers clinically, in combination with standard chemotherapy regimens, to circumvent the effects of Pgp [16–18]. Although initial results have been encouraging, there is a need for new regimens with improved efficacy and lower toxicity. At the biochemical level, chemosensitizers are observed to inhibit drug transport via Pgp and greatly stimulate the ATPase activity (see, for example, Refs. 6–9 and 19). They appear to compete with drugs, although in a

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†*Abbreviations*: ALLM, *N*-acetyl-leucyl-leucyl-methioninal; MDR, multidrug resistance; and Pgp, P-glycoprotein.

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complex and poorly understood way, for the drug-binding site(s) on the transporter.

One of the major obstacles that has emerged in the testing of various chemosensitizers in a clinical setting has been that of toxicity [13–18]. One potential solution to this problem is the development of new chemosensitizers that are more effective and less toxic. Recent work in our laboratory has shown that certain hydrophobic peptides, both linear (Sarkadi B and Sharom FJ, unpublished observations) and cyclic (Lu P and Sharom FJ, unpublished observations), are excellent chemosensitizers in intact cell systems. Hydrophobic peptides are relatively nontoxic, and therefore this newly identified class of Pgp substrates may offer the possibility of lower toxicity when used as chemosensitizers. Another approach to MDR reversal, which aims to circumvent toxicity, is the use of combinations of chemosensitizers, each at concentrations below their toxic level [20]. This strategy has been employed previously to inhibit replication of drug-resistant human HIV-1 virus isolates [21]. The use of chemosensitizer combinations with additive interactions is satisfactory if the toxicity of the combination is significantly lower than that of the individual drugs, at the dose required to reverse resistance. Clearly, it would be advantageous to identify drug combinations whose effects reinforce each other, i.e. are synergistic, rather than being simply additive. However, antagonistic combinations of compounds will obviously not be suitable for clinical use.

The absence of any rigorous kinetic information for either binding or transport of drugs and chemosensitizers is a major obstacle to gaining a better understanding of the way in which drugs and chemosensitizers interact with Pgp. We recently demonstrated that the median effect analysis can be used successfully to analyze equilibrium drug uptake by Pgp in a plasma membrane vesicle model system [9]. The present study explores the application of the median effect analysis to inhibition of Pgp-mediated colchicine transport by various combinations of drugs, chemosensitizers, and hydrophobic peptides, compounds that are (individually) substrates for the multidrug transporter. We show that the median effect analysis can be used to identify mutually exclusive and mutually nonexclusive combinations of compounds. The use of several combinations of peptides with conventional chemosensitizers of other structural classes was also evaluated for synergism, antagonism, or additivity.

MATERIALS AND METHODS

Drugs, Chemosensitizers, and Peptides

Colchicine, vinblastine, verapamil, reserpine, ALLM, leupeptin, pepstatin A, and valinomycin were purchased from the Sigma Chemical Co. (St. Louis, MO). Cyclosporin A was a gift of Pfizer Central Research (Groton, CT).

Isolation of Plasma Membrane Vesicles from MDR Cells

The colchicine-selected MDR cell line CH^RC5 (96-fold resistant) has been described previously [22]. Cells were maintained at 37° in a humidified atmosphere of 5% CO_2 in α-minimal essential medium (α-MEM) (Canadian Life Technologies, Burlington, Ontario) supplemented with 10% heat-inactivated bovine calf serum (defined/supplemented, Hyclone Laboratories, Logan, UT), penicillin (1000 U/mL), streptomycin (1 mg/mL), and 2 mM L-glutamine (all from Canadian Life Technologies). Cells were grown to confluence in tissue culture roller bottles and harvested by trypsinization. Cells were stored at −70° in 10% DMSO/α-MEM prior to use. Plasma membrane vesicles from CH^RC5 cells were isolated using nitrogen cavitation, followed by centrifugation over a sucrose cushion, as described previously [6, 23]. Plasma membrane was stored in aliquots at −70° for no more than 3 months before use.

