0026-895X/94/040763-10$03.00/0

MOLECULAR PHARMACOLOGY, 45:763–772

Kinetic Evidence Suggesting that the Multidrug Transporter Differentially Handles Influx and Efflux of Its Substrates

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Received April 12, 1993; Accepted January 4, 1994

SUMMARY

A kinetic approach was used to analyze the mechanism by which a substitution of valine for glycine at position 185 in the multidrug transporter alters its substrate specificity so that colchicine and etoposide transport is increased, daunorubicin transport is unchanged, and vinblastine transport is decreased. Time courses for uptake and efflux of colchicine, vinblastine, etoposide, and daunorubicin for NIH/3T3 mouse cells transfected with wild-type (MDR1-G185) and mutant (MDR1-V185) strains of the human *mdr1* gene were determined at room temperature in the presence and absence of an energy supply. The initial rate of vinblastine uptake was reduced approximately 5-fold by glucose feeding of ATP-depleted wild-type (MDR1-G185) cells but was only halved in MDR1-V185 transfectants. In contrast, glucose feeding decreased the initial rate of colchicine uptake approximately 4-fold in the MDR1-V185 (mutant) transfectant but not in the MDR1-G185 (wild-type) transfectant. Efflux of colchicine was accelerated >5-fold in both the MDR1-V185 (mutant) and MDR1-G185 (wild-type) transfectants when glucose was given to raise ATP levels. The effects on initial rates of colchicine uptake accounted semiquantitatively for the increased colchicine resistance of MDR1-V185 (mutant) transfectants. Similar effects were found for etoposide in the MDR-V185 transfectants. Quinidine in the external medium greatly inhibited drug entry rates but had little effect on efflux, whereas verapamil inhibited both uptake and efflux. A possible interpretation of these data is that the multidrug transporter extracts drugs from the external and internal halves of the membrane bilayer by different paths, which are distinguishable by mutation and inhibitors.

Tumor cells, both in patients and in cell culture, can develop resistance to a variety of chemotherapeutic drugs. This is often brought about by enhanced expression of the *mdr1* gene (1–7), which encodes the P170, P-glycoprotein, or multidrug (MDR1) transport protein. Introduction of a cloned *mdr* gene into drug-sensitive cells renders the recipients resistant to a variety of amphiphilic molecules (8–10), the substrates of the P170 glycoprotein. The *mdr* gene is expressed in several normal tissues, with highest levels in intestine, kidney, liver, and adrenal gland (11). The physiological role of P-glycoprotein is unknown, but it may help to protect the organism against xenobiotic substances and toxic endogenous metabolites (7). The presence of the MDR protein, together with a source of metabolic energy, leads to reduced accumulation of MDR drugs (1–3). The system thus appears to function as an energy-driven pump for the net efflux of its substrates.

The *mdr* gene belongs to a superfamily of ATP-dependent transport proteins (12) that includes a number of biologically interesting and medically significant members, such as the cystic fibrosis transmembrane regulator (13), a *Falciparum* malaria chloroquine resistance (*pfmdr*) gene (14), genes apparently involved in peptide transport during antigen presentation (15), and a transmembrane transporter (STE6) in yeast of a-factor mating pheromone, a hydrophobic substituted dodecapeptide (16). P-glycoprotein has been isolated from cell membranes and shown to be active as a drug-stimulated ATPase (17, 18). Understanding the bioenergetics of P-glycoprotein might shed light on general mechanisms of action for this superfamily of transporters.

Although the MDR system possesses a broad specificity towards its substrates, it has been shown that the wild-type *mdr1* gene codes for a protein that is more effective in expelling from the cell the protonatable, highly hydrophobic substrate vinblastine than the relatively water-soluble, uncharged colchicine (5). In contrast, selection of cells resistant to colchicine selects for a mutant *mdr1* gene that is relatively more effective against colchicine than against vinblastine (5, 19–21). The difference between the two strains arises from the substitution of a valine residue in place of the wild-type glycine residue at position 185 in the polypeptide chain (19–21). Direct transfec-

W.D.S. was the recipient of an American Cancer Society International Cancer Research Fellowship administered by the International Union against Cancer.

ABBREVIATIONS: MDR, multidrug resistance or resistant; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Az/DoG, 10 mM sodium azide plus 10 mM deoxyglucose in phosphate-buffered saline; PBS/Glu, 5 mM glucose in phosphate-buffered saline; VP-16, etoposide.

tion of the two DNA sequences showed that the Val-185 mutant was 3.9 times more resistant to colchicine than was the wild-type strain but was, in contrast, 2.6 times more sensitive to vinblastine (19). We describe here a detailed investigation of the time course of uptake and efflux of charged and uncharged substrates of P-glycoprotein by wild-type and mutant strains of the human *mdr1* gene transfected into mouse NIH/3T3 cells. It appears from our analysis that P-glycoprotein can handle the influx and efflux of its substrates somewhat differently and that it is the influx of drug that is differentially affected by the glycine to valine substitution at position 185.

Materials and Methods

Cell culture. Three lines of mouse NIH/3T3 cells, previously characterized, were used in these studies (21). One, termed here the sensitive strain, was the standard NIH/3T3 cell line (a gift of Charles Scher, University of Pennsylvania). The second, termed MDR1-G185, was a derivative of these NIH/3T3 cells that had been transfected, using the $CaPO_4$ technique, with a wild-type *mdr1* cDNA under control of a Harvey promoter (8) and then selected and maintained in the presence of 60 ng/ml colchicine. The third, MDR1-V185, was a derivative of the same NIH/3T3 line that had been transfected with pHaMDR1-V185, carrying a mutant human *mdr1* gene (8), selected initially in the presence of 60 ng/ml colchicine, and then exposed to increasing concentrations of colchicine in four steps, resulting in resistance to 1 μg/ml colchicine, in which MDR1-V185 transfectants were maintained. Fluorescence-activated cell sorting analysis demonstrated that MDR1-G185 and MDR1-V185 express the same amounts of cell surface P-glycoprotein under these growth conditions (21).

Cells were grown as monolayer cultures in DMEM with 10% fetal calf serum and an appropriate concentration of colchicine. Before use, the cells were seeded at 1×10^5 cells/2.5 ml of DMEM plus 10% fetal calf serum, without any drug, in 60-mm plastic tissue culture dishes (Falcon) and were grown to confluence (3–5 days) at 37° in a CO_2 incubator. The mean cell counts at confluence, determined in duplicate, over numerous weeks of experimentation were $1.60 \pm 0.62 \times 10^6$ cells/dish (mean ± standard error, seven experiments), $2.25 \pm 0.43 \times 10^6$ cells/dish (mean ± standard error, 11 experiments), and $3.06 \pm 0.53 \times 10^6$ cells/dish (mean ± standard error, 13 experiments) for the sensitive, MDR1-G185 (wild-type transfectant), and MDR1-V185 (mutant transfectant) cell lines, respectively. In certain experiments (see text), cells were starved for 20–60 min at 37° in Az/DoG. Direct counting showed that dishes containing such starved cells held at least 90% as many cells as did dishes containing the same strains but incubated in parallel in PBS/Glu.

