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Decreased Drug Accumulation without Increased Drug Efflux in a Novel MRP-Overexpressing Multidrug-Resistant Cell Line

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ABSTRACT. KB/7D cells represent a multidrug-resistant subclone of human nasopharyngeal carcinoma KB cells generated by continuous exposure to the topoisomerase II inhibitor VP-16 (etoposide). KB/7D cells also show cross-resistance to doxorubicin and vincristine. Phenotypic traits of the cell line include a 2-fold decrease in topoisomerase II levels and a decrease in the uptake of VP-16 without an increase in the rate of drug efflux or expression of P-glycoprotein, suggesting a novel mechanism associated with the uptake of anticancer drugs. This study demonstrated that the multidrug-resistance associated protein (MRP) is overexpressed in KB/7D cells, and that the loss of resistance in revertant cells correlates with the loss of MRP. The resistance to VP-16 and doxorubicin could be overcome, partially, and resistance to vincristine could be overcome completely, by the L-enantiomer of verapamil, but not by the D-enantiomer or by BIBW 22 (4-[*N*-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis[*cis*-2,6-dimethylmorpholino)-6-phenylpteridin), an inhibitor of MDR-1. L-Verapamil was shown to be significantly more potent than D-verapamil in modulating the accumulation defect in KB/7D cells towards doxorubicin, as measured by flow cytometry and confocal microscopy, and towards VP-16, as measured by increases in protein-linked DNA strand breaks. This suggests that KB/7D cells are multidrug resistant due to decreases in topoisomerase II levels and the overexpression of MRP, that MRP leads to a decrease in drug accumulation, and that L-verapamil can modulate the MRP-associated accumulation defect and drug-resistance phenotype. This contrasts with previous studies that suggest that MRP causes multidrug resistance by exporting cytotoxic drugs out of the cell and that did not show modulation of MRP by verapamil. BIOCHEM PHARMACOL **55**;8:1199–1211, 1998. © 1998 Elsevier Science Inc.

KEY WORDS: MRP; MDR; drug efflux; VP-16; verapamil; confocal microscopy

A major impediment to the successful treatment of human neoplasms is the development of multidrug resistance. The multidrug-resistance phenotype occurs when a tumor becomes resistant to a spectrum of structurally and functionally unrelated antineoplastic agents to which the patient has not been exposed previously.

A molecular mechanism for multidrug resistance was suggested with the discovery of P-gp§ [1]. P-gp, the product of *mdr*-1, was found to be overexpressed in certain multidrug-resistant cell lines and in clinical samples [2]. Its homology to bacterial transport proteins, and the observation that P-gp-overexpressing multidrug-resistant cell lines accumulate lesser amounts of cytotoxic drugs than the drug-sensitive parental lines, suggested that P-gp is a transporter protein that actively pumps cytotoxic drugs out of the cell, leading to drug resistance [1, 3]. Several agents have been shown to modulate P-gp-mediated drug resistance, presumably by blocking the transport activity of P-gp. These compounds include dipyridamole and its analogs [4], the immune suppressor cyclosporin A [5, 6], the calmodulin inhibitor trifluoperazine [7], and several calcium channel blockers such as verapamil [7, 8].

Other multidrug-resistant cells that did not express P-gp were characterized [9–13], suggesting that other mechanisms could exist. Mechanisms proposed to explain non-P-gp-mediated multidrug resistance included topo II alterations [9, 14, 15], inhibition of apoptosis [16], enhanced DNA repair [17], glutathione detoxification [18], and overexpression of the MRP [19–24]. Some MRP-expressing drug-resistant cells contain a defect in the accumulation of antineoplastic agents [20, 21], but MRP-expressing cells are poorly sensitized by agents capable of modulating P-gp, including verapamil [10].

This study characterizes an MRP-expressing cell line, KB/7D cells, generated by continuous exposure to VP-16 (etoposide). The cells show cross-resistance to the Vinca

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§ *Abbreviations:* P-gp, P-glycoprotein; MRP, multidrug-resistance associated protein; topo II, topoisomerase II; PLDBs, protein-linked DNA strand breaks; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; DEPC, diethylpyrocarbonate; FBS, fetal bovine serum; and dox, doxorubicin.

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alkaloid vincristine and several topo II inhibitors including dox. The KB/7D cells display an accumulation defect towards dox and VP-16 without an increase in the rate of drug efflux, in addition to topo II alterations [9]. The L-enantiomer of verapamil, but not the D form, is able to sensitize the KB/7D cells to dox, VP-16, and vincristine. Treatment of the KB/7D cells with the L-enantiomer, but not the D form, is also able to modulate the MRP-associated accumulation defect towards dox, as measured by confocal microscopy and flow cytometry, and VP-16, as measured by increased [^{3}H]VP-16 accumulation and PLDBs. This suggests that KB/7D cells are resistant to anticancer agents, in part due to overexpression of MRP, and that the MRP phenotype can be stereospecifically modulated by the L-enantiomer of verapamil.

MATERIALS AND METHODS

Cells

Human nasopharyngeal carcinoma KB cells were obtained from the American Tissue Culture Collection. Drug-resistant KB/7D cells and revertant KB/7D^r cells were developed in this laboratory by continuous exposure to drug-free medium and have been described previously [9]. KB/7D^{pr} cells are an independently derived revertant line derived from KB/7D cells by continuous culture in drug-free medium. They were used for experiments after 6 weeks in drug-free medium and retained partial MRP overexpression and multidrug resistance. P-gp-expressing KB/V20C [4] cells and KB/MDR cells [25] also have been described previously. All cells were maintained in RPMI 1640 medium with 5% FBS and 100 μg/mL of kanamycin except for KB/MDR cells that required 10% FBS and 1 mM pyruvate. Drug-resistant cells were maintained in drug-containing medium (7 μM of VP-16 for KB/7D cells, 20 nM of vincristine for KB/V20C cells, and 37 nM of doxorubicin for KB/MDR cells). Drug-resistant cells were maintained in drug-free medium for 3 days prior to experiments. During cytotoxicity experiments, when cells were exposed to drug, all cell lines were in RPMI 1640 medium with 5% FBS.

Chemicals

Vincristine sulfate and VP-16 were purchased from the Sigma Chemical Co.; verapamil HCl enantiomers were a gift from Searle Laboratories. Fresh clinical samples of doxorubicin HCl were obtained from Dr. John Murren, Yale Cancer Center, Yale University. BIBW 22 (4-[*N*-(2-hydroxy-2-methyl-propyl)-ethanolamino]-2,7-bis[*cis*-2,6-dimethylmorpholino)-6-phenylpteridin) was a gift from Dr. Karl Thomae, Biberach/Riss. [^{3}H]VP-16 was obtained from Moravek Biochemicals. Doxorubicin stock solutions were prepared in H_2O, while other stock solutions were prepared in DMSO. Drug solutions were stored at −20°.

Drug Sensitivity

Cellular growth inhibition was determined by direct counting of cells, or by methylene blue staining of cellular proteins. The IC_{50} values are the drug concentrations that inhibit cell growth to 50% of the growth seen in drug-free controls.

