

Characterization of Two Pharmacophores on the Multidrug Transporter P-Glycoprotein

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ABSTRACT

The multidrug transporter P-glycoprotein is a plasma membrane protein involved in cell and tissue detoxification and the multidrug resistance (MDR) phenotype. It actively expels from cells a number of cytotoxic molecules, all amphiphilic but chemically unrelated. We investigated the molecular characteristics involved in the binding selectivity of P-glycoprotein by means of a molecular modeling approach using various substrates combined with an enzymological study using these substrates and native membrane vesicles prepared from MDR cells. We determined affinities and mutual relationships from the changes in P-glycoprotein ATPase activity induced by a series of cyclic peptides and peptide-like compounds, used alone or in combination. Modeling of the intramolecular distribution of the hydrophobic and polar surfaces of this series of molecules made it possible to superimpose some of these

surface elements. These molecular alignments were correlated with the observed mutual exclusions for binding on P-glycoprotein. This led to the characterization of two different, but partially overlapping, pharmacophores. On each of these pharmacophores, the ligands compete with each other. The typical MDR-associated molecules, verapamil, cyclosporin A, and actinomycin D, bound to pharmacophore 1, whereas vinblastine bound to pharmacophore 2. Thus, the multispecific binding pocket of P-glycoprotein can be seen as sites, located near one another, that bind ligands according to the distribution of their hydrophobic and polar elements rather than their chemical motifs. The existence of two pharmacophores increases the possibilities for multiple chemical structure recognition. The size of the ligands affects their ability to compete with other ligands for binding to P-glycoprotein.

P-glycoprotein, the product of the *MDR1* gene in humans, is an ATP binding cassette transporter and is located in the plasma membrane of mammalian cells. It actively expels a number of amphiphilic molecules that enter the cell by passive diffusion (Stein, 1997). Because its transport substrates are often toxic xenobiotics, P-glycoprotein fulfills a cellular detoxification function (Schinkel, 1997). It is present in several tissues, such as intestinal mucosa, brain capillary endothelium, biliary canaliculus, and kidney tubules, and is therefore thought to be involved in the protection of the whole organism. The overproduction of P-glycoprotein in some cancer cells is responsible for the MDR phenotype, reducing the effectiveness of various cytotoxic drugs used in

anticancer chemotherapy (Gottesman and Pastan, 1993). Therefore, understanding P-glycoprotein functioning is of importance for controlling the bioavailability of many drugs of pharmaceutical interest and for improving anticancer chemotherapy. Also, the ability of P-glycoprotein to recognize a large number of chemically unrelated molecules, all amphiphilic and neutral or cationic, is of great theoretical interest because it contradicts the classical view of specific ligand-receptor interactions.

P-glycoprotein may recognize its various transport substrates by means of either one unique adaptable binding site or several different specific sites. The unique specific site hypothesis, in which all substrates compete with each other for binding to P-glycoprotein, was initially favored. It was thought that such a mechanism would account for the MDR-reversing effect of verapamil, which resensitizes MDR cells to vincristine (Naito and Tsuruo, 1989). The unique site mechanism was also proposed for the bonding of verapamil and

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ABBREVIATIONS: MDR, multidrug resistance; CSA, cyclosporin A; ACD, actinomycin D; BCT, bromocriptine; PI_A, pristinamycin I_A; PII_A, pristinamycin II_A; TTX, tetroxin; cLF, cyclo-leucylphenylalanine; VRP, verapamil; PRG, progesterone; VBL, vinblastine; BSe-TTX, (benzoyl-4-benzoyl)methylserine-tetroxin.

cyclosporin A, another MDR-reversing agent (Rao and Scarborough, 1994), and then for a larger series of P-glycoprotein ATPase modulators (Borgnia et al., 1996). However, an increasing amount of experimental evidence is consistent with the existence of different specific sites in the drug binding pocket of P-glycoprotein, as recently reviewed by Orlowski and Garrigos (1999). Indeed, drug-binding measurements (Tamai and Safa, 1991; Ferry et al., 1995; Boer et al., 1996; Martin et al., 2000), transport inhibition experiments (Ayesh et al., 1996), fluorescent dye uptake determinations (Shapiro and Ling, 1997), and ATPase modulation analyses (Orlowski et al., 1996; Garrigos et al., 1997; Litman et al., 1997a; Pascaud et al., 1998; Wang et al., 2000) all led to the same conclusion of several different specific sites on P-glycoprotein. However, the number of sites, their chemical selectivities, and their mutual relationships remain unclear. The molecular mechanism underlying the "multispecific" recognition of such a large number of amphiphilic molecules by P-glycoprotein is still poorly understood.

We investigated changes in P-glycoprotein ATPase activity as a means of studying the binding characteristics of P-glycoprotein to a series of cyclic peptide or peptide-like compounds. This group of molecules was chosen because they display steric constraints more marked than those of linear peptides, with the consequence that the various conformations that can be adopted by cyclic molecules are more restrained than those of linear peptides. These steric constraints are useful for explorations of the topology of specific binding sites (Gilon et al., 1991). We used the following molecules: 1) cyclosporin A (CSA), a cyclic undecapeptide; this immunosuppressant is a potent MDR reversing agent (Twentyman et al., 1987) that interacts directly with P-glycoprotein, as demonstrated by specific photoaffinity labeling (Foxwell et al., 1989), and is transported by P-glycoprotein (Saeki et al., 1993). 2) Actinomycin D (ACD), a polycyclic structure of the actinocin series bearing two cyclopentapeptides; this antibiotic is subject to cross-resistance in MDR cells (Biedler and Riehm, 1970), and inhibits the specific photolabeling of P-glycoprotein by various photoactive derivatives of known P-glycoprotein modulators, such as azidopine and verapamil (Safa, 1988). 3) Bromocriptine (BCT), a semi-synthetic ergot alkaloid composed of a tetracyclic nucleus (ergoline) bearing a hydrophobic cyclic tripeptide moiety; the entry of this dopaminergic receptor agonist into the central nervous system is limited by the blood-brain barrier, which is known to bear significant amounts of P-glycoprotein (Granveau-Renouf et al., 2000). It has also recently been reported that BCT can partially reverse MDR (Orlowski et al., 1998). 4) Pristinamycin I_A (PI_A) and pristinamycin II_A (PII_A), two cyclic macrolactones; PI_A is a hexapeptide and PII_A is polyunsaturated, and both have bacteriostatic activity. PI_A has been reported to be subject to basolateral-to-apical transport across an epithelial cell monolayer expressing P-glycoprotein at its apical membrane (Phung-Ba et al., 1995). 5) Tentoxin (TTX), a cyclic tetrapeptide with some host-specific phytotoxicity, probably caused by specific inhibition of the chloroplast ATP-synthase (Santolini et al., 1999). 6) Cycloleucinyphenylalanine (cLF), a cyclic dipeptide found in the media of cultures of fungi that produce tentoxin; it is the smallest natural cyclopeptide structure.

In this work, we combined enzymological analysis of the changes in P-glycoprotein ATPase activity induced by these

molecules, used alone or in combination, with a molecular modeling approach allowing superimposition of the three-dimensional hydrophobic and polar elements of these molecules. This made it possible to characterize two different types of pharmacophore in the drug-binding pocket of P-glycoprotein, which seems to be composed of several specific sites located close together. In this model, the binding of drugs to P-glycoprotein is governed by the distribution of their hydrophobic and polar elements rather than by their chemical motifs.

Materials and Methods

Chemicals. Na₂ATP (<1 ppm vanadium), phosphoenolpyruvate (monocyclohexyl ammonium), ouabain, verapamil (hydrochloride), progesterone (pregn-4-ene-3,20-dione), vinblastine (sulfate), bromocriptine, and actinomycin D were obtained from Sigma (Saint-Quentin Fallavier, France); cycloleucinyphenylalanine was obtained from Bachem (Bubendorf, Switzerland); pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Mannheim (Mannheim, Germany). Pristinamycins I_A and II_A, cyclosporin A, and tentoxin were kindly provided by Rhône-Poulenc-Rorer (Vitry, France), Galena State Corporation (Opava, Czech Republic), and B. Liebermann (Friedrich Schiller Universität, Jena, Germany), respectively. All other products were reagent grade.

