Competition of Hydrophobic Peptides, Cytotoxic Drugs, and Chemosensitizers on a Common P-glycoprotein Pharmacophore as Revealed by Its ATPase Activity*

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The aim of the present study was to demonstrate that the modulation of P-glycoprotein (Pgp) ATPase activity by peptides, drugs, and chemosensitizers takes place on a common drug pharmacophore. To this end, a highly emetine-resistant Chinese hamster ovary cell line was established, in which Pgp constituted 18% of plasma membrane protein. Reconstituted proteoliposomes, the Pgp content of which was up to 40% displayed a basal activity of 2.6 ± 0.45 μmol of Pi/min/mg of protein, suggesting the presence of an endogenous Pgp substrate. This basal ATPase activity was stimulated (up to 5.2 μmol of Pi/min/mg of protein) by valinomycin and various Pgp substrates, whereas, to our surprise, gramicidin D, an established Pgp substrate, was inhibitory. Taking advantage of this novel inhibition of Pgp ATPase activity by gramicidin D, a drug competition assay was devised in which gramicidin D-inhibited Pgp ATPase was coincubated with increasing concentrations of various substrates that stimulate its ATPase activity. Gramicidin D inhibition of Pgp ATPase was reversed by Pgp substrates, including various cytotoxic agents and chemosensitizers. The inhibition of the basal ATPase activity and the reversal of gramicidin D inhibition of Pgp ATPase by its various substrates conformed to classical Michaelis-Menten competition. This competition involved an endogenous substrate, the inhibitory drug gramicidin D, and a stimulatory substrate. We conclude that the various MDR type substrates and chemosensitizers compete on a common drug binding site present in Pgp.

Inherent as well as acquired antitumor drug resistance continue to pose major obstacles toward the successful chemotherapeutic treatment of various human malignancies (1). Resistance to a broad spectrum of hydrophobic cytotoxic drugs, including Vinca alkaloids, anthracyclines, epipodophyllotoxins, actinomycin D, taxoids (eg, taxol), actinomycin D, and dolastatin 10 has been termed multidrug resistance (MDR); for reviews, see Refs. 2–5). Mammalian cells with the MDR phenotype, express increased levels of a heavily glycosylated 170-kDa plasma membrane protein known as P-glycoprotein (Pgp).

Pgp is a tandemly duplicated 1280-amino acid polypeptide, each half of which contains six transmembrane α-helices and one cytoplasmic nucleotide triphosphate binding site (6, 7). It has been shown that MDR cells contain markedly decreased intracellular drug concentrations (8–10); this was shown to result from an ATP-dependent drug efflux (for reviews, see Refs. 2–5 and 11). Pgp-containing plasma membrane vesicles from tissue-cultured cells (12) or from rat liver canalicular membrane vesicles (13) displayed an ATP-dependent drug transport.

Recently, Sharom et al. (14) showed an ATP-dependent transport of colchicine into proteoliposomes reconstituted with hamster Pgp, whereas Eytan et al. (15) demonstrated an ATP-driven transport of the hydrophobic peptide ionophores gramicidin D and valinomycin into proteoliposomes reconstituted with rat and hamster Pgp. Pgp has been shown to bind the photoaffinity ATP analogue 8-azido-ATP (16, 17), and amino acid substitutions of either or both the ATP-binding domains resulted in abolished function of drug transport. In this respect, several initial reports have demonstrated that Pgp has a low ATPase activity (18, 19), whereas more recent studies have shown Pgp to contain a high drug-stimulatable ATPase activity (20–23). Recently, Smit et al. (24), used targeted MDR1 gene disruption in transgenic mice and found these mice to be 100-fold more sensitive to ivermectin, a central nervous system neurotoxin, thus strongly suggesting that the physiological overexpression of Pgp in the blood brain barrier endothelium (25) is presumably responsible for cytotoxins extrusion. Taken together, these results strongly suggest that Pgp functions as an ATP-dependent efflux transporter of multiple hydrophobic cytotoxic drugs.

A major unresolved problem in the multidrug resistance field is how a single integral membrane transporter can transport various hydrophobic peptides, cytotoxic drugs, and chemosensitizers bearing a wide array of molecular structures (4). Several genetic and biochemical approaches have been taken in an attempt to resolve this major question; these included an examination of the relationship between the structure and function of Pgp using mutational analysis of this transporter, as well as its photoaffinity labeling with substrate and substrate analogues (for a recent review, see Ref. 26). The first point mutation described in Pgp, involving a Gly → Val substitution at position 185, was detected in a highly colchicine-selected MDR KB-C1 cell line; this single amino acid substitution was shown to change the specificity of the transporter so that colchicine transport was improved, whereas vinblastine and actinomycin D transport was decreased (27, 28). Similarly, mutations of Gly → Ala and Ala → Pro at positions 338 and 339, respectively, in transmembrane domain 6 of Chinese hamster Pgp (29), appeared to decrease the resistance to several drugs, while maintaining normal resistance to actinomycin D. Furthermore, a site-directed mutation leading to conversion of Ser...
→ Phe at residue 941 in the mouse mdr1 gene in transmembrane domain 11 (30, 31) altered drug specificity such that the transport of vincristine was intact, whereas that of colchicine and doxorubicin was drastically decreased.