For the methylene blue staining experiments, 1×10^4 cells were plated in 24-well tissue culture plates (Corning) and allowed to attach overnight at 37° with 5% CO_2. Drugs were added, and the cells were allowed to grow for 3 days. The medium was removed, the cells were washed twice with PBS, and cellular proteins were stained as described in Finlay *et al.* [26]. For the cell counting experiments, 1.5 to 2×10^4 cells were added to 6-well plates (Corning) and allowed to grow overnight. Drugs were added, and the cells were allowed to grow for 3 days. Medium was removed, and the cells were released with pancreatin. Cells were then suspended in PBS and counted by Coulter Counter (Coulter Electronics).

Confocal Microscopy

Cells (5×10^4) were loaded onto glass coverslips that had been sterilized by soaking in 70% ethanol and then air dried. Cells were allowed to attach overnight in the incubator. The medium was removed, cells were washed with PBS, and new medium was added containing 10 μg/mL (17 μM) of dox with or without sensitizers. Cells were allowed to accumulate dox for 1.5 hr at 37° with 5% CO_2. Immediately before observing, the medium was removed and the cells were washed twice with ice-cold PBS. Then cells were examined with the confocal scope (Zeiss/Bio-Rad) under ice-cold PBS.

Flow Cytometry

Cells (2.5 to 3×10^5) were loaded into 24-well tissue culture plates (Corning) and allowed to attach overnight in the incubator. The medium was removed, and new medium was added containing the sensitizers or DMSO vehicle alone. Then the cells were incubated for 15 min at 37° with 5% CO_2, dox was added, and the cells were incubated for an additional hour as before. The dox-containing medium was removed, and then cells were washed with 1 mL of drug-free 37° medium and returned to the incubator for another 20 min with 1 mL of medium containing the sensitizers or DMSO alone. The medium was removed, and 0.5 mL of a 37° release buffer containing 2.6 g/L of KH_2PO_4, 11.4 g/L of Na_2HPO_4, and 0.5 g/L of pancreatin was added. The bulk of the release buffer was removed, the plates were returned to the incubator for 2.5 min, and then the cells were washed off with 1 mL of ice-cold medium. Cells were pelleted at 1000 *g*, resuspended in 100 μL of ice-cold medium, and examined by flow cytometry (FACS IV, Becton/Dickinson).

Nucleic Acid Isolation

DNA was prepared from log phase cells in drug-free medium by the standard phenol:chloroform preparation for adherent cells [27]. The only deviation from the published procedure was that 50:49:1 phenol:chloroform:isoamyl alcohol was used in place of phenol. RNA was prepared from log phase cells in drug-free medium by the standard guanidinium thiocyanate method [28]. All aqueous solutions were made with DEPC-treated H_2O and autoclaved before use except for the guanidinium stock solution. Final RNA pellets were resuspended in DEPC-treated H_2O.

Protein Isolation and Western Blot

Confluent cells in drug-free medium were harvested by mechanical scraping into ice-cold PBS, and membrane protein was isolated by the method of Naito *et al.* [29]. Twenty micrograms of protein was separated on a 6% acrylamide gel, and proteins were transferred to nitrocellulose membranes with XCell II Mini-Cell Blot Module™ (NOVEX) as per manufacturer's instructions. Western blot analysis was performed with MRP-specific QCRL-1 antibodies [30].

Nucleic Acid Blots

SOUTHERN BLOT. DNA was digested with 10 U of *Eco*RI/μg DNA, EtOH precipitated, and resuspended in TE (10 mM of Tris-HCl, 1 mM of EDTA, pH 8.0) buffer. Ten micrograms of cut DNA was added to each well, run through a 0.7% agarose gel in 1× TAE (40 mM of Tris-acetate, 2 mM of EDTA), and transferred to a nylon membrane by capillary action [27]. Blots were air dried, and the DNA was cross-linked to the membrane with a UV light cross-linker (Stratagene). An MRP 10.1 probe [19] was radiolabeled with [^{32}P]dCTP using the Prime-It II™ kit (Stratagene), purified through a Quick Spin™ G-50 Sephadex column (Boehringer Mannheim), and 1×10^7 cpm were added to the blots prehybridized with Quick-Hyb™ (Stratagene) and salmon sperm DNA. Blots were hybridized overnight at 65°, washed three times with 2× SSC/0.5% SDS at room temperature and three times for 10 min with 0.1× SSC/0.5% SDS at 65° with shaking. Then blots were exposed to x-ray film and developed.

SLOT BLOTS. Known amounts of undigested DNA or total RNA were prepared in 10× SSC. DEPC-treated solutions were used for the RNA blots. DNA or RNA was added to the wells of a Minifold II™ slot blot system (Schleicher & Schuell) and transferred under vacuum to a nylon membrane. DNA was denatured by laying the membrane over a sheet of Whatman 3MM paper soaked in denaturation solution (1.5 M of NaCl/0.5 M of NaOH) and then neutralized by laying on Whatman 3MM paper soaked in 1.5 M of NaCl/1M of Tris-HCl at pH 7.4. The blots were air dried, UV cross-linked, and probed as above except that an *alu* probe was used to standardize the DNA slot blot. Values were quantified by densitometer reading of the x-ray film or read directly by a PhosphoImager system (Molecular Dynamics).

NORTHERN BLOT. Ten micrograms of total RNA was denatured by heating to 65° for 15 min in loading buffer (0.72 mL of formamide, 0.16 mL of 10× MOPS, 0.26 mL of 37% formaldehyde, 0.18 mL of H_2O, 0.1 mL of 80% glycerol, and 0.08 mL of saturated bromphenol blue), 4 parts buffer: 1 part RNA. RNA was run through a 1% agarose gel with 2% formaldehyde in 1× MOPS buffer. The RNA was UV nicked, and the gel was soaked twice in 10× SSC to remove the formaldehyde. RNA was transferred to a nylon membrane [27] and treated as described for the Southern blot.

PLDBs

Log phase cells (5×10^6) were labeled with [^{14}C]thymidine for 18–24 hr. The medium was removed, and unincorporated label was washed away with two 10-mL washes of PBS. Cells were released with trypsin, suspended in nonradioactive medium, plated at 2×10^5 cells/mL, and incubated at 37° with 5% CO_2 for at least 1 hr. Then the cells were incubated with or without drugs for 60 min. Cells were harvested and assayed for PLDBs by the K-SDS method of Rowe *et al.* [31].

VP-16 *Accumulation and Efflux*

[^{3}H]VP-16 was HPLC purified using a C_{18} column (Alltech) on an LC Plus system HPLC (Perkin Elmer) with a 235C Diode Array Detector. The column was run with 70:30:2 water:acetonitrile:acetic acid at 1 mL/min, isocratic. [^{3}H]VP-16 was evaporated to drynes and resuspended in unlabeled VP-16, and specific activity was determined. Cells were treated with [^{3}H]VP-16 as described [9]. Briefly, cells were exposed to VP-16 in duplicate T25 flasks for 4 hr to achieve steady-state intracellular levels. Drug-containing medium was aspirated and replaced with 37° drug-free medium, and drug efflux was allowed to proceed for various times. To stop the experiment, medium was removed from the flasks, cells were washed twice with ice-cold PBS, harvested with pancreatin, and added to scintillation vials containing scintillation fluid. The flasks were washed with water, and the wash was added to the same vials. Radioactive counts were determined and expressed as picomoles VP-16 per million cells. Cell number was determined in replicate flasks treated with unlabeled VP-16. Nonspecific background counts were determined by adding [^{3}H]VP-16 in ice-cold medium to flasks stored on ice, quickly removing the medium, washing twice with ice-cold PBS, and harvesting cells.

