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BENZIMIDAZOLES, POTENT ANTI-MITOTIC DRUGS: SUBSTRATES FOR THE P-GLYCOPROTEIN TRANSPORTER IN MULTIDRUG-RESISTANT CELLS

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Abstract—P-glycoprotein is thought to mediate the energy-dependent efflux of many structurally and functionally unrelated lipophilic compounds. Presently, the molecular mechanism underlying the binding and efflux of drugs by P-glycoprotein is not well understood. However, it has been suggested that two planar benzene ring structures and a cationic charge are commonly found in many drugs that interact with P-glycoprotein. The benzimidazoles (BZs) are potent anti-tumour, anti-fungal and anti-parasitic agents, whose mode of action is thought to result from their inhibition of microtubule functions. Although other classes of microtubule inhibitors, such as colchicine and vinblastine, have been studied extensively with respect to their interaction and efflux by P-glycoprotein, the BZ group of drugs has not been characterized. In this study, we have characterized the interaction of BZ with multidrug-resistant cells and found that resistant cells accumulated substantially less BZ compared with drug-sensitive cells. Furthermore, BZ was more toxic to sensitive than to drug-resistant cells, suggesting that BZ is likely to be a substrate for the P-glycoprotein drug efflux pump. In addition, we used a photoactive analogue of BZ ([^{125}I]ASA-BZ) to demonstrate a direct binding between BZ and P-glycoprotein. Results showing that a molar excess of vinblastine, unmodified BZ, verapamil and rhodamine 123, but not colchicine, inhibited the photoaffinity labelling of P-glycoprotein by [^{125}I]ASA-BZ confirmed the binding specificity of BZ to P-glycoprotein. Protease digestion of [^{125}I]ASA-BZ photoaffinity labelled P-glycoprotein yielded two peptides that were similar to those obtained with other P-glycoprotein-associated drugs, e.g. azidopine and iodoaryl azidoprazosin. Taken together, these results demonstrate a direct and specific interaction between P-glycoprotein and BZ in a manner that is probably similar to other previously characterized P-glycoprotein-associated drugs.

Key words: multidrug resistance; P-glycoprotein; photoaffinity labelling; benzimidazole

The chemotherapeutic treatment of cancer patients with a single cytotoxic agent often induces a state of MDR† in which the tumour becomes tolerant to a large number of structurally and functionally unrelated drugs. A similar MDR phenotype was observed when tumour cell lines were selected *in vitro* with cytotoxic lipophilic drugs [1]. The development of the MDR phenotype has been linked to the over-expression of a 170 kDa membrane glycoprotein, termed P-gp [2, 3]. It is now believed that P-gp functions as an ATP-dependent drug efflux pump that results in a net decrease in the cellular accumulation of cytotoxic drugs [4]. Hence, the role of P-gp in tumour cell lines has been demonstrated to mediate the MDR phenotype [5–7]. P-gp is a member of the ABC superfamily of membrane transporters that include the cystic fibrosis transmembrane conductance regulator (CFTR; [8]), the antigenic MHCII peptide transporter [9] and the pigment uptake proteins in *Drosophila* [10]. Although, the normal function of P-gp remains to be determined, P-gp is believed to encode for an evolutionary conserved transport mechanism [11]. For example, P-gp homologues were shown to be over-expressed in chloroquine-resistant *Plasmodium falciparum* (*pfmdr*; [12]), emetine-resistant *Entamoeba histolytica* (*Eh mdr*; [13]), and vinblastine-resistant *Leishmania donovani* (*ldmdr*; [14]).

Direct and specific binding between P-gp and certain MDR-associated drugs has been demonstrated using photoactive drug analogues [15]. Thus, it is now believed that P-gp drug efflux is mediated by direct interactions between P-gp and cytotoxic drugs. Furthermore, certain non-toxic drugs, which include calcium channel blockers [16], quinines [17] and cyclosporins [18], have also been shown to interact directly with P-gp and reverse the MDR phenotype. The mechanism of MDR reversal remains to be clarified; however, it was suggested that MDR-reversing agents compete for the drug binding site in P-gp [19, 20].