Several photoaffinity labels including Pgp substrates such as anticancer drugs, hydrophobic peptides, and chemosensitizers bind to two symmetrical sites on Pgp (i.e. transmembrane domains 6 and 12; see Ref. 4). Various hydrophobic peptides, drugs, and chemosensitizers were shown to competitively inhibit the photoaffinity labeling of Pgp with radiolabeled substrate analogues, or chemosensitizers’ analogues, in isolated plasma membrane vesicles, or in whole MDR cells (reviewed in Ref. 26). Using Pgp-reconstituted proteoliposomes, we have recently shown an ATP-driven transport of valinomycin and gramicidin D (15); this active intravesicular accumulation of peptide ionophores was blocked by established Pgp substrates including the antracyclines doxorubicin, daunorubicin, the Vinca alkaloid vincristine as well as the tripeptide N-acetyl-leucyl-leucyl-norleucinal (ALLN; see Ref. 32).

Taken collectively, these different studies pointed out that a common drug pharmacophore may exist in Pgp, which binds the structurally and functionally distinct drugs and chemosensitizers that participate in the MDR phenomenon (2−4, 33, 34). Indeed, using proteoliposomes reconstituted with Pgp ATPase, we show here that various hydrophobic peptides, cytotoxic drugs, and MDR chemosensitizers compete on a common drug pharmacophore present in Pgp. We therefore suggest that substrate competition using a drug-modulatable Pgp ATPase activity, is a functional assay useful for the screening of Pgp substrates and MDR chemosensitizers.

**EXPERIMENTAL PROCEDURES**

**Drugs**—The peptides gramicidin D (Dubos), valinomycin, alamethicin, ALLN, ALLM, n-formyl-norleucyl-leucyl-phenylalanine-methylester (f-NL-ML-ME), n-formyl-methionyl-leucyl-phenylalanine-methylester (f-ML-ME), N-acetyl-Ala-Ala-Ala-methylester, and viscosin, the cytotoxic agents colchicine, doxorubicin hydrochloride, emetine hydrochloride, taxol, as well as the chemosensitizers (21). The routine yields for microsomes and plasma membrane vesicles were, respectively: 0.4−0.6 mg of protein/10^8 cells and 50−100 μg of protein/10^8 cells. The membrane fractions were frozen in liquid nitrogen and stored at −75°C until analysis.

**Extraction and Reconstitution of Pgp-rich Membrane Fractions**—The extraction and reconstitution of Pgp were performed according to our recently published protocol (15) except for minor modifications. A liposome suspension was prepared from a mixture containing the acetonitrile-soluble,ether-soluble fraction of soybean phospholipids, phosphatidylserine, and cholesterol in a ratio of 5:1:1, respectively. The lipid solutions were mixed, and the solvents were removed under a stream of nitrogen and exposure to vacuum for 30 min. The lipids were suspended in solubilization buffer in a round-bottom 12-ml glass test tube (Corning, Hicksville, NY) to a concentration of 50 mg/ml in a reconstitution medium containing 25 mg HEPES-Tris, pH 7.4, 85 mM K_2SO_4, 1 mM dithiothreitol, and 1 mM PMSF. Microsomal fraction or plasma membrane vesicles obtained from parental AA8 and EmtR1 cells were incubated for 20 min on ice at a protein concentration of 1 mg/ml in a solubilization buffer containing: 35 mM HEPES-Tris, pH 7.4, 13.3% octylglucoside, 0.7% liposome suspension, 15% glycerol, 3 mM dithiothreitol, 3 mM PMSF, 1 mM aprotinin, 100 μg/ml pepstatin, and 50 μg/ml leupeptin. The detergent-soluble proteins were obtained as the supernatant after centrifugation for 30 min at 130,000 × g, and a liposome suspension was added to a final lipid concentration of 17 mg/ml and incubated for 20 min on ice in a centrifugation buffer containing: 100 mM K_2SO_4, 10 mM NaPO_4, 1 mM Na_2EDTA at pH 7.0, 1% SDS, and 10 μg/ml of polyvinylpyrrolidone 360, 20 mg of Ficoll, and 20 mg of bovine serum albumin (100 ml of distilled water) Ficoll. Following hybridization, blots were washed twice for 30 min under high stringency conditions (0.1× SSPE, 0.1% SDS at 65°C). Autoradiography was performed for various exposures to visualize highly sensitive x-ray films (XAR-5 and XAR, Eastman Kodak Co.). Gene copy numbers were estimated by scanning densitometry (Cliniscan 2 scanning densitometer, Helena Laboratories) of linear film exposures. Scanning densitometry of the ethidium bromide-stained gels was used to correct for any slight differences in DNA loading.

