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Competitive, non-competitive and cooperative interactions between substrates of P-glycoprotein as measured by its ATPase activity.

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Abstract

We have studied the interaction between verapamil and other modulators of the P-glycoprotein ATPase from membranes of CR1R12 Chinese hamster ovary cells. Four major categories of interaction were identified. (i) Non-competitive inhibition of verapamil's stimulation of enzyme activity was found with vanadate. (ii) Competitive inhibition of the ATPase was found for the pair verapamil and cyclosporin A. (iii) Allosteric inhibition with an increase in the Hill number for verapamil was found in the cases of daunorubicin, epirubicin, gramicidin S and D, vinblastine, amiodarone, and colchicine. (iv) Cooperative stimulation of verapamil-induced ATPase activity was found with progesterone, diltiazem, amitriptyline, and propranolol. At high levels, progesterone and verapamil mutually enhanced each other's inhibitory action on the ATPase. Our data show that the substrate binding behavior of P-glycoprotein is complex with more than one binding site being present. This information could form the basis for the development of improved modulators of P-glycoprotein.

Keywords: Chinese Hamster ovary cell; Multidrug resistance; P-glycoprotein; ATPase; Drug interactions

1. Introduction

A major effort is being made to find ways to block the action of P-glycoprotein which is believed to be the main mechanism behind multidrug resistance in cancers refractory to chemotherapy [1–4]. Many modulators of P-glycoprotein have been identified and some have entered clinical trials, including D-verapamil [5], cyclosporin A [6], and amiodarone [7]. One strategy for improving clinical responses has been to search for more effective modulators, e.g. Sandoz PSC-833 [8] and Glaxo's dihydropyridine analogue teludipine [9]. Another strategy has been to investigate the use of pairs of reversers in the hope of finding synergistic effects between them. This latter approach has been pursued at the cellular level [10–12], and also at the clinical level [2]. In order to provide a molecular basis for the further advances in the study of synergy between reversers, it is necessary to explore reverser-reverser interactions on the P-glycoprotein itself.

In the preceding paper in this series (Litman et al., Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity, BBA, this issue) we investi-

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gated the effect of a variety of drugs on the ATPase activity of P-glycoprotein from highly multidrug resistant Chinese hamster ovary cells. We characterized the kinetic behavior of 34 substances and found that the affinity of these drugs for P-glycoprotein was positively correlated with the molecular surface area of the drugs. In the present paper we deepen the analysis by studying some of these drugs in pairs so as to test for interactions between them. Ayesh et al. [13] studied the effect of pairs of modulators on the accumulation of daunorubicin in a multidrug resistant P388 cell line and showed how it was possible to analyze interactions between reversers in terms of cooperative, competitive and non-competitive models. We have applied their analysis at the level of the ATPase activity of P-glycoprotein, and find that in addition to these three categories of interaction, allosteric effects can also be demonstrated. In certain cases, binding of one substrate is associated with a marked increase in the Hill number that describes the stimulation by a second substrate molecule. Thus, effects beyond mere additivity have been demonstrated and could be useful in developing modulator strategies based on the combining of reversers of P-glycoprotein.

2. Materials and methods

2.1. Tumor cells

The CHO cell line CR1R12 (kindly made available for us by Dr Alan E. Senior, University of Rochester Medical Center, New York, USA), was used. The cells were maintained in α-MEM (minimal essential medium) containing 10% fetal calf serum and 5 μg/ml colchicine.

2.2. Isolation of microsomal membranes

The cells were washed with Ringer's medium (NaCl, 148.7 mM; K_2HPO_4, 2.55 mM; KH_2PO_4, 0.45 mM; $MgSO_4$, 1.2 mM; pH 7.4) before being resuspended in 10 ml lysis buffer (Hepes-Tris, 10 mM; EDTA, 5 mM; EGTA, 5 mM; dithiothreitol, 2 mM; pH 7.4) containing protease inhibitors (phenylmethylsulfonyl fluoride, 2 mM; aprotinin, 5 μg/ml; pepstatin, 10 μg/ml; leupeptin, 10 μg/ml). This and all subsequent steps were performed at 4°C. Cells were lysed by nitrogen cavitation (Parr Instrument Co., Moline, IL, USA) at 1200 psi for 20 min. Nuclei were sedimented by centrifugation at $300 \times g$ for 10 min, and subsequently mitochondria were removed by centrifugation at $4000 \times g$ for 10 min. In a third and final centrifugation ($45\,000 \times g$, 60 min) the microsomal fraction was sedimented. The pellet was resuspended in lysis buffer and homogenized by aspiration ten times through a 27 gauge syringe. Aliquots of 150 μl (containing 1–2 mg protein/ml) were frozen in $N_{2(l)}$ and stored at −80°C until use.

