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Reversal of P-Glycoprotein-Associated Multidrug Resistance by Ivermectin

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ABSTRACT. P-Glycoprotein (P-gp) causes a multidrug resistance (MDR) phenotype in tumour cells. In some cancers, the expression of P-gp has been correlated with low clinical response to chemotherapy and survival of patients. Previous studies have shown that certain lipophilic drugs bind to P-gp and reverse the MDR phenotype of tumour cells. In this study, we extend that list of compounds and present evidence for the capacity of a potent and clinically safe anthelmintic, ivermectin (IVM), as an MDR-reversing drug. Using a highly drug-resistant human cell line, we compared IVM with other MDR-reversing agents and showed that IVM is 4- and 9-fold more potent than cyclosporin A and verapamil, respectively. The capacity of IVM to inhibit iodoaryl-azidoprazosin photolabeling of P-gp is consistent with direct binding to P-gp. Studies showed that [^{3}H]IVM binding to membranes from resistant cells is specific and saturable with K_D and B_{max} values of 10.6 nM and 19.8 pmol/mg, respectively. However, while cyclosporin A or vinblastine inhibited [^{3}H]IVM binding to membranes from drug-resistant but not drug-sensitive cells, neither verapamil nor colchicine had any effect. Furthermore, both IVM and cyclosporin A and, to a lesser extent, verapamil also inhibited [^{3}H]vinblastine binding to membranes from drug-resistant cells. Drug transport studies showed that [^{3}H]IVM is a substrate for the P-gp drug efflux pump. However, it was transported less efficiently by P-gp than [^{3}H]vinblastine. Moreover, only cyclosporin A was effective in potentiating the accumulation of [^{3}H]IVM in drug-resistant cells. Taken together, the high efficiency of MDR reversal by IVM combined with its low toxicity are consistent with the properties of an ideal MDR-reversing agent. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:17–25, 1997.

KEY WORDS. ivermectin; P-glycoprotein; multidrug resistance

Selection of tumour cells with cytostatic hydrophobic drugs has been shown to result in the development of an MDR† phenotype and in the overexpression of P-gp [1, 2]. P-gp is a member of the ABC (ATP binding cassette) superfamily of membrane transporters that includes the multidrug-resistance-associated protein (MRP) [3], the cystic fibrosis transmembrane conductance regulator (CFTR) [4], and several bacterial periplasmic membrane proteins [5]. Although P-gp has been shown to cause MDR in tumour cells, its function in normal tissues is less certain. The P-gp gene family in rodents and humans consists of three (I, II, and III) and two (I and III) classes, respectively. Moreover, while classes I and II have been shown to cause MDR, class III of both rodents and humans does not. Using homologous recombination, it was shown that class I P-gp is involved in drug transport in normal tissues while class III P-gp mediates phosphatidylcholine transport [6] and may be "a flipase" [7].

Various levels of P-gp expression have been shown in tumours from different cancers [8]. However, more studies are needed to determine if changes in P-gp levels in tumour cells are prognostic of MDR. Recently, P-gp expression in sarcomas of children and in neuroblastomas was shown to correlate with low response to chemotherapy and the long-term survival of patients [9, 10]. Moreover, other studies using MDR-reversing drugs have implicated P-gp-associated MDR in some cancers [11–13]. Nevertheless, a clear clinical benefit of MDR-reversing drugs remains to be demonstrated. Earlier attempts to use verapamil as an MDR-reversing drug have been hampered by its high cardiotoxicity [14, 15]. The results obtained with cyclosporin A and the non-immunosuppressive analog SDZ-PSC 833 have been more encouraging, but some toxic effects were also observed when cyclosporin A was used in clinical studies [16, 17]. Hence, the identification of MDR-reversing drugs with low toxicity is a major concern for the clinical treatment of MDR tumours.

Avermectins are naturally occurring macrocyclic lactones produced by the soil microorganism *Streptomyces avermitilis*. IVM (Fig. 1) is a semisynthetic avermectin analogue

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† *Abbreviations:* MDR, multidrug resistance (resistant); IVM, ivermectin; IAAP, iodoaryl-azidoprazosin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; P-gp, P-glycoprotein; α-MEM, α-Minimal Eagle's Medium; VLB, vinblastine.

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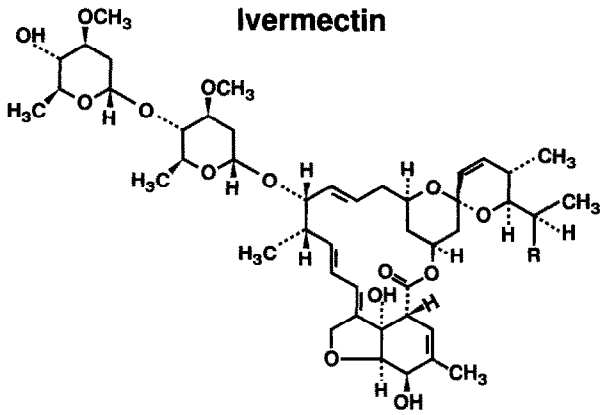

FIG. 1. Chemical structure of IVM. IVM was obtained by the selective hydrogenation of avermectin B_1. This compound contains at least 80% of 22, 23-dihydroavermectin B_{1a} (R = CH_2CH_3) and no more than 20% of 22,23-dihydroavermectin B_{1b} (R = CH_3).

shown to be an anthelmintic of unprecedented efficiency and low toxicity [18]. IVM has been successfully used orally, by subcutaneous injection, or by transdermal uptake to cure nematode infections in animals and has also been used in humans to treat several types of infections, such as onchocerciaisis (river blindness) [18]. Although the molecular mechanism of the antiparasitic effects of IVM is not understood completely, it is thought that IVM binds with high affinity to a glutamate-gated chloride channel in nematodes [19]. Indeed, IVM is highly selective for the invertebrate chloride channel but binds with only low affinity to the γ-aminobutyric acid-gated chloride channel in vertebrate brain [20, 21]. The binding of IVM to the invertebrate glutamate-gated chloride channel, which is essentially irreversible, keeps the chloride channel open and prevents membrane depolarization, leading to the paralysis of the nematode. The low host toxicity of IVM is due to both the low affinity towards the host receptor and the compartmentalization of the receptor in the brain. IVM, which is very hydrophobic, does not effectively cross the blood–brain barrier at low concentrations [22]. In a recent study using transgenic mice that had their P-gp I function disrupted by homologous recombination, IVM accumulation in the brain and in several other organs was increased dramatically [23]. This led the authors of that study to postulate that the P-gp in normal tissues mediates IVM transport. However, no direct biochemical evidence was shown to support their conclusion. The results of our study show that IVM is a substrate for the P-gp drug efflux pump in human MDR cells. Moreover, we show that IVM interacts with P-gp and is a potent MDR-reversing agent.