Cell Culture and Preparation of P-Glycoprotein-Containing Membrane Vesicles. The MDR cell line used, DC-3F/ADX, was selected from spontaneously transformed DC-3F Chinese hamster lung fibroblasts on the basis of resistance to actinomycin D (Biedler and Riehm, 1970). The DC-3F/ADX cells were highly resistant to actinomycin D, vincristine, and colchicine, and their MDR phenotype was caused by overexpression of the *pgp1* gene. DC-3F/ADX cells and DC-3F cells (their sensitive parental counterparts) were grown to confluence in roller bottles (in the absence of actinomycin D), harvested by scraping (in the presence of protease inhibitors), and frozen until used for the preparation of membrane vesicles, as described previously (Garrigos et al., 1993).

Membrane vesicles were prepared from DC-3F and DC-3F/ADX cells as described previously (Garrigos et al., 1997), except that the final pellet of membrane vesicles was homogenized in phosphate-buffered saline supplemented with 2 mM MgCl₂ and 1 mM dithiothreitol, by passage through a 25-gauge needle. Membrane protein concentrations were determined by the method of Bradford (1976), using the Bio-Rad protein assay kit, with bovine serum albumin as the standard. In vesicles prepared from DC-3F/ADX cells, P-glycoprotein accounted for about 15% of membrane proteins, whereas in control vesicles prepared from the sensitive DC-3F cells, P-glycoprotein levels were too low to be detected with the monoclonal antibody C219 (Garrigos et al., 1993).

Determination of Drug Interaction with P-Glycoprotein by ATPase Modulation Analyses. The ATPase activity of P-glycoprotein is tightly correlated with its transport function, and the stimulation of P-glycoprotein ATPase by a given molecule strongly suggests that this molecule is a substrate transported by P-glycoprotein (Stein, 1997). Changes in P-glycoprotein ATPase activity after the addition of an amphiphilic drug can therefore be used to study the interaction of this drug with P-glycoprotein (Litman et al., 1997b; Schmid et al., 1999). In addition, analysis of the mutual effects on ATPase activity of the tested molecules and some well-known P-glycoprotein transport substrates can be used to detect competitive and noncompetitive inhibition, which strongly indicates that the interaction of these molecules with P-glycoprotein is specific (Borgnia et al., 1996; Garrigos et al., 1997). We used verapamil (VRP) and progesterone (PRG), two well known MDR-reversing agents, and vinblastine (VBL), a typical cytotoxic drug transported by P-glycoprotein, as typical P-glycoprotein substrates, because these compounds have been shown to bind to three different sites on P-glyco-

protein, with apparent affinities of about 1.5, 20, and 0.5 μM , respectively (Garrigos et al., 1997).

The theoretical kinetic analysis of changes in P-glycoprotein ATPase activity has been described elsewhere (Garrigos et al., 1997). Briefly, the kinetic data can be described according to a simple Michaelis-Menten model involving MgATP as the substrate S, with one or two ligands modulating the catalytic reaction. In the presence of a modulator M, the ATP hydrolysis rate changes, either increasing or decreasing. The rate of ATP hydrolysis after the addition of M will hereafter be referred to as "stimulated activity", in contrast to the "basal activity" measured in the absence of any added drug. The enzymatic activity of P-glycoprotein can be modeled as shown in Scheme 1.

We have shown previously (Garrigos et al., 1997) that the apparent dissociation constant K_d for M is determined by estimating the half-stimulating concentration of M in the reaction medium. When the enzyme binds two different modulators, M_1 and M_2 , this can be modeled by the general kinetic Scheme 2.

In the case of competitive inhibition between M_1 and M_2 (i.e., the form M_1M_2E does not exist), the inhibition constant for M_2 , K_I , is calculated according to the equation:

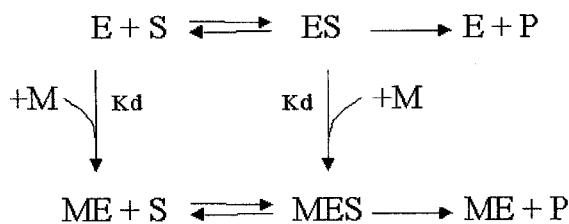
$K'_{1/2} = K_{1/2} (1 + [M_2]/K_I)$, where $K_{1/2}$ and $K'_{1/2}$ are the ATPase half-stimulating modulator M_1 concentrations in the absence and presence of the modulator M_2 , respectively. In the case of noncompetitive inhibition between M_1 and M_2 (i.e., the form M_1M_2E does exist), the inhibition constant for the modulator M_2 is simply deduced from the concentration inducing a 50% decrease in the maximal ATPase activity stimulated by M_1 . Practically, the determination of whether two compounds are competitive or noncompetitive requires the test of at least two concentrations of the first compound on the concentration dependence of the second compound on P-glycoprotein ATPase activity. The order of addition of the two compounds seems to be of no importance for the conclusion about their mutual relationships for modulating P-glycoprotein ATPase (Garrigos et al., 1997).

The ATPase activity of the membrane vesicle suspension was determined at 37°C using a coupled enzyme assay comprising an ATP-regenerating system and continuous spectrophotometric detection of NADH absorbance at 340 nm. We were therefore able to continuously monitor NADH consumption in the reaction medium, in 1:1 stoichiometry with ADP production, as described previously (Garrigos et al., 1997). The reaction medium consisted of 30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM KCl, 2 mM MgCl_2 , 1 mM dithiothreitol, and 1 mM MgATP, supplemented with 1 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, 0.5 mM NADH, and 0.1 mg/ml lactate dehydrogenase. ATPase activity was determined for 14 to 22 μg of membrane protein/ml. The assay medium was supplemented with 10 mM sodium azide, 0.5 mM ouabain and 1 mM EGTA to inhibit FOF1-ATPase, Na^+/K^+ -ATPase, and Ca^{2+} -ATPase, respectively. These ATPases account for 80 to 90% of the ATPase activity of membrane vesicles from sensitive cells devoid of P-glycoprotein, whereas in membrane vesicles from MDR cells, they account for only about 50% of total activity. The residual ATPase activity measured in the presence of the specific inhibitors of these enzymes and in the absence of any added drug is essentially due to the basal activity of P-glycoprotein (Garrigos et al., 1993). Successive additions of the compound tested were made in the same optical cuvette, with

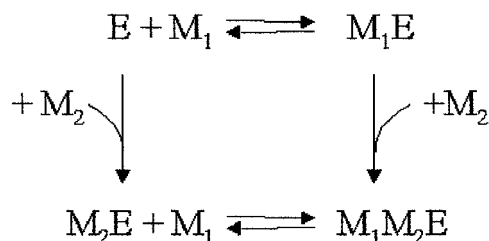
continuous stirring. Dilution effects were taken into account for the final ATPase activity calculations. The compounds tested (at the highest concentration used in the tested ranges on P-gp basal activity) were without inhibitory effects on the coupled enzymes, pyruvate kinase, and lactate dehydrogenase (as directly assayed by addition to the reaction medium of an ADP pulse). The compounds were added to the reaction medium from stock solutions prepared either in DMSO (for VRP, VBL, BCT, PI_A , PII_A , and cLF) or ethanol (for PRG, CSA, ACD, and TTX), or from 1:100 dilutions in distilled water. The solvent alone, the concentration of which never exceeded 1% (v/v) in the assay medium, had no effect on P-glycoprotein ATPase activity. Data were obtained from four different membrane vesicle preparations. The accuracy of ATPase activity measurement, evaluated from the accuracy of determination of the slope of the curve of absorbance over time, was about ± 10 nmol/mg/min (i.e., relative accuracy of about 5%), displayed as error bars on the graphs. Curves were fitted with SigmaPlot software, assuming Michaelian equations for the drug concentration dependences of ATPase activity.