2.3. ATPase activity assay

The ATPase activity of P-glycoprotein was determined according to Borgnia et al. [14], by quantitating the release of inorganic phosphate from ATP. Each series of experiments was carried out in a 96-well microtiter plate, with reaction volumes of 50 μl/well ATPase assay medium (ATP, 3 mM; KCl, 50 mM; $MgSO_4$, 2.5 mM; dithiothreitol, 3 mM; Tris-HCl, 25 mM; EGTA, 0.5 mM; ouabain, 2 mM; azide, 3 mM; pH 7.0). Incubation with the various drugs was started by transferring the plate from ice to 37°C for 30 min, and terminated by addition of 200 μl ice-cold stopping medium (ammonium molybdate, 0.2% (w/v); sulfuric acid, 1.3% (v/v); SDS, 0.9% (w/v); trichloroacetic acid, 2.3% (w/v); freshly prepared ascorbic acid, 1% (w/v)) to each well. After 30 min incubation at room temperature, the released phosphate was quantitated colorimetrically in a micro-plate reader (Bio-Tek Instruments, VT USA) at 620 nm. Samples incubated with 500 μM vanadate were obtained in parallel and subtracted from the measurements.

2.4. Chemicals

Verapamil (D-verapamil) was kindly provided by Knoll AG (Ludwigshafen, Germany). Cyclosporin A was a kind gift from Sandoz (now Novartis, Basel, Switzerland). All other chemicals were of analytical grade, purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, MO, USA).

3. Results

Fig. 1A depicts the dependence of the ATPase activity of P-glycoprotein on the concentration of verapamil at four different concentrations of vanadate. The same data transformed to the linear forms of $1/V$ versus $1/S$ are shown as the inset. At all concentrations of verapamil, increasing levels of vanadate inhibited the ATPase. As is clear from the inset, the K_m for verapamil's effect in activating the ATPase does not change with the vanadate concentration, showing that vanadate is a non-competitive inhibitor of verapamil activation. In contrast, Fig. 1B shows an analogous plot and replot for the pair cyclosporin A and verapamil. Here, the Lineweaver-Burke plot shows that cyclosporin acts as a competitive inhibitor of verapamil-stimulated ATPase activity.

A different pattern of behavior is found for the pair daunorubicin and verapamil (Fig. 2A). Here, increasing daunorubicin changes the nature of the dependence of activity on verapamil concentration, in that