MATERIALS AND METHODS

Materials

IVM and [^{3}H]IVM were supplied by the American Cyanamid Co. (Princeton, NJ, U.S.A.). Cyclosporin A and its non-immunosuppressive analogue, SDZ-PSC 833, were a gift from Sandoz Inc. (East Hanover, NJ, U.S.A.). Vinblastine was from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), while verapamil and colchicine were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{125}]IAAP (2200 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Drug-sensitive human lymphoma cells (CEM) [24] were a gift from Dr. W. Beck at St. Jude Children's Research Hospital (Memphis, TN, U.S.A.). The CEM/VLB$^{1.0}$ line was established from the CEM/VLB$^{0.1}$ line and was obtained from Dr. V. Ling at the B.C. Cancer Research Centre (Vancouver, B.C.). All other chemicals used were of the highest grade available.

Tissue Culture and Plasma Membrane Preparation

CEM and CEM/VLB$^{1.0}$ cells were grown in α-MEM as previously described [24]. The CEM/VLB$^{1.0}$ cells are resistant to 1 μg/mL vinblastine and express high levels of P-gp compared with the sensitive cells [25]. Plasma membranes were prepared using a calcium precipitation procedure essentially as described by Lin *et al.* [26]. Briefly, CEM and CEM/VLB$^{1.0}$ cells were washed three times in ice-cold PBS and resuspended in a hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl, pH 7.4) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 30 μM leupeptin and pepstatin). Cells were homogenized using a Dounce glass homogenizer, and the cell lysate was centrifuged at low speed (3000 *g*) to remove unbroken cells and nuclei. The resultant supernatant was made up to 10 mM $CaCl_2$ final concentration and mixed on ice. The calcium-induced membrane aggregates were precipitated by high speed centrifugation at 100,000 *g* for 1 hr at 4° using a Beckman SW50 rotor. The enriched plasma membrane pellet was washed with 10 mM Tris–HCl, pH 7.4, and 250 mM sucrose and stored at −80° until needed. The protein concentration was measured by the method of Bradford [27].

MTT Cytotoxicity Assay

Cells, cultured without drug for at least 1 week, were harvested in the exponential growth phase, and 100-μL aliquots 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 VLB plus/minus MDR-reversing agents. The cells were then cultured for 4 days, and MTT dye was added to a final concentration of 0.5 mg/mL. The plates were incubated for 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 repetitively pipetted. The 96-well plates were heated in the microwave oven for 1 min at the minimal power setting, and 10 μL of ethanol was added to disperse the bubbles formed during pipetting. Plates were read at 570 nm using an ELISA micro titer plate reader.

Photoaffinity labeling and SDS–PAGE

CEM and CEM/VLB$^{1.0}$ cells (1×10^6) were washed three times in α-MEM and incubated in the dark for 30 min at

25° in the presence of 20 nM [^{125}I]IAAP with or without 1-, 10-, and 100-fold molar excess of IVM, SDZ-PSC 833, cyclosporin A, VLB, or verapamil. The cells were set on ice for 10 min and irradiated with a 254 nm UV source (Stratagene UV crosslinker, Stratagene, La Jolla, CA, U.S.A.). Cells were centrifuged at 500 *g* for 5 min, the supernatant was removed, and the pellet was lysed in Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ and 1% NP-40. The proteins were resolved by SDS–PAGE using the Fairbanks buffer system [28]. After electrophoresis, the gels were fixed in 40% methanol and 10% acetic acid for at least 1 hr. The gels were dried and exposed to an XAR Kodak film for 16 hr at −80°.

Drug Binding Assays

Plasma membranes from CEM and CEM/VLB$^{1.0}$ cells (20 μg) were preincubated in 10 mM Tris, pH 7.4, containing 250 mM sucrose (TS) for 30 min at 37° in the presence of a 300-fold molar excess of unlabelled drugs (IVM, cyclosporin A, VLB, verapamil, or colchicine). The cells were then incubated for 30 min at 37° in the presence of 20 nM [^{3}H]IVM or [^{3}H]VLB. The incubation was stopped with the addition of 1 mL of ice-cold TS. The membranes were washed twice with the same volume of TS, and the membrane pellet was resuspended in 1 M NaOH and neutralized with the same volume of 1 M HCl 4 hr later. Drug binding was evaluated by liquid scintillation counting.

Drug Transport

For drug accumulation, CEM and CEM/VLB$^{1.0}$ cells (1 × 10^6) were washed three times in PBS containing 1 mg/mL glucose and preincubated for 30 min at 37° in the presence of 300-fold molar excess of unlabelled drugs (IVM, cyclosporin A, VLB, verapamil, or colchicine). Cells were then incubated for 30 min at 37° in the presence of 0.2 μM [^{3}H]IVM or [^{3}H]VLB in a final volume of 100 μL. The incubation was stopped with the addition of 1 mL of ice-cold PBS containing 60 μM unlabelled IVM or VLB. Cells were then washed twice with the same stop solution and lysed in 100 μL of 1 M NaOH followed by neutralization with the same volume of 1 M HCl, 4 hr later.

For drug efflux, cells were preincubated for 30 min at 37° in the presence of 2 μM [^{3}H]IVM or [^{3}H]VLB and 10 mM sodium azide to inhibit drug efflux. Cells were washed and resuspended in PBS solution containing 1 mg/mL of glucose at 37°. Samples were removed following 0- to 30-min incubations. Cells were washed in 10 vol. of ice-cold PBS containing a 2 μM concentration of unlabeled drug. The final cell pellet was resuspended in 1 M NaOH followed by neutralization with the same volume of 1 M HCl, 4 hr later. The accumulation of labelled drugs was measured by liquid scintillation counting.