Chemicals and radiochemicals. Media used (DMEM and PBS) were from Bio-Whittaker. The drugs used were colchicine, vinblastine, daunorubicin, and quinidine from Sigma, and VP-16 was a gift from Bristol-Myers Laboratories (Syracuse, NY). The tritiated radiochemicals were from the following sources: [^{3}H]colchicine, NEN DuPont; [^{3}H]vinblastine, Amersham (Arlington Heights, IL); [^{3}H]daunorubicin, NEN; and [^{3}H]VP-16, Moravek Biochemicals Inc. (Brea, CA). Trypsin (0.25%) (GIBCO), 7 mM EDTA, in Tris-dextrose buffer, was from the National Institutes of Health Media Unit.

Transport assays. Dishes containing a confluent layer of cells were taken from the 37° incubator in batches, the growth medium was removed by suction through a fine pipette, and this medium was replaced with 2 ml of either Az/DoG or PBS/Glu, followed by an additional incubation at 37° for the period specified in Results (generally 20–60 min). For measurements of drug uptake, which were always performed at room temperature, this initial incubation medium was replaced at time 0 with 1.5 or 2 ml of a solution of the labeled drug (generally 0.5 μCi/ml) at a concentration shown to be nontoxic for the sensitive strain of cells. After the desired incubation period (ranging from 12 sec to 360 min), uptake was stopped by rapid washing of the dishes, three times, in ice-cold PBS. "Time 0" blanks were prepared by adding ice-cold labeled solution and washing immediately. Where noted in Results, these time 0 blanks were subtracted from the counts at the measured room temperature time points. Dishes containing the labeled washed cells were left for at least 20 min at 37° in 1 ml of a medium containing 0.25% trypsin and 7 mM EDTA in Tris-dextrose buffer, to remove the cells from the dishes. This suspension of cells was aspirated quantitatively from the dishes, transferred to counting vials, and counted by liquid scintillation counting. It was shown directly that approximately 6% of the original cells still remained on the dishes after a second extraction in trypsin/EDTA. No correction was subsequently made for this small loss of cells. In every case, duplicate aliquots (generally 100 μl) of the labeled solutions were counted under the same conditions. Wherever possible, counts were accumulated until the counting error was <2%. In preliminary experiments in which determinations of the counts per dish were made after 360 min of incubation (i.e., at steady state), the average standard deviation in six different triplicate determinations was 11%.

Data are reported as the volume (in microliters) of the extracellular medium cleared by 10^6 cells during the reported time interval (22). This measure, which it will be convenient to term "clearance," was obtained by first dividing the counts/dish by the mean number of cells/dish (in millions, measured in duplicate parallel samples) and then by the number of counts in the incubation medium/1 μl of solution. Clearance (Q) (in microliters of cell water/10^6 cells), the internal concentration of drug (C_i) (in moles/liter of cell water), and the external concentration of drug (C_e) (in moles/liter of solution) are related by the formula $C_i = Q \times C_e/V$, where V is the volume of cell water (in microliters) associated with 10^6 cells. In the legends to the figures, we quote the drug concentration (in nanomolar) in the extracellular medium. This is the simple numerical factor by which clearance must be multiplied to obtain the conventional uptake units of femtomoles/10^6 cells. For a hypothetical drug that would be neither affected by any active transport system nor bound to any intracellular constituent, the steady state value of the uncoupled clearance, Q_{uc}, is merely the internal water space $V/10^6$ cells. Reporting the data as clearances enables a direct comparison to be made of the effectiveness of a particular cell line in allowing the uptake of one or another drug, independently of the particular concentration at which that drug happens to be present.[1] In addition, it allows an immediate appreciation to be made of whether the drug is accumulated within the cells. For the cells used in the present study, the intracellular volume happens to be approximately 1 μl/10^6 cells. A clearance of 1 μl therefore implies that the drug has reached approximately the same concentration in the intracellular fluid as is present in the external medium.

Results

Evidence that the kinetics of accumulation of colchicine and vinblastine are affected differently in MDR1-G185 (wild-type) and MDR1-V185 (mutant) NIH/3T3 transfectants. Fig. 1 depicts the time courses of uptake of colchicine (Fig. 1A) and vinblastine (Fig. 1B) into NIH/3T3 (wild-type parental) cells and into the MDR1-G185 (wild-type P-glycoprotein) and MDR1-V185 (mutant P-glycoprotein) lines of mouse NIH/3T3 cells that have been used in this study. In this and subsequent experiments, the concentrations of colchicine (100 nM or 40 ng/ml) and vinblastine (1.3 nM or 1 ng/ml) were approximately 1/200 and 1/400, respectively, of the concentration of drug reported as half-maximally stimulating the ATPase activity of the pump, measured as the isolated ATPase (18). In each case, both *mdr1*-expressing strains ac-

[1] Clearance is used here in a somewhat different way, compared with that of the kidney physiologists. There, the meaning is of a rate, the volume (of blood) cleared in unit time. Here, we refer to the amount cleared, at a particular time. Our usage is closer to the conventional one of "the action of clearing, or making clear (1563)" [*Shorter Oxford English Dictionary*, Ed. 3., 322 (1955)].

