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BIBW22 BS, Potent Multidrug Resistance-Reversing Agent, Binds Directly to P-Glycoprotein and Accumulates in Drug-Resistant Cells

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SUMMARY

The expression of P-glycoprotein (P-gp) in tumor cells causes a multidrug resistance (*MDR*) phenotype. P-gp has been shown to mediate the transport of structurally dissimilar drugs across the cell membrane in an energy-dependent manner. In this report, we show that BIBW22 BS, a phenylpteridine analog, reverses the MDR phenotype of CEM human lymphoma cells in a dose-dependent fashion. Using a photoactive analog of BIBW22 BS {[^{3}H]azido-4-[*N*-(2-hydroxy-2-methylpropyl)-ethanolamino]-2,7-bis(*cis*-2,6-dimethyl-morpholino)-6-phenylpteridine}, we show the photoaffinity labeling of a 170-kDa protein in drug-resistant cells immunoprecipitated with P-gp-specific monoclonal antibodies. The photolabeling of P-gp by [^{3}H]azido-BIBW22 BS was specific and saturable. Furthermore, BIBW22 BS, vinblastine, and verapamil, but not colchicine, inhibited the photolabeling of P-gp by [^{3}H]azido-BIBW22 BS. Drug binding studies showed that membranes from *MDR* cells bound more BIBW22 BS than parental drug-sensitive cells, and this binding was inhibited with vinblastine and, to a lesser extent, with uridine. However, drug transport studies demonstrated that BIBW22 BS is not a substrate for P-gp efflux pump. Interestingly, BIBW22 BS was shown to accumulate more in resistant cells. Also, BIBW22 BS accumulation in drug-sensitive and -resistant cells was not energy dependent. These results are in contrast with the observed decrease in accumulation or enhanced efflux of [^{3}H]vinblastine seen in the same *MDR* cells. A comparison of [^{3}H]azido-BIBW22 BS or [^{3}H]azidopine photolabeled P-gp by Cleveland mapping with *Staphylococcus aureus* V8 protease showed differences in the photolabeled peptides. Taken together, the results of this study show that BIBW22 BS is a potent *MDR*-reversing agent that binds directly to P-gp but is not effluxed from drug-resistant cells.

The development of multidrug resistance in tumor cells selected with lipophilic cytotoxic drugs has been shown to correlate with the expression of a membrane phosphoglycoprotein called P-gp (for reviews, see Refs. 1 and 2). P-gp is a member of the ABC family of membrane transporters, which includes the multidrug resistance-associated protein (MRP; Ref. 3), the yeast a-mating factor (STE6; Ref. 4), the cystic fibrosis transmembrane regulator (CFTR; Ref. 5), and the bacterial periplasmic membrane transporters (6). The P-gp gene family consists of three genes in rodents (classes I, II, and III) and two in humans (classes I and III) (7). Transfection studies of P-gp genes have shown that although the P-gp classes I and II cause drug resistance (8, 9), the class III P-gp does not mediate the MDR phenotype (10). More recently, studies using homologous recombination to inactivate the class I or III P-gp genes in mice (11, 12) have shown that the class I gene is involved in the blood-brain barrier, whereas the class III gene mediates phosphatidyl choline transport in liver and may be a translocase (13). Thus, the normal function of P-gp is likely to involve the transport of xenobiotics and normal cell metabolites (14). P-gp has been shown to mediate the transport of ATP, oligopeptides, and chloride ions (15–17).

Energy-dependent drug transport has been shown using purified P-gp reconstituted into defined lipid vesicles (18–20). Although the molecular mechanism by which P-gp mediates drug efflux remains to be determined, photoactive analogs of cytotoxic drugs and other lipophilic compounds were shown to bind directly to P-gp (21–24). Furthermore, certain nontoxic drugs that bind to P-gp have been shown to potentiate the accumulation of cytotoxic drugs by competing

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ABBREVIATIONS: MDR, multidrug resistance; P-gp, P-glycoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

for P-gp drug binding site (25) or by modulating the activity of protein kinase C (26, 27).

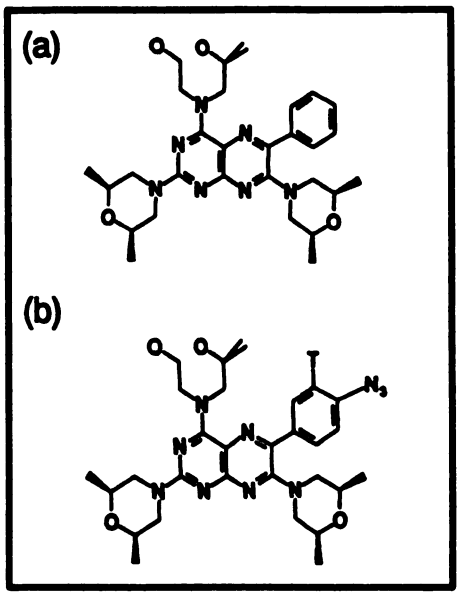
High levels of P-gp have been found in normal tissues and in tumors of various cancers (28–31). More recently, P-gp expression in tumors from children with sarcomas or neuroblastomas has been shown to correlate with the outcome of chemotherapeutic treatment and the long term survival of patients (32, 33). Clinical trials using *MDR*-reversing agents (e.g., verapamil or quinidine) have shown some response in tumors that were otherwise nonresponsive to chemotherapy (34). However, high pharmacological toxicity associated with several *MDR*-reversing agents has prevented their use at effective concentrations (35). A better clinical response has been observed using other *MDR*-reversing agents (i.e., cyclosporin A and its nonimmunosuppressive analog, PSC833); some toxic effects have also been seen with cyclosporin A (36). Therefore, other *MDR*-reversing agents with tolerable pharmacological toxicity are needed to develop effective chemotherapeutic treatment of drug-resistant tumors.

Dipyridamoles are general nucleoside transport inhibitors (37) shown previously to reverse the MDR phenotype of P338 murine cells (38). BIBW22 BS, a dipyridamole-related compound, was shown to be more active than dipyridamoles in inhibiting nucleoside transport and in reversing the MDR phenotype of P-gp-positive cells (39). The reversal of *MDR* with BIBW22 BS was postulated to be due to its interaction with P-gp because BIBW22 BS inhibited the photoaffinity labeling of P-gp by [^{3}H]azidopine. However, no direct evidence was presented to support this hypothesis. In this study, we investigated further the mechanism of *MDR* reversal by BIBW22 BS in P-gp-positive cells. Our results show that BIBW22 BS reverses the MDR phenotype of highly drug- resistant CEM human lymphoma cells in a dose-dependent manner. In addition, using a photoactive analog of BIBW22, [^{3}H]azido-BIBW22 BS, we show a direct and specific binding between P-gp and [^{3}H]azido-BIBW22. Interestingly, BIBW22 BS is not a substrate for P-gp drug efflux pump, and surprisingly, it accumulates more in resistant cells. The latter finding was unexpected, but it was consistently observed in two different cell lines. These findings are discussed with respect to BIBW22 BS binding affinity and drug binding domain(s) in P-gp.

Experimental Procedures

Materials. [^{3}H]Azidopine (53–75 Ci/mmol) was purchased from Amersham Biochemical (Mississauga, Ontario, Canada). BIBW22 BS (Fig. 1a) was synthesized at Dr. Karl Thomae GmbH (Biberach/Riss, Germany). The photoactive derivative of BIBW22 BS {[^{3}H]azido-4-[*N*-(2-hydroxy-2-methylpropyl)-ethanolamino]-2,7-bis-(*cis*-2,6-dimethyl-morpholino)-6-phenylpteridine) was synthesized at DuPont-New England Nuclear (Boston, MA; specific activity, 7.0 Ci/mmol; Fig. 1b). Drug-sensitive and -resistant human lymphoma cells (CEM or CEM/VLB$^{0.1}$, respectively) (40) were a gift from Dr. W. Beck (St. Jude Children's Research Hospital, Memphis, TN). The CHO drug-sensitive (Aux b1) and colchicine selected drug-resistant cells (CHO^r C5 and CHO^r B30) were a gift from Dr. V. Ling (Ontario Cancer Institute, Toronto, Ontario, Canada). All other reagents were of the highest grades available.