Octanol Fractionation

Drugs were solubilized in octanol, mixed with an equal volume of PBS, and strongly vortexed. After a 30-min agitation, the mixture was centrifuged at 1000 *g* for 5 min. The upper (octanol) phase was separated from the lower (PBS) phase using a Pasteur pipette. The amount of drug in both phases was determined by measuring the UV absorbance. In some cases, after partitioning, the drugs were separated by HPLC to increase the sensitivity of the detection.

RESULTS

IVM Transport in MDR Cells

IVM is presently the most potent anthelmintic and is nontoxic to humans and animals [18]. The recent demonstration that the disruption of P-gp class I gene in mice by homologous recombination resulted in IVM accumulation in the brain and other P-gp-positive tissues [23] led us to examine the possibility of whether P-gp directly transports IVM. Figure 2 shows the accumulation and efflux of [^{3}H]IVM or [^{3}H]VLB in drug-sensitive (CEM) and -resistant (CEM/VLB$^{1.0}$) cells. [^{3}H]IVM initial uptake and

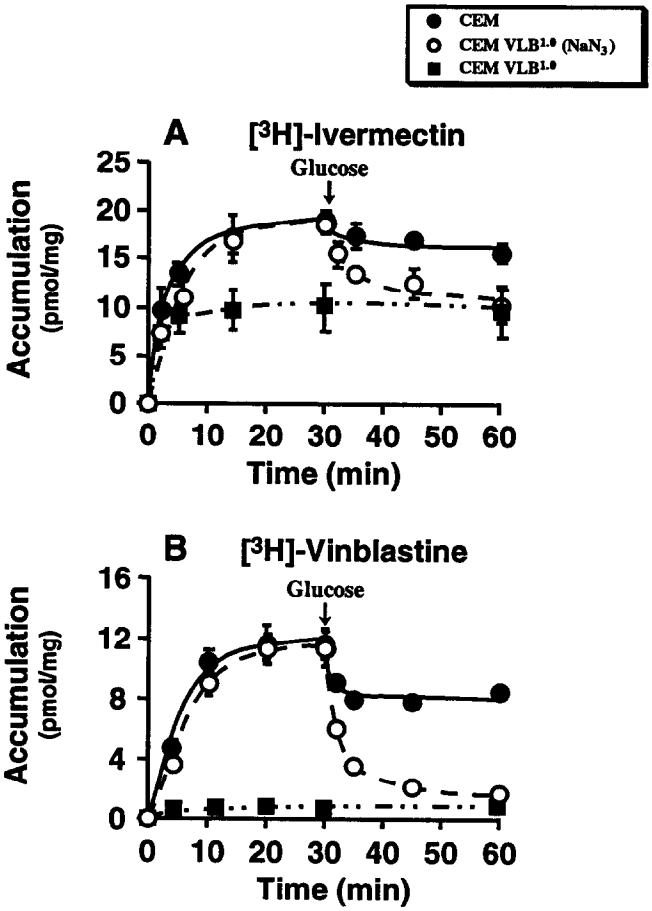

FIG. 2. IVM and VLB accumulation and efflux by cancer cells. Drug-sensitive (CEM) and -resistant (CEM/VLB$^{1.0}$) cells were incubated at 37° in α-MEM for 0–60 min in the presence of 200 nM [^{3}H]IVM (A) or [^{3}H]VLB (B) alone or in the presence of 10 mM sodium azide. The cells were then washed, and the efflux was carried out at 37° in α-MEM for times varying from 0 to 30 min. The accumulation values represent means ± SD of three experiments.

steady-state accumulation were lower in CEM/VLB$^{1.0}$ than in CEM cells (Fig. 2A). Similar results were obtained with [^{3}H]VLB, a known substrate of P-gp (Fig. 2B). The accumulation of [^{3}H]IVM in resistant cells, was much higher than the accumulation of [^{3}H]VLB. Moreover, the results in Fig. 2 show that the accumulation of [^{3}H]VLB and [^{3}H]IVM were energy dependent since the addition of metabolic inhibitor (10 mM sodium azide) to CEM/VLB$^{1.0}$ cells increased the accumulation of both drugs to the same level as that of CEM drug-sensitive cells. The results in Fig. 2 also show the efflux of [^{3}H]IVM and [^{3}H]VLB from CEM and CEM/VLB$^{1.0}$ cells. The efflux of [^{3}H]IVM from CEM/VLB$^{1.0}$ cells was much slower than that of [^{3}H]VLB. Indeed, 40% of [^{3}H]IVM remained in drug-resistant cells following a 30-min incubation. Taken together, these studies show that IVM is transported from drug-resistant cells in an energy-dependent manner.

To determine if the accumulation of IVM in CEM/VLB cells is modulated by P-gp-associated drugs, [^{3}H]IVM levels were measured in CEM and CEM/VLB$^{1.0}$ cells in the absence and in the presence of a 300-fold molar excess of IVM, VLB, colchicine, verapamil, or cyclosporin A (Fig. 3A). As shown earlier, Fig. 2A revealed higher levels of [^{3}H]IVM accumulation in sensitive cells than in resistant cells. However, the presence of unlabelled IVM, cyclosporin A, or verapamil increased the accumulation of [^{3}H]IVM to the same extent as that found in CEM cells, while VLB and colchicine were without effect (Fig. 3A). Similar results were also seen when [^{3}H]VLB accumulation was measured in the presence of the above drugs. Both cyclosporin A and IVM at 300-fold excess completely restored the accumulation of [^{3}H]VLB in CEM/VLB$^{1.0}$ cells (Fig. 3B). Interestingly, the presence of excess IVM resulted in a consistent decrease in [^{3}H]VLB accumulation in sensitive cells (Fig. 3B). This decrease in [^{3}H]VLB accumulation in CEM cells in the presence of IVM was not due to cell death since IVM was not toxic to cells for the duration of the experiment as determined by trypan blue staining (data not shown).