Colchicine Transport Measurements

Colchicine transport measurements were carried out by modification of a method described previously [6, 9, 12]. ATP and an ATP-regenerating system (final concentrations: 1 mM ATP, 30 μg/mL creatine kinase, and 3 mM creatine phosphate) were combined with 0.3 μCi [^{3}H]colchicine (DuPont Canada, Mississauga, Ontario) in a total volume of 90 μL of transport buffer (10 mM Tris-HCl, 0.25 M sucrose, 5 mM $MgCl_2$, pH 7.4). The final colchicine concentration was 1 μM. Transport was initiated by the addition of a 10-μL aliquot in transport buffer of CH^RC5 plasma membrane vesicles, containing 25–35 μg protein as determined by the Bradford protein assay [24]. After 30 min at 23°, transport was terminated by rapid filtration of the entire reaction mixture through a Whatman GF/F filter using a Hoeffer filtration manifold, followed immediately by a wash with 5 mL of ice-cold transport buffer. Filters were oven-dried, and counted for [^{3}H] by liquid scintillation counting. Non-specific [^{3}H]colchicine binding to the filter and the membrane vesicles was determined in the absence of membrane vesicles, and in the absence of the ATP and a regenerating system, respectively. All transport measurements were carried out in triplicate. The compounds used in this work were prepared as stock solutions in DMSO, and controls contained the appropriate DMSO concentration, which never exceeded 0.2% (v/v). This level of DMSO had no inhibitory effects on ATP-dependent colchicine transport.

Median Effect Analysis

The median effect analysis was developed by T-C. Chou in 1976 [25]. The median effect equation [26, 27] permits the experimenter to quantitate the relationship between any concentration of a compound, and its effect on the system under study. In this case, the inhibition of [^{3}H]colchicine

transport into CH^RC5 plasma membrane vesicles was measured at various concentrations of the test compound(s), which included drugs, chemosensitizers, and peptides. The basic median effect equation is as follows:

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m$$

where f_a = fraction of the system that is affected at a concentration D (in this case, the fractional inhibition of equilibrium colchicine uptake); f_u = fraction of the system that is unaffected at concentration D; D_m = the compound concentration causing 50% inhibition; and m = parameter indicating the sigmoidicity of the concentration–effect curve. Note that both f_a and f_u can vary between 0 and 1, and $f_a = (1 - f_u)$.

One transformation of the median effect equation is as follows:

$$\log\left(\frac{f_a}{f_u}\right) = m\log D - m\log D_m$$

A plot of log (f_a/f_u) versus log D generates a straight line with slope m, and an x-intercept equalling log D_m, and is designated as a median effect plot. D_m and m values for individual compounds and various combinations were determined from these plots, which were fitted to straight lines by linear regression analysis. The correlation coefficient (r) determined from linear regression analysis need only exceed 0.95 for the median effect analysis to be applicable.

To display and compare median effect plots for two different compounds with highly dissimilar D_m values, together with the plot for their combination, we normalized the median effect plots to correct for different D_m values. In a normalized median effect plot, a plot of log (f_a/f_u) versus log (D/D_m) for each compound and combination produces a straight line with slope m, with all plots passing through the (0, 0) point.

$$\log\left(\frac{f_a}{f_u}\right) = m\log\left(\frac{D}{D_m}\right)$$

Three sets of measurements were utilized for analysis of the relationship between the effects of two agents used in combination; the median effect plots for each individual compound, and for a combination of the two compounds at a fixed ratio, leading to the determination of m_1 (for compound 1), m_2 (for compound 2), and m_{12} (for the combination of compounds 1 and 2). The exclusivity of drug effects was examined by comparing the m values calculated from the three measurements. There were three possibilities: (i) if $m_1 = m_2 = m_{12}$, then the effects of the two compounds were mutually exclusive; (ii) if $m_1 = m_2 \neq m_{12}$, then the effects were mutually nonexclusive; and (iii) if $m_1 \neq m_2$, then the exclusivity of the effects could not be determined.

To evaluate whether the effects of two compounds were synergistic, antagonistic, or additive, the median effect parameters and the exclusivity of drug effects were employed. For any given concentration of a compound, a comparison of the experimentally determined effect and the theoretical calculated effect yielded a ratio, whose value is termed the combination index, CI [26, 27]. In cases where combinations were shown previously to be either mutually exclusive or mutually nonexclusive, the appropriate equation for the CI was selected. In cases where the exclusivity of the interaction could not be determined (since the m values for individual compounds were not the same), the CI was calculated using both equations [25], and two lines are shown in the CI plot; in all cases, similar plots were obtained. The CI value was calculated for the entire range of f_a values for each combination of compounds, using an iterative algorithm.