Cell culture and plasma membrane preparation. Drug-sensitive (CEM or Aux b1) and -resistant (CEM/VLB$^{0.1}$, CEM/VLB$^{1.0}$, CHO^r C5, or CHO^r B30) cells were grown in α-minimum essential medium containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT). Drug-resistant cells were grown continuously in the presence of 0.1–1 μg/ml vinblastine or 5–30 μg/ml colchicine unless cells were to be used for drug transport studies. Plasma membranes were prepared essentially as described by Riordan and Ling (41). Briefly, CEM or CEM/VLB$^{1.0}$ cells were washed three times with ice-cold PBS and resuspended in a hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride and 30 μM leupeptin. Cells were placed on ice for 15 min and then lysed by passing the cell suspension once through the Standsted pump at 250 psi. The cell lysate was centrifuged at 4000 × *g* in the Sorval centrifuge using the SS34 rotor. The supernatant from the above low-speed spin was loaded onto a discontinuous sucrose gradient consisting of 16%, 31%, 45%, and 60% sucrose in 10 mM Tris·HCl, pH 7.4. The plasma membrane enriched fraction at the 16–31% sucrose interphase was collected and washed with 10 mM Tris·HCl, pH 7.4, containing protease inhibitors. Protein concentrations were determined according to the method of Lowry (42). Membrane fractions were stored at −85° if not used immediately.



Organic structures of BIBW22 and its photoactive derivative

Fig. 1. Organic structures of the BIBW22 BS (a) and its photoactive analog [^{3}H]azido-BIBW22 BS (b).

MTT cytotoxicity assay. The sensitivity of cells to vinblastine, doxorubicin, or colchicine in the absence or presence of BIBW22 BS or verapamil was determined using an MTT assay (43). Cells were harvested during the exponential growth phase, and a 100-μl aliquot of single-cell suspension was plated onto 96-well plates at 0.5×10^4 cells/well for CEM and 1×10^4 cells/well for CEM/VLB. The cells were incubated for 24 hr at 37° before the addition of BIBW22 BS or verapamil (0.125–5.0 μM). Cells were then cultured in the presence of BIBW22 BS or verapamil with or without vinblastine, doxorubicin, or colchicine. The liquid in each well was carefully removed and followed by the addition of 20 μl of MTT solution (5 mg/ml) in PBS. The plates were incubated for an additional 4 hr at 37°, and the colored crystals formed from the tetrazolium salt were solubilized by the addition of 50 μl of 10% Triton X-100 in 0.01 N HCl and vigorously mixed. The cells were microwaved for 1 min, and 20 μl of ethanol was added to disperse the bubbles formed during pipetting. The absorbance in each well was measured at 570 nm using a BioRad (Hercules, CA) microtiter plate reader. Reversal of *MDR* in the presence of verapamil or BIBW22 BS was expressed as cell growth percentage of control.

Photoaffinity labeling. Plasma membranes (20 μg) were incubated with 0.2–1.0 μM [^{3}H]azidopine or [^{3}H]azido-BIBW22 BS (Fig. 1b) in the absence or in the presence of 100- and 500-fold molar

excess of vinblastine, verapamil, BIBW22 BS, or colchicine. The incubation was continued for an additional 30–60 min and then UV irradiated for 10 min at 254 nm on ice (Stratagene UV crosslinker, Stratagene, La Jolla, CA).

Protease cleavage. Plasma membrane fractions from CEM/VLB$^{1.0}$ cells were suspended in 10 mM Tris·HCl, pH 7.4, containing 250 mM sucrose and photoaffinity labeled with [^{3}H]azidopine or [^{3}H]azido-BIBW22 BS as described earlier. Photoaffinity labeled P-gp was digested with *Staphylococcus aureus V8* protease (10–20 μg/slice) in gel slices and loaded onto the wells of a 15% acrylamide gel, essentially as described by Cleveland *et al.* (44). Partial cleavage of P-gp was obtained by incubating plasma membrane fractions from CEM/VLB$^{1.0}$ with *V8* protease (50:1 w/w protein/enzyme) for 30 min in 10 mM Tris·HCl, pH 7.4.

Drug binding and transport. For drug binding studies, 20–50 μg of plasma membranes from CEM or CEM/VLB$^{1.0}$ cells was incubated with 0.1–1 μM [^{3}H]azido-BIBW22 BS or [^{3}H]azidopine in 100 μl of 10 mM Tris·HCl, pH 7.5, containing 250 mM sucrose. Samples were incubated at room temperature for 30 min in the absence and the presence of 100-fold molar excess of BIBW22 BS, vinblastine, or uridine. Ten volumes of ice-cold Tris buffer were added to each fraction, and samples were washed several times with an ice-cold buffer to remove unbound drugs. Drug binding was determined from the radioactivity associated with each sample. It is emphasized that these drug binding studies were done under a safety light to prevent the photodestruction of the photoreactive groups. Furthermore, the integrity of the photoreactive moiety monitored by absorbance at 254 nm and controls to check for nonspecific photolabeling of proteins was included in each experiment (data not shown).

For drug accumulation studies, CEM or Aux b1 and their drug-resistant mutants (CEM/VLB$^{0.1}$ CEM/VLB$^{1.0}$, CHO^r C5, or CHO^r B30) were washed three times in PBS plus glucose and incubated with 0.1–1 μM BIBW22 BS or [^{3}H]vinblastine alone and in the presence of 100-fold molar excess of verapamil, vinblastine, BIBW22 BS, uridine, or colchicine. Drug accumulation was stopped after a 0–60-min incubation at 37°, and cells were washed three times with ice-cold PBS. Drug accumulation in ATP-depleted cells was done as outlined above; however, cells were preincubated for 45 min in 10 mM sodium azide and 10 mM 2-deoxyglucose at 37° before the addition of [^{3}H]BIBW22 BS or [^{3}H]vinblastine (45).

For drug efflux, cells were washed with PBS alone and preincubated with 10 mM sodium azide, 10 mM 2-deoxyglucose, and 10 μM BIBW22 BS or [^{3}H]vinblastine for 30–45 min at 37°. Cells were washed twice with ice-cold PBS and resuspended in PBS plus glucose at 37°. Cells were removed after a 0–60-min incubation and centrifuged through 1 ml of ice-cold PBS containing 250 mM sucrose at 4°. The cell pellets were washed twice with 1 ml of ice-cold PBS and then solubilized in 1 N NaOH. An equal volume of 1 N HCl was added to the cell lysate, and 100 μl of the mixture was spotted onto a Whatman filter disc for [^{3}H]vinblastine. To determine the levels of BIBW22 BS after drug accumulation or efflux, BIBW22 BS was extracted from cell lysate by the addition of methanol to a final concentration of 80% (v/v). The precipitated proteins were removed by centrifugation for 30 min at 30,000 × *g*. BIBW22 BS was extracted with C_{18} SepPak cartridges (Waters Associates, Milford, MA) as described by Allan *et al.* (46). Briefly, cartridges were conditioned by washing with 5 ml of 10 mM ammonium acetate, pH 4.0. After the application of BIBW22 BS-containing samples, the cartridges were washed successively with 20 ml of water, 0.5 ml of 40% acetonitrile, and 2.5 ml of 100% acetonitrile. The final 2.5 ml of methanol was removed by vacuum drying, and the products were redissolved in 100 μl of methanol and analyzed by high performance liquid chromatography using a Vydac 201HS54 C_{18} reverse-phase column. The chromatographic procedure consisted of a 30-min gradient of 20–100% acetonitrile in 10 mM ammonium acetate, pH 4.0. The concentration of BIBW22 BS was determined by measurement of the peak area compared with the BIBW22 BS standard curve. For [^{3}H]vinblastine, the radioactivity was measured by liquid scintillation spectrometry using a Beckman LKB1219 Rackbeta counter (Beckman Instruments, Palo Alto, CA). The results are expressed as pmol/mg of proteins and show representative data from three experiments.