**Isolation of Microsomal and Plasma Membrane Fraction—Cells** (10^9) growing under suspension culture conditions were harvested by sedimentation at 3,000 rpm in a Sorvall GSA rotor, washed with phosphate-buffered saline, and resuspended in 10 ml of lytic buffer containing 10 mM Heps-Tris, pH 7.4 at 4°C, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, as well as the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 2 mg), aprotinin (1 mg), pepstatin (10 μg/ml), and leupeptin (10 μg/ml). Following a 5 min incubation on ice, cells were lysed using a Teflon-glass homogenizer and diluted 3-fold in lysis buffer. Nuclei and mitochondria were removed by consecutive 10 min centrifugations at 300 and 4,000 × g, respectively, and the microsomal fraction was recovered by a 30 min centrifugation at 100,000 × g, and finally resuspended in 1 ml of lysis buffer.

Plasma membrane vesicles were isolated from the microsomal fraction by discontinuous density gradient centrifugation as described by Gray and Ling (38) with the modification of Camp et al. (39). The routine yields for microsomes and plasma membrane vesicles were, respectively: 0.4−0.6 mg of protein/10^9 cells and 50–100 μg of protein/10^9 cells. The membrane fractions were frozen in liquid nitrogen and stored at −75°C until analysis.
Common Drug Binding Site on Pgp ATPase

Isolation and Characterization of Emetine-resistant Mammalian Cells—To establish a Pgp-overexpressing cell line, wild-type Chinese hamster ovary cells (AA8) were subjected to gradually increasing concentrations of emetine, a eukaryotic translation inhibitor used as an antiamebic. Resistant variants stably growing at 0.15 μM (i.e., ~3 times the LD_{50} of wild-type cells; this line was therefore termed Emt^{R0.15}) to 1 μM emetine (termed Emt^{R1}) were established. Southern blot analysis with a highly conserved MDR exon probe (43, 44) showed that the MDR gene copy number rose from 20 copies in Emt^{R0.15} cells to approximately 100 copies in the highly emetine-resistant Emt^{R1} variant (Fig. 1). Consequently, a 170-kDa band constituting 45% of microsomal proteins and 38% of plasma membrane proteins was observed in Emt^{R1} cells, but was absent from parental AA8 cells (data not shown). Immunoblot analysis using C219 monoclonal antibody confirmed the high levels of Pgp overexpression in microsomes and plasma membrane vesicles from Emt^{R1} cells, whereas Pgp was not detectable in microsomal protein from parental AA8 cells (45).

Emt^{R1} cells displayed a 45-fold resistance to the selecting agent emetine, as well as a 15-95 cross-resistance to MDR-type drugs including doxorubicin, colchicine, vinblastine, taxol, and the novel microtubule depolymerizing anticancer peptide, dolastatin 10 (Table I). The ionophore resistance pattern in Emt^{R1} cells (Table I) was consistent with our previous findings of a high level resistance to the channel-forming pentadecapeptide ionophore gramicidin D and a low level resistance to the carrier-type ionophore valinomycin in GD^{R30} cells (36). In contrast, Emt^{R1} cells maintained wild-type sensitivity to cytotoxic agents that are not handled by Pgp including the hydrophobic antifolate methotrexate and the hydrophobic peptide antibiotic viscosin. Taken together these data suggest that upon stepwise selection to the antiamebic drug emetine, mammalian cells acquire a classic MDR phenotype via a prominent MDR gene amplification and consequent Pgp overexpression. The excessive overproduction of Pgp in Emt^{R1} plasma membrane makes this cell line an excellent source for Pgp.

