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Agents which reverse multidrug-resistance are inhibitors of [^{3}H]vinblastine transport by isolated vesicles

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Resistance of human cancer cells to multiple cytotoxic hydrophobic agents (multidrug resistance) is due to overexpression of the *MDR*1 gene whose product is the ATP-dependent multidrug transporter, P-glycoprotein. We have previously reported that plasma membrane vesicles partially purified from multidrug-resistant human KB carcinoma cells, but not from drug-sensitive cells, accumulated [^{3}H]vinblastine in an ATP-dependent manner (Horio, M., Gottesman, M.M. and Pastan, I. (1988) Proc. Natl. Acad. Sci. USA 85, 3580–3584). Certain calcium-channel blockers, quinidine, and phenothiazines are able to overcome multidrug resistance in cultured cells. In this work, the effect of these reversing agents on ATP-dependent vinblastine (VBL) transport by vesicles from drug-resistant KB cells has been characterized. Azidopine was the most potent inhibitor of ATP-dependent VBL uptake tested (ID_{50}: concentration of inhibitor such that the transport of vinblastine is inhibited by 50%, < 1 μM). Verapamil, quinidine, and the tiapamil analogue RO-11-2933 were potent but less effective inhibitors ($ID_{50} < 5$ μM). Diltiazem, nifedipine and trifluoperazine were even less effective. These agents had no effect on Na^+-dependent and Na^+-independent L-leucine uptake by the vesicles, indicating that the inhibition of ATP dependent VBL transport by these agents is not a non-specific effect, as might result from leaks in the vesicle membrane. Verapamil, quinidine, azidopine and trifluoperazine increased the apparent K_m value of vinblastine transport, suggesting that these agents may be competitive inhibitors of vinblastine transport.

Introduction

Resistance of cultured cells to many cytotoxic drugs that are natural products results from expression of the *MDR*1 gene which encodes the 170 kDa P-glycoprotein or multidrug transporter (reviewed in Ref. 1). This transporter uses the energy of ATP to pump drugs with widely varying structures such as adriamycin (doxorubicin), vinblastine, vincristine, taxol, teniposide, etoposide and actinomycin D out of cells [2]. Resistance to these agents resulting from the activity of the transporter can be reversed by other less cytotoxic drugs including calcium channel blockers such as verapamil [3] and azidopine [4], and other agents including quinidine [5] and phenothiazines [6].

To understand how a single transporter can handle so many different drugs, and to determine the mechanism of reversal of drug resistance, we have developed an in vitro ATP-dependent multidrug transport system using vesicles isolated from multidrug-resistant cells [7]. In a preliminary characterization of this system, we showed that ATP was essential for transport of [^{3}H]vinblastine, and that other drugs, including vincristine, actinomycin D, daunomycin, puromycin and colchicine inhibited this transport [7]. In the current work, we have evaluated the effect of agents which reverse multidrug-resistance on in vitro [^{3}H]vinblastine transport. The most potent reversing agents are strong, probably competitive inhibitors of transport, while less potent reversing agents are weaker inhibitors.

Materials and Methods

Cell culture

KB-3-1, the parent cell line for the drug-resistant mutants described in this study, was derived from a single clone of human KB epidermoid carcinoma cells after two subclonings [8]. The Adriamycin (doxorubicin) and vinblastine resistant sublines (KB-A10 and KB-V1, respectively) were selected with increasing con-

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centrations of these respective drugs to 10 μg/ml of adriamycin or 1 μg/ml of vinblastine [9].

Vesicle preparation

Plasma membrane vesicles were prepared from human KB cell lines, KB-3-1, KB-A10 and KB-V1, by nitrogen cavitation as previously described [7].