The use of membrane vesicles prepared from DC-3F/ADX cells for the determination of changes in P-glycoprotein ATPase activity was validated by comparison with P-glycoprotein ATPase measurements performed on membranes prepared from Sf9 cells transfected with the *MDR1* gene (purchased from Interchim, Montluçon, France). These P-glycoprotein-containing membranes exhibited an ATPase activity specifically assigned to P-glycoprotein, with apparent affinities for VRP, PRG, and VBL similar to those obtained with membranes from DC-3F/ADX cells.

Molecular Modeling and Alignment. All the compounds tested were modeled with SYBYL version 6.6 software (Tripos, St. Louis, MO), running on a Silicon Graphics INDY R4400 SC workstation (SGI, Mountain View, CA). For CSA and ACD, minimization calculation was based on the structures given by the PDB, as obtained by NMR and X-ray crystallography, respectively (Protein Data Bank accession numbers 1CYB and 1A7Y, respectively). For TTX, minimization calculation was performed using constraints drawn from previous data obtained by NMR (Pinet et al., 1995). In the absence of available structural data for the other molecule structures, energy minimization was calculated from de novo free molecular dynamics under SYBYL 6.6. Free molecular dynamics calculations were performed at 300 K for 10 ps, with structure snapshots every 5 fs, using a Tripos force field with distance-dependent dielectric function, with a dielectric constant value of 1 as usual, and a cutoff value for nonbonded energy terms of 8 Å. To reach the most stable conformation for each molecule studied, we collected the 1981 structure snapshots between 100 fs and 10 ps. Each snapshot structure energy was minimized using the Boyden-Fletcher-Goldfarb-Shanno gradient method (a quasi-Newton approximation), with an energy gradient limit set to 0.05 kcal/mol/Å. Clusters were generated to determine the most stable conformational family. Owing to the rather simple structure of these molecules, the stable conformation obtained was unique for each molecule, except in the case of VRP. For VRP, molecular dynamics calculations revealed a flexible structure, and the major family of conformations corresponded to the lowest energy structures. This predominant conformation was thus selected for this modeling work. Hydrophobicity and H-bond potential areas were calculated with the MOLCAD routine of the SYBYL program.



Scheme 1.



Scheme 2.

The compounds were superimposed manually and consensus groups selected. The compounds were then aligned using the multifit command of SYBYL. We were unable to identify the common substructures with SYBYL alignment tools because the structural diversity of the compounds tested was too great. The results of the manual superimposition are expressed as a set of mean distances between the various chemical groups. Octanol/water partition coefficients were calculated with the logP routine under SYBYL 6.6, as described by Moriguchi et al. (1992).

Results

Specific Binding of Cyclic Peptides to P-Glycoprotein. The basal ATPase activity of P-glycoprotein was measured using membrane vesicles prepared from the MDR cells DC-3F/ADX, in the absence of added drugs, as described under *Materials and Methods*. The ATPase activity displayed by these vesicles, 150 to 250 nmol/mg of total membrane protein/min, is much higher than the residual activity measured on membrane vesicles prepared from the sensitive parental cells devoid of P-glycoprotein (30–40 nmol/mg of total membrane protein/min; see Fig. 1 at zero drug concentration). This higher level of activity corresponds to the inherent ATPase activity of P-glycoprotein. With increasing concentrations of various hydrophobic cyclic peptide mole-

cules, various types of P-glycoprotein ATPase activity modulation curve were observed (Fig. 1). ACD inhibited P-glycoprotein ATPase activity, with 50% inhibition at a concentration of $2 \pm 0.2 \mu\text{M}$, to reach a turnover rate of $20 \pm 3\%$ of the initial activity, near the level of the control vesicles. In contrast, TTX activated P-glycoprotein ATPase up to a maximum of $141 \pm 4\%$ of the basal activity, with a half-activating TTX concentration of $10 \pm 3 \mu\text{M}$. PI_A had no effect at concentrations below $50 \mu\text{M}$ (Fig. 1). The control experiments performed with membrane vesicles devoid of P-glycoprotein showed that ACD, TTX, and PI_A had no effect on ATPase activity in the presence (Fig. 1) or absence (data not shown) of ionic pump inhibitors. This demonstrates the specificity of the modulations observed with respect to P-glycoprotein. Table 1 (column "Effect on Basal ATPase Activity") summarizes the data collected with the various drugs tested. CSA moderately activated P-glycoprotein ATPase at low concentrations, with a half-activating concentration of 20 nM , and inhibited it at much higher concentrations, with a half-inhibiting concentration of 5 to $20 \mu\text{M}$, consistent with the results of a previous report (Litman et al., 1997b). Like ACD, BCT inhibited P-glycoprotein ATPase, but with a lower 50% inhibition concentration ($0.3 \mu\text{M}$), as reported previously (Orlowski et al., 1998). PII_A and cLF had no effect on basal ATPase activity at concentrations below 30 and $40 \mu\text{M}$, respectively.

Mutual Relationships between Drugs for Binding to P-Glycoprotein. We extended the analysis of drug interactions with P-glycoprotein by investigating the mutual effects on ATPase activity of the molecules tested and the typical P-glycoprotein transport substrates VRP, PRG, and VBL. If the ATPase activity of P-glycoprotein is first stimulated by a compound, subsequent changes induced by a typical substrate provide information about competition between the two molecules; this competition reveals an interaction of the compound tested with P-glycoprotein (Rao and Scarborough, 1994; Borgnia et al., 1996; Orlowski and Garrigos, 1999). For instance, Fig. 2A shows the effects of TTX and PI_A on the VRP-induced stimulation of P-glycoprotein ATPase. TTX, at a concentration of $100 \mu\text{M}$, had no significant effect on the control curve for the VRP-induced stimulation of P-glycoprotein ATPase activity. In contrast, PI_A modified the control curve by a competitive mechanism, as shown by the increase in the half-activating concentration of VRP in the presence of 15 or $30 \mu\text{M}$ PI_A (see Fig. 2B and legend to 2A), giving an inhibition constant of about $5 \mu\text{M}$. However, PI_A inhibited the PRG-induced stimulation of P-glycoprotein ATPase activity by a noncompetitive mechanism, as shown by the unchanged half-activating concentration of PRG (Fig. 2C). As another example, ACD at $5 \mu\text{M}$ increased the half-activating concentration of VBL by a factor of 4, which corresponds to a competition with an inhibition constant of about $1.5 \mu\text{M}$ (Fig. 2D). The whole data set for the seven molecules tested on VRP-, PRG-, or VBL-stimulated P-glycoprotein ATPase activity is shown in Table 1 (column "Effect on Stimulated ATPase Activity"). In particular, we observed a specific interaction between PI_A and P-glycoprotein, with an apparent affinity constant of about $10 \mu\text{M}$, although PI_A had no effect on basal ATPase activity. We also observed specific binding of TTX, which increased ATPase activity from basal levels and competed with VBL for this activation. A possible specific interaction between PII_A and P-glycoprotein is suggested by