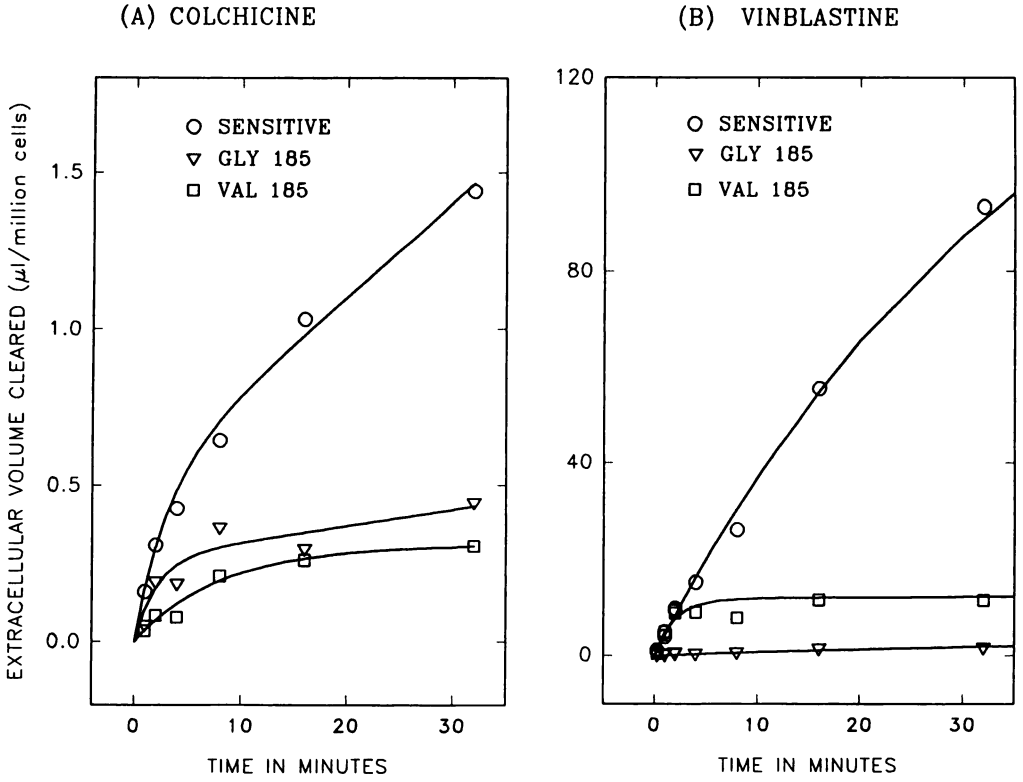

Fig. 1. Time course of uptake of radiolabeled drugs into three strains of NIH/3T3 cells at room temperature in the presence of 5 mM glucose. A, Colchicine (100 nM or 40 ng/ml, in this and subsequent experiments) uptake at 24°; B, vinblastine (1.3 nM or 1 ng/ml, in this and subsequent experiments) uptake at 25°. Cells either were the parental NIH/3T3 drug-sensitive strain (○) or had been transfected with *mdr1* vectors, as described in Materials and Methods, to yield the MDR1-G185 (wild-type) strain (▽) or the MDR1-V185 (mutant) strain (□) (see text). Each *point* represents a single Petri dish. In this and subsequent figures, the *ordinate* gives the amount of drug accumulated per 10^6 cells, in terms of the volume of the external medium that had been cleared of the drug. Multiplying this number by the drug concentration (in nanomolar) gives the number of femtomoles of drug that have entered 10^6 cells at the times indicated. The *lines* drawn have no theoretical significance for the purposes of the present paper.

cumulated markedly less drug than did the drug-sensitive strain. The MDR1-V185 (mutant) transfectant accumulated less colchicine than did the MDR1-G185 (wild-type) transfectant (Fig. 1A), whereas the reverse was true for vinblastine, which showed virtually no accumulation in MDR1-G185 (Fig. 1B). For the drug-sensitive strain, the extent of accumulation of vinblastine at 40 min, measured in terms of clearance (see Materials and Methods), was approximately 100 times higher than that of colchicine, whereas its uptake at the earliest times was approximately 30 times higher.

Uptake and efflux of vinblastine. Fig. 2 depicts similar data on the early time course of vinblastine entering MDR1-G185 (wild-type) cells (Fig. 2A) and MDR1-V185 (mutant) cells (Fig. 2B). The cells were incubated with glucose or starved in Az/DoG and the uptake of drug was then measured in PBS/Glu or Az/DoG. Note here the essentially linear time course of uptake of the drug into cells that had been starved in Az/DoG. Note also that the initial rate of vinblastine entry from PBS/Glu into cells was far slower than from Az/DoG for the MDR1-G185 (wild-type) transfectants, although these rates were comparable in the case of the MDR1-V185 transfectant (for regression data, see the legend to Fig. 2). Note also that it required at least 2 min before the full effect of re-energization could be observed in the wild-type cells (Fig. 2A).

Effect of energy deprivation on colchicine and vinblastine uptake. Fig. 3A explores the effect of energy deprivation on the initial rate of entry of colchicine. The data here were accumulated at short times (up to 60 sec). Fig. 3A compares colchicine uptake in cells starved in Az/DoG and then incubated in Az/DoG with that in cells incubated in parallel in PBS/Glu, i.e., with an adequate energy source, with transport then being followed in PBS/Glu. Energy deprivation greatly increased the initial rate of colchicine uptake in the MDR1-V185 transfectant (about 4-fold, by visual inspection of the lines), while having only little, if any, effect on uptake by the MDR1-G185 (wild-type) transfectant. Note that at 60 sec the clearance was only 0.2–0.3 μl/10^6 cells for the more rapid uptakes and <0.1 μl/10^6 cells for the slowest set, whereas with such cells the water space (and hence the equilibrium value of free colchicine uptake) would be about 1 μl/10^6 cells.

Fig. 3B presents a similarly designed study of vinblastine uptake but comparing the uptake in the sensitive strain with that in the two drug-resistant strains, rather than examining the effect of energy deprivation. There was an obvious effect of the presence of an active *mdr1* gene on the initial rate of vinblastine uptake. For the MDR1-G185 transfectant uptake was roughly 20% that of the sensitive strain and for the MDR1-V185 transfectant it was about half as great (for regression data, see the legend to Fig. 3). At the first data point, the clearance equaled or approached the value that would be predicted from the water space if vinblastine were present as the free drug.

Analysis of uptake and efflux of colchicine. Fig. 4A shows the time course of accumulation of colchicine by MDR1-

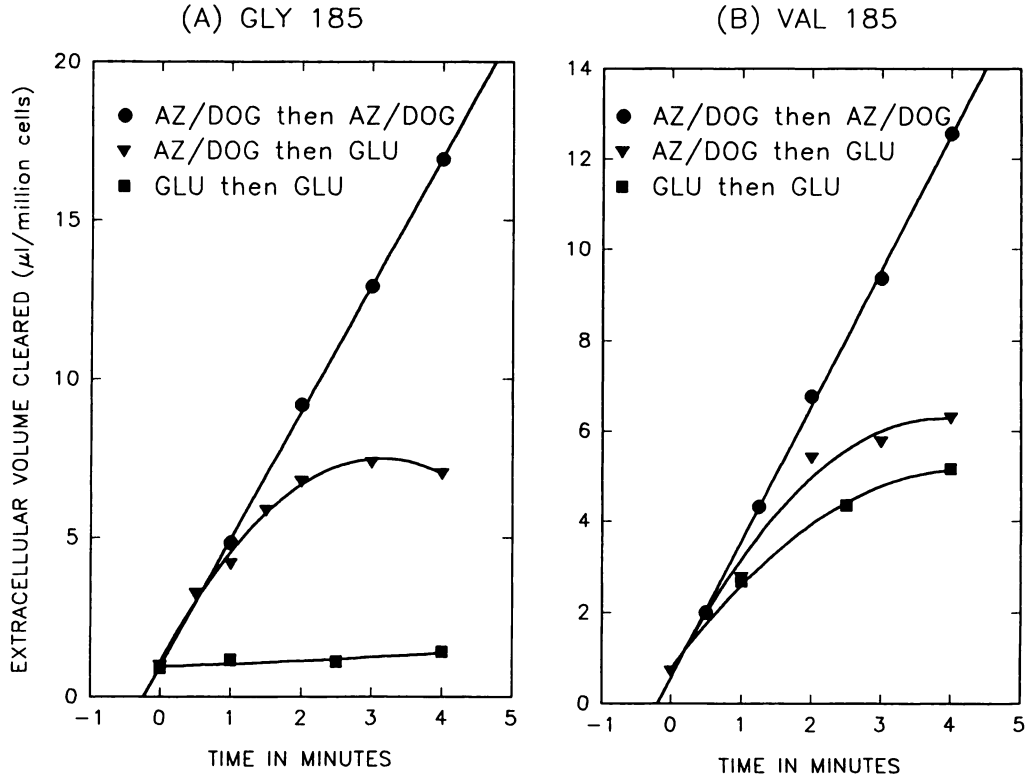

Fig. 2. Time course of the uptake of vinblastine into wild-type (MDR1-G185) (A) and mutant (MDR1-V185) (B) transfectants at 25°. Cells were starved in Az/DoG for 10 min at 37°, followed by 60 min at 25° (●, ▼), or were held in parallel in PBS/Glu (■) before uptake of labeled drug was commenced, this being from Az/DoG (●) or from PBS/Glu (▼, ■). The *solid lines* were calculated as follows: (i) for cells maintained in Az/DoG and then Az/DoG, first-order with slopes of 2.99 and 4.00 and correlations of $r = 0.999$ and 0.999 for A and B, respectively, (ii) for cells starved in Az/DoG and then measured for uptake in PBS/Glu, second-order with initial slopes of 2.93 and 4.08 for A and B, respectively, and, (iii) for cells held and measured in PBS/Glu, second-order with initial slopes of 2.07 and 0.068 for A and B, respectively.

