Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents

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P-Glycoprotein, the plasma membrane protein responsible for the multidrug resistance of some tumour cells, is an active transporter of a number of structurally unrelated hydrophobic drugs. We have characterized the modulation of its ATPase activity by a multidrug-resistance-related cytotoxic drug, vinblastine, and different multidrug-resistance-reversing agents, verapamil and the dihydropyridines nicardipine, nimodipine, nitrendipine, nifedipine and azidopine. P-Glycoprotein ATPase activity was measured by using native membrane vesicles containing large amounts of P-glycoprotein, prepared from the highly multidrug-resistant lung fibroblasts DC-3F/ADX. P-Glycoprotein ATPase is activated by verapamil and by nicardipine but not by vinblastine. Among the five dihydropyridines tested, the higher the hydrophobicity, the higher was the activation factor with respect to the basal activity and the lower was the half-maximal activating concentration. The vinblastine-specific binding on P-glycoprotein is reported by the inhibitions of the verapamil- and the nicardipine-stimulated ATPase. These inhibitions are purely competitive, which means that the bindings of vinblastine and verapamil, or vinblastine and nicardipine, on P-glycoprotein are mutually exclusive. In contrast, verapamil and nicardipine display mutually non-competitive interactions. This demonstrates the existence of two distinct specific sites for these two P-glycoprotein modulators on which they can bind simultaneously and separately to the vinblastine site. The nicardipine-stimulated ATPase activity in the presence of the other dihydropyridines shows mixed-type inhibitions. These dihydropyridines have thus different binding sites that interact mutually to decrease their respective, separately determined affinities. This could be due to steric constraints between sites close to each other. This is supported by the observation that vinblastine binding is not mutually exclusive with nifedipine or nitrendipine binding, whereas it is mutually exclusive with nicardipine. Moreover, verapamil binding also interacts with the five dihydropyridines by mixed inhibitions, with different destabilization factors. On the whole our enzymic data show that P-glycoprotein has distinct but interacting binding sites for various modulators of its ATPase function.

INTRODUCTION

Cellular resistance to the lethal effects of cytotoxic drugs can be observed *in vitro* for cultured cells as well as *in vivo* for tumour cells. Resistance to chemotherapeutic agents restricts their effectiveness for treating patients suffering from tumours. Among the various cell resistance mechanisms described, P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) is one of the most intensely studied because it has been clearly involved in clinical situations [1]. P-gp is a plasma membrane responsible for the efflux of various cytotoxic drugs out of the MDR tumour cells. P-gp converts the energy produced by ATP hydrolysis to transport a number of amphiphilic molecules of very different chemical structures [2]. Indeed, the transport substrates of P-gp include different cytotoxic drugs (such as anthracyclines, *Vinca* alkaloids and actinomycin D), as well as various compounds (such as verapamil, cyclosporin A and steroids) that are capable of reversing the MDR phenotype to a greater or lesser degree [3]. Moreover, the direct molecular interaction between P-gp and its transport substrates comes from P-gp-specific photoaffinity labelling by various amphiphilic compounds [4], and from recent spectroscopic studies reporting tertiary structure changes [5,6]. However, the mechanisms of P-gp-mediated cytotoxic drug transport inhibition by the MDR-reversing agents are still unclear, in particular with respect to whether it involves a true competition when these agents are transported by P-gp.

The basic puzzling functional characteristic observed with P-gp is the unusual, very broad recognition of molecular structures for the amphiphilic molecules that are transported by P-gp. It is thus of interest to elucidate whether MDR-related cytotoxic drugs and MDR-reversing agents interact either with one universal, adaptable binding site or with several distinct, more or less specific, binding sites. However, few such studies, with various experimental techniques and different tested molecules, have been published and they are not even always self-consistent. Indeed, it can be stated that: (1) ATP-dependent transport of *Vinca* alkaloids into P-gp-containing membrane vesicles is competitively inhibited by verapamil, cyclosporin A, azidopine, nicardipine, nifedipine, quinidine, diltiazem and trifluoperazine [7–9], whereas ATP-dependent binding of azidopine to P-gp-containing membrane vesicles is non-competitively inhibited by vinblastine [10]; (2) verapamil-stimulated P-gp ATPase is competitively inhibited by vinblastine, and progesterone-stimulated P-gp ATPase is non-competitively inhibited by vinblastine [11]; (3) vinblastine net secretory flux across transfected cells expressing P-gp in a polarized epithelial layer is inhibited competitively by verapamil and nifedipine, and non-competitively by dideoxyforskolin [12]; (4) tetrahydropyranyl-adriamycin efflux from MDR cells is inhibited by verapamil by a (pure or mixed) non-competitive mechanism [13]. In addition, it was recently reported that vinblastine and verapamil non-competitively interact to inhibit daunomycin efflux from MDR cells [14]. An understanding of MDR reversion, and eventually its optimization, by amphiphilic molecules also requires clarification of the mutual relationship between the binding sites on P-gp of the various MDR-reversing agents. In this context, cyclosporin

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Abbreviations used: BZDC-DHP, benzoyldihydrocinnamic acid-dihydropyridine; MDR, multidrug resistance/resistant; P-gp, P-glycoprotein.
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A has been shown to inhibit non-competitively the ATP-dependent association of azidopine to P-gp-containing plasma membrane vesicles [10], providing the first experimental indication of distinct binding sites on P-gp. Recently we also reported non-competitive effects in the modulation of P-gp ATPase activity by verapamil, progesterone and closely related steroids [15].