TABLE 1. IC_{50} **Values of antiproliferative agents**

Drug combination	KB	KB/7D	KB/7D^r	KB/MDR
VP-16 (μM)	0.23 ± 0.035	28 ± 4.5	0.45 ± 0.15	6.1 ± 0.14
+10 μM Vrp	0.14 ± 0.021	6 ± 3.2	0.28 ± 0.09	0.33 ± .011
+10 μM L-Vrp	0.14 ± 0.071	5 ± 3.3	0.22 ± 0.08	0.25 ± 0.007
+10 μM D-Vrp	0.19 ± 0.014	15 ± 3.3	0.28 ± 0.11	0.40 ± 0.014
+1 μM BIBW 22	0.26 ± 0.021	21 ± 2.1	0.29 ± 0.12	0.20 ± 0.014
Vincristine (nM)	1.4 ± 0.3	22 ± 5.7	2.1 ± 0.45	170 ± 28
+10 μM Vrp	0.5 ± 0.2	3.0 ± 0.88	0.6 ± 0.14	4.0 ± 0.0
+10 μM L-Vrp	0.4 ± 0.1	1.4 ± 0.62	0.5 ± 0.07	2.7 ± 0.5
+10 μM D-Vrp	0.7 ± 0.1	10 ± 5.6	0.8 ± 0.07	5.3 ± 0.4
+1 μM BIBW 22	1.4 ± 1.0	12 ± 0.71	1.9 ± 0.92	6.9 ± 0.2
Doxorubicin (nM)	16 ± 3.9	550 ± 92	32 ± 4.9	355 ± 7.0
+10 μM Vrp	9.1 ± 1.4	184 ± 31	22 ± 6.4	12 ± 3.5
+10 μM L-Vrp	8.0 ± 2.5	84 ± 19	25 ± 2.1	6.0 ± 2.8
+10 μM D-Vrp	11 ± 2.8	305 ± 69	25 ± 7.8	7.5 ± 0.7
+1 μM BIBW 22	12 ± 1.4	400 ± 71	32 ± 6.4	17 ± 16

IC_{50} Values (see Materials and Methods) of cytotoxic drugs are expressed as means ± SD. Vrp = Verapamil. The table contains many experiments performed using cell counting and methylene blue staining. Since no significant differences were observed between the two methods, the results have been combined. All values represent at least three experiments.

RESULTS

Drug Resistance Profile of KB Cell Variants

KB/7D cells have been shown previously to possess 50% of the topo II enzyme and 33% of the topo II activity of KB cells [9]. In a previous study, KB/7D cells accumulated approximately 40% of the [^{3}H]VP-16 accumulated by the KB cells, but they did not express P-gp or display a measurable increase in the rate of drug efflux [9]. Cytotoxicity studies indicated that KB/7D cells are approximately 120-fold resistant to VP-16, and are also cross-resistant to dox (35-fold) and vincristine, an inhibitor of mitotic spindle formation vincristine (16-fold) (Table 1). The revertant KB/7D^r cells had lost resistance to dox, VP-16, and vincristine (Table 1), but did maintain topo II enzyme and activity levels comparable to those of KB/7D cells [9]. The similarity in topo II status in KB/7D and KB/7D^r cells, along with resistance to vincristine, suggests that topo II alterations could not account for the entire drug resistance phenotype in KB/7D cells.

MRP Expression in KB Cell Variants

Northern blot analysis (Fig. 1A) was performed to examine the expression of MRP in the cell lines. A partial revertant cell line, KB/7D^{pr}, which showed approximately 40-fold resistance to VP-16 and 4-fold resistance to vincristine when compared with KB cells, and known P-gp-expressing cell lines, V20C and KB/MDR, were included in these studies. The KB/7D cells showed a large overexpression of MRP RNA, the KB/7D^{pr} cells lost the majority of the MRP transcript, and the KB/7D^r cells did not show overexpression when compared with KB cells. V20C cells also showed no significant MRP expression at the RNA level.

Amplification of the MRP gene itself was demonstrated by Southern blotting (Fig. 1B). KB/7D cells showed amplification of the MRP gene when compared with the parental KB cells. The KB/7D^{pr} cells lost most of this overexpression, while KB/7D^r and V20C cells did not show any amplification of the MRP gene. Amplification of *mdr*-1 was not seen in KB/7D cells (data not shown), in agreement with previous data showing no expression of P-gp in KB/7D cells [9]. The level of MRP expression was quantified by slot blot analysis. Slot blots of total RNA were probed with MRP and actin specific probes, the intensity of the blots was quantified, and the MRP:actin ratios were compared. The KB/7D cells showed a 20-fold increase in MRP RNA when compared with KB cells. Consistent with their reduced resistance to anticancer agents, the KB/7D^{pr} cells exhibited only a 6-fold overexpression of MRP, whereas KB/7D^r cells lost all overexpression of MRP RNA. DNA slot blots were probed with *MRP* and *alu* specific probes. When the *mrp*/*alu* ratios were compared, the MRP gene was found to be amplified 40-fold in KB/7D cells when compared with KB cells. Consistent with their loss of resistance to the cytotoxic drugs tested, KB/7D^{pr} cells showed only a 3-fold increase and KB/7D^r cells actually showed a nominal decrease in the copy number of the MRP gene.

The anti-MRP QCRL-1 antibody [30] recognizes a protein of approximately 200 kDa in KB/7D cells (Fig. 1C). The size of this band corresponded to the reported 190 kDa size of MRP [19]. The KB cells did not show this amplified band, and the KB/7D^r cells had lost the band along with the MDR phenotype (Fig. 1C, compare lanes 1, 2, and 3). P-gp control KB/MDR cells also did not show MRP expression (lane 6).

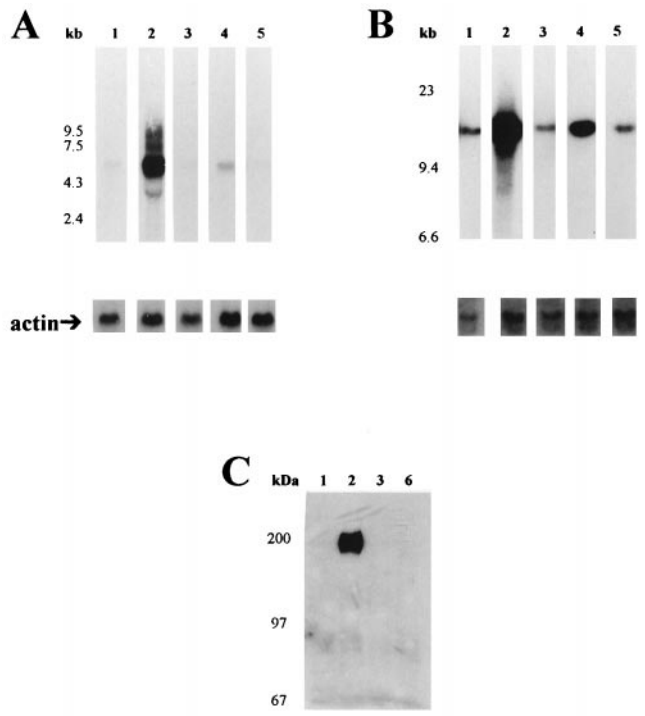

FIG. 1. MRP expression in KB cell variants. (A) Ten micrograms of total RNA was separated on a 1% agarose/formaldehyde gel, transferred to nylon, and probed for MRP or actin. (B) Ten micrograms of DNA was separated on a 0.7% agarose gel, transferred to nylon, and probed for MRP or actin. (C) Twenty micrograms of membrane protein was separated on a 6% acrylamide gel, transferred to nitrocellulose, and probed with MRP specific antibodies. In all blots, 1 = KB, 2 = KB/7D, 3 = KB/7D^r, 4 = KB/7D^{pr}, 5 = KB/V20C, and 6 = KB/MDR cells.

