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Effect of Modulators on the ATPase Activity and Vanadate Nucleotide Trapping of Human P-Glycoprotein

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ABSTRACT. P-Glycoprotein (Pgp) is responsible for the energy-dependent efflux of many natural product oncolytics. Overexpression of Pgp may result in multidrug resistance (MDR). Modulators can block Pgp efflux and sensitize multidrug resistant cells to these oncolytics. To study the interaction of modulators with Pgp, Pgp–ATPase activity was examined, using plasma membranes isolated from the multidrug-resistant cell line CEM/VLB_{100}. A survey of modulators indicated that verapamil, trifluoperazine, and nicardipine stimulated ATPase activity by 1.3- to 1.8-fold, whereas two others, trimethoxybenzoylyohimbine (TMBY) and vindoline, had no effect. Further evaluation showed that TMBY completely blocked the stimulation by verapamil of ATPase activity by competitive inhibition, with a K_i of 2.1 μM. When the effects of these two modulators on the formation of the enzyme–nucleotide complex important in the catalytic cycle were examined, verapamil increased the amount of vanadate-trapped 8-azido-[α-^{32}P]ATP bound to Pgp by two-fold, whereas TMBY had no effect. Moreover, TMBY blocked the verapamil stimulation of vanadate-8-azido-[α-^{32}P]ATP. Together, these data indicate that verapamil and TMBY bind to Pgp at a common site or overlapping sites, but only verapamil results in enhanced Pgp–ATP hydrolysis and formation of the vanadate–nucleotide–enzyme complex. BIOCHEM PHARMACOL **56**;6:719–727, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. P-glycoprotein; ATPase; trimethoxybenzoylyohimbine; verapamil stimulation; multidrug resistance

A common problem encountered in cancer chemotherapy is the development of MDR§. Tumor cells treated with natural product oncolytics often become resistant not only to the drug initially used during treatment but to many other structurally unrelated drugs [1, 2]. In many cases, the development of MDR may result from the over-expression of Pgp [3, 4]. This protein actively exports drugs from the cell and thereby reduces the intracellular drug concentration, resulting in cell survival. Drugs effluxed by Pgp include certain structurally unrelated hydrophobic oncolytic drugs such as the *Vinca* alkaloids, anthracyclines, taxanes, and others.

Pgp is a 170-kDa integral plasma membrane glycoprotein and is a member of the ATP-binding cassette (ABC) superfamily of transporters that is comprised of many ATP-dependent prokaryotic and eukaryotic transport proteins [1, 5]. Like many other family members, Pgp is a tandemly duplicated molecule with each half containing six putative transmembrane-spanning regions and one ATP binding site on the cytoplasmic side of the protein. The efflux of compounds from the cell by Pgp is ATP dependent [6, 7]. Drug binding is dependent on the presence of ATP; non-hydrolyzable analogs of ATP cannot substitute [6]. Furthermore, drug binding is inhibited by vanadate [8, 9]. ATPase activity immunoprecipitates with Pgp, using Pgp-specific monoclonal antibodies, and copurifies with the 170-kDa protein [10, 11]. Highly purified Pgp retains vanadate-sensitive ATPase activity that can be stimulated by the calcium channel antagonist verapamil, an MDR modulator [12, 13].

Modulators (or chemosensitizing agents) are agents that are used in combination with oncolytics to block their efflux by Pgp, resulting in an increase in intracellular drug concentration and eventual cell death to MDR cells [14, 15]. Pgp modulators bind to Pgp and displace the binding of radiolabeled oncolytics to the ~170-kDa glycoprotein. These include a wide variety of non-cytotoxic lipophilic agents, such as verapamil, trifluoperazine, *Vinca* alkaloid analogs, reserpine, cyclosporin A, and, most recently, LY335979 [14–18].

In the present study, we investigated the effects of modulators on Pgp–ATPase activity associated with multidrug-resistant membranes prepared from CEM/VLB_{100} cells. The effects on Pgp–ATPase activity of verapamil and

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§ *Abbreviations:* ATPase, adenosine triphosphatase; MDR, multidrug resistance; Pgp, P-glycoprotein; TMBY, trimethoxybenzoylyohimbine; and VLB, vinblastine.

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TMBY two modulators, that share several structural features are compared. Previous studies have identified key structural elements that define a pharmacophore necessary for binding to Pgp and the displacement of radiolabeled photo-activated ligands [19]. The present study suggests that the binding of these two modulators to a common or overlapping site(s) may result in different conformations of Pgp that alter their ability to hydrolyze ATP and to form an enzyme–nucleotide complex believed important in catalysis.

MATERIAL AND METHODS

Reagents

Verapamil hydrochloride and ATP were obtained from the Sigma Chemical Co. Paclitaxel (Taxol) was purchased from Calbiochem. All other oncolytics and modulators were obtained from Eli Lilly & Co. Growth medium and cell culture reagents were obtained from Life Technologies, and fetal bovine serum was purchased from HyClone. Fiske and Subbarow reducer and all other reagents were obtained from the Sigma Chemical Co.

Cell Culture

Human lymphoblastic leukemia cell lines were used. Both drug-sensitive parental cells, CCRF-CEM, and MDR cells, CEM/VLB_{100} (selected for resistance to 100 ng/mL VLB), have been described, the resistant line having been developed in one of our laboratories (W.T.B.) [20]. Cells were maintained in Minimum Essential Medium as suspension cultures containing Earle's salts, 2 mM L-glutamine, and 10% fetal bovine serum [20]. For the plasma membrane preparation, cells were grown in a 5-L spinner flask in medium supplemented with penicillin (10 units/mL), streptomycin (10 μg/mL), and bacitracin (141 μg/mL).