[^{3}H]Vinblastine uptake by vesicles

The procedure for measuring the uptake of [^{3}H]vinblastine was previously described [7]. A 20 μl aliquot of vesicles was placed in a glass test tube. 30 μl of ATP buffer (10 mM Tris-HCl (pH 7.5), 1 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 10 mM $MgCl_2$, 0.25 M sucrose) was added. After 30 s, a 50 μl aliquot of reaction medium containing [^{3}H]vinblastine, 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose was added. For measurement of the inhibitory effect of an agent on vinblastine transport, we used a reaction medium containing the agent at an appropriate concentration. After 10 min at 25°C, the reaction was terminated by the addition of 1 ml of ice cold buffer (10 mM Tris-HCl (pH 7.5), 0.25 M sucrose). The vesicles were then applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filters were then washed further with 5 ml of ice cold buffer. The filters were dissolved in scintillation fluid and counted to determine [^{3}H]vinblastine uptake by the vesicles. ATP-dependent association of vinblastine with the vesicles was determined by replacing the ATP with AMP in the incubation medium, and ATP dependent uptake was calculated by subtracting this uptake from that determined in the presence of ATP. We reported the properties of this ATP dependent VBL transport previously [7]. Vesicles from multidrug-resistant human KB carcinoma cells, but not from drug sensitive cells showed ATP-dependent VBL transport, and this 'transport' was osmotically sensitive indicating that it was not due to binding of vinblastine to the vesicles but represented the intravesicular accumulation of vinblastine.

Because vinblastine is a hydrophobic substance and binds non-specifically to the filters giving a high background in the assay, we could not measure uptake after short time to give an accurate initial transport rate. We used the 10 min uptake of vinblastine as a transport rate for the kinetics analysis. This uptake is close to the steady-state uptake, but is not an initial transport rate. The 10 min uptake does not represent a final equilibrium state. Outside to inside transport of vinblastine by the ATP-driven pump is balanced by inside to outside diffusion of vinblastine in this state. The uptake of vinblastine is decreased at 90 min probably by consumption of ATP [7]. The transport rate of the pump at 10 min is thought to be the same as diffusion driven by the vinblastine concentration gradient between the inside and outside of the vesicles. This concentration difference was used as a measure of the ATP dependent uptake. Uptake at 10 min is therefore not a real initial transport rate, but can be thought as an apparent transport rate measured under the conditions observed in this manuscript.

[^{3}H]Vinblastine and L-[^{3}H]leucine were filtered through a Millipore filter (Millex^R GV, 0.22 μm) before use. All experimental points were obtained at least in triplicate. Standard deviations were calculated where possible, and they are indicated as error bars in the figures.

Materials

[^{3}H]Vinblastine and L-[^{3}H]leucine were obtained from Amersham. Tissue culture products were from GIBCO. Azidopine was purchased from Amersham. Verapamil, quinidine, diltiazem and nifedipine were purchased from Sigma. The tiapamil analog RO-11-2933 was a generous gift from Hoffman LaRoche. All other chemicals were of reagent grade.

Results

Agents which reverse multidrug resistance inhibit vinblastine transport by vesicles

It has been reported that certain calcium channel blockers such as verapamil, the phenothiazine calmodulin antagonist trifluoperazine, and quinidine can overcome the multidrug resistant phenotype of tissue culture cells. We reported previously that plasma membrane vesicles from MDR cells had an ATP dependent VBL transport system which was inhibited by verapamil, quinidine and hydrophobic cytotoxic drugs such as daunomycin, vincristine and actinomycin D. These results suggest that this ATP dependent transporter extrudes all these cytotoxic drugs and that the multidrug-resistance phenomenon is due to this transport system.

If multidrug-resistance depends on energy-dependent transport activity, agents which reverse multidrug-resistance might be inhibitors of ATP-dependent VBL transport. To test this hypothesis, ATP-dependent [^{3}H]vinblastine uptake by vesicles from V1 cells was measured in the presence of various concentrations of verapamil, azidopine, the tiapamil analog RO-11-2933, diltiazem, nifedipine, trifluoperazine and quinidine (Fig. 1). Azidopine was found to be the most potent inhibitor of uptake (ID_{50} < 1 μM). Verapamil, quinidine, and RO-11-2933 were potent but less effective (ID_{50} < 5 μM). Diltiazem, nifedipine and trifluoperazine were much less effective.