Enzymological analysis of P-gp ATPase seems well adapted to elucidate the relationships between the binding sites of P-gp modulators. Indeed, the most commonly accepted model for P-gp implies that it functions as an active transporter [2], which means that modulations of P-gp-mediated drug transport and modulations of drug-stimulated P-gp ATPase should be consistent. Moreover, the alterations of P-gp ATPase activity induced by either a point mutation (in the 185 position) or a deletion in the first extracellular loop are consistent with the changes in the resistance profile of the cells overexpressing the corresponding mutated P-gp, when considering colchicine [16,17] or *Vinca* alkaloids and rhodamine [18,19] respectively. In addition, P-gp ATPase studies are probably more reliable than drug transport studies because of the more precise quantifications made possible by a decrease in non-specific signals owing to the amphiphilic modulators' partitioning and diffusion across the vesicle membranes [20]. For the only pair of MDR-reversing agents that have been studied for modulation of either P-gp ATPase or P-gp-mediated drug transport, the results were consistent with a competition between verapamil and cyclosporin A [14,21].

We previously studied P-gp ATPase modulations to demonstrate that vinblastine- and verapamil-binding sites on P-gp are mutually exclusive [11]. These two molecules can be considered to be representative of MDR-related drugs and MDR-reversing agents respectively. However, because they have dissimilar chemical structures, no conclusion about the uniqueness of their binding site(s) can be drawn: a negative allosteric interaction between distant sites must a priori be considered plausible as well as a common site. We thus further studied the mutual relationships with another well-known MDR-reversing agent, nicardipine. This inhibits P-gp function in living cells [22] and also in plasma membrane vesicles [7,23], and stimulates P-gp ATPase activity with a bell-shaped dependence on nicardipine concentration [24]. Other dihydropyridine derivatives such as nimodipine, nitrendipine and nifedipine also display MDR-reversing properties [22,25]. Moreover, a photoactivatable dihydropyridine, azidopine, specifically labels P-gp, and this photoaffinity labelling is displaced by the above dihydropyridines [26]. It is thus likely that dihydropyridines reverse MDR by interacting directly with P-gp. This prompted us to study the effects of a series of five dihydropyridines on P-gp ATPase.

We report here that: (1) vinblastine, verapamil and nicardipine have distinct binding sites on P-gp; (2) the five dihydropyridines tested modulate the basal as well as the drug-stimulated P-gp ATPase activities differently; (3) nicardipine, nimodipine and nitrendipine have distinct but mutually interacting binding sites; (4) verapamil- and vinblastine-binding sites interact differently with the dihydropyridine-binding sites. These results provide new functional characteristics on the complex interaction set between P-gp and its numerous modulators: they lead to a 'sticky paper model' for the P-gp transport sites, which points to various possibilities of independent binding, mutually destabilizing binding or mutually exclusive binding of two P-gp modulators. This can shed light on recent structural results [27] showing a rather large interaction surface between the membrane core and the transmembrane domain of this pharmacologically important membrane transporter. Preliminary data from this study were presented at the Second European Biophysics Congress [28].

MATERIALS AND METHODS

Chemicals and cells

Verapamil hydrochloride, vinblastine sulphate, nifedipine, nicardipine, ATP, ouabain and EGTA were from Sigma (Saint-Quentin Fallavier, France); nitrendipine and nimodipine were from Tocris-Cookson (Bristol, Avon, U.K.), azidopine was kindly donated by J. Striessnig (Innsbruck University, Innsbruck, Austria), sodium azide was from Merck (Meylan, France), and pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim (Darmstadt, Germany); all other products were of reagent grade.

The selection by actinomycin D of the DC-3F/ADX cell line from the spontaneously transformed Chinese hamster DC-3F lung fibroblasts has been described previously [29]. The DC-3F/ADX cells are highly resistant to actinomycin D, vincristine and colchicine [30], and their MDR phenotype is due to the overexpression of the *pgp1* gene [31]. The DC-3F and DC-3F/ADX cells were cultured, harvested and frozen as previously described [11,30].

Preparation of membrane vesicles

Membrane vesicles were prepared from DC-3F or DC-3F/ADX cells as previously described [30], except that the final pellet of membrane vesicles was resuspended in PBS supplemented with 10 mM KCl, 2 mM $MgCl_2$ and 1 mM dithiothreitol, then homogenized with a 25 gauge needle.