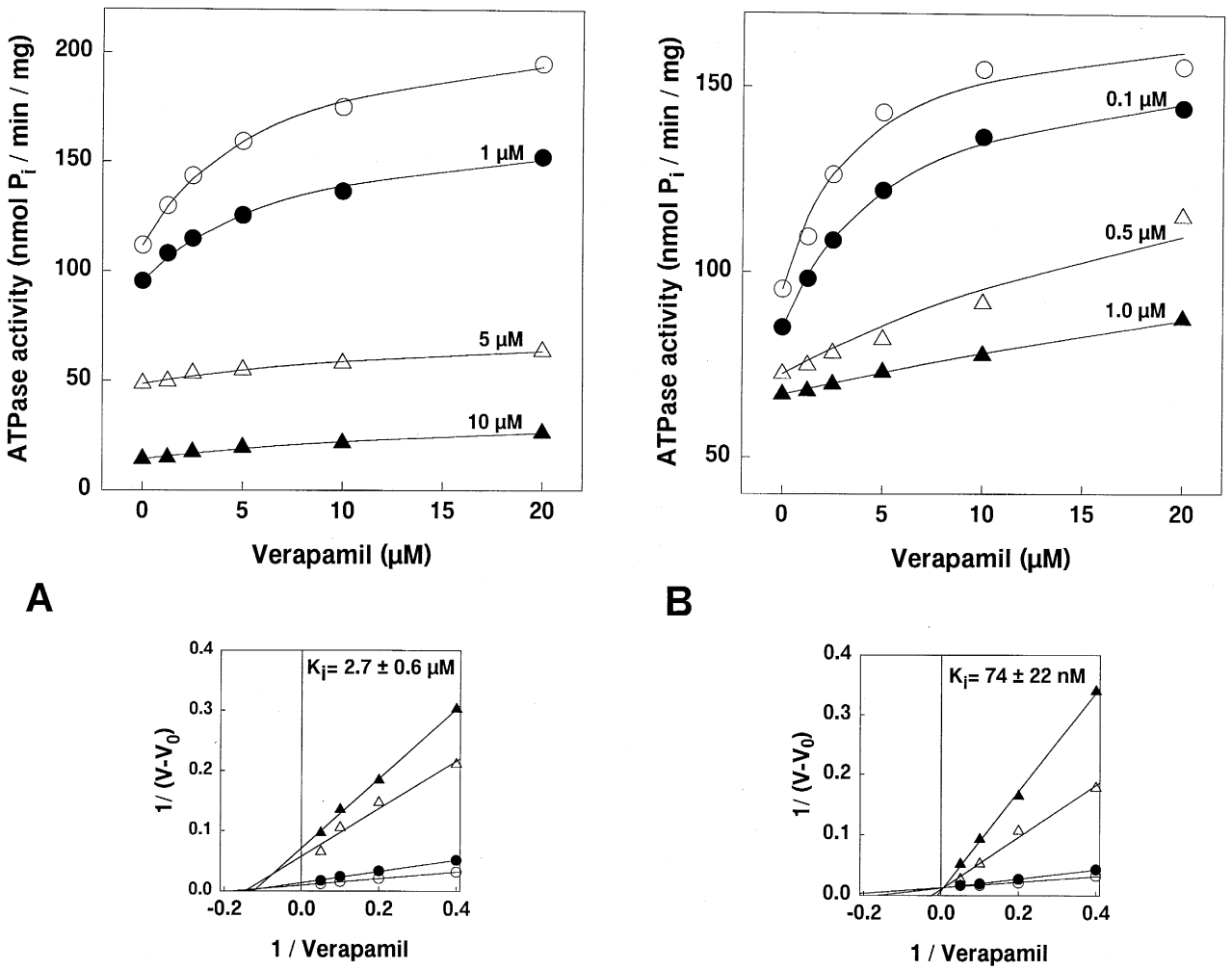


Fig. 1. Kinetics of inhibition of the verapamil-stimulated P-glycoprotein ATPase activity by (A) vanadate at 0 (○), 1 μM (●), 5 μM (△), and 10 μM (▲), and (B) cyclosporin A at 0 (○), 0.1 μM (●), 0.5 μM (△), and 1.0 μM (▲). The curves in (A) and (B) have been fitted to the Michaelis-Menten equation: $V = V_0 + (V_{max} - V_0)S/(S + K_m)$. The insets are Lineweaver-Burk replots ($1/V$ versus $1/S$) of the data. (A) Vanadate, non-competitive inhibition. K_i is obtained from $V^I_{max} = V_{max}/(1 + I/K_i)$, where V^I_{max} is the maximal activity in the presence of the inhibitor, I. (B) Cyclosporin A, competitive inhibition. K_i is given by: $1/V = 1/V_{max} + K_m/V_{max}(1 + I/K_i)(1/S)$.

