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ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells

(cancer chemotherapy/daunomycin/actinomycin D/verapamil/quinidine)

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ABSTRACT **Resistance of human cancer cells to multiple cytotoxic hydrophobic agents (multidrug resistance) is due to overexpression of the *"MDR1"* gene, whose product is the plasma membrane P-glycoprotein. Plasma membrane vesicles partially purified from multidrug-resistant human KB carcinoma cells, but not from drug-sensitive cells, accumulate [^{3}H]vinblastine in an ATP-dependent manner. This transport is osmotically sensitive, with an apparent K_m of 38 μM for ATP and of ≈2 μM for vinblastine. The nonhydrolyzable analog adenosine 5′-[β,γ-imido]triphosphate does not substitute for ATP but is a competitive inhibitor of ATP for the transport process. Vanadate, an ATPase inhibitor, is a potent noncompetitive inhibitor of transport. These results indicate that hydrolysis of ATP is probably required for active transport of vinblastine. Several other drugs to which multidrug-resistant cell lines are resistant inhibit transport, with relative potencies as follows: vincristine > actinomycin D > daunomycin > colchicine = puromycin. Verapamil and quinidine, which reverse the multidrug-resistance phenotype, are good inhibitors of the transport process. These results confirm that multidrug-resistant cells express an energy-dependent plasma membrane transporter for hydrophobic drugs, and establish a system for the detailed biochemical analysis of this transport process.**

Genetic and biochemical evidence based on the study of cell lines selected for resistance to multiple cytotoxic agents, including colchicine, vinblastine, doxorubicin, and actinomycin D, indicates that the multidrug-resistance phenotype results from expression of the *mdr* gene, which encodes the plasma membrane protein P-glycoprotein (refs. 1–8, reviewed in refs. 9 and 10). Analysis of cloned cDNAs for the human *"MDR1"* gene and other *mdr* genes demonstrates that these related genes encode a protein of about 1280 amino acids with 12 transmembrane domains and two potential ATP binding sites homologous to ATP binding sites in bacterial transport proteins (11–13). Because *MDR1*-dependent multidrug resistance results from increased energy-dependent drug efflux (14) and because P-glycoprotein is located in the plasma membrane (15), it seems likely that P-glycoprotein is an energy-dependent drug-efflux system for cytotoxic hydrophobic drugs.

Membrane vesicles derived from multidrug-resistant human KB carcinoma cells bind greater amounts of [^{3}H]vinblastine than do vesicles from drug-sensitive cells (16). Both the vinblastine analog *N*-(*p*-azido-3-[^{125}I]iodosalicyl)-*N*′-(β-aminoethyl)vindesine ([^{125}I]NASV) and the ATP analog 8-azido-[^{32}P]ATP photoaffinity label P-glycoprotein in membrane preparations from multidrug-resistant cells (17–19), indicating that P-glycoprotein is a drug- and ATP-binding protein. In this paper, we demonstrate that P-glycoprotein-containing membrane vesicles prepared from multidrug-resistant KB cells transport [^{3}H]vinblastine in an ATP-dependent manner. ATP hydrolysis appeared to be required for this transport. Vinblastine transport was inhibited by daunomycin, actinomycin D, vincristine, puromycin, and colchicine and also by verapamil and quinidine, agents that overcome multidrug resistance.

MATERIALS AND METHODS

Vesicle Preparation and Characterization. Crude membrane vesicles were prepared from drug-sensitive (KB-3-1) and drug-resistant (KB-V1) human KB carcinoma cell lines (20, 21) by nitrogen cavitation (22) as previously described (16). These preparations were enriched 3- to 4-fold in Na^+,K^+-ATPase activity compared to whole homogenates (16). The crude membrane vesicles were then suspended in buffer A (10 mM Tris·HCl, pH 7.5/0.25 M sucrose), layered onto a 20%/30%/50% discontinuous sucrose gradient in 10 mM Tris·HCl, and centrifuged for 15 hr at 100,000 × *g*. Vesicles at the 30%/50% interface were collected, diluted in buffer A, and centrifuged for 50 min at 100,000 × *g*. The vesicle pellet was resuspended in buffer A by use of a 25-gauge needle. Vesicles were stored at −70°C prior to use. Relative to the homogenate, the activity of 5′-nucleotidase (a plasma membrane marker) in the final vesicle fraction was enriched ≈7-fold. There was no difference in enrichment of this enzyme activity between membrane vesicles from drug-sensitive and drug-resistant cells. By electron microscopic analysis, most of the structures had the appearance of plasma membrane vesicles.

