Analysis of the Steady-State and Initial Rate of Doxorubicin Efflux from a Series of Multidrug-Resistant Cells Expressing Different Levels of P-Glycoprotein[†,‡]

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Received July 27, 1992; Revised Manuscript Received September 28, 1992

ABSTRACT: Continuous monitoring of fluorescence (CMF) has been used to examine doxorubicin efflux from intact human myeloma cells. The time resolution of these measurements has enabled detailed comparison of the initial rates of efflux for the drug-sensitive myeloma line RPMI 8226 and a series of sequentially derived multidrug-resistant (MDR) lines expressing different amounts of human MDR protein (P-glycoprotein). Cells that are 3-, 10-, 60-, or 120-fold resistant to doxorubicin export approximately 10, 20, 30, or 33% more doxorubicin than the parental sensitive cells, respectively, when all are preloaded to the same level of total intracellular drug. Remarkably, however, when cells are loaded to the same level of exchangeable drug the initial rates of efflux are found to be virtually identical. This agreement between rates is apparently not dependent on the drug concentration. Approximately 50% of the increase in the steady-state level of doxorubicin efflux for the resistant cells is abolished upon glucose starvation. However, surprisingly, the apparent initial rates of efflux from the treated and untreated cells are found to be virtually the same. Pretreatment of the resistant cells with verapamil reduces the steady-state level of efflux but increases the apparent initial rate at some concentrations. Conversely, vincristine does not alter steady state but slows the initial rate of efflux from both sensitive and resistant cells by approximately the same extent. Finally, quite interestingly, a nearly linear relationship between pH_i and relative steady state of efflux is found for the series of cell lines. These data are interpreted in terms of existing models for MDR.

The development of multidrug resistance (MDR;[1] cf. Endicott & Ling, 1989; Gottesman & Pastan, 1988; Roninson, 1990) often hampers the treatment of cancer. That is, during chemotherapy tumor cells may develop cross-resistance to drugs that are significantly different, both in structure and mode of action, than the agent(s) initially administered. The MDR phenomenon has been modeled in tissue culture (Biedler & Riehm, 1970; Akiyama et al., 1985; Dalton et al., 1986) with a variety of cell lines and chemotherapeutic drugs. Remarkably, MDR cells appear to retain much lower intracellular levels of both the initial selecting agent and the agents to which they are cross-resistant.

Ling and co-workers (Riordan & Ling, 1979) first documented the overexpression of a membrane protein in MDR cells (denoted P-glycoprotein) with an apparent molecular mass of 170–180 kDa. Molecular cloning and characterization of human, hamster, and mouse genes encoding this protein (Roninson et al., 1986; Van der Bliek et al., 1987; 1988; Scotto et al., 1986; Gros et al., 1986a, 1988) has revealed it is a homologue of components of several well-characterized bacterial membrane transport systems, including those responsible for histidine, phosphate, or maltose uptake and hemolysin export (Ames, 1986; Felmlee et al., 1985; Higgins et al., 1990). This homology, as well as earlier studies that documented lower intracellular levels of chemotherapeutic drug in MDR cells, led to the suggestion that the MDR protein acts as an ATP-coupled active drug efflux pump (Gros et al., 1986a; Gerlach et al., 1986; Ames, 1986). However, direct evidence for active drug transport by the MDR protein via the observation of drug efflux against a concentration gradient in either cells, vesicles, or reconstituted proteoliposomes has not yet been unequivocally demonstrated. Relatedly, alternative support for the active transport hypothesis via the observation of rate enhancement in the efflux process unequivocally attributable to overexpression of the MDR protein is lacking, although one report has noted a slight (1.5–2-fold) increase in the rate of drug efflux for a 3000-fold resistant MDR cell line relative to the sensitive parent (Sirotnak et al., 1986). It should be emphasized, however, that although MDR protein expression generally increases concomitant with selection to higher levels of MDR, overexpression begins to plateau well before thousandfold levels of resistance are acquired. Thus, it is difficult to attribute phenotypic differences between thousandfold resistant cells and the sensitive parent solely to the expression of MDR protein.

Therefore, a sensitive fluorescence-based assay for the export of chemotherapeutic drugs from MDR cells has been developed and has been used to systematically examine the drug efflux properties (rate and steady state) for a series of sequentially derived MDR cells that express increasing amounts of MDR protein concomitant with development of increased levels of resistance (Dalton et al., 1986, 1989). These cells, derived from the parental myeloma line RPMI 8226, are approximately 3-, 10-, 60-, and 120-fold resistant to doxorubicin, are significantly cross-resistant to vincristine and other chemo-

[†] This research was supported by grants from the Raymond & Beverly Sackler Foundation for Cancer Research and the Society of Memorial Sloan Kettering Cancer Center. P.D.R. is a Sackler Biomedical Scholar at MSKCC.

[‡] A preliminary account of this research was presented at the Annual Biophysical Society Meeting, February 9–13, 1992, Houston, TX.

[1] Abbreviations: MDR, multidrug resistance; kDa, kilodaltons, ATP, adenosine 5′-triphosphate; pH_i, intracellular pH; BCECF, 2′,7′-bis-(carboxyethyl)-5,6-carboxyfluorescein; SNARF, seminaphthorhodafluor-1; SNARF-AM, acetoxymethyl ester form of SNARF; HBSS, Hanks' balanced salt solution; CMF, continuous monitoring of fluorescence; D_T, total intracellular doxorubicin; D_{ex}, portion of D_T that is freely exchangeable; D_{cyt}, cytoplasmic concentration of doxorubicin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IR^{10}, initial rate of efflux calculated from the first 10 s of the efflux curve; FU, fluorescence units; [dox], concentration of doxorubicin.

0006-2960/92/0431-12555$03.00/0

therapeutics, and express 1, 9, 50, and 55 relative molar amounts of MDR protein, respectively. They thus represent an ideal system for systematically assaying the contribution of MDR protein expression to any alteration in the initial rate or steady state of drug efflux by searching for *trends* in the series that might qualitatively correlate with expression of MDR protein. Importantly, the presented experiments should be distinguished from previous transport studies which have used MDR cells that were 10^2–10^3-fold resistant or higher, a realm of MDR where direct correlations between the degree of resistance, the concentration of MDR protein, and transport alterations are more difficult to demonstrate and certainly less linear.

In general agreement with other studies, it is found that 120-fold doxorubicin resistant cells efflux nearly 35% more drug than the sensitive parent within 10 min if cells are loaded to the same total level of intracellular drug. Moreover, a steady increase in the relative steady-state level of efflux appears to accompany increased MDR protein expression in the series of 8226-derived resistant cell lines. However, interestingly, no increase in the apparent initial rate of doxorubicin efflux is found for any of the resistant cells, relative to the sensitive parental cells, at a variety of drug concentrations when attention is paid to altered levels of exchangeable drug for the different cells.

In addition, ATP-depletion strategies are found to substantially reduce the steady-state level of efflux from the resistant cells but do not significantly affect the rate. Treatment of resistant cells with verapamil, which has been shown to reverse the MDR phenotype by perhaps inhibiting drug efflux (Kessel & Wilberding, 1984, 1985) also reduces the steady-state level of doxorubicin efflux from the resistant cells but actually increases the rate of efflux at some concentrations. In contrast, efflux measurements made in the presence of excess internal vincristine reveal the vinca alkaloid inhibits the rate of doxorubicin efflux, but not the steady state, and that it inhibits efflux similarly in both sensitive and resistant cells.