V185 (mutant) cells at room temperature in Az/DoG, after the cells had been starved of energy sources by incubation in Az/DoG, and the continuation of this experiment, in which the cells were then freed of the labeled medium and exposed to either Az/DoG, PBS/Glu, or PBS/Glu containing 10 μg/ml quinidine, an inhibitor of the MDR pump (3). The loss of label was subsequently monitored over the next 30 min, as for the uptake studies. In parallel, the medium containing labeled colchicine was removed from two dishes and replaced by fresh medium with labeled colchicine, followed by incubation for 0 or 15 min. The data show that the restoration of an energy source greatly accelerated colchicine efflux. The half-time of efflux was approximately 5 min in the absence of added glucose and <1 min when glucose was added (see the legend to Fig. 4 for data on exponential best fits). Similar data were obtained in four other experiments with the MDR1-V185 (mutant) strain and in two experiments with the wild-type strain (one of which is depicted in Fig. 4B).

It is interesting to observe (by visual inspection of the curves) that the rate of efflux into Az/DoG was apparently not much different from the rate of uptake from this same medium. This might suggest that, in the absence of an energy source, colchicine movements are vectorially symmetric. Quantitative analysis of such influx and efflux curves is not, however, the subject of the present paper.

Because this experiment showed little effect of the P-glycoprotein inhibitor quinidine (at 10 μg/ml) on colchicine efflux, in another experiment the effects of the inhibitors verapamil (at 30 μg/ml) and cyclosporin A (at 10 μg/ml) were studied, both in PBS/Glu. Here, the efflux from cells that had been starved in Az/DoG was rapid into PBS/Glu alone, whereas for efflux into Az/DoG or into PBS/Glu containing verapamil or cyclosporin A the curves were indistinguishable (Fig. 5). Similar efflux rates were found from cells that had been preincubated with this concentration of verapamil in PBS/Glu, with efflux being followed in the presence of verapamil in PBS/Glu (Fig. 5B). These data indicate that cyclosporin A and verapamil, unlike quinidine, are both able to block efflux of colchicine from MDR cells.

Effect of inhibitors on drug uptake. Fig. 6 depicts the effect of the addition of 10 μg/ml quinidine on the uptake of colchicine in the MDR1-V185 transfectant (Fig. 6A) and of vinblastine in the MDR1-G185 transfectant (Fig. 6B). Quinidine increased the initial rate of uptake of colchicine by approximately 4-fold (for regression data, see the legend to Fig. 6). The effect was clear by 2 min, when the first sample was taken. Note from Fig. 4A that this concentration of quinidine did not greatly affect the rate of efflux of colchicine. Fig. 6A also includes data from another experiment, using the inhibitor verapamil at 10 μg/ml. Fig. 6 also depicts the data for the respective control uptake of colchicine when quinidine or verapamil was the inhibitor. Fig. 6B shows data from a similar experiment with vinblastine and quinidine. Here the time scale is in seconds and one can see that quinidine at 10 μg/ml had an immediate effect on increasing the initial rate of vinblastine uptake.[2]

[2] For cyclosporin A acting as an inhibitor of colchicine and vinblastine uptake, we have data only at 37°. These will be discussed in a subsequent paper, together with data on the reversal of cell killing using these and other modulators of the MDR pump.

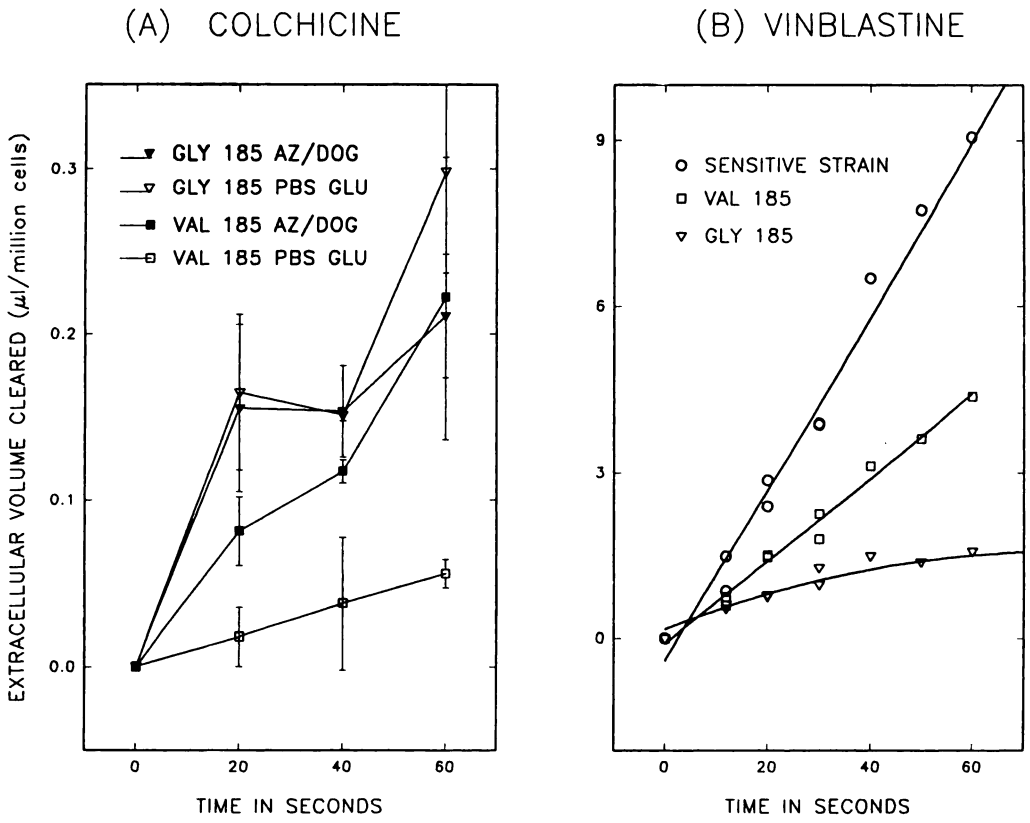

Fig. 3. Initial rates of uptake of colchicine at 25° (A) and of vinblastine at 26° (B) into MDR1-G185 (wild-type) and MDR1-V185 (mutant) transfectants. In A, the cells were starved for 60 min at room temperature in Az/DoG (*closed symbols*) or held in parallel in PBS/Glu (*open symbols*), with subsequent uptake being from the corresponding medium. Time points were determined in triplicate for the time 0 blanks and for the points at 20 sec and in duplicate for the points at 40 and 60 sec. The data represent uptakes with the means of the time 0 blanks subtracted, with these being 0.177, 0.273, 0.454, and 0.454 $\mu l/10^6$ cells for the MDR1-V185 strain in Az/DoG and PBS/Glu and for the MDR1-G185 strain in Az/DoG and PBS/Glu, respectively. The data are depicted as means ± standard errors for the 20-sec data and mean and extremes for the data at 40 and 60 sec. The *lines* drawn have no theoretical significance. In B, the uptakes were in PBS/Glu for the sensitive strain (□), the MDR1-G185 strain (▽), and the MDR1-V185 strain (○). The *lines* are first-order for the sensitive and MDR1-V185 strain data, with slopes of 0.162 and 0.077 $\mu l/10^6$ cells/sec (correlations of $r = 0.997$ and 0.991, respectively), and second-order for the MDR1-G185 strain data, with initial slopes of 0.031 $\mu l/10^6$ cells/sec.