The broad substrate specificity of P-gp toward a large number of structurally diverse lipophilic compounds remains unresolved. Physico–chemical analysis of certain MDR-associated drugs has indicated that two planar aromatic rings and a cationic charge are commonly found in many drugs that interact with P-gp [21]. BZs are a group of structurally similar compounds that have been shown

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† Abbreviations: MDR, multidrug-resistant (resistance); P-gp, P-glycoprotein; BZ, benzimidazole; NHS-ASA, *N*-hydroxysuccinimydyl-4-azidosalicylic acid; IAAP, iodoaryl azidoprazosin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

to possess anti-mitotic activity *in vitro* and *in vivo* [22]. However, their major application is currently in the treatment of veterinary and human helminthiasis where they have demonstrated remarkable efficacy as well as safety [23]. In this study, it was of interest to determine the effects of BZs on MDR cells and their interaction with P-gp since (i) BZs, although structurally different from other anti-cancer drugs, contain planar aromatic rings and a cationic charge common to many MDR-associated drugs; (ii) BZs are remarkably safe at high doses in humans; hence, they may be applied as MDR-reversing agents; and (iii) the availability of a large number of structural analogues of BZ can be used in the structural analysis of P-gp–drug interactions. The results of this study suggest that BZs are substrates for the P-gp drug efflux pump in human MDR cells. Additionally, using a photoactive analogue of BZ ([^{125}I]ASA-BZ), we have demonstrated a direct and specific binding between P-gp and BZ at similar domains, as previously suggested with other MDR-associated drugs.

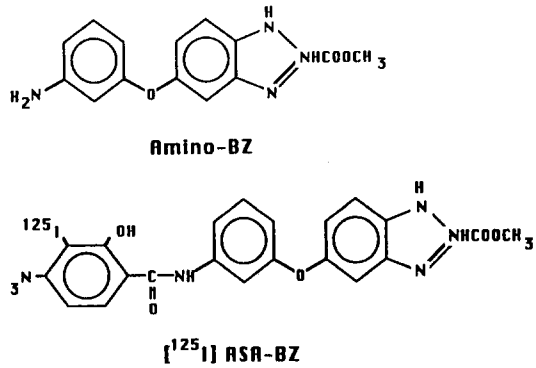

Fig. 1. Chemical structures of unmodified amino-BZ and its photoactive derivative [^{125}I]ASA-BZ. The organic structures of unmodified and modified amino-BZ (photoactive derivative [^{125}I]ASA-BZ) are shown above.

MATERIALS AND METHODS

Materials. Methyl 5-substituted amino phenoxybenzimidazol-2-yl carbamate (amino-BZ) was a gift from Hoechst (Germany). Rhodamine 123 and NHS-ASA were obtained from the Pierce Chemical Co., Rockford, IL. Vinblastine was from the Aldrich Chemical Co., Milwaukee, WI, while verapamil and colchicine were from the Sigma Chemical Co., St. Louis, MO. [^{125}I]IAPP (2200 Ci/mmol) was purchased from Du Pont New England Nuclear, Boston, MA. Drug-sensitive human lymphoma cells (CEM) [24] were a gift from Dr. W. Beck, St. Jude Children's Research Hospital, Memphis, TN. P-glycoprotein-specific monoclonal antibody (C219) and the CEM/VLB$^{1.0}$ drug-resistant cells were a gift from Dr. Victor Ling of the Ontario Cancer Institute, Toronto, Canada. All other chemicals used were of the highest grade available.

Tissue culture and cytotoxicity assays. Drug-sensitive (CEM) and resistant (CEM/VLB$^{0.1}$ or CEM/VLB$^{1.0}$) human lymphoma cell lines were grown in α-minimal Eagle's medium as previously described [24]. The sensitivity of CEM, CEM/VLB$^{0.1}$ or CEM/VLB$^{1.0}$ cells to BZ was measured using the MTT assay [25]. Cells were harvested at the exponential growth phase, and 100 μL aliquots of single cell suspensions were plated into 96-well plates at 0.5×10^4 for CEM and 1×10^4 for CEM/VLB per well. The cells were incubated for 24 hr at 37° before the addition of amino-BZ. Cells were then cultured in the presence of amino-BZ for 4 days. The liquid in each well was carefully removed followed by the addition of 20 μL of 5 mg/mL MTT in PBS. The plates were incubated for 4 hr at 37°, and the coloured 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 vigorous pipetting. The 96-well plates were microwaved for 1 min at power level 1, and 10 μL of ethanol was added to disperse the bubbles formed during pipetting. Samples were read at 570 nm using an ELISA microtitre plate reader. Drug effects were expressed as percent survival of cells incubated in the absence of amino-BZ. The effect of 1–2 μM BZ on CEM, CEM/VLB$^{0.1}$, or CEM/VLB$^{1.0}$ cytotoxicity was determined by the trypan blue dye exclusion method after a 3-hr incubation of cells at 37°.