By immunochemical quantitation of MDR protein, the apparent initial rate and steady state of drug efflux for all cells in the series are compared to levels of putative active drug transport protein. In contrast to many well-characterized transport processes known to be mediated by a single active transport protein, there is no relationship between the initial rate of drug transport and the level of MDR protein, yet importantly, the steady-state level of efflux and the amount of MDR protein are qualitatively related. This suggests that increased MDR protein expression leads to decreased intracellular binding or sequestration of drug, as previously suggested (Beck et al., 1983). Measurement of intracellular pH (pH_i) for the series of cells reveals that it is closely correlated with the relative steady state of efflux, suggesting that the pharmacologically relevant function of MDR protein expression in these cells may be to raise intracellular pH directly or indirectly such that less drug is sequestered in a charge-dependent manner. Thus, the data in their entirety are consistent with the hypothesis that MDR protein acts to *indirectly* lower drug accumulation.

MATERIALS AND METHODS

Materials. Doxorubicin and vincristine were obtained as 2 and 1 mg/mL solutions, respectively, in normal saline from Adria Laboratories (Columbus, OH). 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and carboxyseminaphthorhodafluor-1 (carboxy-SNARF) were purchased from Molecular Probes (Eugene, OR) and used without further purification. All other chemicals were reagent grade or better and purchased from commercial sources.

Tissue Culture. Drug-sensitive RPMI 8226 cells and the 3-fold, (Dox 1) 10-fold, (Dox 6), and 60-fold (Dox 40) doxorubicin-resistant cells were a kind gift of Dr. William Dalton, University of Arizona Cancer Center. The cells were propagated at 37 °C in a 5% CO_2 atmosphere with RPMI medium containing additional 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. Stock cultures were grown in the presence of doxorubicin at the following concentrations: Dox 1 cells, 1×10^{-8} M; Dox 6 cells, 6×10^{-8} M; Dox 40 cells, 4×10^{-7} M. These stock cultures were generally expanded in the absence of drug 1–2 weeks before harvesting for transport studies. The resistant cells do not lose their MDR phenotype or show reduced levels of MDR protein expression (Dalton et al., 1989) when grown for short periods of time in the absence of drug; however, subtle effects on drug efflux are noticed and will be reported on elsewhere. The Dox 120 cell line was developed from the Dox 40 line by single-step selection in 1.2×10^{-6} M doxorubicin. This line was considered stable when it grew approximately as fast as the parental line (about 3 months after selection was begun).

MDR protein was quantitated for this series by western blot analysis using the antibody C219 and peroxidase-enhanced detection, followed by densitometry with a Stratagene Stratascan 7000 densitometer interfaced to an AST personal computer.

Fluorescence Spectroscopy. Fluorescence measurements were made with a Photon Technologies International fluorometer interfaced to an AST personal computer. Methyl acrylate cuvettes (3.0 mL) were jacketed within the sample compartment by an aluminum holder connected to a LKB variable-temperature water bath. For efflux experiments, excitation at 467 nm was with a water-cooled xenon source and emission was usually monitored at 590 nm; other experimental details may be found in the individual figure captions. While scanning was performed in a darkened room, concentrated, washed cells (see below) were fast diluted 60-fold by rapid injection into a 3.0-mL cuvette harboring Hanks' balanced salt solution (HBSS) equilibrated to the desired temperature and pH. The diluted suspension was continuously mixed. Continuous monitoring of fluorescence at one or more wavelengths was controlled by proprietary software written by Photon Technologies.

Transport Measurements. Continuous monitoring of fluorescence (CMF) is an excellent method for assaying transport (cf. Eidelman & Cabantchik, 1989), assuming some fluorescence characteristic of the transported compound changes when it moves between the intracellular and extracellular milieu. As described in Results, fluorescence of extracellular doxorubicin is approximately 6.5-fold more efficient than intracellular; thus transport of the compound can be followed by monitoring the time-dependent change in doxorubicin fluorescence intensity upon rapid dilution of cells [see Eidelman and Cabantchik (1989) for a more complete discussion].

To prepare cells for efflux measurements, approximately 1×10^6 cells were harvested, spun at 1000 rpm for 10 min in a Sorvall RT6000B centrifuge, washed twice in HBSS, pH 7.3, with or without glucose (see below), and resuspended at a density of 1×10^6/mL. The cells were then incubated at 37 °C in the presence of doxorubicin. Since the resistant cells retain less doxorubicin, it was necessary to adjust the external loading concentration for each cell line such that cells harbored the same total internal concentration of drug (D_T) when steady-

state comparisons were desired or the same exchangeable concentration (D_{ex}) when comparison between rates was desired. The appropriate external concentrations were determined for each cell line under a variety of conditions by incubation of aliquots of the cells in various concentrations of drug for 45 min at 37 °C. The concentrated, washed (see below) cells were then fast-diluted into buffer equilibrated to 4 °C to verify equivalent total intracellular loading (see Results) or lysed with an Ultrasonics cell disruptor to determine intracellular, "releasable" doxorubicin as an independent approximate measure of exchangeable drug. In addition, as a independent measure of total intracellular doxorubicin, sensitive and resistant cells were incubated with drug, washed, and sonicated with an Ultrasonics cell disruptor in the presence of 50% EtOH/0.3 N HCl to extract total doxorubicin (Bachur et al., 1970). The cell pellet was extracted three times, the supernatants were pooled, and fluorescence spectra were obtained. After comparison to a calibration curve obtained with the same solvent, total intracellular doxorubicin was calculated. Exchangeable drug was calculated by comparison of the steady-state level of extracellular doxorubicin after efflux was completed at 37 °C to a calibration curve obtained with HBSS/glucose. Fluorescence of doxorubicin was found to be linear in both EtOH/HCl and HBSS at concentrations below 1.5 μM; thus quantitative determination of drug concentrations could easily be made.

For a given external concentration of doxorubicin in the incubation medium, both total internal doxorubicin and exchangeable doxorubicin were found to be reduced for the resistant cells, relative to sensitive cells, by approximately (but not exactly) the same percentage. Furthermore, for a given external concentration of doxorubicin in the incubating medium, doxorubicin released by the resistant cells after sonication in cold buffer was also reduced by roughly the same percentage as was freely exchangeable drug. For example at 12.5 μM external drug in the incubating medium, total and exchangeable drug were 44% and 33% lower, respectively, for the Dox 40 cells relative to the 8226 cells, but incubating concentrations slightly less than 2-fold greater for the Dox 40 cells resulted in nearly identical levels of total intracellular drug as assayed by the two different methods described above. These percent reductions were found to vary slightly, depending on the incubating concentration (between 0.1 and 125 μM). Cytoplasmic concentrations were estimated as explained in footnote 4.

After incubation with drug, the cells were centrifuged for 10 min at 1000 rpm and resuspended in HBSS that was prechilled to 4 °C. The cells were again spun at 4 °C and resuspended at a density of approximately 5×10^7 cells/mL with ice-cold HBSS. Since slight kinetic differences were observed for efflux when cells were left on ice for longer than about 20 min, cells were used within 10–15 min of washing. Comparison between unwashed and washed cells reveals the kinetic character of efflux at 37 °C is essentially unaffected by short exposure to cold buffer. When the effects of various agents were under study (i.e., the presence of verapamil or vincristine or the absence of glucose) treatment was in general performed during incubation with doxorubicin.