RESULTS

Inhibition of Colchicine Transport by Single Agents

Previous work in our laboratory has shown that equilibrium uptake of [^{3}H]colchicine into plasma membrane vesicles from the MDR Chinese hamster ovary cell line CH^RC5 is inhibited in a concentration-dependent manner by various Pgp substrates [6, 9]. Such transport inhibition data cannot be analyzed by enzyme kinetic models, since it represents equilibrium uptake, rather than initial rates of transport. However, we recently showed that the median effect analysis could be used to evaluate equilibrium uptake data of this type [9]. The fractional inhibition of ATP-dependent colchicine accumulation via Pgp was taken as the parameter f_a, and a median effect plot of log (f_a/f_u) versus log D was constructed for each inhibitor species. The data for many different compounds, including chemotherapeutic drugs, chemosensitizers, hydrophobic peptides, and ionophores, fitted well to the median effect equation. Further analysis led to the estimation of values for D_m (the concentration inhibiting transport by 50%; a useful indicator of relative affinity) and m (analogous to the Hill number; a measure of the sigmoidal nature of the interaction) for individual species (see Table 1). D_m values for the individual compounds ranged from very low, in the submicromolar range (vinblastine, cyclosporin A), to relatively high micromolar (ALLM, leupeptin), representing the differences between high affinity and low affinity substrates, respectively. The m values for the various agents were close to either 1 or 3.

Inhibition of Colchicine Transport by Combinations of Two Agents

The median effect approach was extended to several combinations of Pgp substrates, including a chemotherapeutic drug (vinblastine), chemosensitizers (verapamil, reserpine, cyclosporin A), and hydrophobic peptides (valinomycin, ALLM, leupeptin, pepstatin A). Compounds were com-

TABLE 1. Median effect parameters for various compounds and their combinations, describing their ability to inhibit colchicine transport by Pgp

	D_m (μM)	m	m_{12}	Ratio*
Single compounds†				
Vinblastine	0.55	1.1		
Verapamil	10	2.9		
Reserpine	1.0	1.1		
Cyclosporin A	0.7	3.0		
Valinomycin	4.0	2.9		
ALLM	130	1.3		
Leupeptin	80	1.0		
Pepstatin A	14	1.3		
Combinations of compounds				
Vinblastine/verapamil	13 ± 5		1.3 ± 0.2	1:7
Vinblastine/reserpine	0.8 ± 0.3		1.2 ± 0.2	1:1
Vinblastine/pepstatin A	12.5 ± 0.7		1.2 ± 0.2	1:20
Vinblastine/valinomycin	2.5 ± 0.7		1.7 ± 0.4	1:3
Verapamil/valinomycin	15 ± 7		2.7 ± 1.7	5:1
Verapamil/leupeptin	90 ± 10		1.3 ± 0.3	1:10
Verapamil/reserpine	5.0 ± 3		1.6 ± 0.1	7:1
Cyclosporin A/verapamil	4.0 ± 0.05		1.5 ± 0.1	1:7
Cyclosporin A/reserpine	1.0 ± 0.05		1.7 ± 0.2	1:1
Reserpine/leupeptin	55 ± 30		0.8 ± 0.3	1:89
Valinomycin/ALLM	65 ± 0.05		2.2 ± 0.1	1:200
Leupeptin/ALLM	100 ± 40		1.0 ± 0.03	1:1

ATP-dependent transport of [^{3}H]colchicine into CH^{R}C5 plasma membrane vesicles was determined in the presence of increasing concentrations of various combinations of drugs, chemosensitizers, and peptides, and the transport inhibition data were evaluated using the median effect analysis. D_m and m_{12} values in the "Combinations of compounds" group represent the means ± SD for 3 independent experiments.

* Compounds were combined in ratios approximating the ratio of their individual D_m values.

† Values taken from Ref. 9.

bined in ratios appropriate for their relative D_m values, and the combination was tested for its ability to inhibit ATP-dependent colchicine uptake via Pgp at escalating concentrations. Transport inhibition data for four representative compound combinations are shown in Fig. 1 (A–D), and represent the results of several independent experiments for each. All combinations were effective at blocking Pgp transport activity, with maximal transport inhibition of >90%.