Drug accumulation. Aliquots of CEM or CEM/VLB$^{1.0}$ cells (1×10^6) were incubated with 1 μM amino-BZ, 1 μM rhodamine, or 1 μM [^{3}H]vinblastine in the absence or presence of 50 μM verapamil for 1 hr at 37°. Cells were then transferred to ice and washed three times with ice-cold PBS. The cell pellets were solubilized in 1 N NaOH followed by the addition of an equal volume of 1 N HCl 4 hr later. Amino-BZ was extracted from lysed cell protein samples by the addition of methanol to a final concentration of 80% (v/v). Precipitated proteins were removed by centrifugation for 30 min at 30,000 *g*. Amino-BZ was extracted with C_{18} SepPak cartridges (Waters Associates, Milford, MA) as described by Allan *et al.* [26]. Cartridges were conditioned by washing with 5 mL of 0.025 M ammonium acetate buffer, pH 5.5. After application of the sample, the cartridges were washed successively with 20 mL of distilled water, 0.5 mL of 40% methanol, 0.4 mL of 100% methanol, and 2.5 mL of 100% methanol. The final 2.5% methanol was removed by vacuum drying, and the products were dissolved in 100 μL methanol and analyzed by HPLC using a Vydac 201HS54 C_{18} reverse phase column. The chromatographic procedure consisted of a 30-min gradient of 20–100% acetonitrile in 0.025 M ammonium acetate buffer, pH 5.5, at a flow rate of 1 mL/min and detection at 292 nm. The concentration of amino-BZ was determined by measurement of peak areas relative to an amino-BZ standard. The accumulation of rhodamine 123 and [^{3}H]vinblastine was determined by fluorescence spectrophotometry at 515 nm and liquid scintillation counting, respectively.

Plasma membrane preparation and photoaffinity labelling. Plasma membrane vesicles were prepared using a calcium precipitation procedure essentially as described by Lin *et al.* [27]. Briefly, CEM or CEM/VLB$^{1.0}$ cells were washed in phosphate buffer,

pH 7.4, containing 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4 and suspended in ice-cold hypotonic buffer containing protease inhibitors (50 mM mannitol, 5 mM HEPES, pH 7.4; plus 2 mM phenylmethylsulfonyl fluoride and 30 μM leupeptine). Cells were homogenized in a glass Dounce homogenizer, and the cell suspension was made up to 10 mM calcium chloride (final concentration). The calcium-induced membrane aggregates were removed by centrifugation at 3000 *g* in a Sorval centrifuge using an SS34 rotor. The slightly turbid supernatant containing the plasmalemma vesicles was collected by a 100,000 *g* centrifugation for 1 hr using an SW50 Beckman rotor. The resultant pellet was washed with 10 mM Tris–HCl, pH 7.4, and stored at −80° until needed. Protein concentration was measured by the method of Lowry *et al.* [28] using bovine serum albumin as standard. The preparation of [^{125}I]ASA-BZ (Fig. 1) is described elsewhere.* Briefly, amino-BZ was reacted with NHS-ASA in *N,N*-dimethylformamide in the dark for 48 hr. The product, ASA-BZ, was purified by HPLC, iodinated and purified to homogeneity before photoaffinity labelling. For photoaffinity labelling, aliquots (20 μg) of plasma membrane fractions from drug-sensitive (CEM) or resistant (CEM/VLB$^{1.0}$) cells were incubated with 50 nM [^{125}I]ASA-BZ (2×10^6 cpm) for 1 hr in the dark at room temperature. For drug inhibition, membrane fractions were preincubated with 1000-fold molar excess of amino-BZ, rhodamine 123, verapamil, vinblastine, or colchicine for 30 min before the addition of [^{125}I]ASA-BZ. At the end of the incubation period, the mixture was maintained on ice for 10 min followed by UV irradiation for 10 min at 254 nm (Stratagene UV Crosslinker, Stratagene, La Jolla, CA).