Measurement of pH_i. Intracellular pH was calculated by measuring the ratio of fluorescence emission at 587 and 635 nm for cells loaded with seminaphthorhodafluor-1 (SNARF), a pH-sensing dye with two pH-dependent emission maxima and a clear isosbestic point at 605 nm. A linear standard curve was generated by measuring 587/635-nm ratios for aliquots of cells equilibrated with 10 μM nigericin and KNH

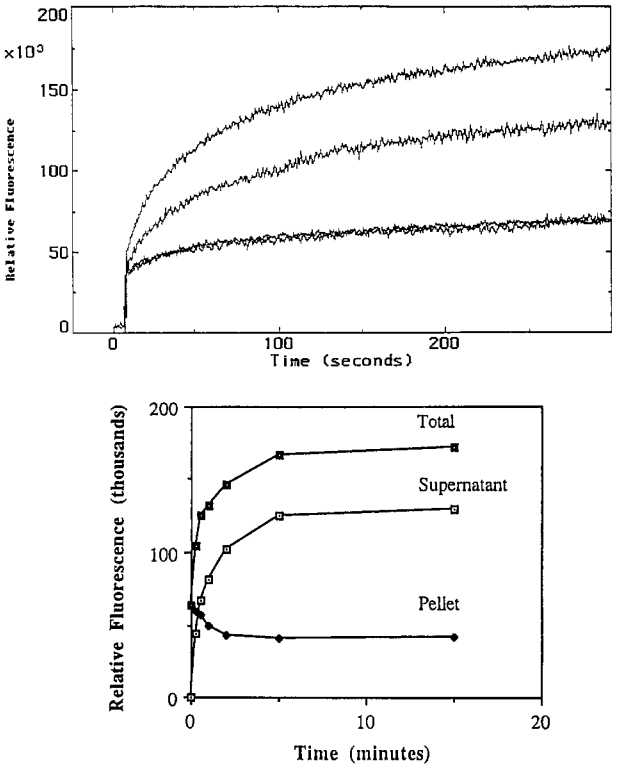

FIGURE 1: (Top panel) Time-dependent fluorescence traces for doxorubicin-sensitive RPMI 8226 and resistant Dox40 cells obtained upon fast dilution of concentrated cells at either 4 °C (bottom two traces) or 37 °C (top two traces). Cells were preloaded to equal D_T (see Materials and Methods) in HBSS/6 mM glucose, pH 7.3, and washed once in HBSS/glucose at 4 °C before rapid dilution. Excitation was at 467 nm; emission was at 590 nm. Excitation and emission slit widths were 6 and 4 nm, respectively, and 20 data points were collected/s. The top trace, Dox 40 diluted at 37 °C, has the greater intensity because more drug is effluxed from these cells than from the 8226 cells (middle trace). Correspondingly, the bottom two traces are flat because no drug efflux occurs at 4 °C, and their similar intensities reflect equivalent drug loading. (Bottom panel) Doxorubicin efflux from resistant Dox 40 cells measured by dilution of aliquots of cells preloaded to the same D_T and incubation in buffer alone for various times followed by fast centrifugation in an Eppendorf centrifuge and quantitation of 590-nm doxorubicin fluorescence intensity in the supernatant (open squares, middle trace) and cell pellet (diamonds, bottom trace). Cells were treated as in the top panel and an aliquot of these cells was also fast-diluted as in the top panel to verify that the magnitude of the curve obtained upon addition of the pellet and supernatant curves (filled squares, top trace) is equal to the magnitude of the trace obtained in the CMF experiment (top panel). The cell pellet data were collected at 4 °C to prevent additional efflux.

buffer (100 mM KCl/20 mM NaCl/20 mM HEPES) at various pH values between 6.7 and 7.6. Cells were washed in appropriate buffer (HBSS in the case of samples, KNH in the case of standards) and incubated with 5 μM SNARF-AM for 30 min at 37 °C. They were washed again with buffer at 25 °C and resuspended in a cuvette at approximately 5×10^5 cells/mL before wavelength-dependent fluorescence spectra were obtained at the same temperature.

In addition, some data were corroborated by use of 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), another pH_i-sensing dye which has been more widely used. Cells were treated as above, and pH_i was monitored by ratioing 439- and 505-nm excitation maxima while measuring 535-nm emission. Standard curves were also obtained by the K^+/nigericin method. Leak of dyes was not different for different cells and was virtually nil during the time course of the measurements, which was <3 min/sample, and scaling to isosbestic points was used to internally control minor sample variation.

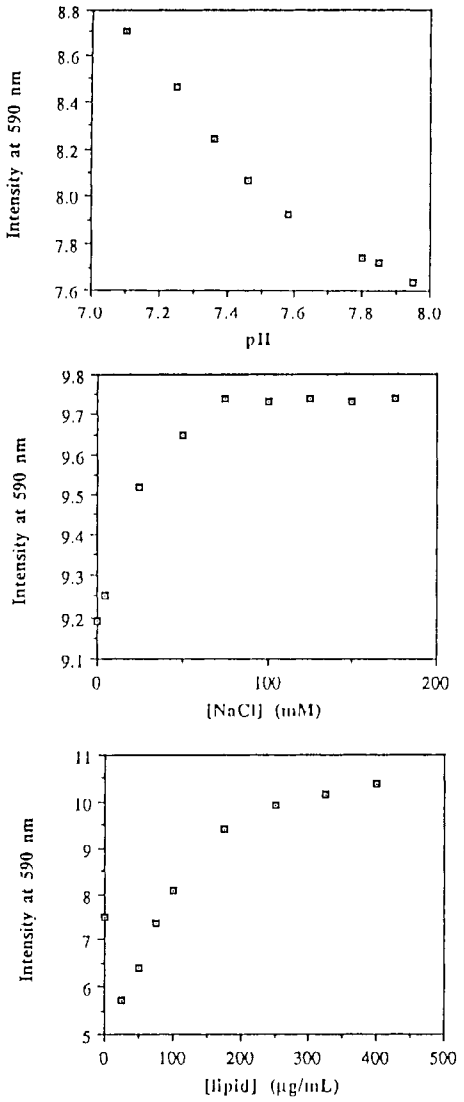

FIGURE 2: Effect of changes in solution pH, ionic strength, and concentration of lipid on the intensity of doxorubicin fluorescence at 590 nm. Approximately 1 μM doxorubicin dissolved in 5 mM Tris buffer was titrated within the fluorometer cabinet with HCl or NaOH (top) or NaCl (middle) while a microelectrode monitored pH. pH was constant at 7.0 for the middle panel. For the bottom panel, washed *E. coli* lipid was suspended at 25 mg/mL in 100 mM potassium phosphate/10 mM $MgCl_2$, pH 7.3, and briefly sonicated. It was then added to doxorubicin dissolved in 10 mM Tris, pH 7.3, to various final concentrations, and the resulting change in 590-nm intensity was noted. Results are plotted vs relative intensity.

Standard curves were generated for each cell preparation, each day measurements were made, and individual determinations were done in triplicate. Excitation of SNARF was at 535 nm, and excitation and emission slits were 6 and 10 nm, respectively.