Inhibitors of vinblastine transport do not affect leucine uptake

To determine whether these inhibitors have non-specific effects on other transport systems, as could occur if they increase the leakiness of the vesicles, we

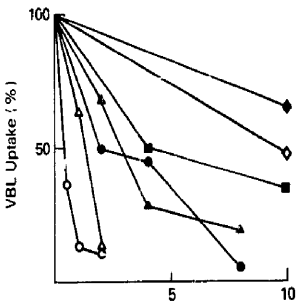

Fig. 1. Inhibition of ATP-dependent vinblastine uptake by reversing agents. ATP-dependent vinblastine uptake by vesicles from V1 cells was measured with verapamil (▲), quinidine (●), azidopine (○), diltiazem (■) trifluoperazine (◇), nifedipine (◆), and the tiapamil analogue RO-11-2933 (△) and compared to ATP-dependent uptake in the absence of inhibitors taken as 100%. The vinblastine concentration was under 10 nM.

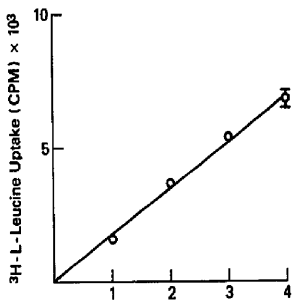

Fig. 3. Effect of extravesicular osmolarity on the equilibrium uptake of L-leucine. Vesicles from V1 cells were preloaded with 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose. The incubation medium was the same medium containing 0.4 μM L-[^{3}H]leucine and 0.25–1.0 M sucrose (final concentration). Uptake was measured after 30 min of incubation.

measured the effect of these inhibitors on L-[^{3}H]leucine uptake by the vesicles. The time course of L-leucine association with vesicles from KB-V1 cells is shown in Fig. 2. NaCl was used to attain an initial 60 mM Na^+ gradient between extravesicular and intravesicular space. KCl was used to replace NaCl to measure Na^+-independent L-leucine uptake. When NaCl was used, the uptake of L-leucine at 30 s, 1 min and 2 min into vesicles was higher compared to that when KCl was used, indicating that the vesicles from multidrug-resistant cells show Na^+ dependent L-leucine uptake that is about 30% of total uptake at 30 s. The same ratio between Na^+-dependent and Na^+-independent L-leucine uptake was reported for L-leucine transport in Chinese hamster ovary cells [10].

True transport of L-leucine into vesicles should be sensitive to the intravesicular space which can be manipulated by varying extravesicular osmolarity. The osmolarity of the extravesicular medium was varied by changing its sucrose concentration. We measure L-leucine uptake by vesicles from KB-V1 cells for 30 min at 25°C in the presence of 60 mM NaCl. When the uptake is plotted against the inverse of sucrose molarity (Fig. 3), a straight line is obtained. The non-zero intercept on the vertical axis represents 'uptake' when the extravesicular osmolarity is extrapolated to infinity. This component of 'uptake' is probably due to binding to membranes and was negligibly small. This result indicates that L-leucine is transported into the intravesicular space and binding of L-leucine to the membranes is very low.

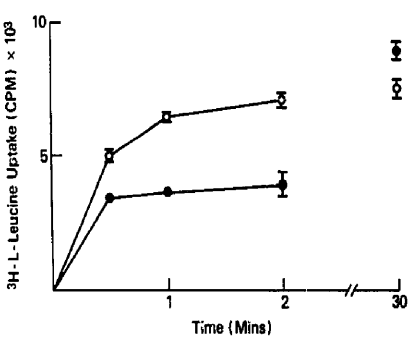

Fig. 2. Time-course of L-leucine uptake into membrane vesicles from V1 cells. Membranes vesicles were preloaded with 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose. Incubation medium was the same medium containing 0.3 μM L-[^{3}H]leucine with 84 mM NaCl (○) or 84 mM KCl (●).

