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Intracellular pH and the control of multidrug resistance

(cancer chemotherapy/daunomycin/doxorubicin)

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ABSTRACT **Many anticancer drugs are classified as either weak bases or molecules whose binding to cellular structures is pH dependent. Accumulation of these drugs within tumor cells should be affected by transmembrane pH gradients. Indeed, development of multidrug resistance (MDR) in tumor cells has been correlated with an alkaline shift of cytosolic pH. To examine the role of pH in drug partitioning, the distribution of two drugs, doxorubicin and daunomycin, was monitored in fibroblasts and myeloma cells. In both cell types the drugs rapidly accumulated within the cells. The highest concentrations were measured in the most acidic compartments—e.g., lysosomes. Modifying the cellular pH in drug-sensitive cells to mimic reported shifts in MDR caused an immediate change in the cellular drug concentration. Drug accumulation was enhanced by acidic shifts and reversed by alkaline shifts. All of these effects were rapid and reversible. These results demonstrate that the alkaline shift observed in MDR is sufficient to prevent the accumulation of chemotherapeutic drugs independent of active drug efflux.**

Chemotherapy takes advantage of the phenomenon that tumor cells are ≈5-fold more sensitive to anticancer drugs than are healthy cells. This narrow therapeutic window permits the use of cytotoxic agents to destroy malignancies. However, during chemotherapy, tumor cells often lose this sensitivity and become no more vulnerable than normal cells. This diminished sensitivity to the original drug also extends to a broad class of other drugs, diverse in their structures and targets. This acquired multidrug resistance (MDR) is a major challenge to successful chemotherapy of malignant tumors.

Different drug-resistant cells overexpress a variety of membrane proteins, including a subunit of a vacuolar H^+-ATPase (1), multiple-resistance protein, which has homology to the cystic fibrosis transmembrane conductance regulator (CFTR) (2), and P-glycoprotein, a 170- to 180-kD plasma membrane glycoprotein (3). The most generally accepted hypothesis for MDR suggests that P-glycoprotein uses ATP to power a molecular pump that removes chemotherapeutic molecules from the cell (ref. 4; reviewed in ref. 3). This model proposes that chemotherapeutic agents diffuse down a concentration gradient into the cell and that the pump either transports the drugs out of the cytosol or serves as a "flippase" to expel them from the bilayer (5).

The data presented in this communication support an additional mechanism of MDR by which changes in intracellular pH alter the transmembrane partitioning or intracellular sequestration of drugs. Cytosolic pH affects protonation of these drugs (typically weak bases, with pK values between 7.4 and 8.2; refs. 6–8), affinity of intracellular sites for drug binding, and/or secretion from organelles which accumulate the drugs. The pH of tumor cells is considerably more acidic than that of normal (9) or multidrug-resistant (10) cells. Drugs which partition across the membrane would be protonated and ionically trapped in the cytosol in their biologically active form [the charged form of these drugs binds to their targets such as DNA (11–15), RNA (15, 16), and tubulin (17, 18)]. The acidic pH of tumor cells would increase their sensitivity to the drugs. P-glycoprotein, as well as other proteins that are correlated with MDR, could affect the activity of chemotherapeutic agents by modification of pH homeostasis.

Gradients of pH have been used to trap these drugs in liposomes and erythrocytes. To test whether the pH gradients observed in tumors and multidrug-resistant cells significantly change intracellular drug concentration, we examined the kinetics of accumulation in drug-sensitive cells where the pH was manipulated experimentally to mimic that observed in resistant cells.

MATERIALS AND METHODS

Cells. NIH 3T3 cells (mouse fibroblasts) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) with 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA). NIH 3T3 cell lines that were transfected with mdr-1 cDNA were supplemented with 100 nM vincristine sulfate. Myeloma cells (8226, the parental drug-sensitive line, and DOX-40, the drug-resistant line) were grown in RPMI 1640 (GIBCO) with 10% fetal bovine serum. The drug-resistant line was supplemented with 100 nM doxorubicin hydrochloride (Calbiochem). All media were supplemented with penicillin, streptomycin, antimytopic, and 2 mM L-glutamine (all from GIBCO) and, unless indicated otherwise, maintained in a 5% CO_2 atmosphere.