Plasma Membrane Preparation

Plasma membranes were prepared by nitrogen cavitation and differential centrifugation. Cells $\sim 3 \times 10^9$ in logarithmic growth phase were centrifuged and washed as described by Lever [21]. The pellet was resuspended to $\sim 3 \times 10^7$ cells/mL in 0.2 mM $CaCl_2$, 0.25 M sucrose, 0.02 mM phenylmethylsulfonyl fluoride, and 0.01 M Tris–HCl, pH 7.4. Cells were equilibrated on ice for 15 min with nitrogen at 175 p.s.i. in a cell disruption bomb (920 mL, Parr Instrument Co.) prior to release through the exit orifice. Nitrogen cavitation of both cell lines gave approximately 90% cell lysis with 92% recovery of intact nuclei. After removal of nuclei and unbroken cells by centrifugation, EDTA was added to give a final concentration of 1 mM, and the homogenate was layered onto a 35% sucrose gradient and centrifuged for 1 hr at 16,000 g, as described [21]. Membranes collected at the interface were subsequently pelleted at 100,000 *g* for 1 hr, resuspended in 0.20 M sucrose and 0.05 M Tris–HCl (pH 7.4), passed through a 25-gauge needle, and stored for up to 2 months at −70°. Protein was determined with bicinchoninic acid and BSA as the standard [22].

ATPase Assay

Pgp–ATPase activity was measured by the release of inorganic phosphate from ATP using a colorimetric method [23]. The assay contained 8–10 μg of plasma membrane protein in a total assay volume of 100 μL. Membranes were added last and were incubated at 37° in a 96-well plate with 0.05 M Tris–HCl (pH 7.4), 0.2 M sucrose, 1 mM $MgCl_2$, 5 mM sodium azide, 1 mM ouabain, 1 mM EGTA, 3 mM ATP, and an ATP-regenerating system of 5 mM phosphoenolpyruvate and 3.6 units/mL of pyruvate kinase. Unless otherwise noted, the assay was conducted for 90 min in the presence or absence of 1 mM sodium orthovanadate. The release of orthophosphate was detected by the addition of 100 μL of the Fiske and Subbarow solution to each well [23] and read at 690 nm in a Titertek Multiskan MCC/340 microtiter plate reader. The amount of phosphate released was calculated from a standard curve. Samples were measured in triplicate, and values are given as means ± SEM. Inhibitors were present in the assay buffer to eliminate any residual activity due to other common vanadate-sensitive ATPases, such as ouabain for Na^+/K^+-ATPase, EGTA for Ca^{2+}-ATPase activity, and sodium azide for any contaminating mitochondrial ATPase. The vanadate-sensitive ATPase activity was calculated by subtracting the vanadate-inhibited ATPase activity from the total activity.

Photoaffinity Labeling of Pgp

A vanadate trapping method using 8-azido-[α-^{32}P]ATP was employed to photolabel Pgp [24]. Membranes (50 μg) were preincubated for 1 hr at 37° in the presence or absence of the indicated modulator (10 μM) in a reaction buffer containing 600 μM vanadate, 1 mM $MgCl_2$, 5 mM sodium azide, 1 mM ouabain, 1 mM EGTA, 200 mM sucrose, and 50 mM Tris–HCl (pH 7.5). Subsequently, the labeling was begun by the addition of 10 μM 8-azido-[α-^{32}P]ATP (20 Ci/mmol), incubated for 10 min, and stopped with 400 μL of ice-cold reaction buffer. Unbound 8-azido-[α^{32}P]ATP was removed by centrifugation at 12,000 g for 10 min at 4°. The pellet was washed in the same buffer, resuspended in 50 μL of reaction buffer, and irradiated for 5 min (254 nm) on ice. After UV irradiation, the membranes were precipitated with 1 mL of ice-cold 80% methanol for 1 hr at 4° and collected by centrifugation at 12,000 *g* for 20 min at 4°. The pellet was resuspended in 80% methanol overnight at 4° and then centrifuged at 12,000 *g* for 20 min. Methanol was removed by vacuum centrifugation, and the pellet was resuspended in gel sample buffer consisting of 3.58 M urea, 10% SDS, 25 mg/mL of dithiothreitol and 50 mM Tris–HCl (pH 6.8). Next, the samples were warmed to 50° for 5 min, loaded onto an 8% Laemmli gel, and electrophoresed at 100 mA/gel. The gel was silver stained using the Pierce

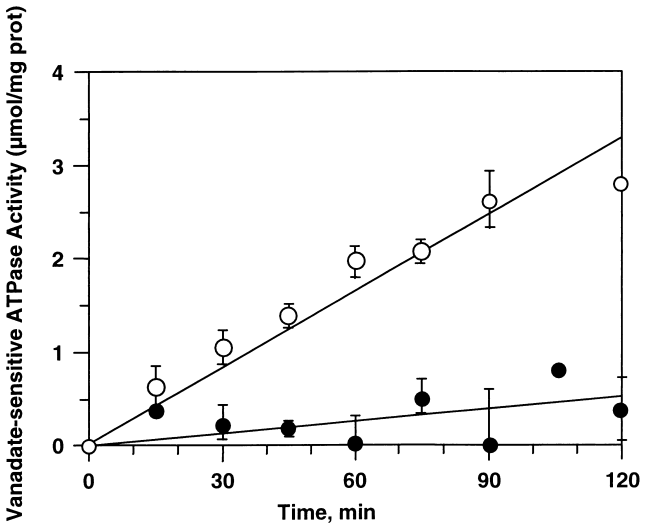

FIG. 1. Time dependence of vanadate-sensitive ATPase hydrolysis by MDR CEM/VLB$_{100}$ (○) and drug-sensitive CCRF–CEM plasma membranes (●). The reaction mixture contained 3 mM ATP, 1 mM $MgCl_2$,1 mM EGTA, 1 mM ouabain, 5 mM sodium azide, and 8 μg of membrane protein as described in Materials and Methods. Data represent the averages ± range of duplicate determinations; curves are representative of three independent experiments.

Gel Code kit, dried, and exposed to film overnight at −70°. Bands were quantitated by integration of the intensity of the beta emission from the volume corresponding to the ~170-kDa Pgp protein using a Molecular Dynamics PhosphorImager. Each band was corrected for non-specific 8-azido-[α-^{32}P]ATP labeling by subtraction of the value obtained for the non-UV-treated sample.

