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Role of Differential Drug Uptake, Efflux, and Binding of Etoposide in Sensitive and Resistant Human Tumor Cell Lines: Implications for the Mechanisms of Drug Resistance

PEDRO M. POLITI and BIRANDRA K. SINHA

Clinical Pharmacology Branch, Clinical Oncology Program, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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SUMMARY

In order to study the mechanism of etoposide (VP-16) resistance in human tumor cells and to assess the role of P-170 glycoprotein in VP-16 accumulation, we have examined the uptake and efflux of VP-16 in both sensitive and multidrug-resistant MCF-7 human breast and HL60 human promyelocytic leukemia cells. The drug-resistant cells, MCF-7/ADR and HL60/ADR, were selected for resistance to adriamycin and were 200- to 250-fold resistant to VP-16. Whereas MCF-7/ADR cells overexpress the P-170 glycoprotein and show the multidrug-resistant phenotype, HL60/ADR cells do not overexpress the P-170 glycoprotein. Although there was a 2-fold decrease in accumulation of VP-16 in MCF-7/ADR cells, this decrease did not correlate with a 250-fold resistance to the drug. VP-16 efflux was rapid and almost complete from MCF-7 cell lines and it was decreased at 4°. Further, there was a significant increase in VP-16 accumulation in the MCF-7/ADR cells in the presence of glucose-free medium supplemented with sodium azide. However, no change in the pattern of VP-16 efflux was observed. Under these conditions, addition of glucose caused release of VP-16 from MCF-7/ADR cells, suggesting energy-dependent modifications in the drug binding. Coincubation of vincristine with VP-16 also increased the drug accumulation and decreased the rate of efflux of VP-16 in both sensitive and resistant MCF-7 cells, suggesting that vincristine and VP-16 may compete for similar binding and efflux mechanisms in these cell lines. In contrast, daunorubicin increased VP-16 accumulation only in the sensitive MCF-7 cell line, whereas the efflux rate of VP-16 was not significantly changed in either cell line. HL60 sensitive cells accumulated 4- to 5-fold more VP-16 than the resistant subline. Both sensitive and resistant cells showed an important noneffluxable pool of the drug, 3-fold larger for sensitive cells (79 ± 12 versus 25 ± 2 pmol of VP-16/mg of protein, for sensitive and resistant cells, respectively). The efflux of VP-16 was temperature dependent only in sensitive cells. VP-16 accumulation in HL60/ADR cells was increased in glucose-free medium supplemented with sodium azide; however, the noneffluxable pool of VP-16 was not significantly changed. In contrast, although these conditions had no effect on the drug accumulation in the parental line, they caused a decrease in the noneffluxable pool of VP-16, suggesting an energy-dependent binding and retention of VP-16. Coincubation with a 100-fold molar excess of vincristine resulted in a significant increase in VP-16 accumulation in both sublines. However, vincristine increased retention of VP-16 in sensitive but not in HL60/ADR cells. Although daunorubicin had no significant effects on either VP-16 accumulation or efflux in sensitive cells, it enhanced the release of VP-16 from HL60/ADR cells. These data, taken together, indicate that a selective efflux pump for VP-16 in MCF-7/ADR cell line is not readily apparent under our experimental conditions and that it may not be the only factor in the mechanism of resistance to VP-16. Further, our data suggest that energy-dependent modifications in drug binding may play a role in the phenomenon of resistance to VP-16 in MCF-7 and HL60 tumor cell lines.

Resistance to antineoplastic agents in the clinic is a major cause of treatment failure (1, 2). Recently, a MDR phenotype has been described for several animal and human tumor cell lines. This phenomenon is characterized by the appearance of cross-resistance to a variety of naturally occurring antitumor drugs with completely dissimilar chemical structures, after selection for resistance to a single agent (3). The classes of drugs involved in the MDR phenotype include vinca alkaloids, anthracyclines, and epipodophyllotoxin derivatives. Most MDR cell lines overexpress a membrane glycoprotein of 170,000 Da called P-glycoprotein (4, 5). This glycoprotein has been reported to bind to drugs and to actively pump them outwards, in an ATP-dependent process, resulting in decreased net cell accumulation of drugs (6, 7). The analysis of the nucleotide sequence of cDNA of the glycoprotein has shown similarities to structures of known bacterial transport proteins (8, 9), and

ABBREVIATIONS: MDR, multidrug-resistant; VP-16, etoposide, (VP-16-213); P-170, glycoprotein, M_r 170,000 Da; PBS, phosphate-buffered saline (containing in mM: KH_2PO_4, 120.4; NaCl 153.8; Na_2HPO_4, 5.5, pH 7.40); PEG, polyethyleneglycol (M_r = 200).

the intracellular domain of the glycoprotein appears to have ATP binding sites as well as ATPase activity (10, 11). However, these are not universal findings for all the MDR cell lines studied to date. In fact, "atypical" MDR cell lines have been described, which do not differ from the parent line in drug accumulation (12).

VP-16, an epipodophyllotoxin derivative, has shown considerable activity against leukemia, lymphoma, germ cell tumors, and small cell carcinoma of the lung (13). Moreover, high doses of VP-16 (2 to 4 g/m^2) are now frequenly used in the setting of bone marrow transplantation for patients with either refractory or high-risk leukemia or solid tumors, with encouraging results (14, 15). As with other antitumor agents, the clinical usefulness of VP-16 has been compromised by the appearance of drug resistant and MDR cells (16). However, it is not clear at present whether VP-16 shares P-170-dependent mechanism for resistance.