Fluorescence and Confocal Microscopy. Fibroblasts (NIH 3T3 cells) were grown on coverslips (VWR Scientific; 25 mm; thickness, 0.15 mm) which were placed in a Leiden coverslip chamber (Medical Systems, Greenvale, NY). Myeloma cells were adhered to the same coverslips with Cell-Tak (Collaborative Biomedical Products, Bedford, MA) according to the manufacturer's instructions. The chamber and solutions were kept at 37°C. Solutions equilibrated with ambient (0.033%), 2%, 5%, or 10% CO_2 perfused at a constant velocity. Warmed air (at appropriate CO_2 concentration) was perfused across the surface. For fluorescence microscopy, the coverslip chamber with the cells was mounted on a Nikon Diaphot inverted microscope and illuminated with a 100-W mercury lamp (Nikon) with a 97% neutral density filter. To quantify cell-associated fluorescence, the chamber was mounted on a Zeiss Axiovert 135 inverted microscope with a 100-W mercury light source and a 97% neutral density filter and a Hamamatsu cooled charge-coupled device camera (no. C4880). For confocal microscopy, the chamber was mounted on an inverted InSight confocal microscope (Meridian Instruments, Okemos, MI) which used an argon laser for excitation at 488 nm.

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Abbreviation: MDR, multidrug resistance.

Chemicals. Daunomycin and doxorubicin (Calbiochem) were made as 10 mM stock solutions in water and stored at 4°C. SNARF-1 AM (Molecular Probes) was made as a 20 mM stock in anhydrous dimethyl sulfoxide (Aldrich) and stored at −20°C.

pH Measurement. The pH of cells was measured using SNARF-1 AM according to the manufacturer's instructions. Fibroblasts grown on coverslip dishes in DMEM with 10% fetal bovine serum were rinsed in DMEM without serum and then incubated in DMEM with 10 μM SNARF-1 AM for 15 min. The cells were then placed on an InSight confocal microscope and excited at 488 nm with emission recorded at 570/30 nm and 630/1p nm. A pH calibration curve was constructed by rinsing the cells with 150 mM KCl with 6 μM nigericin and 50 mM sodium phosphate buffered to pH 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, or 8.0. Myeloma cells were harvested and then resuspended in medium without serum. SNARF-1 AM was added to a final concentration of 10 μg/ml for 15 min at 37°C. The cells were then placed in a dialysis bag (Spectrapor, Fisher Scientific; M_r cutoff, 12,000–14,000; diameter, 1.6 cm) suspended in a 200-ml beaker with RPMI 1640. The RPMI 1640 in the beaker was maintained at 37°C and kept aerated with an aquarium airstone with 0.03%, 2%, 5%, or 10% CO_2 in air. The stirred bathing medium could be changed to vary the concentrations of CO_2 or drugs in the dialysis bag. For measurement in a spectrofluorometer an emission scan was taken from 520 to 700 nm with excitation at 488 nm and 514 nm. The cells were calibrated, as described above for the fibroblasts, for both excitations and the results at each were compared. For measurement on a fluorescence-activated cell sorter (Becton Dickinson FACStar^{PLUS}) the cells were pumped at 0.38 ml/min with an Ismatec peristaltic pump (Cole–Palmer) and excited with an argon laser at 514 nm, and emission was monitored with filters at 570/26 nm and 630/30 nm. For measuring daunomycin concentration, the cells were excited at 488 nm and emission monitored at 570 nm.