RESULTS

Time Course for ATP Hydrolysis

ATPase activity associated with plasma membranes was measured at 37° over a 120-min time course in the absence or presence of 1 mM sodium vanadate. Figure 1 compares the hydrolysis of ATP by plasma membranes prepared from drug-resistant CEM/VLB$_{100}$ membranes and drug-sensitive CCRF-CEM, illustrating that membranes from resistant cells had enhanced vanadate-sensitive hydrolysis of ATP due to the expression of Pgp. The effects of modulators on ATP hydrolysis were evaluated with both drug-resistant CEM/VLB$_{100}$ and drug-sensitive CCRF-CEM membranes. With CEM/VLB$_{100}$ membranes, ATP hydrolysis was linear throughout the 120-min time period (Fig. 2A). The presence of TMBY had no effect on the time–course, whereas verapamil increased the phosphate release throughout this time period. By contrast, the presence of vanadate in the assay buffer reduced markedly the formation of phosphate in both the absence and presence of modulators. Figure 2B illustrates the vanadate-sensitive portion of ATP hydrolysis and is the ATPase activity associated with Pgp. ATPase activity was enhanced throughout the time period in the presence of verapamil, when compared with that of the control or that of TMBY-treated membranes. By contrast, there was very little detectable ATPase activity or vanadate-sensitive ATPase activity associated with the drug-sensitive CCRF-CEM cells (Fig. 2, C and D). This residual ATPase activity was not affected significantly by verapamil or TMBY throughout the 120-min time course (Fig. 2D). A comparison of the vanadate-insensitive ATPase activity between the drug-resistant CEM/VLB$_{100}$ and the drug-sensitive CCRF-CEM membranes (Fig. 2, A and C) shows that there also was an enhancement of vanadate-insensitive ATPase in drug-resistant membranes. This may result from the overexpression of other ATPases unrelated to Pgp. In the experiments described below, ATPase activity was routinely measured for 90 min in the absence or presence of vanadate.

Effects of Modulators on Pgp–ATPase Activity Associated with Drug-resistant Membranes

Several modulators were examined for their effects on Pgp–ATPase activity associated with CEM/VLB$_{100}$ plasma membranes. Drugs were evaluated at three concentrations: 5, 10, and 20 μM; the concentration listed in Table 1 is the lowest concentration that gave the maximal effect. The compounds can be divided into three categories: a) those that stimulated Pgp–ATPase activity by 1.3- to 1.8-fold such as verapamil, nicardapine, and trifluoperazine; b) those that had no effect on activity (TMBY, amiodarane, and vindoline); and c) one modulator, cyclosporin A, that reduced ATPase activity to 0.7-fold. Moreover, verapamil, trifluoperazine, and TMBY had no significant effect on the residual vanadate-sensitive ATPase in the drug-sensitive CCRF-CEM membranes (Fig. 2D, data not shown).

To further examine the effect of TMBY on Pgp–ATPase activity, the concentration of TMBY was increased from 1 to 100 μM and compared with that of verapamil (Fig. 3). Verapamil stimulated Pgp–ATPase activity maximally between 10 and 25 μM, by ~1.6-fold. By contrast, TMBY had little effect up to 5 μM and inhibited at higher concentrations. At concentrations that modulate Pgp-mediated drug resistance in cytotoxicity assays with CEM/VLB$_{100}$ cells, 10 μM verapamil increased ATPase activity, while 5 μM TMBY was without effect [18, 25].

Effect of TMBY on Verapamil Stimulation of Pgp–ATPase

To evaluate the effects of TMBY and verapamil on Pgp, the interactions of TMBY and verapamil were examined on Pgp–ATPase activity. As shown in Fig. 4A, the presence of 5 or 10 μM TMBY blocked the stimulation of ATPase by verapamil. A Dixon–Webb plot indicated that TMBY competitively inhibited verapamil stimulation of Pgp–ATPase activity with an apparent K_i of 2.1 μM (Fig. 4B).