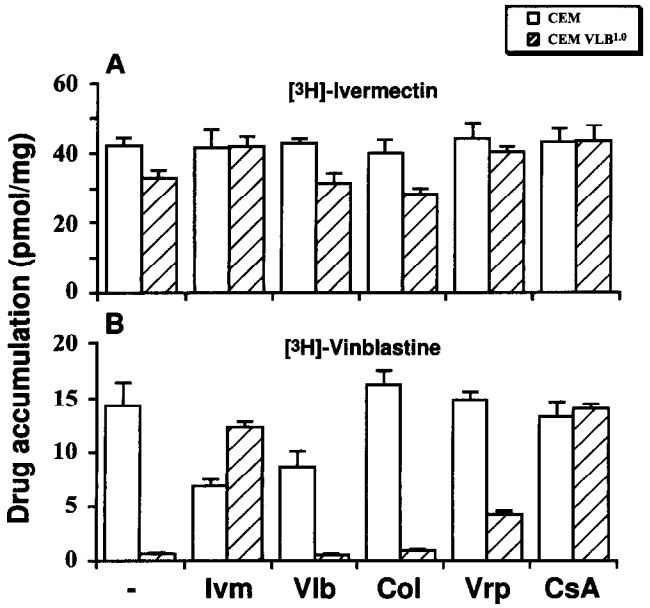

FIG. 3. Competitive inhibition of IVM and VLB uptake. Drug-sensitive (CEM) and -resistant (CEM/VLB$^{1.0}$) cells were incubated with 200 nM [^{3}H]IVM (A) or [^{3}H]VLB (B) in α-MEM for 30 min at 37° in the absence or presence of a 300-fold molar excess of ivermectin (Ivm), vinblastine (Vlb), colchicine (Col), verapamil (Vrp), or cyclosporin A (CsA). The accumulation values represent means ± SD of three experiments.

IVM Binding to Membranes from MDR Cells

To determine if IVM interacts specifically and saturably with membranes from drug-resistant cells, the binding of [^{3}H]IVM was measured over a large range of concentrations. The results in Fig. 4 show that IVM binding to membranes from CEM/VLB$^{1.0}$ is specific and saturable. The Scatchard transformation gave a single regression curve that yielded an apparent K_D of 10.6 nM and a B_{max} value of 19.87 pmol/mg, respectively. To investigate further the nature of IVM interactions with MDR cells, [^{3}H]IVM binding to membranes from CEM or CEM/VLB$^{1.0}$ in the absence and in the presence of drugs was measured and compared with that of [^{3}H]VLB. Figure 5 shows that [^{3}H]IVM bound to a greater extent to membranes from CEM/VLB$^{1.0}$ than to CEM cell membranes. The presence of excess IVM and cyclosporin A completely inhibited [^{3}H]IVM binding to CEM/VLB$^{1.0}$ membranes but had no effect on its binding to CEM membranes (Fig. 5A). Similarly, molar excess of VLB also inhibited [^{3}H]IVM binding to CEM/VLB$^{1.0}$ membranes but to a lesser extent than unlabelled IVM or cyclosporin A. Interestingly, verapamil or colchicine had no effect on [^{3}H]IVM binding. The results of [^{3}H]VLB binding to membranes from CEM and CEM/VLB$^{1.0}$ in the absence

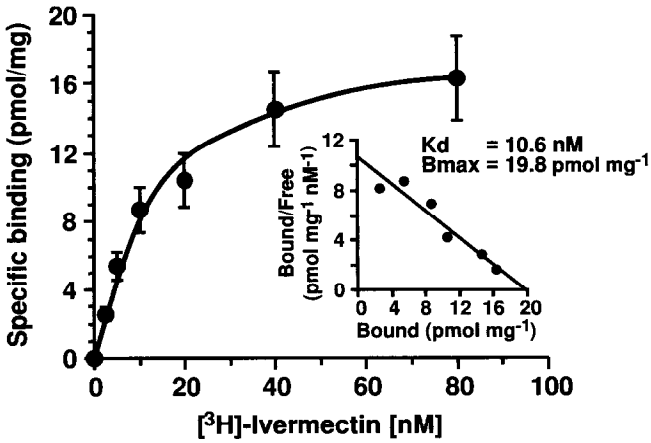

FIG. 4. Saturation binding of IVM to resistant cell plasma membranes. Plasma membranes (20 μg) were incubated with increasing concentrations (0–80 nM) of [^{3}H]IVM. The nonspecific binding was half of total binding. A Scatchard plot was used to calculate the dissociation constant (K_D) and maximal binding value (B_{max}). Each value is the mean ± SEM from three experiments.

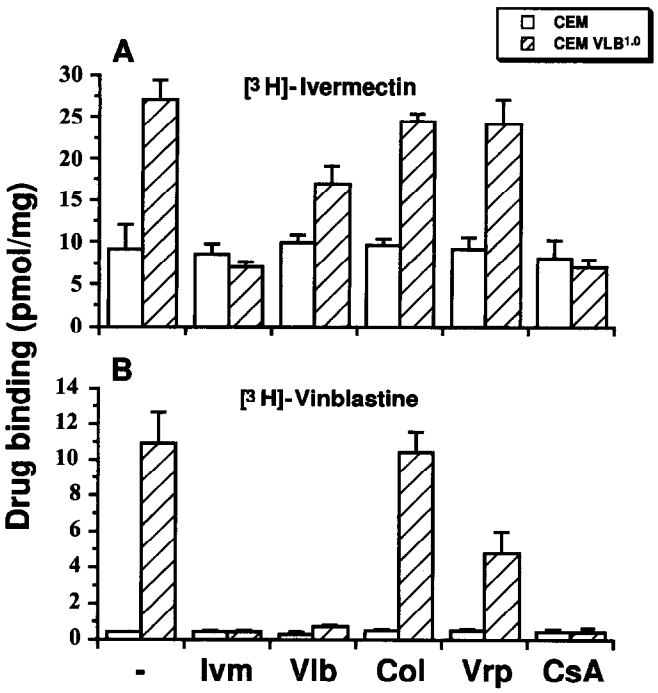

FIG. 5. Competitive inhibition of IVM and VLB binding to membranes from drug-sensitive and -resistant cells. Plasma membranes (20 μg) from CEM or CEM/VLB$^{1.0}$ cells were incubated with 20 nM [^{3}H]IVM (A) or [^{3}H]VLB (B) in the absence or presence of a 300-fold molar excess of ivermectin (Ivm), vinblastine (Vlb), colchicine (Col), verapamil (Vrp), or cyclosporin A (CsA). The values for drug binding to membrane fractions are given as means ± SD of three experiments.