Modulation of MDR in KB/7D Cells by Verapamil

Verapamil is a known modulator of P-gp-mediated multidrug resistance [7]. Verapamil, however, was only able to sensitize MRP-expressing cells to a modest degree [9, 10]. Two stereoisomers of verapamil, the L/(−) and D/(+) forms, exist, and the ability of verapamil to modulate MRP-mediated MDR could be stereospecific. Therefore, the two separate enantiomers were tested, along with racemic verapamil, for their ability to sensitize the KB/7D cells to dox, VP-16, and vincristine (Table 1). Ten micromolar verapamil was chosen because this concentration of racemic verapamil causes some modulation in KB/7D cells, but is not highly toxic to the cells [9]. At 10 μM, all forms of verapamil decreased the IC_{50} values for the KB/7D cells, but the L-enantiomer was more potent than the D-enantiomer (Table 1). With vincristine, the L-enantiomer was able to decrease KB/7D cell resistance to levels comparable with those seen in the KB cells. With dox and VP-16, the L-enantiomer also reduced significantly the IC_{50} values in KB/7D cells, but it could not reduce the IC_{50} values to levels comparable with the KB cells. One micromolar BIBW 22, a known inhibitor of P-gp-mediated multidrug resistance [4], had no detectable effect on KB/7D cell resistance. This contrasts with the P-gp-expressing KB/MDR cells, which were sensitized effectively by both verapamil enantiomers and BIBW 22 (Table 1). The IC_{50} values of parental KB cells and revertant KB/7D^r cells also decreased with verapamil, but the magnitude of the decreases was small when compared with the resistant KB/7D cells (Table 1).

The ability of the enantiomers to overcome MRP-associated MDR is further illustrated by the ability of the two enantiomers to decrease the IC_{50} value of KB/7D cells to a single concentration of a cytotoxic drug (Table 2). Four micromolar L-verapamil was able to decrease the IC_{50} of KB/7D cells from 550 nM of dox to 100 nM, and the IC_{50} values for vincristine decreased from 22 to 1 nM when treated with 6.2 μM of L-verapamil. These concentrations of L-verapamil were approximately half of those required for the racemic mixture, and did not cause significant toxicity in KB/7D cells. The D-enantiomer was not able to sensitize the cells to these same IC_{50} values until concentrations approaching the IC_{50} for verapamil alone in KB/7D cells, approximately 40 μM, were achieved.

MRP has been postulated to cause multidrug resistance by exporting the drugs out of the cell [32, 33], or by causing sequestering of the drugs into vacuoles where they cannot interact with their intracellular targets [33, 34]. The accumulation of the naturally fluorescing drug dox was directly measured by confocal microscopy (Fig. 2). When exposed to 10 μg/mL of dox (17 μM), the KB/7D cells accumulated significantly less dox than the KB or KB/7D^r cells (compare panels A, B, and C of Fig. 2). In both KB and KB/7D cells, the vast majority of the dox was located in the nucleus and not in cytosolic vacuoles. Consistent with the cell toxicity studies (Table 1), addition of 10 μM of L-verapamil led to an increase in dox accumulation in KB/7D cells (Fig. 2, B vs E), whereas 10 μM of D-verapamil had no obvious effect on dox accumulation or distribution (Fig. 2, B vs H). One micromolar BIBW 22 also had no effect on the accumulation of dox in KB/7D cells, yet this concentration effectively overcame the drug-uptake defect in P-gp-expressing KB/MDR cells (data not shown). No obvious change in

TABLE 2. Sensitization potency of verapamil enantiomers

Doxorubicin	550 to 100 nM	Vincristine	22 to 1 nM
+L-Vrp	4.0 ± 1.0 (3)	+L-Vrp	6.2 ± 1.8 (3)
+D-Vrp	34 ± 4.0 (3)	+D-Vrp	41 ± 7.8 (3)
+Racemic Vrp	7.3 ± 2.1 (4)	+Racemic Vrp	16 ± 7.2 (3)

Concentration of verapamil (μM) required to reduce the IC_{50} values in KB/7D cells from 550 nM of doxorubicin to 100 nM of doxorubicin or from 22 nM of vincristine to 1 nM of vincristine. Error bars represent the SD between experiments; (*N*) = number of separate experiments. Vrp = verapamil.