Measurement of ATPase activity

ATPase activities of the membrane vesicle suspensions were measured at 37 °C by continuous monitoring of NADH absorbance at 340 nm, with a coupled-enzyme assay composed of 0.1 mg/ml pyruvate kinase, 1 mM phosphoenolpyruvate, 0.1 mg/ml lactate dehydrogenase and 0.5 mM NADH, allowing ATP regeneration in the reaction medium as described previously [11]. Some membrane ATPases are inhibited by the addition of 10 mM sodium azide, 0.5 mM ouabain and 1 mM EGTA. Under these conditions, the residual ATPase activity measured with the membrane vesicles prepared from DC-3F cells (devoid of P-gp) is 20–40 nmol/min per mg of membrane protein, whereas the activity measured with the membrane vesicles prepared from DC-3F/ADX cells (containing approx. 15 % of membrane proteins as P-gp [30]) is 120–280 nmol/min per mg of membrane protein. Approx. 80 % of this latter value is vanadate-sensitive [30], and it is thus mainly attributed to P-gp. This constitutes its 'basal ATPase activity', i.e. determined in the absence of any added drug. Its exact value depends on the cell batch and vesicle preparation considered, but it is similar to previously published results [11,15,30]. In the presence of an activator, P-gp exhibits a 'drug-stimulated ATPase activity', which can be characterized by an activation factor with respect to the basal activity and a half-maximal activating concentration. These two parameters are fairly constant from one vesicle batch to another [11,15,30].

Successive additions of the drug whose concentration influence was studied were made in the same optical cuvette under continuous stirring. The drug was added either from a stock solution prepared in DMSO or from a solution diluted 1:100 in distilled water. The most hydrophobic dihydropyridines tested, nicardipine, nimodipine and nitrendipine, were also added to the reaction medium from a series of dilutions 1:2 in DMSO to improve their solubility. In any case, comparisons did not show

a qualitative change in the results. Final DMSO concentrations in the assay medium never exceeded 0.4 % (v/v), a concentration that had no effect on the measured ATPase activity. No ATPase inactivation was observed over the duration (approx. 1 h) of a complete concentration-dependence curve determination. Each experimental curve is presented with an error bar showing the inaccuracy of the determination of the slope of the absorbance variations. Owing to variations in the absolute values of the P-gp ATPase activity observed from one vesicle batch to another, the inhibition patterns, i.e. the concentration-dependence curves in the presence of various concentrations of a given modulator, were always measured the same day in one experimental run. The results presented are representative of two to four identical experiments. This allows accurate determinations of the enzymological parameters characterizing P-gp ATPase modulations, such as apparent affinities and inhibition types.

Treatment of enzymological data

The enzymological behaviour of P-gp ATPase can be described by a simple kinetic model [11]. In brief, it is based on $MgATP^{2-}$ hydrolysis, which can be modulated (either activated or partly inhibited) by molecules binding P-gp on sites distinct from the nucleotide catalytic sites. This ATPase activity is fully defined by the V_{max} and K_m parameters, and the curve of P-gp ATPase activity variations as a function of the concentration of a given modulator allows a determination of the apparent affinity of this modulator for P-gp as the half-effective concentration, which can easily be evaluated graphically. When two modulators are present simultaneously in the reaction medium, they can bind either in a mutually exclusive or in a mutually non-exclusive manner. These two types of mutual relationship can easily be distinguished by simple visual inspection of the inhibition pattern (competitive or non-competitive) obtained when P-gp ATPase activity variations are recorded as a function of the concentration of a given modulator in the presence of different concentrations of the second modulator. According to the kinetic equations previously derived [11], secondary plots of concentration of the second modulator against the apparent affinity of the first modulator or against the maximal stimulated activity, respectively, then allow a determination of the inhibition constant for the second modulator (which can be considered to be its apparent affinity) with a reasonable accuracy.

RESULTS

Comparison of effects of five dihydropyridines on the basal P-gp ATPase activity

The ATPase activity of P-gp-containing membrane vesicles displayed progressively deformed bell-shaped concentration-dependence curves for its stimulations by nicardipine, nimodipine, nitrendipine, nifedipine and the photoactivatable derivative azidopine (Figure 1). P-gp ATPase was maximally activated by a factor of 2.2 ± 0.4 in the presence of 3–4 μM nicardipine, with a half-maximal activating nicardipine concentration of 0.5 ± 0.2 μM (means $\pm$ S.D.), in agreement with previous studies [24]. Nimodipine and nitrendipine had an activation factor with respect to the basal activity of 1.6 ± 0.1 and 1.3 ± 0.1 respectively, whereas azidopine stimulated $MgATP^{2-}$ hydrolysis approx. 1.25-fold before vesicle aggregation prevented observation of inhibition. Nifedipine had no stimulating effect. Compared with nicardipine, the various ATPase stimulations were observed for higher half-maximal activating concentrations, 2.5–3 and 3–5 μM for nimodipine and nitrendipine respectively,
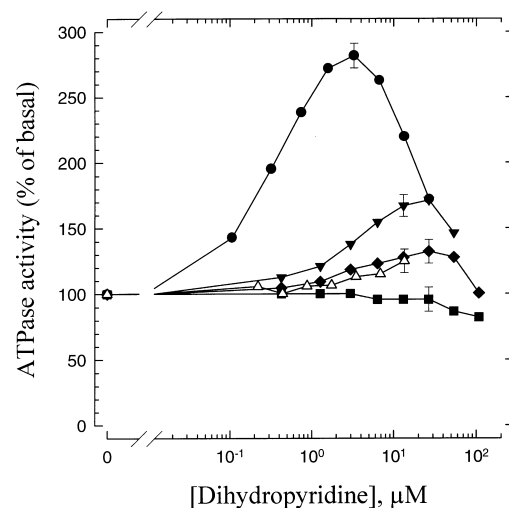

Figure 1   Comparison of effects of five dihydropyridines on the basal P-gp ATPase activity

