0026-895X/90/060790-07$02.00/0

MOLECULAR PHARMACOLOGY, 37:790–796

P-Glycoprotein-Independent Mechanism of Resistance to VP-16 in Multidrug-Resistant Tumor Cell Lines: Pharmacokinetic and Photoaffinity Labeling Studies

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Received June 20, 1989; Accepted February 6, 1990

SUMMARY

The interaction of etoposide (VP-16), *Vinca* alkaloids, and verapamil with the P-glycoprotein (P-gp) was studied in human breast (MCF-7) and Chinese hamster lung (DC3F) cell lines and the corresponding multidrug-resistant MCF-7/ADR and DC3F/ADX tumor cell lines, selected for resistance to Adriamycin and actinomycin D, respectively, and overexpressing P-gp. Verapamil (10 μM) markedly reversed resistance to vincristine (11-fold in DC3F/ADX and 125-fold in MCF-7/ADR; 1-hr exposure), but it had a very modest effect on resistance to VP-16 (3- to 4-fold; 1-hr exposure). Resistant cells accumulated 2- to 4-fold less VP-16 and vincristine than the parental cell lines. Verapamil (10 μM) significantly increased accumulation and retention of vincristine, but not of VP-16, in resistant cell lines. Photoaffinity labeling of resistant cell lines with radioactive analogs of verapamil [*N*(*p*-azido-3-^{125}I-salicyl)-*N'*-β-aminoethylverapamil (NASVP)] and vinblastine[*N*-(*p*-azido-3-^{125}I-salicyl)-*N'*-β-aminoethylvindesine (NASV)] showed distinctly labeled P-gp bands in both resistant cell lines, compared with wild-type cells. Excess nonradioactive vinblastine or verapamil effectively competed with the P-gp photolabeling by either NASVP or NASV, with IC_{50} levels of 0.6 and 10 μM, respectively. In contrast, nonradioactive VP-16 was 100- to 500-fold less potent than vinblastine in competing with P-gp photolabeling, suggesting that VP-16 has significantly lower affinity for P-gp than *Vinca* alkaloids have. Taken together, our data indicate that P-gp glycoprotein by itself may not be important in the transport/efflux of VP-16 and, thus, in the mechanism of resistance to VP-16 in these cells.

Multidrug resistance to cytotoxic agents has been associated with the presence of a membrane glycoprotein M_r 170,000–180,000 (P-gp) in both tumor cell lines and human tumors (1, 2). This glycoprotein has been proposed to confer resistance to structurally unrelated compounds such as *Vinca* alkaloids, anthracyclines, colchicine, and epipodophyllotoxin derivatives (2, 3). Energy-dependent drug export of vincristine and anthracyclines has been demonstrated in MDR cell lines (3); furthermore, pharmacologic inhibition of the P-gp transporter by calcium antagonists, calmodulin inhibitors, and monoclonal antibodies against P-gp resulted in increased accumulation of vincristine and anthracyclines, due to decreased drug efflux (4, 5). However, the involvement of P-gp in the mechanism of resistance to VP-16 remains unclear. For example we have shown recently that defective drug accumulation, considered to be a hallmark of P-gp-mediated resistance, is very modest (2–3-fold) for VP-16 in MCF-7/ADR cells, which are highly cross-resistant to VP-16 and which overexpress P-gp (6). Furthermore, although energy depletion by means of glucose-free medium supplemented with sodium azide increased VP-16 accumulation in resistant cells, it had no effect on the drug efflux rate (6). Moreover, MCF-7/ADR cells accumulated 170-fold more VP-16 and retained 530-fold more drug than the parental cell line when exposed to equitoxic (IC_{50}) concentrations of the drug (6).

With the aim of more clearly defining the role of P-gp in the mechanism of resistance to VP-16, we have examined the effects of verapamil on both cellular pharmacokinetics and cytotoxicity of VP-16. Verapamil has previously been shown to dramatically reverse drug resistance by enhancing drug accumulation in MDR cell lines, presumably through its competitive binding to P-gp (4). We have shown here that verapamil has an insignificant effect on VP-16 accumulation and only a modest effect on VP-16 cytotoxicity, compared with vincristine. In addition, we have used quantitative competition of P-gp photoaffinity labeling with radioactive photoactivatable ana-

ABBREVIATIONS: P-gp, M_r 170,000–180,000 glycoprotein; MDR, multidrug-resistant; VP-16, etoposide (VP-16-213); PBS, phosphate-buffered saline (containing 120.4 mM KH_2PO_4, 153.8 mM NaCl, 5.5 mM Na_2HPO_4, pH 7.40); PEG, polyethyleneglycol (M_r = 200); DMSO, dimethylsulfoxide; ^{125}I-NASVP, *N*-(*p*-azido-3-^{125}I-salicyl)-*N'*-β-aminoethylverapamil; ^{125}I-NASV, *N*-(*p*-azido-3-^{125}I-salicyl)-*N'*-β-aminoethylvindesine; WT, wild-type; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

logs of both verapamil and vinblastine (7–9) to demonstrate a markedly lower affinity of VP-16 for P-gp, compared with vinblastine. The low affinity of VP-16 for P-gp is consistent with the absence of an effect by verapamil and suggests that VP-16 resistance occurs primarily by P-gp-independent mechanisms.