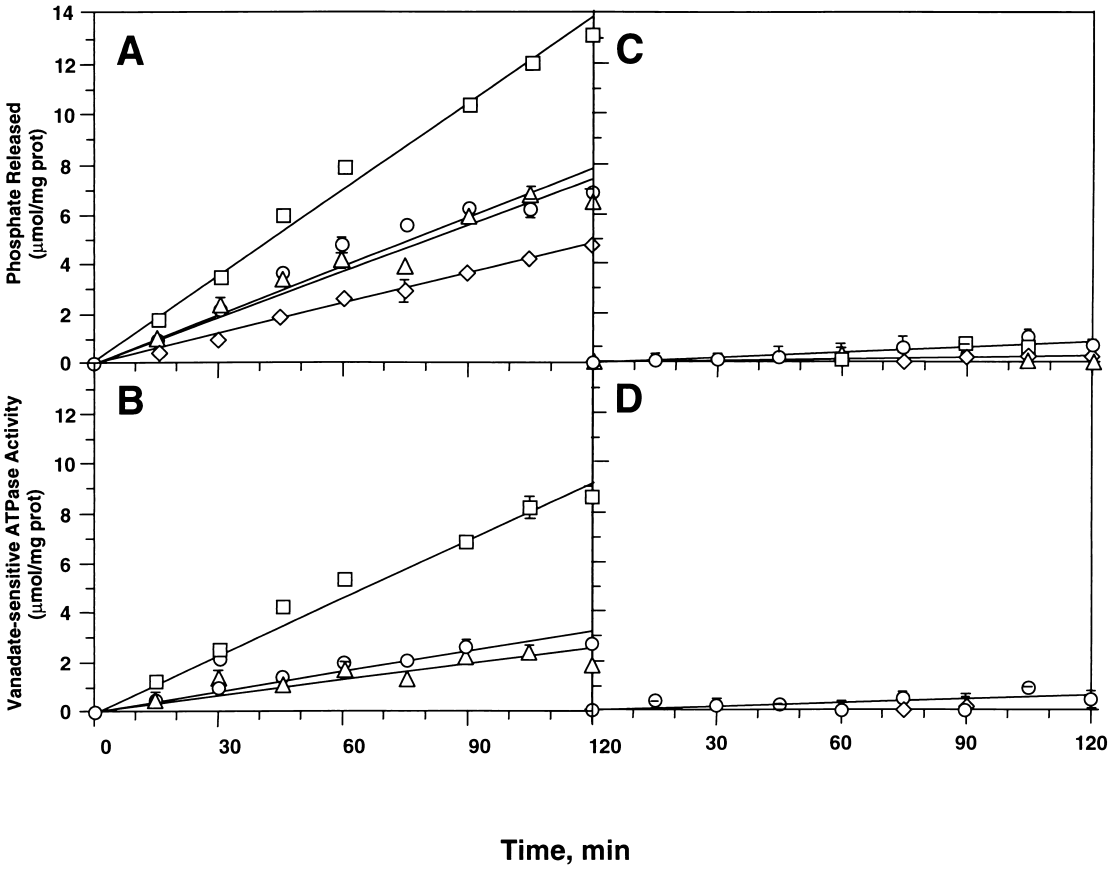

FIG. 2. Effect of modulators on the time course for ATPase hydrolysis by multidrug-resistant CEM/VLB$_{100}$ and drug-sensitive CCRF-CEM plasma membranes. The reaction mixture contained 3 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM ouabain, 5 mM sodium azide, and 8 μg of membrane protein as described in Materials and Methods. ATPase hydrolysis was measured in the absence of drug (○) or in the presence of 10 μM verapamil (□), 10 μM TMBY (△), or 1 mM vanadate (◇). Data represent the averages ± range of duplicate determinations; curves are representative of two or three independent experiments. (A) Phosphate released by ATPase hydrolysis associated with CEM/VLB$_{100}$ membranes. ATP hydrolysis was measured in the absence or presence of vanadate. (B) Pgp–ATPase activity associated with CEM/VLB$_{100}$ membranes determined from the data in panel A. This activity represents the vanadate-sensitive ATPase activity associated with Pgp. (C) Phosphate released by ATPase activity associated with drug-sensitive CCRF-CEM membranes. ATP hydrolysis was measured in the absence or presence of vanadate. (D) Vanadate-sensitive ATPase activity associated with drug-sensitive CCRF-CEM membranes determined from data in panel C.

TABLE 1. Effect of modulators on Pgp–ATPase activity in CEM/VLB$_{100}$ membranes*

Drug	Concn† (μM)	Pgp–ATPase activity (μmol/min/mg/protein)	Stimulation‡ (fold)	N
No drug	0	0.056 ± 0.005	1.00	18
Verapamil	10	0.102 ± 0.008	1.82§	18
Nicardapine	10	0.074 ± 0.004	1.32§	3
Trifluoperazine	10	0.085 ± 0.005	1.52§	8
TMBY	10	0.051 ± 0.005	0.91	8
Amiodarone	10	0.058 ± 0.003	1.04‖	5
Vindoline	10	0.054 ± 0.007	0.96‖	3
Cyclosporin A	10	0.039 ± 0.007	0.70§	5

*Pgp–ATPase activity was measured using CEM/VLB$_{100}$ plasma membranes. Data represent the means ± SEM from 3 to 18 independent experiments, as indicated.

†The drug concentrations tested were 5, 10, and 20 μM except for amiodarone and vindoline, which were tested at 10 and 20 μM. The indicated concentration is the drug concentration that gave the maximal effect.

‡Enhancement of Pgp–ATPase activity in drug-treated membranes compared with untreated membranes.

§Significantly different from the control by Student's *t*-test ($P < 0.05$).

‖Not significantly different from the control by Student's *t*-test ($P > 0.5$).

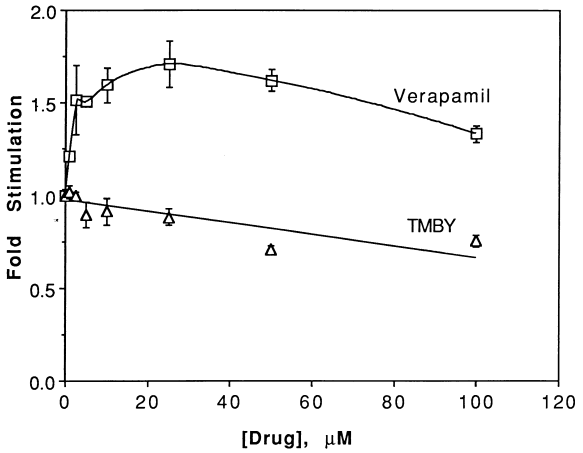

FIG. 3. Effect of the concentration of verapamil and TMBY on Pgp–ATPase activity of CEM/VLB$_{100}$ plasma membranes. Activity was assayed as described in Materials and Methods. Each point represents the mean ± SEM of triplicate determinations and is representative of three independent experiments.

Effects of Modulators on 8-azido-[α-^{32}P]ATP Photolabeling of Pgp

Urbatsch and coworkers [24] have demonstrated that Pgp–ATPase activity is inhibited by vanadate-trapped 8-azido-[α^{32}P]ATP and that photolabeling of Pgp is specifically at the ATP binding sites. We asked if modulators might also affect the binding of 8-azido-[α^{32}P]ATP to Pgp. CCRF-CEM and CEM/VLB$_{100}$ membranes were incubated in the presence of vanadate, $MgCl_2$, and 8-azido-[α^{32}P]ATP, and were irradiated with UV light. Proteins were separated by SDS–PAGE, and an autoradiogram was prepared. As shown in Fig. 5A, a ~170-kDa protein was labeled in CEM/VLB$_{100}$ membranes but not in CCRF-CEM membranes, consistent with labeling of Pgp. To ascertain if modulators might alter photolabeling of Pgp under these conditions, the same incubation conditions were used in the absence or presence of 10 μM verapamil, 10 μM TMBY, or both modulators. As shown in panels B and C of Fig. 5, verapamil enhanced the vanadate-trapping of 8-azido-ATP by 2.1-fold ($P < 0.001$) whereas, TMBY had no effect (0.98-fo1d, $P > 0.5$). When membranes were incubated with both 10 μM TMBY and 10 μM verapamil, there was no effect on Pgp photolabeling (0.94-fold; $P > 0.5$). Control experiments indicated that photolabeling was dependent upon UV activation and that excess ATP blocked photolabeling of Pgp, as shown in Fig. 5.