With the aim of defining the role of VP-16 uptake and efflux in the phenomenon of resistance to VP-16, two human tumor cell lines, MCF-7 (breast carcinoma) and HL60 (promyelocytic leukemia), were studied. The resistant cell lines, MCF-7/ADR and HL60/ADR, were selected in the presence of adriamycin (16–18) and were found to be significantly cross-resistant to VP-16 and to other natural products. The MCF-7/ADR cell line exhibits amplification of the MDR gene and overexpression of P-170 (19). In contrast, HL60/ADR does not overexpress P-170 (18). Because ATP is needed for the proposed P-170-mediated drug export (6, 7), the effects of energy-depleting conditions on VP-16 accumulation and efflux in these cell lines were examined. Furthermore, because vincristine and daunorubicin compete for binding to P-170 (20), we evaluated the potential of these agents to modulate cellular pharmacokinetics of VP-16. Some of the data have been recently published in abstract form (21).

Materials and Methods

Drugs and chemicals. VP-16 was a gift from Bristol-Myers Co. (Syracuse, NY) and was dissolved in PEG (Sigma Chemical Co., St. Louis, MO) and stored at −70°. [^{3}H]-VP-16, labeled only in the aromatic rings (900 mCi/mmol; >99.5% pure by HPLC and nonexchangeable), was obtained from Moravek Biochemicals (Brea, CA). Daunorubicin and vincristine were obtained from the Drug Development Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Cells. MCF-7 human breast tumor cells (provided by Dr. K. H. Cowan, Clinical Pharmacology Branch, National Cancer Institute, Bethesda, MD) 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. HL60 leukemia cells (provided by Dr. S. Grant, Division of Medical Oncology, Columbia University, New York, NY) were grown in RPMI 1640 medium supplemented with 2 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum, and 20 μg/ml gentamycin. Cells used in these studies were in exponential growth phase.

Uptake of [^{3}H]VP-16. MCF-7 cells were grown to 50–70% confluency in 25-cm^2 flasks and were treated with a drug mixture containing 0.5 μCi/ml [^{3}H]VP-16, plus unlabeled VP-16 in PEG, dissolved in fresh, complete tissue culture medium (final PEG concentration, <0.1%; final VP-16 concentration, 10 μM, unless otherwise stated). Cells were incubated at 37° under a 5% CO_2 atmosphere. Uptake studies were carried out similarly to those described by Schilsky *et al.* (22). 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. For radioactivity determinations, aliquots were neutralized with 1 N HCl, dissolved in 10 ml of Hydrofluor (National Diagnostics, Manville, NJ), and processed in a Packard 2000 CA TriCarb liquid scintillation counter (Packard Instrument Co, Sterling, VA) with appropriate quenching corrections. The protein concentration was determined by the method of Lowry *et al.* (23) 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, both for the uptake and efflux studies. All manipulations except incubations were performed on ice. Incubation medium was prewarmed to 37°.

Uptake studies in HL60 were carried out according to the method described by Hamza *et al.* (24). Briefly, cells were harvested, washed three times with PBS, and resuspended in the complete RPMI medium containing 0.5 μCi/ml [^{3}H]VP-16 and unlabeled VP-16 to a final drug concentration of 10 μM, unless otherwise specified. At the end of the incubations (at 37° with gentle shaking), 1-ml aliquots were transferred to plastic Eppendorf tubes on ice and spun for 30 sec in an Eppendorf 3200 microcentrifuge. Cell pellets were washed three times with 1 ml of ice-cold PBS and processed for radioactivity and protein content. Zero time values for drug uptake were obtained by adding the radioactive mixture to cells on ice and immediately centrifuging and washing as indicated above. The zero time values (10 and 6 pmol of VP-16/mg of protein for sensitive and resistant cells, respectively) were subtracted from each subsequent determination.

Drug efflux studies. Efflux experiments were carried out after loading the cells with the above-mentioned radiolabeled drug mixture for 60 min. The drug was then removed by washing the cells two times with ice-cold PBS and incubating in an equal volume of fresh drug-free medium at 37°. At the prescribed times, flasks (or 1-ml aliquots, for HL60 cells) were removed and processed as indicated for uptake studies. Experiments to assess the temperature dependence of VP-16 efflux were similarly carried out at 4° after the cells were loaded with the radiolabeled drug mixture at 37° for 60 min.

Effects of different concentrations of VP-16 (including the IC_{50} values) on drug accumulation (60 min) and retention (at 60 min efflux time) were also examined. At the highest drug concentrations, e.g., 650 and 250 μM, final PEG concentration was 0.65% for MCF-7 and 0.25% for HL60 cells, respectively, which did not significantly affect the drug transport.

Studies under metabolic blockade. The effects of energy depletion on both VP-16 accumulation and efflux were determined by incubating the cells with 10 mM sodium azide in glucose-free medium (Medium A) for 30 min at 37° and carrying out uptake and efflux as described above. For these experiments, however, previously dialyzed (24 hr at 4° against PBS) fetal bovine serum was used. Sodium azide is a well known inhibitor of cytochrome oxidase (25), and its effects on glucose utilization and ATP levels in MCF-7 sensitive and resistant cell lines have been reported (26). In order to evaluate the effects of glucose restoration, drug accumulation experiments were carried out in Medium A for 120 min, with addition of glucose at 60 min (final concentration, 10 mM). Controls received an equal volume of medium A at 60 min (2.5%, v/v). Drug levels were determined at prescribed times as described above.