The ATPase activity of a suspension of membrane vesicles at 13–27 μg/ml membrane proteins, prepared from DC-3F/ADX cells, was measured at 37 °C in a reaction medium composed of 10 mM Tris/HCl (pH 7.8 at 20 °C), 100 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM sodium azide, 0.5 mM ouabain, 1 mM EGTA and 1 mM $MgATP^{2-}$. Increasing concentrations of nicardipine (●), nimodipine (▼), nitrendipine (◆), nifedipine (■) or azidopine (△) were obtained by successive additions to the reaction medium under continuous stirring. ATPase activities have been normalized with respect to the basal activity, whose mean value was 220 ± 10 nmol/min per mg of membrane protein and set at 100 %; it was determined for each curve before the addition of any drug. The graph depicts typical curves, each being recorded two to five times, independently of the control curves included in the inhibition patterns. Error bars are an evaluation of the measurement inaccuracy due to the determination of the slope of the aborbance variations; these were similar for a whole curve, so only one bar is drawn on each curve for clarity.

and above 3–4 μM for azidopine because the maximal activation was not reached in this case.

The specificity of these dihydropyridine-induced ATPase modulations with respect to P-gp was demonstrated by the use of control membrane vesicles lacking P-gp, prepared from the parental sensitive DC-3F cell line [11,15,30]. Indeed, no significant ATPase activity change in these control vesicles was observed when each of the five dihydropyridines was assayed under the same conditions as in Figure 1 (results not shown). Moreover, when the above experiments were performed in the absence of the specific ionic pump inhibitors azide, ouabain and EGTA, only a slight global ATPase inhibition was detected at high concentrations (50–100 μM for all the dihydropyridines tested except azidopine, which could not be used above 20 μM): this suggests that, in the concentration range tested, these dihydropyridines do not induce membrane perturbation that could be reported by these ionic membrane ATPases (results not shown).

Relationships between nicardipine and vinblastine or verapamil for P-gp ATPase modulation

When the nicardipine concentration dependence of the P-gp ATPase activity was measured in the presence of increasing concentrations of vinblastine, added before the nicardipine, the nicardipine concentration necessary to activate P-gp ATPase to the control curve level measured without vinblastine increased
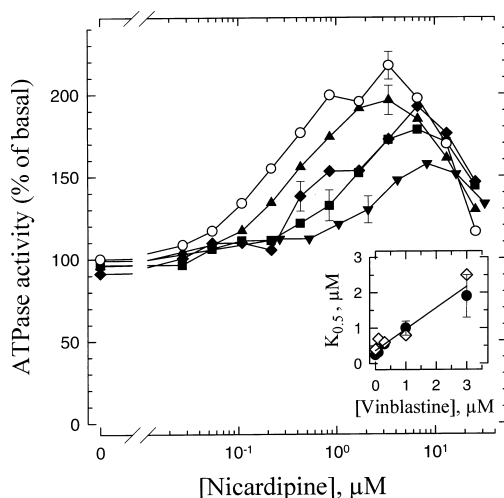
Figure 2 Effect of vinblastine on the nicardipine concentration dependence of the P-gp ATPase activity

The ATPase activity of a suspension of membrane vesicles at 15–18 μg/ml membrane proteins was measured, as described for Figure 1, in the absence (○) or presence of 0.1 μM (▲), 0.3 μM (◆), 1 μM (■) or 3 μM (▼) vinblastine. Increasing nicardipine concentrations were obtained by successive additions to the reaction medium under continuous stirring. The graph depicts one typical experiment out of two, all the curves being recorded during the same experimental run (same vesicle batch, same day). Error bars are an evaluation of the measurement inaccuracy due to the determination of the slope of the absorbance variations; these were similar for a whole curve, so only one bar is drawn on each curve for clarity. ATPase activities have been normalized with respect to the basal activity, whose mean value was 280 ± 30 nmol/min per mg of membrane protein and set at 100%; it was determined for each curve before the addition of any drug. Inset: secondary plot, drawn from the two similar experiments depicted in the main graph (●) or not shown (◇), showing the vinblastine concentration present in the reaction medium plotted against the half-maximal activating nicardipine concentration; the line is the regression fitted from all the experimental points.

Figure 3 Effect of verapamil on the dependence of P-gp ATPase activity on nicardipine concentration

The ATPase activity of a suspension of membrane vesicles at 18–20 μg/ml membrane proteins was measured, as described for Figure 1, in the absence (○) or presence of 1 μM (▲), 3 μM (◆) or 10 μM (■) verapamil. Increasing nicardipine concentrations were obtained by successive additions to the reaction medium under continuous stirring. ATPase activities have been normalized with respect to the basal activity, whose mean value was 230 ± 25 nmol/min per mg of membrane protein and set at 100%; it was determined for each curve before the addition of any drug. Error bars are an evaluation of the measurement inaccuracy for a given experimental curve; only one bar is drawn on each curve for clarity. Inset: secondary plot of verapamil concentration present in the reaction medium against the initial ATPase activity in the absence of nicardipine (◇), and the activity measured for each experimental curve at the nicardipine concentration corresponding to the maximum of the control curve (●).

with increasing initial vinblastine concentration (Figure 2). The secondary plot of vinblastine concentration against the half-maximal activating nicardipine concentration was linear over one and a half decades (Figure 2, inset). This reveals a competition between vinblastine and nicardipine, with a 1:1 stoichiometry, for P-gp ATPase stimulation, which means a mutual exclusion of these two molecules for their binding to P-gp. The calculated inhibition constant for vinblastine, approx. 0.6 μM, is in agreement with the apparent affinity of vinblastine for P-gp (0.5 ± 0.2 μM) determined previously [11]. Thus the vinblastine- and nicardipine-binding sites on P-gp might be common, or distinct but overlapping, or distant but in negative allosteric interaction.