Materials and Methods

Drugs. VP-16 was a gift from Bristol-Myers Co. (Syracuse, NY) and was dissolved in PEG (Sigma, St. Louis, MO) and stored at −70°. [^{3}H]VP-16, labeled in the aromatic rings (900 mCi/mmol; nonexchangeable and 99.5% pure by high pressure liquid chromatography), was obtained from Moravek Biochemicals, Inc. (Brea, CA). Vincristine was obtained from the Drug Development Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD). Vinblastine sulfate was purchased from Sigma. [^{3}H]Vincristine sulfate (6.2 Ci/mmol; 99% pure by HPLC) and [^{3}H]vinblastine sulfate were from Amersham Corp. (Arlington Heights, IL). (±)-Verapamil hydrochloride was purchased from Sigma. The radioactive photoactive verapamil derivative ^{125}I-NASVP was prepared by procedures similar to those previously described for the vinblastine analog ^{125}I-NASV (8, 10). Briefly, *N*-hydroxysuccinimidyl-4-azidosalicylate (0.5 nmol) (Pierce Chemical Co.) was iodinated and purified as described (11). The iodinated compound was dried with a stream of nitrogen gas, dissolved in 0.200 ml of tetrahydrofuran, mixed with 0.56 μmol of (±)-5-[(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylpentylamine (12), and incubated 60 min at room temperature. The reaction mixture was then absorbed to a silica gel column (0.4 × 5 cm) equilibrated in chloroform, the column was washed with 1% methanol in chloroform to remove impurities, and the ^{125}I-NASVP was eluted with 2% methanol in chloroform. The purified ^{125}I-NASVP was dried with a stream of nitrogen gas and stored in chloroform at −70°. Thin layer silica gel chromatography (chloroform/methanol/40% methylamine, 80:20:4) indicated that >80% of the radioactivity cochromatographed with authentic nonradioactive material having an R_F = 0.675.

Cells. MCF-7 human breast tumor cells, sensitive (MCF-7 WT) and MDR (MCF-7/ADR), were provided by Dr. Kenneth H. Cowan, Medicine Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD). Both cell lines were grown in monolayer in improved minimum essential medium supplemented with 2 mM glutamine, 5% fetal bovine serum (GIBCO, Grand Island, NY), and 50 μg/ml gentamycin, under an atmosphere containing 5% CO_2. MCF-7/ADR cells had been selected for resistance to Adriamycin and displayed cross-resistance to a number of antitumor drugs including VP-16, vincristine, and vinblastine (13). Further, MCF-7/ADR overexpress P-gp (14). Resistance was stable for about 52 weeks in the absence of drugs (13).

DC3F Chinese hamster lung cancer cells and the resistant line DC3F/ADX (provided by Dr. June Biedler, Memorial Sloan Kettering Cancer Center, New York, NY) were cultured in monolayer in a mixture of equal volumes of Ham's F-12 and minimum essential Eagle medium (modified) with Earle's salts and glutamine (both from Flow Laboratories, McLean, VA), supplemented with nonessential amino acids, 5% fetal bovine serum and 20 μg/ml gentamycin. DC3F/ADX was selected for resistance to actinomycin D and displays cross-resistance to a variety of cytotoxic drugs (15). Moreover, these cells overexpress P-gp (16). Because resistance in this cell line is not stable in the absence of drugs, DC3F/ADX cells were grown in the presence of 5 μg/ml actinomycin D. However, resistant cells were grown in drug-free medium for at least two passages before being used in experiments. All cells were in exponential growth.

Uptake of VP and vincristine. Cells were grown to 50–70% confluency in 25-cm^2 flasks and treated with a drug mixture containing 0.5 μCi/ml [^{3}H]VP-16, plus nonradioactive VP-16 in PEG, dissolved in fresh complete tissue culture medium (final PEG concentration, <0.1%; final VP-16 concentration, 1 μM unless otherwise stated). Cells were incubated at 37° under a 5% CO_2 atmosphere. Uptake studies were carried out as previously reported (6). Briefly, at the appropriate times, flasks were removed and, after the medium was aspirated, cells were washed three times with 10 ml of ice-cold PBS (Biofluids, Rockville, MD), added carefully to the side. Cell monolayers were treated with 1 N NaOH to break up cell membranes and to dissolve the protein. For radioactivity determinations, aliquots were neutralized with 1 N HCl and dissolved in 10 ml of Hydrofluor (National Diagnostics, Manville, NJ), and the radioactivity was counted with a Packard 2000 CA Tri-Carb liquid scintillation counter (Packard Instrument Co, Sterling, VA), with appropriate quenching corrections. The protein concentration was determined by means of the Lowry assay (17), with albumin as the standard. Zero time values for drug uptake were obtained by adding the radiolabeled mixture to cells on ice, immediately aspirating the medium, and washing, as indicated above. Zero time values were substracted from each subsequent determination of cellular drug content, for both the uptake and efflux studies. All manipulations except incubations were performed on ice. Incubation medium was prewarmed to 37°.

Vincristine (both tritiated and nonradioactive) was substituted for VP-16 at the same final concentrations indicated for the latter, and vincristine uptake and efflux studies were performed as described for VP-16. Verapamil, dissolved in glass-distilled water (final concentration, 10 μM; 0.025% distilled water, v/v) or an equivalent volume of the solvent, was preincubated for 30 min at 37° under a CO_2 atmosphere. Verapamil was present throughout the procedure in the appropriate incubations.