Competition with vincristine and daunorubicin. In order to evaluate the effects of structurally unrelated cytotoxic drugs on cellular accumulation and retention of VP-16, a series of uptake and efflux studies were carried out by coincubating 100 μM levels of either daunorubicin or vincristine with 1 μM VP-16. These concentrations were chosen to provide vincristine and daunorubicin with a large concentration advantage, so as to be able to observe an eventual competition for the efflux mechanism. Cells were preincubated with the appropriate

drug for 30 min at 37°. Either vincristine or daunorubicin was also present in the medium during both uptake and efflux experiments.

Regression curve-fitting was performed with a computer graph software (Cricket Graph; Cricket Software, Philadelphia, PA) using a Macintosh computer. Data for only the initial rate (up to 30 min) of otherwise biphasic efflux curves were fitted to the following monoexponential equation:

$$C(t) = Ae^{at} + B$$

where C is the VP-16 concentration at a defined time, A and B are the zero-time coefficients, and a is the pseudo-first order rate constant for efflux (27). Correlation coefficients (r) were also obtained and regression analysis was performed. The one-way analysis of variance was used to assess the statistical significance of the difference between mean values for each experimental group. p Values of 0.05 or less were considered significant.

Results

Cytotoxicity studies. Both MCF-7 and HL60 resistant cell lines were selected for resistance to adriamycin and were 200- to 250-fold cross-resistant to VP-16. The respective IC_{50} values obtained during 1-hr or continuous exposure are presented in Table 1.

VP-16 uptake and efflux. Steady state concentrations of VP-16 were achieved within 30 min in both MCF-7 cell lines; however, accumulation of VP-16 was significantly higher at 60 min in sensitive cells (2–3 fold; Fig. 1A) than in MCF-7/ADR cells. On the other hand, VP-16 efflux from MCF-7 cells was rapid and almost complete; the sensitive cells retained approximately 12% of the drug after 90 min. The amount of retained or "noneffluxable" drug at this time point was approximately similar in both sensitive and MCF-7/ADR cells. VP-16 efflux was markedly decreased when cells were exposed to drug-free medium at 4°.

VP-16 uptake was rapid in HL60 sensitive cells, and cellular levels of the drug for each time point were significantly higher than those in the resistant cell line (4–5-fold higher at 60 min; Fig. 1B). Efflux of VP-16 was biphasic in HL60 sensitive cells, rapidly reaching a plateau such that 38.5 ± 5.8% of the accumulated VP-16 was retained 90 min after resuspension in drug-free medium. This noneffluxable drug was 3-fold higher in sensitive cells than in HL60/ADR cells (72.0 ± 12.0 versus 24.7 ± 2.1 pmol/mg of protein, respectively; $F = 16.85$; $p = 0.0045$).

Whereas VP-16 efflux from the parental line was markedly decreased when cells were exposed to drug-free medium at 4°, no temperature dependence could be observed in HL60/ADR cells.

Effects of VP-16 concentration on accumulation and retention. Both accumulation (at 60 min) and retention (after 60-min exposure to drug-free medium) of VP-16 showed concentration dependence in sensitive and resistant MCF-7 cells, the relationship being linear throughout the range of extracellular drug levels tested (Table 2). The sensitive cells exhibited a 2- to 3-fold higher drug accumulation and retention than the resistant cells in this range of concentrations. Furthermore, when sensitive and resistant MCF-7 cells were exposed to equitoxic concentrations of VP-16 (IC_{50} for 1-hr exposure, 4 and 650 μM, respectively), the resistant cells accumulated 171-fold more drug and retained 536-fold more VP-16 than the parental cell line. When the complete time sequence of drug uptake and efflux was carried out, differences in cellular drug levels remained higher in MCF-7/ADR by a factor of 100 or more (data not shown). Under these conditions, PEG (0.65%, v/v) did not significantly effect either uptake or efflux of VP-16.

Similarly, both HL60 sensitive and HL60/ADR cells also showed a concentration-dependent accumulation and retention of VP-16; the sensitive cells accumulating 3–5-fold more VP-16 and retaining up to 12-fold more drug than HL60/ADR (Table 3). Exposure to equitoxic concentrations of VP-16 (IC_{50} for 1-hr exposure, 0.5 and 140 μM, respectively) yielded higher drug accumulation (43-fold) and retention (16-fold) in the resistant cells.

Effects of energy depletion on VP-16 uptake and efflux. Treatment of MCF-7 cells with sodium azide in glucose-free medium (Medium A) resulted in an increase in VP-16 accumulation only in the resistant cells (Fig. 2, A and C). Efflux of VP-16 was again rapid for both the sensitive and MCF-7/ADR cells, and most of the accumulated drug (about 90%) was released within 90 min in drug-free medium. MCF-7/ADR cells displayed similar drug efflux patterns both in Medium A and in the standard, glucose-containing, medium. No significant difference between these two conditions was noted when the percent VP-16 retained was plotted against efflux time (data not shown). Furthermore, a logarithmic plot of cellular VP-16

TABLE 1

Relative cytotoxicity of VP-16 in MCF-7 and HL60 cell lines during 1-hr and continuous exposures

IC_{50}, concentration of VP-16 required for 50% cell kill as determined by growth inhibition assay following drug treatment. WT, wild type.