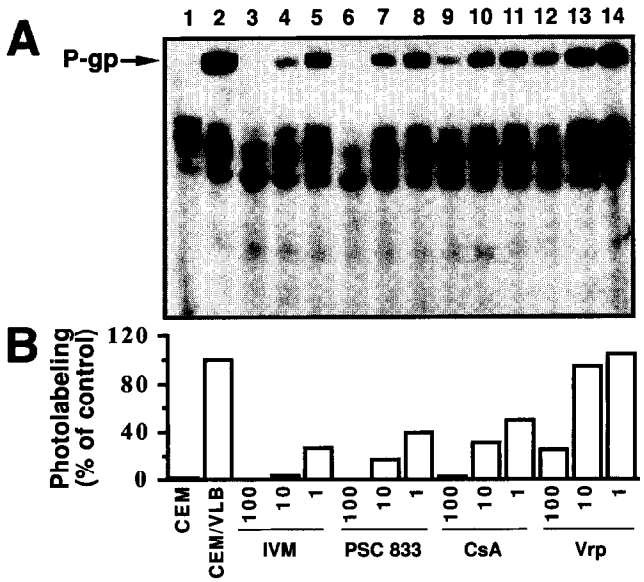

FIG. 6. Competitive inhibition of IAAP photoaffinity labelling. (A) Drug-sensitive (CEM) and -resistant (CEM/VLB$^{1.0}$) cells were photoaffinity labelled with 20 nM [^{125}I]IAAP. Drug-sensitive (lane 1) and -resistant (lanes 2–14) cells were incubated in the absence (lanes 1 and 2) or the presence of a 1-fold (lanes 5, 8, 11, and 14), 10-fold (lanes 4, 7, 10, and 13), or 100-fold (lanes 3, 6, 9, and 12) molar excess of IVM (lanes 3–5), SDZ-PSC 833 (lanes 6–8), cyclosporin A (lanes 9–11), or verapamil (lanes 12–14). (B) Percent inhibition of IAAP P-gp photolabelling from Fig. 6A as determined from densitometric scanning of the P-gp photolabelled band. The signal in lane 2 (photolabelled P-gp in CEM/VLB$^{1.0}$ cells in the absence of drugs) was taken as control or 100%.

and in the presence of drugs were similar to those obtained with [^{3}H]IVM but with some differences. For example, although [^{3}H]VLB bound more to CEM/VLB$^{1.0}$ than to CEM membranes, much less binding was seen with membranes from CEM cells (Fig. 5B). Moreover, verapamil but not colchicine also inhibited [^{3}H]VLB binding to CEM/VLB$^{1.0}$ membranes (Fig. 5B). Taken together, these results show higher levels of [^{3}H]IVM binding to membranes from resistant cells and that this binding was inhibited by drugs that interact directly with P-gp.

Inhibition of P-gp Photoaffinity Labelling

Several drugs have now been modified with a photoactive moiety and have been shown to interact directly with P-gp. The α_1-adrenergic receptor ligand IAAP was shown previously to specifically photolabel P-gp [29]. Moreover, it also has been suggested that drugs that inhibit photoaffinity labelling of P-gp by IAAP are likely to interact directly with P-gp and compete for its drug binding site(s) [30]. Thus, to determine if IVM interacts directly with P-gp, membranes from CEM or CEM/VLB$^{1.0}$ cells were photolabelled with 20 nM IAAP in the presence of 1-, 10-, and 100-fold molar excess of IVM and other P-gp-associated drugs (Fig. 6). The results in Fig. 6A show a 170 kDa photolabelled protein in membranes from resistant- but not from drug-sensitive cells (lanes 2 and 1, respectively). The presence of 100-fold excess of IVM and SDZ-PSC 833 completely inhibited the photolabelling of P-gp with IAAP (lanes 3 and 6). A similar molar excess of cyclosporin A or verapamil was less effective (lanes 9 and 12). Interestingly, IVM at 10-fold molar excess was more effective than SDZ-PSC 833 in inhibiting the photolabelling of P-gp with IAAP (lanes 4 and 7).

Reversal of MDR with IVM

Given the above results and the low host toxicity of IVM, it was of interest to determine if IVM potentiates the toxicity of VLB or doxorubicin in highly drug-resistant human lymphoma cells. Therefore, CEM or CEM/VLB$^{0.1}$ cells were incubated with increasing concentrations of VLB or doxorubicin in the absence or in the presence of IVM, SDZ-PSC 833, cyclosporin A, or verapamil (0, 0.1, 0.5, or 2 μM). The potentiation of drug toxicity by IVM and other MDR-reversing agents was determined by the MTT cytotoxicity assay (Fig. 7). The IC_{50} values for VLB or doxorubicin for CEM and CEM/VLB$^{0.1}$ were 3.5 and 500 ng/mL or 25 and 1100 ng/mL, respectively. The presence of IVM, cyclosporin A, and verapamil alone had no significant effect on the viability of CEM or CEM/VLB$^{0.1}$ cells (Fig. 7).