Drug efflux measurements. Efflux experiments were carried out after the cells were loaded with the above-mentioned radiolabeled drug mixture (either VP-16 or vincristine) for 60 min. The drug was then removed by two washes of the cells with ice-cold PBS and incubation in an equal volume of fresh drug-free medium at 37°. At the prescribed times, flasks were removed and processed as indicated for uptake studies. Verapamil (10 μM) was present in the medium for the appropriate incubations. We recently studied the cellular pharmacokinetics of VP-16 in detail with this technique and reported that the efflux of VP-16 is both temperature and energy dependent in MCF-7 cell lines under these conditions (6).

Cytotoxicity determinations. Because the MTT assay allows a rapid evaluation of a large number of wells, this technique was used to assess the ability of verapamil to reverse resistance to VP-16 and vincristine in all four cell lines. The MTT colorimetric assay relies on the reduction of the MTT tetrazolium salt to a colored formazan product by enzymes present only in metabolically active cells (18). At least part of this metabolic activity depends on the mitochondrial function of the cells, and succinate dehydrogenase is but one of the identified participating enzymes (18). The MTT assay is being used with increasing frequency in order to evaluate the cytotoxic effects of antitumor agents. Moreover, it has been adopted as part of the drug-screening program at the National Cancer Institute (18). A slightly modified version of the published protocol was used (19). Cells were harvested from exponential-phase cultures by trypsin-EDTA, suspended in medium, and plated in 96-well plates (Costar) at 2000 cells/well. After 24 hr of incubation at 37° in a 5% CO_2 humidified atmosphere, cells were treated in the presence or absence of verapamil, following exactly the same protocol used for transport studies, a 30-min preincubation, a 60-min exposure to either VP-16 or vincristine, plus a final 60-min incubation without VP-16 or vincristine. Medium with either verapamil (to a final concentration of 10 μM) or solvent (glass-distilled water, 0.05%, v/v) was added in a volume of 50 μl/well and incubated for 30 min. After addition of 25 μl/well of either verapamil or medium to adjust the final volume, VP-16 or vincristine was added in a broad range of concentrations, at 50 μl/well, and then the plates were incubated for 60 min. Control wells received similar concentrations of solvents (DMSO for experiments with VP-16, glass-distilled water when vincristine was used). Medium was then carefully removed with a multichannel pipette and replaced with 200 μl/well of

either verapamil (10 μM) or medium with solvent, for an additional 60-min incubation. At that time, the medium was again removed, 200 μl of fresh drug-free medium were distributed to each well, and cells were allowed to grow for 120 hr. MTT (from Sigma; 2 mg/ml in PBS; 50 μl/well) was added and then incubated for 3 hr. The medium was removed by decanting and blotting after the plates were centrifuged for 10 min at 1850 rpm, and 120 μl of DMSO/well were added. The plates were then agitated on an orbital shaker for 30 min to solubilize the formazan grains, and the absorbance of each well was measured at 570 nm with a microplate reader (Molecular Devices, CA). Data were collected as replicates of six wells and the cytotoxicity was defined as the survival fraction of cells, determined by the formula: survival fraction = (absorbance of treated cells − absorbance of medium)/(absorbance of control cells − absorbance of medium).

Photoaffinity labeling. Mixed membrane vesicles were prepared from drug-free cultures of the four cell lines essentially as previously described (20). Aliquots were stored at −70°. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) (21). Ten micrograms of membrane vesicle protein in 20 mM HEPES buffer (pH 7.4) were incubated with 1 μl of stock solution (0–25 mM) of VP-16 or vinblastine and 1 μl of ^{125}I-NASV (specific activity, 1.64×10^{12} cpm/mmol) or ^{125}I-NASVP (specific activity, 2.47×10^{12} cpm/mmol), for 10 min at 25°, as described previously (8–10). The assay was performed in a total volume of 50 μl in polystyrene microtiter plates. ^{125}I-NASVP, ^{125}I-NASV, and VP-16 were dissolved in DMSO and vinblastine was dissolved in deionized water. Appropriate solvent controls were included in all experiments. The photolabeled samples were solubilized in sodium dodecyl sulfate sample buffer, counted, and separated on 5–15% polyacrylamide gradient gels containing 4.5 M urea. Gels were stained with Coomassie blue R-250, dried, and exposed to Kodak XAR-2 film, and specific P-gp labeling was quantitated by cutting the portion of the gel corresonding to P-gp and counting it in a γ-counter. The possibility that the apparent inhibition was due to inner filtering of the activating UV light was checked by cutting and counting a portion of each gel corresopnding to several nonspecifically labeled proteins (M_r 66,000–115,000) and verifying that there was no change in radioactivity with increasing concentrations of drug competitors. Nonspecific inhibition was not observed for any of the drugs up to at least 100 μM.

Statistical analysis. Student's t test and one-way analysis of variance were used when appropriate to evaluate statistical significance of differences. Values of p smaller than 0.05 were considered significant.