Functional Reconstitution of Pgp ATPase into Proteoliposomes—Analysis of the proteins from proteoliposomes reconstituted with the n-octylglucoside-soluble extract from Emt^{R1} microsomes and plasma membrane vesicles showed that Pgp constituted 18% and 40%, respectively; this Pgp enrichment by its reconstitution into proteoliposomes was confirmed by Western blot analysis using C219 monoclonal antibody (data not shown). In contrast, Western blot analysis failed to detect Pgp

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V = \frac{V_{\text{max}} \times S}{S + K_{\text{m}i} \times \left(1 + \frac{S_1}{K_{\text{m}1}} + \cdots + \frac{S_n}{K_{\text{m}n}}\right)} + \frac{V_{\text{max}1} \times S_1}{S_1 + K_{\text{m}1} \times \left(1 + \frac{S_0}{K_{\text{m}0}} + \frac{S_2}{K_{\text{m}2}} + \cdots + \frac{S_n}{K_{\text{m}n}}\right)} + \cdots + \frac{V_{\text{max}n} \times S_n}{S_n + K_{\text{m}n} \times \left(1 + \frac{S_0}{K_{\text{m}0}} + \frac{S_1}{K_{\text{m}1}} + \cdots + \frac{S_{n-1}}{K_{\text{m}n-1}}\right)}
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(Eq. 1)

Regression analysis and iterative best-fit computer-simulated curves were generated using Sigmaplot software distributed by Jandel Scientific Software.

RESULTS

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in either AA8 microsomes, or in proteoliposomes reconstituted with microsomal proteins from parental AA8 cells (45).

When examined with established Pgp substrates, the ATPase activity of Pgp-constituted proteoliposomes was linear for at least 1 h at 37 °C (Fig. 2). The basal (i.e. when no substrates were added) ATPase activities obtained with proteoliposomes reconstituted with EmtR1 microsomal and plasma membrane proteins were: 1.1 ± 0.25 and 2.6 ± 0.45 μmol of P/mg of protein (mean ± S.E.), respectively. Thus, in accord with the 2.2-fold increased Pgp content in proteoliposomes reconstituted with plasma membrane proteins was their 2.3-fold elevated ATPase activity (Fig. 3). This basal ATPase activity was vanadate sensitive (see below), and under the sodium-free assay conditions used, the ATPase activity was ouabain-insensitive. Furthermore, this ATPase activity was inhibited by the well established Pgp substrate gramicidin D and stimulated by a variety of known Pgp substrates including hydrophobic peptides, cytotoxic drugs, and chemosensitizers (see below). In contrast, proteoliposomes reconstituted with AA8 cell membrane proteins did not display any drug-modulatable ATPase activity. Thus, the basal ATPase activity present in proteoliposomes reconstituted with EmtR1 membrane proteins is attributable to Pgp.

Modulation of Pgp ATPase Activity with Hydrophobic Peptide Ionophores—Recently we have shown that proteoliposomes reconstituted with mammalian Pgp actively transport the pentadecapeptide channel-forming ionophore gramicidin D, and the decapptide carrier-type K+_ionophore, valinomycin (15). We also reported that acquisition of gramicidin D resistance was a result of Pgp overexpression due to MDR gene amplification (36). Thus, the ability of these well established Pgp peptide substrates to stimulate its ATPase activity was examined. As expected, valinomycin stimulated the ATPase activity of proteoliposomes reconstituted with proteins extracted from EmtR1 microsomes but not of those reconstituted with microsomal proteins from wild type AA8 cells (Fig. 3A). A 2-fold stimulation with an apparent K_m of 1 μM was observed in proteoliposomes reconstituted with a protein extract from EmtR1 microsomes (Fig. 3A and Table I), or from plasma membrane vesicles (Fig. 3B). Surprisingly, however, gramicidin D inhibited the ATPase activity of proteoliposomes rather than stimulating it (Fig. 3, A and B). The gramicidin D inhibition of the ATPase activity of proteoliposomes reconstituted with microsomal (Fig. 3A) and plasma membrane protein extract (Fig. 3B) reached minima values of 0.5-fold of basal activity with an apparent K, of 20 μM (Fig. 3, A and B, and Table I).

A quantitative description of the inhibition of basal Pgp ATPase by gramicidin D and stimulation by valinomycin is best described in terms of competition between these substrates and

| Drug             | AA8 LD_{50}a | EmtR1 LD_{50}a | Resistance |
|------------------|---------------|----------------|------------|
| Vincristine      | 2.3 ± 0.05    | 1.1 ± 0.03     | 2.2        |
| Vinblastine      | 2.6 ± 0.04    | 1.3 ± 0.02     | 2.0        |
| Taxol            | 3.9 ± 0.1     | 1.5 ± 0.05     | 2.6        |
| Colchicine       | 5.9 ± 0.2     | 2.9 ± 0.1      | 2.0        |
| Doxorubicin      | 11.0 ± 0.2    | 5.5 ± 0.2      | 2.0        |

*LD_{50} values represent the means of at least two clonogenic cytotoxicity determinations.

**FIG. 2.** Linearity of ATPase activity of Pgp-reconstituted proteoliposomes incubated with various stimulatory and inhibitory Pgp substrates. Proteoliposomes were incubated for variable times with 33 μM gramicidin D, 100 μM verapamil, 100 μM vinblastine, or with 5 μM dolastatin 10. After incubation at 37 °C for up to 1 h, the ATPase activity was measured colorimetrically by following the production of phosphate (see “Experimental Procedures”).