Fig. 4 shows the effect of reversing agents on Na^+-dependent and Na^+-independent L-leucine uptake by vesicles from KB-V1 cells. 2 μM azidopine, 8 μM quinidine, 8 μM verapamil, 20 μM nifedipine, 20 μM diltiazem and 20 μM trifluoperazine did not affect Na^+-independent L-leucine uptake by the vesicles, indicating that these agents did not non-specifically inhibit transport or compromise the integrity of the membrane vesicles at the concentrations used in these transport studies. Azidopine, quinidine, verapamil, diltiazem and trifluoperazine also did not inhibit Na^+-dependent L-leucine uptake.

Reversing agents are probably competitive inhibitors of vinblastine transport

To determine whether inhibition by these agents was competitive or non-competitive, a more detailed kinetic analysis was conducted for verapamil, quinidine, azidopine and nifedipine inhibition of ATP-dependent vinblastine uptake.

Fig. 5 shows Eadie-Hofstee plots of ATP-dependent vinblastine uptake by the vesicles from A10 cells and

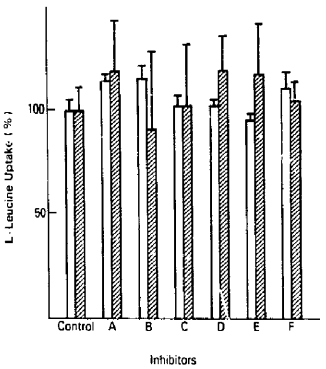

Fig. 4. Effect of reversing agents on Na^+-dependent and Na^+-independent L-leucine uptake by vesicles from V1 cells. Vesicles were preloaded with 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose. The Na^+ independent L-leucine uptake (open bar) was measured at 15 s using the incubation medium which contained 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, and 0.3 μM L-[^{3}H]leucine with or without reversing agent. Na^+-dependent L-leucine uptake (cross-hatched bar) was calculated from the total flux with the incubation medium containing 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose and 84 mM NaCl by subtracting the Na^+-independent uptake measured with choline replacing sodium. A, 2 μM azidopine; B, 8 μM quinidine; C, 8 μM verapamil; D, 20 μM nifedipine; E, 20 μM diltiazem; F, 20 μM trifluoperazine.

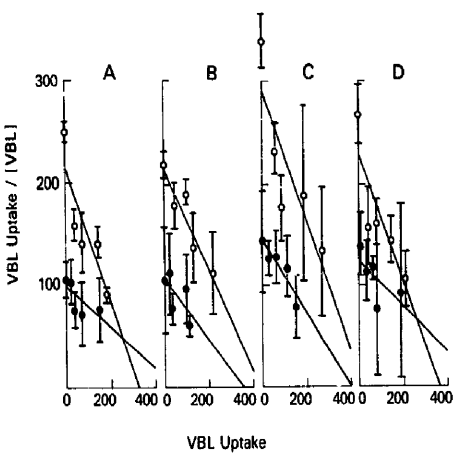

Fig. 5. Kinetics of inhibition of vinblastine uptake by verapamil, quinidine, nifedipine and azidopine. ATP-dependent vinblastine uptake by vesicles from A10 cells (A, B, C) and V1 cells (D) was measured as a function of VBL concentration (0.01, 0.25, 0.5, 1.0 and 2.0 μM) with inhibitors (●) or without inhibitors (○). Data are shown as Eadie-Hofstee plots. The units of VBL uptake are pmol/mg protein per 10 min. (A) 4 μM verapamil, (B) 4 μM quinidine, (C) 20 μM nifedipine, (D) 0.5 μM azidopine. A least-square plot of uptake in the absence of inhibitors yields an apparent $K_m = 1.77 \pm 0.32$ μM (A, B, C; vesicles from A10 cells) and 1.68 μM (D; vesicles from V1 cells). Inhibitors increase the apparent K_m values and least-squares plots of the uptake in the presence and absence of inhibitors give lines which intersect near the abscissa, suggesting that these agents are competitive inhibitors of ATP-dependent VBL uptake.