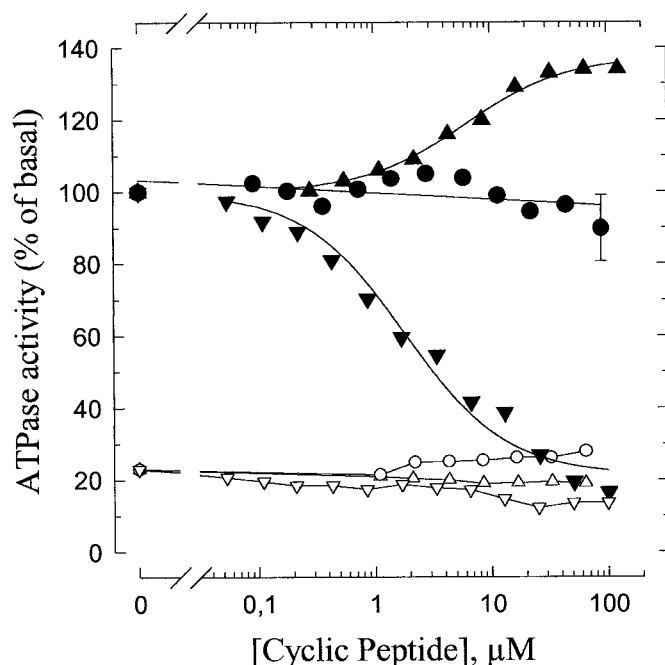


Fig. 1. Modulation of basal P-glycoprotein ATPase activity by hydrophobic cyclic peptide compounds. The ATPase activity of a suspension of P-glycoprotein-containing membrane vesicles (closed symbols) or of control P-glycoprotein-devoid membrane vesicles (open symbols) was measured spectrophotometrically using a coupled enzyme assay, as described under *Materials and Methods*. The reaction medium contained about 20 (closed symbols) or about 35 (open symbols) μg of membrane proteins/ml and 1 mM MgATP, in the presence of 10 mM sodium azide, 0.5 mM ouabain, and 1 mM EGTA as ionic membrane ATPase inhibitors. Increasing concentrations of TTX (triangles), PI_A (circles), or ACD (inverted triangles) were obtained by sequential additions to the medium in the optical cuvette under continuous stirring (at 37°C). ATPase activities are normalized with respect to the activity measured in the absence of any added drug for P-glycoprotein-containing vesicles ("basal activity"). The Michaelian parameters V_{max} and K_m were calculated as described under *Materials and Methods*: for TTX, $K_m = 10 \pm 3 \mu\text{M}$ and $V_{\text{max}} = 141 \pm 4\%$; for ACD, $K_m = 2 \pm 0.2 \mu\text{M}$ and $V_{\text{max}} = 20 \pm 3\%$.

the inhibition of PRG-induced ATPase activation, with an inhibition constant of about 5 μM . In contrast, cLF had no measurable effect on P-glycoprotein ATPase activity, either basal or stimulated, suggesting the absence of any specific interaction with P-glycoprotein. The other three drugs tested, CSA, ACD, and BCT, which have already been re-

ported to bind to P-glycoprotein (Safa, 1988; Foxwell et al., 1989; Orlowski et al., 1998), were included in this series to enable us to characterize quantitatively their interactions with their specific sites in our experimental system. Their affinities, calculated from both modulation of the basal ATPase activity and mutual interactions with the typical P-

TABLE 1
 Binding of various hydrophobic cyclic peptide molecules on P-glycoprotein as measured by modulation of its ATPase activity
 Apparent K_a values are determined as the means of the half-maximal activating concentration for the basal ATPase activity and of the inhibition constants for the stimulated ATPase activities.

	MM	LogP ^a	Effect on Basal ATPase Activity	Effect on ATPase Activity Stimulated by			Apparent K_a
				VRP (455 Da) (logP = 3.2)	PRG (314 Da) (logP = 4.1)	VBL (811 Da) (logP = 3.0)	
	Da		μM		μM	μM	μM
CSA	1203	-0.32	Act (0.020) Inh (5-20)	C (0.015)	N (0.04)	C (0.05-0.1)	0.03
ACD	1255	-2.1	Inh (2)	C (0.5)	None (<5)	C (1-2)	1
BCT	750	3.1	Inh (0.3)	C (0.2)	N (0.1)	N (< 4)	0.2
PI _A	854	0.07	None (<50)	C (5)	N (15-20)	N (< 60)	10
PII _A	524	1.2	None (< 30)	None (< 60)	N (5)	None (< 60)	5
TTX	415	0.77	Act (10)	None (<100)	None (< 30)	C ^b	10
cLF	260	1.6	None (< 40)	None (<40)	None (<40)	None (<40)	

Act, activation of the basal ATPase activity of P-glycoprotein (half-maximal activating concentration in brackets) Inh, inhibition of the basal ATPase activity of P-glycoprotein (half-maximal inhibitory concentration in brackets); None: The basal ATPase activity of P-glycoprotein is unchanged; highest drug concentration tested in brackets; C, competitive inhibition of the stimulated ATPase activity of P-glycoprotein (inhibition constant in brackets); N, noncompetitive inhibition of the stimulated ATPase activity of P-glycoprotein (inhibition constant in brackets); MM, molecular mass.

^a Calculated according to SYBYL software.
^b Competition observed by the effect of VBL on the ATPase activation induced by TTX.

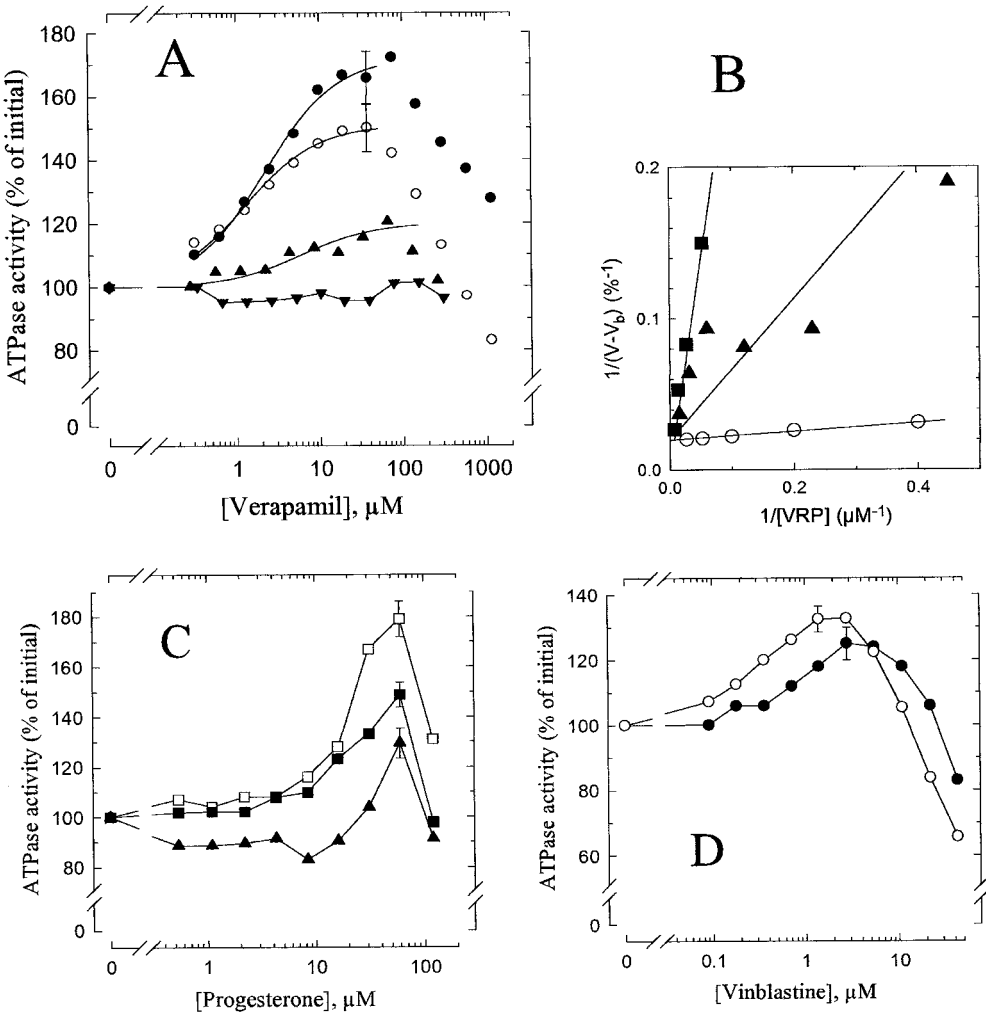


Fig. 2. Modulation by hydrophobic cyclic peptides of the P-glycoprotein ATPase activity stimulated by verapamil, progesterone, or vinblastine. The ATPase activity was measured in a suspension of P-glycoprotein-containing membrane vesicles as described in Fig. 1. ATPase activities were normalized with respect to the basal activity value for the control curves (open symbols) or to the initial activity value measured after adding the modulating compound (before VRP, PRG, or VBL additions) for the other curves. A, increasing concentrations of VRP were obtained by sequential additions to the medium, in the absence (○) or presence of TTX at 100 μM (●), or of PI_A at 15 μM (▲) or 60 μM (▼). The two error bars drawn, representing the measurement accuracy, show the weakness of the significance for the effect of 100 μM TTX. The Michaelian parameters V_{max} and K_m were calculated as described under *Materials and Methods*: for VRP alone, $K_m = 1.3 \pm 0.2 \mu\text{M}$ and $V_{\text{max}} = 151 \pm 2\%$; for VRP in the presence of 100 μM TTX, $K_m = 1.3 \pm 0.2 \mu\text{M}$ and $V_{\text{max}} = 165 \pm 2\%$; for VRP in the presence of 15 μM PI_A, $K_m = 6 \pm 1 \mu\text{M}$ and $V_{\text{max}} = 120 \pm 1\%$. B, double-reciprocal plot for the dependence of P-glycoprotein ATPase activity on VRP concentration in the absence (○) or presence of PI_A at 15 μM (▲) or 30 μM (■). V_b is the basal level, set at 100%. C, increasing concentrations of PRG were obtained by sequential additions to the medium, in the absence (□) or presence of PI_A at 12 (■) or 30 μM (▲). D, increasing concentrations of VBL were obtained by sequential additions to the medium, in the absence (○) or presence of ACD at 5 μM (●).

glycoprotein substrates, were 30 nM, 1 μ M, and 0.2 μ M, respectively (Table 1, last column).

