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# Effects of Membrane Potential Versus $pH_i$ on the Cellular Retention of Doxorubicin Analyzed via a Comparison Between Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and Multidrug Resistance (MDR) Transfectants*

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**ABSTRACT.** Recently (Wei *et al.*, *Biophys J* **69:** 883–895, 1995), several 3T3/hu cystic fibrosis transmembrane conductance regulator (CFTR) transfectant clones were found to exhibit a low-level multidrug resistance (MDR) phenotype. This phenotype is similar, but not identical to that found for MDR transfectants not previously exposed to chemotherapeutic drugs. Both MDR and CFTR transfectants are depolarized (exhibit lower plasma membrane $\Delta\Psi$), but the former have alkaline $pH_i$ whereas the latter are acidic. It has been proposed (Roepe *et al.*, *Biochemistry* **32:** 11042–11056, 1993) that both decreased $\Delta\Psi$ and increased $pH_i$ contribute to altered cellular retention of chemotherapeutic drugs in MDR tumor cells, but the relative contribution of each to altered cellular drug accumulation, drug retention, and drug efflux has not been studied in detail. We therefore examined doxorubicin transport for hu CFTR and mu MDR 1 transfectants using sensitive continous monitoring of fluorescence techniques. Both CFTR and MDR transfectants exhibited significantly reduced doxorubicin accumulation, relative to drug-sensitive control cells. Plots of the initial rate of accumulation versus doxorubicin concentration were linear for the control cells and the CFTR and MDR transfectants between 0.1 to 0.5 μM drug, but better fit by a quadratic between 0.1 to 1.5 μM drug. The slopes of these curves were proportional to measured $\Delta\Psi$. Low-level selection of either CFTR or MDR transfectants with chemotherapeutic drug did not decrease further the initial rate of drug accumulation or change $\Delta\Psi$. Accumulation experiments for control cells performed in the presence of various concentrations of $K^+$ further suggests that the rate of accumulation is related to $\Delta\Psi$. By measuring the kinetics of doxorubicin release for CFTR and MDR transfectants preloaded with drug, we concluded that alkaline $pH_i$ perturbations are more important for determining relative intracellular binding efficiency. We also concluded, similar to the case previously made for MDR protein (Roepe, *Biochemistry* **31:** 12555–12564, 1992) that CFTR overexpression does not enhance the rate of drug efflux. These data better define the role of lowered $\Delta\Psi$ and elevated $pH_i$ in altering the cellular retention of doxorubicin in MDR tumor cells. BIOCHEM PHARMACOL 52;7:1081–1095, 1996.

**KEY WORDS.** multidrug resistance; membrane potential; intracellular pH; efflux; drug accumulation

Overexpression of the MDR‡ protein confers broad resistance to dozens of structurally divergent compounds by decreasing the efficiency of their intracellular retention. Elucidation of the molecular mechanism behind this type of MDR remains an important goal. Models for how MDR protein overexpression leads to MDR essentially fall into two categories: the direct "pump" or active transporter models, wherein the protein directly binds and actively transports, flips, or "vacuums" the plethora of agents to which MDR cells are resistant [1, 2], and the "altered partitioning" models, wherein overexpression of the protein influences electrical membrane potential ($\Delta\Psi$), volume, and/or $pH_i$ homeostasis, thereby indirectly perturbing the efficiency of intracellular drug partitioning and retention via several different mechanisms [3]. If one entertains the altered partitioning model, it is not yet defined how MDR

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‡ *Abbreviations:* MDR, multidrug resistance (resistant); CFTR, cystic fibrosis transmembrane conductance regulator; $\Delta\Psi$, plasma membrane electrical potential; $pH_i$, intracellular pH; CMF, continuous monitoring of fluorescence; ABC, ATP-binding cassette; MRP, multidrug resistance related protein; LUV, large unilamellar vesicles; G418, geneticin; RT-PCR, reverse transcriptase-polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; HBS, Hanks' balanced salts (140 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/10 mM glucose/10 mM HEPES, pH 7.33); $D_T$, total intracellular concentration of drug; $D_{ex}$, concentration of intracellular exchangeable (osmotically sensitive) drug; $^{fast}D_{ex}$, component of $D_{ex}$ that is immediately exchangeable (i.e. cytoplasmic) [see Ref. 20]; and $TPP^+$, tetraphenylphosphonium.

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protein overexpression leads to the $\Delta\Psi/pH_i$ changes that have been measured [3], but several possibilities from the recent literature include: (1) as a $Cl^-$ channel or channel regulator [4], (2) as a component of an autocrine pathway involving transport of ATP [5, 6], and (3) as an inhibitor/regulator of $Cl^-/HCO_3^-$ exchangers [7]. Recently, other ABC transporters homologous to the MDR protein (MRP and CFTR) have been found to confer MDR phenomena when they are overexpressed in cells. Considerable effort is currently expended to understand any similarities among these MDR phenotypes, which may include similar changes in $\Delta\Psi$ [8].

Regardless of whether one entertains the altered partitioning model for MDR protein function, the fact remains that significant $\Delta\Psi$ and $pH_i$ perturbations have been measured for many MDR cells by a number of laboratories (reviewed in Ref. 3). It is not yet precisely clear how these perturbations affect various aspects of cellular drug transport and retention, or even what level of drug resistance could conceivably be conferred by these perturbations, although some data bearing on the latter have been reported recently [8, 9]. With regard to the former, for example, it is not trivial to predict how substantially altered $\Delta\Psi$ should alter accumulation and intracellular retention of chemotherapeutic drugs since most are hydrophobic weak bases and one or two (e.g. colchicine) are neutral. A prediction from biophysical theory is that the neutral form of a weak base will diffuse faster than the charged form and thus, simplistically, $\Delta\Psi$ might not be expected to substantially alter "intracellular" (the implication being equilibrium cytoplasmic) concentrations of drug. However, significant $\Delta\Psi$ perturbations do indeed appear to alter passive diffusion and/or membrane binding/partitioning of chemotherapeutic drugs [3, 9]. Effects could either be "direct" (i.e. electrostatic) or "indirect" (e.g. a consequence of altered bilayer properties secondary to the $\Delta\Psi$ perturbation). Along these lines, it has also been observed that $\Delta\Psi$-dependent accumulation of certain hydrophobic anesthetics does not necessarily conform to simplified biophysical predictions [10]. In addition, since chemotherapeutic drugs are concentrated enormously in cells (up to 50- to 100-fold over external incubating concentrations), it is debatable whether cytoplasmic drug concentrations are ever in true "equilibrium" with the extracellular milieu under many reported experimental conditions. Changes in $\Delta\Psi$ may have major implications for the altered *cellular* retention of certain drugs over time in MDR cells that extend beyond effects on cytoplasmic drug concentrations.

Indeed, a variety of model LUV studies have shown that $\Delta\Psi$ and $\Delta pH$ significantly affect passive diffusion of the chemotherapeutic drugs doxorubicin and vincristine (reviewed in Ref. 3). However, the effects in the liposomes may not be exactly analogous to the behavior of whole cells for several reasons. First, these drugs are hydrophobic with relatively high octanol/saline partitioning coefficients (log P = 0.2 to 3.0) that are pH dependent [9], and the relative percent volume of an LUV that is membrane volume is orders of magnitude higher than the relative percent volume of a cell that is plasma membrane volume; thus membrane partitioning in LUVs versus cells is very different, and this makes comparison between transport in LUVs versus cells difficult. Second, many studies with LUVs have used high concentrations of chemotherapeutic drugs (e.g. up to 200 $\mu$M doxorubicin) at which unusual behavior may occur (e.g. doxorubicin aggregates at concentrations above 2 $\mu$M) and thus complicate interpretation. These concentrations are also outside the realm of concentrations of drug to which MDR, MRP, or CFTR overexpression confers resistance. Thus, it is necessary to examine these phenomena using whole cells and lower levels of drug if we are to fully assess the possible contributions of $\Delta\Psi$ and $pH_i$ perturbations to the MDR phenotype.

Comparison between CFTR and MDR transfectants presents a unique opportunity to distinguish between the effects of decreased $\Delta\Psi$ and altered $pH_i$ on the cellular retention of chemotherapeutic drugs. This is because both MDR protein and CFTR overexpression lead to similar significant plasma membrane depolarization [3, 11], but $pH_i$ changes in opposite directions [8]. Thus, unless one envisions that CFTR is also a drug pump (see below), features common to both transfectants may be attributable to decreased $\Delta\Psi$ and not elevated $pH_i$ whereas features specific to the MDR transfectants are more likely due to elevated $pH_i$ or some combined effect of decreased $\Delta\Psi$ and elevated $pH_i$.

From a different point of view, if drug pump models for MDR protein are entertained even though we do not favor them for several reasons [3, 9], precise description of the altered drug transport unequivocally mediated by MDR protein is very important. Surprisingly, however, no kinetic drug transport studies have been performed previously for MDR cells not exposed to chemotherapeutic drug but engineered to overexpress the MDR protein by other means (i.e. "true" transfectants; see Refs. 3, 8, and 9). It is likely that growth of MDR cells in the presence of chemotherapeutic drugs further affects diffusion and retention of chemotherapeutic drugs independent of MDR protein overexpression, and that different MDR cell lines exhibiting similar overexpression of MDR protein but selected with different chemotherapeutic drugs may exhibit very different drug retention/transport (see Refs. 3, 12, and 13). Relatedly, a key unresolved issue and point of some controversy surrounding putative active drug transport by the MDR protein is the apparent presence (e.g. Ref. 14) versus absence (e.g. Ref. 15) of inhibition of altered drug transport mediated by MDR protein upon treatment with excess extracellular chemotherapeutic drug. It is possible that this apparent discrepancy is due to the different drug selection conditions used to create the different cell lines studied, since chemotherapeutic drugs are powerful compounds with complex effects.