Results

Cytotoxicity

Both MCF-7/ADR and DC3F/ADX were highly cross-resistant to VP-16 and vincristine (MCF-7/ADR were 55- and 5567-fold resistant to VP-16 and vincristine, respectively; DC3F/ADX were 160- and 360-fold resistant to these drugs, in the same order) (Table 1). A high degree of resistance was confirmed with the growth-inhibition assay by cell counts (data not shown). Moreover, under conditions that were comparable to those for cellular pharmacokinetic assays (30-min preincubation, 60-min exposure to cytotoxic drugs, and 60-min incubation after removal of cytotoxic drugs), verapamil (10 μM) reduced the IC_{50} of vincristine by 11- and 125-fold in DC3F/ADX and MCF-7/ADR cell lines, respectively. In contrast, verapamil decreased the IC_{50} of VP-16 by only 3- to 4-fold in these resistant cells. Increasing the concentration of verapamil to 50 μM failed to reduce the IC_{50} of VP-16 any further (data not shown). Verapamil (10 μM) had little or no effect on the cytotoxicity of either VP-16 or vincristine in sensitive MCF-7 cells. However, verapamil decreased the IC_{50} of vincristine and VP-16 by 9- and 12-fold, respectively, in the parental DC3F cell line. Verapamil (up to 50 μM) did not inhibit cell proliferation by more than 10% under these conditions (data not shown).

Cellular Pharmacokinetics

Uptake and efflux of VP-16 in resistant cell lines: lack of any effect of verapamil. Because both MCF-7/ADR and DC3F/ADX cells were highly cross-resistant to VP-16 and *Vinca* alkaloids, we next studied uptake and retention of drugs to evaluate the role of defective drug transport in the phenomenon of resistance. Whereas cellular levels of VP-16 apparently reached a plateau at 30 min in MCF-7/ADR (values not significantly different from those at 60 min), DC3F/ADX continued to accumulate VP-16 even after 60 min (Fig. 1). Most of the accumulated VP-16 (80–90%) was rapidly released from both resistant cell lines after removal of the drug-containing medium and exposure to drug-free medium. However, that VP-16 is not simply washed out from the cells was previously demonstrated by the fact that most of the drug was retained when MCF-7 cells (sensitive and resistant) were washed using the same protocol and then incubated in drug-free medium on ice for up to 90 min (6). Further, the efflux pattern of VP-16 in these cell lines is similar to previously published results with VP-16 and VM-26 by other investigators in several tumor cell lines (22–25). Under these conditions, verapamil (10 μM) had no significant effect on the cellular accumulation of VP-16 in either resistant cell line. Moreover, verapamil did not affect the efflux pattern or the retention of VP-16 in either resitant cell line. Increasing the concentration of verapamil to 50 μM increased accumulation of VP-16 by a factor of 2 in both MCF-7 WT and MCF-7/ADR, such that the transport defect remained at 2-fold (14.65 and 5.84 pmol/mg of protein, respectively; average of two experiments). However, no effect on retention of VP-16 or on the pattern of drug efflux was observed under these conditions (data not shown).

Uptake and efflux of vincristine in resistant cell lines: effects of verapamil. In contrast to what was observed with VP-16, verapamil (10 μM) significantly enhanced (2–3-fold) the cellular accumulation of vincristine in both MCF-7/ADR and DC3F/ADX (Fig. 2). Furthermore, verapamil increased the retention of vincristine 4-fold in both resistant cell lines. Increasing the concentration of verapamil to 50 μM failed to elicit a more pronounced effect (mean of three experiments within 10% range of values obtained with 10 μM; data not shown). Similar effects of verapamil (10 μM) on cellular pharmacokinetics were observed when vinblastine was substituted for vincristine under similar conditions (data not shown).

Pharmacokinetics of VP-16 and vincristine in parental cell lines: effects of verapamil. Accumulation of VP-16 in sensitive MCF-7 cells was 2-fold higher than in MCF-7/ADR cells (5.62 ± 0.36 versus 2.68 ± 0.41 pmol/mg of protein; $p < 0.05$), whereas sensitive DC3F cells accumulated only 48% more VP-16 than DC3F/ADX (10.96 ± 0.11 versus 7.39 ± 0.77 pmol/mg of protein). As noted with both resistant lines, VP-16 was rapidly and extensively (75 to 94%) released from both sensitive cell lines (Fig. 3, A and B). Verapamil (10 μM) did not modify the accumulation of VP-16 at 60 min in MCF-7 and DC3F parental cell lines (Fig. 3, A and B). However, significant increases in cellular levels of VP-16 were observed earlier during the incubation in MCF-7 WT cells only (at 5, 15, and 30 min). As shown for the resistant cells, verapamil did not alter the efflux pattern of VP-16 in either sensitive cell line.

TABLE 1

Cytotoxicity of VP-16 and vincristine in MCF-7 and DC3F tumor cell lines

MTT growth-inhibition assays were performed as previously described (26); IC_{50} is the drug concentration that inhibits growth by 50%. The numbers in parentheses represent the ratio of IC_{50} without verapamil/IC_{50} with verapamil (10 μM), for the resistant cells. Values are mean ± standard error of at least three separate experiments performed in six replicates. See Materials and Methods for details.