**FIG. 3.** Modulation by valinomycin and gramicidin D of the ATPase activity of proteoliposomes reconstituted with Pgp. Proteoliposomes reconstituted with detergent-soluble proteins from EmtR1 microsomal (A) or plasma membrane fraction (B) were incubated for 1 h at 37 °C in an ATP-containing medium (for details see “Experimental Procedures”) in the presence of increasing concentrations of the peptide ionophores valinomycin and gramicidin D, after which the ATPase activity was determined. Data shown in panel A were derived from 10 independent experiments, and the lines depicted were obtained by a computer-aided best-fit analysis using Equation 1, described under “Experimental Procedures,” for a two-substrate competition system consisting of a putative endogenous substrate and an added MDR drug, a putative endogenous substrate responsible for the basal ATPase activity (see “Discussion”). Both the endogenous and added substrates are described in classical Michaelis-Menten terms of K_m and V_{max}. A stimulatory substrate such as valinomycin exhibits a high V_{max}, whereas an inhibitory substrate...
such as gramicidin D is characterized by a relatively low V_{\text{max}}. As shown in Fig. 3A, the experimental data could be best-fit to a computer simulation of classical competition between the putative endogenous substrate and either gramicidin D or valinomycin (see “Experimental Procedures” for Equation 1 and explanations thereof).

Stimulation of ATPase Activity with Various Pgp Substrates—In order to establish an ATPase stimulation pattern that is characteristic to Pgp (17, 20–23), the ability of various hydrophobic peptides, anticancer drugs, and MDR chemosensitizers to modulate its ATPase activity was examined (Fig. 4 and Table II). Dolastatin 10, which participates in the MDR phenomenon (46), achieved a 1.9-fold stimulation of ATPase activity with an apparent K_{m} of 0.9 \mu M (Fig. 4A and Table II). Among the established non-peptide Pgp substrates, the anticancer drug vinblastine stimulated the ATPase activity by 1.6-fold with an apparent K_{m} of 0.8 \mu M (Fig. 4A), whereas the selecting agent emetine yielded a 1.8-fold stimulation of ATPase activity (Fig. 4A) with an apparent K_{m} of 6 \mu M (Table II). Other established MDR drugs, including oligochicine, doxorubicin, and leupeptin, a polar protease inhibitor increased the ATPase activity even at millimolar concentrations (Fig. 4A). Among the chemosensitizers of the Pgp-dependent MDR phenotype, verapamil and progesterone were 2 and 30 \mu M, respectively (Fig. 4B and Table II). In contrast, quinine and quinidine gave a 1.3-fold maximal increase of ATPase activity (Fig. 4B). The various MDR drugs and chemosensitizers displayed optimum curves of stimulation. Pgp substrates, which are hydrophobic by nature, partition into the membrane leading to a high intramembranous concentration relative to that present in the aqueous medium. This concentration alters the physical properties of the membrane (45), thereby interfering with Pgp function such as its ATPase activity. This type of inhibition is particularly most pronounced at high concentrations of vinblastine, dolastatin 10, and progesterone (Fig. 4, A and B).

As a control, the effect of drugs that do not participate in the MDR phenomenon, including methotrexate, metoprine, and the peptide ionophore alamethicin, was also examined. These cytotoxic agents had no effect on the ATPase activity even at high concentrations (Table II). It should be emphasized that no drug-dependent stimulation of ATPase activity was detected in proteoliposomes reconstituted with microsomal and plasma membrane proteins extracted from wild type AA8 cells.

We have examined the relationship between the polarity of peptides and their ability to stimulate the Pgp-associated ATPase activity. A series of bioactive linear tripeptides bearing increasing polarity was chosen. At a 1 \mu M concentration, the highly hydrophobic (i.e. due to formylated NH_2 terminus and esterified carboxyl terminus) white blood cell chemotaxtractants f-NLP-ME and f-MLP-ME potently stimulated Pgp ATPase activity by 2.6- and 2.3-fold, respectively (Fig. 4C). At a 1 \mu M concentration, the less hydrophobic calpain inhibitors ALLN and ALLM (Fig. 4C), which are established Pgp substrates (32), as well as leupeptin, a polar protease inhibitor increased the ATPase activity by 1.7-, 1.3-, and 1.2-fold, respectively (Fig. 4C). The less hydrophobic tripeptides (Fig. 4C) and n-acetyl-Ala-Ala-Ala-p-nitroanilide failed to increase the basal ATPase activity. These results suggest an inverse relationship between