RESULTS

Figure 1 (top) presents single-wavelength fluorescence intensity traces obtained at either 4 or 37 °C upon fast dilution of doxorubicin-sensitive (RPMI 8226) or 60-fold doxorubicin-resistant (Dox 40) cells preloaded to the same level of total internal doxorubicin (D_T). Rapid dilution at 4 °C results in an instantaneous increase in doxorubicin fluorescence at 590 nm (due to the sudden appearance of the preloaded cells), the intensity of which remains relatively constant. If the 4 °C suspension is centrifuged 20 min after dilution of cells, no doxorubicin is found in the supernatant, indicating that no efflux occurs under these conditions. The similar magnitude of the 4 °C traces reveals that the samples harbor nearly equivalent D_T, which can be further verified by extraction of the cells with 50% EtOH/0.3 N HCl and quantitation in the same solvent (see Materials and Methods). Agreement between these two methods indicates that no significant net intensity change in cell-associated doxorubicin fluorescence occurs for Dox 40 vs 8226 cells due to differences in ionic strength, pH_i, etc. (see also Figure 2).[2]

In contrast, upon dilution of the preloaded cells harboring equivalent D_T into drug-free medium at 37 °C (top two traces, top panel of Figure 1), a sudden increase in fluorescence is seen that is approximately the same magnitude as that observed upon dilution at 4 °C, followed by a more gradual increase in intensity. This increase, specific to cells diluted at temperatures >10–15°C, is due to the appearance of extracellular doxorubicin during efflux. Direct proof of this is obtained by performing the experiment presented in Figure 1 (bottom), wherein aliquots of Dox 40 cells loaded to identical D_T are fast-diluted and doxorubicin fluorescence is measured for both the cell pellet and supernatant separated at various times after dilution [cf. Figure 1 (bottom); see also Skovsgaard (1977)]. Data from this experiment may be plotted either as a loss of drug from the cells (bottom trace) or as a gain of drug in the supernatant (middle trace) vs time. If the two curves are added, the sum (top trace) yields a curve of the same magnitude and curvature as the trace produced by the CMF experiment (compare top curves in top and bottom panels of Figure 1). Thus, the entire fluorescence intensity change revealed in the CMF experiment is due to the sum of these two processes.[3]

[2] Doxorubicin in aqueous solution (pH 7.3) exhibits an excitation maximum at 467 nm and emission maxima at 557 and 590 nm. No appreciable shift in the 590-nm band is observed upon comparing spectra of doxorubicin in HBSS solution to spectra of loaded cells, thus, a change in fluorescence over time in these experiments is due to intensity alterations at 590 nm upon efflux of the compound. Ionic strength, pH, and interaction with lipid also effect doxorubicin fluorescence. But model studies (cf. Figure 2) show that virtually no change in 590-nm intensity accompanies the change in ionic strength associated with titrating solution NaCl between 50 and 175 mM. A 6.4% decrease in 590-nm intensity accompanies titration of solution pH from 7.25 to 7.58 and an approximately 30% increase accompanies titration from pH 7.25 to 4.0. Calculations which take into account pH_i differences of 0.4 pH unit, a 20% increase in the relative population of acidic vesicles as well as 20 mM changes in ionic strength, reveal that these possible changes would not lead to major net 590-nm fluorescence intensity differences in cell-associated doxorubicin for the different cell types.

[3] Although other minor fluorescence intensity changes likely occur upon partitioning between various intracellular compartments, by quantitating free drug from data analogous to that presented in Figure 1 (bottom), it is determined that >95% of the fluorescence change over time is due to efflux. Thus these other minor changes can essentially be neglected. Since the fluorescence quantum yield for extracellular drug is 6.5 times that for intracellular drug (cf. Figure 1, bottom), the time-dependent increase in total fluorescence is due to the appearance of extracellular drug. However, since fluorescence due to internal doxorubicin *decreases* during the course of the experiment, the *net* change in total fluorescence in these traces actually *underestimates* extracellular doxorubicin. In any case, it is important to realize that the rate of change in total fluorescence is equal to the rate of efflux. Thus, if appearance of extracellular drug [analogous to the middle curve, Figure 1 (bottom)] is expressed as $F_a = a_0e^{kt}$ and disappearance (bottom curve) as $F_d = c - d_0e^{kt}$, the total fluorescence change (top curve) is $F_t = F_a + F_d = c + (a_0 - d_0)e^{kt}$. Although terms that fit the trace representing the total fluorescence change are multiplied by different damping factors, rate constants describing the trace are equal to the rate constant(s) which describe efflux. Since the collected fluorescence data is in digital form, exponential and linear fits are simple operations. To calculate absolute levels of extracellular drug appearing over time, the appropriate constants need only be inserted into the expression for F_t or measured as in Figure 1 (bottom).

It is emphasized that although a myriad of possible cellular/doxorubicin interactions might be expected to affect the efficiency of doxorubicin fluorescence (partitioning between intracellular sites, membrane fluidity changes, etc.) the huge net change in the quantum yield of doxorubicin fluorescence that accompanies dilution of preloaded cells into buffer alone (cf. Figure 1) is due almost completely to efflux of the drug. This is verified by comparing the magnitude of the top trace in Figure 1 (top panel) to the top trace in Figure 1 (bottom panel) and confirming that the sum of the curves obtained for the cell pellet and supernatant data is equal in magnitude to the trace obtained in the CMF experiment. Therefore, although other smaller changes likely occur, the huge net change measured in these experiments is indeed due to efflux and likely occurs simply because both excitation and emission are more efficient for extracellular drug. Thus, the time-dependent increase in fluorescence seen at 37 °C is greater for the resistant Dox 40 cells (Figure 1, top panel, top trace) due to the fact that more drug is effluxed.

There is no significant difference in traces obtained for cells continuously kept at 37 °C by omitting the washing step noted in Materials and Methods (except for a baseline increase due to residual drug from the incubating medium); thus, changes in membrane fluidity upon dilution do not contribute significantly to the measured increase in fluorescence. Furthermore, measurements with model liposomes or untreated cells fast-diluted into doxorubicin solution support assignment of the fluorescence change to efflux (unpublished data). Thus, when liposomes made of pure *E. coli* lipid are fast-diluted, a small, very rapid (<1 s) increase in fluorescence is seen due to fast association of drug with lipid (see also Figure 2), followed by a slower ($t_{1/2}$ = 15–20 s) *decrease* in fluorescence intensity upon diffusion of doxorubicin *into* the intraliposomal space. When the experiment is repeated in the presence of 1% Triton X-100 such that liposomes are disrupted upon dilution, no decrease in intensity associated with internalization of drug is seen.

These data, along with previous results which suggest that nuclear association proceeds very rapidly (Danø, 1973; Skovsgaard, 1977) with very high affinity, permit calculation of initial rates of efflux from traces like those presented in Figure 1 (top). As suggested previously (Skovsgaard, 1978), other events are very unlikely to be rate-limiting with respect to efflux and do not overwhelm the increase in fluorescence due to efflux of cytoplasmic doxorubicin (compare top and bottom panels of Figure 1). In a previous independent study which used different methods to assay transport, Skovsgaard also concluded that the initial rapid cellular efflux of anthracyclines involves cytoplasmic or loosely associated drug, whereas a later slower component involves nuclear-bound drug (Skovsgaard, 1978).