Thus, whether or not they are caused by MDR protein,

$\Delta\Psi$ and $pH_i$ perturbations will complicate analysis of any putative drug transport believed to be catalyzed by MDR protein. Comparison between CFTR and MDR transfectants would help separate $\Delta\Psi$ from $pH_i$ effects. Therefore, in either distinguishing between models for MDR protein function, or in analyzing these models further, more detailed analysis of drug transport with "true" (non-chemotherapeutic-drug selected) MDR, MRP, and CFTR transfectants is important.

In this study, we analyzed doxorubicin transport for true MDR and CFTR transfectants. We analyzed several clones exhibiting different $pH_i$ and $\Delta\Psi$ in order to assess the importance of these two key parameters for altered accumulation (influx) versus retention and efflux. The results suggest a relationship between initial rates of doxorubicin accumulation and $\Delta\Psi$ as well as a relationship between alkaline $pH_i$ and altered intracellular retention of the anthracycline.

## MATERIALS AND METHODS

### *Materials*

Doxorubicin was obtained as a 2 mg/mL solution in saline from Adria Laboratories (Columbus, OH). For accumulation experiments, 100 μM stock solutions of doxorubicin were prepared, divided into aliquots, and stored in siliconized tubes at −20° in the dark. Deviations from our linear fluorescence intensity versus concentration plot (see Results) were noted if stock solutions of higher concentration (e.g. 1 mM) were used in these experiments. For incubations prior to efflux, fresh 2 mg/mL solutions of doxorubicin from Adria Laboratories, provided by the Sloan–Kettering pharmacy, were used to achieve 50–100 μM concentrations in the incubating medium. We routinely handled doxorubicin with siliconized plastic tips, vials, and tubes. Vinblastine sulfate was the gift of Lilly Research Laboratories (Indianapolis, IN). G418 (geneticin) was from Sigma (St. Louis, MO); stock solution concentrations were adjusted to account for variable percent active G418 in different lots. All other chemicals were reagent grade or better and used without further purification.

### *Tissue Culture*

Construction of the MDR cell line EX4N7, which overexpresses mu MDR 1 protein, has been described [16]. Northern, RT-PCR, and western blot analyses verified overexpression of mu MDR 1 mRNA and protein for cells used in these experiments as well as their absence for the parental LR73 cell line (data not shown; see Ref. 7). LR73 and EX4N7 were grown at 37° in a 5% $CO_2$ atmosphere in DMEM supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL streptomycin; 500 μg/mL active G418 was included in the EX4N7 growth medium until the last splitting prior to transport measurements. For transport measurements (see below), cells were harvested by trypsinization, washed, and resuspended gently in fresh medium. For northern and western blot analyses, the cells were harvested by either trypsinization or scrapping with a sterile rubber policeman.

Construction of the CFTR transfectants and the 3T3 control transfectant (3T3/c) used in this work has been described previously [11]. Several clones (C3, C5, and C10) were found previously to exhibit significant multidrug resistance as defined by drug resistance to doxorubicin, vincristine, and colchicine in colony formation assays; reduced electrical membrane potential via both electrophysiologic and fluorometric $K^+$/valinomycin titration methods; altered $pH_i$ homeostasis via single-cell photometry; reduced [$^3$H]vinblastine accumulation; and the ability to select rapidly expanding colonies exhibiting increased resistance to a variety of drugs via continuous exposure to one chemotherapeutic drug [8].

C3 and C5 (harboring CFTR) and control 3T3/c transfectants were grown in DMEM supplemented with antibiotics and 250 μg/mL active G418 as described above. For both MDR and CFTR transfectants, cells were not used in transport measurements if they had been passed more than ten times, since we have noted that overexpression can be unstable [7].

The cell line C3D1 was created by single-step selection of the C3 CFTR clone on 100 nM doxorubicin [8]. C3D1 exhibits increased CFTR expression but no measurable MDR protein, as well as elevated $pH_i$ but no significant change in $\Delta\Psi$ relative to C3 (see Table 2, Results, and Ref 8). 1-1 Cells (EX4N7 selected on 50 ng/mL vinblastine) have been described previously [7, 16]. Both C3D1 and 1-1 were grown in the presence of chemotherapeutic drug until the last passage prior to transport measurements.

### *Drug Resistance Assays*

For growth inhibition assays, cells were subcultured in 96-well plates and incubated overnight at 5% $CO_2$. Drug at various concentrations was added the following day, and cells were grown for 3 days more at 5% $CO_2$. Cell growth was assayed by crystal violet staining. We verified that staining was a linear function of both cell number and total cell-associated protein for these cell lines grown under the different conditions. Colony formation assays were performed as described previously [8].

### *Fluorescence Spectroscopy*

Fluorescence spectra were obtained with a Photon Technologies Inc. (P.T.I., New Brunswick, NJ) fluorometer interfaced to a Dell 433/L computer. Cuvette temperature was controlled by a circulating water bath. Cell suspensions were mixed rapidly with a magnetic stirrer situated beneath the cuvette. Excitation/emission wavelengths and the other parameters of various experiments may be found in the individual figure captions.

### *Drug Transport Assays*

Accumulation of doxorubicin was measured essentially as described [17, 18] using our previously described CMF tech-

niques [19, 20] with several modifications. Cells were cultured in 100 $cm^2$ plates, harvested by trypsinization, and washed in DMEM pre-equilibrated at 5% $CO_2$ before performing the assay. Aliquots of cells [$1.50 \times 10^6$ ($\pm 3 \times 10^4$)] were washed once in HBS at 37° and resuspended in a final volume of 30 μL. The cells were counted by eye with a hemocytometer. We verified that protein content/cell was nearly identical for the different cell types analyzed using the Bradford assay with BSA as a standard. These data were also verified with an amido black protein assay, again with BSA as a standard.

Cells were fast-injected into rapidly mixed methylacrylate cuvettes harboring 3 mL of pre-warmed HBS and various concentrations of doxorubicin (see Results and Ref. 19). In some cases, additional drugs (vinblastine, forskolin) were added prior to the cells, and the transport medium was allowed to equilibrate for several minutes. These additional drugs were titrated against doxorubicin to test whether they affected the fluorescence properties of the anthracycline; within the range of concentrations used in this work, they did not. The cuvette harboring transport medium was jacketed and temperature controlled. Baseline doxorubicin fluorescence was verified to be stable before injecting the cells. Accumulation was monitored as a decrease in total fluorescence of the continuously mixed suspension over time (CMF; see Refs. 17, 18, and 21). Data obtained in this fashion (see Results) may be converted to picomoles doxorubicin per microgram cell protein per time via comparison to standard curves for doxorubicin fluorescence versus concentration (linear below 2 μM; see Ref. 19), and knowing the ratio of fluorescence quantum yield for intracellular versus extracellular doxorubicin (see Ref. 19) and the protein content per cell (see Figs. 1, 2, and 4 of Results).

To verify that the CMF experiments properly revealed accumulation, "point-wise" uptake experiments were also performed, essentially as described previously for [$^3$H]daunomycin uptake studies [12] but with several modifications. We chose to take advantage of the natural fluorescence of doxorubicin, the linear relationship between fluorescence intensity and concentration below 2 μM [19], and to avoid the expense of isotopically labeled reagents. Aliquots of cells $1.50 \times 10^6$ were incubated with doxorubicin for different times with intermittent agitation, and the mixture was filtered rapidly through treated Whatman cellulose nitrate filters. Filtering doxorubicin solutions alone provided suitable reference for non-specific association of drug to the filter. The filters were then extracted with 3 mL of 50% EtOH/0.5 N HCl in a 10-mL scintillation vial for 10 min on a rotator, the filters were removed, and fluorescence emission (590 nm emission, 490 nm excitation, 6 and 8 nm slit widths, respectively) was measured. Comparison to a linear calibration curve in the same solvent was used to quantitate the doxorubicin. Additional incubation of the filters with the EtOH/HCl solvent revealed that 10 min was sufficient to elute the drug from the trapped cells.

Efflux of doxorubicin was measured as described in detail previously [19]. Aliquots of cells ($1.50 \times 10^6$) were incubated in medium with doxorubicin (incubating concentrations varied from 1 to 100 μM; see Results), centrifuged, and washed once in HBS pre-chilled to 4°. The cell pellet was resuspended in a 30 μL volume of cold HBS and fast-injected into a rapidly mixed, temperature-controlled cuvette harboring 3 mL of HBS/glucose at 37°. Control injections at 4° were used as an independent measure of relative drug loading. See Refs. 19 and 20 for additional description of the methods.

The percent drug released from a cell in a given time was calculated by separating cells from the transport medium by centrifugation and quantitating medium doxorubicin via comparison to a calibration curve obtained in the same solvent (doxorubicin emission is a linear function of concentration in $H_2O$-based solvents at $\leq 2$ μM). This was then compared to total intracellular doxorubicin calculated for the cell preparation by extraction with 50% EtOH/0.3 N HCl as described [19]. Intracellular volume was calculated as described below.

For both uptake and efflux, quantitation of cell number and cell-associated protein was important. Total cell-associated protein was quantified for cell pellets solubilized in Triton X-100/SDS via a commercial Bradford assay (Bio-Rad). An amido black assay performed with an aliquot of the cell pellet, with BSA used as a standard, verified the accuracy of this assay, and control experiments with detergent at various concentrations verified that 1–2% levels of Triton X-100 or SDS did not interfere with the linearity of the Bio-Rad assay. Higher concentrations of detergent, however, made the Bio-Rad assay less reliable. We verified that the average protein content per cell was similar for the different cell lines by counting cell suspensions by eye and with a Coulter counter.