Effect of Vinblastine on Verapamil Stimulation of Pgp-ATPase

Previously in equilibrium binding studies, we demonstrated that TMBY is a competitive inhibitor of the binding of the natural product oncolytic VLB, which is a known substrate of Pgp [18]. Consequently, the effect of VLB on Pgp–ATPase activity was examined. When CEM/VLB$_{100}$ membranes were incubated with a wide range of VLB concentrations from 0.1 to 50 μM, Pgp–ATPase activity was unaffected (data not shown). Accordingly, the effect of vinblastine on the stimulation of Pgp–ATPase activity by verapamil was examined. A Dixon–Webb plot of these data (Fig. 6) demonstrates that VLB was a competitive inhibitor of verapamil stimulation with an apparent K_i of 2.1 μM.

DISCUSSION

The present study examined the effects of MDR modulators on ATPase activity associated with Pgp expressed by MDR cells. Because the lipid environment has been shown to affect Pgp–ATPase activity [26, 27], studies were conducted with Pgp still associated with native plasma membranes prepared from drug-resistant CEM/VLB$_{100}$ cells. Previous reports have indicated that certain modulators enhance the ATP hydrolytic activity of Pgp, using either partially or fully purified Pgp [9, 11–13, 28, 29]. The present results with plasma membranes are consistent with these earlier findings. Pgp–ATPase activity was stimulated by nicardipine, trifluoperazine, and verapamil by 1.4- to 1.8-fold [29]. Cyclosporin A, on the other hand, reduced Pgp–ATPase activity as previously reported, and, in this respect, cyclosporin A acts similarly to gramicidin D [30, 31]. Unlike

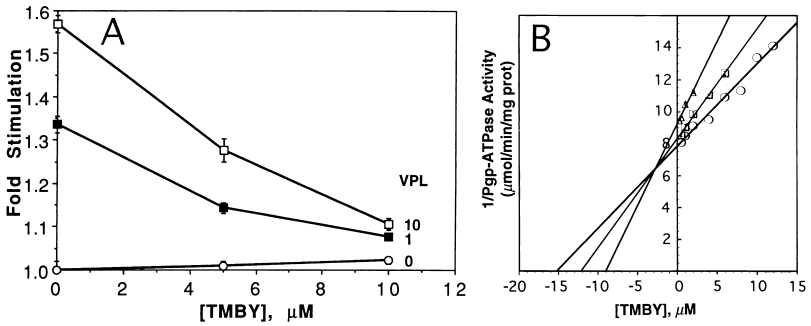

FIG. 4. Effect of TMBY on verapamil stimulation of Pgp–ATPase activity. (A) Effect of increasing concentrations of TMBY on verapamil stimulation of Pgp–ATPase. The concentration of verapamil in the assay was none (○), 1 μM (■), and 10 μM (□). Each point represents the mean ± SEM of triplicate determinations; the curve is representative of two independent experiments. (B) Dixon–Webb plot for the inhibition of verapamil-stimulated Pgp–ATPase activity by TMBY. Verapamil concentrations were 0.5 μM (△), 1 μM (□), and 10 μM (○). The apparent K_i for TMBY was determined to be 2.1 μM.

