HUMAN MYELOID leukemia cells selected for resistance to the anthracyclines doxorubicin or daunorubicin generally display a multidrug-resistance phenotype associated with overexpression of \( \text{MDR1} \) and/or \( \text{MRP} \). Both MDR1 and MRP are members of the ATP-binding cassette superfamily of membrane transporters.\(^{1,3} \) Transfection of the cDNA for either \( \text{MDR1} \) or \( \text{MRP} \) results in cell lines that display diminished sensitivity to the anthracyclines, vinca alkaloids, and epipodophyllotoxins and demonstrate energy-dependent efflux of cytotoxic agents.\(^{4,6} \)

In some anthracycline-selected cell lines, the subcellular drug distribution is strikingly different between parental and drug-resistant cell lines.\(^{7,13} \) Whereas most parental cell lines accumulate anthracyclines predominately in the nucleus, certain drug-resistant cells appear to sequester anthracyclines into a vesicular, cytoplasmic compartment. Because the cytotoxic target of the anthracyclines is the nuclear enzyme topoisomerase II,\(^{14} \) non-nuclear drug redistribution may contribute to diminished sensitivity to these agents. Despite these observations, the contribution of vesicular sequestration to the resistance phenotype has been incompletely characterized.

We described a series of U-937 cell lines selected for doxorubicin resistance.\(^{15} \) Cells that were exposed to low levels of drug overexpressed the multidrug-resistance-associated protein and showed a vesicular redistribution of anthracyclines into a non-nuclear compartment. We now present data that further define this process. The results indicate that vesicular anthracycline sequestration directly contributes to diminished sensitivity to the anthracyclines. These cells possess a distinct constitutive redistribution of the lysosomal compartment associated with the expression of the lysosomal-associated membrane protein, LAMP-1. Staining for the P-glycoprotein and for MRP suggests that these transporters are expressed independently of the vesicular compartment and are primarily on the plasma membrane in U-937 cells. Furthermore, this compartment may be a target for modulating agents to partially reverse this resistance phenotype.

MATERIALS AND METHODS

**Cell culture.** The U-937 human myeloid leukemia cell line was obtained from the American Type Culture Collection (Rockville, MD). U-937 cells were derived from the parental U-937 line by chronic exposure to a final concentration of 10 ng/mL doxorubicin.\(^{12} \) Both cell lines were routinely grown at 37°C and 5% CO\(_2\) in RPMI-1640 medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin.

**Drugs and chemicals.** Daunorubicin was purchased from Wyeth-Ayerst Laboratories (Philadelphia, PA). [\( ^3 \)H]-daunorubicin (specific activity, 1.4 Ci/mmol) and [\( ^3 \)H]-water (specific activity, 6.33 mCi/mL) were from DuPont-New England Nuclear (Boston, MA). Silicone oil (specific gravity, 1.035 to 1.045) was from William F. Nye Co (New Bedford, MA). Lysosensor yellow/blue DND-160 (sulfonylbisisomerase II) was from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma Chemical Co (St Louis, MO).

**Antibodies.** The antisera MRP-6KQ was generously provided by

From the Division of Cancer Pharmacology, The Dana-Farber Cancer Institute and The Harvard Medical School, Boston, MA.

Submitted April 5, 1996; accepted December 29, 1996.

Supported in part by Clinical Investigator Award CA-01613 from the National Cancer Institute (C.A.S.).

Address reprint requests to Christopher A. Slapak, MD, Lilly Research Laboratories, Lilly Corporate Center DC #2133, Indianapolis, IN 46285.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1997 by The American Society of Hematology.

0006-4971/97/8910-0027$3.00/0

M. Center (Kansas State University, Manhattan, KS) and was used at a 1:1,000 dilution. The monoclonal antibodies (MoAbs) QCRL-1 and QCRL-3 were kindly provided by S. Cole (Queen’s University, Kingston, Ontario, Canada) and were used as described. The MoAb C219 was purchased from Signet (Dedham, MA) and was used at 1 µg/mL. Antibodies to human LAMP-1 and LAMP-2 were purchased from the Developmental Studies Hyridomab Bank (University of Iowa, Iowa City, IA) and were used at the supplier’s recommended dilution.

