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Characterization of a Novel Bisacridone and Comparison with PSC 833 as a Potent and Poorly Reversible Modulator of P-Glycoprotein

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SUMMARY

Novel compounds, composed of two acridone moieties connected by a propyl or butyl spacer, were synthesized and tested as potential modulators of P-glycoprotein (P-gp)-mediated multidrug resistance. The propyl derivative 1,3-bis(9-oxoacridin-10-yl)-propane (PBA) was extremely potent and, at a concentration of 1 μM, increased steady state accumulation of vinblastine (VLB) ≈9-fold in the multidrug-resistant cell line KB8–5. In contrast to the readily reversible effects of VRP and cyclosporin A on VLB uptake and similar to the effects of the cyclosporin analog PSC 833, this modulation by PBA was not fully reversed 6–8 hr after transfer of cells to PBA-free medium. Continuous exposure to 3 μM PBA was nontoxic and could completely reverse VLB resistance in KB8–5 cells. Consistent with its effects on VLB transport, the drug resistance-modulating effect of PSC 833 was significantly more persistent than that of VRP. However, the effect of PBA was, like that of VRP, rapidly reversed once the modulator was removed from the extracellular environment. PBA was able to compete with radiolabeled azidopine for binding to P-gp and to stimulate P-gp ATPase activity. However, both the steady state accumulation of PBA and the rate of efflux of PBA were similar in drug-sensitive KB3–1 and drug-resistant KB8–5 cells, suggesting that this compound is not efficiently transported by P-gp. These results indicate that PBA represents a new class of potent and poorly reversible synthetic modulators of P-gp-mediated VLB transport.

Despite advances in the use of chemotherapeutic drugs for the treatment of human cancer, the emergence of resistance to these agents, at either initial presentation or the time of relapse, remains a major problem and has been the subject of numerous investigations. MDR in model cell lines is frequently associated with overexpression of the *MDR1* gene product, P-gp. One characteristic of the MDR phenotype is that tumor cells selected for resistance to a single agent (e.g., a *Vinca* alkaloid or an anthracycline antibiotic) simultaneously become resistant to a large number of structurally and functionally unrelated cytotoxic agents. P-gp is a 170-kDa integral membrane protein proposed to catalyze ATP-dependent drug efflux from cells, and thus effectively reducing intracellular drug accumulation in the resistant cells (1).

MDR1 expression has been detected in several human tumors (2), but the relevance of P-gp and the MDR phenomenon to drug resistance in clinical malignancies is still uncertain (3, 4), and other factors conferring drug resistance are important. There is some evidence to suggest that in childhood cancers and hematological malignancies, P-gp expression is predictive of poor therapeutic outcome (5, 6), although in another study, P-gp expression at diagnosis did not correlate with response in childhood rhabdomyosarcoma (7). In addition, as described in patients with myeloma, the selection of tumor cells with a high level of *MDR1* expression may result from prior therapy with natural product chemotherapeutic agents (8). There seems to be two basic approaches to circumvention of P-gp-associated MDR; the first is to use other effective chemotherapeutic drugs, such as the alkylating agents, in which transport is not mediated by P-gp (1),

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ABBREVIATIONS: MDR, multidrug resistant/resistance; PBA, 1,3-bis(9-oxoacridin-10-yl)-propane; VLB, vinblastine; VRP, verapamil; CsA, cyclosporin A; R123, rhodamine 123; P-gp, P-glycoprotein; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HR, HEPES-Ringer.

and the second is to develop means of inhibiting the function of P-gp (9).

Since the observation that the calcium channel blocker VRP was able to enhance cytotoxicity in MDR cell lines, a large number of structurally unrelated small lipophilic molecules capable of modulating P-gp function *in vitro* have been described (reviewed in Ref. 10); these include other calcium channel blockers, calmodulin antagonists, steroidal agents, phenothiazine and phenoxazine derivatives, CsA, and its nonimmunosuppressive analog, PSC 833 (11, 12). These modulating agents can reverse resistance to the chemotherapeutic agents associated with MDR, presumably by increasing intracellular drug accumulation. In many cases, the mechanism responsible is believed to be competition between modulator and cytotoxic drug for binding to P-gp and, hence, for efflux from cells (13).

The attributes of an optimal modulator remain to be elucidated; however, the agent must be able to reverse drug resistance at concentrations that are nontoxic, a problem that has limited the use of many of the agents that have been evaluated in a clinical setting (reviewed in Ref. 2). For example, although some clinical responses have been achieved in patients with myeloma by treatment with VRP as part of the chemotherapy regimen, cardiovascular toxicity is observed commonly. The addition of CsA to chemotherapy protocols has had limited success, but there are reports of multiple toxicities at doses required to achieve blood concentrations capable of reversing MDR *in vitro*. A nonimmunosuppressive analog, PSC 833, has been shown to be more potent than CsA *in vitro* (14) and is currently undergoing clinical evaluation (15). Although many attempts have been made to identify potent modulators, one aspect that has received less attention is the rate of recovery of P-gp function once the modulator is removed from the extracellular environment. The rapid reversal of the P-gp block by VRP has been described previously, as has the persistence of inhibition of P-gp function conferred by PSC 833 (16, 17).

We demonstrated previously that whereas an acridine derivative had no effect on *Vinca* accumulation in MDR cell lines and phenothiazine had only low-level activity, phenoxazine, in which the C5 position is replaced with oxygen, was extremely effective (18). These data suggest that the electronegativity at C5 may be important for modulating P-gp-mediated drug efflux. In this study, a small series of bisacridones in which two acridone moieties were separated by a propyl or butyl spacer were synthesized, and the compounds were characterized as potential modulators of P-gp-mediated MDR. The propyl derivative, PBA, was found to be extremely specific and effective in enhancing VLB accumulation and inhibiting VLB efflux in the MDR cell line KB8–5. In addition, this modulation of VLB transport by PBA, like that of PSC 833, was found to be only poorly reversible after transfer of cells to modulator-free medium. These results indicate that PBA represents a new class of potent and poorly reversible synthetic modulators of P-gp-mediated VLB transport.

Experimental Procedures

Cell lines and culture conditions. Human epidermoid carcinoma KB3–1 and MDR variants KB8–5 (maintained in 10 ng/ml colchicine) and KB-V1 (maintained in 1 μg/ml VLB) were obtained from Dr. M. Gottesman (National Cancer Institute, Bethesda, MD) (19). They were grown in monolayer culture in antibiotic-free Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone, Logan, UT).

Synthesis of PBA. A suspension of 1 g (4.4 mmol) of acridone and 0.172 g (4.4 mmol) of sodium amide in 150 ml of anhydrous xylene was heated at reflux with stirring. After the addition of 4 g (24 mmol) of 1-bromo-3-chloro propane, the reflux was continued for 3 days. The reaction mixture was filtered under hot conditions and roto-evaporated, and the residue containing PBA was purified by recrystallizing in an ethanol/water (3:2) mixture containing 1 N KOH. The purified product was characterized by UV, NMR, and mass spectrometry methods. The structural formulas of the parent compound acridone and the propyl (PBA) and butyl bisacridone derivatives are shown in Fig. 1.