Cell line	IC_{50}			
	VP-16		Vincristine	
	−Verapamil	+Verapamil	−Verapamil	+Verapamil
	μM			
MCF-7 WT	4.33 ± 0.67	2.75 ± 0.25	0.030 ± 0.012	0.030 ± 0.010
MCF-7/ADR	237 ± 12 (3.3)	72 ± 5*	167 ± 43 (125)	1.33 ± 0.33*
DC3F WT	9.67 ± 2.67	0.77 ± 0.06*	3.78 ± 0.93	0.42 ± 0.09*
DC3F/ADX	1550 ± 166 (3.9)	400 ± 202*	1360 ± 103 (11.3)	120 ± 65*

*$p < 0.05$ versus drug without verapamil.

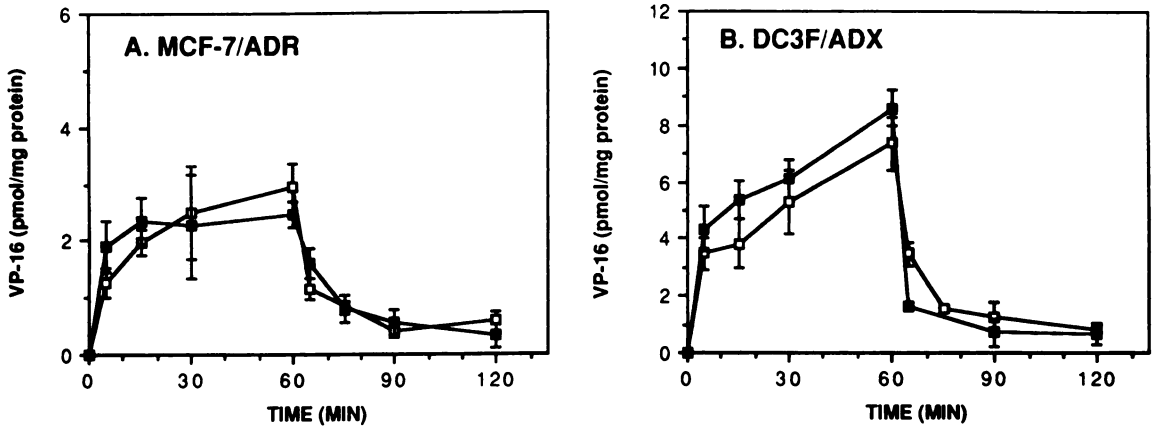

Fig. 1. Uptake and efflux of VP-16 in MCF-7/ADR (A) and DC3F/ADX (B) MDR cell lines: effects of verapamil. Incubations were performed in complete medium at 37°, in the presence of 1 μM VP-16, with (■) or without (□) the addition of 10 μM verapamil. At 60 min, cells were washed and exposed to medium free of VP-16. However, 10 μM verapamil was present throughout in the appropriate group (■). *Bars*, standard errors for at least three separate experiments. See Materials and Methods for details. Note different vertical scales.

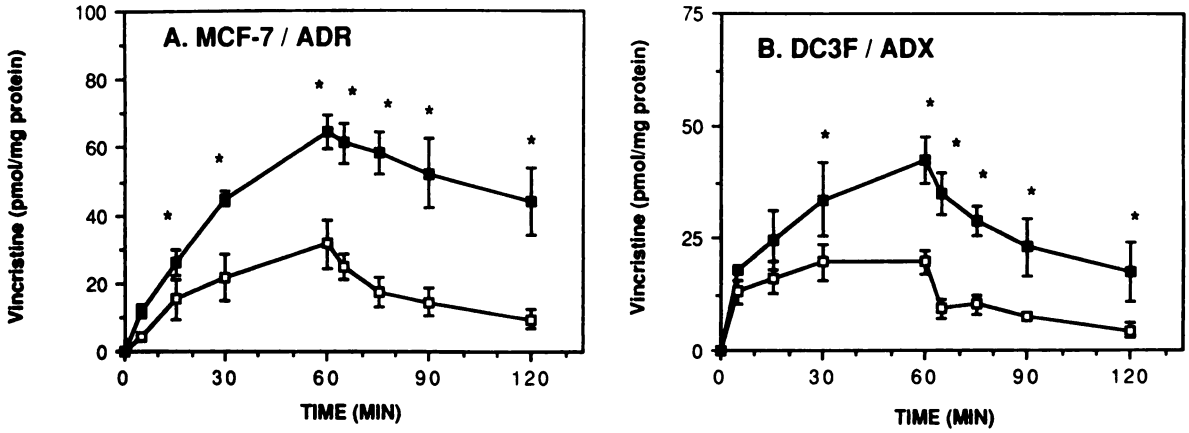

Fig. 2. Effects of verapamil on the uptake and efflux of vincristine (1 μM) in MCF-7/ADR (A) and DC3F/ADX (B) MDR cell lines. Conditions and symbols as for Fig. 1. See Materials and Methods for details. Note different vertical scales. * $p < 0.05$ versus experiments without verapamil.

The accumulation of vincristine did not reach steady state levels in either sensitive cell line by 60 min of incubation (Fig. 3, C and D). At this time point, however, cellular levels of vincristine were 3- to 4-fold higher in sensitive cells than in resistant cells (148.98 ± 26.1 versus 36.68 ± 5.13 pmol/mg of protein for MCF-7 and MCF-7/ADR, and 65.49 ± 2.82 versus 19.64 ± 2.13 pmol/mg of protein for DC3F and DC3F/ADX). A higher percentage of the accumulated vincristine was retained in sensitive MCF-7 and DC3F cells (65 and 50%, respectively), compared with the resistant cells, resulting in 9- and 7-fold higher noneffluxable drug pools in the parental cell lines. Although verapamil (10 μM) had no significant effect on the uptake and efflux of vincristine in sensitive MCF-7 cells, this calcium channel blocker increased both accumulation and retention of vincristine in the DC3F parental cell line.