Fluorescence photomicrographs. Cells or cytoplasts were suspended in RPMI medium and incubated with daunorubicin (500 ng/mL final concentration) for 1 hour. They were then washed twice in phosphate-buffered saline (PBS; 0°C) and kept on ice until examination by fluorescence microscopy. In some experiments, chloroquine (0 to 100 µmol/L) was added to cell suspensions for 2 hours before daunorubicin exposure to examine its effect on vesicular accumulation. Cells or cytoplasts were photographed rapidly to minimize the effect of photo bleaching. Photographs were taken through an Olympus BH-2 fluorescence microscope (Olympus Optical Co, Tokyo, Japan). Exposures were for 4 seconds with Kodak TMAX ASA 3200 film or for 8 seconds with Kodak ASA 1600 colorprint film (Eastman Kodak, Rochester, NY).

Vital staining with pH indicator. Cells (1 x 10⁷/mL) were exposed to the pH sensitive fluorescent dye, LysoSensor yellow/blue DND-160, at a final concentration of 10 µmol/L for 2 hours in PBS-glucose. Cells were washed twice with cold PBS (0°C) and then kept on ice until fluorescence microscopy. After an excitation period of 5 seconds, exposures of 20 seconds were performed with Kodak ASA 400 colorprint film.

Immunofluorescence studies. Cells (1 x 10⁷) were washed with PBS, fixed in 70% ethanol at 0°C for 1 hour, and then incubated in 5% goat serum for 30 minutes while on ice. Primary antibody incubation occurred for 1 hour at 0°C before washing in PBS and incubating with either a goat antimouse or goat antirabbit fluorescein isothiocyanate (FITC)-conjugated second-step antibody (Boehringer Mannheim, Indianapolis, IN) for 45 minutes (0°C). The cells were then washed and kept on ice in the dark until examination by fluorescence microscopy using an Olympus BH-2 fluorescence microscope (Olympus Optical Co, Tokyo, Japan). Cells were photographed using Kodak Tri-PanX ASA 400 film.

Measurement of daunorubicin accumulation. All drug incubations were performed at 37°C. Cells or cytoplasts were resuspended at 10⁷/mL and incubated for 1 hour in RPMI medium containing 3[H]-daunorubicin at final concentrations of 0 to 750 ng/mL as indicated. Drug accumulation was terminated by sedimenting cells or cytoplasts through silicone oil using a previously described technique. Briefly, after drug incubations, 200 µL of cell suspensions were pelleted through silicone oil and radioactivity determined as described above.

Preparation of cytoplasts. Cytoplasts were prepared as described. Two to three times were washed in Dulbecco’s PBS, and resuspended in 1 mL of 10% Ficoll-400 previously prepared in RPMI medium containing 10 µg/mL of cytochalasin B. This cell suspension was spun through a discontinuous Ficoll gradient (10% to 25%) at 25,000 rpm for 1 hour at 30°C. Cytoplasts were recovered at the 15% to 16% Ficoll interface, washed, counted, and resuspended in RPMI medium (10⁷ cytoplasts/mL) and incubated at 37°C for 1 hour before exposure to drugs. Aqueous volumes of cytoplasts prepared from U-937 and U-A10 cells were determined by measuring the 2-hour accumulation of tritiated water at 37°C in RPMI medium.

Measurement of cellular accumulation of chloroquine. After 2 hours of exposure to chloroquine, cells (10⁶) were pelleted and washed twice in ice-cold PBS and resuspended in 50 µL of PBS containing 8-hydroxyquinoline as an internal standard. Cells were lysed by adding 800 µL of a 50% solution of acetonitrile in methanol, and the lysate was left on ice for 1 hour before centrifuging on a desk top centrifuge at high speed for 10 minutes. Samples (10 µL) were injected onto a high-performance liquid chromatography (HPLC) column. The HPLC system consisted of a system controller (C-R3A; Shimadzu, Kyoto, Japan), two pumps (LC-6A; Shimadzu), a UV/Vis detector (SPD-6AV; Shimadzu) set at 330 nm, and an integrating recorder (SPD-6AV; Shimadzu). A MOS hypersil C8, 5m, 100 x 2.1 mm column was used (Hewlett-Packard, Palo Alto, CA). The mobile phase contained 80% methanol, 20% water, 50 mM/L sodium acetate, and 30 mM/L octadecyl sulfonate adjusted to pH 5.0. Retention times were 2.3 and 4.3 minutes for 8-hydroxyquinoline and chloroquine, respectively. This assay was linear for chloroquine in the range used (r² > .999; 5 to 500 µmol/L).