When the nicardipine-concentration dependence of the P-gp ATPase activity was measured in the presence of increasing concentrations of verapamil instead of vinblastine, there was no shift toward higher values of the nicardipine activating concentrations (Figure 3). In contrast, there was a decrease in the activation factor measured at approx. 3 μM nicardipine, after the addition of 5–10 μM verapamil. This decrease was observed for verapamil concentrations above those inducing stimulation of the basal P-gp ATPase (half-maximal activating concentration approx. 1–2 μM verapamil), but below those inducing the inhibition of the basal P-gp ATPase (above approx. 30 μM verapamil) (Figure 3, inset).

When similar P-gp ATPase determinations were performed with the reverse order of addition of the two modulators, the same activity levels were measured and the same set of inhibition
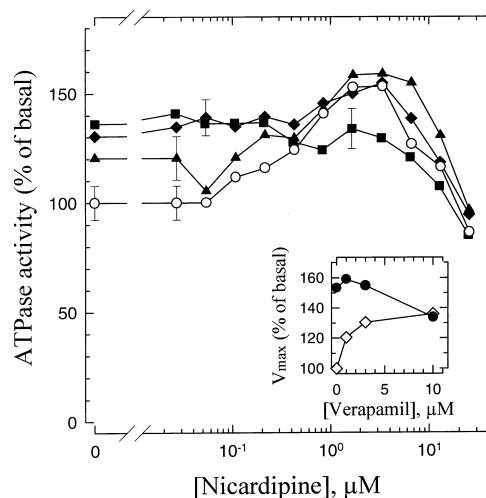
curves was obtained (results not shown). In particular, the ATPase activity measured at approx. 30 μM verapamil (the maximum of the bell-shaped curve in the absence of nicardipine) decreased for nicardipine concentrations above 1 μM, which was above those inducing stimulation of the basal P-gp ATPase (half-maximal activating concentration approx. 0.5 μM nicardipine), but below those inducing the inhibition of the basal P-gp ATPase (above approx. 5 μM nicardipine). These similarities to the results in Figure 3 indicate that these two modulators interact with P-gp by equilibrium binding to their different sites.

Effects of dihydropyridines on the P-gp ATPase activity stimulated by various drugs

The effects of azidopine in place of nicardipine on the verapamil concentration dependence of the P-gp ATPase activity were more convenient to analyse owing to the low activation factor of azidopine on the basal activity. As the initial azidopine concentration increased, the bell-shaped verapamil concentration dependences were altered, with a progressive decrease in the activation factors with respect to the basal activity level, whereas the half-maximal activating verapamil concentration remained constant (Figure 4). This reveals a pure non-competitive inhibition of the verapamil-stimulated P-gp ATPase and means that azidopine and verapamil can simultaneously bind to P-gp. The non-competitive inhibition constant for azidopine, approx. 7 μM, could be estimated from the secondary plot of azidopine concentration against the maximal ATPase activity of the bell-shaped curves (Figure 4, inset).
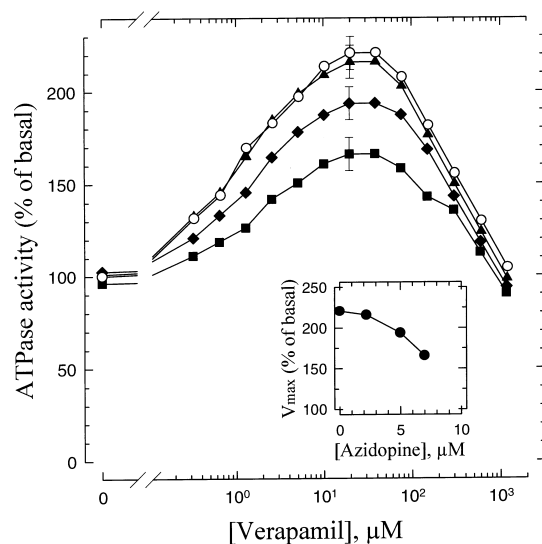
When the verapamil concentration dependence of the P-gp ATPase activity was measured in the presence of nitrendipine instead of azidopine, the inhibition pattern was representative of a mixed inhibition. Indeed, when the initial nitrendipine concentration increased, we observed both a progressive decrease in the activation factors with respect to the basal activity level and an increase in the half-maximal activating verapamil concentrations (results not shown). The secondary plot of nitrendipine concentration against the maximal ATPase activity of the bell-shaped curves permitted the estimation of the non-competitive inhibition constant for nitrendipine of $K_{\text{INC}} \approx 20$–$30$ μM. The secondary plot of nitrendipine concentration against the half-maximal activating verapamil concentration was linear, and gives the competitive inhibition constant (assuming a 1:1 competition stoichiometry) for nitrendipine of $K_{\text{IC}} \approx 25$–40 μM. The good agreement between these two evaluations of the inhibition constant for nitrendipine argues in favour of a mixed inhibition by nitrendipine of the verapamil-stimulated P-gp ATPase. These results show that nitrendipine can bind to P-gp simultaneously to verapamil, but with a decreased affinity, 25–30 μM, compared with the affinity observed in the absence of verapamil, 3–5 μM, with which it activates the basal P-gp ATPase. We can thus define a destabilization factor as the ratio of the apparent affinities determined in the presence and in the absence of a modulator: this destabilization factor is approx. 7 for verapamil on nitrendipine binding.