### Table II

| Drug                  | Apparent K_{m} \text{\,(\mu M)} | V_{\text{max}}^{\text{b}} | Fold of basal ATPase activity |
|-----------------------|-------------------------------|---------------------------|--------------------------------|
| Gramicidin D          | 2                             | 0.5                       | 0.5                            |
| Valinomycin           | 1                             | 2                         | 2                              |
| Emetine               | 6                             | 1.8                       | 1.8                            |
| Doxorubicin           | Silent \text{\,(ND)}          | 1                         | 1                              |
| Calchicine            | Silent \text{\,(ND)}          | 1                         | 1                              |
| Vinblastine           | 0.8                           | 1.6                       | 1.6                            |
| Dolastatin 10         | 0.9                           | 1.9                       | 1.9                            |
| f-MLP-ME              | >100                          | >2.2                      | >2.2                           |
| f-NLP-ME              | >80                           | >2.5                      | >2.5                           |
| ALLN                  | >70                           | >1.6                      | >1.6                           |
| ALLM                  | >100                          | >1.3                      | >1.3                           |
| Leupeptine            | >100                          | >1.2                      | >1.2                           |
| nAc-AAA-ME            | Silent \text{\,(ND)}          | 1                         | 1                              |
| Alamethicin           | Silent \text{\,(ND)}          | 1                         | 1                              |
| Methotrexate          | Silent \text{\,(ND)}          | 1                         | 1                              |
| Metoprine             | Silent \text{\,(ND)}          | 1                         | 1                              |
| Verapamil             | 2                             | 1.8                       | 1.8                            |
| Progestrone           | 30                            | 2                         | 2                              |
| Quinine               | 30                            | 1.3                       | 1.3                            |
| Quinidine             | ND \text{\,(ND)}              | 1.3                       | 1.3                            |

\text{a} Mean apparent K_{m} values calculated graphically from 3–10 independent experiments.

\text{b} Values are the means of 3–10 independent experiments.

\text{c} Silent refers to a substrate that does not alter Pgp ATPase activity.

\text{d} V_{\text{max}} could not be achieved even at 1 mM drug concentrations.

\text{e} ND, not determined.

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![Figure 4](image-url)
Common Drug Binding Site on Pgp ATPase

The Km lastatin 10, vinblastine, and emetine (Fig. 6), drugs and substrates including valinomycin, progesterone, doxorubicin, and trimetrexate did not modulate Pgp ATPase activity. Substrates with an intermediate \( V_{\text{max}} \) partially inhibit it, whereas substrates with a high \( V_{\text{max}} \) appear silent as they do not alter Pgp ATPase activity.

In summary, the effect of Pgp substrates and chemosensitizers varied between partial inhibition as displayed by gramicidin D and stimulation as shown with valinomycin, doxorubicin, and trimetrexate. The basal Pgp ATPase activity and the reversal of gramicidin D inhibition of Pgp ATPase by its various substrates were studied. Several lines of evidence establish that gramicidin D is a substrate of the multidrug transporter, Pgp. Single-dose exposure or stepwise selection with increasing gramicidin D concentrations result in a prominent MDR gene amplification and Pgp overexpression. Mammalian cells stably transfected with the human MDR1 cDNA display a typical MDR phenotype with a collateral resistance to gramicidin D. Various MDR cell lines obtained by selection to different established pleiotropic drugs exhibit a marked cross-resistance to gramicidin D; this gramicidin D resistance was attributed to a consistent decrease in the number of gramicidin D channels in the plasma membrane. We have recently described an ATP-driven transport of gramicidin D and valinomycin into proteoliposomes reconstituted with Pgp from rat liver or from highly MDR cells. Interestingly, however, instead of stimulating the Pgp ATPase activity like valinomycin and like a variety of other Pgp substrates, gramicidin D was inhibitory. Taking advantage of this ATPase inhibition by gramicidin D, a drug competition assay was devised in which we established Pgp substrates, which stimulate its ATPase activity, were examined for their ability to competitively overcome the gramicidin D inhibition of Pgp ATPase. We demonstrated that various established Pgp substrates including the cytotoxic agents valinomycin, vinblastine, doxorubicin, and emetine, the chemotactic tripeptides f-NLP-ME and f-MLP-ME, the calpain tripeptide inhibitors ALLN and ALLM (data not shown), as well as the chemosensitizers verapamil and progesterone reverse the gramicidin D inhibition of Pgp ATPase. The basal ATPase activity and the reversal of gramicidin D inhibition of Pgp ATPase by its various substrates conformed to classical Michaelis-Menten competition. This competition involved an endogenous substrate, the inhibitory drug gramicidin D, and a stimulatory substrate. We therefore conclude that the various Pgp substrates including peptides, cytotoxic agents, and chemosensitizers compete for a common pharmacophore (i.e. drug binding site) present in Pgp.