Figure 3 (top panel) presents traces obtained for a period of 30 min after dilution at 37 °C that demonstrate doxorubicin efflux from both resistant Dox 120 and sensitive 8226 cells reaches steady state in approximately 12 min. Upon multiplication of the 8226 trace by a constant such that the two are superimposed (to reflect similar steady state, see inset to Figure 3, top panel), it can be appreciated that the kinetics of net efflux from both cells are very similar (i.e., the approach to steady state for either cell occurs with nearly identical rate). Scaling of traces is necessary before comparison of initial rates of efflux, since the levels of exchangeable drug (D_{ex}) differ between cell types loaded to equivalent total intracellular levels (D_T, cf. Figure 1, top panel). Since D_{ex} can be calculated for the cells (see Materials and Methods), comparison between

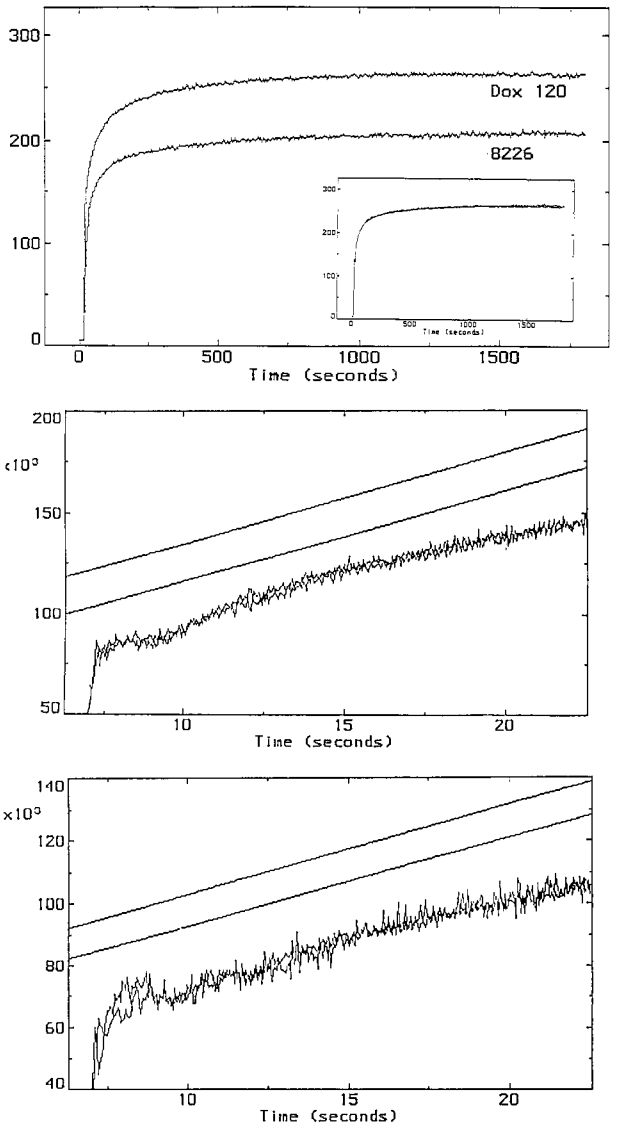

FIGURE 3: (Top panel) Efflux traces obtained over a 30-min period to illustrate the nearly identical approach to steady state for the sensitive and resistant cells loaded to similar D_T. (Inset) When the trace for the sensitive cells is multiplied by a constant to reflect similar steady state (i.e., similar D_{ex}), the traces are virtually superimposable. (Bottom panels) Expansion of the traces presented in the top panel in the region below 30 s after dilution. A duplicate set of data (20 data points/s) obtained with different cell preparations is also shown to illustrate reproducibility of this effect at different $[dox]_{in}$. Raw data are superimposed after scaling, and linear fits (to the region <10 s after dilution) of these data are above. Pairs of the linear fits are identical within 1.60% when the same time windows are used, and this agreement also holds for fits to data obtained <5 s, and even <3 s if artifacts are ignored. The slight overshoot within the first second for the sensitive cell spectrum in the bottom comparison is presumably due to cell clumping and was neglected when performing the linear fit.

rates can be made after correcting for these differences. Alternatively, comparison between rates can be made with unscaled traces if equivalent D_{ex} is known to reside within different cell preparations.

The bottom panels of Figure 3 expand the properly scaled 8226 and Dox 120 traces in the region below 30 s after dilution. By fitting the nearly linear early portion (<10 s after dilution) to a straight line and comparing the computed slopes, it is determined that the initial rate of efflux (IR^{10}) from the two cell types are virtually identical. Furthermore, when data are obtained with cell preparations harboring equivalent D_{ex}, IR^{10} values computed from unscaled traces are virtually indistin-

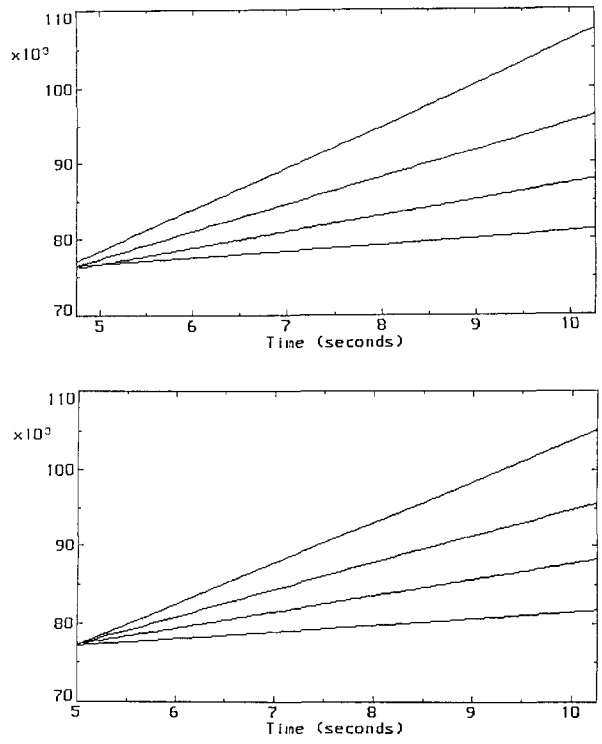

FIGURE 4: Linear fits to the region below 10 s for efflux curves obtained for resistant Dox 40 cells (top panel) and sensitive 8226 cells (bottom panel) preloaded to (from top) 125, 50, 2.5, and 0.5 μM cytoplasmic doxorubicin (see Materials and Methods). Calculated rates (in ΔFU/s) are 5310, 3520, 2090, and 860 for the resistant cell traces and 5270, 3580, 2130, and 850 for the sensitive cell traces (SE = <1.5%). Note the increase in initial rate of efflux upon loading to higher concentrations, as well as the excellent agreement between the rates for the sensitive and resistant cells at similar concentrations of exchangeable intracellular drug. Slit width settings differ between scans; thus quantitation curves at different settings were required for computing K_m from these data (see Materials and Methods). Traces were scaled relative to the 0.5 μM curve before fitting for ease of visual comparison.