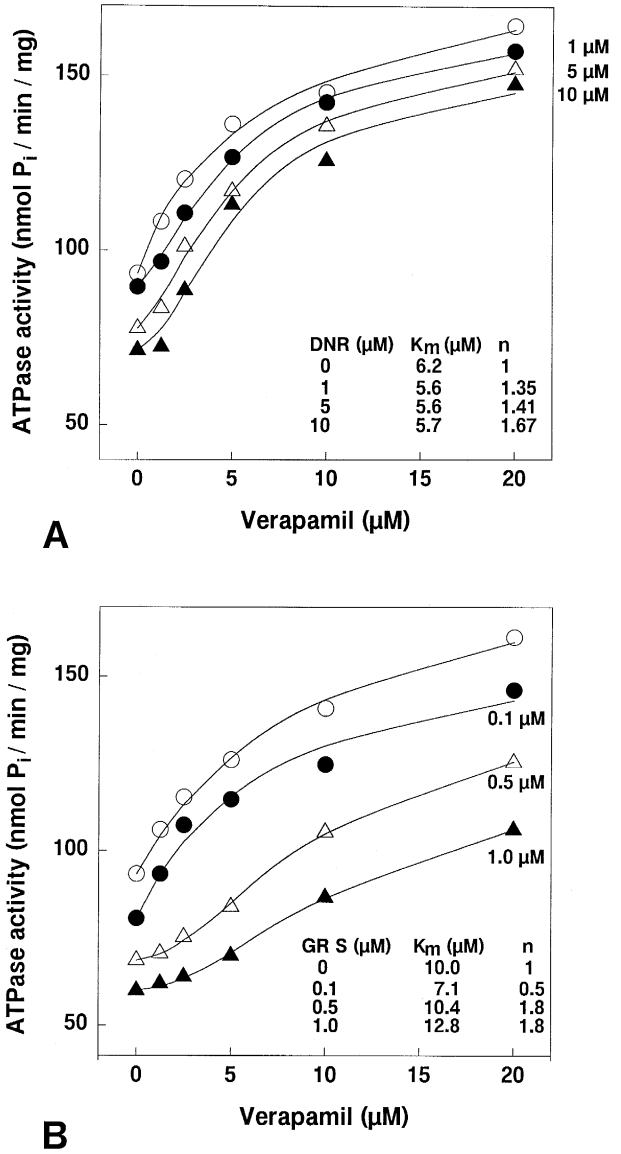


Fig. 2. Kinetics of inhibition of the verapamil-stimulated ATPase activity of P-glycoprotein by (A) daunorubicin (DNR) at 0 (○), 1 μM (●), 5 μM (△), and 10 μM (▲), and (B) gramicidin S (GR S) at 0 (○), 0.1 μM (●), 0.5 μM (△), and 1.0 μM (▲). The curves are obtained by curve-fitting to a modified form of the Michaelis-Menten equation: $V = V_0 + (V_{max} - V_0)S^n/(S^n + K_i)$, which includes the Hill number, n, to be fitted. The listed K_m, and Hill coefficients are obtained from the sigmoidal curve fits.

the Hill coefficient increases from $n = 1$ to $n = 1.7$ with daunorubicin. The Michaelis constant for verapamil, however, remains unchanged as daunorubicin increases. Thus, daunorubicin and verapamil interact non-competitively, but binding of daunorubicin to P-glycoprotein affects the kinetic interaction between verapamil and P-glycoprotein. A very similar pattern is observed for the pair gramicidin S and verapamil (Fig. 2B). Again, interaction between these two modulators is non-competitive, but the binding of gramicidin S affects the kinetics of interaction between