VLB accumulation and efflux studies. Cell suspensions (2 ml, 2×10^6 cells) were plated onto 35-mm dishes and allowed to attach overnight. Medium was then aspirated, and the cells were washed twice with 2 ml of physiological Tris buffer (120 mM NaCl, 20 mM Tris base, pH 7.4, 3 mM K_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose). After the second 10-min wash, monolayers were incubated at room temperature with 1 ml of serum-free RPMI/25 mM HEPES, pH 7.4, containing 37.5 nM [^{3}H]VLB (0.5 μCi/ml; specific activity, 12 Ci/mmol; Moravek Biochemicals, Brea, CA) with or without modulating agents. In the control experiment in which both uptake and efflux were in the absence of modulator, a radioactive concentration of 5 μCi/ml was used. This would result in a "steady state" radiolabeled VLB uptake similar to that achieved with 0.5 μCi/ml in the presence of modulator. VRP was obtained from Sigma Chemical (St. Louis, MO), CsA (Sandimmune injection) was purchased from Sandoz (Basel, Switzerland), and the cyclosporin analog PSC 833 was a gift from Dr. D. Cohen (Sandoz, East Hanover, NJ). After the appropriate period of incubation at room temperature, the medium was rapidly aspirated to terminate VLB accumulation or, for efflux studies, replaced with 3 ml of radiolabel-free medium with or without modulator. At the end point of the experiment, the monolayers were washed four times with ice-cold phosphate-buffered saline, and the cell-associated radiolabel was determined by scintillation counting of trypsinized cells. Cell number per dish was determined by a cell lysis procedure (20).

To determine the reversibility of the effects of the modulating agents on VLB uptake, cell monolayers were first incubated with modulators for 1 hr in serum-free DMEM at 37°. The cells were then washed and incubated in complete medium without modulator at 37° for ≤12 hr after the beginning of preincubation until the start of a 2-hr VLB accumulation study as described above. To determine the long-term reversibility of the effects of modulators on VLB efflux, cells were first preincubated with PBA or PSC 833 as described for the accumulation studies. VLB accumulation (1 hr) in modulator-

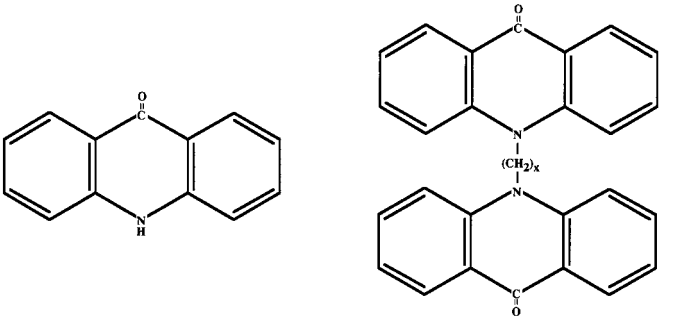

Fig. 1. Structural formulas of acridone and the PBA and butyl bisacridone derivatives.

free medium was carried out at 2 or 4 hr after the beginning of the preincubation; then, the percent retention of VLB was determined after a 2-hr efflux in modulator-free medium.

Effect of acridone derivatives on the cytotoxicity of VLB. KB3–1 or KB8–5 cells were plated at a density of 1000 cells/well in six-well tissue culture dishes. After 24 hr, incubation medium was replaced with medium containing serial dilutions of VLB (Lyphomed, Deerfield, IL) in the absence or presence of nontoxic concentrations of modulators. In the initial studies, cells were incubated with VLB either continuously or for 2 hr, with continuous exposure to the modulators in both sets of experiments. After 7 days, colonies were stained with crystal violet and counted using an automated colony counter. The IC_{50} values (defined as the concentration of VLB required for 50% reduction in colony number compared with controls) were determined from cell survival curves.

Photoaffinity labeling of P-gp. Crude membranes were prepared from drug-sensitive KB3–1 cells and the MDR variants KB8–5 and KB-V1 essentially as described previously (21). Briefly, cells were homogenized in ice-cold medium containing 20 mM Tris base, pH 7.2, 250 mM sucrose, and 0.5 mM DTT and then centrifuged at $1,000 \times g$ to remove nuclei and unbroken cells. The supernatant was further centrifuged at $10,000 \times g$ to separate mitochondria; then, a membrane fraction was obtained by centrifugation at $270,000 \times g$. This pellet was suspended in medium containing 25 mM Tris base, pH 7.2, and 100 mM mannitol, and the suspension was stored at − 80° until use. Membrane fractions (170 μg of protein) were preincubated with dimethylsulfoxide (0.1%) or 3 μM of either PBA or VLB for 30 min. [^{3}H]Azidopine (specific activity, 55 Ci/mmol; Amersham, Arlington Heights, IL) was added to a final concentration of 0.4 μM, and the incubation was continued for 60 min in the dark. The samples were then irradiated for 2 min at 254 nm (Stratalinker; Stratagene, La Jolla, CA). Finally, the samples were centrifuged at $270,000 \times g$ for 30 min, and the pellets were resuspended and electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel. After fixation, the gels were incubated for 30 min with AMPLIFY (Amersham) and dried. Autoradiography was performed at −80°.

Measurement of P-gp-associated ATPase activity. Sf9 cells growing in suspension culture in Grace's insect medium (0.5×10^6/ml) were infected with baculovirus containing either recombinant *LacZ* or *MDR1* genes (22). Membranes were prepared as described above for the KB cells. The ATPase activity of isolated Sf9 cell membranes was estimated by measuring inorganic phosphate liberation essentially as described by Sarkadi *et al.* (23). Briefly, membrane suspensions (20 μg of protein) were incubated at 37° in 0.1 ml of buffer containing 50 mM Tris-2-(*N*-morpholino)ethanesolfonic acid, pH 6.8, 2 mM EDTA, 2 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 5 mM sodium azide, and 1 mM ouabain. The reaction was started by addition of 5 mM ATP and stopped after 20 min by addition of 0.1 ml of 5% SDS solution. ATPase activity was determined from the difference obtained in inorganic phosphate levels at 0 and 20 min. Inorganic phosphate was measured immediately by a colorimetric assay, and the vanadate-sensitive ATPase activity, linear for >20 min and attributable to P-gp ATPase, was determined.