We also studied the mutual effects on P-glycoprotein ATPase of pairs of hydrophobic cyclic peptides, to characterize further their interaction with P-glycoprotein as a means of investigating the topology of the binding domain of this protein. Because TTX was the most effective activator of P-glycoprotein ATPase in this series, we investigated the ability of the other compounds to affect stimulation by TTX (Fig. 3). PI_A , at a concentration of 30 μ M, markedly inhibited TTX-induced ATPase activation, whereas 30 μ M PII_A had no effect. In contrast, CSA, at a concentration of 30 nM, increased TTX-induced ATPase stimulation, without affecting the half-activating concentration of TTX (Fig. 3). Because CSA alone only slightly activates P-glycoprotein ATPase (even less than VBL), this increasing effect is synergistic (see *Discussion*). In addition, this activation by CSA could not be attributed to a nonspecific effect because it was not observed with the other molecules tested or with control vesicles.

In conclusion, some of the molecules tested (PRG, PII_A) displayed only noncompetitive interactions with the other molecules and therefore must bind to different sites on P-glycoprotein. Other molecules (VRP, CSA, ACD, VBL), however, displayed competitive effects; such mutual exclusions between two molecules for binding to P-glycoprotein may be caused by binding to a common site or by steric constraints between separate but close sites, preventing the two molecules from binding simultaneously (see *Discussion*).

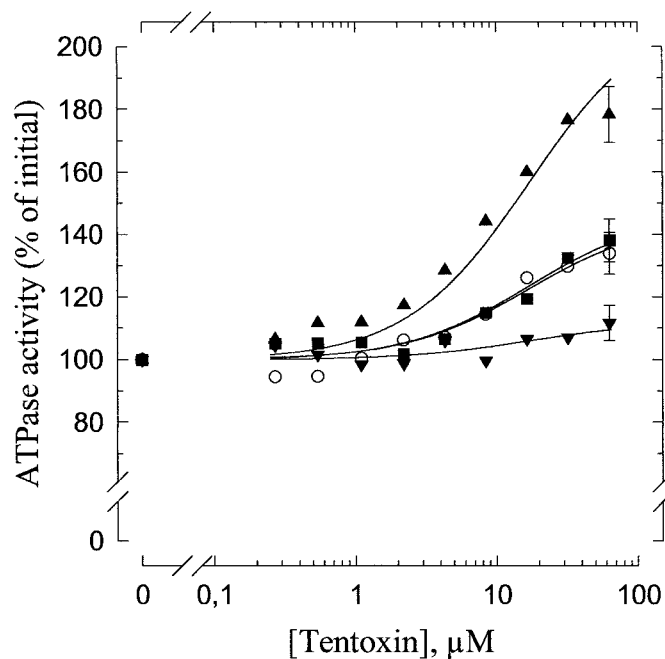


Fig. 3. Modulation by hydrophobic cyclic peptides of the P-glycoprotein ATPase activity stimulated by TTX. The ATPase activity was measured in a suspension of P-glycoprotein-containing membrane vesicles as described in Fig. 1. Increasing concentrations of TTX were obtained by sequential additions to the medium, in the absence (\circ) or presence of 30 μ M PI_A (\blacktriangledown), 30 μ M PII_A (\blacksquare), or 30 nM CSA (\blacktriangle). ATPase activities were normalized with respect to the basal activity value for the control curve (\circ) or to the initial activity value measured after addition of the modulating compound (before TTX additions) for the other curves. The Michaelian parameter V_{\max} was calculated as described under *Materials and Methods*: for TTX alone, $V_{\max} = 145 \pm 7\%$; for TTX in the presence of 30 μ M PI_A , $V_{\max} = 112 \pm 2\%$; for TTX in the presence of 30 μ M PII_A , $V_{\max} = 147 \pm 3\%$; for TTX in the presence of 30 nM CSA, $V_{\max} = 213 \pm 5\%$.

Potential Area Calculations and Correlation with Binding to P-Glycoprotein. Molecular modeling was used to determine the three-dimensional structures of all the compounds studied (the cyclic peptides and the three typical P-glycoprotein substrates). In particular, we studied the distribution of hydrophobic surfaces (caused by aromatic, alkyl chains and double-bond moieties) and polar surfaces (caused by electron donor or acceptor groups, such as carbonyl, hydroxyl, or amino groups) of these molecules, as depicted in Fig. 4. The hydrophobic and polar elements of each molecule were then compared with those of the other molecules in an attempt to identify consensus elements correlated with the enzymological data (Table 1). Because VRP binding to P-glycoprotein excludes the binding of a large proportion of the molecules tested, we investigated whether the molecules concerned have structural elements in common with VRP. The MOLCAD routine of SYBYL software allowed us to superimpose the hydrophobic and polar surface of VRP with those of CSA, ACD, BCT, and PI_A . In each case, a good match was observed, within 0.8 Å of precision for the aromatic planes and electron donors and 1.5 Å of precision for the alkyl areas (Fig. 4A). In addition, all five molecules displayed three consensus elements, as shown in Fig. 5, top. CSA, ACD, and VRP also displayed a fourth consensus alkyl area (Fig. 5, top). In contrast, if VRP and VBL were compared, no common element set was identified between their hydrophobic or polar surfaces (Fig. 4C). However, experimental evidences for competition between VRP and VBL have been previously reported (Garrigos et al., 1997; Shepard et al., 1998), and such a competition has been suggested from molecular modeling (Zamora et al., 1988).

The competition between VBL and TTX led us to compare the hydrophobic and polar surfaces of these molecules. We observed a good match (within 0.8 Å for the aromatic planes and electron donors and 1.5 Å for the alkyl areas) between the two structures when superimposed (Fig. 4B). The corresponding consensus elements are shown in Fig. 5, bottom. In contrast, no common hydrophobic or polar element could be