Median Effect Analysis

Combination transport inhibition data of the type shown in Fig. 1 were then transformed into median effect plots. Figure 2 (A–D) presents such plots for the four combinations shown in Fig. 1, together with the median effect plots for each component of the combinations tested individually. Since the D_m values for the individual components of some of the combinations are very different in magnitude, the median effect plots were normalized to allow easier comparison. In a normalized median effect plot, a plot of log (f_a/f_u) versus log (D/D_m) for each compound and their combination produces straight lines with slope m, passing through the origin.

The D_m and m_{12} values for a total of twelve combinations were determined (see Table 1). For three of the combination plots displayed in Fig. 2 (B, reserpine/leupeptin; C, vinblastine/reserpine; and D, vinblastine/pepstatin A), the combined median effect plot (closed circles) was superimposable on the median effect plots for the individual components (open symbols), and the slope of the combination plot (m_{12}) was very similar to those of the individual plots (m_1, m_2) (Table 1). However, for the combination shown in Fig. 2A (cyclosporin A/verapamil), the combined plot was nonsuperimposable on the plots for the individual compounds, and the slope of the combined plot was clearly different; in this case, $m_{12} = 1.5$, whereas the m values for each of the two agents separately were close to 3.

For those six combinations where the m values for the two individual agents were similar (Groups 1 and 2, Table 2), the exclusivity of their interactions was determined, by comparison with the m_{12} value for their combination. The various combinations of agents were categorized into three groups (see Table 2). For five of these combinations, $m_{12} \cong m_1 \cong m_2$ (Group 1 in Table 2), indicating that the interactions of the two agents with Pgp are mutually exclusive, i.e. their effects on Pgp are similar, and not independent of each other. It should be noted that the actual values of m_1, m_2, and m_{12} for these combinations varied from close to 1 (vinblastine/reserpine, vinblastine/pepstatin A, reserpine/leupeptin, leupeptin/ALLM) to close to 3 (verapamil/valinomycin). Group 2 consists of a single combination (cyclosporin A/verapamil) where $m_{12} \neq m_1 \cong m_2$, which is apparent from examination of Fig. 2A. This combination is therefore mutually nonexclusive, which indicates that the effects of the two compounds on Pgp are different, and independent of each other. Group 3 in Table 2 comprises combinations whose exclusivity could not be established, since the m values of the individual

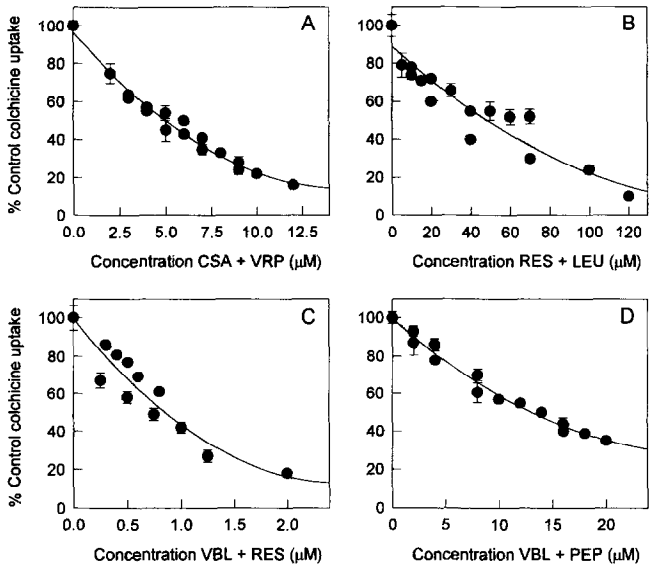

FIG. 1. Inhibition of [^{3}H]colchicine uptake into CH^{R}C5 plasma membrane vesicles by various combinations of drugs, chemosensitizers, and peptides. (A) Cyclosporin A (CSA)/verapamil (VRP); (B) reserpine (RES)/leupeptin (LEU); (C) vinblastine (VBL)/reserpine; (D) vinblastine/pepstatin A (PEP). The data points shown represent means ± SEM for triplicate determinations, and are compiled from several independent experiments using different batches of plasma membrane. The concentration ratios for the various combinations are given in Table 1.