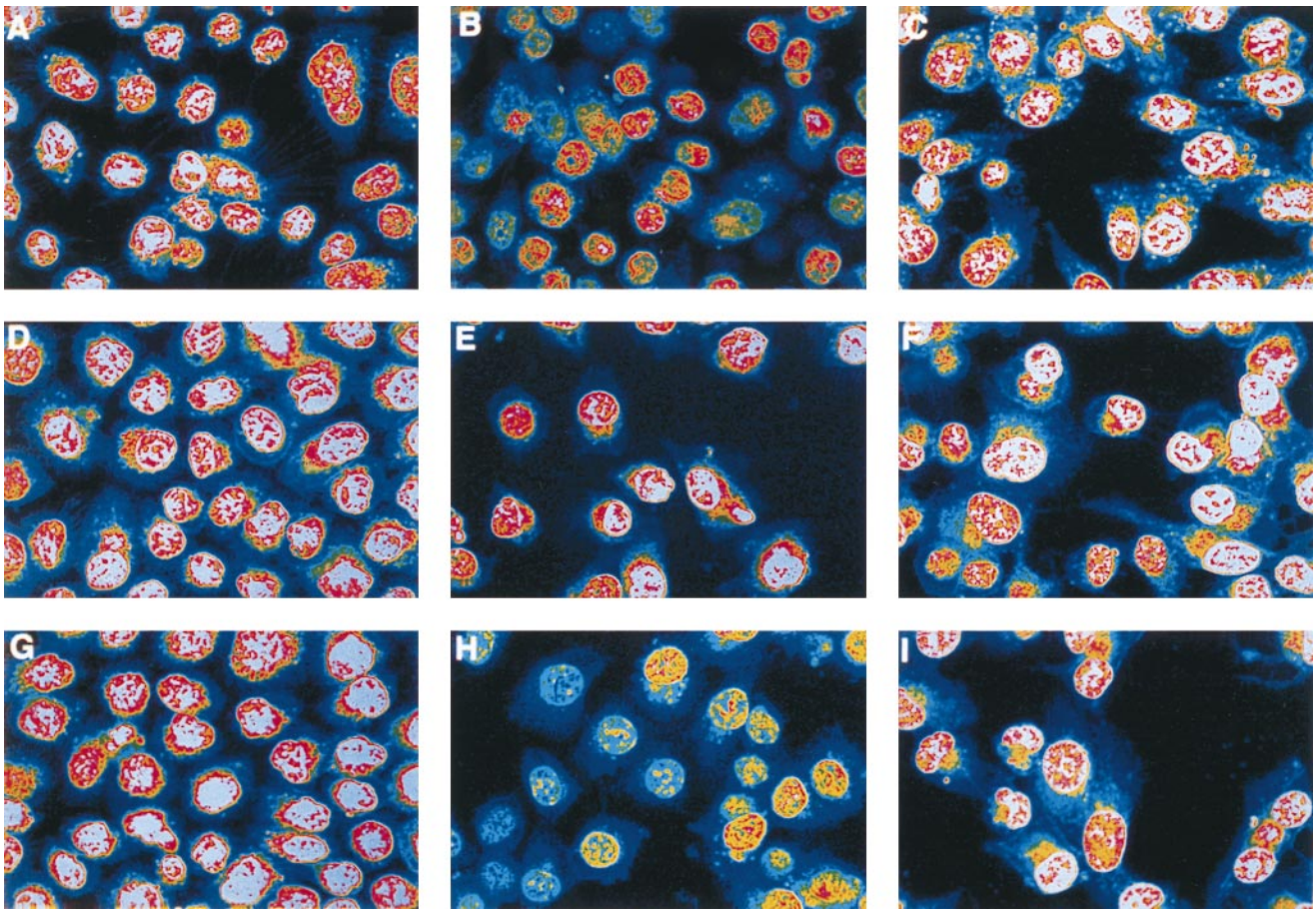

FIG. 2. Doxorubicin accumulation in KB cell variants as measured by confocal microscopy. Cells were exposed to 10 μg/mL of dox with or without verapamil enantiomers, as described in Materials and Methods. Dox accumulation was recorded immediately after removal of dox-containing medium. A = KB, B = KB/7D, C = KB/7D^r, D = KB + L-verapamil, E = KB/7D^r + L-verapamil, F = KB/7D^r + L-verapamil, G = KB + D-verapamil, H = KB/7D + D-verapamil, and I = KB/7D^r + D-verapamil. Confocal scope conditions were as follows: black level = 4.9, enhancement = 0, gain = 9.0, and slit width = 1/3 open. Dox location is presented on a pseudocolor scale with the highest concentration white, red, orange, green, blue, and black being the least intense.

fluorescence was observed when the sensitizers were added to the KB cells along with the dox (Fig. 2, A, D, and G), or the KB/7D^r cells (Fig. 2, C, F, and I). Confocal experiments were repeated with 5 μg/mL (8.5 μM) or 1 μg/mL (1.7 μM) of dox, and similar results were obtained.

Fluorescence-activated cell sorting (FACS) experiments further demonstrated that L-verapamil is more potent than D-verapamil in its ability to reverse the MRP-associated drug accumulation defect. Cells were exposed to 5 μM of dox, and the amount of dox in the cells was quantified on a fluorescence intensity scale. Then the fluorescence intensity of other cell types exposed to the same concentration of dox was compared with that of the original cells. Values were reported as a fold increase in dox fluorescence. KB/7D and KB/MDR cells accumulated less dox than KB cells, while KB/7D^r cells accumulated approximately the same amount of dox (Fig. 3A and Table 3). Even though KB/MDR cells were less resistant to dox than KB/7D cells, the accumulation defect in the KB/MDR cells was greater than in the KB/7D cells (Fig. 3A and Table 3). Since the KB/7D cells show a marked decrease in topo II activity [9], it is not unexpected that they could accumulate more dox and still show a greater resistance to topo II poisons than the KB/MDR cells, which are P-gp-transfected cells.

None of the sensitizers tested had a significant effect on the accumulation of dox in parental KB or revertant KB/7D^r cells (Table 4). Coincubation of KB/7D cells with dox and 10 μM of racemic verapamil or either enantiomer resulted in a significant increase in dox accumulation. The magnitude of the increase with D-verapamil was much less than with L or racemic verapamil, and the difference between L- and D-verapamil was significant ($P < 0.05$; Student's *t*-test; Fig. 3B and Table 4). As expected, the accumulation defect of P-gp-expressing KB/MDR cells was reversed by all forms of verapamil and BIBW 22 (Table 4). FACS experiments were repeated with 10 μg/mL of dox (17 μM), as this was the concentration used in our initial confocal experiments, and similar results were obtained (data not shown).

The ability of MRP to cause a defect in drug accumulation and the ability of L-verapamil to circumvent this defect were further strengthened by examining the effect of verapamil on PLDBs in KB/7D cells treated with VP-16 (Fig. 4). KB/7D and V20C cells accumulated fewer PLDBs

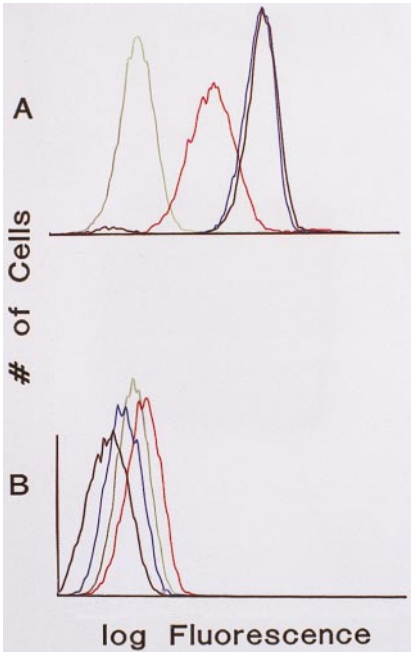

FIG. 3. Doxorubicin uptake in KB cell variants as measured by flow cytometry. For all experiments, 20,000 cells were excited at 488 nm and fluorescence was collected above 520 nm. (A) Uptake of 5 μM of dox in KB cell variants. Black = KB, Blue = KB/7D^r, Red = KB/7D, and Green = KB/MDR. (B) Differential increase in dox uptake in KB/7D cells with verapamil enantiomers. Black = 5 μM of dox alone, Blue = dox + D-verapamil, Red = dox + L-verapamil, and Green = dox + racemic verapamil. Note, fluorescence scales are different in panels A and B.

than did KB cells (Fig. 4A). When the different enantiomers of verapamil were coincubated with VP-16 in KB/7D cells, the L-enantiomer was clearly more potent than the D form in inducing VP-16-mediated PLDBs (Fig. 4B). Treatment of KB/7D cells with VP-16 and L-verapamil did not increase the number of PLDBs to levels seen in KB cells. This suggests that a mechanism other than the MRP-mediated drug-uptake defect, such as topo II alterations, is contributing to VP-16 resistance in KB/7D cells.

TABLE 3. Relative accumulation of doxorubicin in sensitive and resistant cells without resistance modifiers as measured by FACS analysis

	KB	KB/7D	KB/7D^r	KB/MDR
5 μM Dox alone	1.0	0.3 ± 0.08*	1.3	0.05*

Five experiments were performed for KB/7D cells and an average of two for KB/7D^r and KB/MDR cells. Error bars represent SD between experiments.

*Significant change in dox fluorescence as compared with KB cells (Student's *t*-test with $\alpha = 0.05$).