Steady state intracellular PBA concentration. KB3–1 and KB8–5 cells plated onto rectangular coverslips were loaded with 1 μM PBA in HR for 1 hr at room temperature and then washed with ice-cold HR for 30 sec. HR contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 8 mM glucose, and 10 mM HEPES/NaOH, pH 7.4. For PBA accumulation experiments in energy-depleted KB8–5 cells, accumulation of 3 μM PBA was conducted in HR containing 1 μM antimycin A and no glucose. The coverslips were washed, placed in a cuvette, and lysed in ethanol. The concentration of PBA was determined on a SPEX spectrofluorometer (CMT1; SPEX Industries, Edison, NJ). The excitation and emission wavelengths were 390 and 430 nm, respectively. Cell numbers were determined from a nuclei count (20).

Unidirectional efflux of R123 and PBA. Cells plated onto round coverslips were loaded with R123 or PBA for 1 hr at room temperature. The concentration of R123 in HR was 2 μM for KB3–1 cells and 10 μM for KB8–5 cells, and the concentration of PBA for both cell lines was 1 μM. Once loading was complete, the cells were placed on the stage of an inverted microscope (Diaphot; Nikon, Tokyo) and superfused with HR at room temperature, and the decrease in intracellular fluorescence of ≈150 cells was followed as a function of time. All measurements were carried out with a SPEX spectrofluorometer using a high-numerical aperture oil-immersion objective (NA = 1.3, 40×, Nikon model 78820). For R123 measurements, the excitation wavelength was 488 nm, the dichroic mirror was a 515DRLP (Omega Optical, Brattleboro, VT), and the emission filter was a 535DF25 (Omega Optical). For PBA measurements, the excitation wavelength was 390 nm, the dichroic mirror was a 400DCLP (Omega Optical), and the emission filter was a long-pass 420 nm (Nikon).

The fluorescence decay of R123 is well fit by a single exponential, and the rate constant (k) was obtained from fitting the data to $F_t = F_0 + F(0)e^{-kt}$, where F_t is the fluorescence of R123 at time t, F_0 is the background fluorescence, and F(0) is the fluorescence at time zero. The decrease in intracellular PBA fluorescence was fit by two exponentials, and the rate constants k_A and k_B were obtained by fitting the data to $F_t = F_0 + F_A(0)e^{-k_A t} + F_B(0)e^{-k_B t}$, where A and B denote compartments A and B. Data were acquired at 1 Hz, and analysis was performed using commercial software (Sigmaplot; Jandel, San Rafael, CA).

Results

VLB accumulation and efflux. Steady state accumulation of VLB, a substrate for P-gp-mediated efflux (24), was studied in the MDR cell line KB8–5 in the presence and absence of novel MDR modulators. Both the parent molecule, acridone, and the bisacridone derivative composed of two acridone moieties connected by a butyl spacer (Fig. 1, where x = 4) were totally ineffective at increasing VLB steady state accumulation (data not shown). However, the propyl bisacridone (PBA) with one less methylene group in the spacer (Fig. 1) enhanced VLB accumulation in a concentration-dependent manner (Fig. 2). The maximal effect of PBA (800–900% increase relative to control) was achieved at a concentration of 1 μM. Very little increase in VLB accumulation was seen at higher PBA concentrations, probably due to the low aqueous solubility of PBA; the maximal concentration that can be

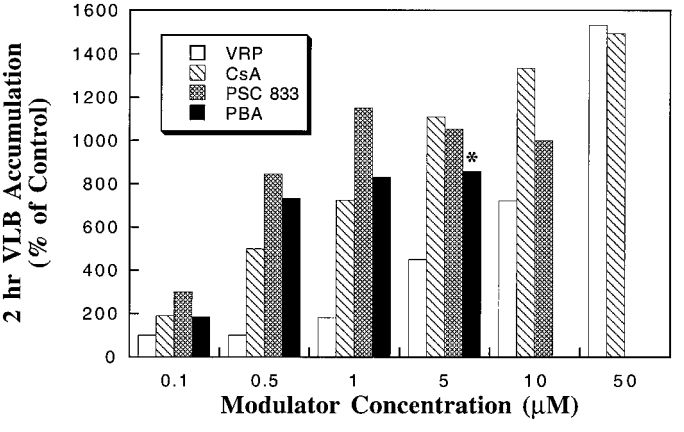

Fig. 2. Dose-dependent effects of VRP, CsA, PSC 833, and PBA on the steady state accumulation of VLB in KB8–5 cells. Monolayers of cell were incubated with [^{3}H]VLB for 2 hr at room temperature in the presence or absence of various concentrations of modulating agents as described in Experimental Procedures. Values represent the means of at least two independent determinations. ∗, Due to the limited solubility of PBA, the maximum aqueous concentration is 3 μM.

achieved in tissue culture medium is ≈3 μM. When compared with the effectiveness of other well-documented MDR modulators under the same conditions, a concentration of 1 μM PBA was found to be equivalent to 15 μM VRP, 2 μM CsA, and 0.5 μM PSC 833 (Fig. 2). PBA had no effect on VLB accumulation in the parental drug-sensitive line KB3–1 or in an unrelated P-gp-negative line, Rh30 (data not shown).

To determine whether the increase in VLB accumulation was due to reduced VLB efflux, KB8–5 cells were loaded with [^{3}H]VLB in the presence of modulator and incubated with VLB-free medium containing the same modulator, and the decrease in intracellular radiolabel was followed over time (Fig. 3). At a concentration of 1 μM, PBA was able to slow VLB efflux to the same extent as 100 μM VRP (Fig. 3 and Table 1), 2 μM CsA, and 0.5 μM PSC 833 (Table 1). As shown in Table 1, only 9% of the VLB accumulated was retained after 2-hr efflux in the absence of modulator, whereas 50–60% of the accumulated VLB was associated with the cells at 2 hr in the presence of any of the four modulators. In contrast, PBA, like VRP, had no effect on the 2-hr efflux of VLB from the parental drug-sensitive cell line KB3–1 (data not shown). This suggests that PBA, like the well-characterized MDR modulators VRP, CsA, and PSC 833, is specifically able to inhibit P-gp-mediated efflux of VLB from MDR cells, thus eliciting an increase in intracellular VLB levels.

Reversibility of the effects of modulators on VLB accumulation and retention. To study modulator reversibility, cells were incubated for 1 hr with modulators at concentrations resulting in equivalent effects on VLB uptake (8–9-fold increase in accumulation; Fig. 2) washed, and then incubated in complete medium without modulator for ≤12 hr until the start of a 2-hr VLB accumulation experiment. The effects of both VRP (15 μM) and CsA (2 μM) on VLB accumulation in KB8–5 cells were rapidly reversed once the modulator was removed (Fig. 4). After only 2 hr in VRP- or CsA-free medium following the initial preincubation with the modulator, VLB accumulation was similar to control values. In contrast, the enhanced VLB accumulation seen as a result of a 1-hr exposure to either PBA or PSC 833 was very poorly reversible (Fig. 4). After a 4–6-hr incubation in modulator-free medium, VLB accumulation was still elevated to approximately half of the level seen when cells are coexposed to modulator and VLB. After 8 hr, the enhancing effects were still evident, but by 12 hr, accumulation was not different from that in control incubations without modulator (data not shown).