When the same experiments were performed with nimodipine instead of nitrendipine, a similar mixed inhibition pattern was observed, with an inhibition constant for nimodipine of approx. 10 μM (results not shown). This represents a destabilization
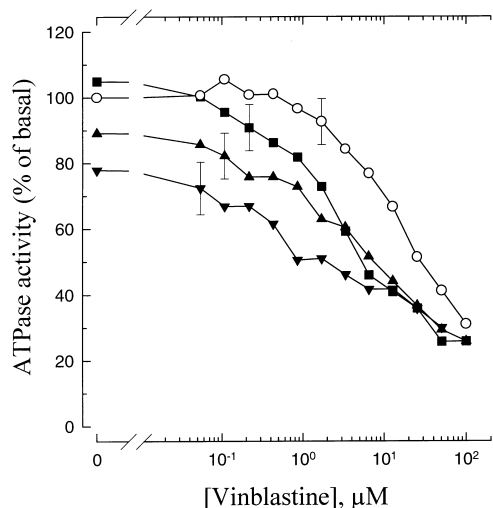
factor for verapamil on nimodipine binding of approx. 3–4 with respect to the half-activating nimodipine concentration of the basal P-gp ATPase activity, which is approx. 2.5–3 μM. In contrast, when the same experiments were performed with nifedipine instead of nitrendipine, only a decrease in the activation factor was observed, with no change in the half-maximal activating verapamil concentration (results not shown). This shows a pure non-competitive type of inhibition, with an evaluated inhibition constant for nifedipine of approx. 5 μM.

We have also performed similar experiments in which we studied the effects of increasing concentrations of nitrendipine, nimodipine or nifedipine on the nicardipine concentration dependence of the P-gp ATPase activity. In all cases the inhibition patterns closely resembled that observed for the influence of nitrendipine on the verapamil concentration dependence of the P-gp ATPase activity, i.e. a mixed-inhibition type pattern (results not shown). The same type of enzymic data analysis led to the evaluations of the corresponding inhibition constants: approx. 25 μM for nitrendipine, approx. 20–25 μM for nimodipine, and approx. 10 μM for nifedipine. These dihydropyridines can thus bind to P-gp simultaneously with nicardipine, but with decreased affinities compared with the affinities observed in the absence of nicardipine. The corresponding destabilization factors for nicardipine on nitrendipine, nimodipine or nifedipine binding were approx. 6, approx. 8 and approx. 2 respectively.

We then studied the effects of nitrendipine (50 μM) or nifedipine (13 or 50 μM) on the vinblastine concentration dependence of the P-gp ATPase activity. In the presence of nitrendipine, vinblastine induced an inhibition of the P-gp ATPase above approx. 0.1–0.2 μM instead of approx. 2 μM for the control curve without nitrendipine (Figure 5). This means that there is no competition between vinblastine and nitrendipine for the P-gp ATPase modulation. Conversely, the additional

inhibition induced by nitrendipine indicates the simultaneous binding of vinblastine and nitrendipine on P-gp. Similarly, in the presence of 13 or 50 μM nifedipine, vinblastine induced an inhibition of the P-gp ATPase above approx. 0.2–0.3 μM (Figure 5). This also indicates the simultaneous binding of vinblastine and nifedipine to P-gp. In contrast, the vinblastine concentration dependence of the P-gp ATPase activity in the presence of 50 μM nimodipine was similar to the control curve without nimodipine (results not shown).

DISCUSSION

Broad recognition by P-gp of various modulators of its ATPase activity

The stimulation of the P-gp ATPase activity by several MDR-reversing agents often results in a bell-shaped dependence on the reversing agent concentration. For verapamil, which has been widely studied, the exact value of the activation factor with respect to the basal activity can vary depending on the experimental system [30,32–34]. This is consistent with P-gp ATPase's modulating factors such as the nature of the lipid environment of P-gp [35]. In contrast, the half-maximally activating concentration of verapamil is largely independent of the experimental system; this characterizes its apparent affinity for P-gp (in the micromolar range). Like verapamil, nicardipine is an activator of P-gp ATPase, and the direct interaction between P-gp and these reversing agents is confirmed by the fact that, at their respective maximally activating concentrations, they decrease the Michaelis constant of P-gp for MgATP^{2-} (350–400 μM; results not shown) with respect to that measured for the basal activity (650–700 μM) [30]. In spite of there being no detectable stimulation of basal ATPase and no change in Michaelis constant for MgATP^{2-} induced by vinblastine, a cytotoxic drug that is effluxed out of the MDR cells via P-gp, the specific interaction of vinblastine with P-gp can be identified by its inhibitory effects on the drug-stimulated ATPase activity, as demonstrated previously on the verapamil- and progesterone-stimulated P-gp ATPase [11], and confirmed here by the consistent value of the inhibition constant (in the sub-micromolar range) calculated from the inhibition of the nicardipine-stimulated ATPase.