PAGE. Membrane proteins (~20 μg) were resolved on SDS-PAGE using the gel system of Fairbanks *et al.* (47) with some modifications. Briefly, proteins were dissolved in 1/5 volume of 5× SDS buffer 1 (2% SDS, 50 mM DTT, 1 mM EDTA, and 10 mM Tris·HCl, pH 8.0) and mixed with an equal volume of buffer 2 (2× buffer 1 and 9 M urea). For Cleveland maps, samples were resolved on a 15% Laemmli gel system (48). Gel slabs containing the resolved membrane proteins were fixed in 40% methanol/10% acetic acid and incubated for 30 min in an Amplify solution (Amersham) before drying. Dried gels were exposed to Kodak X-ray films.

Results

Modulation of *MDR* with BIBW22 BS. The sensitivity of CEM and CEM/VLB$^{0.1}$ cells to vinblastine or doxorubicin and the potentiating effect of drug cytotoxicity by BIBW22 BS are shown in Fig. 2A. The IC_{50} values of vinblastine or doxorubicin for CEM and CEM/VLB$^{0.1}$ are 1.6 and 38 ng/ml or 650 and 1350 ng/ml, respectively. This shows that CEM/VLB$^{0.1}$ is ~406- or ~35-fold resistant to vinblastine or doxorubicin. The presence of BIBW22 BS at 0.125–2.0 μM decreased the IC_{50} of CEM/VLB$^{0.1}$ cells to the same level as that of CEM cells (Table 1). BIBW22 BS caused a small decrease in the IC_{50} of CEM cells to vinblastine and doxorubicin. The decrease in IC_{50} of CEM by BIBW22 BS is likely due to low levels of P-gp in these cells, which were previously shown to mediate a verapamil-inhibitable efflux of rhodamine 123 (49). Furthermore, similar concentrations of BIBW22 BS alone were not toxic to CEM cells (Fig. 2B). The results in Fig. 2B compare the *MDR* reversal of CEM, CEM/VLB$^{0.1}$, or CEM/VLB $^{1.0}$ to vinblastine in the presence of verapamil or BIBW22 BS. Verapamil was less effective than BIBW22 BS in potentiating the toxicity of vinblastine in CEM/VLB$^{0.1}$ and CEM/VLB$^{1.0}$ cells (IC_{50} = 1.25 versus 0.25 μM and 5 versus 0.6 μM, respectively). Of interest was the ability of BIBW22 BS to reverse the *MDR* of highly resistant cells (CEM/VLB$^{1.0}$) where verapamil was much less effective.

Similar cytotoxicity experiments using CHO drug-sensitive (Aux b1) and colchicine-resistant mutants (CHO^r C5 and CHO^r B30) incubated in the presence of increasing concentrations of colchicine with or without BIBW22 BS (0.5–1.0 μM) are shown in Fig. 3. These results show that BIBW22 BS at 1.0 μM increases the susceptibility of CHO^r C5 and CHO^r B30 cells to colchicine. Interestingly, BIBW22 BS alone was more toxic to CHO cells than CEM cells (compare Fig. 2A with Fig. 3).

BIBW22 BS binding to membranes containing P-gp. The above results demonstrated that BIBW22 BS does reverse the *MDR* of tumor cells. However, given that BIBW22 BS is a general inhibitor of nucleoside transport, it could not be concluded that *MDR* reversal by BIBW22 BS is mediated by its binding to P-gp. To investigate further the mechanism of BIBW22 BS *MDR* reversal, the binding of [^{3}H]azido-BIBW22 BS to membranes from CEM or CEM/VLB$^{1.0}$ cells was compared in the absence and the presence of *MDR*-associated drugs and nucleosides (e.g., uridine). The binding of BIBW22 BS to plasma membranes from CEM and CEM/VLB$^{1.0}$ cells was measured over a wide range of drug concentrations and was saturable with K_D of 0.16 μM and B_{max} of 13.84 pmol/mg (Fig. 4A). The dissociation constant (K_D) of

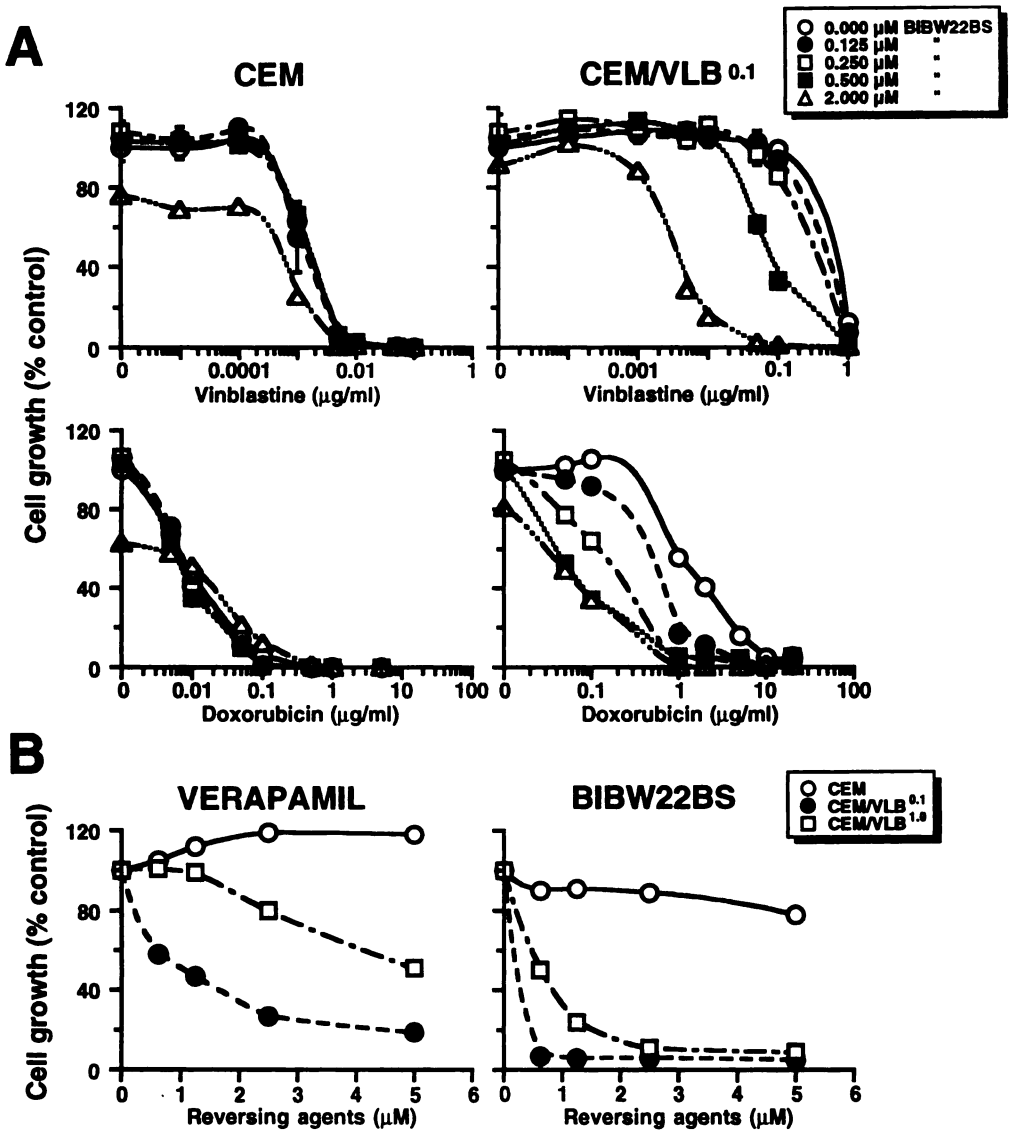


Fig. 2. Modulation of vinblastine and doxorubicin toxicity in CEM/VLB$^{0.1}$ cells with BIBW22 BS. The cytotoxicity of vinblastine and doxorubicin on CEM or CEM/VLB$^{0.1}$ cells was determined by an MTT assay. A, Viability of CEM and CEM/VLB$^{0.1}$ cells after their incubation in increasing concentrations of vinblastine or doxorubicin in the presence of 0.0, 0.125, 0.250, 0.500, or 2.0 μM BIBW22 BS. B, CEM, CEM/VLB$^{0.1}$, or CEM/VLB$^{1.0}$ incubated in the presence of 0, 0.1, or 1.0 μg of vinblastine in the absence or the presence of increasing concentrations of BIBW22 BS (0.312, 0.625, or 1.25 μM). The growth curves in B are expressed as percentage of CEM control.