In efflux measurements, it was important to have knowledge of relative total intracellular and exchangeable intracellular drug concentrations ($D_T$ and $D_{ex}$, respectively). After determining average cell volume (see below) and extracting total doxorubicin [19], $D_T$ could be calculated. Exchangeable drug was determined by allowing efflux to reach steady state (30–90 min, depending on $D_{ex}$) and then measuring the fluorescence of the collected supernatant and comparing to a fluorescence versus concentration calibration curve obtained in the same solvent. "Fast-exchangeable" (cytoplasmic) drug, $^{fast}D_{ex}$, was determined as described [20]. This fraction represents that portion of intracellular drug that is immediately osmotically sensitive (i.e. presumably not including drug reversibly bound to intracellular target) as defined by exponential curve fitting of zero-trans efflux as described [20] (see also "kinetic analysis of transport data" below).

### *Measurement of Intracellular Volume ($V_i$)*

Total cell volume was calculated after determining the mean particle size of cell suspensions by the single threshold Coulter method performed with a ZM Coulter coun-

ter (Coulter Scientific Instruments, Hialeah, FL) as described [21].

Intracellular water volume was estimated by calculating the ratio of [$^{14}$C]inulin versus $^{3}H_2O$ dpm as described [22]. Radioactivity in the supernatant and the cell pellet was quantitated with a dual channel $^{14}C/^{3}H$ dpm Beckman LS 5801 program. Using the auto quench correcting capabilities of the LS 5801, counting efficiency was maximized and channel spillover was decreased (see Ref. 21).

### *Kinetic Analysis of Transport Data*

CMF transport curves were collected as described above and initially stored as P.T.I. data files on our Dell 433/L PC. They were converted to ASCii files, translated by Apple file exchanger, and stored on a Macintosh IIci. These were then imported as Sigma plot files (Jandel Scientific) for further analysis. This program uses the Marquardt–Levenberg iterative least squares algorithm. Tolerance was initially set to 0.00001. Raw doxorubicin fluorescence counts were converted to a more convenient form via the equation $Y = C + [(\Delta F)/100{,}000]$, where C is a constant, and $\Delta F$ is change in fluorescence units. Each transport curve was fit initially to a single exponential function of the form $y = c - a \exp(-b/x)$, where $c$ is a constant, $a$ and $b$ are variables, and $x$ and $y$ represent time and doxorubicin emission, respectively. If this converged with tolerance satisfied in <20 iterations, with reasonable variable dependencies, the fit was assumed to be satisfactory (see Results). If not, a second exponential term was added (see Ref. 20).

Having information pertaining to mono- or bi-exponential character of drug translocation, rates could also be reliably estimated with a linear fit to the first 60 sec of the efflux curve (IR60) (see Ref. 20).

## RESULTS

Ramu and colleagues [17] first used CMF methods to compare doxorubicin accumulation for drug-resistant and drug-sensitive P388 murine leukemia cells. It was shown that the large decrease in quantum efficiency of doxorubicin fluorescence over time in a continuously mixed cell suspension was due to accumulation of the drug. Along with previous data and arguments [14, 23–25] it may be concluded from this study that inward transport of the drug is rate-limiting for the quenching phenomena associated with binding to nucleic acid (see Ref. 19). Thus, if the relative quantum efficiency of intracellular versus extracellular drug fluorescence is known, proper analysis of CMF curves is an accurate measure of the rate and extent of drug influx.

Note that unlike most previous work the present study measures drug transport for continuously mixed suspensions of cells. Since chemotherapeutic drugs are hydrophobic, this is an important practice when performing detailed kinetic analysis.

### *Reduced Doxorubicin Accumulation for True MDR and CFTR Transfectants*

Figures 1 and 2 compare accumulation of doxorubicin at several external concentrations for true MDR (Fig. 1) or CFTR (Fig. 2) transfectants versus control LR73 and 3T3/c cells, respectively. Importantly, and in contrast to all previous studies that we are aware of, none of the transfectants were exposed previously to chemotherapeutic drugs prior to these transport measurements.* Thus, importantly, the effects of ABC transporter overexpression are separated from other possible effects associated with drug selection (see below). This is not a trivial distinction, since altered cellular drug transport can be influenced greatly by selection with various chemotherapeutic drugs, independent of the CFTR or MDR protein (e.g. Ref 13).

As described in detail in two previous papers [19, 20] "pointwise" transport assays, wherein cells and transport medium are separated at various time after dilution and fluorescence for each quantitated separately, revealed that greater than 95% of the difference signal in these CMF traces is due to doxorubicin transport into the cell (data not shown). This is because the quantum efficiency of intracellular doxorubicin fluorescence is about seven times lower than extracellular (see Ref. 19).

Although absolute levels of MDR protein and CFTR differ in the transfectants [11, 26], it is clear that overexpression of either transporter is associated with a significantly decreased initial rate of doxorubicin accumulation, relative to the corresponding sensitive cells (which accumulate similar levels of drug in similar time). Previously, Gros and colleagues noted that a mu MDR 1 transfectant grown in the presence of 0.1 μg/mL doxorubicin accumulates [$^{3}$H]vinblastine at about 50% the rate of control LR73 cells (at several external concentrations from 10 nM–10 μM, see Ref. 15). Similarly, Stein and coworkers [28] recently showed that hu MDR1/3T3 transfectant selected on 60 ng/mL colchicine accumulate about 25–30% of the colchicine accumulated by the 3T3 parental line within 30 min. In both cases, prior exposure of the transfectants to chemotherapeutic drug precludes assigning reduced drug accumulation solely to the overexpression of MDR protein (see Discussion); thus, to the best of our ability to ascertain, the data in Fig. 1 are the first direct demonstration that MDR protein overexpression in and of itself leads to a significantly reduced initial rate of chemotherapeutic drug accumulation.

Moreover, CFTR overexpression in and of itself also led

* Hammond *et al.* [15] were the first to study drug transport characteristics for cells harboring transfected MDR cDNA; however, the cell lines used in this study were grown in the presence of 0.1 μg/mL doxorubicin. Thus, it is difficult to separate the possible effects of drug exposure from those due to MDR protein overexpression [3, 9]. Devault and Gros [26] and Guild *et al.* [27] are, to the best of our ability to ascertain, the only investigators that have previously analyzed the phenotype of true MDR transfectants (where transfectants were not exposed to chemotherapeutics prior to analysis), but neither study analyzed drug transport characteristics for the true transfectants.

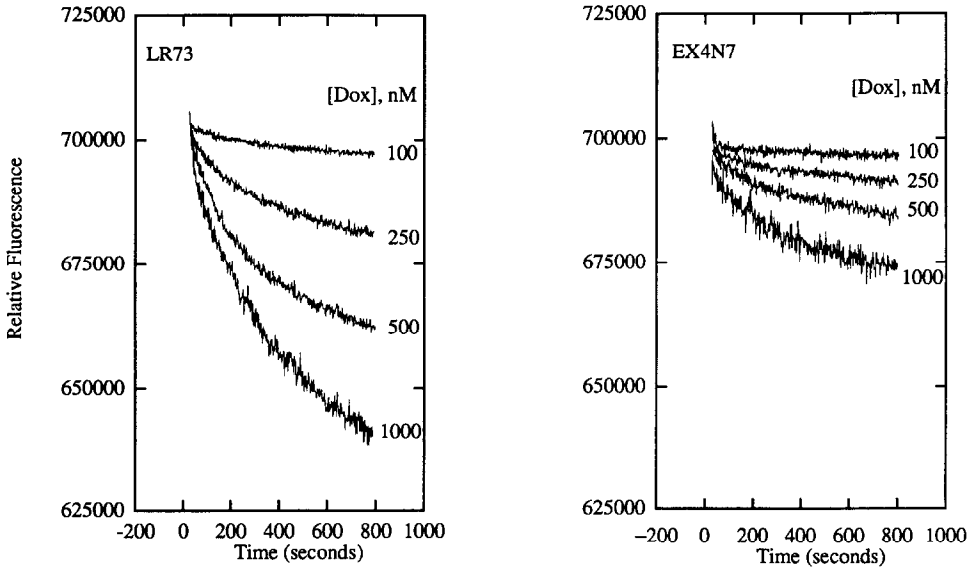


**FIG. 1. Doxorubicin accumulation in LR73 (left) and EX4N7 (right) cells at several concentrations (from top, 100, 250, 500 and 1000 nM) of drug in the transport medium. Each trace shown is the average of 3 individual traces obtained for $1.50 \times 10^6$ ($\pm 3 \times 10^4$) cells prepared as described in Materials and Methods and fast-injected into a methylacrylate cuvette containing HBS equilibrated to 37° and pH 7.33 and pre-equilibrated with doxorubicin for 5 min. Fluorescence was excited at 490 nm using a xenon arc lamp and a Czerny-Turner monochromator and emission at 590 nm monitored with a photon multiplier tube at 90° relative to the excitation beam. Data were collected using Photon Technologies Inc. software and a Dell 433/L computer. Cell suspensions were mixed continuously after injection with a teflon stirbar and a stir plate situated beneath the cuvette. Temperature was regulated for an aluminum-jacket surrounding the cuvette with a circulating water bath. Five data points/sec were collected. Data were not smoothed; however, only every fifth data point is plotted for clarity. Initial rates of accumulation calculated from these data were converted to picomoles doxorubicin per microgram cell protein in Fig. 4 (see Materials and Methods). Nearly identical data, relative to LR73, were obtained for 88-8, a mu MDR 1 transfectant harboring MDR protein unable to confer an MDR phenotype (see Ref. 16) and exhibiting similar $\Delta\Psi$ relative to LR73 (see Ref. 7). Assuming samples are prepared in an identical fashion, and assuming fresh stock concentrations of doxorubicin are used and equilibrated under exactly the same conditions for exactly the same amount of time, both the initial rate and steady state of accumulation for a given cell line at a given concentration of drug are highly reproducible by this method (SEM for rates computed from the 3 individual traces before averaging was <7%, SEM for steady states calculated at 20 min was <5%; see also Refs. 19 and 20).**

to a reduced initial rate of doxorubicin accumulation (Fig. 2). The relative rate of accumulation appeared to be inversely related to relative expression of the cyclic AMP-regulated, low conductance $Cl^-$ channel, since clone C5 (which expresses about 40% less CFTR, relative to C3 [11]) accumulated more doxorubicin in the same time period, relative to C3. These data are better appreciated by examination of Fig. 3, which overlays accumulation data for C5 (solid lines) on data for C3 (dashed lines) obtained at the same external concentration of doxorubicin. C10, which exhibits similar levels of CFTR expression and similar membrane depolarization relative to C3 [8, 11], accumulated doxorubicin very similarly relative to C3 (not shown; see Fig. 7 below). A decreased rate of [$^3$H]vinblastine accumulation has also been observed for clone C3 relative to controls [8].