VP-16 Retention in KB/7D Cells

MRP has been postulated to be a transport protein that actively pumps cytotoxic drugs or drug conjugates out of the cell [32, 33, 35–38]. We have directly examined the accumulation and retention of [^{3}H]VP-16 in KB and KB/7D cells. When KB and KB/7D cells were exposed to 1 μM of VP-16, KB/7D cells accumulated 27% the amount of VP-16 seen in KB cells (Fig. 5). When KB and KB/7D cells were loaded with [^{3}H]VP-16 to approximately equal intracellular concentrations, no significant differences in VP-16 efflux were seen (Fig. 6A). Replotting the data on a log scale resulted in a linear pattern with increasing efflux time and decreasing drug concentration. This is consistent with passive diffusion of VP-16 from both cell types and not with active transport of drug (Fig. 6B). When analogous experiments were performed with KB and P-gp-expressing KB/MDR cells, large differences were seen, demonstrating that we can detect differences in drug efflux in spite of the rapid loss of VP-16 from KB cell variants (data not shown). In this experiment, it appears that VP-16 efflux was, if anything, slightly faster in KB cells than in KB/7D cells. However, experiments on different days demonstrated virtually identical efflux rates, and we do not feel that the rates differed significantly.

DISCUSSION

KB/7D cells were one of the first human tumor cell lines shown to be multidrug resistant without significant expression of P-gp or an enhancement in the rate of drug efflux [9]. Although topo II alterations have occurred in KB/7D cells, this cannot be the only source of resistance in these cells for two reasons. First, KB/7D cells show significant resistance to vincristine, which inhibits mitotic spindle formation, a process not affected by changes in topo II; and second, KB/7D^r cells have lost most of their resistance to

TABLE 4. Relative increase in doxorubicin accumulation in sensitive and resistant cells when treated with resistance modifiers

Drug combination	KB	KB/7D	KB/7D^r	KB/MDR
5 μM Dox	1.0	1.0	1.0	1.0
+10 μM Vrp	0.93 ± 0.10	2.0 ± 0.18*	0.94 ± 0.06	17 ± 1.7*
+10 μM L-Vrp	0.91 ± 0.16	2.3 ± 0.35*	0.96 ± 0.04	17 ± 1.0*
+10 μM D-Vrp	0.83 ± 0.12	1.4 ± 0.13*	0.88 ± 0.04	15 ± 1.1*
+1 μM BIBW 22	0.83	1.2	0.92	15*

Error bars represent SD between experiments. All values are the average of three separate experiments except for the BIBW 22 values, which are the average of two.

*Significant change in fluorescence in cells treated with modulators and 5 μM of dox as compared with cells treated with 5 μM of dox alone (Student's *t*-test with $\alpha = 0.05$).

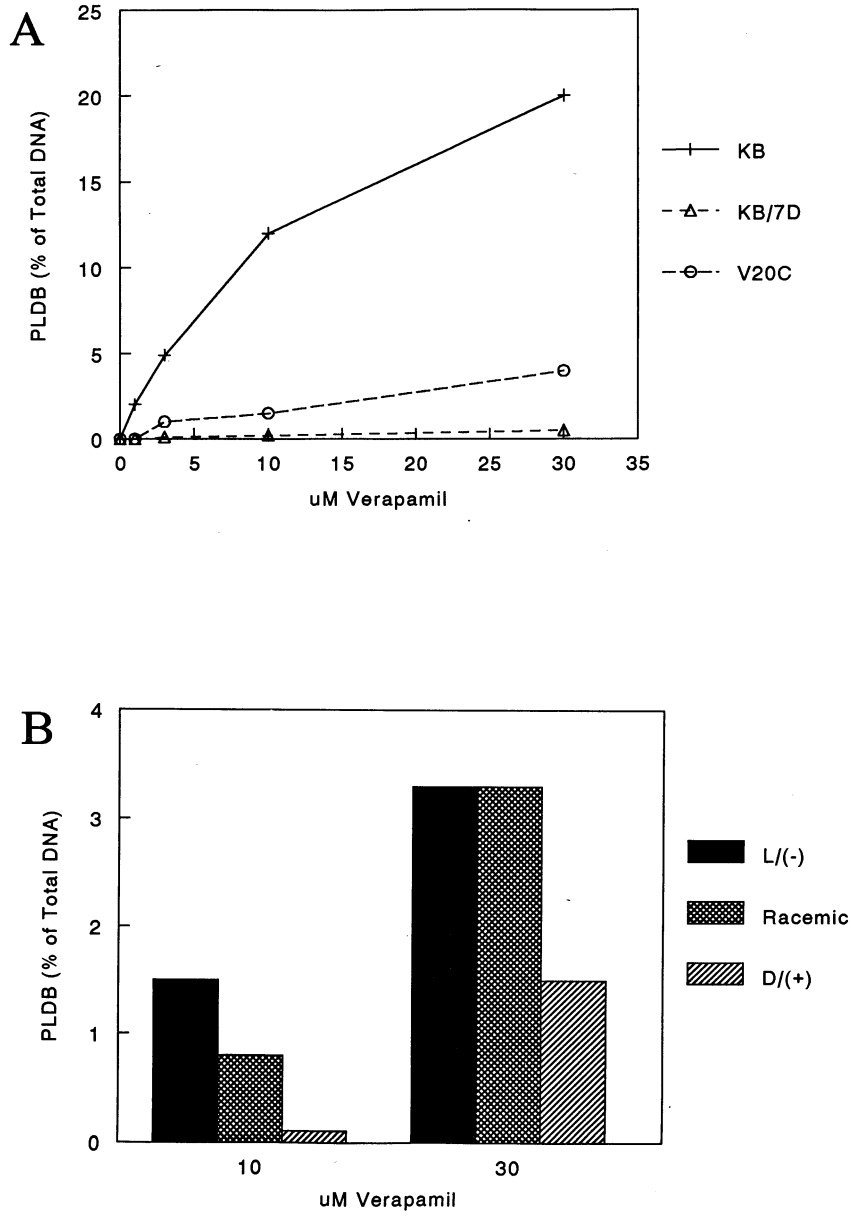

FIG. 4. Induction of VP-16-mediated PLDBs in KB cell variants. (A) Percentage of total DNA present in PLDBs in drug-sensitive and -resistant KB cells with increasing concentrations of VP-16. (B) Increase in VP-16-mediated PLDBs in KB/7D cells treated with 30 μM of VP-16 and verapamil enantiomers. Results are the averages of two experiments and are reported as the percentage of DNA in PLDBs in cells treated with VP-16 and verapamil minus that in cells treated with VP-16 alone.

vincristine, dox, and VP-16, yet they still show decreases in the level of topo II protein and activity [9]. This suggested that a mechanism other than P-gp expression or topo II alterations was mediating multidrug resistance in KB/7D cells, and that this mechanism resulted in a decreased accumulation of anticancer agents.

MRP was shown recently to be associated with the multidrug-resistance phenotype [19–24]. Our results (Fig. 1) demonstrated that MRP is overexpressed in KB/7D cells. MRP overexpression is likely due to gene amplification, as loss of the MRP gene correlated with loss of the transcript, the protein itself, and the multidrug-resistance phenotype, although our experiments do not rule out the possibility that other mechanisms, such as increased translational efficiency or protein stability, contribute to MRP overexpression. Given that MRP expression is a recently well-documented source of MDR [19–24], and that the resistance phenotype is lost along with the protein, MRP is likely a significant source of resistance in KB/7D cells. This is further strengthened by the fact that KB/7D cells are not resistant to mitoxantrone (1.7-fold resistant) or topotecan (1.2-fold resistant), which are not known to be affected by MRP ([9] and data not shown).