TABLE 1

Modulation of resistance to vinblastine and doxorubicin in CEM/VLB$^{0.1}$ cells by BIBW22 BS

BIBW22 BS (μM)	IC_{50}[a]	Modulation ratio[b]
	ng/ml	
Vinblastine alone	650.0 ± 12	
+BIBW22 BS (0.125)	470.0 ± 11	1.4
+BIBW22 BS (0.250)	320.0 ± 16	2.0
+BIBW22 BS (0.500)	70.0 ± 15	9.3
+BIBW22 BS (2.000)	3.4 ± 0.8	191
Doxorubicin alone	1350 ± 14	
+BIBW22 BS (0.125)	580 ± 12	2.3
+BIBW22 BS (0.250)	180 ± 11	7.5
+BIBW22 BS (0.500)	54 ± 6	25
+BIBW22 BS (2.000)	47 ± 1.2	29

[a] An IC_{50} drug concentration was obtained from Fig. 2A and represents 50% inhibition of MTT dye formation. Each value represents the mean ± standard deviation of at least three determinations.

[b] The modulation ratio is calculated from the IC_{50} for the drug alone versus the IC_{50} in the presence of the modulating agent.

BIBW22 BS to membranes from CEM/VLB$^{1.0}$ cells was severalfold lower than that of vinblastine (0.4–0.5 μM) (50, 51). These results suggest that BIBW22 BS binding affinity to CEM/VLB$^{1.0}$ membranes is higher than that of vinblastine.

To determine whether P-gp or the nucleoside transporter is responsible for BIBW22 BS binding, membranes from CEM or CEM/VLB$^{1.0}$ cells were incubated with 0.1–1 μM [^{3}H]azido-BIBW22 BS alone or in the presence of 100-fold molar excess of unmodified BIBW22 BS, vinblastine, or uridine. The results in Fig. 4B show that membranes of CEM/VLB$^{1.0}$ bound more [^{3}H]azido-BIBW22 BS than CEM cells. The presence of BIBW22 BS, vinblastine, and uridine inhibited the binding of [^{3}H]azido-BIBW22 BS to membranes from CEM/VLB$^{1.0}$ cells (Fig. 4B). Similar concentrations of BIBW22 BS, vinblastine, or uridine did not affect [^{3}H]azido-BIBW22 BS binding to CEM membranes (Fig. 4B). As mentioned in Experimental Procedures), the binding of [^{3}H]azido-BIBW22 BS to CEM/VLB$^{1.0}$ membranes was not due to nonspecific photolabeling of P-gp or other membrane proteins because the above experiments were done in the dark under a safety light. Furthermore, the addition of BIBW22 or vinblastine caused a large reduction in [^{3}H]azido-BIBW22 BS binding to membranes from drug-resistant but not drug-sensitive cells. Therefore, the observed increase in [^{3}H] BIBW22 BS binding to CEM/VLB$^{1.0}$ membranes is likely due to P-gp. However, because uridine also inhibited BIBW22 BS binding to CEM/VLB$^{1.0}$

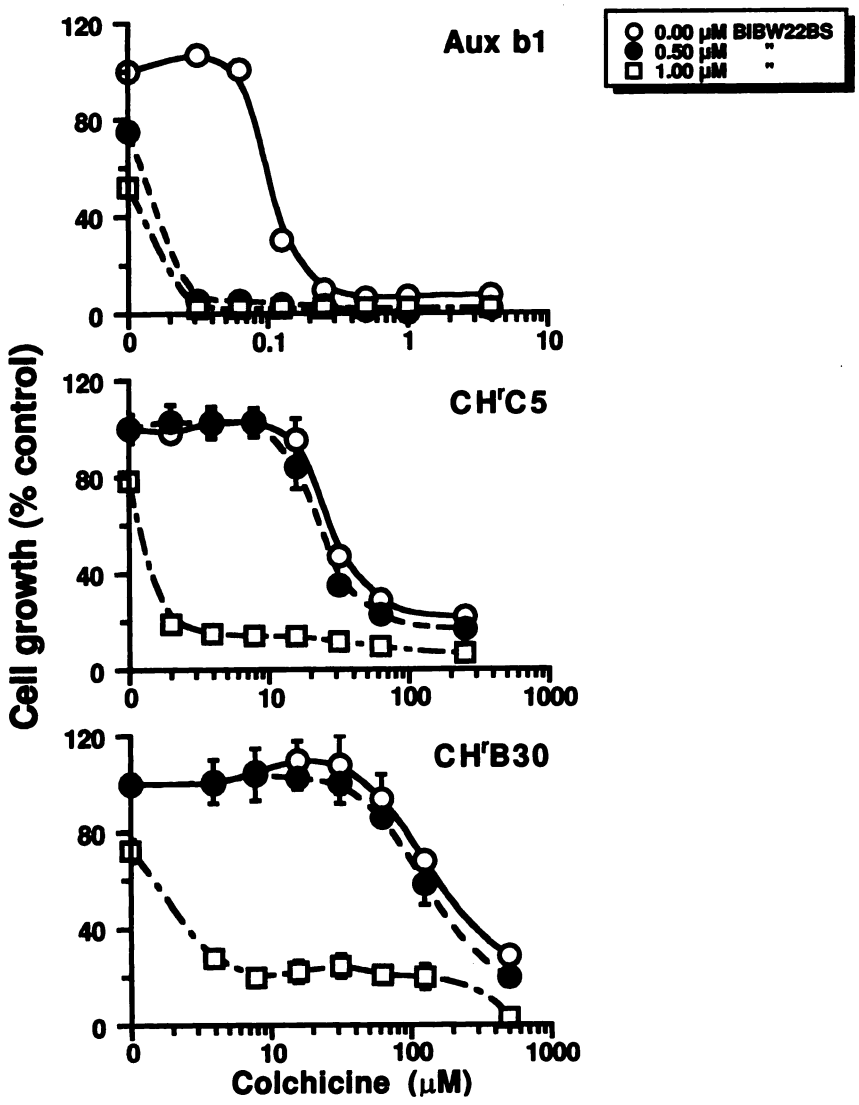


Fig. 3. Sensitization of CHO cells to colchicine with BIBW22. The susceptibility of drug-sensitive (Aux b1) and -resistant CHO cells (CHO^r C5 or CHO^r B30) to colchicine without and with BIBW22 BS was determined by MTT assay. Cells are incubated in increasing concentrations of colchicine in the presence of 0.0, 0.50, and 1.00 μM of BIBW22 BS.

membranes, it is likely that CEM/VLB$^{1.0}$ cells have higher levels of the nucleoside transporter. An earlier study using [^{3}H]dipyridamole (a general nucleoside inhibitor) has suggested that there is a higher number of a nucleoside transporter in membranes of MCF7/ADR drug-resistant versus MCF7 drug-sensitive cells (52).