RESULTS

Daunomycin Accumulates in Cells. Daunomycin, a chemotherapeutic agent, fluoresces maximally at 595 nm when excited at 488 nm. These optical properties enable monitoring the drug in living cells. NIH 3T3 fibroblasts were incubated in the presence of 5 μM daunomycin for 30 min and examined on an inverted fluorescence microscope. Since the fluorescence spectrum of daunomycin is not affected by pH (data not shown), the fluorescent images of increasing cytosolic daunomycin fluorescence reflect accumulation of the drug. The concentration of daunomycin in the cytosol (Fig. 1) was higher than in the surrounding medium, with the highest concentration in the nucleoli and two of the major acidic compartments of the cell (trans-Golgi and lysosomal), as previously reported (19). Similar patterns of intracellular accumulation were observed for cells incubated with doxorubicin and with several strains of NIH 3T3 fibroblasts and with myeloma cells growing in suspension. Daunomycin binds DNA with great affinity and RNA to a lesser extent (15, 16). Binding to tightly packed DNA in the chromatin results in quenching of the daunomycin fluorescence, whereas binding to nucleoli yields fluorescent structures.

The pH Is Different in Drug-Sensitive and Drug-Resistant Cells. The NIH 3T3 fibroblasts and myeloma cells were loaded with SNARF-1 AM, a dye whose fluorescence emission is pH sensitive. When excited at 514 nm, its emission maximum is at 630 nm in a basic environment and at 570 nm when acidic. Ratioing of fluorescence emission is used as a quantitative measure of the pH, independent of cell volume or dye concentration. The pH of the myeloma cells, as measured in a fluorescence-activated cell sorter or spectrofluorometer, was 7.1 for the drug-sensitive cells (8226) and 7.45 for the drug-resistant cells (DOX-40). The pH of the drug-sensitive NIH 3T3 cells (mock transformed with a neomycin marker) was 6.8 while that of those transfected with mdr-1 cDNA was 7.25 as measured with a fluorescence confocal microscope.

Changing the CO_2 Concentration Rapidly and Reversibly Shifts Cytosolic pH. To mimic the alkaline cellular pH shift that occurs in MDR, the CO_2 concentration was lowered. CO_2 quickly equilibrates across cellular membranes. The rapid activity of cytosolic carbonic anhydrase and the numerous cellular mechanisms to regulate bicarbonate exchange ensures that changes in CO_2 concentration rapidly affect cellular pH (20). NIH 3T3 fibroblasts were loaded with SNARF-1 AM and mounted on an inverted microscope. Changing the partial pressure of CO_2 from 5% to 2% resulted in a rapid alkaline shift of intracellular pH in the fibroblasts from 6.8 to 7.2. This pH shift was reversible, changing the

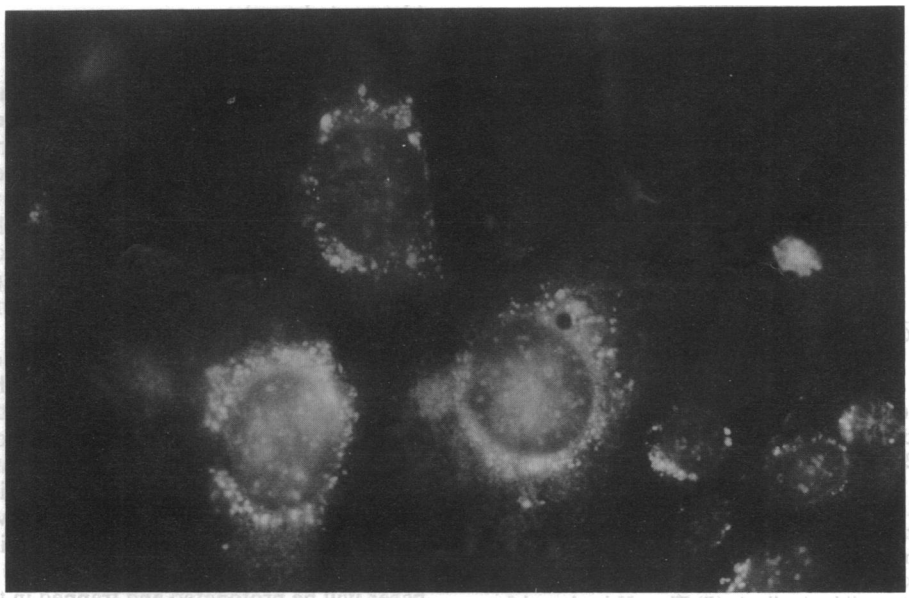

FIG. 1. Fluorescence photomicrograph of daunomycin fluorescence in NIH 3T3 fibroblasts. Daunomycin (5 μM) was added to the medium 45 min prior to viewing with fluorescence microscopy. (×500.)