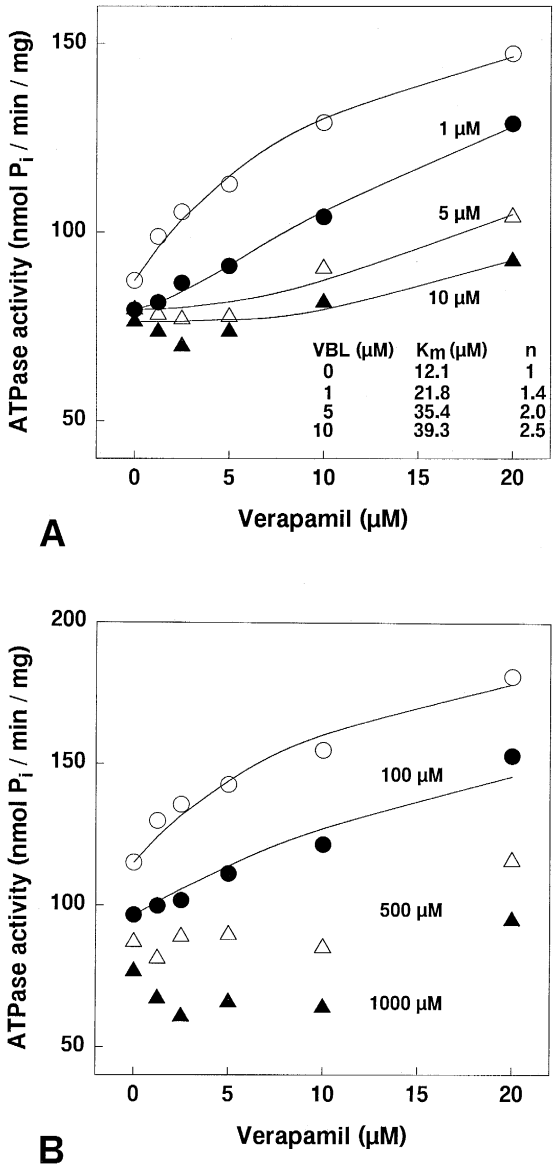


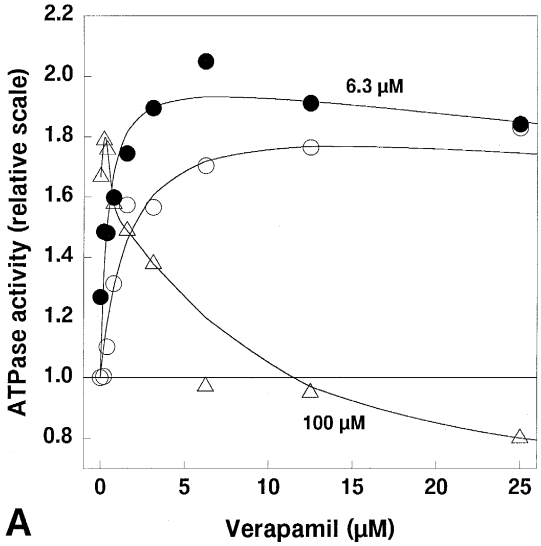
Fig. 3. Kinetics of inhibition of the verapamil-stimulated P-glycoprotein ATPase activity by (A) vinblastine at 0 (○), 1 μM (●), 5 μM (△), and 10 μM (▲), and (B) colchicine at 0 (○), 100 μM (●), 500 μM (△), and 1000 μM (▲). It has only been possible to fit the curves without additional drug (○) to the Michaelis-Menten equation.

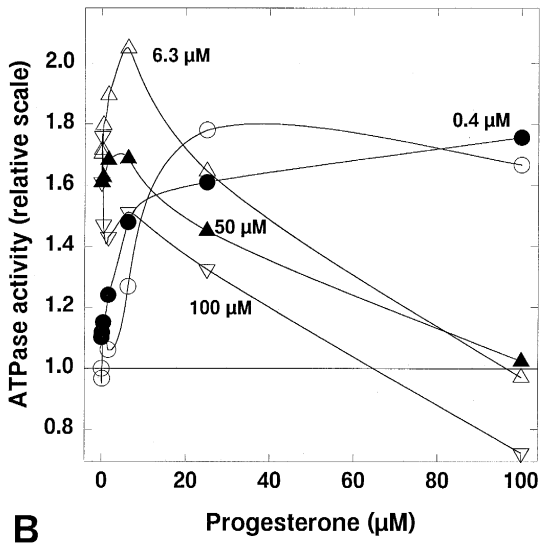
verapamil and P-glycoprotein, the Hill number increasing from 1 at zero gramicidin S to 1.8 at 1 μM.

The data depicted in Fig. 3 display a variation on this pattern. Both for vinblastine (Fig. 3A) and for colchicine (Fig. 3B), at the highest concentrations of these transport substrates verapamil apparently exerts an inhibitory effect at low concentrations, while activation occurs at higher verapamil levels.