Immunoprecipitation of photoaffinity labelled proteins. Immunoprecipitation of [^{125}I]ASA-BZ photolabelled P-gp was carried out as previously described [29]. Briefly, [^{125}I]ASA-amino-BZ photolabelled plasma membranes (100 μg) from CEM or CEM/VLB$^{1.0}$ cells were incubated with 10 μg of C219 or C494 monoclonal antibody [30] or an irrelevant second antibody. Proteins bound to C219 mAb or IgG_{2a} were isolated using protein-A Sepharose beads (Pharmacia). The [^{125}I]ASA-BZ photoaffinity labelled proteins were eluted from protein-A Sepharose beads with 5% SDS and 10 mM dithiothreitol and resolved on SDS–PAGE.

Protease cleavage. Plasma membrane fractions from CEM/VLB$^{1.0}$ cells were suspended in 10 mM Tris–HCl, pH 7.4, 250 mM sucrose and photoaffinity labelled with 50 nM [^{125}I]ASA-BZ or 20 nM [^{125}I]-IAAP as described above. Following SDS–PAGE, [^{125}I]ASA-BZ or [^{125}I]IAAP photoaffinity labelled P-gp was digested with *Staphylococcus aureus* V8 protease (5–10 μg) in gel slices loaded in wells of a 15% acrylamide gel essentially as described by Cleveland *et al.* [31].

Polyacrylamide gel electrophoresis. Membrane protein fractions were resolved by SDS–PAGE using the Fairbanks gel system [32]. For Cleveland maps, samples were run on a 15% Laemmli gel system [33]. After electrophoresis, gel slabs were fixed in 40% methanol, 10% acetic acid and dried. Dried slabs were exposed to an XAR film at −80° overnight.

* Nare *et al.*, submitted for publication.

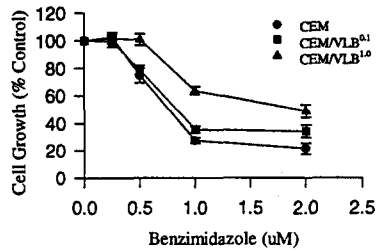

Fig. 2. Sensitivity of CEM and CEM/VLB cells to amino-BZ. Drug-sensitive (CEM) and resistant (CEM/VLB$^{0.1}$ or CEM/VLB$^{1.0}$) cells were plated and incubated for 24 hr before the addition of 0.25–2.0 μM amino-BZ. The viability of cells in the presence of BZ was determined by the MTT assay following a 96-hr exposure to amino-BZ. The survival values are expressed as a percent of control cells that were incubated in the absence of amino-BZ. Each point is the mean (±SD) of three independent experiments. Differences in EDC_{50} values for the above three cell lines were statistically significant to a P value equal to 0.000511.

RESULTS

BZs are potent anti-neoplastic agents, and nocodazole, a BZ carbamate, has been used the most in this respect [23]. However, the effects of this group of drugs on cancer cells with acquired multidrug resistance phenotype have not been examined. In this study, it was of interest to determine the sensitivity of CEM human lymphoma cells and their vinblastine-resistant derivatives (CEM/VLB$^{0.1}$ and CEM/VLB$^{1.0}$) to amino-BZ. Cells were incubated in the absence or the presence of amino-BZ (0.25 to 2.0 μM), and the viability of cells was assessed using an MTT assay. The results in Fig. 2 show the effect of amino-BZ on the viability of drug-sensitive and resistant cells. The EC_{50} values for BZ were estimated to be 0.78, 0.89 and 2 μM in CEM drug-sensitive, CEM/VLB$^{0.1}$ and CEM/VLB$^{1.0}$ drug-resistant cells, respectively (Fig. 2). These results demonstrate that human cell lines selected for resistance to vinblastine are also cross-resistant to BZ compounds, which are structurally dissimilar. Interestingly however, the relative cross-resistance of CEM/VLB cells toward amino-BZ was only 1.2 and 2.6-fold for CEM/VLB$^{0.1}$ and CEM/VLB$^{1.0}$, respectively. In contrast, these resistant cell lines are ~70-fold and ~800-fold resistant to vinblastine [24]. Similar differences in the cross-resistance profile of MDR cells toward different cytotoxic drugs have been observed previously and remain unclear [34]. The presence of 1–5 μM verapamil led to the reversal of BZ drug resistance in CEM/VLB$^{0.1}$ and CEM/VLB$^{1.0}$, while similar concentrations of verapamil alone had no effect on drug-sensitive or resistant cells (data not shown).