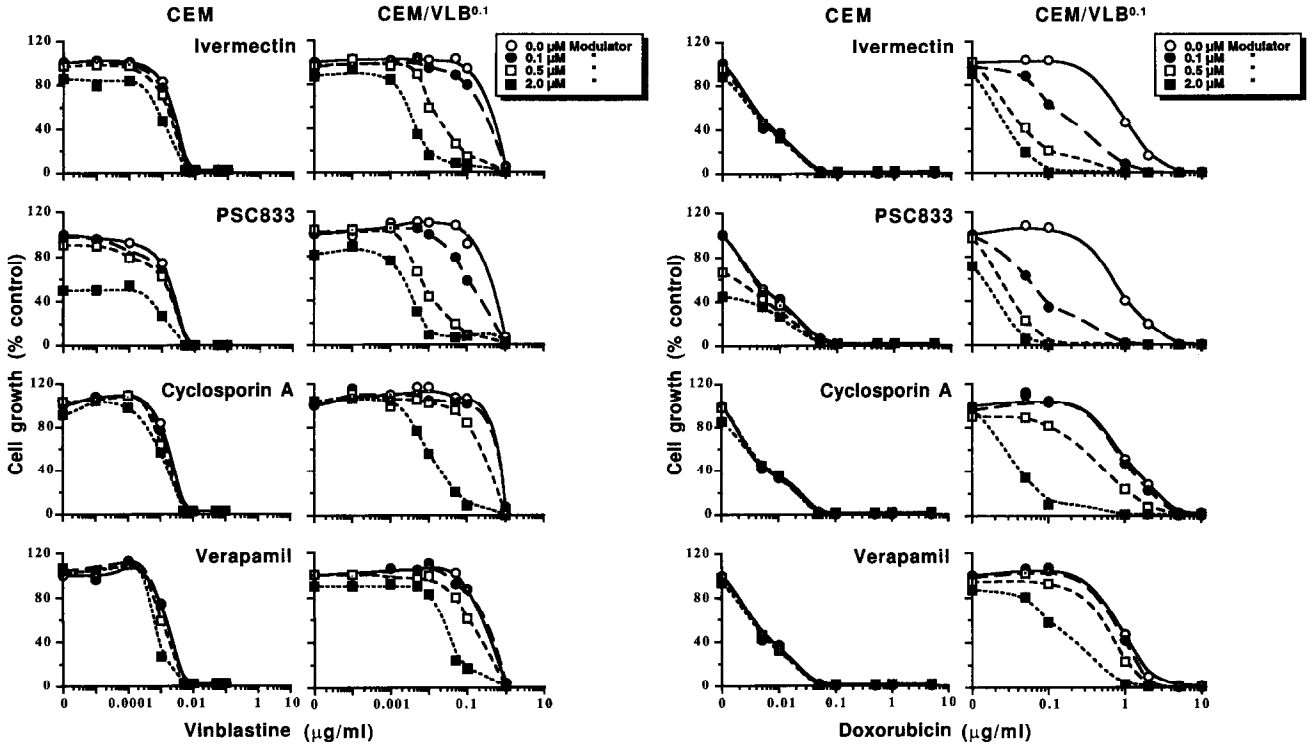

FIG. 7. Modulation of VLB (left panel) and doxorubicin (right panel) resistance by IVM, SDZ-PSC 833, cyclosporin A, and verapamil. Drug-sensitive (CEM; 0.5 × 10^4) and -resistant (CEM/VLB$^{0.1}$; 1 × 10^4) cells were plated and incubated for 24 hr. VLB or doxorubicin was then added (0–5 or 0–10 µg/mL, respectively) in the absence and in the presence of IVM, SDZ-PSC 833, cyclosporin A, or verapamil (0.1, 0.5, or 2 µM). After a 96-hr exposure, the viability of CEM and CEM/VLB$^{0.1}$ cells was estimated by measuring the absorbance at 450 nm. Each point is the mean (± SD) of three independent experiments.

SDZ-PSC 833, a potent MDR reversing agent [31], at 2 µM was toxic to both CEM and CEM/VLB$^{0.1}$ cells (Fig. 7). Table 1 shows the IC_{50} of CEM/VLB$^{0.1}$ cells to VLB or doxorubicin in the presence of increasing concentrations of IVM, SDZ-PSC 833, cyclosporin A, and verapamil. A comparison between IVM and the other MDR-reversing agents (e.g., verapamil, cyclosporin A, and SDZ-PSC 833) showed that IVM at 2 µM was ~9- and ~4-fold better than verapamil and cyclosporin A in potentiating the toxicity of VLB or doxorubicin (Fig. 7 and Table 1). SDZ-PSC 833 was ~1.2-fold better than IVM in potentiating the toxicity of VLB or doxorubicin. However, SDZ-PSC 833 alone was much more toxic to CEM and CEM/VLB$^{0.1}$ cells (Fig. 7).

Relative Hydrophobicity of IVM

To further analyse the effect of IVM as an MDR-reversing agent, the hydrophobicity of IVM was evaluated using an octanol/water fractionation coefficient and compared with other P-gp-associated drugs or MDR-reversing agents. The results in Table 2 show the octanol/water fractionation coefficients for colchicine, VLB, verapamil, cyclosporin A, and IVM to be 14, 30, 154, 518 and 1358, respectively. When the latter coefficients were compared with respect to the ability of drugs to inhibit binding, transport, or reverse the MDR of CEM/VLB$^{1.0}$ cells (Table 2); a strong correlation between hydrophobicity and these parameters could be observed. However, some exceptions were seen. For example, verapamil which is more hydrophobic than VLB, was a better inhibitor of drug transport, although photolabelling and binding were preferentially inhibited by VLB (photoaffinity labelling results with VLB or colchicine are not shown).

DISCUSSION

In this study, we have shown that IVM is a substrate for the P-gp drug efflux pump. Our results demonstrated the [^{3}H]IVM accumulates to a lesser extend in drug-resistant than in drug-sensitive cells. Moreover, efflux studies showed that [^{3}H]IVM is removed rapidly from resistant cells and this transport is energy dependent. These results are consistent with an earlier report [23] which suggested that IVM may be a substrate for P-gp, since the disruption of murine P-gp gene I from the mouse genome by homologous recombination led to the accumulation of IVM in the brain and other tissues that intrinsically overexpress P-gp [23]. Thus, although the results of this and a previous study do support the contention that IVM is transported from MDR cells, IVM was transported less efficiently than [^{3}H]VLB. It is not clear why these drugs are differentially transported from MDR cells; however, such differences may be due to the high hydrophobicity of IVM as compared with that of VLB (octanol to water fractionation IVM/VLB

TABLE 1. Modulation of resistance to VLB and doxorubicin by different reversing agents

Reversing agent	Concentration (μM)	IC_{50}* (ng/mL)	Modulation ratio†
VLB alone		500 ± 22	
Ivermectin	0.1	310 ± 17	1.6 ± 0.09
	0.5	16 ± 1.1	31.2 ± 2.15
	2.0	3.4 ± 0.6	147.0 ± 25.9
PSC 833	0.1	165 ± 12	3.0 ± 0.22
	0.5	9 ± 0.7	55.0 ± 4.28
	2.0	2.8 ± 0.1	178.0 ± 6.35
Cyclosporin A	0.1	500 ± 16	—
	0.5	300 ± 14	1.7 ± 0.08
	2.0	13 ± 0.9	38.5 ± 2.67
Verapamil	0.1	500 ± 16	—
	0.5	350 ± 16	1.4 ± 0.06
	2.0	30 ± 2.2	16.7 ± 1.22
Doxorubicin alone		1100 ± 43	
Ivermectin	0.1	164 ± 5.0	6.7 ± 0.20
	0.5	34 ± 1.5	32.4 ± 1.43
	2.0	24 ± 2.0	45.8 ± 3.84
PSC 833	0.1	80 ± 3.6	13.8 ± 0.62
	0.5	29 ± 2.1	38.0 ± 2.75
	2.0	18 ± 1.2	61.1 ± 4.07
Cyclosporin A	0.1	930 ± 41	1.2 ± 0.05
	0.5	410 ± 19	2.7 ± 0.12
	2.0	35 ± 1.5	31.0 ± 1.33
Verapamil	0.1	1000 ± 58	1.1 ± 0.06
	0.5	664 ± 43	1.7 ± 0.11
	2.0	153 ± 11	7.2 ± 0.52