Photoaffinity labeling of P-gp with [^{3}H]azido-BIBW22 BS. To determine whether BIBW22 BS interacts directly with P-gp, a photoactive analog of BIBW22 BS ([^{3}H]azido-BIBW22 BS; Fig. 1) was used to photoaffinity label plasma membranes from drug-sensitive (CEM) and -resistant (CEM/VLB$^{1.0}$) cells. Membrane fractions from CEM or CEM/VLB$^{1.0}$ cells were incubated with 0.5 μM [^{3}H]azido-BIBW22 BS and UV irradiated (see Experimental Procedures). The results in Fig. 5 (*lane 2*) show the photolabeling of two polypeptides (~150 kDa and ~170 kDa) with [^{3}H]azido-BIBW22 BS in drug-resistant cells. Similar photoaffinity labeling of CEM membranes revealed two photolabeled proteins with apparent molecular masses of 55 and 150 kDa (Fig. 5, *lane 1*). The identity of the 170-kDa [^{3}H]azido-BIBW22 BS photolabeled polypeptide was confirmed by immunoprecipitation with P-gp-specific monoclonal antibodies (C219 or C494). Fig. 5 (*lanes 3 and 4*) shows the immunoprecipitated polypeptides from [^{3}H]azido-BIBW22 BS photolabeled CEM/VLB$^{1.0}$ plasma membranes with IgG_{2a} or C219 monoclonal antibody, respectively. A 170-kDa [^{3}H]azido-BIBW22 BS photolabeled protein was immunoprecipitated with C219 monoclonal antibody (*lane 4*), whereas no photolabeled proteins were immunoprecipitated with an irrelevant IgG_{2a} (*lane 3*). These results show that [^{3}H]azido-BIBW22 BS interacts directly with P-gp. The other (55 and 150 kDa) photolabeled proteins are unlikely to represent a deglycosylated or degraded product of P-gp because both forms are recognized by C219 monoclonal antibody (28) and are not found in CEM cells. The 55-kDa photolabeled protein may be the nucleoside transporter shown earlier to migrate with a molecular mass of 55 kDa on SDS-PAGE (53). However, previous photoaffinity labeling of CEM cells with nitro benzyl mercapto-purine riboside, a photoactive nucleoside transport inhibitor, was shown to photolabel a 75-kDa protein (54). The 75-kDa protein was postulated to represent a highly glycosylated form of 55-kDa nucleoside transporter (54). It is interesting that photolabeling of CEM with BIBW22 BS did not reveal a 75-kDa membrane protein. Thus, differences in the molecular mass of the two photolabeled nucleoside transporters are not clear but should be further investigated. The

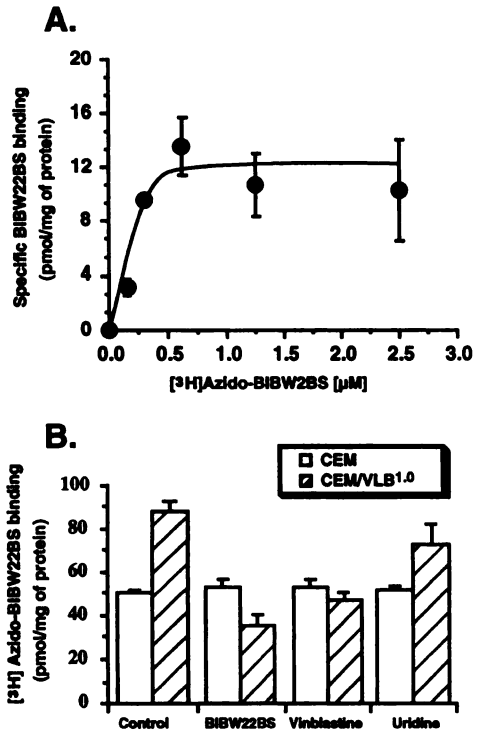


Fig. 4. Equilibrium binding of [^{3}H]azido-BIBW22 BS to CEM/VLB$^{1.0}$ membranes. A, Specific binding of [^{3}H]azido-BIBW22 BS in the absence and the presence of 50-fold excess of unlabeled BIBW22 BS. These results represent at least three independent experiments. B, [^{3}H]azido-BIBW22 BS binding to membranes from CEM or CEM/VLB$^{1.0}$ cells in the absence and the presence of 50-fold molar excess of BIBW22 BS, vinblastine, or uridine.

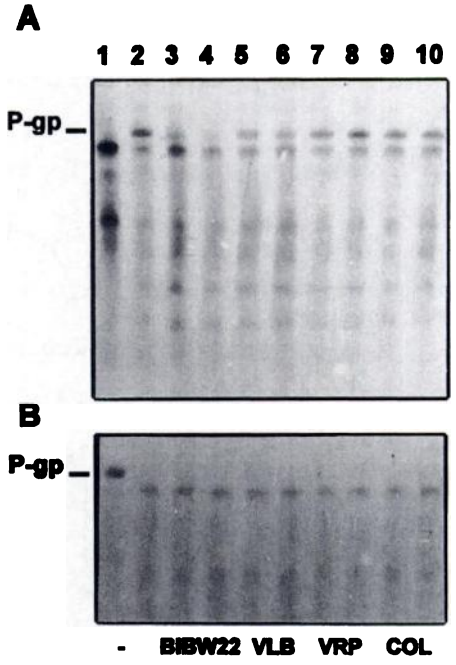


Fig. 6. Effects of *MDR*-associated drugs on [^{3}H]azido-BIBW22 BS photolabeling of P-gp. Membrane fractions from CEM/VLB$^{1.0}$ (A) and CEM (B) cells were photoaffinity labeled with 0.5 μM [^{3}H]azido-BIBW22 BS alone (*lane 2*) or in the presence of 50 and 250 μM of BIBW22 BS, vinblastine, verapamil, or colchicine (*lanes 3–10*, respectively). B, Similar photolabeling of CEM membranes in the absence (*lane 2*) or the presence (*lanes 3–10*) of the above drugs. *Lane 1* (A and B), photolabeled proteins from CEM or CEM/VLB$^{1.0}$ membranes in the absence of drugs, respectively.

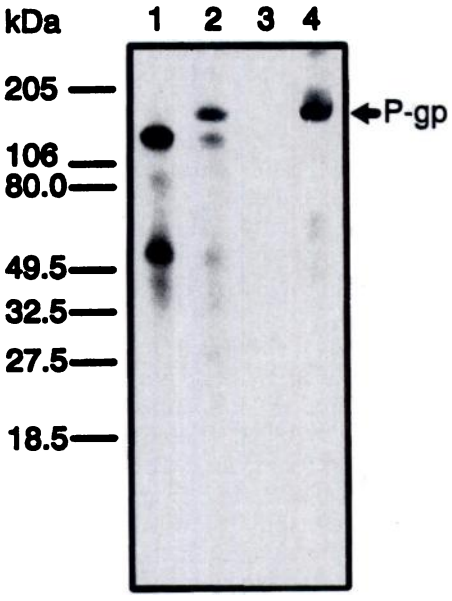


Fig. 5. Photoaffinity labeling of P-gp with [^{3}H]azido-BIBW22 BS. Plasma membranes from CEM and CEM/VLB$^{1.0}$ cells are photolabeled with [^{3}H]azido-BIBW22 BS and immunoprecipitated with P-gp-specific monoclonal antibody C219 or an irrelevant IgG_{2a}. *Lanes 1 and 2*, [^{3}H]azido-BIBW22 BS photolabeled proteins in CEM and CEM/VLB$^{1.0}$ membranes, respectively. *Lanes 3 and 4*, immunoprecipitated proteins from CEM/VLB$^{1.0}$ cells using an IgG_{2a} or a C219 monoclonal antibody, respectively. *Left*, molecular mass marker proteins.