[^{3}H]Vinblastine Uptake by Vesicles (Rapid Filtration Technique). Unless otherwise noted, the procedure for uptake of [^{3}H]vinblastine was as follows. A 20-μl aliquot of vesicles (2–2.5 mg of protein per ml) was placed in a glass test tube and 30 μl of ATP buffer (10 mM Tris·HCl, pH 7.5/1 mM ATP/10 mM $MgCl_2$/10 mM creatine phosphate/0.25 M sucrose containing creatine kinase at 100 μg/ml) was added. After 30 sec, a 50-μl aliquot of reaction medium containing [^{3}H]vinblastine, 10 mM Tris·HCl (pH 7.5), and 0.25 M sucrose was added. After an appropriate time at 25°C, the reaction was terminated by the addition of 1 ml of ice-cold buffer A. The vesicles were then applied to a Millipore filter (HAWP, 0.45-μm pore size) under light suction. The filters were then washed further with 5 ml of cold buffer A. The filters were dissolved in scintillation fluid and [^{3}H]vinblastine uptake by the vesicles was measured by liquid scintillation counting. ATP-independent association of vinblastine with the vesicles was determined by replacing ATP with

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Abbreviations: AdoPP[NH]P, adenosine 5′-[β,γ-imido]triphosphate; [^{125}I]NASV, *N*-(*p*-azido-3-[^{125}I]iodosalicyl)-*N*′-(β-aminoethyl)vindesine.

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AMP in the incubation medium, and ATP-dependent uptake was calculated by subtracting this uptake from that determined in the presence of ATP. [^{3}H]Vinblastine, diluted in buffer A, was filtered through a Millipore filter (Millex-GV, 0.22 μm) before use. All experimental points were carried out at least in triplicate. Standard deviations were calculated where possible, and these are indicated as error bars in the figures.

Materials. [^{3}H]Vinblastine was obtained from Amersham. Tissue culture products were from GIBCO. Unlabeled drugs were purchased from Sigma and sodium metavanadate was obtained from Aldrich. Adenosine 5'-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*) was from Pharmacia. All other chemicals were of reagent grade.

RESULTS

ATP Dependence of Vinblastine Uptake in Vesicles from Drug-Resistant Cells. The time course of vinblastine association with plasma membrane vesicles from drug-sensitive and drug-resistant KB cells is shown in Fig. 1. Total accumulation includes vinblastine binding to the vesicles (16) as well as specific transport into the vesicles. In plasma membrane vesicles from drug-resistant cells, ATP stimulated vinblastine uptake severalfold at 10 min, but there was no stimulation of vinblastine association with vesicles from drug-sensitive cells. In vesicles from drug-resistant cells, uptake at 90 min was lower than at 30 min, suggesting that depletion of ATP occurred by 90 min and vinblastine diffused out of the vesicles. When ATP medium was used without the ATP-regenerating system (creatine kinase and phosphocreatine), no stimulation of vinblastine uptake was observed at 10 min (data not shown). These data indicate that a continuous supply of ATP is needed for vinblastine transport.

Osmotic Sensitivity of ATP-Dependent Transport. True transport of vinblastine into vesicles should be sensitive to the intravesicular space, which can be manipulated by varying extravesicular osmolarity. The extravesicular osmolarity was varied by preincubating vesicles with different concentrations of sucrose for 60 min at 25°C. Incubation medium for the [^{3}H]vinblastine uptake studies in the presence or absence of ATP contained the same concentration of sucrose as the preincubated vesicles. When ATP-dependent uptake was plotted against the inverse of sucrose molarity (Fig. 2), a straight line was obtained. This result indicates that ATP stimulates the intravesicular transport of vinblastine. Since vinblastine appears to diffuse out of vesicles after 90 min when ATP is depleted (Fig. 1), intravesicular transport must be against a concentration gradient.