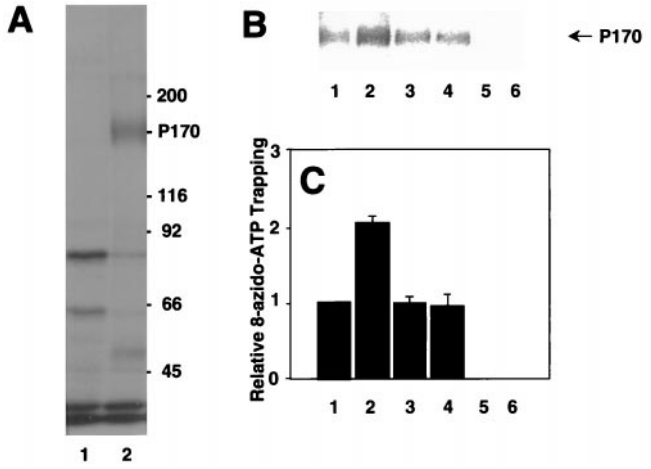

FIG. 5. Vanadate trapping of 8-azido-[α-^{32}P]ATP of Pgp. Plasma membranes were incubated with 600 μM vanadate, 1 mM $MgCl_2$, and 10 μM 8-azido-[α-^{32}P]ATP as described in Materials and Methods. Photolabeling of drug-resistant CEM/VLB_{100} membranes in the absence of vanadate was barely detectable (data not shown). In control experiments, immunoblots probed with the anti-Pgp antibody C219 detected the presence of a 170-kDa protein only in the drug-resistant CEM/VLB_{100} membranes and not in the drug-sensitive CCRF-CEM membranes (data not shown). (A) Vanadate-trapping of 8-azido-[α-^{32}P]ATP by drug-sensitive CCRM-CEM and drug-resistant CEM/VLB_{100} plasma membranes. Plasma membranes were incubated in the absence of any modulator. Proteins were separated by SDS–PAGE, and an autoradiogram was prepared. Lanes 1 and 2 were UV-treated CCRF–CEM and CEM/VLB_{100} membranes, respectively. The P170, 170-kDa protein, corresponds to the Pgp associated with the membranes. (B) Membranes were incubated in the absence of modulator (lane 1) or in the presence of 10 μM verapamil (lane 2), 10 μM TMBY (lane 3), or 10 μM verapamil plus 10 μM TMBY (lane 4). Two control incubation conditions were also examined. Lane 5 represents the effect of 3 mM ATP on membranes incubated in the absence of modulator and in the presence of 3 mM ATP, and lane 6 shows the effect of no UV treatment. Silver staining of gels prior to quantitation with the PhosphorImager showed uniform staining for each lane (data not shown). (C) Graph of the relative 8-azido-[α-^{32}P]ATP trapping for each condition presented in panel B. Lane 1 was the control with no modulator; lane 2, 10 μM verapamil (2.11 ± 0.005 fold enhancement, $P < 0.001$); lane 3, 10 μM TMBY (0.98 ± 0.07 fold enhancement, $P > 0.5$); lane 4, 10 μM verapamil and 10 μM TMBY (0.94 ± 0.14 fold enhancement, $P > 0.5$); lane 5, 3 mM ATP; and lane 6, no UV treatment. Data represent the mean ± SEM, N = 3 for lanes 1 and 2 and the average ± the range, N = 2 for lanes 3–6. Data are from 2–3 independent experiments. Data were analyzed by a Student's *t*-test where $P < 0.05$ is significantly different and $P > 0.5$ is not significantly different from the control.

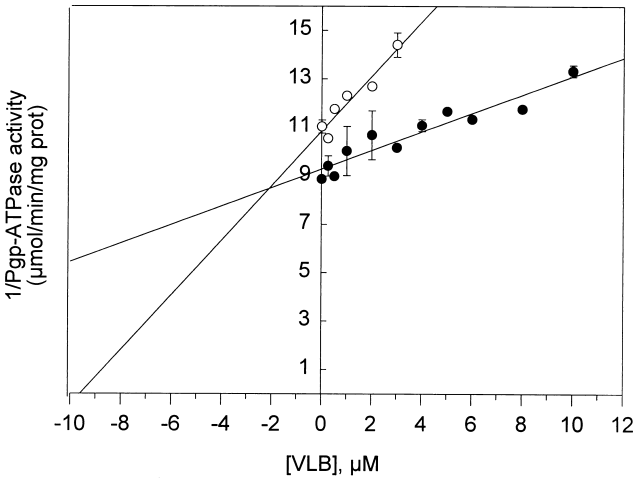

FIG. 6. Dixon–Webb plot for the inhibition of verapamil-stimulated Pgp–ATPase activity by vinblastine. Verapamil concentrations were 1 μM (○) and 10 μM (●). The apparent K_i for vinblastine was determined to be 2.1 μM. Data are the average ± range, N = 2, for 1 μM verapamil. The curves are representative of two independent experiments.

previous reports, amiodarone had no effect on Pgp–ATPase activity [13, 32]. Two other agents, neither of which has hitherto been examined, were also shown to have little or no effect on Pgp–ATPase activity: the yohimbine analog TMBY and the *Vinca* alkaloid analog vindoline. It is interesting that concentrations of TMBY that fully modulate drug sensitivity in cytotoxicity assays (5 μM and above) had little or no effect on Pgp–ATPase. This has also been observed for the highly potent Pgp modulator LY335979 that fully modulates multidrug resistance at 0.1 μM and has no effect on Pgp–ATPase activity up to 0.4 μM [18]. Thus, the ability to alter the activity of Pgp–ATPase does not indicate how well a compound may modulate drug resistance.

An unexpected finding of the present study was that TMBY and verapamil have different effects on Pgp–ATPase activity. Both drugs are effective modulators of a number of MDR drugs in cytotoxicity assays [18]. They have been shown to displace the photolabeling of Pgp with photoactivatable analogs of vinblastine [25] and to displace the binding of radiolabeled oncolytics to Pgp in equilibrium binding studies [18]. The modulators share several structural features critical for Pgp binding and chemosensitizing of MDR cells. Molecular modeling studies indicated that the two compounds are readily superimposable (Fig. 7) within their lower conformational energy surface and possess similar calculated molar refractivity [25]. Most importantly, verapamil and TMBY share a common pharmacophore consisting of two aromatic rings and a basic nitrogen necessary for binding to Pgp (Fig. 7). Thus, similar effects on Pgp–ATPase activity would be anticipated. However, verapamil stimulated ATPase activity by 1.8-fold, whereas TMBY had no effect on this activity at a concentration that modulates MDR in cytotoxicity assays [18]. It is curious, therefore, that TMBY blocked the stimulation of Pgp–ATPase activity by verapamil. A Dixon–Webb plot demonstrated that TMBY is a competitive inhibitor of verapamil-stimulated ATPase with an apparent K_i of 2.1 μM, in close agreement with the previously reported K_i of 1.1 to 3.2 μM measured by the displacement of the radiolabeled oncolytics vinblastine, paclitaxel, and etoposide to Pgp in equilibrium binding assays [18]. Moreover, identical results were obtained when the effects of these two modulators were examined on vanadate-trapping of 8-azido-[α-

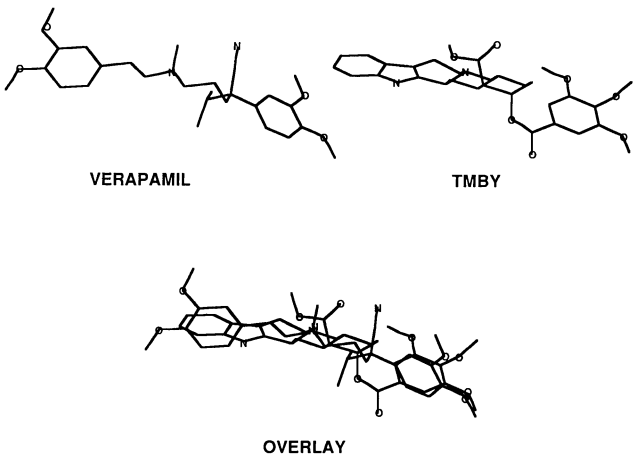

FIG. 7. Calculated three-dimensional structures of verapamil, TMBY, and their overlay. The overlay was generated by minimizing the least-squares differences in the basic nitrogen atom and the position of the aromatic domains, as previously reported [25].