Within the dihydropyridine chemical family, nicardipine, nimodipine, nitrendipine, nifedipine and azidopine are all recognized by P-gp and modulate its basal and/or its drug-stimulated ATPase activity. The values of their apparent affinities are in fair agreement with the constants reported from kinetics experiments [10,36]. Moreover, the estimated affinity for nifedipine (approx. 5 μM) agrees with the half-maximal activation concentrations (from 5 to 20 μM) reported previously [32,33,37]. The dihydropyridine concentrations that stimulate P-gp ATPase are roughly correlated with their hydrophobicities, suggesting that these molecules should partition into the membrane before they interact with P-gp, as proposed from different experimental results [38–40] (see below). However, the existence of different activation factors demonstrates the specific character of the interaction between P-gp and these dihydropyridines, similarly to observations on a series of steroids [15].

P-gp is thus able to bind, on specific sites, molecules possessing various chemical structures and inducing modulations of its ATPase activity. This broad recognition by P-gp of various modulators of its ATPase activity is reminiscent of its broad recognition for various transport substrates, as should be expected for an active transport ATPase. The binding of P-gp modulators must associate several properties such as multiplicity,

high affinity and selectivity, which seems hard to reconcile with the simple model of a unique binding site.

Experimental evidence supporting a multi-site model for the binding of P-gp modulators

When both are in the presence of P-gp, the two P-gp ATPase activators verapamil and nicardipine do not induce additive stimulation, nor do they display mutually exclusive behaviour. Conversely, each of them induces non-competitive-type inhibitory effects with respect to the bell-shaped stimulation curve observed in the presence of the other alone. Because the first modulator is added at specifically activating concentrations, avoiding non-specific membrane effects, we can thus conclude that there are two distinct and specific binding sites for verapamil and nicardipine on P-gp.

We also demonstrate here a competitive interaction between vinblastine and nicardipine, but this result alone does not allow us to distinguish between the possibilities of either a unique common binding site or two mutually exclusive but distinct binding sites. A further insight into this question comes from the previous demonstration of a competitive interaction between vinblastine and verapamil [11]. Indeed, because nicardipine- and verapamil-binding sites are mutually non-exclusive, we can now conclude that the vinblastine-binding site is distinct both from the verapamil-binding site and from the nicardipine-binding site, because if it were common with either of these sites it could not be mutually exclusive with the other one. These distinct sites might be either distant sites linked by allosteric effects or overlapping sites related by steric constraints (see below). Experimental evidence of a vinblastine-binding site distinct from a dihydropyridine-binding site has been recently reported, with other experimental techniques [36,41], which strengthens our conclusions deduced from enzymological studies. Thus we arrive at the conclusion of a binding site for vinblastine separate from the binding sites for three MDR-reversing agents when including results obtained with progesterone [15], taking into account non-competitive effects between nicardipine and progesterone (results not shown). This is in agreement with the multi-site model for P-gp modulation previously developed [11].

The bell shape of the curve of P-gp ATPase stimulation by nicardipine allows a characterization of the type (competitive or non-competitive) of inhibition induced by other P-gp modulators because we can observe each type as well: pure competition by vinblastine and pure non-competition by nifedipine. When the dihydropyridine derivatives are tested, we never observe pure competitive effects for the modulation of the nicardipine-stimulated P-gp ATPase activity, showing that, although sharing closely related chemical structures, these dihydropyridines cannot bind at the same specific site. This conclusion agrees with a recent report demonstrating the simultaneous reversible binding of two newly synthesized dihydropyridines, benzoyldihydrocinnamic acid-dihydropyridine (BZDC-DHP) and B9309-012, at separate sites on P-gp [41]. Taken together, these experimental observations support the multi-site model for P-gp modulator-binding sites and extend similar conclusions drawn from a series of structurally related steroids [15]. In any case, additional photo-affinity labelling experiments should provide further arguments favouring the multi-site model for P-gp.

An interesting consequence of the binding of various MDR-reversing agents at distinct, mutually non-exclusive sites on P-gp separate from the binding site(s) for MDR-related cytotoxic drugs would be a synergistic inhibiting effect on P-gp-mediated drug transport when two (or more) of these MDR-reversing

agents were associated. There could be indications of such a possibility when considering the effects of associations of verapamil with either quinine [42] or cyclosporin A [43] for MDR reversion as assayed by cytotoxicity tests, although it was not proved in these reports that direct interactions with P-gp were the mechanism responsible for the observed synergies. In addition, we always observed in our experimental system that the simultaneous binding of two modulators to P-gp induced an inhibition of the ATPase activity in comparison with the activity measured in the presence of either one of these modulators. This could reflect the higher energetic barrier that P-gp encounters, as an active transporter, to translocate two different molecules across the plasma membrane than to translocate only one. This functional aspect of interactions between the modulator-binding sites of P-gp should require further analysis with transport experiments.