Figure 4 plots the linear initial rate of accumulation determined after curve fitting of the raw data (see Materials and Methods) versus the external concentration of doxorubicin for the MDR (EX4N7) and CFTR (C3, C5) transfectants, as well as the controls (LR73 and 3T3/c cells, respectively). Similar to the case for accumulation of vincristine [29] or vinblastine [15], the accumulation rate was nearly linearly dependent on external drug concentration for all cell types, and reduced significantly for the MDR cells. In addition, C5, which harbors less CFTR than C3 and which has higher $\Delta\Psi$ than EX4N7 or C3, yielded the steepest curve of the three MDR cell lines. C5 is also less resistant to doxorubicin, relative to C3 (see Ref. 8).

### *Lack of Competition by Vinblastine and Effects of Low Level Drug Selection*

Somewhat similar to previous work which showed that excess external vincristine or daunomycin did not affect significantly the rate of [$^3$H]vinblastine accumulation for

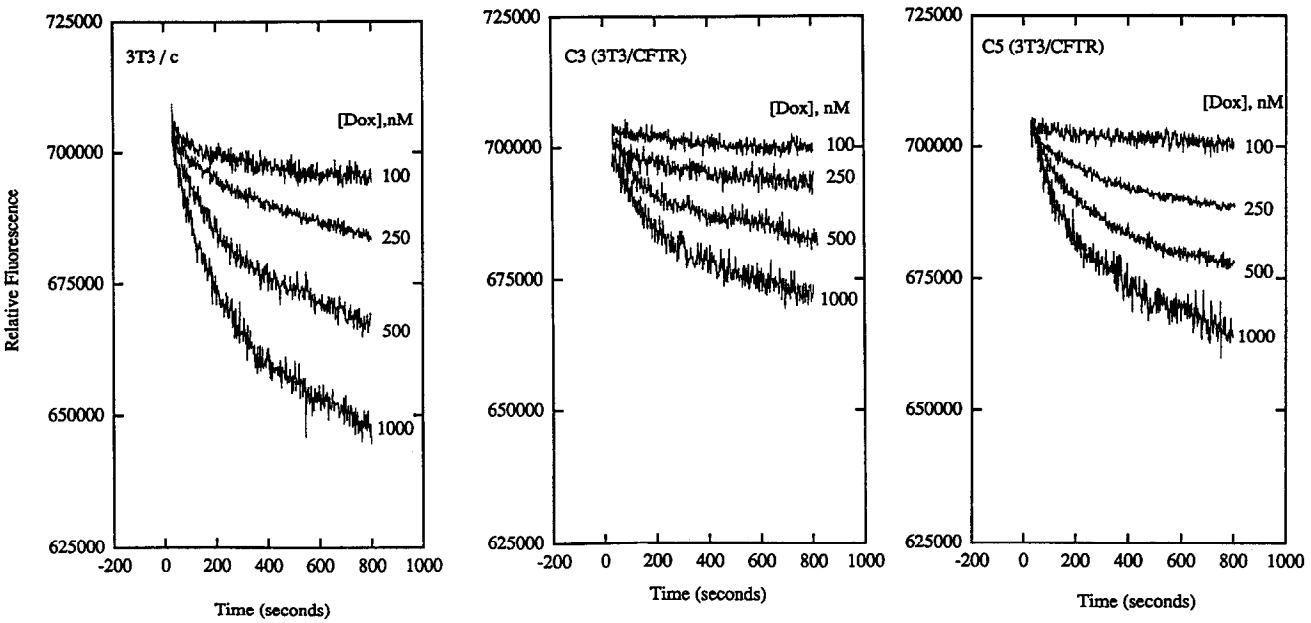


**FIG. 2. Doxorubicin accumulation as described in Fig. 1 in 3T3 control transfectants (left), and C3 (middle) and C5 (right) CFTR transfectants. Note the decreased rates of accumulation for the cells overexpressing functional CFTR, and the poorer rate of accumulation in C3, relative to C5. Again, each trace shown is the average of data from 3 individual experiments; SEM was <6% and <4% for calculated initial rate and steady state, respectively.**

MDR cells [15], we found that 10- to 100-fold molar excess of external vinblastine only mildly affected the rate of doxorubicin accumulation for the MDR 1 or CFTR transfectants (Table 1). A very small (<5%) increase in the rate of accumulation was seen for *both* the MDR 1 transfectants *and* the parental LR73 cells in the presence of 100-fold excess vinblastine as was also the case for 3T3/c and C3. This effect did not vary significantly as a function of doxorubicin concentration (not shown), and was essentially identical for both sensitive control cells and transfectants; thus, we do not attribute it to competitive inhibition of any putative drug-pumping activity.

As described [8], we also selected the C3 CFTR transfectant on 100 ng/mL doxorubicin to create a stable cell line exhibiting an increased MDR phenotype (C3D1). We compared accumulation for C3 and C3D1 in order to estimate the effects of chemotherapeutic drug selection (Fig. 5A). Previously, Gros and colleagues selected a mu MDR 1/LR73 transfectant with 50 ng/mL vinblastine (1-1 cells; [16]); doxorubicin accumulation data for this drug-selected MDR transfectant was also superimposed on the data for EX4N7 (Fig. 5B). Both the doxorubicin- and vinblastine-selected variants exhibited significantly increased drug resistance and either increased CFTR or MDR protein expression, respectively, along with increased $pH_i$ but similar $\Delta\Psi$ (Table 2; [7, 8, 16]) relative to their true transfectant parents. However, as demonstrated in Fig. 5, the drug-selected variants exhibited similar rates of doxorubicin accumulation relative to the true transfectants. Since 1-1 cells express about 10-fold more MDR protein relative to EX4N7 [7] and are significantly more drug resistant, these data suggest that decreased rates of accumulation in MDR-expressing cells are not necessarily related to relative expression of MDR protein or relative resistance. A similar disagreement between relative resistance and relative altered transport also was reported recently by Wadkins and Houghton [30]. However, note that EX4N7 and 1-1 exhibit similar membrane depolarization [7] as do C3 and C3D1 (Table 2). Thus, these data support the observation (Figs. 1 and 2, Table 2) that decreased accumulation is more closely related to decreased $\Delta\Psi$ (but not necessarily related to relative MDR protein or CFTR expression, or to relative drug resistance). They also suggest that any additional altered retention or partitioning in the drug-selected variants that further contribute to resistance may be due to factors other than altered rates of accumulation (see below).

### *Possible Relationship between $\Delta\Psi$ and Accumulation Rates*

Since doxorubicin is cationic at physiologic pH, and since both the CFTR and MDR transfectants exhibit decreased $\Delta\Psi$ but $pH_i$ perturbations in opposite directions [7, 8], and since lower $\Delta\Psi$ decreases the efficiency of doxorubicin accumulation in model systems [31, 32; see also Ref. 9], we further investigated whether the decreased rates of accumulation at these concentrations of doxorubicin were indeed related to lowered $\Delta\Psi$. Although only an approximation, one way to assess this is accumulation experiments in the presence of increasing concentrations of $K^+$, since eukaryotic plasma membrane $\Delta\Psi$ is dominated by the $K^+$ term in the Goldman/Hodgkin/Katz equation, and elevated extracellular $[K^+]$ will thus lower $\Delta\Psi$. Since the time resolution of our CMF experiments is quite good, the results of these experiments are informative.