Kinetics of accumulation of VP-16 and daunorubicin. Fig. 7 explores further the substrate specificity of the MDR system. Fig. 7A shows the time course of uptake (from 85 nM) and efflux of the epipodophyllotoxin VP-16 for the MDR1-V185 and MDR1-G185 transfectants, with and without energy deprivation. The rate of uptake of VP-16 (Fig. 7A) was approximately 10 times that of colchicine (Fig. 4A). Uptake of VP-16 by the MDR1-V185 transfectant was far less than that by the MDR1-G185 (wild-type) transfectant, just as was the case for colchicine. The rate of the efflux of VP-16 could not be determined with any accuracy, because VP-16 had mostly left the cell by the first efflux point at 30 sec. However, the uptake and efflux time courses, measured in the MDR1-G185 transfectant under conditions of starvation, are not noticeably asymmetric. This might suggest that uptake of VP-16 under these conditions takes place by simple diffusion. This was the conclusion for colchicine from the similar data of Fig. 4. For the case of VP-16, however, efflux of the drug from starved cells was so rapid (with a half-time of <30 sec; for best-fit exponential rate constant, see the legend to Fig. 7) that no effect of energy metabolism on this process could be seen (see Fig. 7A).

Finally, Fig. 7B depicts the time course of uptake of another substrate of the MDR system, daunorubicin, at 1.9 nM. Again, the data were obtained for cells starved in Az/DoG and, in parallel, for cells maintained in PBS/Glu, for both transfected lines. It is apparent from inspection of Fig. 7B that the clearance values for daunorubicin were far higher than those for colchicine or for VP-16 (compare with Figs. 4 and 7A). Energy deprivation had no apparent effect on the initial rate of drug uptake in the case of daunorubicin, for either transfectant. There was, however, a marked effect of energy deprivation on the slope of the later linear stage.

Discussion

It was shown previously that the MDR1-V185 mutant multidrug transporter renders cells relatively more resistant to colchicine and VP-16, with reduced resistance to vinblastine and little change in daunorubicin resistance, compared with the wild-type multidrug transporter (19–21). Our kinetic analysis of drug accumulation and efflux from MDR cells transfected with vectors encoding wild-type (MDR1-G185) and mutant (MDR1-V185) multidrug transporters reveals some additional insights about the transport process. Although we confirm that the accumulation of the drugs is in each case reduced in these cells, a kinetic dissection of the initial uptake step from the efflux process indicates that it is the influx of drug that is specifically affected, depending on whether the wild-type or mutant transporter is expressed. The mutant transporter is more efficient at reducing the initial rate of uptake of colchicine and VP-16 than is the wild-type transporter, less efficient at

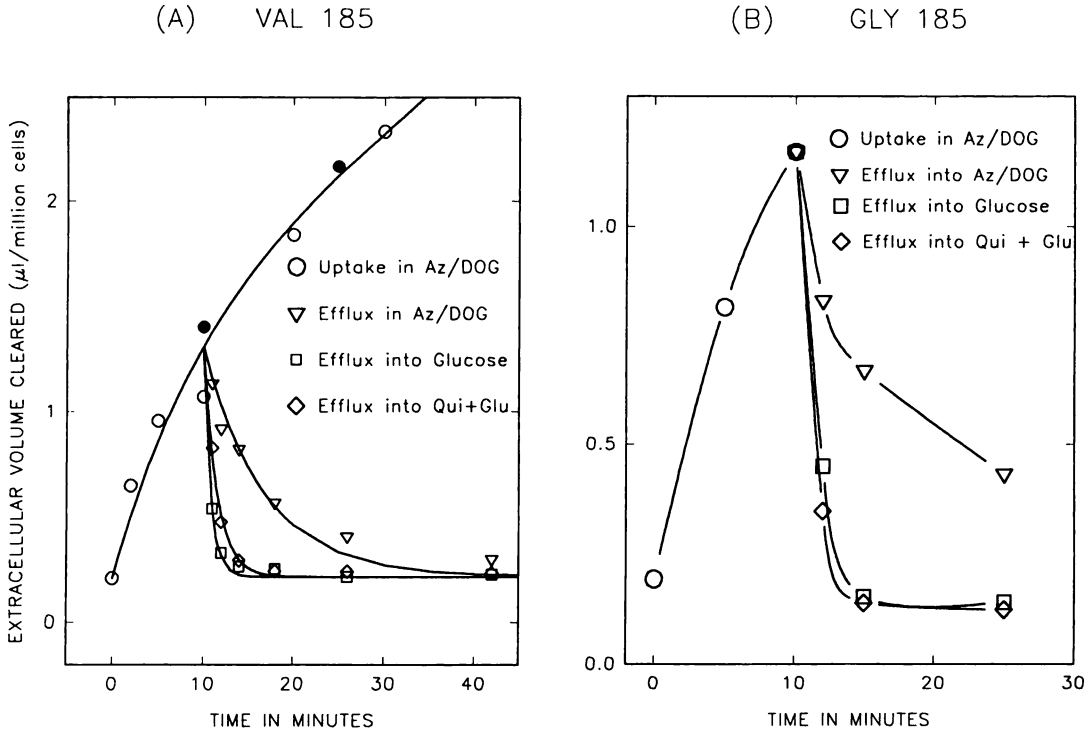

Fig. 4. A, Time course of the uptake and efflux of colchicine at 26° into the MDR1-V185 (mutant) NIH/3T3 transfectant. Cells were starved for 20 min at 37° in Az/DoG before uptake of colchicine at room temperature was commenced. Each *point* represents a single Petri dish. ● and ○, Drug influx in two different experiments. The *line* drawn for the influx data has no theoretical significance for the purposes of the present paper. Efflux was from dishes incubated in parallel with colchicine in Az/DoG for 10 min; the medium was then changed to colchicine-free Az/DoG (▽), PBS/Glu (□), or PBS/Glu plus quinidine (10 μg/ml) (◇). The *solid lines* for the efflux data are the best fit first-order exponential functions, with rate constants of 0.150, 1.19, and 0.655 min^{-1} for efflux into Az/DoG, PBS/Glu, and PBS/Glu plus quinidine, respectively. B, Similar experiment with the MDR1-G185 (wild-type) strain.