Finally, a quite different pattern is found when one studies the interaction between two modulators, both of which stimulate the ATPase activity. Fig. 4A shows a plot similar to those of Figs. 1–3 but now for the pair progesterone and verapamil. Here, a low concentration of progesterone acts additively with verapamil in stimulating the ATPase activity, but at the highest progesterone level verapamil acts as an inhibitor, even at the lowest verapamil concentration. For clarity, in Fig. 4A only three concentrations of progesterone are depicted, although intermediate concentrations in this range were studied; the ATPase activity profiles changed continuously from stimulatory (up to 6.3 μM progesterone) to pure inhibitory (above 25 μM progesterone). From this same series of experiments the data have also been plotted as ATPase activity against progesterone concentration (Fig. 4B). With increasing verapamil, the apparent affinity of P-glycoprotein for progesterone increases in that stimulation occurs at lower concentrations of progesterone. This is illustrated in the inset of Fig. 4B. Verapamil decreases the K_m of progesterone with a K_i of 0.61 μM. Thus, verapamil and progesterone act cooperatively. Higher levels of progesterone and verapamil induce inhibition of the ATPase activity.

Similar experiments have been performed with six other transport substrates and modulators of the P-glycoprotein ATPase (data not shown). Those behaving like daunorubicin and gramicidin S (i.e. with a profile similar to Fig. 2) were amiodarone, epirubicin, and gramicidin D. Those behaving like progesterone (Fig. 4) were amitriptyline, diltiazem, and *R*-propranolol.





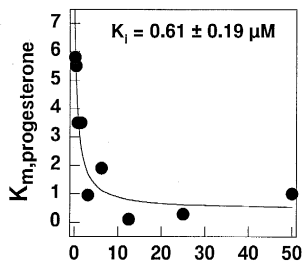


Fig. 4. Kinetics of the combined effect of verapamil and progesterone on the P-glycoprotein ATPase. (A) The fractional ATPase activity as a function of verapamil concentration at 0, 6.3 μM, and 100 μM progesterone. (B) The ATPase activity as a function of progesterone concentration at 0 (○), 0.4 μM (●), 6.3 μM (△), 50 μM (▲), and 100 μM (▽) verapamil. The inset shows how the K_m of progesterone varies with the verapamil concentration.

4. Discussion

The interactions between verapamil and other transport substrates and modulators of P-glycoprotein are complex but can be classified into four major categories. The first, and simplest, is represented by the pair in Fig. 1A. Here, verapamil and vanadate behave in a strictly non-competitive fashion, in that the Michaelis constant, K_m, for verapamil's stimulation of the ATPase does not change with the vanadate concentration. This is consistent with the mode of action of vanadate, interacting with the catalytic domain of the P-glycoprotein ATPase [15] and not with the substrate binding site. Vanadate is a known transition state analog in phosphoryl transfer reactions as it easily forms a pentacovalent bipyramidal structure similar to that of a phosphate ester during hydrolysis [16]. Thus, enzymes, such as the P-type ATPases (e.g. the Na^+/K^+-, Ca^{2+}-, and Mg^{2+}-ATPases), which employ a covalent phosphorylated intermediate, are most sensitive to vanadate inhibition with K_i values in the nanomolar concentration range [16,17]. That the P-glycoprotein ATPase is inhibited by vanadate could indicate, that a pentacoordinate phosphorus intermediate exists during the catalytic cycle, but the relatively low sensitivity towards vanadate inhibition (high K_i) suggests that the reaction intermediate is not of the *covalent* phosphorylated type. This notion is supported by our failure to identify a covalent, phosphorylated intermediate (data not shown), in agreement with previous such studies [18,19].

The next-simplest category is represented by Fig. 1B, the pair verapamil and cyclosporin A. Here the K_m of verapamil increases with the cyclosporin concentration. Cyclosporin A behaves as a strict competitive inhibitor with a K_i of 74 ± 22 nM, comparable to that found for the inhibition of daunorubicin pumping in Ehrlich ascites cells (Litman et al., ATPase activity of P-glycoprotein related to emergence of drug resistance in Ehrlich ascites tumor cell lines, BBA, this issue) and multidrug resistant P388 cells [13] and the values reported Rao et al. [20] and by ourselves (Litman et al., Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity, BBA, this issue) for inhibition of P-glycoprotein ATPase activity. These findings suggest that cyclosporin A overlaps with verapamil on the same site on P-glycoprotein, confirming the suggestion of Ayesh et al. [13].