CO_2 back to 5% returned the pH to 6.8. The basal pH value of 6.8 is somewhat more acidic than previously reported values for the NIH 3T3 cells. However, in those experiments, pH was measured with the cells at an ambient concentration CO_2 of 0.033%. The pH rises as the CO_2 changes from 5% to 0.033% (21). Our results are consistent with published measurements in 5% CO_2 (22).

The pH of myeloma cells, grown in suspension, was examined in both a FACS and a spectrofluorometer. The emission spectrum of SNARF-1 in drug-sensitive cells (Fig. 2*A*, black line) and MDR cells (gray line) is shown at 5% CO_2 (solid line) or 2% CO_2 (dotted line). The pH of sensitive cells incubated with a 2% CO_2 (measured as the ratio of the emission at 630 nm to that at 585 nm, Fig. 2*A*, dotted black line) was indistinguishable from the pH of the resistant cells at a CO_2 of 5% (solid gray line). This demonstrates that varying CO_2 concentration can be used to shift the pH of the drug-sensitive cells to a value as alkaline as that of the resistant cells. Likewise, the pH of the drug-sensitive cells in 5% CO_2 (Fig. 2*B*) was comparable to the pH in resistant cells at 10% CO_2 (Fig. 2*B*). Alternatively, the resistant cells could have their intracellular pH shifted to that of the more acidic, sensitive cells.

The pH of the drug-sensitive myeloma cells was 7.1 and that of the drug-resistant cells in 5% CO_2 was 7.45. The CO_2 concentration was modified in the following manner: 5%, 2%, 0.03%, 2%, 5%, 10%, 5%. The pH values were measured for each level of CO_2. At lower CO_2, the intracellular pH was more alkaline and at higher CO_2 it was more acidic. At 2% CO_2, the pH was 7.45 for the sensitive cells and 7.75 for the resistant cells. This was accompanied by a shift of only 0.04 pH unit in the extracellular pH. Cells at 0.03% CO_2 demonstrated pH values of 7.85 (sensitive) and 7.9 (resistant). Returning the cells to 5% CO_2 caused the pH to rapidly revert to the starting level. Further, increasing the CO_2 concentration to 10% caused an increased acidification to 6.65 (sensitive) and 7.05 (resistant). This sequence of cycling CO_2 was repeated each time, yielding the same intracellular pH values shown in Fig. 2*B*.