Interactions between the binding sites of P-gp modulators

The new characteristics here reported of the multi-site model for P-gp raise the possibility of mutually destabilizing interactions between the binding sites of P-gp modulators. Indeed, the modulation of the nicardipine-stimulated P-gp ATPase activity by other dihydropyridines is a mixed-type inhibition. This means that when two different dihydropyridine-binding sites are occupied, they interact with each other by decreasing their respective affinities. The corresponding destabilization factors (approx. 2, approx. 6 and approx. 8 for nifedipine, nitrendipine and nimodipine respectively) in the presence of nicardipine depend on the dihydropyridine considered, and especially on the length of its lateral chain. This could indicate subtle steric constraints between very close and interacting, more or less overlapping, specific binding sites: the larger the molecules, the higher the mutual destabilization factor. The effect of the exact structure of the dihydropyridine bound to P-gp was recently exemplified by the comparison between two reports by Boer et al. [41,44], who determined by binding inhibition experiments of either [³H]BZDC-DHP or [³H]B9209-005, two newly synthesized dihydropyridines, clearly different affinity constants for the other dihydropyridines nicardipine (0.21 and 0.55 μM), azidopine (0.11 and 1.0 μM) and B9109-012 (7.2 nM and 0.1 μM).

Mutual interactions are also observed for the binding by P-gp of compounds belonging to different chemical families. Indeed, verapamil and nicardipine interact on their binding sites by mixed-type effects, with the same destabilization factor (approx. 6) independently of the order of addition of these two modulators. Moreover, verapamil binding on P-gp is also subject to mixed inhibition by nimodipine and by nitrendipine, which indicates that the verapamil-binding site is distinct from, but interacts with, the dihydropyridine-binding sites to decrease their affinities (destabilization factors approx. 7 and approx. 4 for nitrendipine and nimodipine respectively). However, nifedipine and azidopine are purely mutually non-exclusive with respect to verapamil for P-gp ATPase stimulation. Thus the mutual interactions observed between verapamil and the dihydropyridine-binding sites are not exactly the same as those observed with nicardipine and the other dihydropyridine-binding sites. This suggests that the verapamil-binding site has specific interactions with each dihydropyridine-binding site. In addition, the binding of vinblastine and nicardipine to P-gp are mutually exclusive, whereas the binding of vinblastine is mutually non-exclusive with the binding of nitrendipine or nifedipine. This could be interpreted by a vinblastine-binding site partly overlapping the dihydropyridine-binding sites where, by steric hindrance, vinblastine prevents the binding of the dihydropyridine with the longest lateral chain
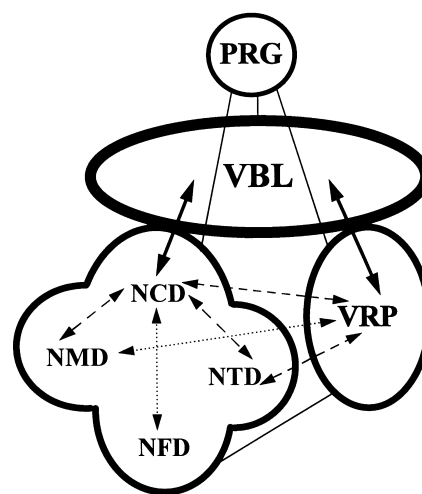
Scheme 1   Functional model of the relationships between the binding sites of some P-gp ATPase modulators

Arrows linked by bold lines represent mutual exclusivity, arrows linked by broken or dotted lines represent mutual destabilization of decreasing strength, and single lines represent mutual non-exclusivity. Abbreviations: VBL, vinblastine; PRG, progesterone; VRP, verapamil; NCD, nicardipine; NMD, nimodipine; NTD, nitrendipine; NFD, nifedipine.

but accommodates the dihydropyridines with the shortest lateral chain. Specific mutual interactions between dihydropyridines and verapamil or vinblastine have been reported as different inhibition constants for the displacement from P-gp of either [³H]BZDC-DHP or [³H]B9209-005 (14.6 and 3.6 μM respectively for verapamil, and 0.054 and 0.46 μM for vinblastine) [41,44].

Finally, our enzymic results allow us to confirm and further characterize the multi-site model for the binding of P-gp modulators. This means that P-gp has different binding sites for (1) MDR-related cytotoxic drugs and MDR-reversing agents, (2) MDR-reversing agents belonging to various chemical families, and (3) P-gp modulators belonging to the same chemical family. In addition, these different binding sites exhibit all the possible mutual relationships: pure mutual exclusion, mutual destabilization (with various destabilization factors) or pure mutual non-exclusion. A functional model of the relationships between the binding sites of some of the P-gp ATPase modulators is depicted in Scheme 1, which includes the interactions, always mutually non-exclusive, of progesterone with verapamil [15], with vinblastine [11] and with nicardipine. This functional model of P-gp displaying multiple, interacting binding sites for its modulators, at least some of which are subject to steric constraints, can be conveniently assigned to the analogy of a 'sticky paper model'. This tentative description of the molecular basis for the broad recognition by P-gp of various amphiphilic modulators is in good agreement with the three-dimensionally reconstructed low-resolution structure of P-gp reported recently, which shows a rather large intra-membranous opening to the central pore that could constitute the translocation pathway [27]. In this description there would be a relatively large surface of the intra-membranous part of P-gp that could bind amphiphilic molecules from the internal leaflet of the plasma membrane after their partition from the cytosol, and could thus accommodate a number of distinct specific binding sites. This model could shed light on the molecular mechanisms of this pharmacologically important membrane transporter.

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