Drug sensitivity assays. Cell sensitivity to daunorubicin, doxorubicin, chloroquine, and anthracycline/chloroquine combinations was determined after 96 hours of exposure to drugs using an MTT assay performed in quadruplicate at least three times. The IC₅₀ values were determined by fitting the data to a sigmoidal inhibitory effect model with a baseline effect parameter using a nonlinear least squares regression program (Model 108, PCNONLIN ver 4; SCI Software, Stateline, PA). The fold resistance was calculated from the ratio of IC₅₀ value for U-A10 cells to the IC₅₀ value for the parental U-937 cells. The effect of chloroquine on anthracycline

Fig 1. Comparison of daunorubicin and Lysosensor yellow/blue DND-160 distribution in U-937 cells. Parental U-937 cells (A and B) and U-A10 cells (C and D) were exposed to 500 ng/mL of daunorubicin for 1 hour, and the cells were washed, visualized by fluorescence microscopy, and photographed. Parental U-937 cells (E and F) and U-A10 cells (G and H) were exposed to Lysosensor yellow/blue DND-160 (10 μmol/L) for 2 hours, and the cells were washed, visualized by fluorescence microscopy, and photographed. Shown are photographs of typical single cells centered within the frame such that the perimeter of the cell approximately reaches the edge of the photograph.
cytotoxicity was determined by adding the modulating agent just before the anthracycline. A chloroquine concentration of 10 \( \mu \text{mol/L} \) is minimally toxic to cells and is clinically achievable.\(^{25}\)

Statistical analyses. Standard error bars are presented for each value on the drug accumulation graphs. Student’s paired \( t \)-test was used to compare differences in accumulation between parental and drug-resistant sublines as indicated. All statistics were calculated with software programs from StatView (Brainpower Inc, Calabasas, CA).

RESULTS

The U-A10 cell line was derived by selecting the human myeloid leukemia cell line, U-937, in 10 ng/mL of doxorubicin.\(^{12}\) Studies using \( ^{3} \text{H} \)-daunorubicin showed that U-A10 cells accumulate net levels of daunorubicin that are almost equal to that observed in parental U-937 cells. However, fluorescence microscopy of U-937 and U-A10 cells after 1 hour of exposure to 500 ng/mL of daunorubicin showed marked differences in the subcellular distribution of drug between the two cell lines (Fig 1). In U-937 cells, drug-associated nuclear fluorescence predominated, although a faint, punctate cytoplasmic accumulation of daunorubicin was observed (Fig 1A and B). By contrast, in U-A10 cells, daunorubicin was sequestered into expanded cytoplasmic vesicles, generally located to one side of the nucleus (Fig 1C and D). U-A10 cells have markedly less drug-associated nuclear fluorescence than the parental cell line.

To examine for the presence of acidic vesicles and to characterize further the site of vesicular anthracycline accumulation, the pH-sensitive vital stain, LysoSensor yellow/blue DND-160, was used. This indicator fluoresces yellow in highly acidic organelles. After 2 hours of accumulation of dye, a distinct punctate pattern of yellow fluorescence consistent with vesicular dye accumulation was observed in parental U-937 cells (Fig 1E and F). The vesicles were distributed uniformly throughout the cell. By contrast, U-A10 cells were observed to accumulate dye into large expanded clustered vesicles that were eccentrically placed in the cells (Fig 1G and H). The intense yellow fluorescence confirmed the acidic nature of these organelles. With the exception of a lack of nuclear staining, the pattern of dye accumulation in U-937 and U-A10 cells was essentially identical to that observed with daunorubicin.