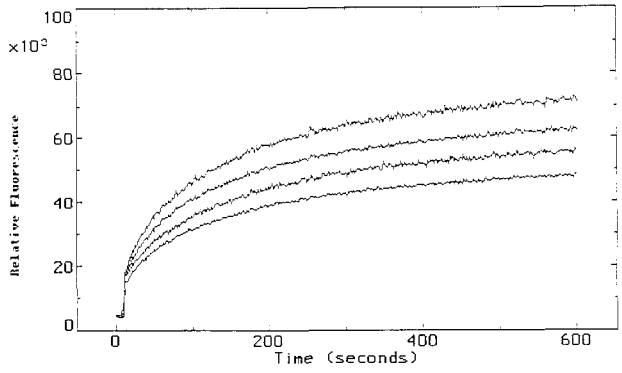

FIGURE 5: Traces obtained for Dox 40 and RPMI 8226 cells before and after glucose starvation to reduce intracellular ATP levels. From top: Dox 40 + glucose, Dox 40 – glucose, 8226 + glucose, 8226 – glucose. Cells were washed in HBSS ± 12 mM glucose prior to incubation with doxorubicin. After a 10-min incubation at 37 °C, doxorubicin was added and incubation was continued for an additional 30 min. The cells were then pelleted and washed in HBSS ± glucose at 4 °C. Traces were obtained in the same buffer, equilibrated to 37 °C. All traces represent cells loaded to the same D_T.

guishable (not shown). This can be further verified by comparing supernatant data obtained as in Figure 1 (bottom), although the initial time resolution in this case is limited to 2–3 s (not shown).

Figure 4 shows that when 8226 and Dox 120 cells are loaded with equivalent D_{ex} or when traces are properly scaled to reflect similar D_{ex}, the IR^{10} computed for a variety of $[dox]_{in}$ are virtually identical. Therefore the excellent agreement between rates seen in Figure 3 is not dependent on drug concentration. Furthermore, it is noteworthy that the IR^{10} for either cell is highly dependent on D_{ex}, indicating that careful standardization of loading conditions is necessary before comparison of efflux rates. In addition, although it could be argued that at relatively high D_{ex} intrinsic passive permeability might mask any kinetic effects due to MDR protein, at low concentrations of drug where the IR^{10} is considerably slower (0.5–2.5 μM, cf. Figure 4), no rate enhancement is detected either.

Eadie–Hofstee plots of these and additional data reveal similar K_m values for the sensitive and resistant cells (17.1 and 17.3 μM, respectively) when calculated D_{cyt} is used as the pertinent [S], but non-Michaelis–Menten behavior is illustrated when D_T or D_{ex} is taken as the pertinent value of [S] (see Discussion). Truly accurate kinetic parameters await more exact definition of D_{cyt} at t_0, but in any case, in terms of identifying rate enhancement for efflux due to the presence of MDR protein these estimates are less important than detailed empirical comparison between the IR^{10} values obtained for cells loaded to the same D_{ex}.[4]

When the Dox 40 cells are ATP-depleted by glucose starvation for 30 min during loading with doxorubicin (cf. Figure 5), the increase in the steady-state level of efflux is reduced by approximately 50%, in general agreement with other studies (Yang et al., 1990; Hammond et al., 1989). A slightly less dramatic change for the 8226 cells is observed upon ATP depletion (Figure 5). When traces for ATP-depleted cells are either scaled to reflect similar steady state or obtained for cells with equivalent D_{ex}, the computed IR^{10} does not change substantially (not shown). Therefore, although decreased intracellular ATP levels may affect the steady-state level of efflux, they do not necessarily alter the IR^{10} values from resistant cells harboring MDR protein.

Figure 6 presents traces obtained for the sensitive and 2–3-fold (Dox 1), 10-fold (Dox 6), 60-fold (Dox 40), and 120-fold (Dox 120) resistant cells, which harbor 1, 9, 50, and 55 relative molar [MDR protein], respectively, as determined by western blot analysis with the antibody C219 (Dalton, 1989; Li Yong Wei and P.D.R., unpublished work). It is seen that the degree

[4] Cells concentrate doxorubicin during incubation with the drug [see also Ganapathi et al. (1989)] such that at equilibrium D_T can be up to 50 times greater than the extracellular concentration. Since some of this drug is reversibly bound, freely exchangeable drug (D_{ex}) is greater than the initial cytoplasmic concentration (D_{cyt}) and is generally 40–60% of D_T, depending on the cell line and the incubating concentration. Experiments involving rapid lysis and measurement of "releasable" drug give estimates for D_{cyt}, but these are likely somewhat inaccurate due to the methods required for efficient lysis. In the present work, D_{cyt} for the sensitive cells was assumed to be the concentration of drug in the incubating medium after equilibrium was reached (calculated from the uptake curve). D_{cyt} for resistant cells was calculated on the basis of the percent differences observed for D_T or D_{ex} when similar loading incubations were compared for the sensitive and resistant cells (see Materials and Methods). Therefore, although accurate comparison of IR^{10} can be made empirically after assuring that equivalent D_{ex} resides within cell preparations, calculation of kinetic parameters dependent on D_{cyt} are only internally accurate approximations. If one assumes that D_{cyt} is higher than calculated for the resistant cells, efflux would be less efficient (higher K_m). A lower K_m for the resistant cells would require significantly lower D_{cyt} than is calculated and, therefore, significantly more drug bound at equilibrium. Since higher levels of exchangeable and releasable drug (at fixed D_T) and lower levels of retained drug are actually observed, this possibility appears unlikely. Furthermore, to explain the presented data, that component of D_{ex} which is not D_{cyt} would need to be effluxed by a non-MDR protein-mediated mechanism that coincidentally has the same kinetics as the MDR protein-mediated mechanism. Thus, the simplest explanation is that the K_m values for efflux of D_{cyt} from resistant and sensitive cells are the same.

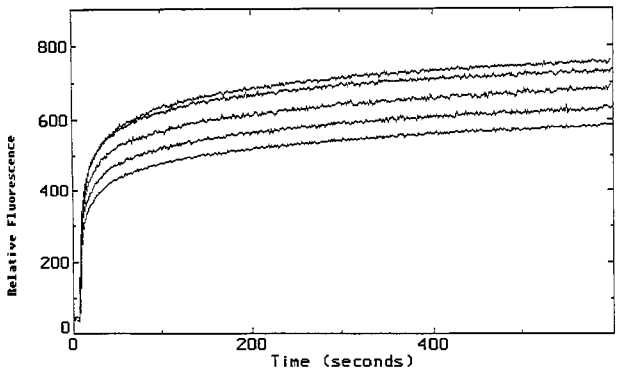

FIGURE 6: Traces over a 10-min region for (from bottom) RPMI 8226, Dox 1, Dox 6, Dox 40, and Dox 120 cells. Note the increase in the steady-state level of efflux concomitant with increased resistance and increased MDR protein expression in the cell lines. All traces represent cells loaded to the same D_T; SE < 5%.

Table I: Relative Steady State and IR^{10} for Doxorubicin Efflux[a]

cell line	doxorubicin alone	+ verapamil (μM) 1	10	100	+ vincristine (μM) 1	10	100
		Apparent Initial Rate					
RPMI/8226	2620	2740	2580	2720	2370	2200	2150
Dox 40	2625	2730	2590	2680	2400	2280	2180
		Relative Steady State					
RPMI/8226	100	95.4	90.8	89.7	100	100	98
Dox 40	130	114	109	106	130	130	127

[a] As determined by fitting the linear portion of the efflux curve below 10 s. Rate data are expressed as ΔFU/s. Note that although rates are slower in the presence of vincristine, similar steady state is reached (in slightly longer time), whereas rates are slightly faster in the presence of verapamil even though a lower steady state is maintained. SF is <1.5% for rates, <4% for steady states.

of resistance for these cells is qualitatively related to the steady-state level of doxorubicin efflux. Thus, each 5–6-fold increment in resistance is accompanied by an increase of nearly 10% in the extent of efflux (cf. Figure 6). However, remarkably, when the initial rates of efflux for the different cells are compared as described above, they are found to be virtually identical (not shown).