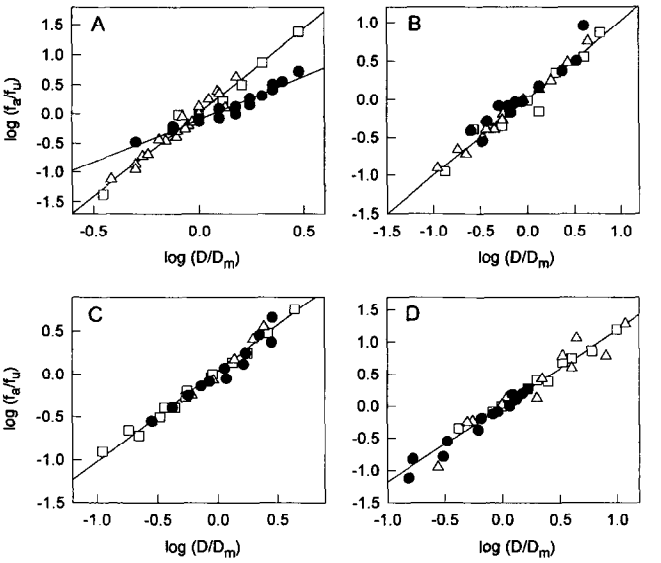

FIG. 2. Normalized median effect plots for drugs, chemosensitizers, and peptides, used alone and in combination. (A) Cyclosporin A (□), verapamil (△), and cyclosporin A/verapamil (●), a mutually nonexclusive combination; (B) reserpine (□), leupeptin (△), and reserpine/leupeptin (●); (C) vinblastine (□), reserpine (△), and vinblastine/reserpine (●); and (D) vinblastine (■), pepstatin A (△), and vinblastine/pepstatin A (●). (B), (C), and (D) are all mutually exclusive combinations. The concentration ratios for the various combinations are given in Table 1.

agents were not similar. There was no apparent correlation between the chemical class(es) of compounds used in combination and their exclusivity.

Analysis of Synergism and Antagonism

To determine the concentration ranges over which the various combinations of compounds tested displayed synergism, antagonism, or additivity, further analysis was carried out using combination index plots. Effects were considered to be strongly synergistic if $CI < 0.8$, and strongly antagonistic if $CI > 4$. At values of CI between these two limits, the effect of a compound combination was considered to be approximately additive. Several different patterns were observed for the CI plots for the twelve combinations tested, which are shown in Figs. 3 and 4. The combinations of vinblastine/verapamil and verapamil/valinomycin, presented in the left-hand panels of Fig. 3, were the only two to show antagonism over the entire range of f_a values. The combinations of cyclosporin A/verapamil and valinomycin/ALLM (right-hand panels of Fig. 3) displayed both synergistic and antagonistic regions, depending on the value of f_a. The two curves were almost mirror images of each other, with strong synergism at low f_a values and strong antagonism at high f_a values for cyclosporin A/verapamil, and weak antagonism at low f_a values and strong synergism at high f_a values for valinomycin/ALLM. The other eight combinations (shown in Fig. 4) showed approximately additive effects ($0.8 < CI < 4$) over most of the range of f_a, except for some combinations that showed antagonism at the extremes of the plot (reserpine/leupeptin, vinblastine/valinomycin, verapamil/leupeptin).

TABLE 2. Exclusivity of interactions between various drugs, chemosensitizers, and peptides in their ability to inhibit colchicine transport by Pgp

Compound combination	Exclusivity of drug effects	
	Mutually exclusive	Mutually nonexclusive
Group 1		
Vinblastine/reserpine	+	
Vinblastine/pepstatin A	+	
Verapamil/valinomycin	+	
Reserpine/leupeptin	+	
Leupeptin/ALLM	+	
Group 2		
Cyclosporin A/verapamil		+
Group 3		
Vinblastine/verapamil	±	±
Vinblastine/valinomycin	±	±
Verapamil/leupeptin	±	±
Verapamil/reserpine	±	±
Cyclosporin A/reserpine	±	±
Valinomycin/ALLM	±	±

Combinations of compounds with similar m values (see Table 1) were defined as mutually exclusive (Group 1) if $m_{12} \cong m_1 \cong m_2$, and as mutually nonexclusive (Group 2) if $m_{12} \neq m_1 \cong m_2$. The exclusivity of interactions could not be determined for Group 3 combinations (indicated as ±), since the m values of the individual compounds were not similar.