Efflux of VLB from KB8–5 cells in the absence of modulator (after also loading in the absence of modulator) resulted in <10% retention of cell-associated VLB after 2 hr (Table 1). In addition, efflux of VLB in the presence of any of the four modulators, after loading in the presence of equieffective concentrations of the same modulator, resulted in 50–60% of the accumulated VLB being retained at 2 hr (Table 1). Efflux of VLB for 2 hr in the absence of modulator, after loading in the presence of PSC 833 or PBA, resulted in the greatest retention (Table 1). The lowest VLB retention was observed after accumulation with VRP. The difference seen in percent retention after loading in the presence of PBA and VRP was

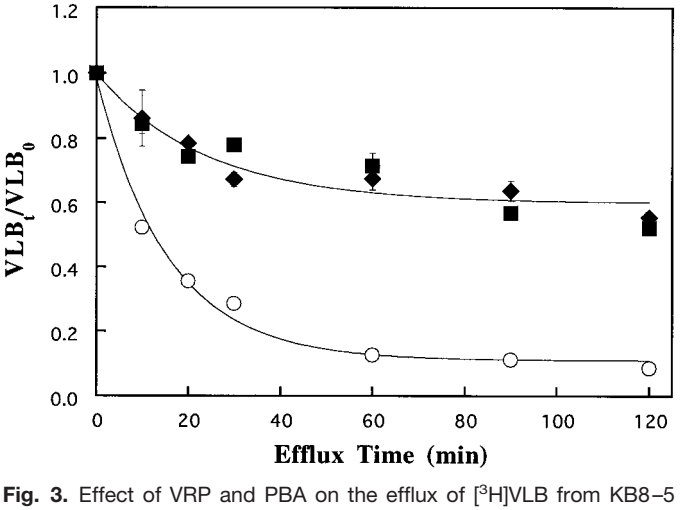

Fig. 3. Effect of VRP and PBA on the efflux of [^{3}H]VLB from KB8–5 cells. Both the initial loading of VLB and the efflux were carried out in the absence (○) or presence of modulator 100 μM VRP (■) or 1 μM PBA (♦). *VLB$_t$*, intracellular level of VLB at efflux time *t*. *VLB$_0$*, intracellular level of VLB at time 0. Values in the absence of modulator are from a single experiment; *time points*, mean of triplicate determinations. Values in the presence of modulators are the mean ± standard error of three independent experiments.

TABLE 1
Reversibility of modulating agent-induced inhibition of VLB efflux from KB8-5 cells

Modulator	Retention of VLB at 2 hr [a,b]	
	Efflux with modulator	Efflux without modulator
	%	
None[c]	8.6	8.6
VRP (100 μM)	57.2 ± 2.5	19.6 ± 3.2
CsA (2 μM)	49.6 ± 1.0	31.4 ± 1.8
PSC 833 (0.5 μM)	57.3 ± 3.2	36.0 ± 1.6
PBA (1 μM)	50.9 ± 3.2	39.8 ± 3.1

[a] VLB accumulation (0.5 μCi/ml) was for 1 hr in the presence of modulator with efflux in the presence or absence of modulator.
[b] Values are expressed as mean ± standard error of three or four independent experiments.
[c] For control experiments, both VLB accumulation (5 μCi/ml) and efflux were conducted in the absence of modulator. The results are from a single experiment performed in triplicate.

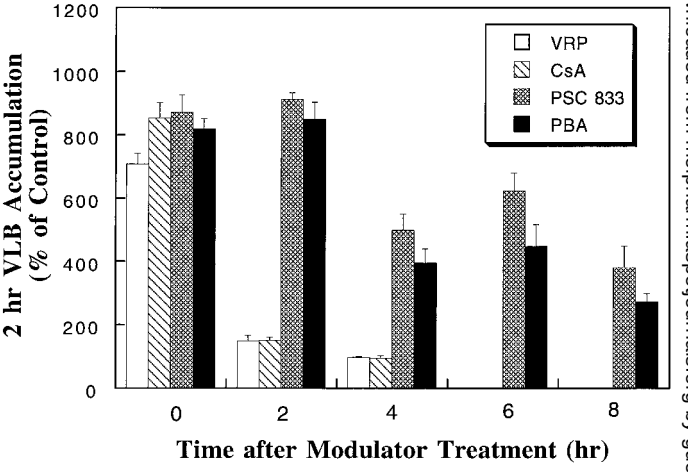

Fig. 4. Reversibility of modulator-enhanced VLB accumulation in KB8–5 cells. Cell monolayers were first pretreated for 1 hr with 15 μM VRP, 2 μM CsA, 0.5 μM PSC 833, or 1 μM PBA. The cells were washed, and [^{3}H]VLB accumulation was measured in the absence of modulators at times 2–8 hr after the beginning of modulator treatment. Accumulation at 2 hr is presented as mean percent of control ± standard error for three to six independent determinations.

statistically significant ($p < 0.05$). These results indicate that after removal of modulator from the medium, the PSC 833- and PBA-induced inhibition of VLB efflux is longer lasting than that of VRP.

To study long term reversibility, cells were first incubated for 1 hr with either PSC 833 or PBA at concentrations resulting in equivalent effects on VLB accumulation; cells were then washed and incubated in complete medium without modulator for 2–4 hr before loading the cells for 1 hr with [^{3}H]VLB. Efflux was then followed for 2 hr. The effects of both PBA and PSC 833 on VLB efflux were very similar and were still evident 2 and 4 hr after preincubation with modulator (Table 2). An experiment using the same protocol was not possible for VRP or CsA because VLB accumulation is not elevated even at 2 hr after preincubation with these modulators, and hence efflux cannot be followed.

Effect of acridone derivatives on the cytotoxicity of VLB. KB8–5 cells were incubated with serial dilutions of VLB either continuously or for 2 hr in the presence or absence of modulator, and the drug sensitivity of the cells was determined by clonogenic assay. As anticipated from the results of the VLB accumulation studies, neither the parent compound acridone nor the butyl bisacridone derivative had any chemosensitizing effect on continuous VLB exposure (Fig. 5A). However, PBA at a concentration of 1 μM was effective at potentiating VLB cytotoxicity (IC_{50} value decreased from 28.1 to 4.2 nM), and at a concentration of 3 μM, PBA completely reversed VLB resistance (IC_{50} = 0.77 nM; Fig. 5A). The IC_{50} value for continuous exposure to VLB in the drug-sensitive parental cell line KB3–1 was found to be 0.76 nM and was not affected by coincubation with 3 μM PBA (data not shown).