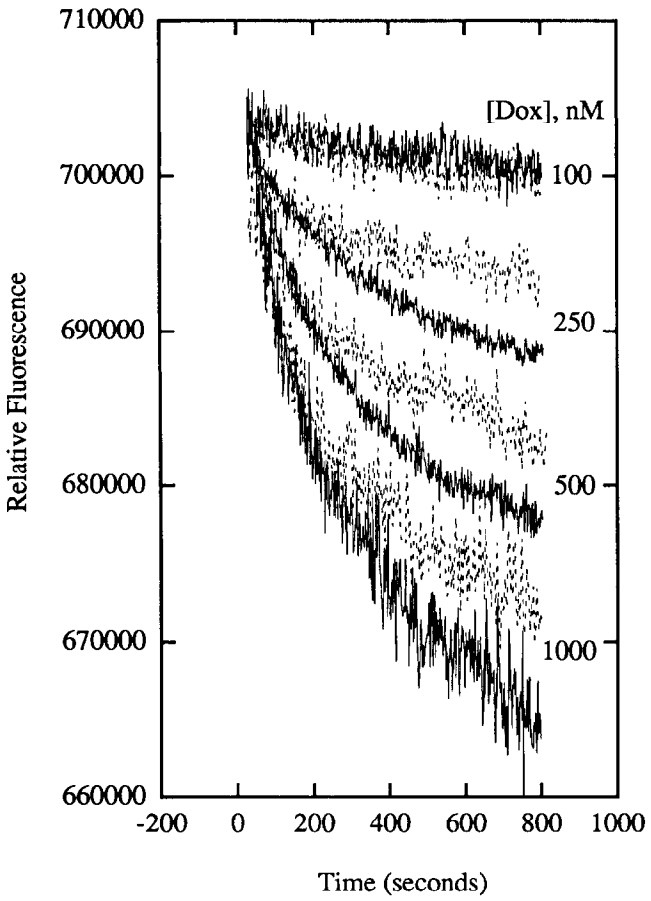


**FIG. 3. Expanded overlay of doxorubicin accumulation data from Fig. 2 for C3 (lighter colored dashed lines) versus C5 (darker colored solid lines) at several concentrations of drug. C5 cells expressed less CFTR and exhibited higher $\Delta\Psi$, relative to C3, and appeared to accumulate doxorubicin more efficiently at each drug concentration (see also Fig. 4).**

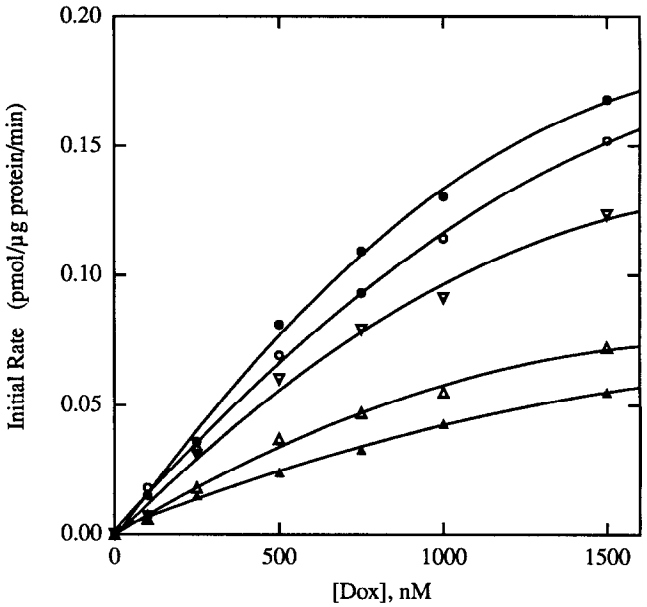


**FIG. 4. Plot of the initial rate of doxorubicin accumulation in the different cells versus the concentration of doxorubicin in the transport medium. Each data set for (from top) LR73 (closed circles), 3T3/c (open circles), C5 (downward open triangles), C3 (upward open triangles), and EX4N7 (closed triangles) was fit by a second order polynomial; $R^2 \geq 0.97$ in each case ($0.91 < R^2 < 0.94$ for straight line fits). Data below 500 nM doxorubicin was very well fit by a straight line ($R^2 > 0.97$ in each case; not shown). The initial rate (IR60) of accumulation was calculated by a straight line fit to the first 60 sec of the accumulation curve, after verifying mono-exponential behavior (see Materials and Methods). Each point is the average of 3 determinations (SEM < 7%). Linear rates of accumulation should not be confused with rate constants for influx (see Ref. 9).**

Figure 6 presents accumulation data for LR73 cells in HBS + glucose containing 5, 50, or 100 mM $K^+$, respectively (balance $Na^+$ to 145 mM monovalent cation, see legend). In these experiments, cells were washed in HBS containing physiologic [$K^+$] before fast injection. Although we cannot eliminate contributions from subtle changes in $pH_i$ or cellular volume that also may accompany this procedure, the results are consistent with a positive relationship between the rate of doxorubicin accumulation and the magnitude of $\Delta\Psi$ (see legend). Somewhat similar data have been obtained by Chen and colleagues in their analysis of $TPP^+$ and rhodamine accumulation for MCF-7 cells [33], as well as by Charcosset *et al.* [34] in their analysis of 2-*N*-methyl-ellipticinium acetate uptake by L1210 cells.

Previously [35] we performed $Cl^-$ substitution experiments on these LR73 cells in the absence of $Na^+$ by replacing $Na^+$ with $K^+$ and noted small (<0.1 pH units) changes in $pH_i$ that were short-lived. Since any transient changes in $pH_i$ that might be caused by $K^+$ substitution are lower than the differences in steady-state $pH_i$ noted for the transfectants [8], it is more likely that decreased $\Delta\Psi$ is responsible for the observed decreased accumulation.

To further test any relationship between $\Delta\Psi$ and doxorubicin accumulation, uptake was assessed for 3T3/c control cells and C3 and C10 in the presence (dashed lines) or absence (solid lines) of 25 μM forskolin (Fig. 7). Stutts and colleagues [11] have shown previously that of five isolated CFTR clones, C3 and C10 exhibit the largest increase in $Cl^-$ conductance upon forskolin stimulation. In contrast, clone C5 curiously does not respond to forskolin [11] even though it exhibits characteristic CFTR-mediated $Cl^-$ conductance. Parenthetically, C5 is also the only CFTR clone that does not exhibit decreased $pH_i$ (see Table 2, [8]); perhaps these phenomena are related. In any case, 25 μM forskolin stimulation (by adding to the transport medium before uptake is initiated) appeared to further mildly lower rates of drug accumulation for C3 and C10 (see Fig. 7), but not for C5 (not shown) or 3T3/c (left side, Fig. 7). It is difficult to quantify precisely how significantly forskolin treatment under these conditions perturbs $\Delta\Psi$ for C3 and C10, based on available data, but since increased $Cl^-$ permeability is predicted to lower $\Delta\Psi$, the results are again consistent with decreased $\Delta\Psi$ conferring reduced rates of doxorubicin accumulation.

**TABLE 1. Effect of excess external vinblastine on the initial rate of doxorubicin accumulation in the different cell lines**

| Cell line | [Vinblastine] in transport medium (μM) | Initial rate of 1 μM doxorubicin accumulation (pmol/μg protein/min) |
|---|---|---|
| LR73 | 0 | 0.128 |
| | 1 | 0.123 |
| | 10 | 0.130 |
| | 100 | 0.134 |
| EX4N7 | 0 | 0.042 |
| | 1 | 0.040 |
| | 10 | 0.043 |
| | 100 | 0.045 |
| 3T3/c | 0 | 0.113 |
| | 1 | 0.111 |
| | 10 | 0.116 |
| | 100 | 0.119 |
| C3 | 0 | 0.050 |
| | 1 | 0.050 |
| | 10 | 0.055 |
| | 100 | 0.053 |

Rates are the averages of 3 determinations (SEM < 7%). Although a small increase in the rate of accumulation was seen at high molar excess of vinblastine, similar effects were seen amoung all cell lines, suggesting that the effect is not related to MDR or CFTR expression.

### *Relative Retention and Rates of Efflux*

It is well appreciated that along with accumulation, retention of chemotherapeutic drugs is also altered in MDR cells. Panels A and B of Fig. 8 compare doxorubicin retention over time for the CFTR and MDR transfectants (dashed lines) relative to the control 3T3/c and LR73 cells (solid lines), respectively. Each panel compares drug retention for cells preincubated in the presence of 100 μM (top), 50 μM (middle) or 10 μM (bottom) doxorubicin for 30 min. Thus, note that the cells are not necessarily loaded to the same total intracellular level of drug ($D_T$), or the same exchangeable level of intracellular drug ($D_{ex}$). In fact, $D_T$ is lower for the MDR and CFTR transfectants due to the fact that they accumulate less drug during the incubation period (see Figs. 1 and 2 above). However, this experiment is informative with regard to analyzing retention phenomena and is the first step in analyzing drug efflux for these cells. Also, since many previous studies have compared MDR cells loaded in similar fashion, the data serve as a useful comparison to previous work.

Although the percentage of $D_T$ released from the MDR transfectants in similar time was greater than the percentage released for the LR73 parental cells (about 20–30% more after 60 min, not shown; see Materials and Methods and Fig. 9 below, as well as Ref.15), the CFTR transfectants released a similar percentage relative to 3T3/c. The kinetic character of this release also appeared different for EX4N7 versus C3. Note how the dashed and solid lines appear to begin to converge at longer time in the comparison between EX4N7 and LR73 (Fig. 8B), whereas the C3 and 3T3/c traces (Fig. 8A) are virtually parallel (see also Fig. 9).

To compare the true rate of doxorubicin efflux for different cells, efflux should be measured under conditions where the concentrations of freely exchangeable drug ($[D]_{ex}$) are the same [19]. We have previously described our methods for determining when $^{fast}[D]_{ex}$ is similar (see Ref. 20). Figure 9 compares retention traces for C3 (dashed) and 3T3/c (solid) cells (top overlay) or LR73 (solid) and EX4N7 (dashed) cells (bottom overlay), predicted to harbor the same $^{fast}[D]_{ex}$ (see Ref. 20). Thus, these cells were not pre-loaded under identical conditions, as was the case for the experiments summarized in Fig. 8. Initial rates of efflux appeared to be nearly identical, if not slightly slower for the CFTR transfectant relative to the parental 3T3 cells (see inset). No significant increase in the initial rate of efflux was noted for EX4N7 relative to LR73 either, although, similar to many other studies, a greater percentage of intracellular drug was released (see above). This similarity in initial rate extended over a wide range of estimated $^{fast}[D]_{ex}$ (Table 3), as previously described for MDR myeloma cells [19] as well as mu MDR 3 transfectants further selected with vinblastine (not shown, see Ref. 20). The rate of the second component for EX4N7 (the increased release that appears nearly linear over time; bottom dashed line Fig. 9), in analogy to the second component recently described for coumarin-vinblastine efflux from MDR35 cells [20] was at least 20-fold slower than passive diffusion at similar concentrations of drug (see Ref. 20) and decayed much more rapidly as a function of $^{fast}D_{ex}$, relative to the fast efflux component (not shown; see Ref. 20). As described in detail previously [12, 20] since it is so much slower than passive diffusion, the behavior of this component is more consistent with slow release of intracellular drug from an altered binding site (see Discussion).