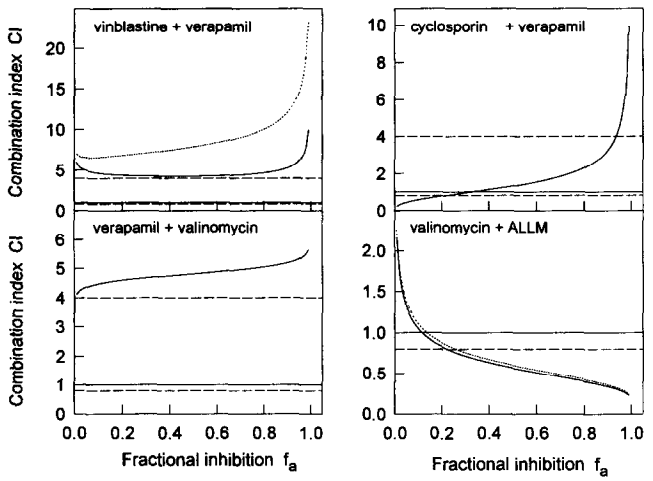

FIG. 3. Combination index plots for four combinations of drugs, chemosensitizers, and peptides. A solid horizontal line marks CI = 1.0, and two dashed horizontal lines define the boundaries of synergism (CI < 0.8) and antagonism (CI > 4). The left panels present two Group 1 combinations that display antagonism over the entire plot. The right panels show two combinations in which both synergism and antagonism are present in different regions of the plot. In those cases where it was not possible to use the median effect analysis to determine the exclusivity of the interactions between compounds, calculations were carried out assuming both mutually exclusive interactions (solid curve) and mutually nonexclusive interactions (dotted curve). The concentration ratios for the various combinations are given in Table 1.

DISCUSSION

The use of plasma membrane vesicles derived from MDR cells to study the interaction of various agents and agent combinations with Pgp offers several advantages over whole cells. Intact cells are complex systems in which drug metabolism, drug sequestration, and specific drug binding to intracellular targets may either alter Pgp–drug interactions, or lead to indirect changes in Pgp transport activity. In addition, other mechanisms of drug resistance not mediated at the level of the plasma membrane (e.g. overexpression of glutathione-*S*-transferases) may be present in the drug-selected lines usually used in intact cell studies, making it difficult to draw firm conclusions. Plasma membrane vesicles derived from MDR cells overexpressing Pgp represent a simple model system that avoids these added complications.

In this study, we have used uptake of colchicine by plasma membrane vesicles from the MDR cell line CH^RC5 for assessing interactions of various substrates and chemosensitizers with the multidrug transporter. The CH^RC5 vesicle system represents one of the best characterized models for measuring active transport by Pgp *in vitro*; the sidedness of the vesicles is known, drug uptake has been shown to represent true transport into the vesicle lumen, transport is active and generates a substrate concentration gradient, and transport is dependent on ATP hydrolysis [6, 12]. We previously characterized active transport of both colchicine and vinblastine using the CH^RC5 vesicle system [6]. Colchicine has been the drug substrate of choice for accurate transport measurements, since it is substantially less hydrophobic than vinblastine, and nonspecific colchicine uptake in the absence of ATP (basically "sticking" of the drug to the membrane) is much lower than for vinblastine [6]. The use of this single drug has the advantage of providing large amounts of correlated transport data [6, 7, 9, 12].

Compounds that interact with Pgp can be identified by their ability to inhibit the ATP-driven uptake of colchicine into CH^RC5 plasma membrane vesicles [6, 9, 12]. We have shown that such inhibitors act by one of two mechanisms; either they interact with Pgp directly, and thus compete for transport of the drug, or they may prevent drug accumulation in the vesicle lumen by permeabilizing the membrane bilayer [9]. Some membrane-active peptides, which do not interact specifically with Pgp, were found to disrupt colchicine transport by the latter mechanism. However, MDR