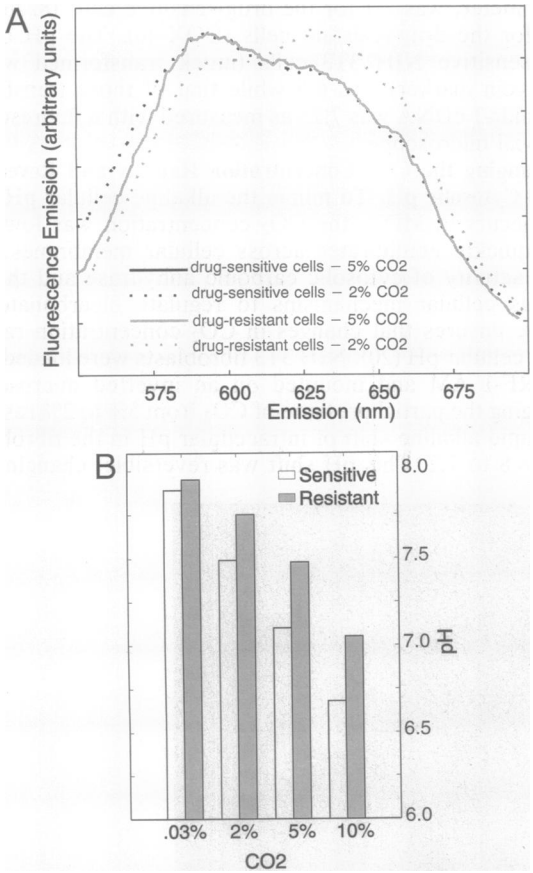

FIG. 2. Effect of CO_2 concentration on cytosolic pH. (*A*) Myeloma cells were loaded with SNARF-1 and the CO_2 in the medium was shifted between 2% (dotted lines) and 5% (solid line). The fluorescence emission was recorded between 520 nm and 700 nm with an excitation of 514 nm for both the drug-sensitive cells (black line) and the drug-resistant cells (gray line). The pH (as indicated by the ratio of the emission at 630 nm to 585 nm) is indistinguishable between the sensitive cells in 2% CO_2 (dotted black line) and the resistant cells in 5% CO_2 (solid gray line). (*B*) The pH is plotted for the drug-sensitive cells (open bars) and drug-resistant cells (filled bars) at 0.03%, 2%, 5% and 10% CO_2.

Changing CO_2 Concentration Rapidly and Reversibly Shifts Intracellular Daunomycin Fluorescence. NIH 3T3 cells at 5% CO_2 were incubated with 5 μM daunomycin until the intracellular levels were approximately at a steady state (Fig. 3*A*, red background). The partial pressure of CO_2 perfusing the solution was shifted from 5% to 2% (Fig. 3*A*, blue background). The daunomycin fluorescence rapidly decreased in the cells. Upon returning the cells to 5% CO_2 (red background), the daunomycin fluorescence increased to its starting level. The pattern remained unchanged upon repeated cycling between 2% and 5% CO_2. The intracellular daunomycin fluorescence was quantified for a number of cells (Fig. 3*B*). In all cases, the cellular fluorescence decreased when the CO_2 level was lowered (more alkaline pH) and the fluorescence increased when CO_2 was increased. These changes were rapid, repeatable, and reversible.

The experiment was repeated with both drug-sensitive (8226) and drug-resistant (DOX-40) myeloma cells with similar results. Cells were loaded with 20 μM daunomycin in medium equilibrated with 5% CO_2. While the daunomycin fluorescence was monitored, CO_2 was sequentially shifted to 2%, 0.03%, 2%, 5%, 10%, and 5% (Fig. 4). This cycle was repeated. The cellular daunomycin fluorescence decreased when the CO_2 level was lowered and increased when the CO_2 level was raised. These changes were completely reversible and occurred at the same rate in all intracellular compartments.

Similar reversible increases of cellular drug levels were also observed when the pH was transiently shifted to alkaline with 20 mM NH_4Cl for 2 min (data not shown). Reversible increases of cytosolic drug levels were observed when the pH was transiently shifted to acidic with 2.5 mM NaN_3 for 2 min (data not shown).

DISCUSSION

Fluorescent chemotherapeutic agents accumulate in tumor cells (see Fig. 1). This could be a consequence of decreased drug influx, increased intracellular trapping, and/or increased drug efflux. There are two general mechanisms for drug transport: active and passive. An active-transport model for MDR has been proposed based on the observations that transport is blocked by metabolic inhibitors such as azide and that transport is associated with the expression of P-glycoprotein, an ATP-binding protein which is a member of a family of membrane transporters.

The passive-diffusion models are based on the observation that these drugs are sufficiently hydrophobic to cross membranes. The asymmetric distribution of the drugs is assumed to be the consequence of an asymmetry of chemical potential (such as ΔpH, voltage, and ionic concentrations). For example, the higher rate of aerobic glycolysis in tumors and the hypoxic conditions surrounding cells within a tumor mass cause an acidic environment (9). This increased proton concentration has two effects. First, the drugs that are weak bases will be protonated and trapped in the cytosol. Second, the binding of each of these drugs to their cytosolic targets, such as tubulin (17, 18) or DNA (11–15), has an acidic pH