## DISCUSSION

Previously, we found that even though MDR cells release more drug relative to sensitive cells, they do not necessarily efflux the drug at an increased rate [19, 20]. Further analysis revealed a reduced initial rate of drug accumulation (unpublished observations) as well as perturbed $\Delta\Psi$ and $pH_i$ [7, 9, 21] for MDR cells, including transfectants not previously exposed to chemotherapeutic drugs. These data along with other work (reviewed in Ref. 3) suggested to us that MDR protein overexpression might indirectly lead to decreased intracellular drug retention via reducing the statistical likelihood of intracellular binding and lowering initial rates of drug accumulation. However, the degree and manner in which these $\Delta\Psi$ and $pH_i$ perturbations may affect different aspects of chemotherapeutic drug transport and retention for whole cells is not described. In addition, since we recently identified an MDR phenotype for CFTR transfectants [8], we were also interested to see if reduced initial rates of accumulation were also found in these cells.

One suggestion for reduced rates of drug accumulation in MDR cells has been that exposure to drugs alters membrane fluidity and/or lipid composition that might then affect

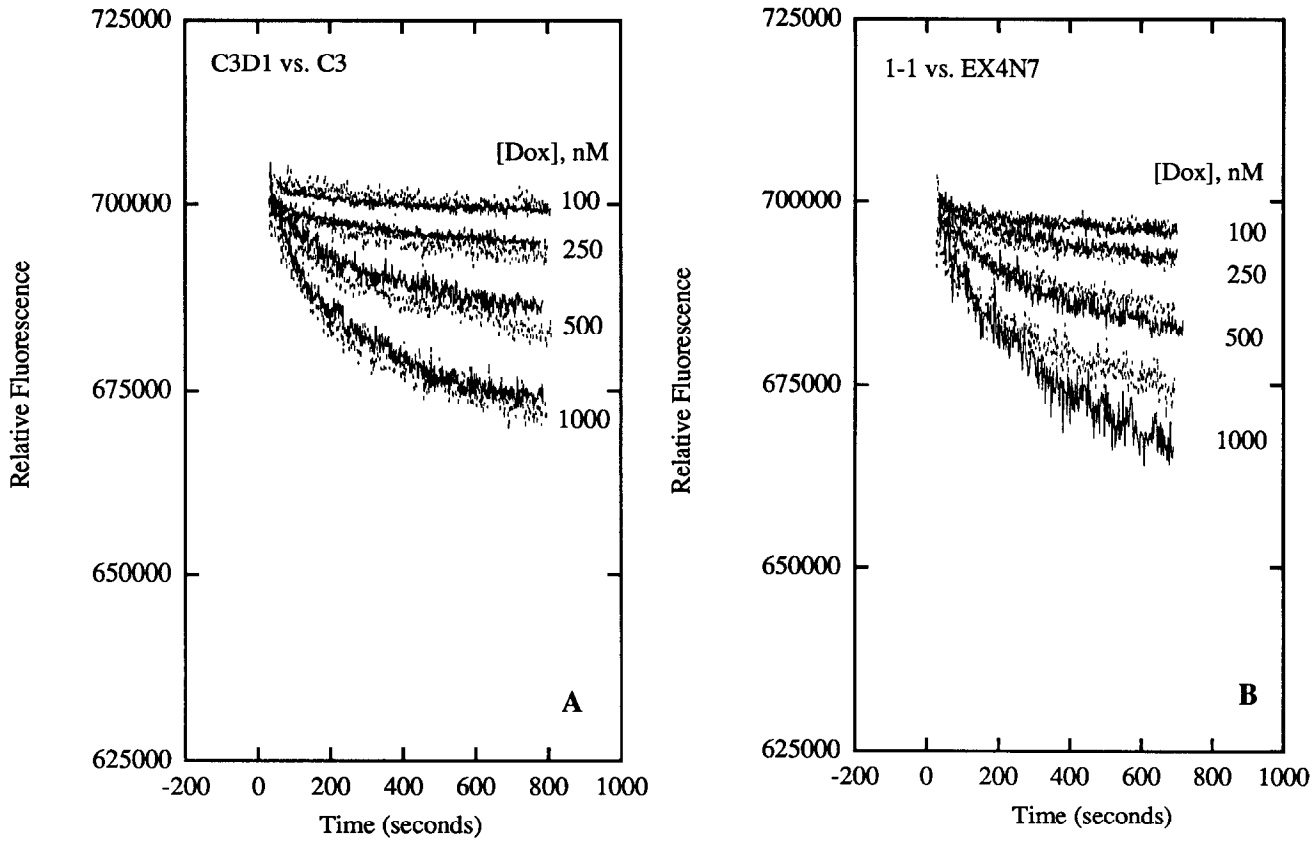


**FIG. 5. Comparison of doxorubicin accumulation for (A) C3 (lighter colored dashed lines) and C3D1 (darker colored solid lines), which is C3 selected for 2 weeks on 100 nM doxorubicin, and (B) EX4N7 (lighter colored dashed lines) and 1-1 (darker colored solid lines), which is EX4N7 selected with 50 ng/mL vinblastine [16]. Traces shown are the average of data from 3 experiments (SEM < 7% with respect to calculated initial rate). This level of drug selection of the true transfectants with either anthracycline or Vinca alkaloid, respectively, did not appear to further decrease the rates of doxorubicin accumulation or further alter $\Delta\Psi$ (Table 2), although it did increase resistance and expression of the relevant ABC transporter.**

drug diffusion. The present study using true transfectants (non-chemotherapeutic drug-selected transfectants) is thus quite informative, since effects of drug selection have been eliminated.

We previously suggested that perturbations in both $pH_i$ and plasma membrane $\Delta\Psi$ may be responsible for the unusual drug transport characteristics of MDR cells [21; see also Ref. 9]. Based on the present data, we now further suggest that decreased $\Delta\Psi$ is responsible for a substantial portion of the reduced initial rate of drug accumulation seen for MDR cells when the MDR is clearly due to MDR protein or CFTR overexpression, since CFTR transfectants are acidic whereas true MDR transfectants are alkaline. In contrast, $pH_i$ may more significantly affect other aspects of drug transport and retention (see below).

Alternatively, similar to recent proposals for MDR protein [36], one could speculate that CFTR is both a drug pump and a $Cl^-$ channel, although we do not favor this idea.* If this model is entertained, the caveat must be included that, similar to the apparent effect of MDR protein [19, 20], the "pumping" function substantially reduces rates of accumulation without necessarily altering rates of efflux, which would be extremely curious. Also, to reduce accumulation as observed, the CFTR (and also MDR 1) would need to translocate several drug molecules per second to compete with passive diffusion.† Under the initial rate conditions used in the present work, inward passive diffusion of doxorubicin is very fast. Thus, the turnover of this putative

* Channels and pumps are thermodynamically and kinetically distinct. Channels transport $10^6$–$10^7$ ions/sec down electrochemical gradients, whereas pumps translocate 10–$10^3$ ions/sec (or small molecules/sec) against a substrate concentration gradient. We consider the possibility that a single transport protein could catalyze both processes to be unlikely, due to these fundamental differences, although other investigators argue differently (see Ref. 36). Regardless, to test this possibility for CFTR, additional kinetic and thermodynamic studies of drug transport are required.

† Based on these data (see Fig. 4) and our estimates of mu MDR 1 protein site density, this would also be the approximate required turnover for MDR protein (at nanomolar to micromolar levels of external drug) if we invoke some permutation of a pump model to completely explain observed reduced initial rates of drug accumulation [3]. Conspicuously, hypothetical turnover for MDR protein estimated based on available measurements with proteoliposomes or vesicles is at least 3–5 orders of magnitude slower at similar drug concentrations (reviewed in Ref. 3). Thus, if a pump model is invoked, available data do not support the existence of a process with sufficient capacity to completely explain these and other [9, 15, 17, 29] data regarding lower initial rates of drug accumulation in MDR cells.

**TABLE 2. Electrical membrane potential ($\Delta\Psi$) and intracellular pH ($pH_i$) determined for cultures of the different cell lines**

| Cell line | $\Delta\Psi$ (mV ± 4) | $pH_i$ |
|---|---|---|
| 3T3/c | 61 | 7.37 ± 0.05 |
| C3 | 27 | 7.21 ± 0.04 |
| C3D1 | 26 | 7.38 ± 0.05 |
| C5 | 43 | 7.43 ± 0.05 |
| LR73 | 46 | 7.18 ± 0.03 |
| EX4N7 | 24 | 7.31 ± 0.04 |
| 1-1 | 27 | 7.52 ± 0.06 |

$\Delta\Psi$ was measured by $K^+$/valinomycin null point titration using di-4-ANEPPS as described [7,8,21], and $pH_i$ was determined by single cell photometry performed under constant perfusion in the presence of $HCO_3^-/CO_2$ as described [7]. Data shown are means (for $\Delta\Psi$, SEM < 9%, N = 3; for $pH_i$ data are shown ± SD, N = 20–25). C3 and C5 are 3T3/hu CFTR transfectants selected with G418; 3T3/c is a control transfectant also selected with G418 [11]; and C3D1 is C3 selected on 100 nM doxorubicin as described [8]. LR73 is a Chinese hamster ovary fibroblast, EX4N7 is an LR73/mu MDR 1 transfectant selected with G418, and 1-1 cells are LR73/mu MDR 1 transfectants further selected with 50 ng/mL vinblastine (see Refs. 16 and 26). LR73 transfected with inactive mu MDR 1 and selected with G418 (88-8 cells) exhibited behavior essentially identical to LR73 (see Ref. 7).