Our results, thus, indicate that, although verapamil effectively competes for P-gp-dependent export of vincristine in resistant cells, this calcium channel blocker does not modify

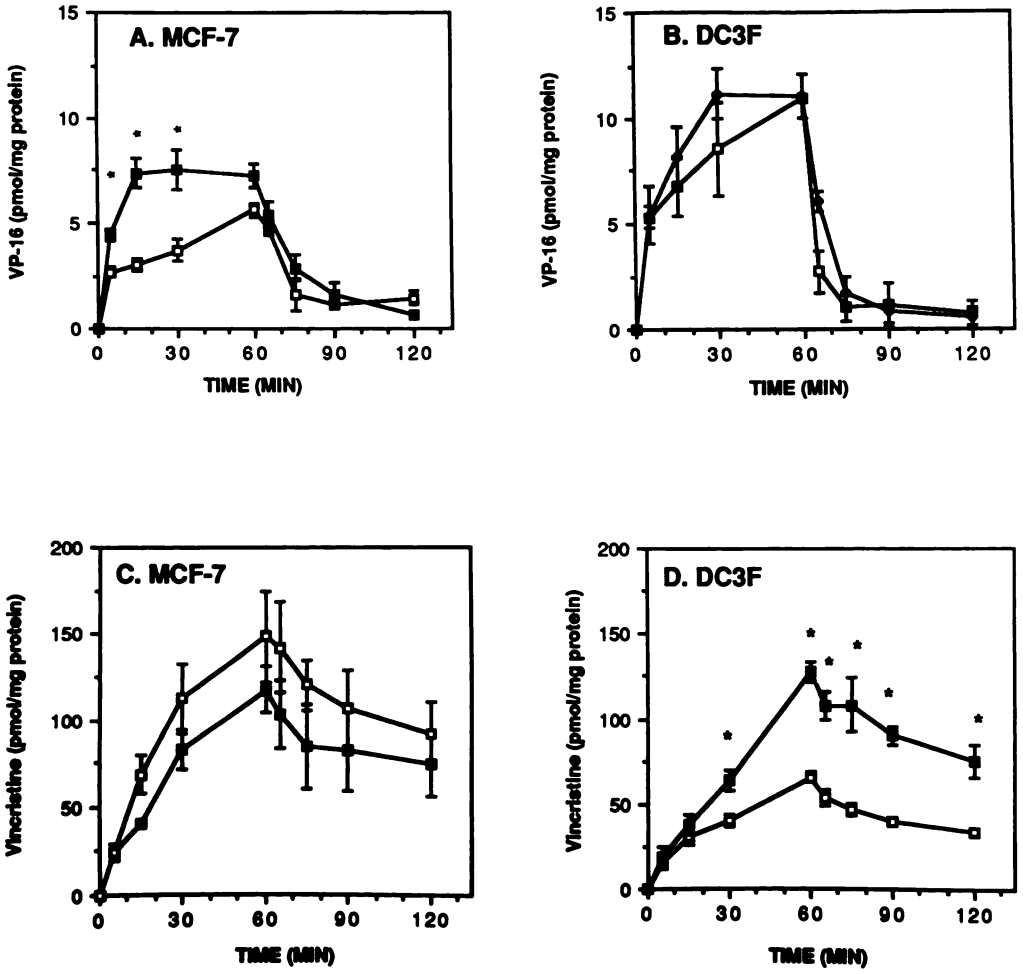

Fig. 3. Uptake and efflux of VP-16 and vincristine in drug-sensitive MCF-7 and DC3F cell lines: effects of verapamil. Incubations were performed in complete medium at 37°, in the presence of 1 μM of either VP-16 (A and B) or vincristine (C and D), with (■) or without (□) the addition of 10 μM verapamil. At 60 min, cells were washed and exposed to medium free of VP-16 or vincristine. However, 10 μM verapamil was present throughout in the appropriate group (■). $^*p < 0.05$ versus medium without verapamil. *Bars*, standard errors for at least three separate experiments. See Materials and Methods for details.

the cellular pharmacokinetics of VP-16 in the MDR cell lines studied here. This suggests that P-gp may play a much less important role, if any, in the cellular pharmacokinetics of VP-16, compared with those of vincristine.