The current studies as well as previous data have suggested that the anthracyclines accumulate into lysosomes.\(^{20,26}\) To define further this compartment, the expression of the lysosomal-associated membrane proteins, LAMP-1 and LAMP-2, was examined by immunofluorescence.\(^{18}\) In parental U-937 cells, staining for LAMP-1 showed the protein to be expressed in a fine, punctate pattern throughout the cytoplasm of fixed, permeabilized cells (Fig 2A and B). However, staining of U-A10 cells showed enlarged ring-like structures that were tightly clustered in the perinuclear area (Fig 2C and D). The staining for LAMP-1 closely resembled the vesicular compartment that accumulates anthracyclines or the LysoSensor dye in these cells. When U-A10 cells were grown in the presence of doxorubicin (10 ng/mL for 30 days), there was no appreciable difference in the staining for LAMP-1 as assessed by immunofluorescence (data not shown). Staining for LAMP-2 appeared identical to that for LAMP-1 in both cell lines (data not shown).

To examine the contribution of MRP and the P-glycopro-
tein to vesicular anthracycline accumulation in the drug-resistant cells, the cells were fixed, permeabilized, stained for MRP or P-glycoprotein, and observed by fluorescence microscopy. Parental U-937 cells showed extremely faint MRP staining (data not shown). Expression of the P-glycoprotein could not be detected in parental cells by this method. When examined for MRP staining with either polyclonal antisera 6KQ or MoAbs QCRL-1 and QCRL-3, MRP expression was observed primarily along the perimeter of the cell (Fig 3A and B). Likewise, the P-glycoprotein was observed to be expressed primarily in the cytoplasmic membrane (Fig 3C and D). Taken together, these studies show that U-A10 cells express MRP and the P-glycoprotein largely independently of the lysosomal compartment in a pattern consistent with a plasma membrane location.

To assess more thoroughly how the expanded lysosomal compartment observed in U-A10 cells affected subcellular daunorubicin distribution, anthracycline accumulation was quantified in various compartments using [3H]-daunorubicin. To measure drug in the nucleus, cells were exposed to [3H]-daunorubicin (0 to 500 ng/mL) for 1 hour, the nuclei were then isolated, and nuclear-associated radioactivity was determined. The results demonstrate that nuclei from U-A10 cells, as compared with U-937 cells, contain 2.5-fold to threefold less daunorubicin at each extracellular daunorubicin concentration (Fig 4A). The observed differences were statistically significant (eg, at 500 ng/mL, P < .005).

To define further the differences in nuclear drug accumulation, nuclei were first isolated from cells and then exposed to drug for 15 minutes. Over this time, essentially no difference could be detected in the net nuclear accumulation of daunorubicin (Fig 4B). Longer periods of drug exposure were not possible due to nuclear degeneration at 37°C. These results suggested that the diminished nuclear drug accumulation observed when whole cells were incubated in daunorubicin was due to extranuclear factors, specifically cytoplasmic sequestration.

Because net daunorubicin accumulation is essentially equal in U-937 and U-A10 cells,12 but net nuclear accumulation in U-A10 cells is about twofold to threefold less than in U-937 cells, then drug accumulation into the cytoplasmic compartment must be greater in U-A10 cells. To measure cytoplasmic drug accumulation, enucleated cells or cytoplasts were prepared. Controls for these experiments included fluorescence microscopy of cytoplasts exposed to 500 ng/mL of daunorubicin (Fig 5). In cytoplasts from parental U-937 cells, fine punctate granules were seen to accumulate daunorubicin (Fig 5A and B), whereas in cytoplasts from U-A10 cells large expanded vesicles were observed to accumulate drug (Fig 5C and D). The finding that accumulation of tritiated water is nearly identical in cytoplasts from U-937 and U-A10 cells supports the presence of similar aqueous volumes (data not shown). Incubation of U-937 or U-A10 cells with cytochalasin B for 1 hour (10 μg/mL) did not affect daunorubicin accumulation in intact cells (<5% difference), suggesting that this agent did not affect anthracycline accumulation in these cell lines.