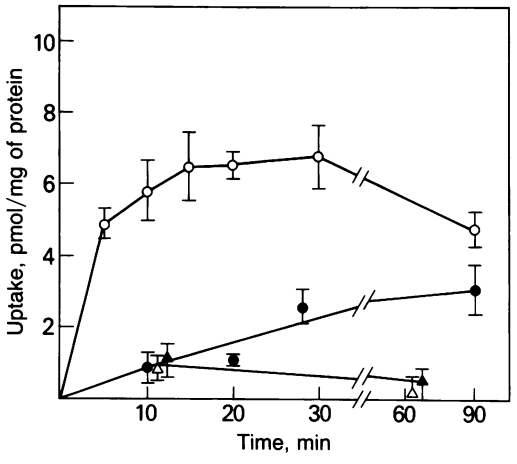

FIG. 1. Time course of ATP-dependent vinblastine uptake in vesicles from drug-sensitive and drug-resistant KB cells. Membrane vesicles from drug-resistant cells (○, ●) and drug-sensitive cells (△, ▲) were suspended in buffer A. An ATP-regenerating system composed of ATP, creatine kinase, and creatine phosphate (final concentrations 0.3 mM, 3 μg/100 μl, and 3 mM, respectively) was added (○, △). After 30 sec, reaction medium containing [^{3}H]-vinblastine (final concentration, 4.5 nM) was added and vinblastine uptake was measured as a function of time. Uptake when ATP was replaced with AMP was also measured (●, ▲).

ATP Hydrolysis Is Required for Vinblastine Transport. ATP-dependent vinblastine uptake was measured as a function of ATP concentration to determine an apparent K_m for ATP in the transport process. ATP stimulated vinblastine uptake in a saturable manner (Fig. 3*A*). These data were replotted as uptake/[ATP] vs. uptake (Eadie–Hofstee plot; Fig. 3*B*). From this linear plot it is possible to calculate that ATP reacts with the transporter with Michaelis–Menten kinetics with an apparent K_m = 38 μM.

Table 1 shows the effect of a variety of ATPase inhibitors on ATP-dependent vinblastine transport. Although the Na^+,K^+-ATPase inhibitor ouabain did not inhibit vinblastine uptake (data not shown), both the nonspecific ATPase inhibitor vanadate and the nonhydrolyzable ATP analog Ado*PP*[NH]*P* were potent inhibitors of the transport activity and acted in a dose-dependent manner. Fig. 4 shows a kinetic analysis of inhibition of vinblastine transport by vanadate and Ado*PP*[NH]*P*. Eadie–Hofstee plots of vinblastine uptake in the presence of 0.4 mM Ado*PP*[NH]*P* and ATP yield the same apparent V_{max} as for ATP alone, but the apparent K_m value is different. These data show that Ado*PP*[NH]*P* is a competitive inhibitor of ATP for vinblastine transport, with an apparent K_i of ≈0.1 mM. Fig. 4 also shows that vanadate at 0.01 mM is a noncompetitive inhibitor of ATP for this transport system. These results indicate that hydrolysis of ATP is probably required for vinblastine transport into vesicles.

Inhibition of Vinblastine Transport by Other Cytotoxic Drugs and Reversing Agents. The binding of [^{125}I]NASV to P-glycoprotein is inhibited by a variety of cytotoxic drugs to which multidrug-resistant cells are resistant (17), as well as by agents such as verapamil and quinidine, which reverse drug resistance (18). These results suggest that there are only a small number of sites on P-glycoprotein to which drugs bind and that the presence of one drug may interfere with transport of other drugs. To test this hypothesis, ATP-dependent [^{3}H]vinblastine transport was measured in the presence of various concentrations of other cytotoxic drugs