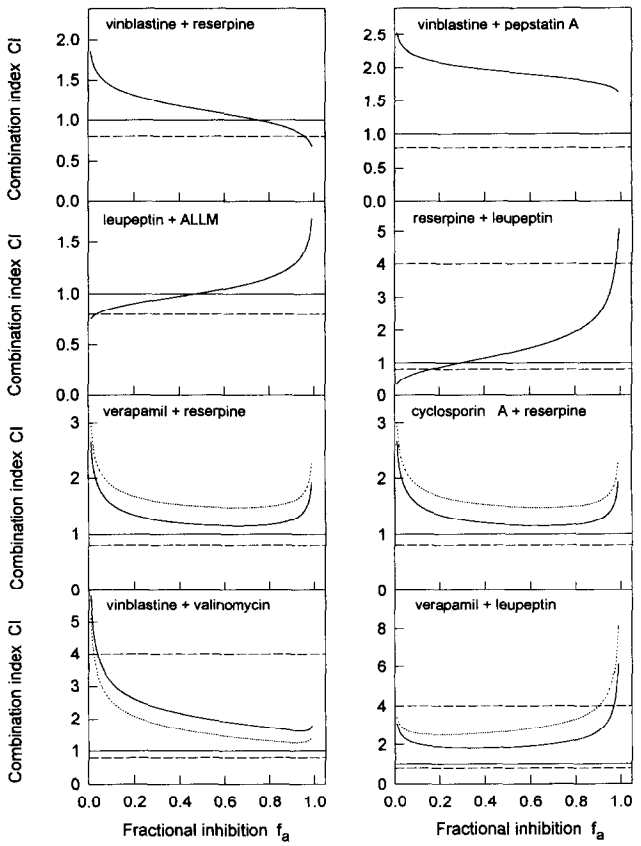

FIG. 4. Combination index plots for eight other combinations of drugs, chemosensitizers, and peptides. A solid horizontal line marks CI = 1.0, and two dashed horizontal lines define the boundaries of synergism (CI < 0.8) and antagonism (CI > 4). All panels show Group 3 combinations, in which drug effects are mainly additive (0.8 < CI < 4). In those cases where it was not possible to use the median effect analysis to determine the exclusivity of the interactions between compounds, calculations were carried out assuming both mutually exclusive interactions (solid curve) and mutually nonexclusive interactions (dotted curve). The concentration ratios for the various combinations are given in Table 1.

cells are not cross-resistant to these peptides, and they do not stimulate Pgp ATPase activity [9, 11]. We have established that the agents employed in the present study do not compromise the integrity of the plasma membrane vesicle system over the concentration range used [9]. Any changes in Pgp transport activity that occur in the presence of a particular test compound, therefore, are attributable to specific interactions with Pgp.

The compounds chosen for investigation include a widely used chemotherapeutic drug (vinblastine), several chemosensitizers of different structural classes, and several linear and cyclic hydrophobic peptides or peptide-like compounds. Within the chemosensitizers (verapamil, reserpine, cyclosporin A) and peptides (ALLM, leupeptin, pepstatin A, valinomycin), compounds were selected with various affinities for Pgp, as indicated by their D_m values (e.g. cyclosporin A > valinomycin > verapamil). All of these agents are likely to be transported substrates; vinblastine, cyclosporin A, and verapamil are known to be transported, and we recently reported that hydrophobic linear peptides are transported very efficiently by Pgp in an ATP-dependent fashion, with the generation of a peptide concentration gradient [19].

It has not yet proved possible to determine true initial rates of transport for Pgp in a model system because of the rapidity with which drugs are transported, and equilibrium, or steady-state, uptake of drug is routinely measured. While inhibition of equilibrium drug uptake by plasma membrane vesicles cannot be subjected to kinetic analysis, previous work in our laboratory has demonstrated the usefulness of the median effect analysis for evaluation of equilibrium drug uptake data [9]. This method of analysis is mechanism independent, and requires no estimates of kinetic constants. Colchicine uptake inhibition data fitted very well to the median effect equation for a large number of Pgp substrates of widely differing chemical structure and affinity (Fig. 2). D_m values extracted from the median effect plots for individual compounds (Table 1) can be used as a quantitative measure of the efficacy of each compound in blocking drug transport. The m values extracted from the median effect plots indicate the extent of the sigmoidal nature of the inhibition of colchicine transport. The significance of the various values of m determined for interaction of the various compounds with Pgp is not yet understood, but these values presumably represent fundamental differences in the way these agents interact with Pgp. Pgp photoaffinity labelling experiments using an azido derivative of the chemosensitizer dexniguldipine [28] have also noted differences between the shape of the inhibition curves (incorporation vs log inhibitor concentration). Certain compounds, especially cyclic peptides such as cyclosporin A, gave a very steep slope, which was interpreted as indicating possible allosteric interaction of these drugs with another site on Pgp.