Similarly, continuous exposure to PBA was able to sensitize KB8–5 cells to a 2-hr exposure to VLB (Fig. 5B). At nontoxic concentrations of 1 and 3 μM, the VLB IC_{50} values decreased from 15.9 to 1.28 and 0.38 μM, respectively. VRP at a concentration of 15 μM and PSC 833 at a concentration of 0.5 μM were also able to completely reverse VLB resistance in the KB8–5 cell line (Fig. 5B). In contrast, the sensitivity of KB3–1 cells to a 2-hr exposure to VLB was not changed by continuous exposure to 3 μM PBA (IC_{50} = 0.42 μM; Fig. 5B).

Reversibility of the effects of modulators on the cytotoxicity of VLB. As discussed above, complete reversal of resistance to a 2-hr VLB exposure was afforded by continuous incubation with 15 μM VRP, 0.5 μM PSC 833, or 3 μM PBA (IC_{50} = 0.4–0.5 μM; Fig. 5C). In experiments in which both modulator and VLB were removed at 2 hr, the IC_{50} values for coexposure of VLB and either VRP or PBA were similar to those obtained for a 2-hr exposure to VLB alone (≈16 μM; Fig. 5C). Thus, the modulatory effects of both VRP and PBA on VLB cytotoxicity were completely reversed after removal of

TABLE 2

Long term reversibility of modulating agent-induced inhibition of VLB efflux from KB805 cells

Modulator	Time after treatment[a]	Retention of VLB[b,c]
	hr	*%*
PSC 833 (0.5 μM)	2	26.8 ± 3.2
	4	17.7 ± 2.3
PBA (1 μM)	2	27.6 ± 1.4
	4	15.7 ± 1.8

[a] Cells were pretreated with modulator for 1 hr, washed, and then incubated for the indicated time without modulator until the start of the experiment.
[b] Cells were incubated with VLB (0.5 μCi/ml) for 1 hr and transferred to VLB-free medium, and percent retention after 2 hr was determined in the absence of modulator.
[c] Values are expressed as mean ± standard error of three or four independent experiments.

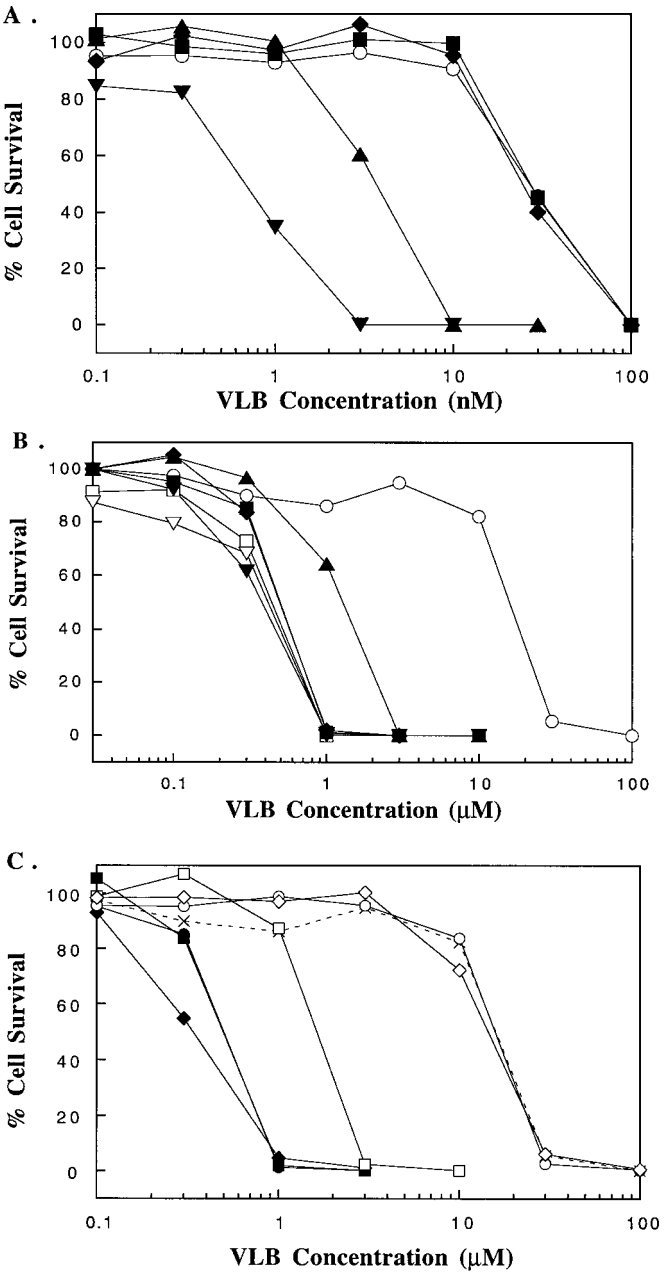

Fig. 5. Effects of acridone derivatives and MDR modulators on the cytotoxicity of VLB in KB3–1 and KB8–5 cells and the reversibility of these effects. Drug sensitivity was determined by clonogenic assay as described in Experimental Procedures. A, Continuous VLB exposure. KB8–5 cells were exposed to VLB in the absence (○) or continuous presence of 1 μM acridone (■), 1 μM butyl bisacridone (♦), 1 μM PBA (▲), or 3 μM PBA (▼). B, 2-hr VLB exposure. KB3–1 cells were exposed to VLB in the absence (□) or continuous presence of 3 μM PBA (▽). KB8–5 cells were exposed to VLB in the absence (○) or continuous presence of 1 μM PBA (▲), 3 μM PBA (▼), 15 μM VRP (■), or 0.5 μM PSC 833 (♦). C, Reversibility. KB8–5 cells were exposed to VLB for 2 hr in the absence (X) or presence of modulators; 15 μM VRP (○, ●), 0.5 μM PSC (□, ■), or 3 μM PBA (◇, ♦). Modulators were present during the 2-hr VLB treatment (○, □, ◇) or continuously (●, ■, ♦).

the modulator from the medium. In similar experiments using PSC 833, KB8–5 cells become more resistant to VLB after removal of the modulator; however, some of the sensitizing effect was retained (IC_{50} = 1.6 μM compared with 0.5 μM). Therefore, KB8–5 cells treated for 2 hr with both VLB and PSC 833 were still 10-fold more sensitive to VLB than were cells exposed to only VLB. The IC_{50} value of 1.6 μM is lower than that obtained in experiments in which cells were exposed to VLB for 8 hr without modulator (IC_{50} = 6.7 μM), suggesting that the 2-hr incubation with PSC 833 allowed retention of a cytotoxic concentration of VLB in KB8–5 cells for >8 hr.