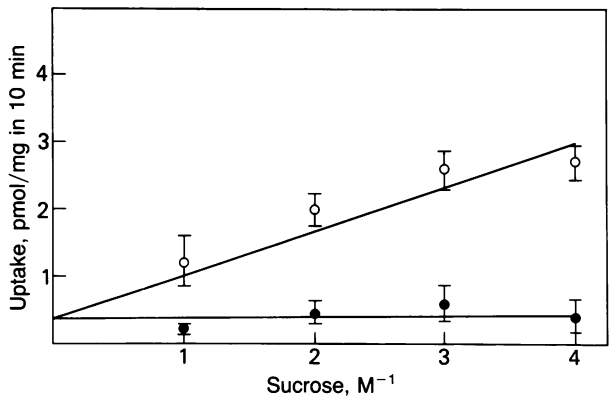

FIG. 2. Osmotic sensitivity of ATP-dependent vinblastine uptake. Vesicles from drug-resistant cells suspended in buffer A were incubated with the same buffer containing 0.25–1.0 M sucrose (final concentration) for ≈60 min at 25°C. The sucrose concentration of the ATP medium and the reaction medium was adjusted to be the same as that of the vesicle suspension. Vinblastine uptake was measured after 10 min of incubation with ATP (○) or with AMP (●) instead of ATP. The vinblastine concentration was 2.3 nM.

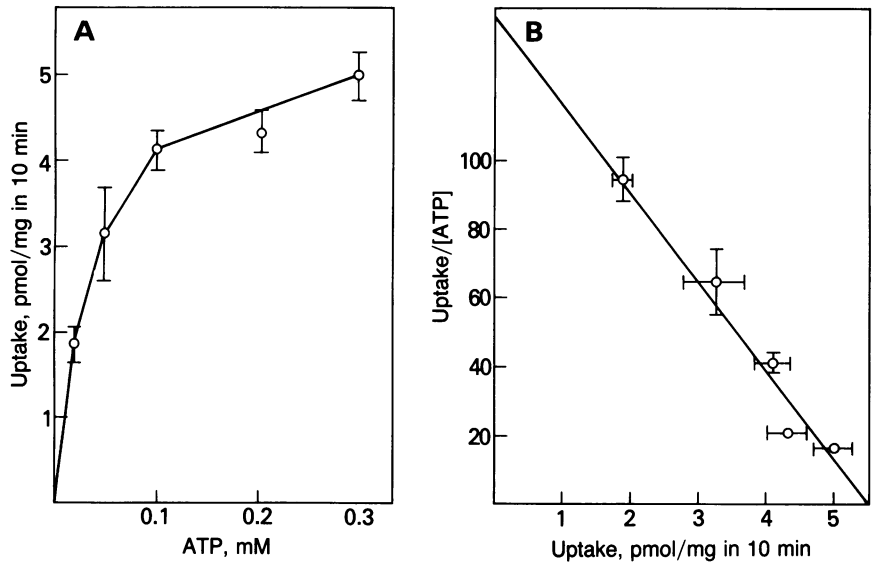

FIG. 3. ATP concentration dependence. (*A*) ATP-dependent vinblastine uptake was measured as a function of ATP concentration in vesicles from drug-resistant cells. Vinblastine concentration was 7.6 nM. (*B*) Eadie–Hofstee plot of the data shown in Fig. 3*A*. A least-squares fit of this plot yields an apparent K_m = 38 μM.

known to be handled by the *mdr* transport system and in the presence of verapamil and quinidine. Vincristine, actinomycin D, verapamil, and quinidine were all found to be potent inhibitors of uptake (IC_{50} < 5 μM), whereas daunomycin was less effective (IC_{50} ≈30 μM). Puromycin and colchicine inhibited transport at >100 μM. These data are summarized in Table 1.

To determine whether inhibition by drugs was competitive or noncompetitive, a more detailed kinetic analysis was conducted for daunomycin inhibition of vinblastine uptake. Fig. 5*B* shows Eadie–Hofstee plots of vinblastine transport in the absence and in the presence of 20 μM and 40 μM daunomycin. The linear plots indicate that the vinblastine transport site has Michaelis–Menten kinetics with an apparent K_m, obtained by least-squares analysis, of 2.18 ± 0.33 μM (four independent experiments). Daunomycin increased the apparent K_m value in a dose-dependent manner but did not affect the apparent V_{max}. These data show that daunomycin is a competitive inhibitor of vinblastine uptake, with an apparent K_i of ≈29 μM.