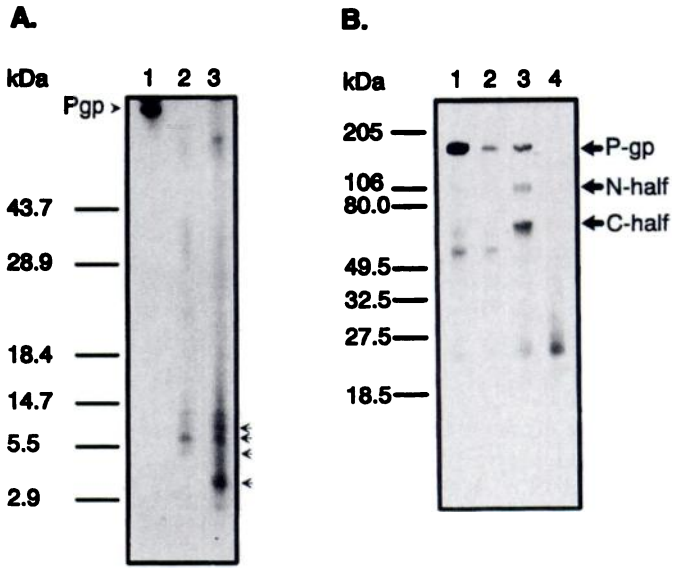


Fig. 7. Cleveland's map of [^{3}H]azido-BIBW22 BS or [^{3}H]azidopine photolabeled P-gp. A, [^{3}H]Azido-BIBW22 BS or [^{3}H]azidopine photolabeled P-gp was digested in a gel slice with 10 μg/gel of *V8* protease. *Lanes 2 and 3*, photolabeled *V8* cleaved peptides of [^{3}H]azidopine or [^{3}H]azido-BIBW22 BS photolabeled P-gp, respectively. *Arrowheads*, positions of *V8* photolabeled peptides. *Lane 1*, undigested [^{3}H]azido-BIBW22 BS photolabeled P-gp. B, Photoaffinity labeling of native and partially digested P-gp with [^{3}H]azidopine in the absence (*lanes 1 and 3*) and the presence (*lanes 2 and 4*) of 100-fold molar excess of BIBW22 BS, respectively.

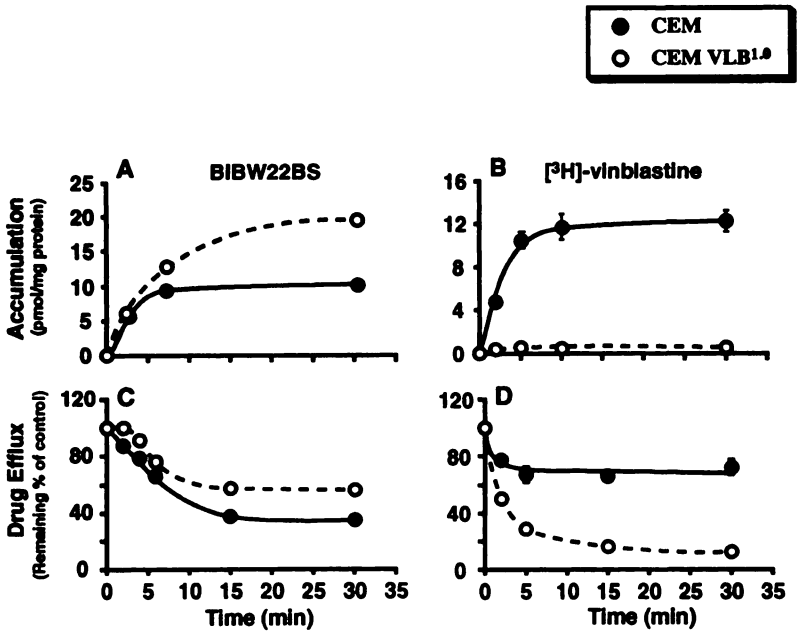


Fig. 8. Accumulation and efflux of BIBW22 BS and [^{3}H]vinblastine in CEM and CEM/VLB$^{1.0}$ cells. Cells were incubated with 0.1 μM BIBW22 BS or [^{3}H]vinblastine for 0, 2, 7, and 15 min at 37°. For BIBW22 BS, the drug concentration in CEM and CEM/VLB$^{1.0}$ cells was determined after the extraction of the drug with organic solvent and high performance liquid chromatographic analysis (see Experimental Procedures). For [^{3}H]vinblastine, the drug accumulation and efflux are determined by quantification of the radiolabel. Drug accumulation values are expressed as pmol/mg of protein. *Points*, mean ± standard deviation of three independent experiments.

identity of the 150-kDa protein, also photolabeled by [^{3}H]azido-BIBW22 BS but not recognized by the monoclonal antibody C219, is not known.

The binding specificity of [^{3}H]azido-BIBW22 BS toward P-gp was determined by photoaffinity labeled of plasma membranes from CEM/VLB$^{1.0}$ with 0.5 μM [^{3}H]azido-BIBW22 BS in the absence and the presence of BIBW22 BS, vinblastine, verapamil, and colchicine. The results in Fig. 6A show that BIBW22 BS completely inhibited P-gp photolabeling by [^{3}H]azido-BIBW22 BS (*lanes 3 and 4*). Vinblastine also inhibited the photolabeling of P-gp by [^{3}H]azido-BIBW22 BS but to a lesser extent than BIBW22 BS (Fig. 6A, *lanes 5 and 6* versus *lanes 3 and 4*). Verapamil or colchicine did not significantly inhibit P-gp photolabeling (*lanes 7–10*). The photolabeling of the 150-kDa protein in CEM/VLB$^{1.0}$ (*lanes 3–10*) or CEM (*lanes 3–10*) membrane was not inhibited with molar excess of BIBW22 BS or the other drugs. Taken together, these results show that the binding of [^{3}H]azido-BIBW22 BS to P-gp is specific and occurs at a physiologically relevant drug binding site or sites.

Cleveland mapping of [^{3}H]azido-BIBW22 BS photolabeled P-gp. The drug binding site in P-gp is not known. Domain mapping studies (55) using photoactive analogs of various drugs or *MDR*-reversing agents have demonstrated the photolabeling of one or two *V8* peptides in P-gp (23, 24, 55). To determine whether similar peptides in P-gp are photoaffinity labeled with [^{3}H]-azido-BIBW22 BS, membranes from CEM/VLB$^{1.0}$ cells were photolabeled with [^{3}H]azidopine or [^{3}H]azido-BIBW22 BS and digested with *V8* protease in gel slices. Fig. 7A shows the *V8* maps (*lanes 2 and 3*) of [^{3}H]azidopine and [^{3}H]azido-BIBW22 BS photolabeled P-gp, respectively. Two peptides with apparent molecular masses of 6.5 and 5.5 kDa were photolabeled with [^{3}H]azidopine (Fig. 7A, *lane 2*), whereas [^{3}H]azido-BIBW22 BS photolabeled three peptides (7.5, 6.5, and 4.7 kDa). Only one of [^{3}H]azido-BIBW22 BS peptides (6.5 kDa) comigrated with [^{3}H]azidopine photolabeled peptides (Fig. 7A, *lane 3*). The other two [^{3}H]azido-BIBW22 BS photolabeled peptides (7.5 and 4.7 kDa) might be different. The possibility that the 7.5-kDa BIBW22 BS photolabeled peptide is an incomplete cleavage of the 6.5-kDa or the 4.7-kDa peptide cannot be completely ruled out. However, *V8* titration experiments using 10–30 μg of *V8* protease/gel slice did not decrease the intensity of the 7.5-kDa peptide (data not shown). Thus, differences in the *V8* profile of the photolabeled peptides suggest that BIBW22 BS is likely to interact with different sequences in P-gp than [^{3}H]azidopine.

Earlier studies have shown that [^{3}H]azidopine interacts with both halves of P-gp (56).[1] Although it remains to be determined whether the two photolabeled sequences of P-gp represent two or one drug binding site(s), it was of interest to learn whether BIBW22 BS interacts with both domains in P-gp. The results in Fig. 7B show the photolabeling of native or partially cleaved P-gp with [^{3}H]azidopine in the absence (*lanes 1 and 3*) and in the presence of 100-fold molar excess of BIBW22 BS (*lanes 2 and 4*). The results in Fig. 7B (*lanes 1 and 2*) confirm an earlier report that demonstrated the capacity of BIBW22 BS to inhibit the photolabeling of native P-gp with [^{3}H]azidopine (39). Furthermore, the results in Fig. 7B show that BIBW22 BS interacts with both halves of P-gp as previously shown using other photoactive drugs that bind directly to P-gp.