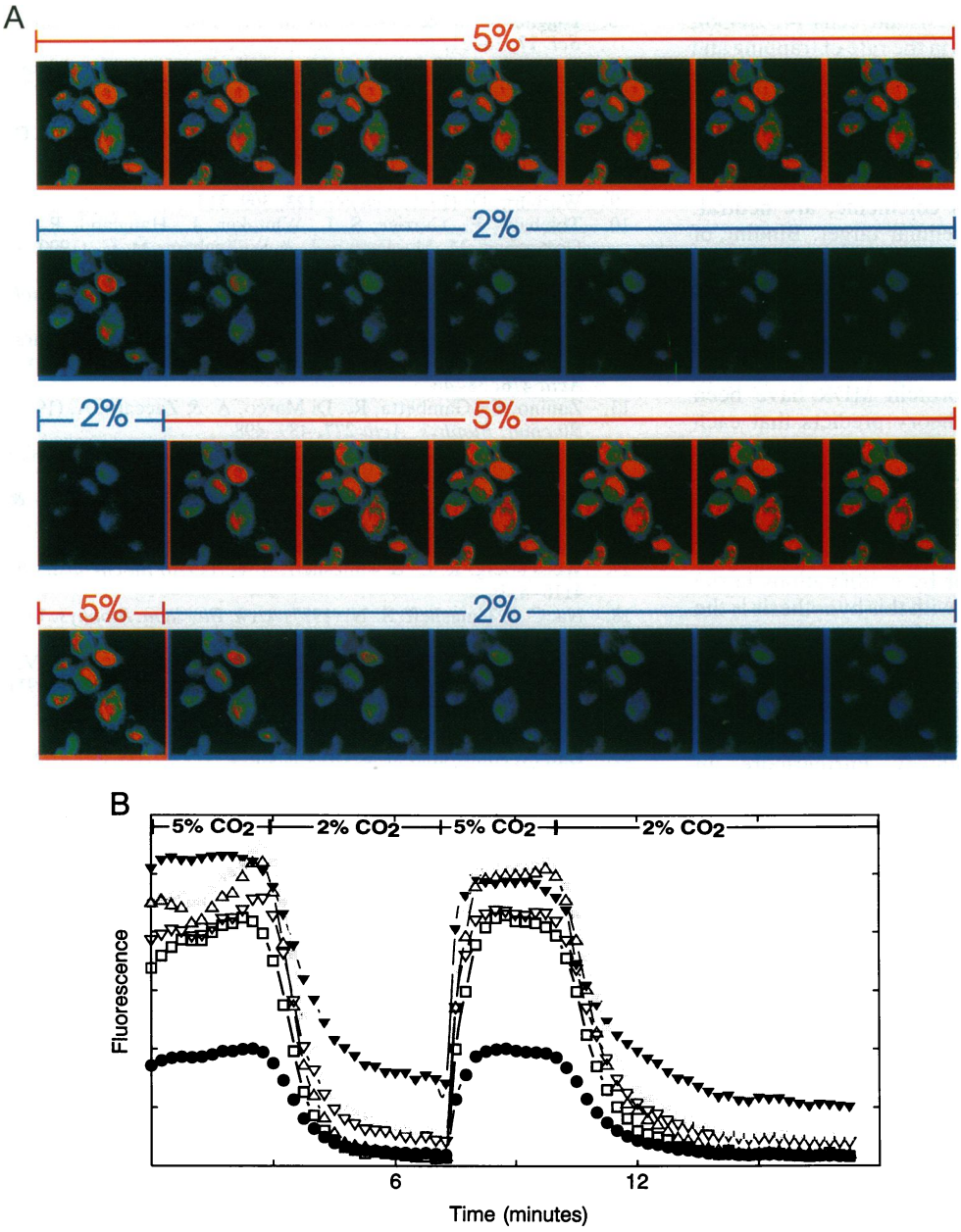

FIG. 3. Effect of shifting the partial pressure of CO_2 on the daunomycin fluorescence in NIH 3T3 cells. (*A*) Fibroblasts were incubated in 2 μM daunomycin and excited at 488 nm, and emission was recorded at 570 nm every 15 sec. The medium initially was equilibrated with 5% CO_2 (the first 7 frames, red background). CO_2 was shifted to 2% for 2 min (blue background) and there was a substantial decrease in the cellular daunomycin fluorescence. Upon returning to 5% CO_2 (red background) the cellular daunomycin fluorescence returned. The cells were repeatedly cycled between 5% CO_2 (red background) and 2% CO_2 (blue background). The daunomycin concentration is pseudocolored with the lowest level in black and increasing concentrations in blue, green, red, and yellow. (×50.) (*B*) Quantification of the effect of changes in CO_2 concentration on daunomycin fluorescence in NIH 3T3 cells. The daunomycin fluorescence was quantified for six different cells as CO_2 was shifted between 2% and 5%. Reducing the CO_2 level raises the cytosolic pH and reduces the cell-associated daunomycin fluorescence. These effects were completely reversible and were repeated on the same cells many times.