Exposure of cytoplasts from U-937 cells to increasing concentrations of [3H]-daunorubicin for 1 hour resulted in a nonlinear drug accumulation into cytoplasts (Fig 6). By contrast, cytoplasts from U-A10 cells accumulated [3H]-daunorubicin in a linear fashion over a broad concentration range.
The studies presented here provide clear evidence that the U-A10 cell line exhibits an enhanced drug-resistance phenotype associated with the doxorubicin-selected U-A10 cell line. Fluorescence microscopy studies have shown that the U-A10 cell line exhibits an enhanced vesicular accumulation of anthracyclines and diminished nuclear drug uptake (Fig 1). Although similar observations have been reported for other drug-selected cell lines, the identification of this compartment has remained unclear. Examination for the expression of the LAMP-1 and LAMP-2 antigens showed that these vesicles were part of the lysosomal compartment (Fig 2). LAMPs are transmembrane proteins whose expression is restricted primarily to lysosomes. Dramatic differences in the staining of this protein between parental U-937 and doxorubicin-selected cell lines were observed.

**DISCUSSION**

The finding of increased daunorubicin accumulation into a functionally expanded vesicular or lysosomal compartment suggested that U-A10 cells may preferentially accumulate alkaline lysosomotropic agents, such as chloroquine. Cells were exposed to various chloroquine concentrations for 1 hour, the cells were lysed, the chloroquine was extracted, and the amount was quantified by HPLC analysis. U-A10 cells accumulated 1.5-fold to twofold more chloroquine compared with U-937 cells at extracellular chloroquine concentrations of 10 and 50 µmol/L ($P < .005$ and $P < .05$, respectively; Fig 7). The finding of increased chloroquine accumulation suggested that U-A10 cells may demonstrate enhanced sensitivity to this agent as compared with parental U-937 cells. Cells were exposed to increasing amounts of this agent for 96 hours and the concentration that produced 50% decreased cell survival was determined in an MTT assay (Table 1). These studies showed that U-A10 cells are approximately twofold more sensitive to chloroquine than the U-937 cells.

The ability of chloroquine to modulate resistance to daunorubicin and to doxorubicin in parental and U-A10 cells was also examined. U-A10 cells were exposed to chloroquine for 2 hours before 1 hour of treatment with daunorubicin. Chloroquine at 10 µmol/L caused enlargement of daunorubicin-containing vesicles and greater anthracycline-associated nuclear fluorescence (Fig 8A and B). Higher chloroquine concentrations (100 µmol/L) also resulted in enlargement of vesicles, however, with diminution of daunorubicin-associated fluorescence in the vesicles (Fig 8C and D). There was also an accompanying greater anthracycline-associated nuclear fluorescence. That chloroquine was affecting lysosomal pH was demonstrated by the elimination of yellow fluorescence associated with Lysosensor yellow/blue when cells were treated with 10 µmol/L chloroquine (data not shown). When used at a nontoxic concentration of 10 µmol/L, chloroquine did not increase the cytotoxicity of either daunorubicin or doxorubicin in U-937 cells as determined by the MTT assay (Table 1). However, the same concentration of chloroquine markedly sensitized U-A10 cells to daunorubicin and to doxorubicin. For example, sensitivity to daunorubicin, measured as 35-fold resistant, was restored to nearly the same level as in parental cells (Table 1).

**DISCUSSION**

The studies presented here provide clear evidence that the lysosomal compartment actively participates in the acquired drug-resistance phenotype associated with the doxorubicin-selected U-A10 cell line. Fluorescence microscopy studies have shown that the U-A10 cell line exhibits an enhanced vesicular accumulation of anthracyclines and diminished nuclear drug uptake (Fig 1). Although similar observations have been reported for other drug-selected cell lines, the identification of this compartment has remained unclear. Examination for the expression of the LAMP-1 and LAMP-2 antigens showed that these vesicles were part of the lysosomal compartment (Fig 2). LAMPs are transmembrane proteins whose expression is restricted primarily to lysosomes.