BIBW22 BS is not a substrate for P-gp efflux pump. To determine whether BIBW22 BS is a substrate for the P-gp drug efflux pump, CEM or CEM/VLB$^{1.0}$ cells were incubated with 0.1–1 μM BIBW22 BS or [^{3}H]vinblastine for 0–30 min at 37° (see Experimental Procedures). The results in Fig. 8 show the accumulation of BIBW22 BS (Fig. 8A) and [^{3}H]vinblastine (Fig. 8B) in CEM and CEM/VLB$^{1.0}$ cells, respectively. BIBW22 BS was found to accumulate more in CEM/VLB$^{1.0}$ than in CEM cells (Fig. 8A). In contrast, [^{3}H]vinblastine accumulated to a greater extent in drug-sensitive cells (Fig. 8B). To demonstrate that the observed accumulation of BIBW22 BS in CEM/VLB$^{1.0}$ cells is not unique to the CEM human lymphoma cells, BIBW22 BS accumulation was measured in drug-sensitive and -resistant CHO cells (Aux b1,

[1] Z. Liu and E. Georges, unpublished observations.

CHO^r C5, or CHO^r B30 cells, respectively). Again, BIBW22 BS was found to accumulate more in resistant CHO^r C5 or CHO^r B30 cells than in drug-sensitive Aux b1 cells (data not shown).

Given the above results, it was of interest to measure the efflux of BIBW22 BS and [^{3}H]vinblastine in CEM and CEM/$VLB^{1.0}$ cells. The results in Fig. 8C) show that BIBW22 BS is retained much longer in CEM/$VLB^{1.0}$ than in CEM cells. BIBW22 BS was released more rapidly from CEM than from CEM/$VLB^{1.0}$ cells (60% versus 40% of BIBW22 released after 15-min incubation, respectively). These results are in contrast with those observed with [^{3}H]vinblastine, in which 50% of the drug was effluxed within 2 min from resistant cells versus 10% of the drug effluxed from drug-sensitive cells (Fig. 8D). Although it is not yet clear why BIBW22 BS accumulates more in drug-resistant than in drug-sensitive cells, it may be due to high affinity binding between BIBW22 BS and P-gp or other receptors in CEM/$VLB^{1.0}$ cells. Furthermore, BIBW22 BS accumulation is not energy dependent. The results in Fig. 9B show that BIBW22 BS accumulation in CEM, CEM/$VLB^{0.1}$, or CEM/$VLB^{1.0}$ is not significantly altered when cells are preincubated in 10 mM sodium azide and 2-deoxyglucose (45). In contrast, [^{3}H]vinblastine accumulation in CEM/$VLB^{0.1}$ or CEM/$VLB^{1.0}$ cells was dramatically increased in the presence of sodium azide and 2-deoxyglucose (Fig. 9A).

To determine the effects of P-gp-associated drugs on BIBW22 BS or [^{3}H] vinblastine accumulation, drug accumulation was done in the absence or the presence of excess (50 μM) BIBW22 BS, colchicine, verapamil, or vinblastine. The results in Fig. 10A show the accumulation of BIBW22 BS in CEM or CEM/$VLB^{1.0}$ cells after 30-min incubation in the absence or the presence of 50-fold molar excess of colchicine, verapamil, or vinblastine. The presence of colchicine or verapamil did not significantly modulate the accumulation of BIBW22 BS, whereas vinblastine at the same drug concentration caused a large decrease in the accumulation of BIBW22 BS in drug-sensitive and -resistant cells (Fig. 10A). The effect of vinblastine on BIBW22 BS accumulation in CEM and CEM/$VLB^{1.0}$ cells is consistent with binding rather than a drug efflux. Thus, it may be suggested that vinblastine and BIBW22 BS compete for binding to the same binding domain in P-gp; however, in the presence of vinblastine, less BIBW22 BS interacts with P-gp. In contrast, similar drug accumulation studies showed that higher [^{3}H]vinblastine accumulation in CEM cells and excess verapamil but not colchicine potentiated the accumulation of [^{3}H]vinblastine in CEM/$VLB^{1.0}$ cells (Fig. 10B). BIBW22 BS also caused a large increase in [^{3}H]vinblastine accumulation in CEM/$VLB^{1.0}$ cells. Taken together, these results suggest that P-gp does not mediate the transport of BIBW22 BS. However, BIBW22 does potentiate the accumulation of [^{3}H]vinblastine in drug-resistant cells.

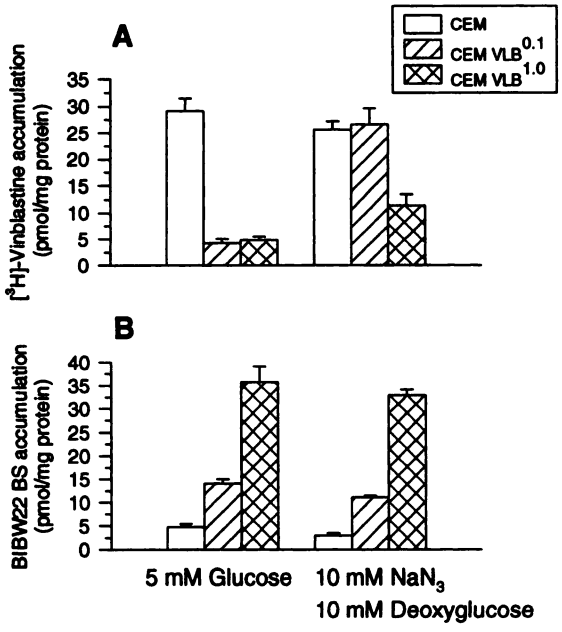


Fig. 9. Accumulation of [^{3}H]vinblastine or BIBW22 BS in cells with or without ATP. Drug accumulation in CEM, CEM/$VLB^{0.1}$, or CEM/$VLB^{1.0}$ cells was measured in cells incubated with 5 mM glucose or preincubated at 37° for 45 min in PBS with 10 mM sodium azide (NaN_3) and 2-deoxyglucose. A and B, Accumulation of [^{3}H]vinblastine or BIBW22 BS after a 45-min incubation of cells that were preincubated for 30–45 min with 5 mM glucose or 10 mM sodium azide and 2-deoxyglucose.

In light of the results shown in Fig. 4B, it was of interest to determine the effect of increasing concentrations of uridine on BIBW22 BS accumulation in CEM and CEM/$VLB^{1.0}$ cells. The results in Fig. 11 show that uridine (10–100 μM) causes a significant increase in BIBW22 BS accumulation in both drug-sensitive (CEM) and -resistant (CEM/$VLB^{1.0}$) cells. The observed increase in BIBW22 BS accumulation in the presence of uridine is not entirely clear; however, excess uridine may compete for BIBW22 BS transport, and therefore more BIBW22 BS is available to interact with P-gp or other membrane proteins. These results are consistent with the effect of uridine on BIBW22 BS binding to membranes from drug-resistant cells seen in Fig. 4B. Therefore, some increase in BIBW22 BS accumulation in CEM/$VLB^{1.0}$ cells may be mediated by the nucleotide transporter.