The median effect analysis was used to examine the exclusivity of interactions between various combinations of compounds. Group 1 compounds had mutually exclusive interactions with Pgp, indicating that their effects on the transporter are similar, and not independent of each other. Inspection of Table 2 indicates that four of the five Group 1 combinations (the exception is verapamil/valinomycin) are linked to each other via mutual exclusivity effects. Thus, it appears that these agents interact with Pgp in a similar fashion, perhaps at a common binding site. Only one combination gave mutually nonexclusive interactions, that of cyclosporin A/verapamil (Group 2 in Table 2), which implies that these two chemosensitizers interact with Pgp in dissimilar ways, and that their interactions are independent of each other. The exclusivity of several additional combinations could not be determined by the median effect analysis, and therefore further analysis for synergism/antagonism was carried out using both assumptions.

Median effect analysis of equilibrium transport data is useful for rapid identification of drug combinations that interact with Pgp additively or synergistically. A limited number of these drug combinations may then be subjected to further testing in more complex intact cell and *in vivo* systems. Two synergistic chemosensitizer combinations have been reported in studies using intact MDR cells *in vitro*; verapamil/cyclosporin A [29, 30] and verapamil/quinine [31]. The present study also identified verapamil/cyclosporin A as a synergistic combination at low f_a values ($f_a < 0.15$); however, antagonism became evident at high f_a. Hu *et al.* [29] demonstrated synergism between verapamil and cyclosporin A in the concentration ranges of 1 to 2 and 0.3 to 1.7 μM, respectively, compared with the somewhat lower synergistic concentrations found in the present study (0.3 to 1.1 μM verapamil and 0.04 to 0.16 μM cyclosporin A). Median effect analysis indicates that at $f_a > 0.2$, this combination becomes antagonistic, a condition that would compromise the ability of either drug to modulate multidrug resistance *in vivo*. It should not be assumed, therefore, that two modulators will be synergistic over the entire concentration range. The median effect analysis can be used to predict not only the type of effect (synergism vs antagonism), but also the concentrations of drugs that would be needed to achieve the desired effect. In addition, it is not restricted to combinations of only two drugs; complex mixtures of chemosensitizers can be assessed using the same methodology.

Two antagonistic combinations (vinblastine/verapamil and verapamil/valinomycin) were also identified by the median effect analysis. Ferry and coworkers [32] reported that the Pgp binding sites for vinblastine and verapamil were allosterically linked to each other in a negative fashion. Clearly, antagonistic combinations would be unsuitable for clinical application in the reversal of MDR. The majority of combinations tested in the present study were considered to be additive over the entire range of f_a values. Additive combinations should also prove suitable for possible clinical use during chemotherapy.

At present, the issue of whether drugs and chemosensitizers interact with Pgp in the same way, and at the same

site, is unresolved. It has been proposed that at least two distinct binding sites are present within Pgp, one for *Vinca* alkaloids, the other for dihydropyridine chemosensitizers [32]. More recently, two different domains for interaction of dihydropyridines have been described, one linked negatively to the vinblastine site, the other positively [33]. Multiple dihydropyridine molecules appear to be able to bind to Pgp simultaneously. Similarly, studies of a series of hydrophobic peptide transport substrates have shown that such compounds are able to stimulate colchicine transport, indicating that both peptide and drug are able to interact simultaneously with Pgp [19]. The existence of synergistic interactions between Pgp substrates, as in the present study, also suggests the concurrent binding of two different compounds to the protein. Ayesh and coworkers [34] recently reported competitive, non-competitive, and cooperative interactions between pairs of drugs and chemosensitizers and Pgp in intact cells. They proposed a two-site model as the basis for these interactions; some drugs interacted with site 1, and others with site 2, while cyclosporin A could interact with both. In contrast, Borgnia and coworkers [35] used kinetic analysis of Pgp ATPase activity to conclude that hydrophobic peptides, chemotherapeutic drugs, and chemosensitizers all interact with a common drug-binding site in a classical competitive fashion. Recent work in our laboratory using site-directed fluorescence labelling of purified Pgp has demonstrated that binding of many different drugs and chemosensitizers leads to conformational changes within the two nucleotide binding domains of the transporter [36]. It seems likely that drugs, chemosensitizers, and hydrophobic peptides interact with different overlapping regions of a flexible drug-binding site that is large enough to accommodate more than one compound at the same time. The observation of competitive or non-competitive interactions might depend on the parameter actually determined experimentally (transport, ATPase activity, etc.). Such a hypothesis may help to explain the conflicting data that exist in the literature on the number of possible binding sites within the Pgp molecule.

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