Exposure time	IC_{50}, MCF-7		IC_{50}, HL60	
	WT	MCF/ADR	WT	HL60/ADR
		μM		
1 hr	4.0	650	0.5	140
Continuous	0.08	20	0.02	5.0

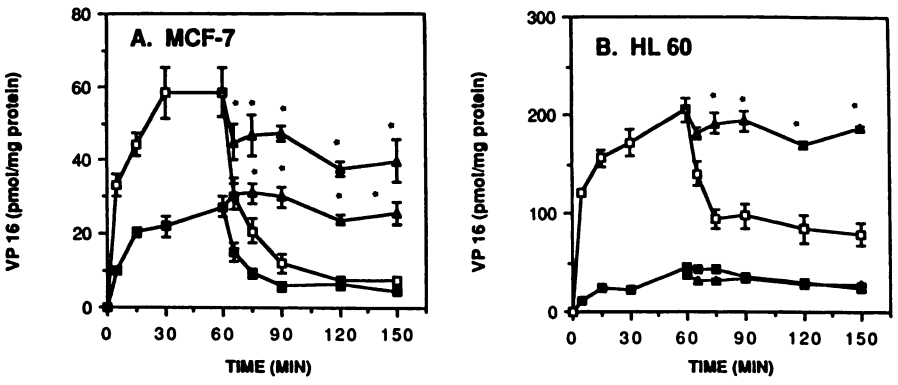

Fig. 1. VP-16 uptake and efflux in MCF-7 and HL60 cell lines. Effects of temperature on drug efflux. Incubation in complete culture medium at 37°, in the presence of 10 μM VP-16. At 60 min, cells were washed and exposed to drug-free medium. □, Sensitive cells, 37°; △, sensitive cells, 4°; ■, resistant cells, 37°; ▲, resistant cells, 4°. *Vertical bars* represent standard error of at least three separate experiments. *$P < 0.05$ versus the corresponding experiments at 37°. Drug accumulation at 37° was significantly higher in sensitive cell lines at all time points ($p < 0.05$ versus the corresponding resistant line), with the exception of data for 120 and 150 min in A (see Materials and Methods for details).

TABLE 2

Concentration dependence of accumulation[a] and retention[b] of VP-16 in sensitive and resistant MCF-7 cell lines

See Materials and Methods for experimental details. VP-16 levels expressed as picomol of the drug/mg of protein. Mean values ± standard error for at least three experiments are given. WT, wild type; ND, not done.

VP-16	MCF-7 WT		MCF-7/ADR	
	Accumulation[c]	Retention[d]	Accumulation[e]	Retention[f]
μM				
1	4.7 ± 0.3	0.5 ± 0.2	2.6 ± 0.4	0.6 ± 0.2
4	15.7 ± 0.7	0.9 ± 0.4	ND	ND
10	52.5 ± 4.9	1.7 ± 0.4	28.1 ± 1.7	1.0 ± 0.5
50	305.6 ± 17.4	7.9 ± 0.6	157.6 ± 20.6	9.3 ± 3.2
100	852.1 ± 71.1	80.1 ± 9.2	341.4 ± 28.8	70.9 ± 6.9
500	4252.3 ± 445.7	466.0 ± 56.4	1890.1 ± 329.4	288.2 ± 80.6
650	ND	ND	2687.5 ± 140.9	483 ± 108

[a] Cellular VP-16 levels after 60-min incubation in the presence of VP-16.
[b] Cellular VP-16 levels after 60-min exposure of preloaded cells to drug-free medium.
Linear regression analysis versus concentration of VP-16: [c] $r = 0.987$, $p < 0.001$; [d] $r = 0.980$, $p < 0.001$; [e] $r = 0.983$; $p < 0.001$; [f] $r = 0.880$; $p < 0.001$.

TABLE 3

Concentration dependence of accumulation[a] and retention of VP-16 in sensitive and resistant HL60 cell lines

VP-16	HL 60 WT		HL 60/ADR	
	Accumulation[b]	Retention[c]	Accumulation[d]	Retention[e]
μM				
0.5	11.9 ± 0.7	6.0 ± 0.6	2.2 ± 0.2	0.5 ± 0.1
1.0	14.4 ± 0.4	4.3 ± 0.1	4.7 ± 0.3	2.5 ± 0.1
10	208.5 ± 8.9	75.1 ± 16.7	43.0 ± 1.9	31.7 ± 1.4
50	710.8 ± 47.4	143.5 ± 39.5	183.1 ± 17.7	54.1 ± 3.4
140	ND	ND	515.3 ± 58.2	95.9 ± 23.7
250	3582.7 ± 366.6	362.3 ± 17.1	990.3 ± 169.0	113.0 ± 21.1

[a] Footnotes as in Table 2.
Linear regression analysis versus VP-16 concentration: [b] $r = 0.98$, $p < 0.001$; [c] $r = 0.784$; $p < 0.01$; [d] $r = 0.907$; $p < 0.001$; [e] $r = 0.791$; $p < 0.01$.

versus efflux time exibited a good linearity by regression analysis and yielded parallel straight lines for both cell lines (Fig. 2D). No modification in uptake and/or efflux of VP-16 was observed in standard medium (with 11 mM glucose) supplemented with 10 mM sodium azide (data not shown).