DISCUSSION

It has been proposed that multidrug resistance results from the efflux of hydrophobic drugs out of resistant cells (23). In this paper we show that there is an active vinblastine-transport system energized by ATP in membrane vesicles from multidrug-resistant cells. The ATP-dependent stimulation of vinblastine uptake and the osmosensitivity of this process indicate that true transport, and not vinblastine binding to membranes, has been measured. Transport occurs from the incubation medium to the interior of the vesicle, suggesting that those vesicles that are transporting drug into their interior are probably inside-out, because drug-resistant cells normally transport drug from the cytoplasm to the extracellular space. Because this transporter was not found in vesicles derived from drug-sensitive cells, these results confirm the hypothesis that multidrug resistance results from the presence of an energy-dependent transport system in plasma membranes of drug-resistant cells. The product of the *MDR1* gene, P-glycoprotein, binds the drugs under study (16–18), as well as ATP (19), and is present in these transporting membrane vesicles (refs. 17 and 18 and data not shown). Hence the *MDR1* gene product

Table 1. Inhibitors of vinblastine transport

Agent	IC_{50}, μM
Ado*PP*[NH]*P*	500*
Vanadate	10*
Vinblastine	2
Vincristine	2
Actinomycin D	4
Daunomycin	30
Colchicine	160
Puromycin	170
Verapamil	3
Quinidine	3

IC_{50} is the concentration of the agent that inhibits vinblastine transport by 50%.
*ATP concentration in these experiments was 100 μM; in all other experiments shown here it was 300 μM.

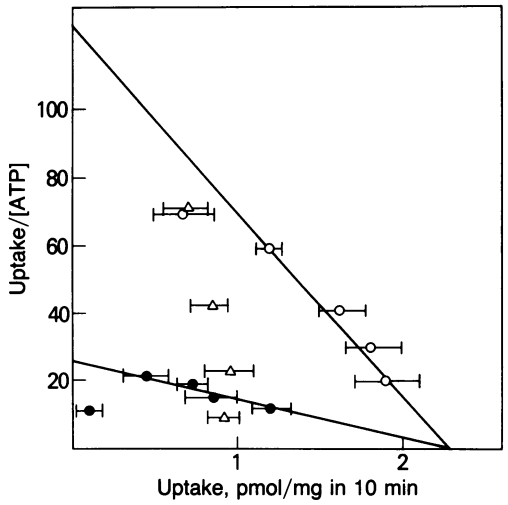

FIG. 4. Kinetics of inhibition of vinblastine uptake by Ado*PP*[NH]*P* and vanadate. ATP-dependent vinblastine uptake by vesicles from multidrug-resistant cells was measured as a function of ATP concentration with 0.01 mM vanadate (△), 0.4 mM Ado*PP*[NH]*P* (●), or without inhibitor (○). Vinblastine concentration was 4.7 nM. The plots of Ado*PP*[NH]*P* and control give lines with similar intercepts on the abscissa. The apparent K_i value for Ado*PP*[NH]*P* inhibition was about 0.1 mM.