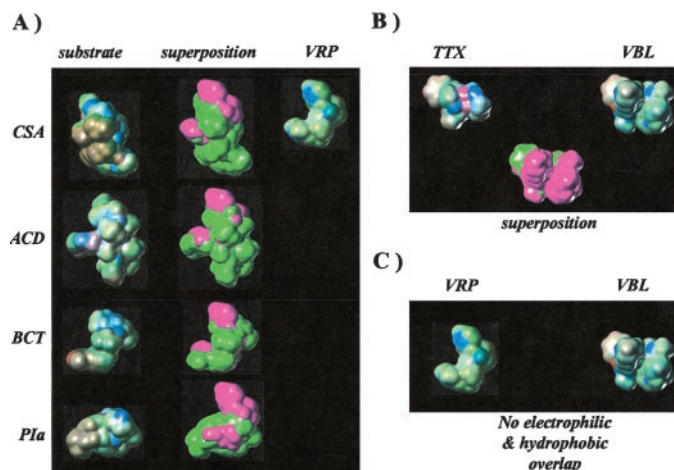
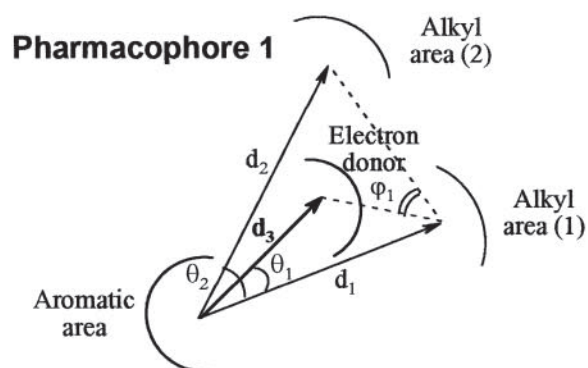
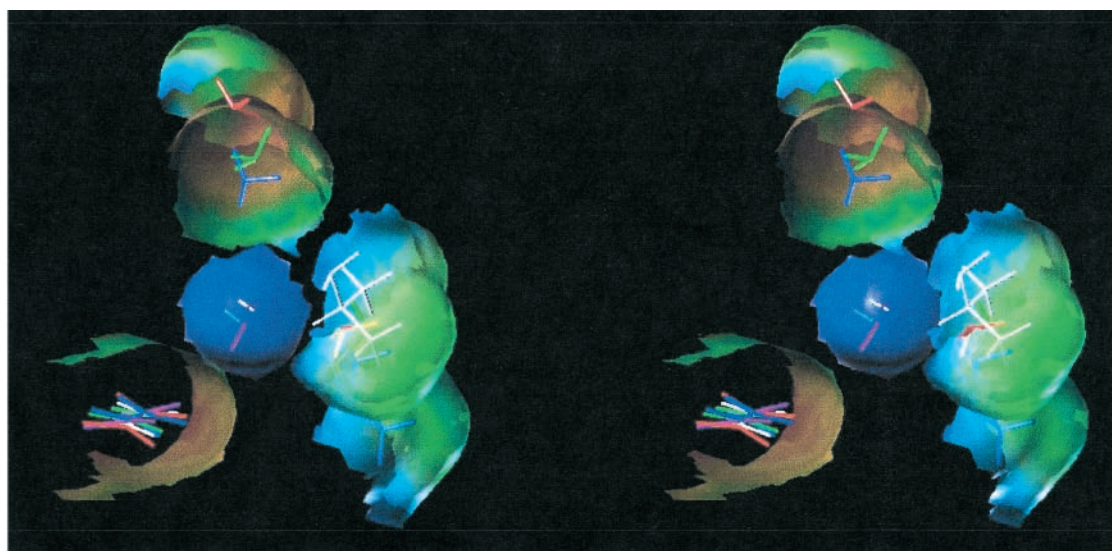


Fig. 4. Molecular modeling of the compounds involved in pharmacophore building. Color-coded hydrophobic and polar surfaces were calculated using MOLCAD under SYBYL 6.6 software (see *Materials and Methods*). The hydrophobic regions are shown from green (low hydrophobicity) to brown (high hydrophobicity), and the polar regions in blue (electron donor groups) and red (electron acceptor groups). The superpositions are shown separately for clarity: VRP with CSA, ACD, BCT, or PI_A (A); VBL with TTX (B); VRP with VBL, without possible superposition (C).



$$d_1 = 8.6 \pm 0.9 \text{ \AA}$$

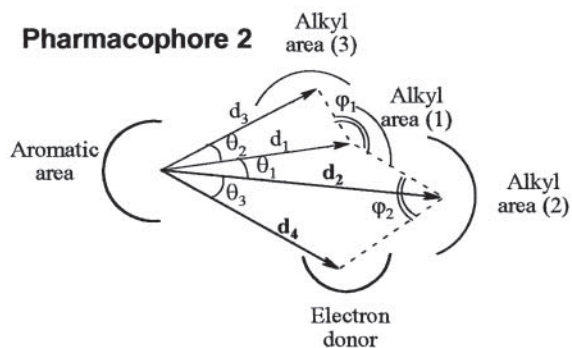
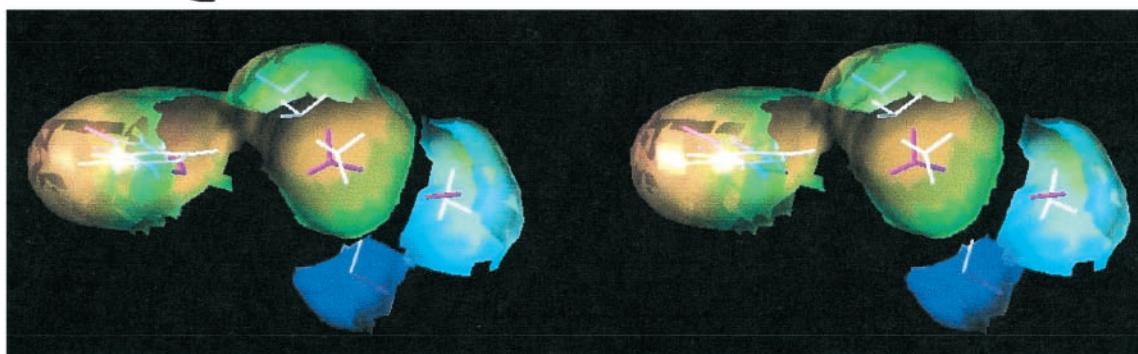
$$d_2 = 9.6 \pm 1.2 \text{ \AA}$$

$$d_3 = 8.6 \pm 0.8 \text{ \AA}$$

$$\theta_1 = 32 \pm 9^\circ$$

$$\theta_2 = 50 \pm 5^\circ$$

$$\phi_1 = 91 \pm 13^\circ$$



$$d_1 = 7.5 \pm 1.5 \text{ \AA}$$

$$d_2 = 10.0 \pm 1.4 \text{ \AA}$$

$$d_3 = 5.5 \pm 1.3 \text{ \AA}$$

$$d_4 = 9.2 \pm 1.4 \text{ \AA}$$

$$\theta_1 = 45 \pm 10^\circ$$

$$\theta_2 = 28 \pm 15^\circ$$

$$\theta_3 = 28 \pm 4^\circ$$

$$\phi_1 = 89 \pm 30^\circ$$

$$\phi_2 = 34 \pm 1^\circ$$

Fig. 5. Two pharmacophores for multidrug recognition by P-glycoprotein. Stereoscopic views of the MOLCAD representation of the pharmacophores depicting drug molecular recognition by P-glycoprotein, as deduced from molecular modeling data shown in Fig. 4, for VRP, CSA, ACD, BCT, and PI_A (top, pharmacophore 1) and for VBL and TTX (bottom, pharmacophore 2). The consensus elements of the molecules tested that can be superimposed are shown to fit the points defining the pharmacophores. Under each is shown a schematic representation of the corresponding pharmacophore, with characteristic angle and distance values connecting the pharmacophoric points in the space. Pharmacophore 1 is described by four elements, knowing that the uppermost pharmacophoric point, the alkyl area (2), fits only three molecules, CSA, ACD, and VRP, of the five molecules giving rise to pharmacophore 1.

found if VBL was compared with either CSA or ACD (not shown). Finally, in contrast to the other molecules of the series, PRG and PII_A, which did not compete with any of the other drugs tested, did not share any common hydrophobic or polar surfaces (not shown).

Discussion

Using a combination of a functional approach based on enzymological analysis and a structural approach based on the molecular modeling of various substrates, we were able to identify molecular properties responsible for a specific interaction with P-glycoprotein. We characterized two different pharmacophores that were responsible for drug recognition, based on three-dimensional consensus hydrophobic and polar elements rather than on chemical motifs. We also found that these two pharmacophores were located very close together and partially overlapped. These data shed light on the molecular mechanisms by which P-glycoprotein specifically recognizes molecules with various chemical structures.