Although incubation in Medium A did not affect VP-16 accumulation in the sensitive HL60 cell line, the noneffluxable drug pool was significantly decreased (Fig. 3). HL60/ADR cells, on the other hand, not only accumulated significantly more VP-16 under these conditions but also released it rapidly. Expressing efflux data as percentage of VP-16 retained, it is evident that sensitive cells and especially HL60/ADR retain less drug under energy depletion (Fig. 4, A to D). The net result was that energy-depleted sensitive and resistant HL60 cells exhibited almost superimposable efflux curves that yielded parallel straight lines in the corresponding log plot (Fig. 4, E and F). Similar experiments carried out in the standard medium (containing 11 mM glucose) supplemented with 10 mM sodium azide showed no modification in either uptake or efflux of VP-16 (data not shown). Restoration of glucose levels to 10 mM, after cells were allowed to accumulate VP-16 in Medium A for 1 hr, induced a release of 52.7% of VP-16 in MCF-7/ADR cells (from 49.2 to 23.3 pmol/mg), and only 20.0% in HL60/ADR cells (from 142.0 to 113.7 pmol/mg; Fig. 5).

Competition studies with other cytotoxic drugs. Coincubation of either daunorubicin or vincristine (100:1 ratio) with sensitive MCF-7 cells enhanced VP-16 accumulation 2-fold. Although vincristine also increased VP-16 accumulation in MCF-7/ADR cells, this increase was not as marked as that observed in the sensitive cells (Fig. 6, A and C). Vincristine (but not daunorubicin) significantly decreased the VP-16 efflux rate, calculated from a curve-fitted logarithmic plot of retained drug versus time, in both MCF-7 cell lines (Fig. 6, B and D). In contrast, daunorubicin had no significant effect on either accumulation or efflux of VP-16 in MCF-7/ADR cells. Similar results were also obtained when higher vincristine and daunorubicin concentrations were used in the presence of 10 μM VP-16 (data not shown).

As shown for MCF-7 cells, vincristine significantly increased VP-16 accumulation in both HL60 cell lines (Fig. 7). However, cellular VP-16 levels rapidly declined and the noneffluxable drug pool was not significantly higher in either cell line. Although daunorubicin did not affect VP-16 accumulation in HL60 cells, it caused a significant increase in the release of VP-16 from HL60/ADR cells. Logarithmic plots of VP-16 retained versus efflux time further showed that vincristine actually increased the rate of VP-16 efflux in HL60/ADR cells and had no effect on the sensitive line (not shown). Similar results were also obtained when higher vincristine and daunorubicin concentrations were used in the presence of 10 μM VP-16 (data not shown).

Discussion

The relative significance of differential drug uptake, binding, and efflux have been studied for a number of tumor cell lines in order to define mechanisms of resistance and MDR (28–31). Our studies with VP-16 in MCF-7 and HL60 cell lines show a moderate decrease in VP-16 accumulation in resistant cells (2-fold in MCF-7 cells and 4- to 5-fold in HL60 cells) as compared with a high degree of resistance to the drug (200–250-fold). Furthermore, at equitoxic (IC_{50}) concentrations of VP-16, drug levels were much higher in both resistant cell lines, suggesting that decreased drug accumulation may not make a major contribution to the mechanism of resistance. Because the accumulation defect in MCF-7/ADR is partially reversed under energy-depleted conditions without significant changes in drug efflux, it is inferred that alterations in drug binding may be responsible for this phenomenon. Glucose-induced drug release in MCF-7/ADR lends further support to the concept of an energy-dependent mechanism. Furthermore, our data show an excellent agreement with ATP levels monitored by magnetic resonance spectroscopy (26) in MCF-7 cells, e.g. (a) exposure to glucose-free medium plus sodium azide for 30 min decreased cellular ATP levels by 75% in sensitive MCF-7 cells and by 90% in MCF-7/ADR, thus ruling out the possibility that MCF-7/ADR may be less affected by sodium azide; (b) after restoration of glucose to 11 mM, ATP levels recovered to 70% of the initial value within 5 min and remained constant for an additional hour, suggesting that the cells were metabolically viable; (c) glucose-containing medium (11 mM) plus sodium azide failed to decrease ATP levels and had no significant effect on the rates of glucose consumption or lactate production by either cell line. These data taken together suggest that accumulation of VP-16 is energy dependent in MCF-7/ADR cells. Although additional effects of energy depletion and sodium azide on cellular metabolism cannot be ruled out, decreased drug efflux does not seem to account for our observation, inasmuch as logarithmic plots of cellular VP-16 against efflux time yielded parallel lines. Similar experimental conditions have been pre-