The conclusion of a common drug pharmacophore present in Pgp agrees with several previous studies. 1) A wide spectrum of Pgp substrates including anticancer drugs and MDR chemosensitizers compete with various photoaffinity labels of Pgp. 2) Various point mutations leading to single amino acid substitutions in mammalian Pgp markedly alter the original
resistance pattern of MDR cells to multiple hydrophobic cytotoxic drugs that participate in the MDR phenomenon (27–31).

3) Structure-activity relationship studies using a photoactivatable vinblastine analog as a probe together with a semi-synthetic series of structurally homologous reserpine and yohimbine analogues pointed out that the Pgp pharmacophore requires two planar aromatic domains and a basic nitrogen atom (50).

A major question arises as to the underlying basis for the surprising gramicidin D inhibition of Pgp ATPase. The most likely explanation is that the bulky three-dimensional α3β6 helical structure of gramicidin D may be difficult to handle by Pgp, thereby leading to a decreased translocation rate. In this respect, this pentadecapeptide that spans a whole hemilayer (51, 52) is to date the largest Pgp substrate (M, 1900) that was shown to be transported.
was then determined as described in Fig. 4 legend. Pgp ATPase inhibitor, orthovanadate, in the simultaneous presence of emetine, which is an ipecac alkaloid currently used in the treatment of severe invasive amebiasis (54, 55). It was recently reported that emetine-resistant Entamoeba histolytica variants overexpress two mRNA species encoded by two P-glycoprotein genes (EhPgp1 and EhPgp2; see Refs. 56 and 57). The open reading frames for these EhPgps showed 40% positional identity with the human mdr1 gene. Furthermore, a phylogenetic tree showed that Entamoeba P-glycoproteins are more related to mammalian Pgps than to those from the parasitic protozoa Plasmodium and Leishmania. This prominent homology between EhPgps and mammalian Pgp led us to examine whether the MDR phenotype emerges as a major protective mechanism upon selection of mammalian cells with emetine. Several lines of evidence support that emetine is an active participant in the Pgp-dependent MDR phenotype. First, like various Pgp substrates, emetine stimulated the ATPase activity of Pgp-reconstituted proteoliposomes. Moreover, consistent with various MDR-type drugs, emetine competitively reversed the gramicidin D inhibition of Pgp ATPase in reconstituted proteoliposomes. Second, a dramatic reversal of gramicidin D inhibition was observed in Chinese hamster ovary cells selected for stepwise resistance to emetine. Consistently, emetine-resistant Entamoeba histolytica overexpressed EhPgps mRNA. Third, EmtR1 cells expressed extremely high levels of Pgp and emetine. Several lines of evidence support that emetine is an active participant in the Pgp-dependent MDR phenotype. First, acquisition of a Pgp-dependent MDR phenotype in mammalian cells and in amebae is an efficient means of functional protection against the cytotoxicity of emetine.

We observe here a strong basal ATPase activity of 2.6 \( \mu \text{mol} \) of P/\( \text{min/mg} \) of protein (\( K_{\text{cat}} = 7 \text{ s}^{-1} \)) in Pgp-reconstituted proteoliposomes under conditions where no exogenous substrate was added. We, as well as others (17–23, 53), have detected this basal ATPase activity in microsomes prepared from Pgp-overexpressing MDR cells. Thus, it appears that the putative endogenous substrate(s) is a lipid-soluble component present in lipid bilayers. This prominent basal ATPase activity has been well documented in plasma membrane vesicles from MDR cells (17, 53) and in functionally reconstituted proteoliposomes (14, 20–23). Thus, we here propose a mechanistic basis for this marked basal ATPase activity involving an endogenous lipid-soluble substrate(s), which continuously stimulates Pgp ATPase activity. In this respect, the various lipids used for reconstitution studies are crude extracts prepared from liver (23), brain (21, 23), soybean (15, 22, 23), and Escherichia coli (20), all of which are rich in a variety of hydrophobic compounds. Thus, lipid-soluble compounds copurifying during lipid extraction will remain associated with the membrane of the proteoliposomes. Some of these lipid-associated hydrophobic compounds could be recognized as substrates by Pgp, thereby serving as endogenous substrate(s) which continuously stimulate Pgp ATPase activity. The latter property may physiologically be crucial, inasmuch as, in vivo, the multidrug transporter is continuously exposed to exogenous (i.e. from the diet) or endogenous Pgp substrates, all of which are hydrophobic. Therefore, once extruded, they easily diffuse back to the membrane, thereby continuously stimulating Pgp ATPase.