MRP is thought to cause multidrug resistance by decreasing the intracellular concentration of the cytotoxic drugs, and although most MRP-expressing cell lines show a defect in drug accumulation [20, 21, 32, and this study], others do not [10]. KB/7D cells accumulated less dox (Figs. 2 and 3) and VP-16 (Fig. 5) than the parental KB cells, while revertant KB/7D^r cells no longer possessed the accumulation defect. Treatment of the KB/7D cells with L-verapamil reversed this drug accumulation defect, suggesting that

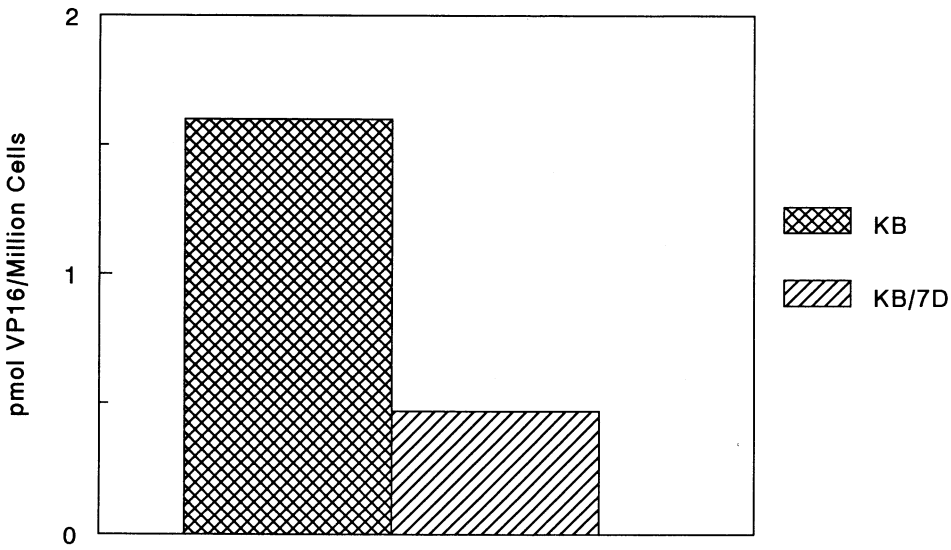

FIG. 5. Accumulation of VP-16 in KB and KB/7D cells. Cells were loaded into 25 cm^2 T flasks so as to give 1.5 to 3 × 10^6 cells per flask after 3 days. Then cells were loaded with 1 μM of [^{3}H]VP-16 (66 μCi/μmol) and allowed to accumulate drug for 4 hr at 37° + 5% CO_2. Radioactivity was determined immediately after removal of medium and washing twice with ice-cold PBS. Background values (see Materials and Methods) were subtracted out from the initial values. Experiments were performed twice, each time in duplicate, and the figure represents a single experiment with duplicate samples.

decreased drug accumulation contributes to MRP-mediated MDR, and that L-verapamil sensitizes the KB/7D cells by overcoming the accumulation defect.

There is strong evidence that MRP can act as an efflux pump for amphipathic anions, including glutathione conjugates of cytotoxic drugs [32, 35–37], but how this transport activity relates to multidrug resistance is unclear. MRP-overexpressing cells are not resistant to electrophilic anticancer drugs, which are often glutathione conjugated *in vivo*, while many natural product drugs that are affected by MRP overexpression, such as doxorubicin, are not glutathione conjugated [37–39]. Two models that could explain MRP-mediated multidrug resistance are given in Fig. 7. In model 1, MRP acts to actively transport cytotoxic drugs or drug conjugates out of the cell, leading to decreased intracellular drug concentrations and multidrug resistance as suggested by others [32, 35, 36, and reviewed in 37]. In model 2, MRP acts to push drugs or drug conjugates back to the extracellular space before entering the cell. MRP overexpression may inhibit entry into the membrane. Drugs that have entered into the cell are not substrates for MRP. This "kickback" model can explain how KB/7D cells become multidrug resistant due to decreased accumulation of drug without showing increased drug efflux. Since all the data presented here involve VP-16, it is possible that other drugs may behave differently in our cell line. It is clear, however, that the kickback model best explains the KB/7D cell response to VP-16.

Our model is clearly distinct from previously proposed models for MRP function [32, 35–37]. It is more similar, but not identical, to the hydrophobic vacuum cleaner model postulated for P-gp action [40]. This model proposes that P-gp removes cytotoxic drugs directly from the cell membrane. The key difference between the models is that P-gp must also act to transport drugs from within the cell, resulting in increased drug efflux, while the kickback model for MRP function suggests that drugs that have entered the cytosol are not able to be transported by MRP. The lack of enhanced efflux is what distinguishes the kickback model from the hydrophobic vacuum cleaner model.

Our model is consistent with experiments using inside-out membrane vesicles generated from MRP-transfected cells, which show natural product drugs to be poor substrates for active transport by MRP [36, 37]. These experiments measure either the transport of drugs into the vesicles or the inhibition of amphipathic anion transport by natural product drugs. Our model predicts that MRP will inhibit the entry of drugs into these vesicles. Furthermore, Versantvoort *et al.* [41] have shown that MRP may have multiple independent activities, one being the transport of amphipathic anions and the other relating to drug resistance. Cytotoxic drugs would then not be expected to inhibit the transport of amphipathic anions. The kickback model best explains the resistance to unconjugated natural product drugs by preventing their entry into the cell. If drug conjugates do form, they could be substrates for energy-dependent drug efflux as suggested by others [32, 35, 36 and reviewed in 37], but if they do not form, efflux will not be seen. This could explain why some groups see efflux and others do not [37].

Our model is also consistent with models suggesting that MRP could act to transport drugs indirectly through cytosolic vesicles [34, 37]. If drugs are endocytosed into the cell or associated with lipids, these complexes could be removed from the membrane before entry into the cell. If the removal of these vesicles was rapid, one would predict that