Photoaffinity Labeling of P-gp

The interactions of these drugs with P-gp were also examined by photoaffinity labeling. Membrane vesicles from sensitive and resistant cell lines were photolabeled with the photoactive analog of vinblastine, ^{125}I-NASV, or verapamil, ^{125}I-NASVP, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Vesicles from both sensitive cell lines, MCF-7 and DC3F, showed little or no labeling in the M_r 170,000 region of the gel. Representative results shown in Fig. 4 include the photolabeling of DC3F vesicles with ^{125}I-NASV (Fig. 4, *lane 1*) and the photolabeling of MCF-7 vesicles with ^{125}I-NASV (Fig. 4, *lane 6*). On the other hand, as expected, vesicles from both resistant cell lines, MCF-7/ADR and DC3F/ADX, exhibited enhanced radiolabeling in the vicinity of M_r 170,000 when photolabeled with ^{125}I-NASVP (Fig. 4, *lanes 2* and *9*) or with ^{125}I-NASV (Fig. 4, *lanes 4* and *7*). The specificity of photolabeling was demonstrated by the substantial inhibition of P-gp labeling in the presence of 100 μM verapamil (Fig. 4, *lanes 3* and *5*) or 10 μM vinblastine (see Fig. 5) in the absence of any effect on background labeling. In contrast, VP-16 appeared to be a much weaker inhibitor of P-gp photolabeling. For example, 500 μM VP-16 apparently only partially blocked the ^{125}I-NASV or ^{125}I-NASVP photolabeling of P-gp in MCF-7/ADR vesicles (Fig. 4, *lanes 8* and *10*). However, because in some experiments the background labeling in the gel also appeared to be reduced (Fig. 4, *lane 8*) and because the absolute level of radioactivity incorporated was too low to accurately quantitate differences, the specificity of VP-16 inhibition was suspect. In order to more accurately assess the relative differences in P-gp affinity for vinblastine, verapamil, and VP-16, these drugs were utilized as competitive inhibitors of ^{125}I-NASV photolabeling of DC3F/ADX membrane vesicles over a broad concentration range (Fig. 5). Because this cell line expresses much higher levels of P-gp than MCF-7/ADR, the inhibition of P-gp labeling relative to the inhibition of background labeling was more accurately determined. Vinblastine exhibited the highest relative affinity for P-gp with a 50% inhibition concentration of 0.6 μM, whereas verapamil exhibited a 50% inhibition concentration of 10 μM. In contrast, VP-16 required concentrations in excess of 100 μM to inhibit P-gp labeling by 50%. These results are in line with the pharmacokinetic data and further support the suggestion that VP-16 has a markedly lower affinity for P-gp than the *Vinca* alkaloids.

Discussion

P-gp has been suggested to play a central role in the development of multidrug resistance, by binding drugs and exporting them by an energy-dependent process (1, 3). Further, all anticancer drugs belonging to the natural product group have been

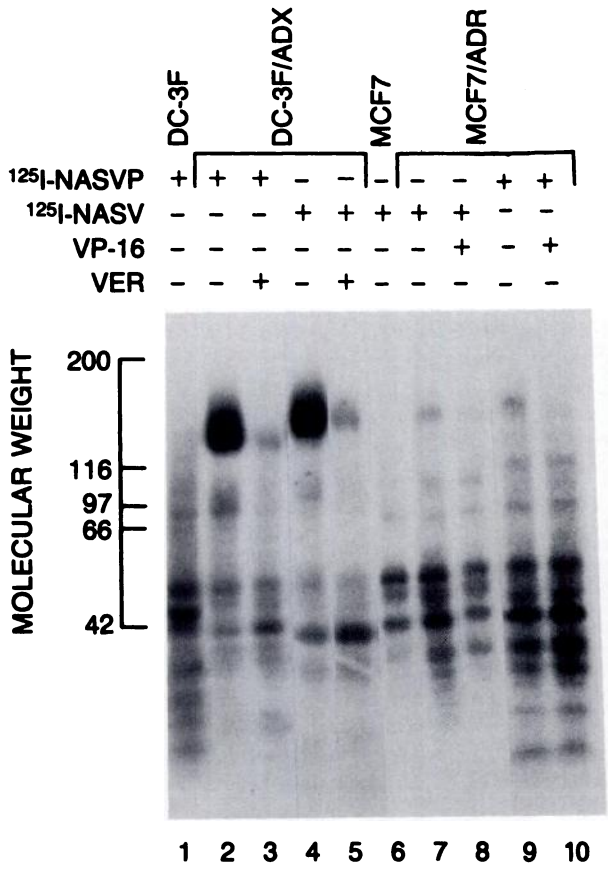

Fig. 4. Photoaffinity labeling with ^{125}I-NASVP and ^{125}I-NASV of P-gp in membrane vesicles from drug-sensitive and -resistant cell lines. Membrane vesicles (10 μg of protein) from DC3F (*lane 1*), DC3F/ADX (*lanes 2–5*), MCF-7 (*lane 6*), and DC3F/ADX (*lanes 7–10*) were photolabeled with ^{125}I-NASVP (*lanes 1–3*, *9*, and *10*) or ^{125}I-NASV (*lanes 4–8*) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Specificity is demonstrated by competition with 100 μM verapamil (*VER*) (*lanes 3* and *5*) or with 500 μM VP-16 (*lanes 8* and *10*). The figure is a composite of several separate experiments, where autoradiograms for *lanes 1–3* were exposed for 2 days, *lanes 4–5* were exposed for 1 day, and *lanes 6–10* were exposed for 8 days. Molecular weight standards are indicated on the *left* (×1000).