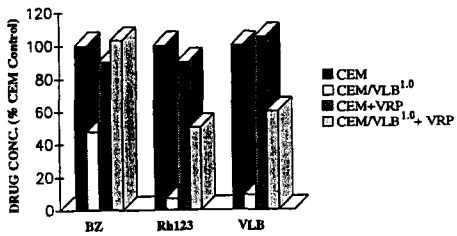

Fig. 3. Amino-BZ drug accumulation. Drug-sensitive (CEM) or resistant (CEM/VLB$^{1.0}$) cells were incubated for 1 hr in α-MEM in the presence of 1 μM amino-BZ, rhodamine 123 (Rh123), or [^{3}H]vinblastine ([^{3}H]VLB) in the absence or presence of 50 μM verapamil (VRP). The total drug accumulation of amino-BZ was determined by HPLC following precipitation of protein with methanol (see Materials and Methods). The accumulation of Rh123 and [^{3}H]VLB was determined by fluorescence spectrophotometry at 515 nm and liquid scintillation counting, respectively. The above drug accumulation values are expressed as percent of CEM control.

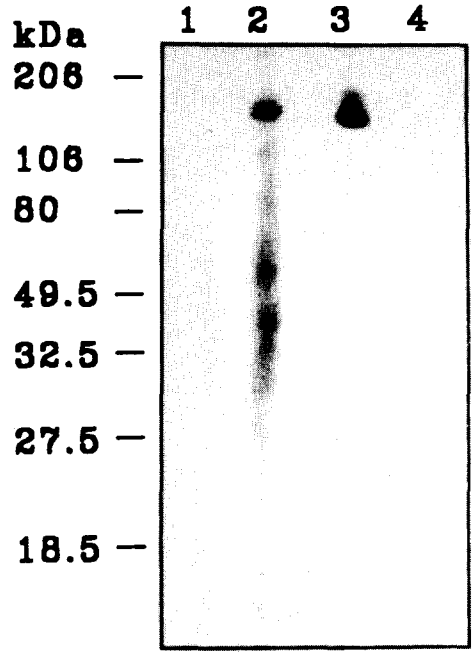

Fig. 4. Photoaffinity labelling of P-glycoprotein with [^{125}I]-ASA-BZ. Plasma membranes from drug-sensitive (CEM) or resistant (CEM/VLB$^{1.0}$) cells were photoaffinity labelled with 50 nM [^{125}I]ASA-BZ. Lanes 1 and 2 show photoaffinity labelling of plasma membranes from drug-sensitive and resistant cells, respectively. A 170 kDa was photoaffinity labelled with ASA-BZ in plasma membranes from drug-resistant cells. Lanes 3 and 4 show immunoprecipitation of [^{125}I]ASA-BZ photoaffinity labelled proteins from CEM/VLB$^{1.0}$ membranes with C219 mAb or an irrelevant IgG$_{2a}$, respectively. The positions of the molecular weight markers are indicated to the left of the figure.

Although the above results demonstrate that CEM/VLB MDR cells are cross-resistant to BZ, it was not clear if the observed cross-resistance is mediated by a P-gp drug efflux pump. For example, it was shown recently that mutations in the microtubule gene rather than over-expression of P-gp may be responsible for the expression of drug resistance in certain cell lines [35]. To examine whether BZ is a substrate for the ATP-dependent P-gp drug efflux mechanism, drug accumulation in CEM and CEM/VLB$^{1.0}$ cells was determined following a 1-hr incubation with 1 μM BZ at 37°. The results in Fig. 3 show that BZ accumulated in CEM drug-sensitive and to a much lesser extend in CEM/VLB$^{1.0}$ drug-resistant cells. To determine if the decrease in the accumulation of amino-BZ in drug-resistant versus drug-sensitive cells is mediated via the P-gp drug efflux pump, amino-BZ drug accumulation was measured in the presence of verapamil, a calcium channel blocker that is known to reverse MDR by competing for the drug transport by P-gp. The results in Fig. 3 show that verapamil caused a dramatic increase in the accumulation of BZ in drug-resistant cells, while the accumulation of amino-BZ in drug-sensitive cells was not affected significantly in the presence of verapamil. These results are interesting since similar drug accumulation studies using [^{3}H]vinblastine or rhodamine 123 demonstrated that drug-resistant cells accumulated significantly lower amounts of these drugs than amino-BZ (Fig. 3). In addition, the presence of verapamil did not reverse completely the accumulation of [^{3}H]vinblastine or rhodamine 123, as was seen for amino-BZ in drug-resistant cells. Taken together, these results suggest that the reduced accumulation of BZ in drug-resistant cells is likely to be mediated via the P-gp drug efflux pump. However, in agreement with the above results from the MTT cytotoxicity assay, amino-BZ is a poor substrate for the P-gp drug efflux pump.