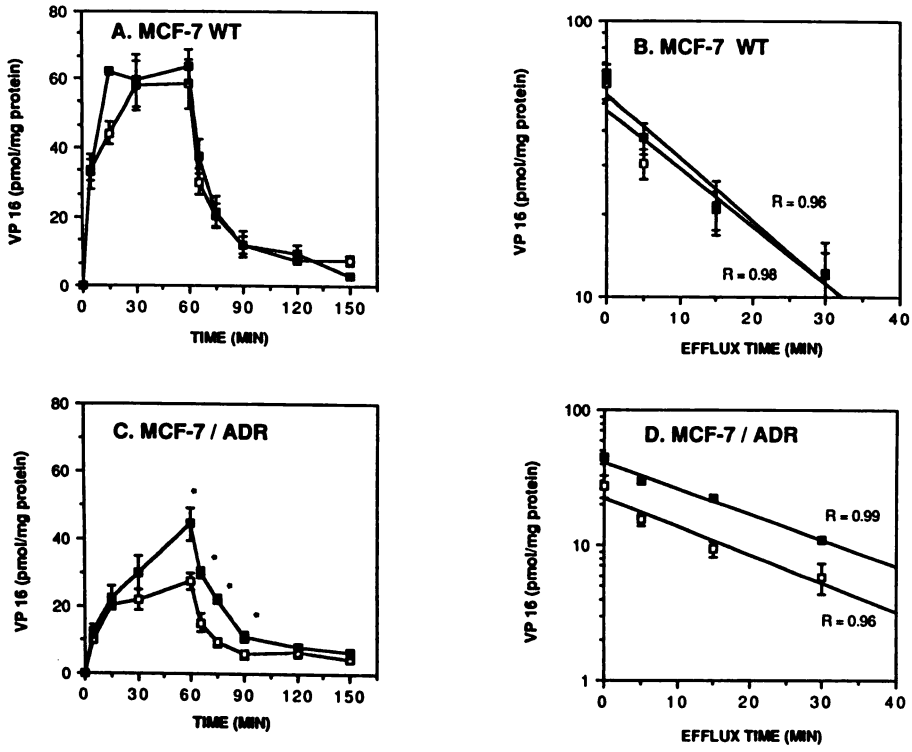

Fig. 2. Effects of glucose-free medium plus sodium azide on the uptake and efflux of 10 μM VP-16 in sensitive (A) and resistant (C) MCF-7 cells. □, Standard, complete culture medium (contains 11 mM glucose); ■, glucose-free medium, with 10 mM sodium azide and dialyzed serum. *Vertical bars* represent standard error of three or more separate experiments. $^*p < 0.05$ versus standard medium. B and D, Computer-generated curve-fits for efflux data from A and B, respectively. *R*; correlation coefficient ($p < 0.001$ for all four computer-generated fits in B and D, when tested by regression analysis). p = not significant for comparison of slopes (*a* values in equation; see Materials and Methods for details).

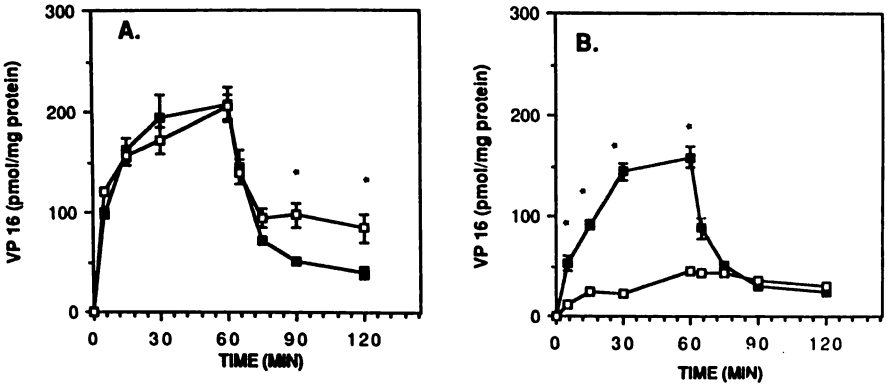

Fig. 3. Effects of glucose-free medium plus sodium azide on the uptake and efflux of 10 μM VP-16 in sensitive (A) and resistant (B) HL60 cells. □, Standard, complete culture medium (contains 11 mM glucose); ■, glucose-free medium, with 10 mM sodium azide and dialyzed serum. *Vertical bars* represent standard error of three or more separate experiments. $^*p < 0.05$ versus standard medium.

viously used to study drug export mechanisms in several tumor cell lines (30, 31).

Because daunorubicin and vincristine bind to P-glycoprotein (6, 7), which acts as an export pump, exposure to high concentrations of vincristine or daunorubicin was expected to compete for the pump and significantly affect VP-16 transport in MCF-7/ADR cells. The effects of vincristine reported here may indicate a competition of vincristine for efflux of VP-16 and also suggest that P-glycoprotein may not be the only or major contributor for resistance to VP-16 in this cell line. Because vinca alkaloid-induced modifications of VP-16 binding to human tumor cell lines have been previously documented (32), it is possible that this phenomenon also plays a role in these cell lines. On the other hand, daunorubicin failed to decrease VP-16 efflux in the cell lines studied. The increase in VP-16 accumulation in the parental cell line may represent daunomycin-induced modification in drug binding and/or possible membrane damage, both leading to increased VP-16 release.

Our data also indicate that VP-16 is poorly retained in both sensitive and resistant MCF-7 cell lines and are, thus, consistent with previous reports (27, 32). In contrast, a large pool of noneffluxable drug was present in HL60 sensitive cells that was only partially released under ATP-depleting conditions, indicating that two different processes, including energy-dependent drug binding and/or sequestration of VP-16, may be involved in the biphasic pattern of drug efflux in HL60 cells. Cellular binding could involve both the parent drug and its metabolites. VP-16 has been shown to undergo rapid *in vitro* metabolism by purified horseradish peroxidase and prostaglandin synthetase (33) and by rat liver microsomes (34–36), giving rise to highly reactive, *O*-demethylated species such as dihydroxy (a catechol) and *o*-quinone derivatives of VP-16. These metabolites irreversibly bind to DNA and proteins and are cytotoxic (37). It is conceivable that myeloperoxidase, present in HL60 cells (38), may catalyze similar reactions. Furthermore, VP-16 binding, sequestration, and redistribution in cell vesicles may also play a role in the mechanism of drug resistance (39). These possibilities are currently under investigation.