V1 cells in the absence and presence of 4 μM verapamil, 4 μM quinidine, 0.5 μM azidopine and 20 μM nifedipine. The plots in the absence of inhibitors are linear indicating that the vinblastine transport site has Michaelis-Menten kinetics with apparent K_m values obtained by least-squares analysis of 1.77 ± 0.32 μM in A10 vesicles (Fig. 5, A, B, C) and 1.68 μM in V1 vesicles ((Fig. 5D). Inhibitors changed the slope of the regression lines. Least-squares plots of the uptake in the presence and absence of inhibitors give lines which intersect near the abscissa. Because of the large standard error in most of the data points, statistical analysis does not indicate whether verapamil, quinidine, nifedipine and azidopine are pure competitive inhibitors or mixed inhibitors of vinblastine uptake.

Discussion

It has been known that many drugs including certain calcium-channel blockers such as verapamil, the calmodulin antagonist trifluoperazine and quinidine are able to reverse multidrug resistance. It is important to study the mechanism of the reversing effect of these agents to understand the multidrug resistance phenomenon and to derive potential therapy for it. In this paper, we show that azidopine, verapamil, quinidine, the tiapamil analogue RO-11-2933, diltiazem, nifedipine and trifluoperazine inhibit ATP dependent VBL transport into vesicles from drug resistant KB cells (Fig. 1). These agents had no effect on Na^+-dependent or Na^+-independent L-leucine uptake. Thus, these data suggest that these agents specifically inhibit ATP dependent VBL transport by vesicles.

It has been proposed that multidrug-resistance results from the efflux of hydrophobic drugs out of resistant cells [1,2]. This efflux pump is apparently the ATP-dependent VBL transport system that we reported previously [7] and have demonstrated in this paper. Agents which are good inhibitors of ATP-dependent VBL transport should be potent reversors of multidrug-resistance and should inhibit the efflux of hydrophobic drugs out of resistant cells.

Azidopine was the most potent inhibitor of ATP dependent VBL transport ($ID_{50} < 1$ μM). Verapamil, quinidine, and RO-11-2933 were somewhat less effective ($ID_{50} < 5$ μM), and diltiazem, nifedipine and trifluoperazine were much less effective. This order of effectiveness is similar to the ability of these drugs to reverse multidrug-resistance in cultured cells [11], except for trifluoperazine, which is a relatively potent reversing agent [6]. We have previously noted that phenothiazines are more potent at reversing resistance than at inhibiting ^{125}I-NASV-labeling of P-glycoprotein, suggesting that these agents may inhibit the multidrug transporter by a mechanism other than direct competition for the pump [12]. Because calmodulin antagonists

strongly bind to plasma membranes it is possible these agents could act by nonspecifically increasing the permeability of the plasma membrane. However, the failure of trifluoperazine to affect L-[^{3}H]leucine uptake appears to rule out this mechanism.

Kinetic studies of ATP dependent VBL transport by vesicles from KB-A10 cells showed that the vinblastine transport site has Michaelis-Menten kinetics with an apparent K_m of 1.77 μM (Fig. 5). This value is almost the same as the apparent K_m value of ATP dependent VBL transport by vesicles from KB-V1 cells that was previously reported [7] and confirmed in the current analysis (Fig. 5D). Azidopine, verapamil, quinidine and nifedipine changed the slopes of the regression lines (Fig. 5) suggesting that these agents are competitive inhibitors of ATP dependent vinblastine uptake. ATP dependent VBL transport was inhibited by daunomycin in a competitive manner, and also inhibited by vincristine and actinomycin D [7]. These results suggest that verapamil, quinidine, azidopine and nifedipine bind to the same site on P-glycoprotein as vinblastine, daunomycin and probably other hydrophobic cytotoxic drugs, and thereby interfere with transport of these drugs.

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