The observed low levels of BZ in MDR cells suggested that BZ may be a substrate for the P-gp drug efflux pump. To further characterize the interaction of amino-BZ with P-gp from human multidrug-resistant cells, a photoactive derivative of BZ ([^{125}I]ASA-BZ) (Fig. 1) was synthesized and used in a photoaffinity labelling assay (see Materials and Methods). For photoaffinity labelling, plasma membrane preparations from drug-sensitive or resistant cells were incubated with [^{125}I]ASA-BZ followed by UV irradiation. Figure 4 (lane 2) shows a 170 kDa protein as the major photoaffinity labelled product in plasma membrane preparations from the CEM/VLB$^{1.0}$ cell line. In contrast, no 170 kDa photoaffinity labelled protein was seen in plasma membranes from CEM drug-sensitive cells (Fig. 4, lane 1). The estimated molecular mass of the photoaffinity labelled protein found in drug-resistant but not drug-sensitive plasma membranes is consistent with that of P-gp. However, the identity of the 170 kDa photoaffinity labelled protein was confirmed by immunoprecipitation using the monoclonal antibody C219 and [^{125}I]ASA-BZ photolabelled proteins from CEM/VLB$^{1.0}$ derived plasma membranes (Fig. 4). The results in lane 3 of Fig. 4 show that a 170 kDa polypeptide specifically

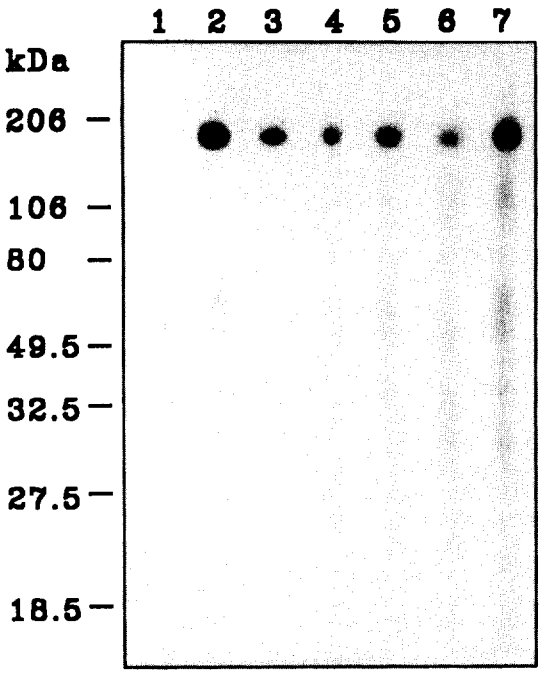

Fig. 5. Effects of amino-BZ, rhodamine 123, vinblastine, verapamil or colchicine on the photoaffinity labelling of P-gp with [^{125}I]ASA-BZ. Plasma membranes from CEM/VLB$^{1.0}$ cells were photoaffinity labelled with 50 nM [^{125}I]-ASA-BZ in the absence (lane 2) and in the presence of 1000-fold molar excess of amino-BZ (lane 3), rhodamine 123 (lane 4), verapamil (lane 5), vinblastine (lane 6) or colchicine (lane 7). Lane 1 shows photoaffinity labelling of plasma membranes from drug-sensitive cells with 50 nM [^{125}I]ASA-BZ.