An energy-dependent mechanism of reduced drug accumulation was apparent in HL60/ADR cells. In fact, energy depletion in these cells increased both the effluxable pool of VP-16 and the rate of drug efflux. These data are not consistent with

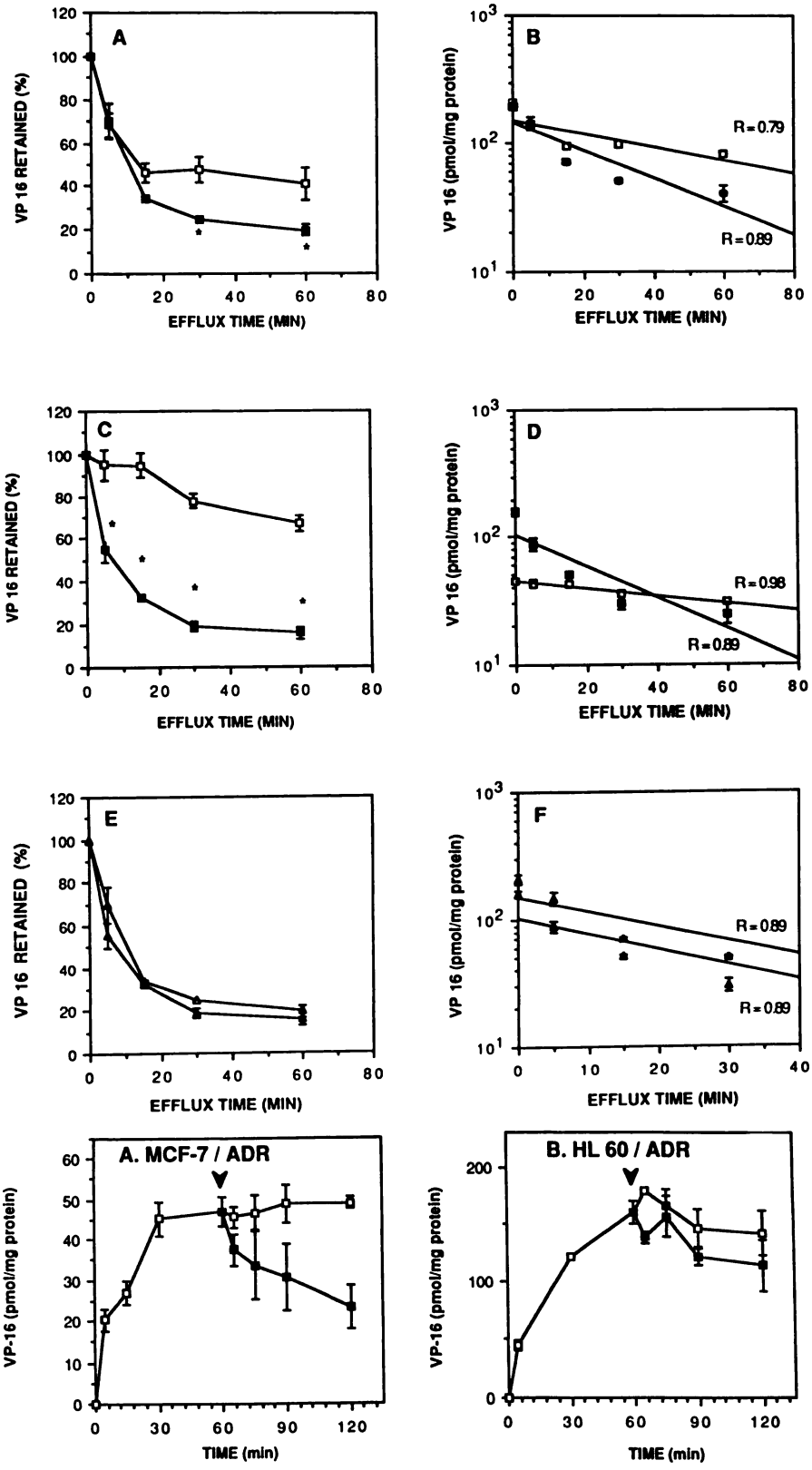

Fig. 4. Effects of energy depletion on the efflux pattern of VP-16. After being loaded with 10 μM VP-16 for 60 min, cells were washed and resuspended in drug-free medium (see Materials and Methods for details). VP-16 retained is expressed as percent (*left*) or as absolute values in log scale (*right*) and plotted versus efflux time. A and B; HL60 sensitive cells; C and D; HL60 resistant cells. □, Standard medium with 11 mM glucose; ■, glucose-free medium with 10 mM sodium azide. *$p < 0.05$ versus standard medium. E and F; sensitive (△) versus resistant cells (▲), both in glucose-free medium plus azide.

Fig. 5. Effects of glucose level restoration on accumulation of VP-16 in MCF-7/ADR (A) and HL60/ADR (B) cell lines. Cells were preincubated in glucose-free medium plus sodium azide (Medium A; □) for 30 min and then allowed to accumulate VP-16 for 60 min. At this time, glucose was added (*arrow*) to some of the incubations to a final concentration of 10 mM (■). The remaining incubations (controls) received an equivalent volume of Medium A (2.5%). Cellular content of VP-16 was determined as indicated in Materials and Methods. *Vertical bars* represent standard error of at least three separate experiments. $p < 0.001$ versus controls only for the 120-min time point in A.

drug export via a pump mechanism but suggest that energy-dependent reduced VP-16 binding may account for decreased drug accumulation in HL60/ADR cells. Because high concentrations of vincristine increased accumulation of VP-16 in both sensitive and resistant HL60 cells without slowing drug efflux, an interaction at binding site(s) is possible, as previously suggested for MCF-7 cells.