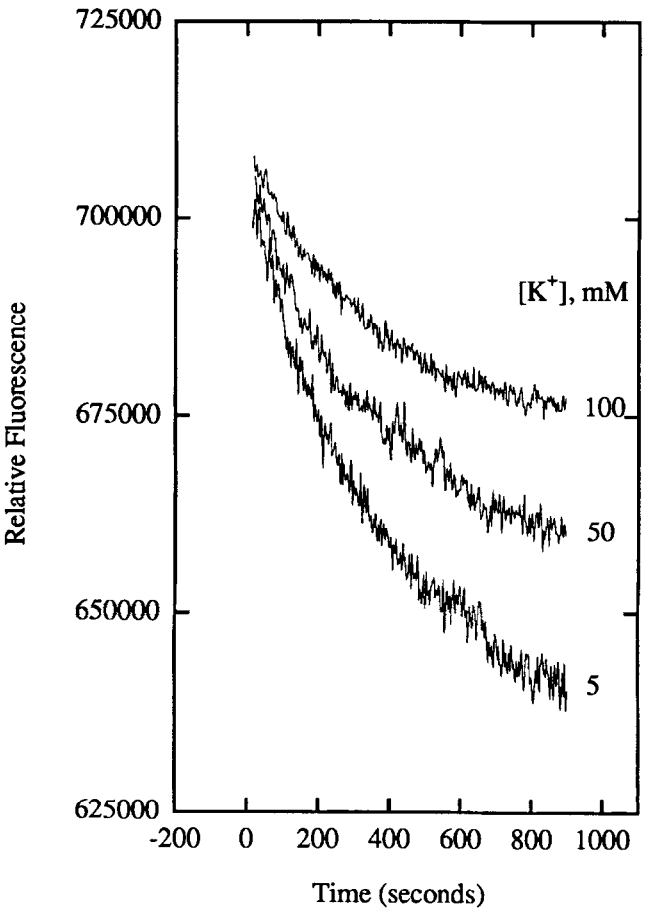


**FIG. 6. Accumulation of 1 μM doxorubicin in 1.50 × $10^6$ LR73 cells fast-injected into HBS containing (from top) 100 mM KCl/45 mM NaCl; 50 mM KCl/95 mM NaCl; 5 mM KCl/140 mM NaCl. In each case, cells were prewashed in HBS containing physiologic (6 mM) $K^+$. Data shown are representative of 3 experiments at each $K^+$ concentration (SEM < 7% with respect to calculated initial rate at a given [$K^+$]). Nearly identical data have been observed for 88-8 cells harboring mutant MDR protein unable to confer MDR (see Ref. 7). Higher concentrations of $K^+$, which are predicted to transiently decrease $\Delta\Psi$ (predicted 12 and 30 mV at 100 and 50 mM $K^+$, respectively, via the Nernst relation) appeared to decrease the efficiency of doxorubicin accumulation.**

outward pumping process would need to compete kinetically with passive diffusion. Based on model LUV studies [31, 32] and the present data (see also Refs. 8 and 9), it is more logical to suggest that decreased $\Delta\Psi$ is responsible for reduced rates of accumulation.

It is stated frequently that MDR cells harbor "lower intracellular levels" of chemotherapeutic drugs, with the implication sometimes being that these decreased levels mean decreased *equilibrium cytoplasmic* levels. As described at the beginning of the paper, it can be difficult to envision how $\Delta\Psi$ perturbations might affect steady-state intracellular retention when one views the MDR phenotype simplistically as a decreased level of cytoplasmic drug. Doxorubicin, for example, is a weak base; thus, some investigators argue that changes in $\Delta\Psi$ will not necessarily alter equilibrium distribution of the compound across the plasma membrane, because passive diffusion of the neutral compound likely far outpaces passive diffusion of the charged compound. Although these rates have never been measured directly for doxorubicin, Skovsgaard and Nissen [25] extrapolated a plot of pH versus diffusion to estimate that they differ by 30- to 40-fold. Thus, some argue that $\Delta\Psi$ cannot have anything to do with altered equilibrium intracellular concentrations of drug (e.g. Ref. 37). However, there are several important points to consider. First, chemotherapeutic drugs are *concentrated* enormously in cells when they are incubated in the drugs. If total intracellular concentration is calculated, it may be up to 50–100 times higher than external at what approximates steady-state. Second, protonation of the drug in the cytoplasm is much faster than passive diffusion of the neutral species, and the protonated species is more pharmacologically relevant. Third, decreased retention of drug in MDR cells may not require drastically altered cytoplasm/extracellular "equilibrium" distribution, but could also be caused by processes that lower the statistical likelihood of intracellular binding over time [3].

We propose that decreased $\Delta\Psi$ lowers the initial rate of accumulation of doxorubicin. Along with possible direct electrostatic effects that will require firm definition of rates of passive diffusion of charged versus neutral doxorubicin to quantify, binding and diffusion could also be influenced by secondary effects of significant $\Delta\Psi$ perturbations. These might include altered lipid distribution and tubulin organization as suggested (see Ref. 8). Some of these secondary perturbations may require long-term perturbation of $\Delta\Psi$ and may not be as apparent in transient perturbations of $\Delta\Psi$ with ionophores.

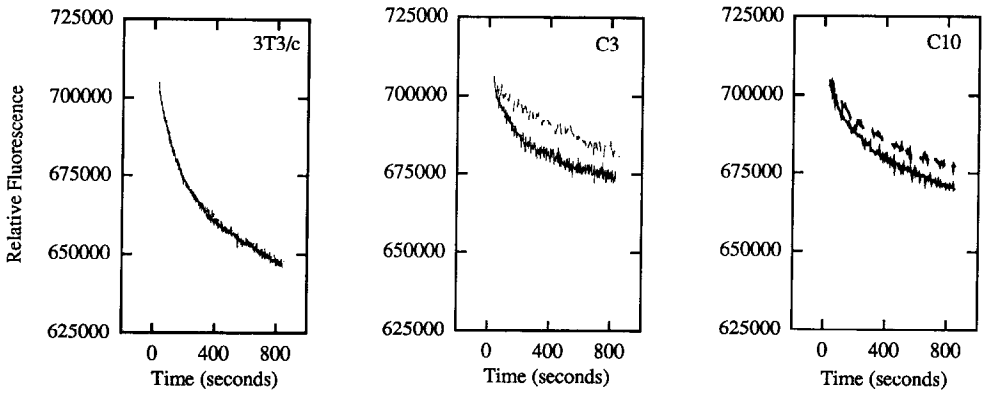


**FIG. 7. Doxorubicin accumulation (at 1 μM external [Dox]) for 3T3/c (left panel), C3 (center panel), and C10 (right panel) cells in the absence (darker solid curve) and presence (lighter dashed curve) of 25 μM forskolin. Note that unlike C3 and C10, C5 did not exhibit further decreased doxorubicin accumulation upon forskolin stimulation, nor did the true mu MDR 1 transfectant EX4N7 (not shown). The data for 3T3/c ± forskolin (left panel) are superimposed. Each trace shown is the average of 3 transport curves. SEM < 1% based on the total fluorescence signal, or <8% based on the change in fluorescence during the entire experiment. After averaging, the data were smoothed using the Savitzky–Golay algorithm (15 pt buffer).**

Also, surface potentials may be included in the Goldman/Hodgkin/Katz expression for the electrical membrane potential difference between cytoplasm and extracellular fluid ($\Delta\Psi$):

$$\Delta\Psi = \frac{RT}{F} \ln \frac{\Sigma(P_{j^+})(C_{jo^+}) + \Sigma(P_{j^-})(C_{ji^-})e^{F(\Psi_o-\Psi_i)/RT}}{\Sigma(P_{j^-})(C_{ji^+}) + \Sigma(P_{j^-})(C_{jo^-})e^{F(\Psi_o-\Psi_i)/RT}} + \Psi_o + \Psi_i$$