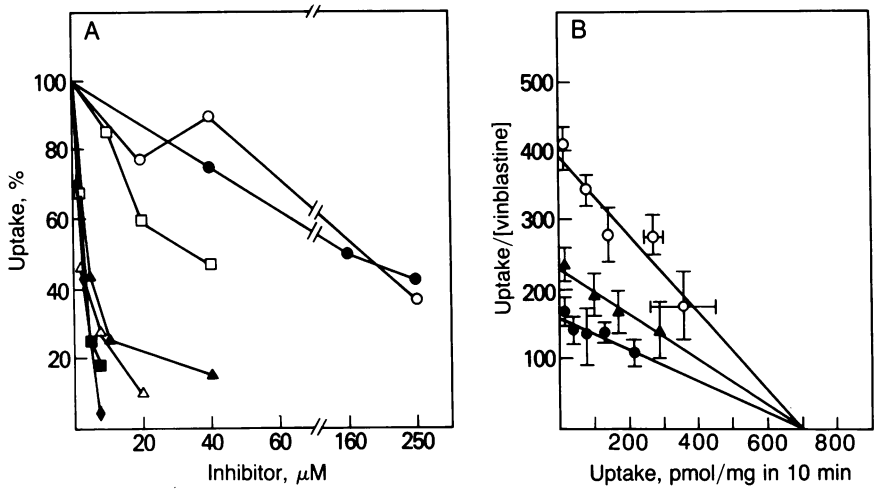

FIG. 5. Inhibition of vinblastine uptake by other cytotoxic drugs and reversing agents. (*A*) Inhibition of ATP-dependent vinblastine uptake by puromycin (○), colchicine (●), daunomycin (□), actinomycin D (▲), vincristine (△), verapamil (■), and quinidine (◆). Vinblastine concentration was <10 nM. (*B*) Eadie–Hofstee plots of a kinetic study of vinblastine uptake and its inhibition by daunomycin. ATP-dependent uptake of vinblastine was measured as a function of vinblastine concentration (0.003, 0.25, 0.5, 1.0, and 2.0 μM) with 0 μM (○), 20 μM (▲), and 40 μM (●) daunomycin. A least-squares plot of uptake in the absence of daunomycin yields an apparent K_m = 1.8 μM in this experiment. The plots of uptake in the presence and absence of daunomycin give lines that intersect at the abscissa, indicating that daunomycin is a competitive inhibitor of vinblastine uptake. The apparent K_i for daunomycin was 29 μM.

is extremely likely to be the vinblastine transporter defined here.

These initial studies have revealed several interesting properties of the transport process. KB cells are killed by 0.5–1.0 μM vinblastine. The apparent K_m for vinblastine transport in vesicles is ≈2 μM. If it is assumed that free intracellular and extracellular vinblastine concentrations are approximately the same in drug-sensitive cells, the acquisition of the transporter could render the cells drug-resistant based on the measured transport affinity of 2 μM.

DNA sequence data show two potential ATP binding sites on P-glycoprotein (11, 12), but the kinetic analysis of transport shown here indicates that the ATP requirement has a hyperbolic concentration curve and a linear Eadie–Hofstee plot, consistent with the use of a single ATP binding site for vinblastine transport. The fact that two very similar ATP binding sites are conserved suggests that both sites have an important function. The nonhydrolyzable ATP analog Ado*PP*[NH]*P* is not a substrate for the enzyme but is a competitive inhibitor of ATP, suggesting that ATP hydrolysis is needed for transport and that ATP is not simply an allosteric activator. The inhibition of transport by the ATPase inhibitor vanadate also supports the conclusion that ATPase activity is required for vinblastine transport.

These studies also provide data indicating that there are a limited number of drug-transporting sites on the transporter. Many cytotoxic drugs to which multidrug-resistant lines are resistant, including vincristine, actinomycin D, and daunomycin, were found to be good inhibitors of the vinblastine transport (Table 1). In the case of daunomycin, we showed that this inhibition was competitive. Colchicine and puromycin were less effective inhibitors of vinblastine transport. Colchicine was also a poor inhibitor of [^{3}H]vinblastine binding to membrane vesicles (17), suggesting the possibility that there may be a second binding and transport site for colchicine on P-glycoprotein, or that colchicine is simply not as good a substrate for the transporter because it does not bind as well to P-glycoprotein. Verapamil and quinidine, which reverse drug resistance and also inhibit [^{125}I]NASV binding to P-glycoprotein (18), were potent inhibitors of the transport process as well. These results indicate that this transport assay may provide an extremely sensitive screening test for agents that reverse multidrug resistance.

Recent data suggest that expression of the *MDR1* gene is associated with intrinsic and acquired multidrug resistance in human cancer (23–26). The complete characterization of the transport system described here will allow the development of new approaches to the circumvention of this impediment to successful cancer chemotherapy.

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