Thus, in contrast to the dramatic increase in the steady-state level efflux as MDR protein content is increased in these MDR cells, computed IR^{10} are virtually superimposable. These data argue that the role of increased MDR protein expression in the increasingly resistant cells is to alter the steady-state distribution of drug without heightening the rate at which doxorubicin efflux occurs.

Table I summarizes the effects of verapamil and excess internal vincristine on efflux from the sensitive and 60-fold resistant cells. In agreement with other studies, verapamil lowers the steady state of efflux for the resistant cells more dramatically than for the sensitive cells. Unlike the effects produced by ATP depletion, increasing concentrations of verapamil also appear to alter the rate of efflux from the resistant cells with a slight *increase* in IR^{10} for either the sensitive or resistant cells upon treatment with 1 μM levels of verapamil (cf. Table I).

Conversely, vincristine does not affect the steady-state level of efflux, but a steady decrease in IR^{10} dependent on the internal concentration of vincristine is seen for both resistant and sensitive cells. These data suggest that vincristine and verapamil inhibit efflux by different mechanisms. Also, since no major difference can be seen in comparison between

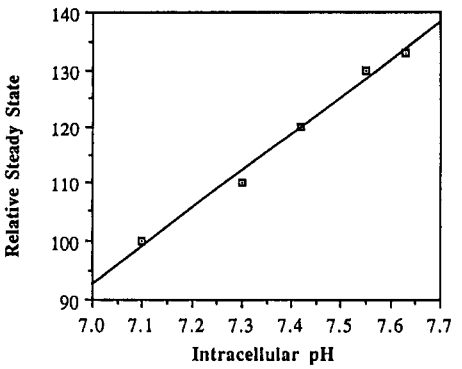

FIGURE 7: Plot of internal pH vs relative steady state of efflux for the series of doxorubicin-resistant cells as determined by internal SNARF fluorescence. Note the linearity of the relationship (SE = <0.8% for pH_i and ± ≤5% for efflux). Note that pH_i was determined for washed cells incubated with SNARF in the absence of doxorubicin. The pH_i values shown represent the mean of multiple determinations made for cells grown either in the presence or in the absence of drug. Points are shown for (from left to right) 8226, Dox 1, Dox 6, Dox 40, and Dox 120 cells.

vincristine-treated sensitive and resistant cells, it is unlikely that vincristine acts directly at any doxorubicin sites specific to resistant cells.

Finally, several recent reports have suggested that pH_i may be altered in MDR cells (Boscoboinik et al., 1989; Keizer & Joenje, 1989; Thiebaut et al., 1990); thus the relationship between pH_i and observed steady-state alterations in efflux for the series of resistant cells was examined. Figure 7 shows that a linear relationship between pH_i and the relative steady state of efflux exists for the series. Thus, each 10% increase in relative steady state correlates with an approximately 0.13–0.18 pH unit rise in intracellular pH. These results are dramatic since no other plot of the various measureable parameters for this series of cell lines (e.g., [MDR protein] vs degree of resistance, [MDR protein] vs relative steady state, or pH_i vs resistance) yields as truly straight a line and thus as quantitative a relationship.

DISCUSSION

Continuous monitoring of fluorescence is a valuable approach for the study of transport phenomena (Eidelman & Cabantchik, 1989), and this study extends the technique to the specific example of doxorubicin efflux from drug-sensitive and MDR cells. At least 95% of the differential fluorescence signal obtained after dilution of preloaded cells is due to drug efflux (Figure 1), which permits estimation of the IR^{10} by fitting digital data collected at 50-ms intervals. Due to increased time resolution and detailed attention to loading conditions, conclusions pertaining to relative rates of efflux for a series of MDR cells and the sensitive parental line can be made and contrasted to conclusions regarding steady-state alterations. These may be summarized as follows: (1) The steady-state level of doxorubicin efflux is related to the level of MDR protein expression in this series of MDR cell lines, but the IR^{10} appears to remain constant. (2) ATP depletion affects the steady-state level of drug efflux but not the rate, whereas treatment with verapamil affects both but in a complex manner. (3) Vincristine, another putative substrate for transport by the MDR protein, does not alter the steady state of doxorubicin efflux but slows the rate of efflux from both the resistant and sensitive cells by a similar amount. Notably, similar unexpected findings have recently been reported by Gros and colleagues (Hammond et al., 1989), who found that vincristine or daunomycin did not compete significantly with

vinblastine for efflux from MDR cells [see also Beck et al. (1983)]. These data suggest that inhibition of anthracycline efflux by vincristine is via some pathway common to both sensitive and resistant cells; thus this pathway does not involve MDR protein. (4) pH_i and the relative steady state of efflux are linearly related for this series of cell lines, whereas other observed relationships are not.

MDR protein has been hypothesized to act as an ATP-coupled active molecular transporter that specifically binds and actively pumps or "flips" (cf. Higgins & Gottesman, 1992) structurally diverse chemotherapeutic drugs (i.e., anthracyclines such as doxorubicin and vinca alkaloids such as vincristine), since increased expression of MDR protein has generally been observed to correlate with what is termed "increased drug efflux". Furthermore, photolabeling data reveal that some drugs bind to MDR protein (Cornwell et al., 1986), although this is indirect evidence for active transport, since many transporters bind compounds specifically but do not transport them (amiloride and Na^+/H^+ exchangers, stilbenes and Cl^-/HCO_3 exchangers, etc.).

Many examples of efflux measurements have appeared in the literature, and all invariably demonstrate that an increased steady-state level of drug efflux is a general characteristic of MDR cells expressing MDR protein. However, few of these studies have been explicitly concerned with kinetic effects. Active transporters perform transport against a substrate concentration gradient at a rate that is faster than passive transport, and thus "increased efflux" by an active efflux pump implies increased pumping efficiency in the outward direction. Thus, for example, increased expression of an active efflux pump should lead to a net increase in the cellular rate of efflux [see Costello et al. (1987)].

Showing that transport occurs against a concentration gradient in the case of MDR protein-mediated transport remains elusive due to the hydrophobicity of the putative substrates, pH_i alterations that accompany resistance (see below), and the fact that these drugs are highly concentrated in cells during loading. Further studies with reconstituted proteoliposomes may prove more informative in this regard. Alternatively, observation of rate enhancement for drug efflux attributable to increased expression of the putative transporter would demonstrate that the protein is indeed an active efflux pump. Series of cells expressing variable amounts of MDR protein offer an ideal model system in this regard, but available data disagree with respect to the significance of the minor rate alterations that have been previously observed. This is perhaps because cells with widely different degrees of resistance have been investigated and usually only one cell line is inspected and compared to the parental sensitive line. One previous careful study has noted a slight rate enhancement for vincristine efflux from a resistant cell line relative to its sensitive parent (Sirotnak et al., 1986); however, it should be noted that the cells used in that work were significantly more resistant than those used here (>3000-fold), yet they harbor roughly the same amount of MDR protein. Thus it is difficult to attribute phenotypic differences solely to the expression of MDR protein.

Other data have also been cited as support for an active drug transporter hypothesis; thus, a recent study (Lankelma et al., 1990) measured transport against a putative concentration gradient but neglected to quantitate internal levels (total, exchangeable, and cytoplasmic) of substrate. An earlier study (Horio et al., 1988) measured osmotically sensitive, ATP-dependent vinca alkaloid influx with an inside-out vesicle preparation but did not rule out ATP effects which might have indirectly promoted internalization of drug (such as acidification of the intravesicular space relative to the incubating medium, see below).