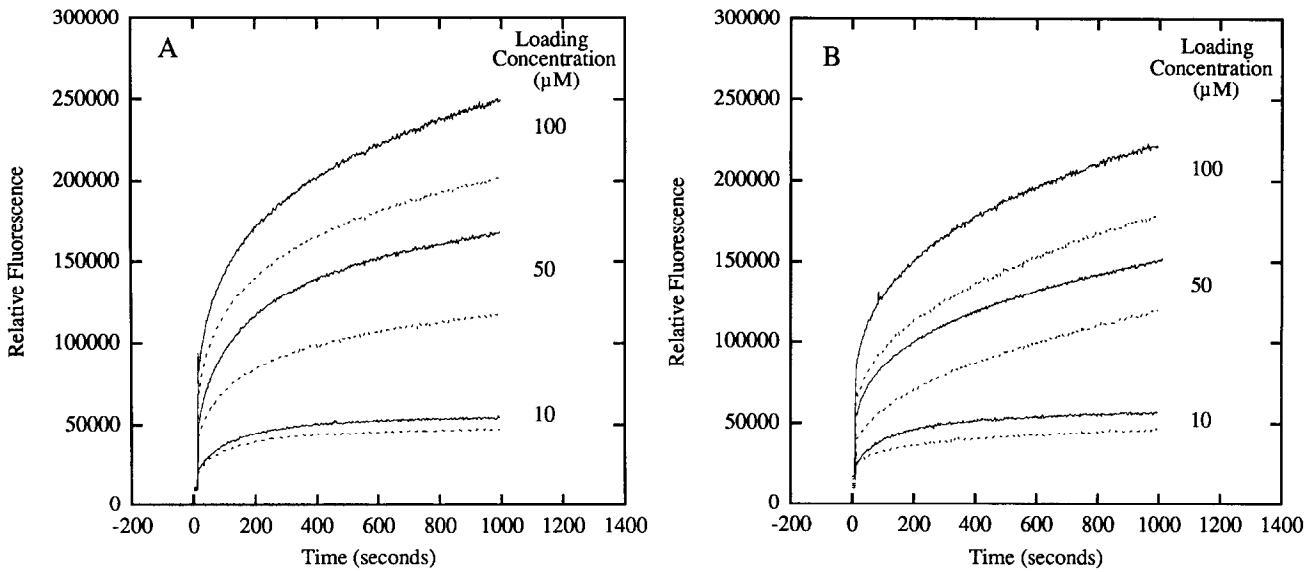


**FIG. 8. Relative retention of doxorubicin for (A) 3T3/c (solid) and C3 (dashed) and (B) LR73 (solid) and EX4N7 (dashed) cells preincubated in different concentrations of doxorubicin; note that these cells did not harbor similar $D_{ex}$. In both A and B, the top two traces were obtained for cells incubated in 100 μM external drug, the middle two traces for cells incubated in 50 μM drug, and the bottom two traces for cells incubated in 10 μM drug. Each trace shown is the average of 3 separate transport curves obtained for cells loaded in the same concentration of drug for the same time (SEM < 5% with respect to initial rate of release, <4% with respect to steady state of release calculated at 20 min), and the data were smoothed using the Savitzky–Golay algorithm. Absolute levels of drug release were greater for the sensitive cells because they accumulated more drug under similar loading conditions. Note that although the heights of the curves for 3T3 and C3 are different, the shape is virtually identical (see also Fig. 9) as defined by exponential curve fitting (see Materials and Methods and Ref. 20). In contrast, the shapes of the LR73 and EX4N7 curves differed (see also Fig. 9), with a more pronounced second linear component present in the EX4N7 trace, somewhat analogous to the case for coumarin-vinblastine efflux from MDR35 cells noted previously [20]. Although absolute levels of drug released from the resistant cells were lower, the percentage released (relative to total intracellular drug at time = 0 sec) was slightly higher (see Results and Fig. 9) for EX4N7.**

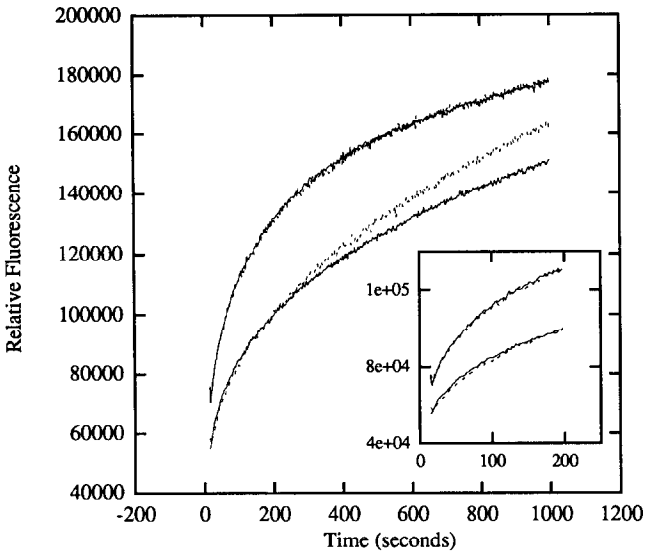


**FIG. 9. Comparison of doxorubicin efflux for 3T3/c (solid) and C3 (dashed) cells (top overlay) and LR73 (solid) and EX4N7 (dashed) cells (bottom overlay) preloaded to similar $^{fast}D_{ex}$ (estimated 2.5 μM) as previously described in detail [20]. Shown in the inset is an expansion of the same data over the first several minutes to highlight the nearly identical initial rates of efflux of $^{fast}D_{ex}$ (1e + 05 = 100,000; see also Table 3). Note the superimposable hyberbolic shape to the transport curves for 3T3 and C3, but the increased second slow component for EX4N7, relative to LR73 (see Ref 20). Traces shown are representative of data from 3 experiments, SEM < 5% with respect to calculated initial rate (see Table 3).**

where $R$, $T$ and $F$ are the gas constant, temperature, and Faraday's constant, respectively, $P$ and C denote permeability and concentration of cationic (+) and anionic (−) species, and $\Psi_0$ and $\Psi_i$ are surface potentials on the outside and inside surface. It is likely that $\Psi_o \neq \Psi_i$ for biological membranes (see Ref. 38). Altered $\Psi_o$ might be expected to mildly alter extracellular binding of the cationic doxorubicin species, and other effects (leading to altered concentration at the cytoplasm/membrane interface) are also possible. That is, Mayer *et al.* [10, 39] have pointed out that some of the concentrative effects due to $\Delta\Psi$ may be heightened for the hydrophobic drug dibucaine due to a propensity of the anesthetic to aggregate at the membrane/cytoplasm interface. Such an effect is also possible for doxorubicin, which may aggregate at concentrations above approximately 2 μM.

We are also curious about the possible combined effects of altered $\Delta\Psi$ and $pH_i$. It is well appreciated at this point that both these parameters have important effects on retention of chemotherapeutic drugs [9, 10, 31, 32, 39–41]. For example, elevated $pH_i$ is predicted to slightly affect the rates of efflux of some drugs [25]. That is, in the presence of a pH gradient (and assuming neutral compound diffuses much faster than charged compound),

$$[Dox]_i/[Dox]_o = (1 + 10^{(pK_b - pH_i)})/(1 + 10^{(pK_b - pH_o)})$$

**TABLE 3. Initial rates of efflux for cells harboring similar $^{fast}D_{ex}$**

| Cell line | $[^{fast}D_{ex}]$ (μM) | Initial rate of efflux (pmol/μg protein/min) |
|---|---|---|
| LR73 | 0.52 | 0.071 |
| | 2.51 | 0.264 |
| | 10.0 | 0.631 |
| EX4N7 | 0.51 | 0.065 |
| | 2.53 | 0.251 |
| | 10.0 | 0.628 |
| 3T3 | 0.51 | 0.113 |
| | 2.51 | 0.413 |
| | 10.0 | 0.772 |
| C3 | 0.54 | 0.107 |
| | 2.53 | 0.395 |
| | 10.0 | 0.763 |

Values for $D_{ex}$ and rates are means of data from 3 experiments (SEM < 5%). Although MDR cells may release a higher percentage of a drug (relative to sensitive cells) when they are diluted into medium without drug (Fig. 8), they do not necessarily efflux the drug at an increased rate when they harbor similar exchangeable concentrations of drug (see also Ref. 19 and 20).

where the subscripts i and o denote inside and outside, respectively.

Thus, a 0.1 unit elevation in $pH_i$ would decrease the steady-state cytoplasmic concentration of doxorubicin ($pK_b$ near 8.2) by about 30%, assuming $pH_i$ for a sensitive cell to be near 7.2 and $pH_o$ near 7.3 (as is the case for LR73 vs EX4N7; see Ref. 7). Thus, a significant change in $pH_i$ may be predicted to alter the rate of *outward* diffusion in a zero trans experiment. Indeed, Skovsgaard and Nissen [25] previously estimated that a 0.1 unit change in pH may alter the rate of simple passive diffusion by as much as 18%. However, we do not find such a significant change in MDR myeloma cells with increased $pH_i$ [19] or these true MDR transfectants with elevated $pH_i$, or in mu MDR 3 transfectants selected with vinblastine [20]. Perhaps this predicted effect is masked to some extent by altered $\Delta\Psi$. Or, we suggest that overlapping $pH_i$-dependent binding phenomena probably complicate the situation (see also Ref. 19). In Ref. 20, we suggested that a second drug efflux component, also apparent for EX4N7 in the present study (Fig. 9), is due to a more loosely bound fraction of drug (see also Ref. 12), that is, the rate-limiting step for efflux of this component is dissociation from target, and the kinetic character of this efflux is thus analogous to the kinetic character of drug/target dissociation. Perhaps elevated $pH_i$ contributes to production of this fraction by altering the drug/target dissociation constant. This is reasonable, since binding of anthracyclines to nucleic acid is highly pH dependent. The kinetic character of release of this fraction may then be superimposed upon the kinetic character of efflux of cytoplasmic drug, and the two will need to be carefully separated (e.g. as in Ref. 20) before any small effects of $pH_i$ on the efflux of cytoplasmic drug can be distinguished unequivocally.

In conclusion, analysis of the doxorubicin transport properties for CFTR and MDR transfectants exhibiting dif-

ferent $\Delta\Psi$, comparison to drug-selected derivatives, and analysis of the effects of elevated $K^+$ and forskolin stimulation suggest that decreased $\Delta\Psi$ contributes to a decreased initial rate of drug accumulation for drug-resistant cells at physiologically relevant levels of doxorubicin. Analysis of drug retention suggests that increased $pH_i$ may be more important for determining binding efficiencies. We note that relative partitioning coefficients for different drugs will, to some extent, dictate the significance of these biophysical effects with regard to resistance to different drugs (see Ref. 9). Continued comparison between CFTR and MDR transfectants will offer one avenue for separating the effects of $\Delta\Psi$ and $pH_i$ in important cellular phenomena.

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