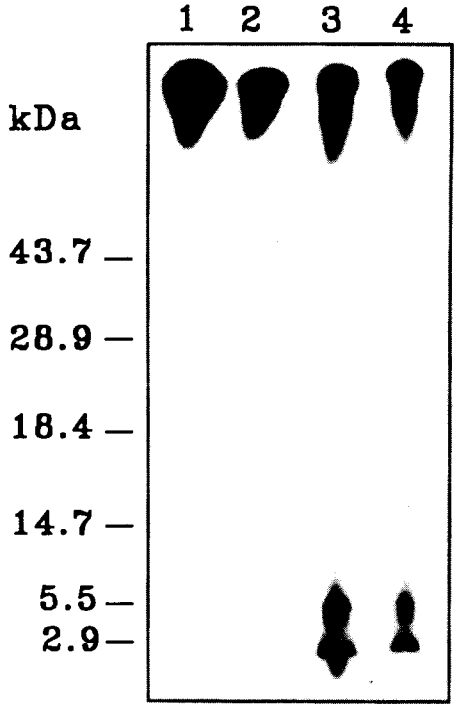

Fig. 6. Cleveland maps of [^{125}I]ASA-BZ or [^{125}I]IAPP photoaffinity labelled P-gp. Plasma membranes from CEM/VLB$^{1.0}$ cell were photoaffinity labelled with [^{125}I]IAPP or [^{125}I]ASA-BZ and exhaustively digested in gel slices with V8 protease. Lanes 1 and 2 show undigested photoaffinity labelled P-gp, while lanes 3 and 4 show V8 digested [^{125}I]IAPP or [^{125}I]ASA-BZ photoaffinity labelled P-gp, respectively. A 6 kDa peptide in P-gp was photoaffinity labelled with [^{125}I]IAPP or [^{125}I]ASA-BZ.

immunoprecipitated with C219 mAb, while an irrelevant IgG_{2a} antibody did not immunoprecipitate a similar 170 kDa protein (lane 4). Identical results were obtained when a class I specific monoclonal antibody (C494) was used in the above immunoprecipitation experiments (data not shown).

To demonstrate that the binding of BZ to P-gp occurs specifically, photoaffinity labelling of P-gp with [^{125}I]ASA-BZ was examined in the presence of excess molar concentrations of certain MDR-associated drugs previously shown to interact specifically with P-gp [16–18]. The results in Fig. 5 show the photoaffinity labelling of plasma membrane fractions from CEM/VLB$^{1.0}$ drug-resistant cells incubated with [^{125}I]ASA-BZ in the absence (lane 2) and in the presence of non-radioactive amino-BZ, various MDR-associated drugs (vinblastine, rhodamine 123 or colchicine) or the chemosensitizing agent verapamil prior to UV irradiation (lanes 3–7). The photoaffinity labelling of P-gp by [^{125}I]ASA-BZ was decreased in the presence of 1000-fold molar excess of amino-BZ, rhodamine 123, verapamil and vinblastine (lanes 3–6, respectively). In contrast, colchicine, a compound to which the CEM/VLB$^{1.0}$ cells are cross-resistant, did not reduce the photoaffinity labelling of P-gp (lane 7). These results demonstrate that amino-BZs bind to P-gp in MDR cells in a specific manner. Moreover, given that BZ photoaffinity labelling of P-gp was inhibited in the presence of excess MDR-associated drugs or reversing agents suggests a common binding domain(s) in P-gp.

The amino acid sequences that encode the P-gp drug binding domain(s) remain a matter of speculation. However, protease digestion studies of photoaffinity labelled P-gp have identified a 6 kDa peptide cross-linked by many photoactive drug analogues [36]. For example, when plasma membranes from MDR cells are labelled with IAAP, an α_1-adrenergic receptor probe, and subjected to protease cleavage, a 6 kDa cross-linked peptide is obtained [37]. A peptide of similar size was also obtained following proteolysis of P-gp photolabelled with azidopine [38] or rhodamine 123 [39]. To determine whether [^{125}I]ASA-BZ interacts with a domain(s) similar to those identified previously [37–39], P-gp photolabelled with [^{125}I]ASA-BZ or IAAP was incubated with *S. aureus* V8 protease, and the digested products were resolved by SDS–PAGE. The results in Fig. 6 show a Cleveland map of [^{125}I]-ASA-BZ or IAAP photoaffinity labelled P-gp. Both drugs appear to interact with the same 6 kDa peptide (lanes 3 and 4 of Fig. 6). Taken together, these results suggest that BZ interacts specifically with P-

gp, and its binding site(s) is likely to be similar to those identified using other MDR-associated drugs.