In the present work, a series of sequentially derived lines expressing increasing amounts of MDR protein concomitant with increased resistance have been examined closely to pinpoint efflux alterations due to increased MDR protein expression, particularly at reasonably low levels of resistance where a strong relationship between the degree of MDR and the level of MDR protein can be demonstrated. At high levels of resistance (thousandfold), the relationship between resistance and MDR protein expression begins to disintegrate and further significant increases in the steady-state level of efflux are not found (Li Yong Wei and P.D.R., unpublished work). Thus, it is difficult to conclude what role MDR expression plays in altered transport in these cells, whereas tabulation of trends upon systematic examination of a series of cell lines may prove more informative.

In this study, increased MDR expression is found to increase steady-state levels of efflux without apparently altering the rate of that efflux. Furthermore, the relative steady state of efflux is correlated most dramatically with pH_i, supporting the contention that efflux alterations are perhaps a consequence of altered charge-dependent sequestration and/or pH_i-dependent binding, as might be suspected since doxorubicin is a weak base with a $pK_a \sim 7.8$ that likely binds to nucleic acid in vivo in a highly pH-dependent manner. At higher pH_i, less internal drug is charged and is thus more freely diffusable and less likely to react with its target. If a putative transporter alters the steady state of transport without altering rate, active transport has not been unequivocally demonstrated. It is also possible that the protein changes the level of freely exchangeable substrate and/or the electrochemical membrane potential directly or indirectly. This is particularly germane for the transport of weak acids or bases.

Elevated pH_i likely contributes to the MDR phenotype in several ways, namely, by titrating the population of pharmacologically relevant drug and/or drug target(s) as well as by lowering ionic sequestration. For example, vinca alkaloids and colchicine are known to bind in a pH-dependent manner to monomeric tubulin, and assembly of polymerized tubulin is known to be pH dependent. Thus, elevations in pH_i may increase the steady state of efflux (i.e., lower sequestration) by lowering the efficiency of binding to target as well as by direct reduction in ionic trapping mechanisms. Therefore, the effects of ionophores such as nigericin are likely to be complex and not easily predictable until more is known about the pH_i dependencies of various binding phenomena (P.D.R., unpublished work).

Relatedly, several other reports have also recently noted pH_i alterations in drug-resistant cells (Boscoboinik et al., 1989; Keizer & Joenje, 1989; Thiebaut et al., 1990), but none of these examined the relationship between pH_i and altered transport characteristics; thus, different interpretations were offered. Furthermore, it has been argued that since some MDR reversal agents do not restore pH_i to "normal" levels, pH_i cannot be responsible for the MDR phenotype (Boscoboinik et al., 1989). However, there are significant differences between the cell lines used in these studies, and the effects of reversal agents on pH_i are complex (Keizer & Joenje, 1989). It is important to realize that, as mentioned earlier, at high levels of resistance, relationships between MDR protein concentration, altered steady state of efflux, and degree of resistance break down. Thus very highly resistant cells (several hundredfold to thousandfold) efflux roughly the same amount

of drug as hundredfold resistant cells and may harbor slightly more MDR protein, but not nearly as much as would be expected on the basis of what is apparently required to confer hundredfold levels of resistance. Altered pH_i is one possible mechanism for lowering the level of retained drug. Very highly resistant cells most certainly develop other mechanisms of resistance that supplement those utilized earlier on, and these may further alter pH_i and/or compromise mechanisms for altered pH regulation that are initially employed by resistant cells (i.e., increased vesicular traffic or overexpression of vacuolar ATPases; Sehested et al., 1987; Ma & Center, 1992). Thus, as in the case of drug transport, examining pH_i for only a single resistant cell line, particularly a highly resistant line, may be less informative than examining a series of cell lines where MDR protein expression is roughly correlated with resistance [see also Keizer and Joenje (1989)] and transport alterations [see also Dalton et al. (1989)]. As in a previous report (Keizer & Joenje, 1989), we find that verapamil significantly affects pH_i in resistant cells, as do glucose starvation and other treatments known to affect the steady-state level of drug efflux (Li Yong Wei and P.D.R., unpublished work).

Thus, an interpretation that is more consistent with all currently available data is that MDR protein modulates intracellular pH either directly or indirectly such that the "MDR family" of drugs (including vinca alkaloids and anthracyclines which have pK_as of 6.8–7.8) are less likely to be retained by ionic effects and/or sequestered in a pH-dependent manner [see also Siegfried et al. (1985)]. That is, MDR protein likely performs "indirect" mediated transport in the sense that it discourages intracellular sequestration by raising pH_i. Possible mechanisms for raising pH_i would include H^+ or Cl^- transport, and many other indirect means are possible. In this regard, recent studies of Valverde et al. (1992), which have associated a Cl^- conductance with the expression of MDR protein, are provocative. Further studies of pH_i homeostasis mechanisms in MDR cells may bear on this point (P.D.R., submitted for publication). It is therefore appropriate to suggest that MDR protein indirectly promotes the efflux of chemotherapeutics without actually performing active drug transport.[5]

Finally, since ΔpH is only one component of membrane potential ($\Delta\mu_H^+ = \Delta pH + \Delta\Psi$), at constant $\Delta\mu_H^+$ and pH_0, elevated pH_i is expected to lead to changes in the other ion gradients represented by $\Delta\Psi$. Relatedly, changes in $\Delta\Psi$ at constant $\Delta\mu_H^+$ may lead to changes in ΔpH. Thus, membrane potential also needs to be examined in MDR cells, and whether pH_i alterations result in $\Delta\Psi$ alterations in MDR cells (or vice versa) remains to be determined. In addition, the relative change in pH_i exhibited by a cell expressing a given amount of MDR protein will depend upon what mechanisms for pH_i homeostasis prevail in the particular cell type. It will also be influenced by additional mechanisms employed by the cell to regulate $\Delta\mu_H^+$, as well as environmental factors such as pH_0. Thus, it is suggested that to fully understand the MDR phenotype a more complete understanding of pH_i homeostasis for a variety of cells is required.

[5] This hypothesis makes the approximate Michaelis–Menten behavior observed for efflux from sensitive and resistant cells when D_{cyt} is used as the pertinent [S] somewhat curious, although the non–Michaelis–Menten behavior observed when D_T or D_{ex} is used in the analysis is easily explained as the result of nonenzymatic processes. Typically, saturation in the rate of transport at some [S] is cited as evidence for a carrier-mediated mechanism. However, this concept has evolved from the study of transport of small, hydrophilic molecules or ions and may not be appropriate for the transport of large, hydrophobic molecules where rather involved membrane transitions may be required for translocation [see Noy et al. (1986)].

ACKNOWLEDGMENT

The author thanks Dr. William S. Dalton, Arizona Cancer Center, University of Arizona, for cell lines used in this work, Diane Carlson for expert technical assistance, and Dr. Francis M. Sirotnak, Laboratory for Molecular Therapeutics, Memorial Sloan Kettering Cancer Center, Dr. John P. Reeves, Roche Institute of Molecular Biology, and Dr. Jonathon Lee, Smith, Kline & Beecham Pharmaceuticals, for helpful discussions. He also thanks the reviewers of the manuscript for their insightful comments during review.

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