Enzymatic Analysis of the Multispecific Recognition of Various Compounds by P-Glycoprotein. In this study, we selected various cyclic peptide compounds, all of which were amphiphilic, a prerequisite for P-glycoprotein substrates, which probably interact with P-glycoprotein after they partition into the lipid phase (Ferté, 2000). The series of molecules tested here included molecules composed of 2 to 11 residues (molecular masses of 260 to 1255 Da). P-glycoprotein has been shown to contain at least two different specific binding sites (Orlowski and Garrigos, 1999). The binding of two different substrates may therefore be either mutually exclusive or nonexclusive. Thus, studies of mutual interactions between pairs of substrates interacting with P-glycoprotein can provide information about the steric constraints between these substrates, especially if they are large or rigid molecules, and thus may reveal overlap between neighboring sites.

Specific interactions between a given substrate and P-glycoprotein are known to induce changes in P-glycoprotein ATPase activity (Litman et al., 1997b; Schmid et al., 1999). The three molecules that mostly increased activity from basal level, VRP, PRG, and TTX, were the smallest molecules in this series. However, changes in P-glycoprotein ATPase activity levels were not correlated with either hydrophobicity or the intramolecular distribution of hydrophobic and polar elements in the molecules tested. In particular, VRP and TTX are representative of the two different pharmacophores described. Thus, the structure-activity relationship for the ATPase does not have the same determinants as that for the recognition of molecules for binding on P-glycoprotein. This may reflect the difference of molecular characteristics between the step of transmembrane translocation coupled to MgATP hydrolysis and the initial step of binding to the transport site.

In this study, we focused on the characteristics of drug recognition by P-glycoprotein, using ATPase modulation as a probe for analyzing specific interactions with P-glycoprotein. Specific interaction with P-glycoprotein was observed for six of the seven compounds tested, and the apparent affinities of these molecules ranged from 30 nM to 10 μ M (Table 1). Taking into account all 10 molecules tested (including VRP, PRG, and VBL), the affinity constant was positively corre-

lated with molecular mass. This suggests that larger molecules interact more strongly with the hydrophobic binding pocket of P-glycoprotein, probably because they have a larger number of interaction points, consistent with a previous report considering the relationship to Van der Waals surface area (Litman et al., 1997b). cLF (260 Da) is therefore probably too small to be recognized (below 40 μ M) by P-glycoprotein. For the sake of comparison, the smallest molecules transported by P-glycoprotein are phenytoin (252 Da) and morphine (285 Da) (Schinkel et al., 1996).

Molecular Alignment of Peptide Compounds Recognized by P-Glycoprotein. In previous studies, the hydrophobicity of molecules was found to be correlated with their interaction with P-glycoprotein (Zamora et al., 1988; Klopman et al., 1997). Molecular shape has also been shown to play a role in recognition by P-glycoprotein, in particular for VRP and VBL, which presented some common features (Zamora et al., 1988), and several attempts have been made to find consensus elements for binding to P-glycoprotein (Klopman et al., 1997; Ecker et al., 1999; Wiese and Pajeva, 2001). We observed no correlation between the overall hydrophobicity of the compounds tested and apparent affinity (Table 1). However, molecular modeling made it possible to superimpose some of the hydrophobic and polar surfaces of the molecules tested (Fig. 4), demonstrating that three-dimensional consensus elements exist for VRP, CSA, ACD, BCT, and PII_A. Our results strongly suggest that these molecules bind to P-glycoprotein at the same site, defining a first consensus binding site, or pharmacophore, in the binding domain of P-glycoprotein (Fig. 5, top, pharmacophore 1). Similarly, molecular alignment of VBL and TTX showed that these molecules defined another pharmacophore (Fig. 5, bottom, pharmacophore 2). Thus, the intramolecular distribution of hydrophobic and polar surfaces is a more relevant parameter for binding to P-glycoprotein than overall hydrophobicity or precise chemical motifs. This view is consistent with two recent theoretical reports indicating the importance of electron donor groups in molecules recognized by P-glycoprotein (Seelig, 1998; Osterberg and Norinder, 2000). In addition, during the termination of our work, two joined papers were published reporting on three-dimensional quantitative structure-activity relationships of various P-gp inhibitors and substrates (Ekins et al., 2002a,b); they performed modeling of different pharmacophores composed of hydrophilic and H-bond acceptor elements, allowing in particular recognition of VRP, BCT, and VBL.

Evidence for Different Specific Binding Sites on P-Glycoprotein. All compounds tested against PRG or PII_A displayed noncompetitive inhibition, suggesting that PRG and PII_A are recognized by two binding sites different from those that recognize the other molecules tested, as previously reported for PRG (Orlowski et al., 1996; Shapiro et al., 1999). This observation is consistent with the fact that molecular modeling identified no consensus elements between PRG or PII_A and the other molecules. In addition, the mutual activation effect between TTX and CSA demonstrates that these two molecules can bind simultaneously to P-glycoprotein. This observation is reminiscent with that reported by Sharom and coworkers for synthetic hydrophobic linear peptides, such as NAc-LLY-amide, activating the P-glycoprotein-mediated transport of colchicine into vesicles (Sharom et al., 1996). Also, Shapiro and Ling (1997) have described a coop-

erative interaction between two transport sites named H and R and characterized by specific recognition of Hoechst 33342 and rhodamine 123, respectively.

Characterization of the Two Pharmacophores Involved in Drug Binding to P-Glycoprotein. Analysis of the mutual interactions between pairs of P-glycoprotein substrates made possible the topological mapping of the P-glycoprotein binding pocket. Mutual exclusion for binding (corresponding to competitive inhibition) was observed for several pairs of molecules: 1) VRP competed with the largest drugs tested (ACD, CSA, BCT, PI_A , and VBL), and 2) VBL competed with large (ACD, CSA, and VRP) and small (TTX) molecules. This observation that large molecules compete for binding with a wider set of substrates than do small molecules may be caused by steric exclusion by binding to largely overlapping sites, rather than binding to allosteric sites with negative heterotrope relationships. This study, combining enzymological data and a three-dimensional modeling ap-

proach, enabled us to draw further conclusions about the mutual relationships between substrates. In particular, taking into account that 1) some ligands (VRP, CSA, and ACD) in the first pharmacophore display competitive inhibition with a ligand (VBL) in the second pharmacophore, 2) the molecular modeling data show a protruding part for the superimposed molecules CSA, ACD, and VRP on one hand and for VBL on the other hand, we suggest that mutual exclusion results from steric constraints caused by the overlapping of these protruding parts (Fig. 6). In this model, the two pharmacophores are located such that the smaller molecules BCT and PI_A on one hand and TTX on the other hand display no mutual exclusion, as experimentally observed. The relative orientation of the two pharmacophores, by axial rotation or lateral bending, cannot be determined (see Fig. 6).

Predictive Capability of the Three-Dimensional Molecular Model for Binding to P-Glycoprotein. We wanted to check the validity of this molecular binding model,