Photoaffinity labeling of P-gp. The VLB transport and cell survival data suggest that the modulatory effects of PBA occur through an interaction with P-gp. To confirm this suggestion, we examined competition between radiolabeled azidopine and PBA. Photoaffinity labeling with [^{3}H]azidopine was not detected under our experimental conditions when we used membranes prepared from drug-sensitive KB3–1 cells (Fig. 6, *lane 1*) or from KB8–5 cells, which have a low level of drug-resistance (data not shown). A highly VLB-resistant and P-gp-expressing KB cell subline, KB-V1, was therefore used for the [^{3}H]azidopine competition studies (Fig. 6, *lanes 2–4*). PBA was able to enhance VLB accumulation in KB-V1 cells, although it was not as effective as in KB8–5 (data not shown). PBA, like VLB, was able to compete with azidopine for labeling of P-gp (Fig. 6, *lanes 3 and 4*). The results therefore confirm that there is an interaction between PBA and P-gp in MDR cells.

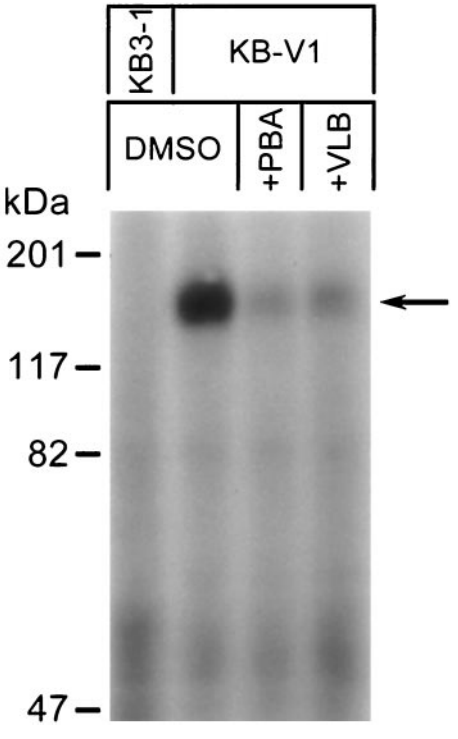

Fig. 6. Effect of PBA on the photoaffinity labeling of P-gp by [^{3}H]azidopine. The autoradiogram illustrates photoaffinity labeling performed as described in Experimental Procedures. Membranes from KB3–1 [*left lane* (*1*)] and KB-V1 (*lanes 2–4*) were labeled with 0.4 μM [^{3}H]azidopine in the absence (solvent alone, *lanes 1 and 2*) or presence of 3 μM PBA (*lane 3*) or 3 μM VLB (*lane 4*). *Arrow*, electrophoretic mobility of P-gp. *DMSO*, dimethylsulfoxide.

Effect of modulators on P-gp ATPase activity. Drug efflux associated with P-gp is ATP dependent, and expression of P-gp results in the appearance of a vanadate-sensitive drug-stimulated ATPase activity (23). It has been shown previously that this P-gp-associated ATPase activity could not be measured in plasma membranes prepared from KB-V1 cells due to the high level of P-type ATPases in this cell line (25). Therefore, we determined the effect of modulators on the ATPase activity in membranes isolated from baculovirus-infected Sf9 cells. Membranes prepared from Sf9 cells infected with LacZ baculovirus had a low level of ATPase activity (1.5 nmol/mg of membrane protein/min), 20% of which was inhibited by 100 μM sodium orthovanadate. Neither VRP nor PBA stimulated this activity (data not shown). Membranes from *MDR1* baculovirus-infected cells had a vanadate-sensitive ATPase activity, attributable to P-gp ATPase, of ≈18 nmol/mg of membrane protein/min. This activity was stimulated 3.6- and 2–7-fold by incubation with VRP or PBA, respectively. However, maximal activity was achieved at a considerably lower concentration of PBA (Fig. 7; 30–100 μM for VRP and 0.3 μM for PBA). Coincubation with VRP and PBA did not result in additive stimulatory effects. In contrast, incubation with PSC 833 (0.03–1 μM) did not result in any stimulation of P-gp ATPase activity (Fig. 7). PSC 833 did, however, completely inhibit the VRP- and PBA-stimulated activity at concentrations of 1 and 0.3 μM, respectively (Fig. 7). These results again suggest there is a direct interaction of the modulators VRP, PBA, and PSC 833 with P-gp in *MDR1*-expressing cells.

Steady state drug accumulation in sensitive and resistant cell lines. When cells were loaded with radiolabeled VLB, there was a statistically significant higher accumulation in drug-sensitive KB3–1 than in drug-resistant KB8–5 cells cells at 2 hr ($p < 0.05$; Table 2). This is consistent with the existence of a P-gp-dependent VLB efflux in the resistant line. In contrast, after loading with PBA, there was a similar steady state accumulation in the two cell lines (Table 2). This result suggests that although PBA can block the P-gp-medi-

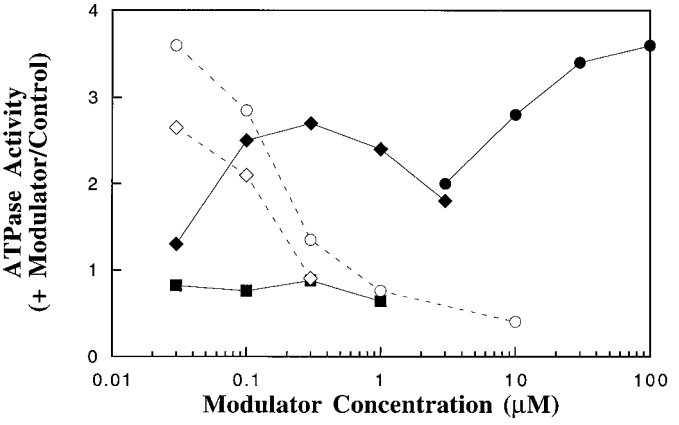

Fig. 7. Effect of VRP, PSC 833, and PBA on P-gp ATPase activity. The vanadate-sensitive P-gp ATPase activity in membranes isolated from *MDR1* baculovirus-infected Sf9 cells was determined in the presence of the indicated concentrations of modulators: VRP (●), PSC 833 (■), and PBA (◆). Effect of PSC 833 is shown in the presence of 100 μM VRP (○) or 0.3 μM PBA (◇). Values represent the means of at least two independent experiments. Activity was estimated by measuring inorganic phosphate liberation as described in Experimental Procedures and expressed as the ratio of activity in the presence and absence of modulator.

ated efflux of VLB, the accumulation of PBA is not P-gp dependent. However, in energy-depleted KB8–5 cells, there was only a low level of accumulation of PBA. The poor loading suggests that the accumulation of PBA is ATP dependent.

Efflux of R123 and PBA from drug-sensitive and -resistant cell lines. The efflux of the fluorescent dye R123 is known to be P-gp dependent and consequently has been used extensively to determine efflux rates from drug-sensitive and P-gp-expressing drug-resistant cell lines (16). To more accurately assess whether efflux of PBA was P-gp dependent, the efflux kinetics of both R123 and PBA were examined in parental and MDR variants of KB cells. After loading cells with R123 and then perfusion with R123-free buffer, there was a significantly faster ($p < 0.05$) efflux of the dye from KB8–5 cells than from KB3–1 cells. Efflux from the MDR cell line KB8–5 could be slowed by the addition of 10 μM VLB, an effective competing substrate for efflux, and by 1 μM PBA (Fig. 8A). PBA was also able to slow efflux of R123 from the highly MDR cell line KB-V1 (data not shown).