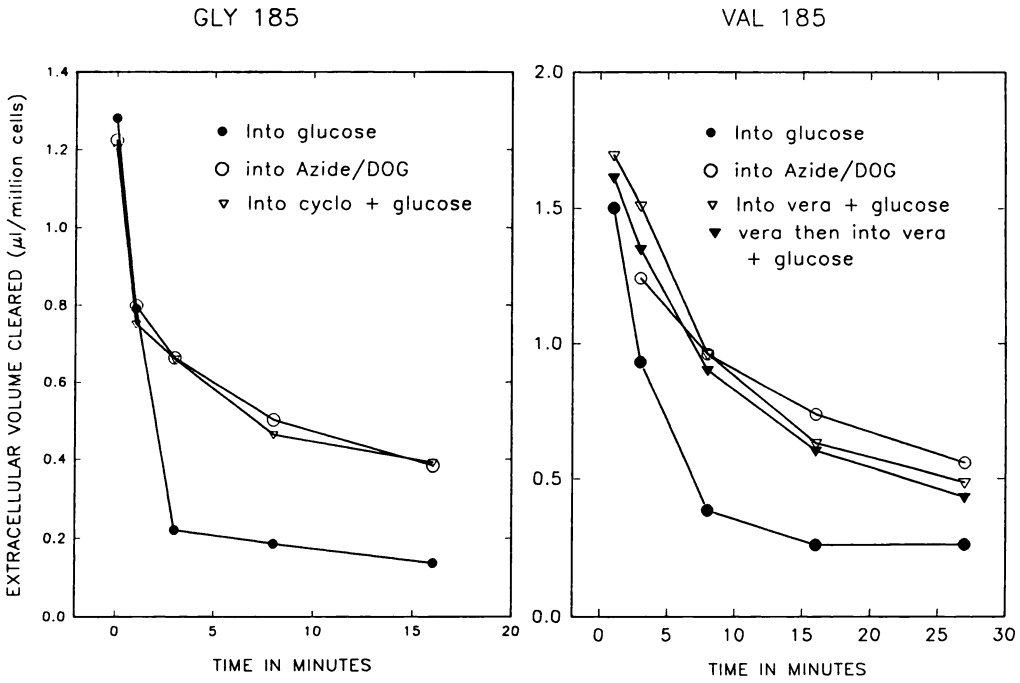

Fig. 5. A, Time course of the efflux of colchicine at 25° from the MDR1-G185 (wild-type) strain. Cells were starved for 20 min at 37° in Az/DoG before uptake of colchicine at room temperature was commenced. Each *point* represents a single Petri dish. Efflux was from dishes incubated in parallel in colchicine in Az/DoG for 10 min; the medium was then changed to Az/DoG without colchicine (○), PBS/Glu (●), or PBS/Glu plus cyclosporin A (10 μg/ml) (▽). B, Similar experiment with the MDR1-V185 (mutant) NIH/3T3 transfectant. Efflux was from dishes incubated in parallel in colchicine in Az/DoG for 10 min; the medium was then changed to Az/DoG without colchicine (○), PBS/Glu (●), or PBS/Glu plus verapamil (30 μg/ml) (▽). In one set of dishes (▼), verapamil, at the same concentration, was added during the preincubation in Az/DoG.

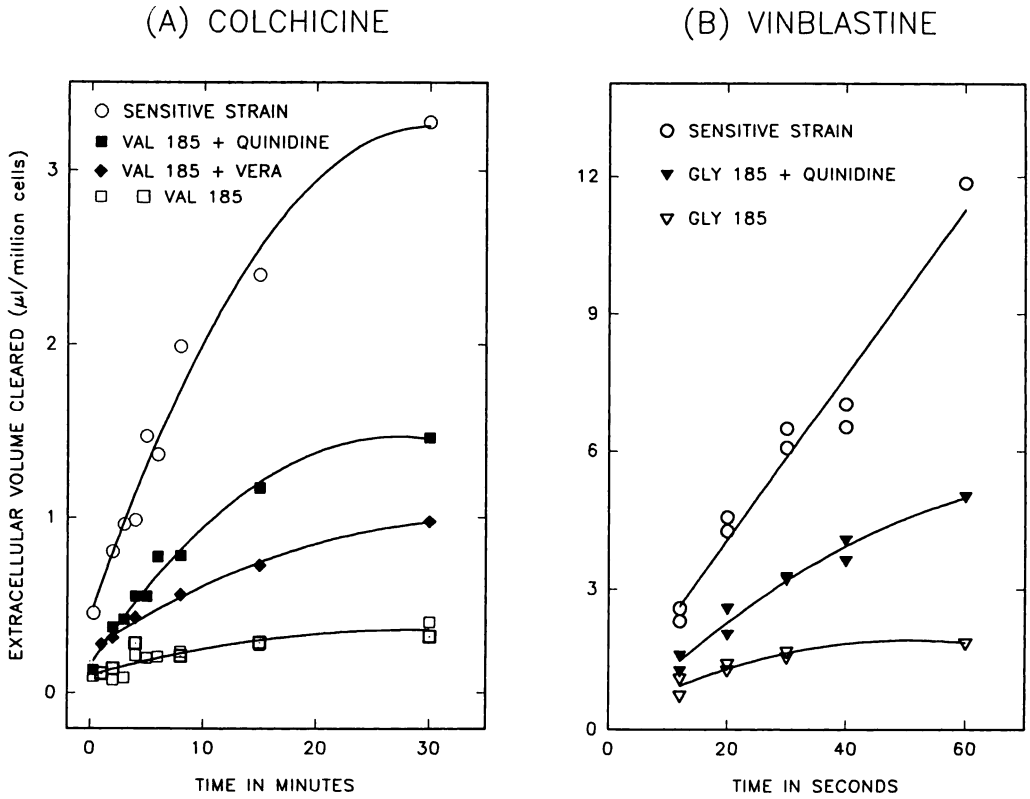

Fig. 6. Effect of immediate exposure to 10 μg/ml quinidine or verapamil on the uptake of colchicine into MDR1-G185 and MDR1-V185 transfectants (A) and of vinblastine into NIH/3T3 cells and MDR1-G185 transfectants of NIH/3T3 cells (B) at 25°. Measurements were in PBS/Glu with or without quinidine or verapamil. ○, Sensitive strain; □, ⊡, and ▽, uptake in the absence of quinidine for Val-185 (□, ⊡) or Gly-185 (▽); ■ and ▼, uptake in the presence of quinidine; ◆, uptake in the presence of verapamil. In A, the *lines* are second-order regressions with initial slopes of 0.188, 0.018, 0.096, and 0.042 μl/10^6 cells/min for the sensitive strain, the combined controls of the MDR1-V185 transfectant, and this line in the presence of quinidine or verapamil, respectively. In B, the *lines* are, for the NIH/3T3 (sensitive) strain, first-order regression with a slope of 0.180 μl/10^6 cells/sec and, for the MDR1-G185 transfectant, second-order regressions with initial slopes of 0.067 and 0.128 μl/10^6 cells/sec in the absence and presence of quinidine, respectively.

reducing initial uptake rates for vinblastine, and without specific effect on the transport of daunorubicin.