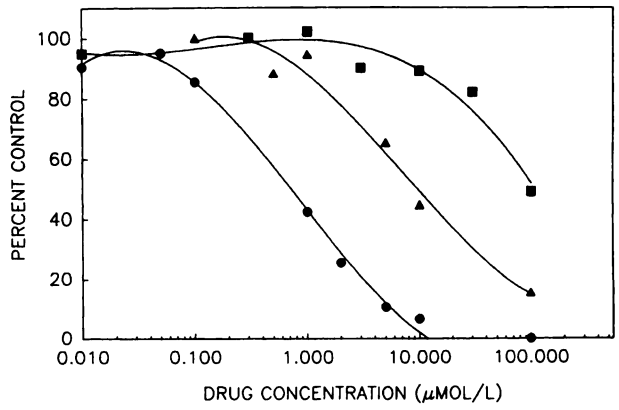

Fig. 5. Competitive inhibition of ^{125}I-NASV photoaffinity labeling of P-gp with vinblastine, verapamil, and VP-16. Membrane vesicles (10 μg of protein) from DC3F/ADX cells were photolabeled with ^{125}I-NASV in the presence of increasing concentrations of vinblastine (●), verapamil (▲), or VP-16 (■) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Radiolabeled P-gp was cut from the gel and counted as described in Materials and Methods. Each data point represents the mean of duplicate determinations.

proposed to be similarly transported, at least qualitatively, by this P-gp transporter (3). However, data reported in this manuscript suggest that P-gp may not play an important role in the efflux of VP-16 and, thus, may not contribute significantly to the resistance to VP-16 in the cell lines examined in this study. In contrast to the *Vinca* alkaloids, we found that VP-16 has a very much reduced affinity for P-gp. Firstly, VP-16 competed weakly for the labeling of P-gp by either the vinblastine or verapamil photoaffinity analogs. Secondly, in the pharmacokinetic studies, verapamil did not elicit a selective increase in the cellular retention of VP-16 in the resistant cell lines examined here, even at high concentrations of the calcium antagonist (50 μM; data not shown). Thirdly, verapamil only modestly enhanced the cytotoxicity of VP-16 in MDR cells. In contrast, verapamil markedly increased accumulation, retention, and cytotoxicity of vincristine in both resistant cell lines.

When results from cytotoxicity, pharmacokinetics, and photoaffinity labeling studies are considered together, the following observations are in order. (a) High levels of drug resistance are not associated with similarly marked alterations in drug transport. Resistant cells exhibit 2- to 4-fold decreased accumulation (compared with parental cells) of VP-16 and vincristine in the presence of a high degree of cross-resistance to both agents. However, this has been reported for several other MDR cell lines and for a number of drugs as well (5, 6, 13). It is not clear at present whether transport defects account for the bulk of the drug resistance phenomenon, in mechanistic terms. (b) In both resistant cell lines, verapamil induced a 3- to 4-fold shift in cytotoxicity of VP-16 without any significant effect on the cellular pharmacokinetics of this drug. Although the mechanism is not clear, several hypotheses are being currently explored in our laboratory, including the possibility of verapamil-induced modifications of DNA damage and repair after cytotoxic treatment. Further, verapamil has been shown to modify the intracellular distribution of cytotoxic drugs (e.g., anthracyclines) in resistant cells, so that more drug is available to the nucleus (26) and it seems reasonable to assess whether this is also true for VP-16. On the other hand, verapamil markedly reverses cross-resistance to vincristine in both MCF-7/ADR and DC3F/ADX, while enhancing vincristine accumulation and retention by a factor of 2 to 4. (c) Verapamil showed different effects on the parental cell lines tested. Verapamil produced modest or no changes in either cytotoxicity or transport of VP-16 and vincristine in MCF-7 WT cells. On the other hand, verapamil enhanced the cytotoxicity of both VP-16 and vincristine by about 10-fold in DC3F WT cells. Interestingly, this was accompanied by no modification in uptake and efflux of VP-16 in this cell line. In contrast, increased vincristine accumulation and retention may account for the enhanced cytotoxicity in DC3F cells. This finding, however, is not without precedence, inasmuch as verapamil has been reported to increase the cellular accumulation and cytotoxicity of VP-16 and other antineoplastic drugs in several other sensitive cell lines, with 10 μM verapamil promoting clear-cut effects (25, 27). Because P-gp is not detectable in sensitive cell lines, these observations suggest that verapamil may have other actions in tumor cells, inasmuch as both *d*- and *l*-isomers of verapamil are equally effective in reversing drug resistance (28). Verapamil has been shown to block calcium channels and, at higher concentrations, sodium channels as well (29). Moreover, verapamil-induced modifications in membrane fluidity have been

reported (30). Further, verapamil, vincristine, and VP-16 undergo cytochrome P-450-mediated metabolism (31–33), thus raising the possibility of still another site of drug interaction. Whether this might also apply to VP-16 is being currently investigated in our laboratory

All these data taken together strongly suggest that P-gp-dependent drug export plays only a minor role in the phenomenon of resistance to VP-16 in these cell lines. This is clearly illustrated by the observations that VP-16 levels are at most 3-fold lower in MDR cells, as compared with the sensitive cells, even though the IC_{50} may be 40- to 160-fold greater than that in the parental cell lines. Resistance to VP-16 has been reported to be associated with a number of mechanisms, including decreased drug accumulation, binding, and retention (4, 34), modifications in the content or activity of its target enzyme, topoisomerase II (35), with subsequent decreases in DNA strand breaks, and differences in activation/detoxification processes (36, 37). As reported with other drugs (37), the mechanisms of resistance to VP-16 appear to be multifactorial (38), and P-gp seems to be just one of the multiple factors involved. Work to delineate alternative mechanisms of resistance to VP-16 is underway in our laboratory.

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