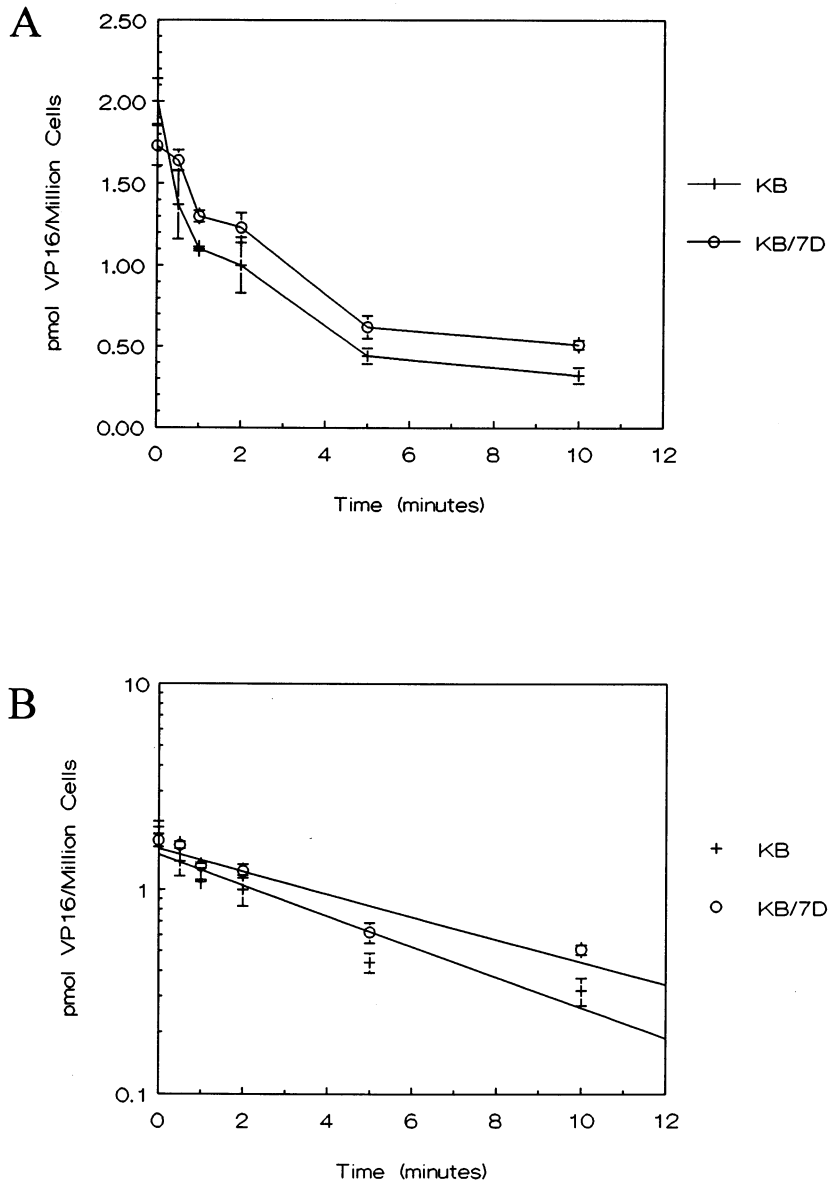

FIG. 6. Retention of VP-16 in KB and KB/7D cells. (A) Cells were treated as described for Fig. 5, using 1 μM of VP-16 for KB cells and 2.5 μM for KB/7D cells. Drug retention was determined as described in Materials and Methods. Experiments were performed three times, and data are from a representative experiment. (B) Identical data as in panel A, but plotted on a log scale.

few such vesicles would be visible by confocal microscopy, as seen in Fig. 2. If removal of the vesicles is slower, they could accumulate, as has been seen by others [34]. The fact that some groups see cytoplasmic vesicles and some do not [reviewed in 37] could simply relate to different kinetics of vesicle removal from different cell lines.

There is a large degree of phenotypic variability seen in MRP-overexpressing cells [37], and direct transport of natural product drugs has been observed in one set of experiments [42]. We do not claim that our model describes the only mechanism by which MRP may cause multidrug resistance, but we do feel that our kickback model can explain much of the experimental data. It is known that sequence variability exists between different MRP-overexpressing cells [19, 33, 36]. Mutations occurring within the MRP gene, other genes, or differences inherent to the tissues from which these tumors were derived could all lead to phenotypic variability. We have shown recently that our KB/7D cells have an MRP mRNA sequence identical to that of peripheral blood mononuclear cells from five healthy individuals [43]. If MRP sequence variability does lead to phenotypic variability, then it is likely that our cells will more closely approximate the phenotype of MRP-overexpressing tumors. Since numerous *mrp*-like genes have now been identified [44], some groups may be working with proteins related to MRP. It is highly unlikely that we are working with a protein other than MRP. First, the QCRL-1 antibody is a monoclonal not known to cross-react with other ABC transport proteins [45]. Second, we have completed comparative genomic hybridization experiments that show specific amplification of chromosome 16p in KB/7D cells, the known location of *mrp*-1, and we have probed northern blots using probes generated by polymerase chain reaction using different sets of primers specific to the

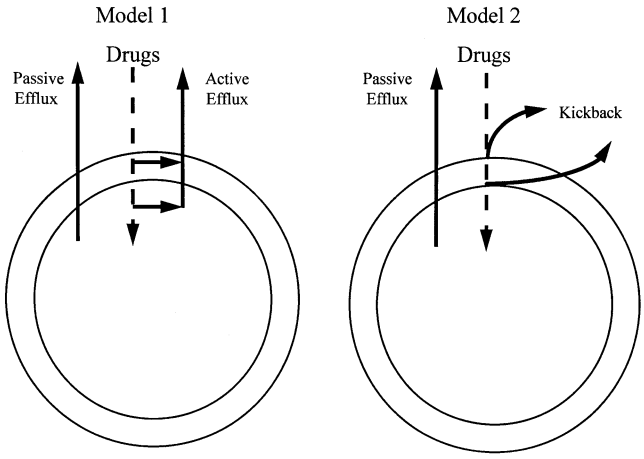

FIG. 7. Potential models for MRP-mediated MDR. Model 1 proposes that MRP can act as a drug efflux pump. Cytotoxic drugs, directly or conjugated, are transported by MRP out of the cell cytosol or plasma membrane, decreasing the intracellular concentration of drug by increasing drug efflux. In model 2, cytotoxic drugs interact with MRP on the exterior of the cell or within the plasma membrane and then are recycled back to the extracellular space, inhibiting drug uptake and decreasing the steady-state levels of intracellular drug without enhancing the rate of drug efflux. The interaction of the drugs with MRP could be direct or involve other carrier molecules. Model 2 better explains the data presented in our study, but we do not discount the possibility that other mechanisms could exist. These models are also not mutually exclusive, as different drugs may interact differently with MRP.

known MRP sequence, and they have always detected the same 6.5–kb transcript without additional bands (data not shown).

While MRP is overexpressed in KB/7D cells, other resistance mechanisms could exist. We believe that overexpression of MRP results in a drug accumulation defect, and this leads to some degree of drug resistance. Additional alterations then result in the full resistance phenotype seen in KB/7D cells. This hypothesis is supported by the MRP transfection experiments that show modest increases in drug resistance [23, 32] along with a slight drug accumulation defect [32]. In KB/7D cells, L-verapamil could overcome the MRP-mediated multidrug-resistance pathway, which would account for resistance to vincristine, but only a portion of the resistance to VP-16 and dox. Topo II alterations likely account for a portion of the resistance to VP-16 and dox, and this mechanism should not be affected by L-verapamil.

The reversal of KB/7D cell resistance by L-verapamil is the first report of stereospecific modulation of the MRP-mediated multidrug-resistance phenotype known to the authors. The reason for the inability of verapamil to show modulation of MRP in previous studies may be that racemic verapamil was used [10, 37]. Since the modulation effect is stereoselective (Tables 1 and 2), the previous studies may not have achieved a sufficient concentration of the L-enantiomer. This hypothesis is supported by our studies showing a modest modulation with 10 μM of racemic verapamil. Recently, it was demonstrated that another MRP-overexpressing cell line could be sensitized by racemic verapamil [41], while most others were not [37]. The ability of other MRP-expressing cells to be sensitized by L-verapamil will need to be examined. While L-verapamil did affect the KB cell response to cytotoxic drugs, the magnitude of the modulation was much weaker than in KB/7D cells (Table 1). Effects on KB cells are likely due to basal levels of MRP expression as BIBW 22 had no effect on drug toxicity in these cells. The possibility of basal levels of MRP causing resistance to toxic compounds is supported by recent experiments that show increased drug sensitivity in *mrp* knockout mouse cells [46].

The major impetus for studying multidrug-resistance-related proteins is their potential effect on chemotherapy in the clinic. Given the mounting evidence that MRP is expressed in human tumors [47–50], understanding the mechanism by which MRP causes multidrug resistance may have a significant impact on our ability to treat human cancer.

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