Drug transport in the presence of energized transporter. Availability of metabolic energy leads to a marked reduction in the accumulation of colchicine, vinblastine, VP-16, and daunorubicin in the *mdr1*-expressing cell lines (Figs. 1 and 7). This effect is largest for the MDR1-V185 (mutant) transfectant in the case of the nonprotonatable drugs colchicine and VP-16 but is largest for the MDR1-G185 (wild-type) transfectant in the case of vinblastine, which is protonatable. For daunorubicin, the effect of energization is much the same in the two strains. For vinblastine, the MDR1-G185 transfectant showed a substantial reduction in the initial rate of drug uptake (Figs. 2 and 3B), with only a moderate reduction for the MDR1-V185 mutant. For colchicine, energization reduced the initial rate of uptake approximately 4-fold for the MDR1-V185 transfectant but not significantly for the MDR1-G185 transfectant (Fig. 3A). The transport of VP-16 was so rapid that no reliable estimate of the initial rate of uptake could be obtained, but it would appear from Fig. 7A that the initial rate of entry of this drug was also reduced greatly in the case of the MDR1-V185 transfectant mutant but not at all for the MDR-G185 transfectant. Interestingly, for daunorubicin the initial rate of entry of drug was hardly affected by energization in either cell line. The direct effect of energization on efflux was studied only for colchicine, where the increase in the efflux rate brought about by energization was at least 5-fold and was much the same for the wild-type strain and for the mutant strain (Fig. 4). The inhibitors quinidine, verapamil, and cyclosporin A[2] appeared to increase initial drug uptakes, but only verapamil and cyclosporin A affected efflux as well (Fig. 5).

A model for drug pumping by P-glycoprotein consistent with the kinetic data. It has been repeatedly documented (1, 2, 23–26) that cells containing an active MDR system maintain a low intracellular concentration of drug and that this is often brought about by a reduction in the initial rate of drug uptake. The possibility that the MDR system can extract drugs directly from the cell membrane has been suggested by Raviv *et al.* (27) on the basis of experiments involving the labeling of membrane components by the lipophilic label iodonaphthalene-1-azide, in the presence of doxorubicin and visible light. This combination of conditions and reagents labeled many of the proteins of the membrane when the multidrug transporter was inhibited but only P-glycoprotein when the system was active. This result suggests that the MDR pump can remove drug from the membrane, concentrating it within P-glycoprotein itself. One possible interpretation of the findings reported in the present paper is that the MDR pump might act by extracting drug from the membrane at its external face and at its internal face, by two related but functionally separable processes. By this interpretation, the rate-limiting barrier for transmembrane transport would be the highly hydrophobic

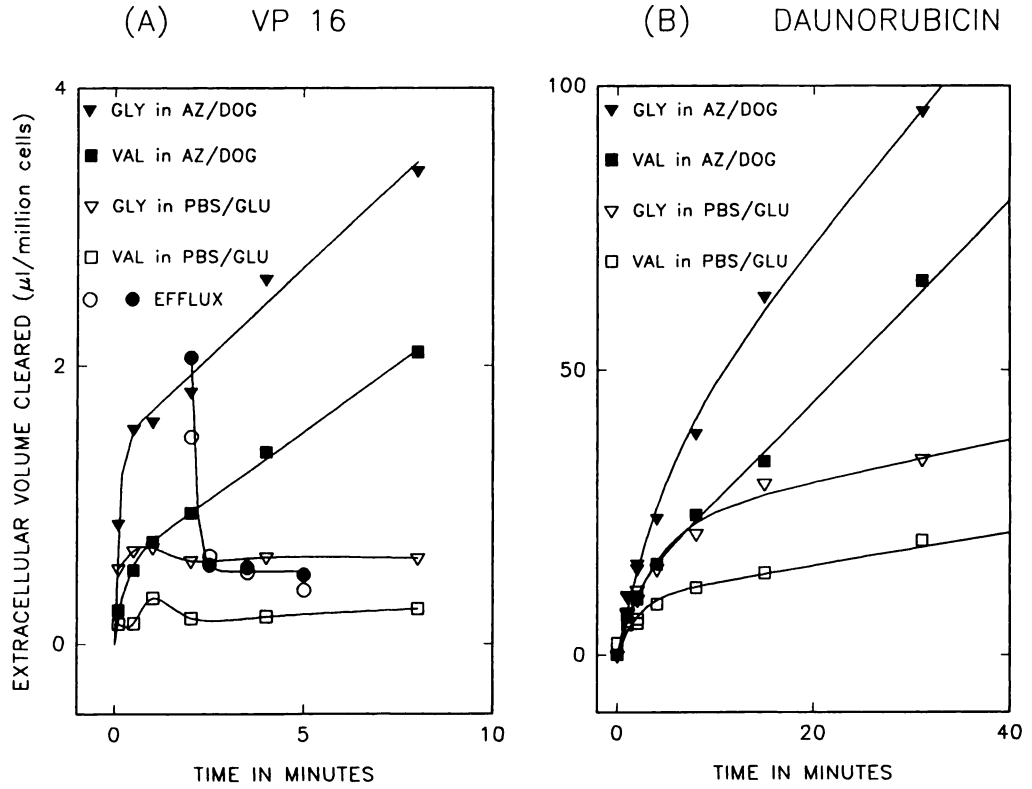

Fig. 7. Time course of uptake and efflux of VP-16 (A) and daunorubicin (B) at 24° into MDR1-G185 (uptake, *inverted triangles*; efflux, *circles*) and MDR1-V185 (*squares*) transfectants of NIH/3T3 cells. In A, the cells were either prestarved in Az/DoG for 60 min at 37° (*closed symbols*) or held in parallel in PBS/Glu under the same conditions (*open symbols*) before uptake was commenced. After 2 min of exposure of MDR1-G185 cells to labeled VP-16 (85 nM) in Az/DoG, the cells were washed with either Az/DoG (●) or PBS/Glu (○) and the loss of label was measured over the subsequent 3 min. The *lines* drawn for the influx data have no theoretical significance for the purposes of the present paper. The *solid line* for the efflux curves was a first-order exponential function fitted to the combined efflux data points, yielding a rate constant of 7.082 min^{-1}. In B, the cells were either prestarved in Az/DoG for 30 min at 37° (*closed symbols*) or held in parallel in PBS/Glu under the same conditions (*open symbols*) before uptake of daunomycin (1.9 nM) was commenced.

interior of the membrane, which might control the flipping of drug between the two halves of the membrane bilayer. This view has been argued at length (22). We can attempt to apply this scheme to the differential ability of the MDR1-V185 and MDR1-G185 transfectants to affect the initial rate of uptake of the various drugs.

The data of Fig. 3A would suggest, by the above analysis, that the MDR1-V185 (mutant) transfectant, but not the MDR1-G185 transfectant, has the ability to extract colchicine from the external face of the membrane bilayer. The initial rate of transport is reduced by energization to approximately one fourth of its value in starved cells. This could be achieved if the MDR pump were able to extract 75% of the drug that is present at any one moment in the external leaflet of the bilayer, reducing the concentration of drug to 25% and hence reducing the effective rate constant for influx to this same 25% of its rate in starved cells.

From the efflux studies (see Fig. 4), energization of the pump appears to accelerate efflux by at least 5-fold in both the wild-type and mutant strains. One might predict that the overall degree of reduction of the intracellular concentration of free colchicine would be given by the product of the separate reduction ratios for influx and for efflux or, for the MDR1-V185 transfectant, by at least 5/0.25- or 20-fold. This is close to the 28-fold increase in resistance to colchicine brought about by the transfection of the MDR1-V185 expression vector into KB epidermoid cells (19). Thus, the combined action of drug removal from the external and internal halves of the membrane bilayer should be to reduce the internal concentration of free colchicine in the MDR1-V185 mutant to <5% of its value in starved cells. Using the data for the MDR1-G185 (wild-type) transfectant, namely ratios of about 1 at the external face of the membrane and at least 5 at the internal face, we predict an overall reduction in the internal concentration of free colchicine in the MDR1-G185 transfectant to <20% of its value in starved cells. These predictions accord well with the experimental data (Fig. 1A) and with the 7.2-fold decrease brought about by the transfection of a MDR1-G185-encoding vector into KB epidermoid cells (19). Thus, the kinetic difference between the two strains appears to lie in the acquired ability of the MDR1-V185 (mutant) transfectant to reduce the rate of colchicine uptake, perhaps by extracting colchicine from the external face of the membrane bilayer, which the wild-type transporter does poorly for colchicine but efficiently for vinblastine. The data for VP-16 (Fig. 7A), although not of such a quantitative nature, support the same interpretation.