The phenomenon of MDR appears to be complex and multifactorial (39–41). Moreover, the main mechanism(s) of drug resistance may vary for each cell line and drug under study. Supporting this contention, resistance to adriamycin in MCF-7/ADR cells includes reduced drug uptake and enhanced drug detoxification (16, 17). Moreover, Hamada and Tsuruo (42)

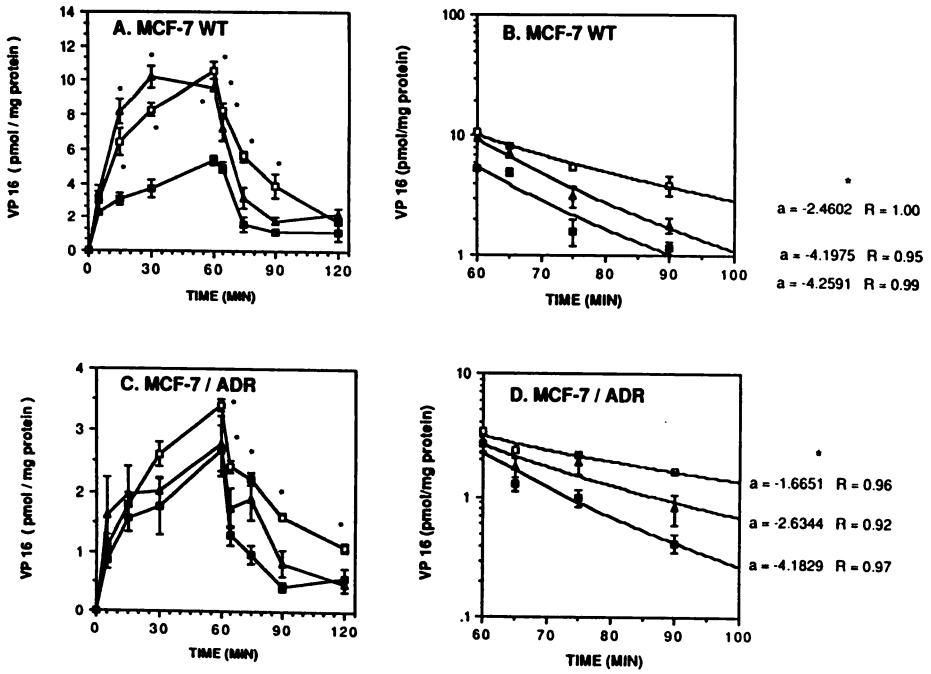

Fig. 6. Effects of vincristine and daunorubicin on the uptake and efflux of 1 μM VP-16 in sensitive (A) and resistant (C) MCF-7 cells. ■, Standard medium (controls); □, 100 μM vincristine; ▲, 100 μM daunorubicin. *$p < 0.05$ versus controls. B and C, Computer-generated curve-fits for efflux data from A and C, respectively, at indicated times after exposure to VP-16-free medium. *R*, correlation coefficient; $p < 0.001$ for all six computer fits in B and C when tested by regression analysis. *a*, calculated exponential coefficient for the curve fit (slope or pseudo-first order rate constant) for efflux times 0–30 min only (see Materials and Methods for equation and details). *a values significantly different from control ($p < 0.05$).

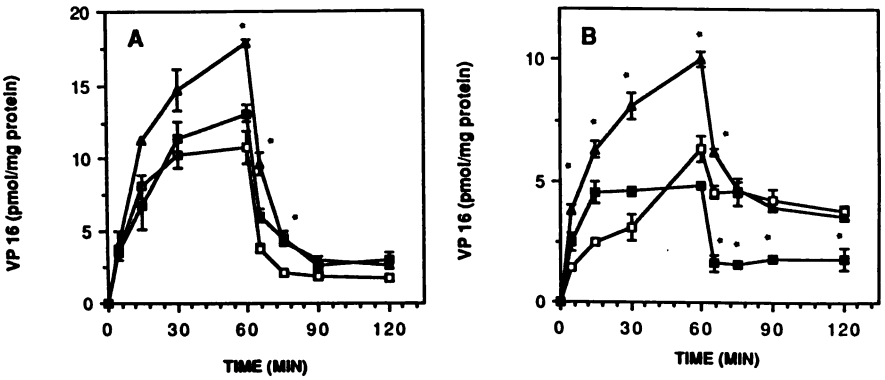

Fig. 7. Effects of vincristine and daunorubicin on the uptake and efflux of 1 μM VP-16 in sensitive (A) and resistant (B) HL60 cells. □, Standard medium (controls); ▲, 100 μM vincristine; ■, 100 μM daunorubicin. *$p < 0.05$ versus controls (see Materials and Methods for details).

reported that a monoclonal antibody against the P-170 glycoprotein increased cellular accumulation of actinomycin D and vincristine (but not adriamycin) in human tumor cell lines selected for resistance to the anthracycline. Decreased drug accumulation and modifications in the interaction of VP-16 with its proposed major target, topoisomerase II, have been reported to mediate acquired resistance to VP-16 and/or its congener VM-26 in tumor cell lines (43). Recent work from our laboratory has shown that MCF-7/ADR cells exhibit a significant decrease in VP-16-induced DNA damage (44). When all this evidence is considered together, a complex picture evolves in which multiple biochemical mechanisms contribute to the MDR phenomenon. Our data suggest that energy-dependent modifications of drug binding may play a role in the mechanism of resistance to VP-16. A similar phenomenon has been reported to account for vinca alkaloid resistance in a human leukemic cell line (28). A major role of P-170 glycoprotein (present in MCF-7/ADR) in VP-16 cellular pharmacokinetics could not be clearly demonstrated.

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Send reprint requests to: Dr. B. K. Sinha, Bldg. 10, Rm. 6N-119, NCI, NIH, Bethesda, MD 20892.