* An IC_{50} drug concentration was obtained from Fig. 7 and represents 50% inhibition of MTT dye formation. Each value is the mean ± SD of at least three determinations.
† The modulation ratio was calculated from the IC_{50} for drug alone (VLB or doxorubicin) versus the IC_{50} in the presence of the modulating agent.

= 45). IVM is poorly soluble in aqueous solution; therefore, it has a tendency to partition in a hydrophobic environment offered by the cell membrane, resulting in a low efflux. Furthermore, due to its higher affinity for P-gp, IVM could possess a slower dissociation constant than VLB (K_D is 10.6 vs 400–500 nM for IVM and VLB [32, 33], respectively).

Earlier reports [30, 34, 35] on the physical–chemical properties of various compounds that are thought to reverse the MDR phenotype have indicated that lipophilicity as exemplified by one or more planar aromatic rings and a cationic charge are common to most MDR-reversing agents. Although these structural moieties are found in many MDR-reversing agents, their role in P-gp-drug bind-

TABLE 2. Effect of MDR-reversing agents on resistant (CEM/VLB$^{1.0}$) cells

Drugs	Octanol (FC)*	IAAP photolabelling (% of control)†	VLB binding (% of control)‡	IVM binding (% of control)‡	VLB transport (% of control)‡	IVM transport (% of control)‡	Increase in VLB sensitivity§ (drugs at 2 μM)
IVM	1358 ± 157	0 ± 0	0 ± 0	0 ± 0	13 ± 4	2 ± 1	147.2x
Cyclosporin A	518 ± 28	5 ± 0	0 ± 0	0 ± 0	4 ± 2	0 ± 0	38.5x
Verapamil	154 ± 11	25 ± 7	42 ± 7	84 ± 12	71 ± 11	22 ± 7	16.7x
VLB	30 ± 5	13 ± 5	4 ± 1	44 ± 15	96 ± 7	100 ± 13	NA‖
Colchicine	14 ± 2	102 ± 6	95 ± 4	86 ± 9	93 ± 9	100 ± 14	ND¶

Each value is the mean ± SD of at least three determinations.
* FC = fractionation coefficient (octanol phase/water phase).
†‡ Competition at: †100× and ‡300× molar excess.
§ Increase in sensitivity = IC_{50} in the absence of reversing agent/IC_{50} in the presence of reversing agent.
‖ NA = not applicable.
¶ ND = not determined.

ing and transport is unknown. Using non-ionic detergents, which also reverse the MDR-phenotype at non-toxic concentrations, we have demonstrated previously that hydrophobic interactions are likely to mediate P-gp drug binding, while the cationic charge associated with some lipophilic MDR-reversing agents may be important in drug transport [25]. In this study, we show that the octanol/water fractionation coefficient of IVM was 3- and 9-fold higher than that of cyclosporin A and verapamil, respectively. Hence, the observed differences in the reversing potential seen with the three MDR-reversing agents with respect to their hydrophobicity are consistent with earlier findings [25, 30, 34, 35].

The molecular mechanism by which P-gp mediates the efflux of drugs is presently not understood; however, using photoactive drug analogues, direct binding between P-gp and drugs has been demonstrated extensively [36–38]. The results of this study show that IVM interacts directly with P-gp, since low molar excesses of IVM completely inhibited the photoaffinity labelling of P-gp with IAAP. Moreover, drugs (e.g. VLB or cyclosporin A) that interact with P-gp were shown to potentiate IVM accumulation in drug-resistant cells and to inhibit the binding of [^{3}H]IVM to membranes from drug-resistant, but not from drug-sensitive cells. The failure of verapamil to inhibit [^{3}H]IVM binding to plasma membranes is not clear. It may be speculated that IVM interacts with different sequences in P-gp than that of verapamil. Competitive inhibition of [^{3}H]VLB and [^{3}H]IVM binding to membranes from resistant cells showed that IVM completely inhibited [^{3}H]VLB binding, while VLB only partially inhibited [^{3}H]IVM binding. Cyclosporin A was the only drug that totally inhibited IVM binding to P-gp. This suggests that these two drugs interact with similar binding sites. Interestingly, IVM was more efficient than SDZ-PSC 833 at inhibiting P-gp photoaffinity labelling with IAAP, while toxicity results demonstrated that SDZ-PSC 833 was more effective in reversing MDR. The IC_{50} for SDZ-PSC 833 was 1.2-fold lower than that of IVM for both VLB and doxorubicin, yet it is also inherently more toxic to drug-sensitive and -resistant cells. Thus, it is likely that some of the enhanced MDR-reversal is due to SDZ-PSC 833 toxicity alone. By contrast, IVM is well tolerated at plasma concentrations exceeding 680 ng/mL [18]. In this study, a 0.5 μM concentration or equivalent to a plasma concentration of 435 ng/mL was sufficient to reverse 96% of the resistance in CEM/VLB$^{0.1}$ cells. In addition, IVM possesses a long half-life that varies between 1 and 8 days according to species [22], and the biological transformation rate is relatively slow (less than 50% after 14 days) [22]. Taken together, IVM may have an advantage over SDZ-PSC 833 in reversing the MDR phenotype of tumours in the clinic.