After loading cells with PBA, a similar rate of efflux was observed in both sensitive and MDR cell lines, and the decrease in intracellular PBA fluorescence was well fit by two exponentials. The fluorophore that effluxed from KB8–5 cells was collected from the bathing solution and had excitation and emission fluorescence spectra indistinguishable from those of PBA (data not shown). This observation strongly suggests that the decrease in intracellular PBA fluorescence in the efflux experiments is due to efflux of PBA and not to metabolic destruction of the chromophore. The initial faster rate of efflux from both sensitive and drug-resistant cell types is fairly rapid (rate constants of 0.1 min^{-1} corresponding to a half-time of ≈7 min; Fig. 8B). In both KB3–1 and KB8–5 cell lines, 76% of the intracellular PBA was in this faster exchange compartment. VLB at a concentration of 10 μM had no effect on the rate of PBA efflux from the faster compartment (Fig. 8B). The rate constants for efflux from the second slower compartment were 0.034 ± 0.013 (n = 8) and 0.018 ± 0.07 (n = 6) for KB3–1 and KB8–5, respectively.

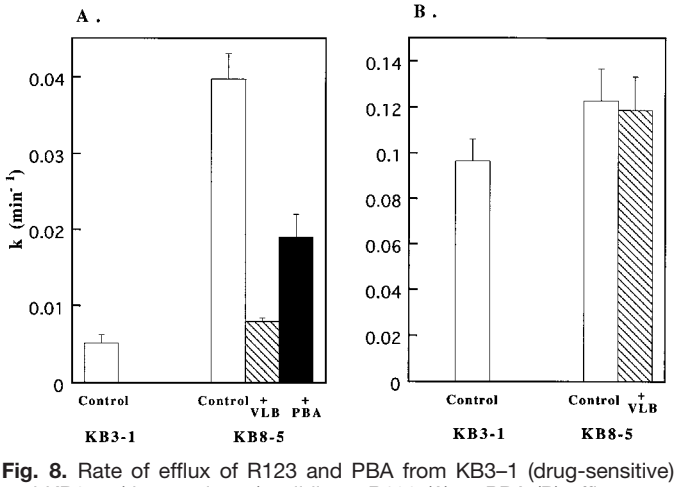

Fig. 8. Rate of efflux of R123 and PBA from KB3–1 (drug-sensitive) and KB8–5 (drug-resistant) cell lines. R123 (A) or PBA (B) efflux were analyzed as described in Experimental Procedures in the absence or presence of 10 μM VLB or 1 μM PBA. Cells were loaded with the fluorescent probe and superfused with buffer at room temperature, and the rate of decrease in intracellular fluorescence was followed. Values are the mean ± standard error average of four or five independent determinations.

These values were significantly different ($p < 0.05$) from the rate constants of the faster compartment of the same cell line, but there were no statistically significant differences observed between the two cell lines. This second slower rate of efflux will allow for prolonged retention of a fraction of accumulated PBA in the cells. These experimental data indicate that although PBA can block P-gp-dependent drug efflux, it is not itself efficiently transported by P-gp.

Discussion

To further investigate MDR modulators, a small series of bisacridones were synthesized in which two acridone moieties were separated by a spacer consisting of three or four carbon units. One of these compounds, PBA, which is composed of two acridones connected by a propyl spacer, was found to be extremely potent, whereas the butyl derivative, like the parent compound acridone, was ineffective. In this limited series, structure-activity analysis was not possible; however, lengthening of the carbon spacer by one methylene group (≈1.5 Å) had a profound effect on biological activity. The acridone derivatives (in particular, the butyl bisacridone) have low aqueous and octanol solubility, making it difficult to determine accurately their high octanol/water partition coefficients by UV spectrophotometry. One possible reason for the inactivity of the butyl derivative is its extreme insolubility in aqueous medium rather than a structure-related change in the interaction with P-gp.

In the model system used in the current study, PBA was more potent than either of the classic modulators VRP or CsA at enhancing the accumulation of VLB and similar in activity to a newer P-gp modulator, PSC 833 (Fig. 2). In addition, a slowing of VLB efflux was observed in the presence of modulators and with the identical order of potency (Fig. 3 and Table 1). These results are in agreement with previous comparative studies showing that PSC 833 is more potent than either VRP and CsA at inhibiting P-gp-mediated transport (26). PBA had no effect in drug-sensitive KB3–1 cells, suggesting that PBA, like VRP (24), CsA, and PSC 833, exerts its effects by inhibiting P-gp-mediated efflux of VLB from drug-resistant cells (Fig. 3 and Table 2). The inhibition of azidopine photoaffinity labeling of P-gp (Fig. 6) and the stimulation of P-gp-associated ATPase activity in the presence of PBA (Fig. 7) confirm that this agent can interact with P-gp. VRP and CsA have been shown previously to interact competitively with the *Vinca* alkaloid drug-binding site of P-gp (27, 28).

In contrast to the results obtained with VLB, similar levels of accumulation were achieved after loading cells with PBA regardless of P-gp status (Table 3). Therefore, either PBA has an extremely fast rate of influx, which efficiently shunts the active efflux by P-gp, or it is not efficiently transported by P-gp. Also, in contrast to the results obtained with R123, a similar rate of PBA efflux, not inhibited by VLB, was observed from MDR KB8–5 cells and the drug-sensitive KB3–1 cells (Fig. 8). Taken together, these data suggest that although PBA can block P-gp-mediated transport of VLB, it either is not a substrate for P-gp or is transported inefficiently, with the bulk of efflux via a P-gp-independent mechanism. This is in contrast to the classic P-gp modulators (i.e., VRP and CsA), in which active transport by P-gp has been demonstrated (29, 30); however, P-gp-mediated efflux of PSC

TABLE 3
Steady state accumulation of VLB and PBA in KB3-1 (drug-sensitive) and KB8-5 (drug-resistant) cell lines

Cell line	Steady state accumulation[a]	
	VLB[b]	PBA[c]
	pmol/10⁶ cells	
KB3-1	3.65 ± 0.12 (n = 3)	125.1 ± 8.7 (n = 13)
KB8-5	0.22 ± 0.03 (n = 3)	127.7 ± 6.5 (n = 26)

[a] Values are expressed as mean ± standard error (n = number of determinations).
[b] Cells were loaded with [^{3}H]VLB (37.5 nM, 0.5 μCi/ml) for 2 hr at room temperature.
[c] Cells were incubated with 1 μM PBA for 1 hr at room temperature.