Category three is represented by Fig. 2A,B. Here, increasing concentrations of daunorubicin or of gramicidin S induce a change in the shape of the dose-response curves for verapamil-stimulated ATPase activity from simple Michaelis-Menten to sigmoidal kinetics. The K_m for verapamil does not change with increasing daunorubicin or gramicidin S, so that their interactions with verapamil are non-competitive. This means that verapamil and daunorubicin (or gramicidin S) are able to bind simultaneously to P-glycoprotein, and at different sites which may interact allosterically. The increase in the Hill coefficient strongly suggests that the allosteric change has, most interestingly, induced the phenomenon of cooperativity between pairs of verapamil molecules. The finding with daunorubicin is in agreement with the work of Pereira et al. [21] and Spoelstra et al. [22], describing non-competitive interactions between verapamil and the anthracyclines.

The next category, which is represented in Fig. 3, displays a variation on the ATPase activity patterns of Fig. 2. Here, both vinblastine (Fig. 3A) and colchicine (Fig. 3B), at high concentrations apparently induce an inhibitory effect of verapamil at low concentrations, while activation occurs at higher verapamil levels. The complexity of the activation profiles in the presence of the higher concentrations of vinblastine and colchicine has prevented us from extracting K_m values for verapamil, so we can not establish quantitatively whether or not the interactions here are competitive. Nevertheless, the fact that the shapes of the verapamil activation profiles have been changed by colchicine (or by vinblastine) strongly suggests that the two drugs are being simultaneously bound to P-glycoprotein (as Ayesh et al. suggested in the case of vinblastine and verapamil [13]). Interestingly, a marked effect of colchicine in inhibiting the ATPase is seen at all levels of verapamil, whereas colchicine on its own seldom displays any inhibitory effect.

The final category is represented by progesterone (Fig. 4), the affinity of which is increased rather than reduced by the presence of verapamil. Such an increase is the very opposite of competition and is, rather, an example of cooperative behavior. From the inset to Fig. 4B, showing the decrease in K_m for

progesterone with increasing verapamil concentration, we derived the K_i value for verapamil which is similar to the K_m for ATPase activation by verapamil alone (Litman et al., Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity, BBA, this issue). This suggests that the site at which verapamil exerts its effect on the affinity of progesterone is the same site at which verapamil stimulates the ATPase. As verapamil increases the affinity for progesterone, the two modulators must be bound simultaneously. Fig. 4A shows that at high levels of progesterone verapamil acts as a very effective inhibitor of the ATPase activity. It would appear that progesterone has increased the affinity of verapamil for its inhibitory site on P-glycoprotein from 100 μM (Litman et al., Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity, BBA, this issue) to 3.5 μM. Correspondingly, verapamil increases the affinity of progesterone for its inhibitory site. Such behavior might indicate that the inhibitory effects found at high substrate concentrations arise from specific interactions with P-glycoprotein, rather than being merely nonspecific membrane perturbations [23].

It might be interesting to speculate that the positive, cooperative interactions between verapamil and progesterone represent an aspect of the relation between affinity and surface area that we demonstrated in Litman et al., Structure-activity relationships of P-glycoprotein interacting drugs: kinetic characterization of their effects on ATPase activity, BBA, this issue. There, we showed that the affinity of a transport substrate or modulator for P-glycoprotein was proportional to the Van der Waals surface area of the drug. It is possible that when two different drugs are present, each binding to the large interacting surface of P-glycoprotein, each can contribute to the overall interaction with P-glycoprotein leading to a combined effect which is greater than that given by either drug alone.

Most of the transport substrates and modulators that we have studied (daunorubicin, epirubicin, vinblastine, the gramicidins, colchicine, and progesterone) seem to bind to P-glycoprotein at a different site or sites from that to which verapamil binds. In contrast, cyclosporin A and verapamil at least share overlapping sites on P-glycoprotein. Our study has underscored the complexity of the substrate binding sites on P-glycoprotein, information which can form the basis for the development of improved modulators for this clinically important protein.

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