Discussion

In this study, we show that low concentrations (0.5–2 μM) of BIBW22 BS completely reverse the resistance of CEM/$VLB^{0.1}$ cells to vinblastine and doxorubicin. These results confirm earlier findings in which BIBW22 BS was shown to reverse the *MDR* of vincristine-resistant KB V20 cells (39). Furthermore, BIBW22 BS was shown to inhibit the nucleoside transport in the latter cell line (39). The capacity of BIBW22 BS to reverse the *MDR* of tumor cells and to inhibit nucleoside transport is not clear. However, our photoaffinity labeling experiments showed a direct binding between BIBW22 BS and P-gp and a 55-kDa membrane protein. The latter protein is likely to encode for the nucleoside transporter previously shown to migrate as 55-kDa protein on SDS-PAGE (58). Furthermore, our drug-binding and -transport studies show that the observed increase in BIBW22 BS binding or accumulation in CEM/$VLB^{1.0}$ cells is likely due to an increase in P-gp and a nucleoside transporter. The increase in the nucleoside transporter in *MDR* cells (MCF7/ADR) has been previously suggested using [^{3}H]dipyridamole (52). In that study (52), the increase in [^{3}H]dipyridamole binding to membranes from MCF7/ADR was postulated to be due to an increase in the number and affinity of the nucleoside transporter. However, [^{3}H]dipyridamole binding to P-gp was not determined in that study (52). Taken together, our

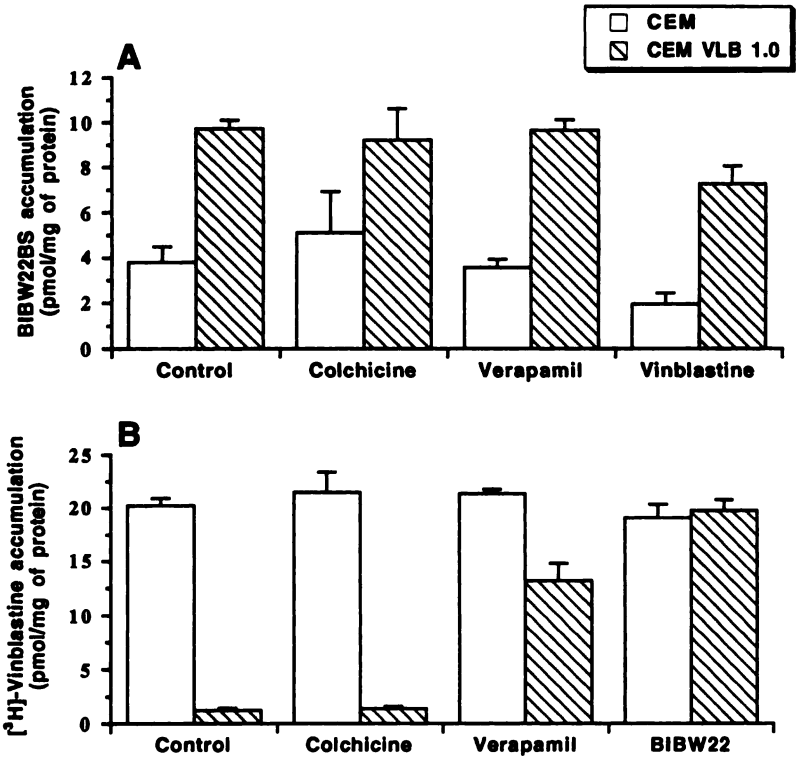


Fig. 10. Effects of drugs on the accumulation of BIBW22 BS or [^{3}H]vinblastine (*VLB*) in CEM and CEM/VLB$^{1.0}$ cells. CEM or CEM/VLB$^{1.0}$ cells were incubated with 0.1 μM BIBW22 BS or [^{3}H]vinblastine for 30 min at 37° in the absence or the presence of 100-fold molar excess of colchicine, verapamil, or cold vinblastine or BIBW22 BS. Estimates of the amount of drug (BIBW22 BS or [^{3}H]vinblastine) accumulation were determined as for Fig. 8.

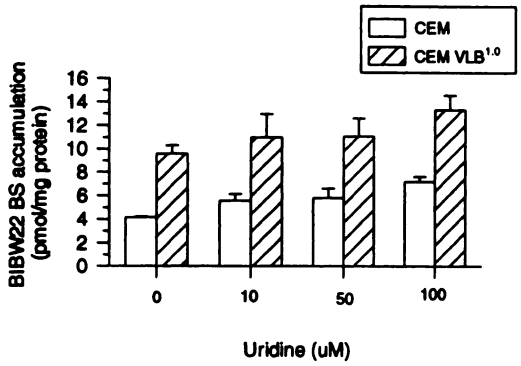


Fig. 11. BIBW22 BS accumulation in CEM or CEM/VLB$^{1.0}$ with increasing concentrations of uridine. Cells were incubated with 0.1 μM BIBW22 BS for 30 min at 37° in the presence of increasing concentrations (0–100 μM) of uridine. BIBW22 BS accumulation was determined as for Fig. 8.

results show that BIBW22 BS binding to CEM/VLB$^{1.0}$ cells is largely due to P-gp because vinblastine was more effective than uridine in inhibiting BIBW22 BS binding to membranes from CEM/VLB$^{1.0}$ cells.

Certain nontoxic compounds are thought to reverse the *MDR* of tumor cells by competing for P-gp drug binding sites (20–24). The effect of BIBW22 BS on P-gp-associated *MDR* is consistent with the latter proposal. BIBW22 BS was shown to inhibit the photolabeling of P-gp with [^{3}H]azidopine and to potentiate the accumulation of [^{3}H]vinblastine in drug-resistant cells. However, BIBW22 BS is not effluxed but accumulates in *MDR* cells. The preferential accumulation of BIBW22 BS in drug-resistant cells was shown to occur in two different cell lines and was not energy dependent. These results are surprising because most *MDR*-reversing agents (e.g., verapamil, azidopine, and cyclosporin A) were shown to be effluxed from *MDR* cells (20–22) or accumulate to the same level in both drug-sensitive and -resistant cells (e.g., progesterone and Triton X-100; Refs. 58 and 59). Thus, BIBW22 BS is the first *MDR*-reversing agent that interacts directly with P-gp and accumulates more in resistant cells. It is presently not clear why BIBW22 BS accumulates in drug-resistant cells. However, it may be speculated that BIBW22 BS binds tightly to P-gp. In fact, the latter suggestion is consistent with our result from the competition of drug accumulation showing that the presence of vinblastine causes a decrease rather than the expected increase in BIBW22 BS accumulation in resistant cells. These results predict that vinblastine competes (competitively or noncompetitively; Ref. 60) with BIBW22 BS for binding to P-gp. Therefore, in the presence of vinblastine, non-P-gp-associated BIBW22 BS is released from drug-resistant cells and is seen as a decrease in BIBW22 BS accumulation. Taken together, our results describe a novel class of *MDR*-reversing agents that are not transported by P-gp and accumulate in *MDR* cells. It remains to be seen whether BIBW22 BS is clinically more effective in reversing the MDR phenotype. BIBW22 BS was shown recently to be better than verapamil in potentiating the antitumor activity of vincristine and doxorubicin in P-gp-positive xenografts (61).

The specificity of BIBW22 BS toward P-gp was confirmed by the ability of vinblastine to inhibit the photoaffinity labeling of P-gp by [^{3}H]azido-BIBW22 BS. Furthermore, the inhibition of P-gp photolabeling and drug binding with vinblastine is consistent with BIBW22 BS binding at a physiologically relevant site in P-gp. The drug binding site in P-gp is presently not known. Recent mapping studies using photoactive drugs have identified two *V8* peptides in P-gp that are photoaffinity labeled with several drugs (e.g., [^{3}H] azidopine, rhodamine 123, iodoazidoprazosin, forskolin, and benzimidazole; Refs. 22, 23, and 55). In this study, we show that similar *V8* mapping of [^{3}H]azido-BIBW22 BS-labeled

P-gp yielded three photoaffinity labeled peptides (7.5, 6.5, and 4.5 kDa). Interestingly, only one of the three [^{3}H]azido-BIBW22 BS photolabeled peptides (the 6.5-kDa peptide) comigrates with the [^{3}H]azidopine photoaffinity labeled peptides. This confirms the interaction of BIBW22 BS with a similar site on P-gp as previously identified for [^{3}H]azidopine and rhodamine 123 (23). However, the photoaffinity labeling of two other *V8* peptides in P-gp with [^{3}H]azido-BIBW22 BS suggests that there are other sites near or distant from the azidopine photolabeled site(s) that may explain the transport properties of BIBW22 BS.

In summary, BIBW22 BS modulates the MDR phenotype of tumor cells by interacting directly and specifically with P-gp. The effectiveness of BIBW22 BS in reversing the MDR phenotype compared with verapamil may be due to its interaction with different sequences in P-gp and/or increased accumulation in drug-resistant cells. Studies are under way to find the amino acid sequences in P-gp that are photoaffinity labeled with [^{3}H]azido-BIBW22 BS.

Acknowledgments

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