833 has not been detected (31). The accumulation of PBA in ATP-depleted KB8–5 cells was extremely low. The most likely explanation for this result is that PBA accumulates in a cell compartment affected by energy-depleting treatment such as mitochondria (loss of electric potential gradient) or endosomes (collapse of pH gradient). These experiments do not allow a definitive conclusion because the intracellular distribution of PBA has not been determined.

Like VRP, PBA was able to stimulate P-gp ATPase activity in *MDR1*-infected Sf9 cells, but it was active at considerably lower concentrations (Fig. 7). In contrast, PSC 833 did not stimulate ATPase activity but was able to inhibit the stimulation affected by both VRP and PBA (Fig. 7), a result similar to that reported previously for CsA (32). Stimulation of P-gp ATPase activity is generally considered to reflect drug transport by P-gp; however, as outlined above, there is no evidence for efficient transport of PBA. Progesterone has also been shown to stimulate ATPase activity (33), but transport by P-gp has not been demonstrated (34). Progesterone is extremely lipid soluble and a fast passive efflux could explain the failure to demonstrate P-gp-mediated efflux (35). A similar situation may exist for PBA because the initial rate of efflux from both sensitive and resistant KB cells is more rapid than the efflux of R123 from the MDR-expressing line (Fig. 8). Alternatively, the interaction of PBA with P-gp may result in ATP hydrolysis without transport, and this uncoupling mechanism is distinct from the effects of VRP, CsA, and PSC 833 on P-gp. The incubation of drug-resistant KB8–5 cells with PBA did not result in depletion of intracellular ATP levels (data not shown). In other MDR human tumor cell lines, there is evidence that the increased ATP hydrolysis as a result of P-gp modulator interaction is compensated for by an increased rate of glycolysis (36).

The increase in VLB accumulation mediated by VRP or CsA in MDR cells was shown to be reversed rapidly once the modulator was removed. In contrast, the enhanced VLB accumulation seen as a result of exposure to PBA or PSC 833 was only slowly reversible (Fig. 4). The inhibitory effects of PBA and PSC 833 on VLB efflux are consistent with the observed effects on VLB accumulation. The results suggest that both PSC 833 and PBA, unlike VRP, are able to induce a strong and poorly reversible inhibition of P-gp-mediated efflux. The rapid reversal of VRP activity has been described previously, and its continuous presence is necessary for substantial inhibition of P-gp function (16, 17). The persistence of P-gp inhibition conferred by PSC 833, with the use of daunomycin or R123 retention as an indicator of P-gp function, also has been demonstrated previously (17, 31).

The long-lasting effect of PBA is consistent with its prolonged retention in cells as determined from measurement of the decrease in intracellular PBA fluorescence. Although a greater part of accumulated PBA effluxed rapidly from sensitive and MDR cell lines ($t_{½}$ ≈7 min), a significant fraction (≈24%) effluxed with a half-time of ≈30 min. In view of the rapid efflux of much of the accumulated PBA from cells (i.e., 76%), the compound must be extremely potent to have such long-lasting effects on P-gp function. However, it should be noted that the critical parameter for the effectiveness of a modulator is the concentration at its target site (P-gp) rather than the total concentration remaining in the whole cell. It is also possible that the persistent VLB retention may be due to an effect of PBA on some other cellular function. The two rate constants obtained for PBA efflux suggest that PBA may accumulate in two compartments within the cell; however, examination of both sensitive and resistant cells by fluorescence microscopy after incubation with PBA indicates that the compound distributes throughout the cytoplasm but apparently not within the nucleus (not shown).

Previous reports have documented the effectiveness of PSC 833 in reversing P-gp-associated drug resistance (37), and it was anticipated that PBA would effectively reverse VLB resistance in the MDR KB8–5 cell line. A concentration of 3 μM PBA is nontoxic, can elicit a complete reversal of VLB resistance in KB8–5 cells, and therefore is at least as effective as an IC_{10} concentration of VRP (i.e., 30 μM) but an order of magnitude more potent. KB8–5 cells, initially selected for resistance to colchicine, are significantly cross-resistant only to the *Vinca* alkaloids VLB and vincristine and to R123 and not to other agents, such as doxorubicin, actinomycin D, and VP-16.[1] Therefore, we were unable to determine whether PBA has a chemosensitizing effect with other types of chemotherapeutic agents in this cell line. In the unrelated MDR cell line MCF-7/Adr (38), which is resistant to *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins, PBA was able to enhance accumulation of radiolabeled daunorubicin and VP-16, but not to the same extent as VLB (data not shown). These results suggest that the inhibitory effects of PBA on P-gp-mediated transport, and therefore the ability to modulate drug resistance, are not limited to the *Vinca* alkaloids.

These results led to the conclusion that the effects of PBA on VLB transport in MDR cells are similar to those of the CsA analog PSC 833 and considerably less reversible than those of VRP. It was therefore expected that the modulatory effects of PSC 833 and PBA on VLB cytotoxicity would also be more persistent than those of VRP. However, the sensitizing effects of VRP and PBA on 2-hr VLB exposure were readily reversed, whereas the effects of PSC 833 were poorly reversible (Fig. 5C). Thus, the recovery of P-gp function after removal of the modulators VRP and PSC 833 was in agreement regardless of whether determined by a study of VLB transport or cytotoxicity. After exposure to PBA, the inhibition of VLB transport was persistent, but the reversal of drug resistance was readily reversible. This discrepancy observed for PBA may be in part due to the presence of serum in the medium used for cytotoxicity experiments and its absence in the transport studies. In VLB accumulation experiments in KB8–5 cells, PBA was found to be equieffective in the presence or absence of 10% serum, whereas PSC 833 was 1.6-fold

[1] J. K. Horton, unpublished observations.

more effective where serum was present (data not shown). A superior inhibition of R123 efflux by PSC 833, when compared with other modulators under serum conditions, has been described previously (39). An explanation may also lie in the fact that the accumulation and cytotoxicity protocols are a measure of two very different things. In the drug accumulation experiments, the total concentration of VLB is measured, both free and that bound to different locations within the cell, whereas in the cytotoxicity experiments, only free intracellular VLB available for interaction with microtubular proteins is assessed. PBA and PSC 833 may have differing effects on the distribution and binding of VLB within the cell, resulting in different levels of free VLB once the modulator is removed.

In conclusion, we characterized PBA, a novel synthetic modulator of MDR, and demonstrated it to be extremely potent compared with the prototype modulator VRP. In contrast to the effects of VRP and CsA, the inhibition of VLB transport by PBA was similar to that of PSC 833 and only slowly reversible. For PSC 833, this slow reversal of the transport function of P-gp could be translated into a poor reversal of the sensitization of an MDR cell line in cytotoxicity assays; however, for PBA the effect on VLB cytotoxicity was readily reversible. These results indicate that although PBA is a more potent chemosensitizer than VRP and CsA, it may not have the attributes of being long lasting, as was observed for PSC 833.

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