optimum. Conversely, an increased pH both decreases intracellular drug accumulation and reduces binding to intracellular targets.

Passive transport of drugs in conjunction with a trapping mechanism is consistent with a number of independent observations. (*i*) In simple systems such as erythrocyte ghosts (23) and phospholipid vesicles (24) the transmembrane distribution of these drugs is determined by the ΔpH. (*ii*) The cytosolic pH of tumor cells increases with increased MDR (25). (*iii*) Transfection of cells with P-glycoprotein causes an alkaline shift of cytosolic pH (10). (*iv*) Verapamil, which reverses MDR, partially reverses this shift of cytosolic pH (25). Verapamil increases the concentrations of anti-cancer drugs even in cells which do not express P-glycoprotein (26,

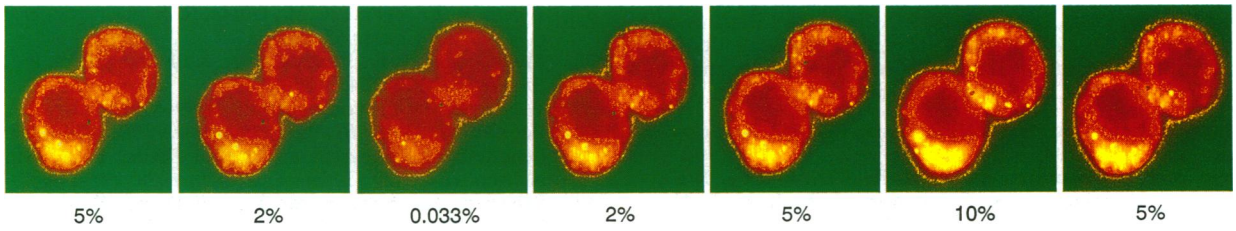

FIG. 4. Effect of shifting the partial pressure of CO_2 on the daunomycin fluorescence in myeloma cells. Myeloma cells attached to glass coverslips were incubated in 6 μM daunomycin for 40 min and monitored under standard fluorescence microscopy. The CO_2 perfusing the surface of the chamber was sequentially switched for 4-min intervals from 5% to 2%, 0.03%, 2%, 5%, and 10% and then returned to 5%. The cycle was repeated. The daunomycin concentration is pseudocolor coded with the lowest value in green and increasing levels in orange, red, and yellow. (×600.)

27). (*v*) Drug influx is slower in resistant cells (4, 28–36), which is consistent with differences in the rate of trapping and inconsistent with an active efflux model. (*vi*) Drugs which acidify the cytosol, such as amiloride, reverse MDR (37).

As shown, the passive-trapping hypothesis can account for changes in cellular accumulation of chemotherapeutic agents that are weak bases. What about the rest? None are negatively charged but some, such as colchicine, are neutral. Each of these drugs has an intracellular target. Binding of colchicine to its target, the extremely acidic carboxyl terminus of α-tubulin (38), is pH dependent with an optimum of pH 6.7–6.8 (39). Any alkaline shift of the pH decreases the binding of colchicine and could protect the cell from this chemotherapeutic agent.