^{32}P]ATP to Pgp. Verapamil increased the amount of vanadate-trapped 8-azido-[α-^{32}P]ATP by 2.1-fold; TMBY again had no effect, and TMBY blocked verapamil stimulation of the vanadate-trapped nucleotide. The present study demonstrated that verapamil enhances the formation of the vanadate–nucleotide complex of Pgp. The vanadate–nucleotide–enzyme complex is thought to be important during the catalytic transition state of Pgp since it is a mimic of the phosphate–nucleotide–enzyme complex [24]. This study is the first demonstration that any modulator affects this critical step in catalysis. A recent study of the effects of verapamil and two other Pgp modulators, cyclosporin A and PSC 833, on 8-azido-[α-^{32}P]ATP photolabeling of Pgp in the absence of vanadate revealed no effect of these three agents [33]. The presence of vanadate enhanced the photolabeling of Pgp dramatically in our studies (data not shown). Consequently, the photolabeling of Pgp in the presence of vanadate enhances the sensitivity of the analysis and is most likely responsible for the discrepancy between these two studies.

We also examined the effect of a known Pgp substrate, vinblastine, on Pgp–ATPase activity. VLB did not affect the Pgp–ATPase activity associated with the CEM/VLB$_{100}$ membranes. When the effect of VLB was examined on the verapamil stimulation of Pgp–ATPase, VLB was a competitive inhibitor of verapamil. VLB had an apparent K_i of 2.1 μM in good agreement with a K_D of 1.5 μM and 6 $\pm$ 2.5 μM (N = 3) determined in equilibrium binding experiments [34] (data not shown). These results are also consistent with certain molecular similarities between these two agents. Although verapamil and VLB differ in their molar refractivities, the two compounds are superimposable with respect to the pharmacophore elements necessary for Pgp binding [35]. Thus, the two aromatic rings and the basic nitrogen used to describe verapamil overlay with similar elements within the larger VLB structure. This suggests that the same structural features in verapamil, VLB, and TMBY are important for mediating their effects on Pgp.

Taken together, these data indicate that TMBY, verapamil, and VLB share a common or overlapping binding site in Pgp [19, 35]. Cyclosporin A, cyclosporin D analog, PSC 833, and gramicidin D have also been shown to be competitive inhibitors of verapamil-stimulated ATPase activity [31, 33, 36]. However, earlier studies indicated that multiple binding sites for modulators do exist. For example, Pgp has a 1,4-dihydropyridine-selective drug site that is distinct from the *Vinca* alkaloid binding site [29, 37]. Also, the effects of progesterone and verapamil on Pgp–ATPase activity are synergistic and result from binding to distinct binding sites [29, 37, 38].

There are at least two plausible explanations for the differential effects of verapamil and TMBY observed in these studies. One possibility is that verapamil and TMBY may possess a common binding site that may be created from two partially overlapping binding sites, as proposed by Garrigos *et al.* [29]. Verapamil, for example, may possess yet another structural element or pharmacophore that TMBY lacks that is necessary for the stimulation of Pgp–ATPase activity. This structural element may interact with a specific portion on the protein, resulting in a conformational change in Pgp necessary for the drug stimulation of the ATP hydrolytic activity [39]. In this scenario, TMBY would lack this putative element, rendering it unable to stimulate ATPase activity but, nevertheless, able to compete with verapamil for binding. Another possibility is that the difference in the effects of TMBY and verapamil on Pgp–ATPase may result from differences in the rigidity of their structures. As a yohimbine analog, TMBY presents a more conformationally restricted structure than verapamil, which has free rotation about many of its bonds (Fig. 7 and [25]). Because the drug binding site of Pgp appears to be formed between the two halves of the Pgp molecule [40–43], binding of a rigid structure such as TMBY may hinder possible conformational changes in Pgp. This may prevent the interactions of the two half portions of the Pgp molecule that are needed for drug stimulation of ATP hydrolysis and/or may interfere with different points in the catalytic cycle of the membrane protein [39, 43]. More studies are clearly needed to distinguish between these possible mechanisms.

References

1. van der Bliek AM and Borst P, Multidrug resistance. *Adv Cancer Res* **52:** 165–202, 1989.
2. Roninson IB, *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*. Plenum Publishing, New York, 1990.
3. Gros P, Neriah YB, Croop JM and Housman DE, Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* **323:** 728–731, 1986.
4. Ueda K, Cardarelli C, Gottesman MM and Pastan I, Expression of a full-length cDNA for the human "MDR1" gene

confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* **84:** 3004–3008, 1987.
5. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58:** 137–171, 1989.
6. Naito M, Hamada H and Tsuruo T, ATP/Mg^{2+}-dependent binding of vincristine to the plasma membrane of multidrug-resistant K562 cells. *J Biol Chem* **263:** 11887–11891, 1988.
7. Horio M, Gottesman MM and Pastan I, ATP–dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc Natl Acad Sci USA* **85:** 3580–3584, 1988.
8. Sarkadi B, Price EM, Boucher RC, Germann UA and Scarborough GA, Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *J Biol Chem* **267:** 4854–4858, 1992.
9. Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM and Pastan I, Partial purification and reconstitution of the human multidrug-resistance pump: Characterization of the drug-stimulatable ATP hydrolysis. *Proc Natl Acad Sci USA* **89:** 8472–8476, 1992.
10. Hamada H and Tsuruo T, Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance. 170- to 180-Kilodalton membrane glycoprotein is an ATPase. *J Biol Chem* **263:** 1454–1458, 1988.
11. Hamada H and Tsuruo T, Characterization of the ATPase activity of the M_r 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res* **48:** 4926–4932, 1988.
12. Sharom FJ, Yu S and Doige CA, Functional reconstitution of drug transport and ATPase activity in proteoliposomes containing partially purified P-glycoprotein. *J Biol Chem* **268:** 24197–24202, 1993.
13. Shapiro AB and Ling V, ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. *J Biol Chem* **269:** 3745–3754, 1994.
14. Ford JM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:** 155–199, 1990.
15. Beck WT, Modulators of P-glycoprotein-associated multidrug resistance. *Cancer Treat Res* **57:** 151–170, 1991.
16. Arceci RJ, Stieglitz K and Bierer BE, Immunosuppressants FK506 and rapamycin function as reversal agents of the multidrug resistance phenotype. *Blood* **80:** 1528–1536, 1992.
17. Mizuno K, Furuhashi Y, Misawa T, Iwata M, Kawai M, Kikkawa F, Kano T and Tomoda Y, Modulation of multidrug resistance by immunosuppressive agents: Cyclosporin analogues, FK506, and mizoribine. *Anticancer Res* **12:** 21–26, 1992.
18. Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, Baughman TM, Bumol T and Starling JJ, Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. *Cancer Res* **56:** 4171–4179, 1996.
19. Pearce HL, Safa AR, Bach NJ, Winter MA, Cirtain MC and Beck WT, Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. *Proc Natl Acad Sci USA* **86:** 5128–5132, 1989.
20. Beck WT, Mueller MJ and Tanzer LR, Altered surface membrane glycoproteins in *Vinca* alkaloid-resistant human leukemic lymphoblasts. *Cancer Res* **39:** 2070–2076, 1979.
21. Lever JE, Active amino acid transport in plasma membrane vesicles from Simian virus 40-transformed mouse fibroblasts. Characteristics of electrochemical Na^+ gradient-stimulated uptake. *J Biol Chem* **252:** 1990–1997, 1977.
22. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Garter FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
23. Fiske CH and Subbarow Y, The colorimetric determination of phosphorous. *J Biol Chem* **66:** 375–400, 1925.
24. Urbatsch IL, Sankaran B, Weber J and Senior AE, P-Glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J Biol Chem* **270:** 19383–19390, 1995.
25. Pearce HL, Winter MA and Beck WT, Structural characteristics of compounds that modulate P-glycoprotein-associated multidrug resistance. *Adv Enzyme Regul* **30:** 357–373, 1989.
26. Doige CA, Yu X and Sharom FJ, The effects of lipids and detergents on ATPase-active P-glycoprotein. *Biochim Biophys Acta* **1146:** 65–72, 1993.
27. Urbatsch IL and Senior AE, Effects of lipids on ATPase activity of purified Chinese hamster P-glycoprotein. *Arch Biochem Biophys* **316:** 135–140, 1995.
28. Al-Shawi MK and Senior AE, Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. *J Biol Chem* **268:** 4197–4206, 1993.
29. Garrigos M, Mir LM and Orlowski S, Competitive and non-competitive inhibition of the multidrug-resistancs-associated P-glycoprotein ATPase–Further experimental evidence for a multisite model. *Eur J Biochem* **244:** 664–673, 1997.
30. Rao US and Scarborough GA, Direct demonstration of high affinity interactions of immunosuppressant drugs with the drug binding site of the human P-glycoprotein. *Mol Pharmacol* **45:** 773–776, 1994.
31. Borgnia MJ, Eytan GD and Assaraf YG, Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J Biol Chem* **271:** 3163–3171, 1996.
32. Litman T, Zeuthen T, Skovsgaard T and Stein WD, Structure–activity relationships of P-glycoprotein interacting drugs: Kinetic characterization of their effects on ATPase activity. *Biochim Biophys Acta* **1361:** 159–168, 1997.
33. Watanabe T, Kokubu N, Charnick SE, Naito M, Tsuruo T and Cohen D, Interaction of cyclosporin derivatives with the ATPase activity of human P-glycoprotein. *Br J Pharmacol* **122:** 241–248, 1997.
34. Cornwell MM, Gottesman MM and Pastan IH, Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J Biol Chem* **261:** 7921–7928, 1986.
35. Zamora JM, Pearce HL and Beck WT, Physical–chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol Pharmacol* **33:** 454–462, 1988.
36. Rao US, Mutation of glycine 185 to valine alters the ATPase function of the human P-glycoprotein expressed in Sf9 cells. *J Biol Chem* **270:** 6686–6690, 1995.
37. Ferry DR, Russell MA and Cullen MH, P-Glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is allosterically coupled to a vinca-alkaloid-selective binding site. *Biochem Biophys Res Commun* **188:** 440–445, 1992.
38. Orlowski S, Mir LM, Belehradek JJr and Garrigos M, Effects of steroids and verapamil on P-glycoprotein ATPase activity: Progesterone, desoxycorticosterone, corticosterone and verapamil are mutually non-exclusive modulators. *Biochem J* **317:** 515–522, 1996.
39. Loo TW and Clarke DM, Drug-stimulated ATPase activity of human P-glycoprotein requires movement between transmembrane segments 6 and 12. *J Biol Chem* **272:** 20986–20989, 1997.
40. Gottesman MM and Pastan I, The multidrug transporter, a double-edged sword. *J Biol Chem* **263:** 12163–12166, 1988.
41. Safa AR, Stern RK, Choi K, Agresti M, Tamai I, Mehta ND

and Roninson IB, Molecular basis of preferential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185->Val-185 substitution in P-glycoprotein. *Proc Natl Acad Sci USA* **87:** 7225–7229, 1987.
42. Bruggemann EP, Currier SJ, Gottesman MM and Pastan I, Characterization of the azidopine and vinblastine binding site of P-glycoprotein. *J Biol Chem* **267:** 21020–21026, 1992.
43. Loo TW and Clarke DM, Reconstitution of drug-stimulated ATPase activity following co-expression of each half of human P-glycoprotein as separate polypeptides. *J Biol Chem* **269:** 7750–7755, 1994.