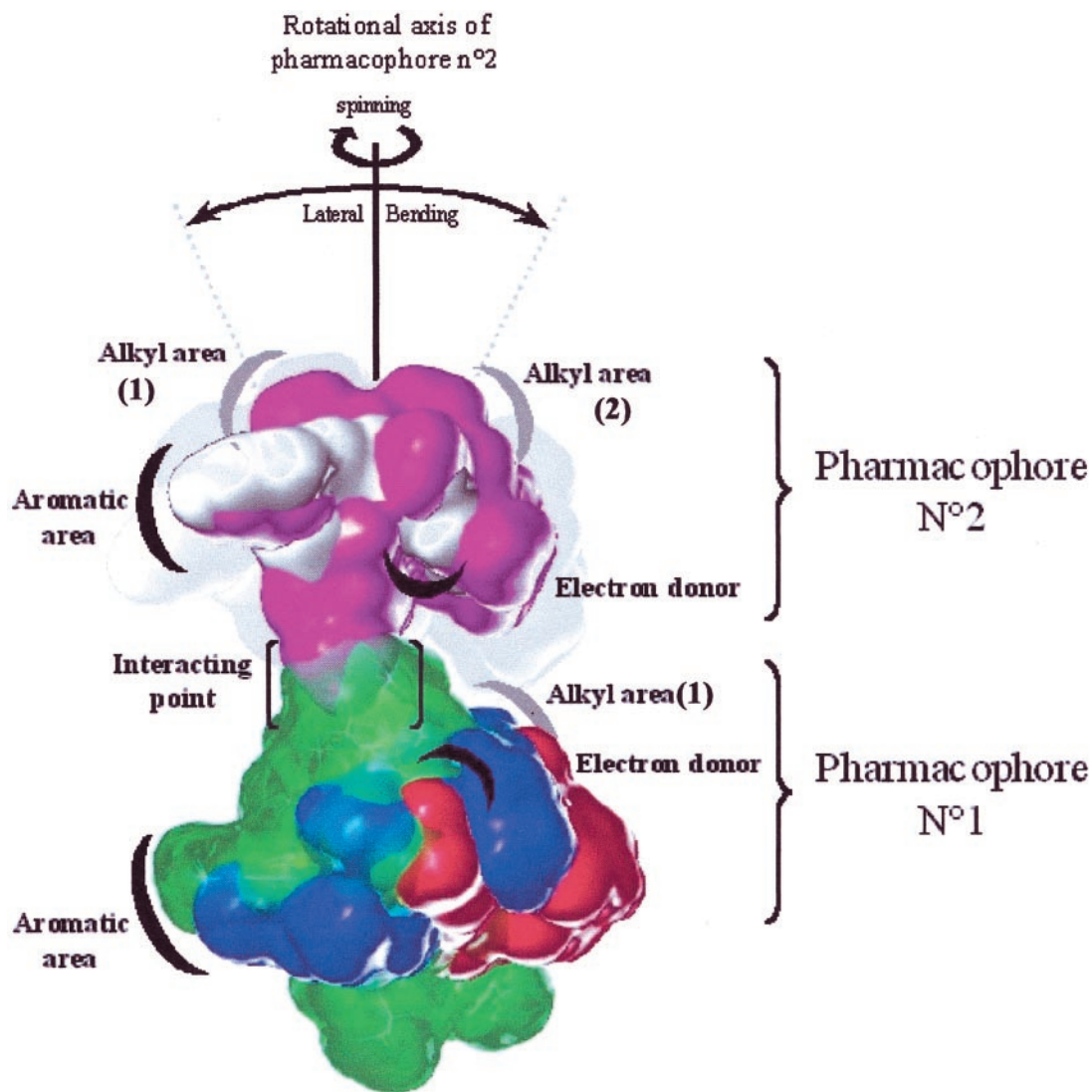


Fig. 6. Multisite model for P-glycoprotein drug binding. MOLCAD representation of the molecules involved in the drug binding model for P-glycoprotein as designed from the closeness of the two characterized pharmacophores shown in Fig. 5, according to the experimental data (see Discussion). CSA, ACD, and VRP are in green, BCT is in blue, PI_A in red, VBL in pink, and TTX in white. The various pharmacophoric points are indicated, except the alkyl area (2) of pharmacophore 1, which is located at the level of the interacting point, and the alkyl area (3) of pharmacophore 2, which is located behind the uppermost part of the model. The arrows indicate the possible freedom for spinning or bending of the two pharmacophores. Otherwise, PRG and PII_A probably bind to separate sites.

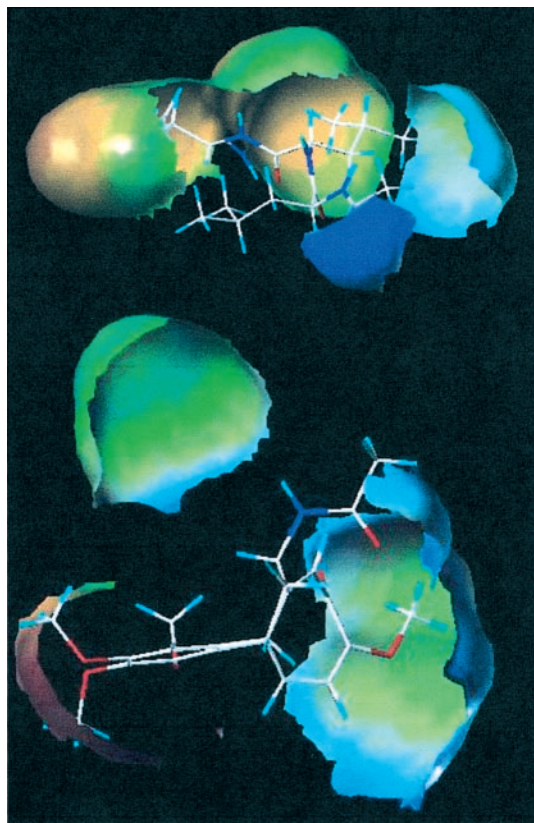


Fig. 7. Molecular fit of colchicine on pharmacophore 1 and NAc-LLY-amide on pharmacophore 2. The two molecule skeletons are shown in regards to the various pharmacophoric points displayed in Fig. 5.

especially as regards the relative location and the closeness of the two pharmacophores. We reasoned that a molecule formed by adding a bulky moiety to the inferior face of TTX (oriented as in Fig. 6) should bind to pharmacophore 2 and display exclusive interactions with the molecules that bind to the upper part of pharmacophore 1 (VRP, CSA, and ACD). We therefore studied (benzoyl-4-benzoyl)-methylserine-TTX (BSe-TTX), a derivative initially synthesized for photolabeling experiments but used here without irradiation. BSe-TTX inhibited the basal ATPase activity of P-glycoprotein with an apparent affinity of about $1\ \mu\text{M}$ (not shown). This molecule therefore has a higher affinity than TTX, consistent with general observations concerning the effects of larger, more hydrophobic molecules, compared with a parent molecule (Pascaud et al., 1998). BSe-TTX and TTX displayed mutual exclusion for P-glycoprotein ATPase activation, with an inhibition constant for BSe-TTX of about $0.3\ \mu\text{M}$ (not shown). BSe-TTX also displayed competitive inhibition of VRP-induced P-glycoprotein ATPase stimulation, with an inhibition constant of about $0.5\ \mu\text{M}$ (not shown). Thus, the results obtained closely matched the predictions made by the model, demonstrating that this molecular model accurately describes drug interactions with P-glycoprotein.

In addition, this model is perfectly consistent with the observation of the *trans*-stimulating effect of the linear peptide NAc-LLY-amide on the transport of colchicine but not VBL (Sharom et al., 1996). Indeed, NAc-LLY-amide, like TTX, displays the consensus hydrophobic and polar elements that determine its binding to the pharmacophore 2, whereas colchicine, like CSA, displays the consensus hydrophobic and

polar elements that determine its binding to the pharmacophore 1 (Fig. 7). Thus, the positive heterotrope effect between NAc-LLY-amide and colchicine seems of the same nature than that between TTX and CSA. In addition, Sharom reported the activation of NAc-LLY-amide transport by VRP and CSA but its inhibition by VBL. These observations reinforce the model describing the respective binding of VRP and CSA to the pharmacophore 1 and the binding of VBL to the pharmacophore 2.

In conclusion, we show in this study that the molecular mechanism underlying the selective recognition of various substrates by P-glycoprotein does not depend on precise chemical motifs, as is typically described for classical specific ligand-receptor interactions. Instead, the binding pocket of P-glycoprotein binds the various substrates at defined sites, according to the shape, size, and distribution of the hydrophobic and polar elements of the substrates, making possible the recognition of various chemical structures. In addition, the various binding sites are distributed between at least two pharmacophores, increasing the number of chemical structures potentially recognized. In this model, the larger molecules compete with most other molecules, and two molecules can bind simultaneously to P-glycoprotein if at least one of the two is small (less than about 750 Da). These results are strikingly similar to the structural data recently reported on QacR, a regulator of the expression of the multidrug export pump QacA, accounting for the ability of this molecule to recognize various drugs (Schumacher et al., 2001). In the absence of a high-resolution structure for P-glycoprotein, we report here the relevant topological mapping of substrate binding sites. These data provide thus a basis for understanding the multispecificity of P-glycoprotein. In addition, this model should be useful for predicting drug-drug interactions caused by P-glycoprotein. This work also can open up the way to the rational design of new molecules optimally adapted for the modulation of P-glycoprotein activity, in particular by considering large molecules able to bind with high affinity to both pharmacophores, fully occupying them and therefore outcompeting a large number of substrates.

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