The assay of competitive reversal of gramicidin D inhibition of Pgp ATPase offers an excellent functional system for the rapid screening of novel Pgp inhibitors, or alternatively, agents that are recognized by Pgp. This functional assay is best suited for the screening of potent MDR chemosensitizers as it combines drug-modulatable ATPase activity and most importantly the ability to obtain quantitative parameters including apparent \( K_m \) and \( V_{\text{max}} \) for each drug and chemosensitizer. Furthermore, their competitive inhibitory or stimulatory effects on Pgp ATPase could be reliably and rapidly determined.

In the present study, we used the eukaryotic protein synthesis inhibitor emetine, which is an ipecac alkaloid currently used in the treatment of severe invasive amebiasis (54, 55). It was recently reported that emetine-resistant Entamoeba histolytica variants overexpress two mRNA species encoded by two P-glycoprotein genes (EhPgp1 and EhPgp2; see Refs. 56 and 57). The open reading frames for these EhPgps showed 40% positional identity with the human mdr1 gene. Furthermore, a phylogenetic tree showed that Entamoeba P-glycoproteins are more related to mammalian Pgps than to those from the parasitic protozoa Plasmodium and Leishmania. This prominent homology between EhPgps and mammalian Pgp led us to examine whether the MDR phenotype emerges as a major protective mechanism upon selection of mammalian cells with emetine. Several lines of evidence support that emetine is an active participant in the Pgp-dependent MDR phenotype. First, like various Pgp substrates, emetine stimulated the ATPase activity of Pgp-reconstituted proteoliposomes. Moreover, consistent with various MDR-type drugs, emetine competitively reversed the gramicidin D inhibition of Pgp ATPase in reconstituted proteoliposomes. Second, a dramatic MDR gene amplification was observed in Chinese hamster ovary cells selected for stepwise resistance to emetine. Consistently, emetine-resistant Entamoeba histolytica overexpressed EhPgps mRNA. Third, EmtR1 cells expressed extremely high levels of Pgp and consequently displayed a typical MDR to multiple hydrophobic cytotoxic agents. Fourth, EmtR1 cells failed to accumulate rhodamine 123, a chromophoric substrate of Pgp; however, upon coincubation with the established MDR chemosensitizer reserpine, a wild type level of rhodamine 123 accumulation was resumed in EmtR1 cells (data not shown). We therefore conclude that acquisition of a Pgp-dependent MDR phenotype in mammalian cells and in amebae is an efficient means of functional protection against the cytotoxicity of emetine.

We note here that, although valinomycin is a bona fide substrate of Pgp, the highly MDR EmtR1 cell line, which displays a strong MDR phenotype including a 100-fold resistance to gramicidin D, exhibited only a modest cross-resistance to valinomycin. We have previously documented various MDR cell lines and found them all to be only marginally resistant to valinomycin but highly resistant to gramicidin D (36). Valinomycin, a cyclic decapptide mobile-carrier ionophore possesses a rapid diffusion rate across membrane (10\(^{-4}\) to 10\(^{-5}\) s). In contrast, the linear pentadecapeptide ionophore gramicidin D forms a functional K\(^+\)-channel only after two gramicidin D monomers residing in opposite membrane leaflets undergo hydrogen bonding and dimerization. Thus, the rate-limiting step in gramicidin D channel formation is the slow (i.e. minutes; see Refs. 51 and 52) flip-flop from the outer hemi-layer to the inner membrane leaflet. Based on the turnover rate of Pgp ATPase at maximal drug stimulation (\( K_{\text{cat}} = 15 \text{ ATP s}^{-1}; \) Ref. 23 and this paper) and an estimated near stoichiometric substrate transport to ATP hydrolysis (see Ref. 58), the multidrug transporter can efficiently expel gramicidin D monomers thus abolishing its cytotoxicity. In contrast, valinomycin is 3 orders of magnitude more rapid in traversing the lipid bilayer than the \( V_{\text{max}} \) of Pgp ATPase (Fig. 3); valinomycin molecules extruded by Pgp rapidly diffuse back into the plasma membrane and rapidly traverse it. Consequently, Pgp overexpression confers upon MDR cells a strong resistance to gramicidin D but only, if at all, a marginal protection from valinomycin cytotoxicity. This study points out that peptide substrates of Pgp, particularly ionophores, which are primarily confined to the membrane, are an invaluable dissection tool for probing the intramembranal mechanism of action of the mammalian multidrug transporter.

**REFERENCES**

1. Frei, E., III (1985) Cancer Res. 45, 6523–6537
2. Gottesman, M. M., and Pastan, I. (1988) J. Biol. Chem. 263, 12163–12166
3. Endicott, J. A., and Ling, V. (1989) Annu. Rev. Biochem. 58, 137–171