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References

1. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58:** 137–171, 1989.
2. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 385–427, 1993.
3. Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM and Deeley RG, Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* **54:** 5902–5910, 1994.
4. Riordan JR, Deuchars K, Kartner N, Alon N, Trent J and Ling V, Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* **316:** 817–819, 1985.
5. Higgins CF, ABC transporters: From microorganisms to man. *Annu Rev Cell Biol* **8:** 67–113, 1992.
6. Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM and Borst P, Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75:** 451–462, 1993.
7. Ruetz S and Gros P, Phosphatidylcholine translocase—A physiological-role for the *mdr2* gene. *Cell* **77:** 1071–1081, 1994.
8. Goldstein LJ, Galski H, Fojo A, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM and Pastan I, Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst* **81:** 116–124, 1989.
9. Chan HSL, Haddad G, Thorner PS, DeBoer G, Lin YP, Ondrusek N, Yeger H and Ling V, P-Glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N Engl J Med* **325:** 1608–1614, 1991.
10. Chan HS, DeBoer G, Haddad G, Gallie BL and Ling V, Multidrug resistance in pediatric malignancies. *Hematol Oncol Clin North Am* **9:** 275–318, 1995.
11. Patel NH and Rothenberg ML, Multidrug resistance in cancer chemotherapy. *Invest New Drugs* **12:** 1–13, 1994.
12. Ozols RF, Clinical reversal of drug resistance—Foreword. *Curr Probl Cancer* **19:** 69–123, 1995.
13. Ford JM, Modulators of multidrug resistance. Preclinical studies. *Hematol Oncol Clin North Am* **9:** 337–361, 1995.
14. Pennock GD, Dalton WS, Roeske WR, Appleton CP, Mosley K, Plezia P, Miller TP and Salmon SE, Systemic toxic effects associated with high-dose verapamil infusion and chemotherapy administration. *J Natl Cancer Inst* **83:** 105–110, 1991.
15. Dalton WS, Crowley JJ, Salmon SS, Grogan TM, Laufman LR, Weiss GR and Bonnett JD, A phase III randomized study of oral verapamil as a chemosensitizer to reverse drug resistance in patients with refractory myeloma. *Cancer* **75:** 815–820, 1995.
16. Murren JR, Ganpule S, Sarris A, Durivage H, Davis C, Makuch R, Handschumacher RE and Marsh JC, A phase II trial of cyclosporin A in the treatment of refractory metastatic colorectal cancer. *Am J Clin Oncol* **14:** 208–210, 1991.
17. Warner E, Tobe SW, Andrulis IL, Pei Y, Trachtenberg J and Skorecki KL, Phase I–II study of vinblastine and oral cyclosporin A in metastatic renal cell carcinoma. *Am J Clin Oncol* **18:** 251–256, 1995.
18. Campbell WC, *Ivermectin and Abamectin*. Springer, New York, 1989.

19. Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LHT, Schaeffer JM and Arena JP, Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. *Nature* **371:** 707–711, 1994.
20. Cully DF and Paress PS, Solubilization and characterization of a high affinity ivermectin binding site from *Caenorhabditis elegans*. *Mol Pharmacol* **40:** 326–332, 1991.
21. Schaeffer JM and Haines HW, Avermectin binding in *Caenorhabditis elegans*. A two-state model for the avermectin binding site. *Biochem Pharmacol* **38:** 2329–2338, 1989.
22. Chiu S-HL, Green ML, Baylis FP, Eline D, Rosegay A, Meriwether H and Jacob TA, Absorption, tissue distribution, and excretion of tritium-labeled ivermectin in cattle, sheep and rat. *J Agric Food Chem* **38:** 2072–2078, 1990.
23. Schinkel AH, Smit JJM, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CAAM, van der Valk MA, Robanus-Maandag EC, te Riele HPJ, Berns AJM and Borst P, Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood–brain barrier and to increased sensitivity to drugs. *Cell* **77:** 491–502, 1994.
24. Beck WT, Vinca alkaloid-resistant phenotype in cultured human leukemic lymphoblasts. *Cancer Treat Rep* **67:** 875–882, 1983.
25. Zordan-Nudo T, Ling GV, Liu Z and Georges E, Effects of nonionic detergents on P-glycoprotein drug binding and reversal of multidrug resistance. *Cancer Res* **53:** 5994–6000, 1993.
26. Lin PH, Selinfreund R, Wakshull E and Wharton W, Rapid and efficient purification of plasma membrane from cultured cells: Characterization of epidermal growth factor binding. *Biochemistry* **26:** 731–736, 1987.
27. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
28. Fairbanks G, Steck TL and Wallach DFH, Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. *Biochemistry* **10:** 2606–2617, 1971.
29. Greenberger LM, Yang C-PH, Gindin E and Horwitz SB, Photoaffinity probes for the α_1-adrenergic receptor and the calcium channel bind to a common domain in P-glycoprotein. *J Biol Chem* **265:** 4394–4401, 1990.
30. Pearce HL, Safa AR, Bach NJ, Winter MA, Cirtain MC and Beck WT, Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. *Proc Natl Acad Sci USA* **86:** 5128–5132, 1989.
31. Watanabe T, Tsuge H, Oh-Hara T, Naito M and Tsuruo T, Comparative study on reversal efficacy of SDZ PSC 833, cyclosporin A and verapamil on multidrug resistance *in vitro* and *in vivo*. *Acta Oncol* **34:** 235–241, 1995.
32. Cornwell MM, Pastan I and Gottesman MM, Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* **262:** 2166–2170, 1987.
33. Naito M, Hamada H and Tsuruo T, ATP/mg^{2+}-dependent binding of vincristine to the plasma membrane of multidrug-resistant K562 cells. *J Biol Chem* **263:** 11887–11891, 1988.
34. Zamora JM, Pearce HL and Beck WT, Physical–chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol Pharmacol* **33:** 454–462, 1988.
35. Nogae I, Kohno K, Kikuchi J, Kuwano M, Akiyama S, Kiue A, Suzuki K, Yoshida Y, Cornwell MM, Pastan I and Gottesman MM, Analysis of structural features of dihydropyridine analogs needed to reverse multidrug resistance and to inhibit photoaffinity labeling of P-glycoprotein. *Biochem Pharmacol* **38:** 519–527, 1989.
36. Nare B, Prichard RK and Georges E, Characterization of rhodamine 123 binding to P-glycoprotein in human multidrug-resistant cells. *Mol Pharmacol* **45:** 1145–1152, 1994.
37. Foxwell BM, Mackie A, Ling V and Ryffel B, Identification of the multidrug resistance-related P-glycoprotein as a cyclosporine binding protein. *Mol Pharmacol* **36:** 543–546, 1989.
38. Greenberger LM, Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. *J Biol Chem* **268:** 11471–11475, 1993.