DISCUSSION

The results of this study demonstrate that human MDR cells selected for resistance against vinblastine are cross-resistant to BZs potent anti-mitotic drugs. Our results suggest that the observed cross-resistance is likely to be mediated by enhanced BZ drug efflux. The above conclusion is consistent with the observed reduction of BZ drug accumulation in CEM/VLB$^{1.0}$ drug-resistant cells compared with that in CEM drug-sensitive cells. Moreover, the capacity of verapamil to potentiate the accumulation of BZ in drug-resistant cells is consistent with a P-gp mediated drug efflux of BZ. Interestingly, the presence of verapamil caused a complete and consistent increase in BZ accumulation in drug-resistant cells to the same level as that observed in drug-sensitive cells. These results are in contrast with the effect of verapamil on the accumulation of rhodamine 123 or vinblastine in CEM/VLB$^{1.0}$ MDR cells [39], which was partial. Thus, BZ may be a poor substrate for the P-gp efflux pump compared with verapamil, vinblastine, or rhodamine 123. The latter suggestion is further supported by the results of the MTT cytotoxicity assay whereby the CEM/VLB$^{1.0}$ cells were only 2.5-fold more resistant to BZ (compared with ~800-fold resistance to the selecting agent vinblastine) than the parental CEM drug-sensitive cells. Similar levels of cross-resistance were obtained using other analogues of BZ (e.g. mebendazole or oxfendazole; data not shown).

To further verify the role of P-gp in mediating BZ resistance, we have demonstrated a direct and specific binding between P-gp and a photoactive derivative of BZ, [^{125}I]ASA-BZ. Photoaffinity labelling of P-gp with [^{125}I]ASA-BZ was preferentially inhibited by vinblastine > rhodamine 123 > amino-BZ > verapamil, suggesting that BZ interact with P-gp domains that are important in the interaction of P-gp with other cytotoxic and non-cytotoxic lipophilic agents. This further indicates that BZ binds specifically to P-gp. The inability of colchicine to inhibit the photoaffinity labelling of P-gp by [^{125}I]ASA-BZ is in agreement with previous observations in which this compound failed to inhibit the photolabelling of P-gp with derivatives of verapamil [16], vinblastine [40], cyclosporin A [18], and rhodamine 123 [39].

The amino acid sequence(s) of the P-gp drug binding site(s) remains largely undefined. Recent studies [41–43], using site-directed mutagenesis, have demonstrated that a single amino acid substitution in transmembrane 6, 11 or 12 modulates P-gp activity and substrate specificity. Moreover, proteolytic cleavage of P-gp photoaffinity labelled with photoactive drug analogues of MDR-associated drugs has yielded a 6 kDa photolabelled peptide thought to contain transmembranes 11 and 12 of P-gp [37]. In this study, we have demonstrated that proteolytic cleavage of [^{125}I]ASA-BZ photoaffinity labelled P-gp also results in a 6 kDa photoaffinity labelled peptide. Thus, although it remains to be determined if the 6 kDa photoaffinity labelled peptide encodes the P-gp drug binding site(s), these results suggest that BZ shares the same P-gp binding domain(s) with other MDR-associated drugs. However, the poor transport of BZ by the P-gp efflux pump suggests the presence of certain amino acid residues in the P-gp drug-binding domain(s), which are incompatible with the overall chemical structure of BZ. Thus, based on the demonstrated effect of a single amino acid substitutions on P-gp substrate specificity [41–43], it may be speculated that a subtle modification of the P-gp drug-binding domain could result in a dramatic change in BZ efflux. Hence, it would be interesting to determine if selection of CEM/VLB$^{1.0}$ cells for higher levels of BZ drug resistance could force similar point mutations in P-gp transmembrane domains or other domains in its sequence (e.g. Val1185 to Gly mutation putative intracellular sequence between transmembranes 2 and 3 [44]).

P-gp homologues have been identified in a number of drug-resistant parasites, which include chloroquine-resistant malaria (*P. falciparum* [12]), emetine-resistant *E. histolytica* [13], and vinblastine-resistant *L. donovani* [14]. Interestingly, all of the above drugs are substrates for the P-gp drug efflux pump [45]. The present demonstration that BZ is a substrate for the P-gp drug efflux pump is of considerable interest, since BZs are widely used helmintics in animals and humans and drug resistance to BZs has been observed [23]. Thus, although current data on BZ drug resistance in parasites and fungi have indicated that point mutations in tubulin are primarily responsible for BZ drug resistance [46, 47], the possibility that a drug efflux mechanism mediated via a P-gp homologue has not been examined yet and may be important.

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