Multiple forms of non-P-glycoprotein MDR have been observed. The passive-transport theory predicts that each affects a common feature—regulation of cellular pH. One protein responsible for non-P-glycoprotein-mediated MDR has been cloned and demonstrated to be a vacuolar H^+-ATPase subunit (1). Other mechanisms for MDR may use pH to affect drug distribution either by selective sequestration—i.e., drug uptake by lysosomes—or by modifications in the secretory pathway (40). Consistent with this hypothesis is the observation of an increase in nonspecific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant cells (41), as well as an increase in membrane traffic in daunomycin-resistant cells (42). In drug-resistant cells, there is a significant rate of exocytosis of lysosomal enzymes, suggesting a modification of the endocytic pathway. Furthermore, an enhanced rate of exocytosis of vesicles containing a H^+-ATPase could be a means by which cytosolic pH is raised, as has been observed in plant and animal cells (43, 44).

These results demonstrate that the passive-trapping model is sufficient to account for the enhanced sensitivity of tumors to anticancer drugs and the decreased sensitivity in MDR. When the pH of drug-sensitive cells is shifted to the level observed in drug-resistant cells, they no longer accumulate chemotherapeutic agents. Likewise, when drug-resistant cells are shifted to the level observed in drug-sensitive cells, they accumulate chemotherapeutics. Although our results neither directly prove nor disprove the hypothesis that P-glycoprotein is an ATP-driven drug efflux pump or "flippase," they demonstrate the existence of alternate pathways for MDR.

There are a number of potential therapeutic implications from this work. If tumor cells are compromised in their ability to regulate pH, they may be more susceptible than healthy cells to pharmacological approaches that modify pH regulation. Thus, approaches that affect pH may potentiate the effects of the chemotherapeutic agents and, in this manner, reverse MDR.

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Cell Biology. In the article "Intracellular pH and the control of multidrug resistance" by Sanford Simon, Deborshi Roy, and Melvin Schindler, which appeared in number 3, February 1, 1994, of *Proc. Natl. Acad. Sci. USA* (**91,** 1128–1132), one of the curves (for drug-sensitive cells in 5% CO_2) was omitted from Fig. 2*A*. The correct figure and its legend are reproduced below.

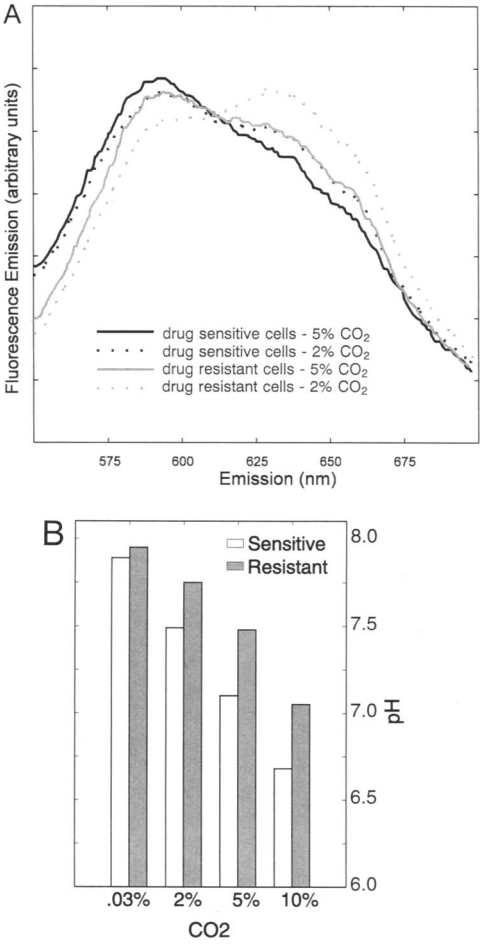

FIG. 2. Effect of CO_2 concentration on cytosolic pH. (*A*) Myeloma cells were loaded with SNARF-1 and the CO_2 in the medium was shifted between 2% (dotted lines) and 5% (solid line). The fluorescence emission was recorded between 520 nm and 700 nm with an excitation of 514 nm for both the drug-sensitive cells (black line) and the drug-resistant cells (gray line). The pH (as indicated by the ratio of the emission at 630 nm to 585 nm) is indistinguishable between the sensitive cells in 2% CO_2 (dotted black line) and the resistant cells in 5% CO_2 (solid gray line). (*B*) The pH is plotted for the drug-sensitive cells (open bars) and drug-resistant cells (filled bars) at 0.03%, 2%, 5% and 10% CO_2.