Our data for the drug vinblastine are not as complete. They do show, however, that the initial rate of uptake of vinblastine is greatly reduced in the MDR1-G185 transfectant with energization (Fig. 2A) and is reduced 5-fold when this strain is compared with the drug-sensitive strain (Fig. 3B) and that the effects with the Val-185 mutant are far smaller. Thus, substi-

tution of valine for glycine at position 185 along the polypeptide chain of P-glycoprotein might be interpreted as having a positive effect on the ability of the mutant to extract colchicine but a negative effect on its ability to extract vinblastine from the outer half of the membrane bilayer. This is consistent with the 2.6-fold increase in sensitivity to vinblastine of the Val-185 strain, compared with the Gly-185 strain (19).

It would appear from Fig. 7B that for neither variant of P-glycoprotein is the initial rate of uptake of daunorubicin affected by the supply of metabolic energy, yet there is a marked effect on the slope of the linear portion of the drug uptake curve for both strains, perhaps suggesting an increase in efflux rate. Published data showed that, in MDR1-V185- and MDR1-G185-transfected lines, there was little differential specificity (resistances of 12- and 8-fold, respectively) towards doxorubicin, an analog of daunorubicin (19–21). The finding that there is little effect of this mutation on the ability of the pump to handle daunorubicin is consistent with our conclusion that it is the process by which drugs enter the cell that is solely affected by the Gly-185 to Val-185 mutation. The data for all four drugs studied thus concur in suggesting that the major effect of the glycine to valine mutation may be to affect the ability of the pump to extract drug from the outer face of the membrane bilayer.

These results can also be interpreted in light of two other studies concerning the mechanism of action of the Val-185 mutation in P-glycoprotein. Using photoaffinity labeling studies, Safa *et al.* (28) concluded that this mutation reduces the rate of release of vinblastine from the transporter, because a photoaffinity analog of vinblastine was found to be a better photoaffinity label of the mutant P-glycoprotein than the wild-type P-glycoprotein, despite the fact that vinblastine is a poorer substrate for transport. The current kinetic analysis does not consider whether the failure of the Val-185 mutant to extract vinblastine from the outer face of the bilayer reflects tighter or weaker binding to the transporter, because it addresses only effective "transport." A recent study by Bruggemann *et al.* (29) reports that *Vinca* alkaloids such as vinblastine are more potent inhibitors of azidopine labeling of P-glycoprotein with the Val-185 mutation than of wild-type P-glycoprotein. This result also suggests that the association of vinblastine with P-glycoprotein may be altered in the mutant P-glycoprotein, and it is consistent with the observed kinetic effects reported here.

Effect of inhibitors on pumping by P-glycoprotein. The data on the effect of quinidine on colchicine efflux and on colchicine and vinblastine uptake can be interpreted in terms of the same model of differential handling of drug influx and efflux. We have repeatedly found that efflux of colchicine into PBS/Glu containing 10 μg/ml quinidine is only slightly slower than that into PBS/Glu alone (Fig. 4). However, the uptake of colchicine or of vinblastine from PBS/Glu containing quinidine is greatly (and immediately) reduced, compared with uptake in PBS/Glu alone (Fig. 6). Thus, efflux and uptake can be differentially blocked by quinidine present in the extracellular medium. We do not have data on the time course of uptake of quinidine, but it is unlikely to enter cells to any great extent during the 2 min that it takes colchicine to leave the cell (Fig. 4). The effect of quinidine is reminiscent of the effects of nonpenetrating inhibitors on transport by membrane carriers (22), where, for example, glucose entry into red blood cells is inhibited by external mannose, whereas its exit is unaffected. The interpretation in such cases has been that substrate and inhibitor can compete only at the same site and, therefore, at the same side of the membrane. For the MDR system, where external quinidine affects drug uptake, this would imply that external quinidine is competing with external colchicine (or vinblastine) while the latter are being pumped out of the cell. This, in turn, implies that the drug is being pumped out before it enters the cell, consistent with our interpretation that it is being removed from the membrane itself. The finding that external quinidine does not, however, effectively block the efflux of colchicine suggests that the extraction of colchicine from the membrane occurs by two dissociable routes, one at the internal half and the other at the external half of the membrane bilayer. This suggestion is compatible with the differential effects of the Gly-185 to Val-185 mutation on drug efflux and influx. The inhibitors verapamil (at 30 μg/ml) and cyclosporin A (at 10 μg/ml) inhibit both uptake and efflux of colchicine (see Results). Presumably, their mechanism of action as inhibitors is somewhat different from that of quinidine, with the latter acting more as a competing substrate for the multidrug transporter. Alternatively, at high concentrations, verapamil and cyclosporin A may be very efficient inhibitors with rapid access to both sides of the membrane, thus being capable of blocking both the uptake and efflux phases of transport.

Differential effects of providing energy on influx and efflux. There appear to be differential effects of providing ATP for the transporter on the efflux and uptake processes for these drugs. Consider again Fig. 4, which shows the effect on the rate of colchicine efflux when Az/DoG is changed to PBS/Glu. This leads to what appears to be an instantaneous stimulation, in that the efflux curve is apparently exponential by the first time point (at 1 min). A similar experiment with vinblastine as substrate (data not shown) showed a marked effect of the addition of PBS/Glu on drug efflux within 20 sec. In contrast, when a similar change of medium precedes the measurement of vinblastine uptake (Fig. 2), at least 2 min are required before the full effect of energization on drug movement can be seen. It appears that the stimulation of drug efflux is more rapidly switched on by energization (i.e., occurs at lower levels of ATP) than is the reduction in the rate of drug entry. This might, again, point to some differences between the processes that affect drug extraction from the outer and inner halves of the membrane bilayer.

Conclusion. The data presented in this paper show that influx and efflux of drugs can be differentially affected by the MDR system, consistent with a model in which P-glycoprotein extracts drug from the two halves of the membrane bilayer independently. These two processes can be separately affected by mutation (the substitution of Gly-185 by Val-185), by inhibitors (quinidine), and by the onset of energization. We have recently proposed a speculative model for the mechanism of action of P-glycoprotein,[3] in which this protein acts as an osmotically driven pump and extracts water-soluble materials directly from the membrane. In this model, the substrates of the pump are assumed to be present in the two halves (leaflets) of the membrane bilayer, in rapid dynamic equilibrium with the aqueous phases that comprise the cytoplasmic and extracellular media. Lateral diffusion within each leaflet of the bilayer brings the substrates into contact with P-glycoprotein,

[3] One version of this model is described in Ref. 7.

which can then pump them out to the external medium. The data presented in the present paper are consistent with these speculations.

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