---

**Fig 4. Assessment of nuclear daunorubicin accumulation after exposure of cells or isolated nuclei to [3H]-daunorubicin.** (A) U-937 and U-A10 cells were exposed to 0 to 500 ng/mL of [3H]-daunorubicin for 1 hour, and the cells were washed, ruptured, and nuclei purified by pelleting through a sucrose cushion. All isolation steps were performed at 4°C to minimize nuclear drug loss. Nuclear-associated radioactivity was determined by scintillation counting. Shown is a plot of the mean (± the standard error) nuclear daunorubicin accumulation versus extracellular drug concentration for three experiments each performed in duplicate. The differences were statistically significant at each concentration tested ($P < .05$ for 50 ng/mL; $P < .005$ for 200 and 500 ng/mL). (B) Nuclei were first isolated by lysing cells in a hypotonic, NP-40–containing buffer and pelleting by centrifugation. They were then exposed to 500 ng/mL of daunorubicin for 15 minutes before sedimenting through silicone oil and radioactivity determined by scintillation counting. Shown are the mean (± the standard error) of three experiments performed in duplicate.
vesiculated anthracycline accumulation in U-937 cells

Fig 5. Daunorubicin distribution in cytoplasts prepared from U-937 and U-A10 cells. Cytoplasts (enucleated cells) were prepared from U-937 cells (A and B) and from U-A10 cells (C and D) by treating cells with cytochalasin B and then pelleting through a discontinuous Ficoll gradient. Cytoplasts were exposed to daunorubicin at 500 ng/mL for 1 hour before being washed, visualized by fluorescence microscopy, and photographed. Shown are photographs of typical single cytoplasts with the images centered within the frame.

Fig 6. Accumulation of 3[H]-daunorubicin into cytoplasts prepared from U-A10 and U-937 cells. Cytoplasts were exposed to 0 to 750 ng/mL of 3[H]-daunorubicin for 1 hour before terminating drug accumulation by pelleting through silicone oil. Shown is a plot of the mean ± the standard error daunorubicin accumulation into cytoplasts versus external drug concentration for three experiments each performed in duplicate. The differences were statistically significant at 500 (P < .005) and 750 ng/mL (P < .01) of daunorubicin.

Fig 7. Cellular accumulation of chloroquine. U-937 and U-A10 cells were exposed to chloroquine (0 to 50 μmol/L) for 2 hours before cells were washed and lysed and chloroquine was extracted. The amount of chloroquine was measured by HPLC analysis using 8-hydroxyquinoline as an internal standard. Shown is the mean ± the standard error net cellular chloroquine accumulation when cells were exposed to 10 or 50 μmol/L chloroquine. The differences between U-937 and U-A10 cells were statistically significant at 10 (P < .005) or 50 μmol/L (P < .05) of chloroquine.

served. Immunofluorescence showed that both LAMP-1 and LAMP-2 stained enlarged vesicles that were present in a perinuclear distribution in U-A10 cells. Parental U-937 cells exhibited a pattern of staining distinct from that observed in U-A10 cells. The vesicular compartment observed in the drug-resistant cell lines is continuously present because there was no difference in the general staining pattern for LAMP-1 expression in the presence or absence of doxorubicin. Because staining for LAMP-1 requires cell permeabilization and fixation, colocalization studies with antibody and drug were not possible.
Table 1. Effect of Chloroquine on Anthracycline Sensitivity in U-937 and U-A10 Cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>U-937 (IC50)</th>
<th>U-A10 (IC50)</th>
<th>Fold Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine (μmol/L)</td>
<td>56.5 ± 8.2</td>
<td>23.0 ± 4.6</td>
<td>0.41</td>
</tr>
<tr>
<td>Daunorubicin (ng/mL)</td>
<td>8.4 ± 0.5</td>
<td>293 ± 23</td>
<td>35</td>
</tr>
<tr>
<td>Daunorubicin/chloroquine</td>
<td>9.8 ± 0.6</td>
<td>11.9 ± 2.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Doxorubicin (ng/mL)</td>
<td>9.5 ± 0.8</td>
<td>683 ± 89</td>
<td>72</td>
</tr>
<tr>
<td>Doxorubicin/chloroquine</td>
<td>11.1 ± 2.0</td>
<td>66.7 ± 2.9</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The sensitivity of the parental U-937 and the doxorubicin-selected U-A10 cell lines to chloroquine, daunorubicin, and doxorubicin was determined in a standard MTT assay after 96 hours of drug exposure. Shown is the average (± the standard mean) IC50 value from three experiments each performed in quadruplicate. The effect of chloroquine on anthracycline cytotoxicity was determined by adding the lysosomotropic agent to a final concentration of 10 μmol/L just before adding the anthracycline. The fold resistance is calculated from the ratio of the IC50 of U-A10 cells/the IC50 of U-937 cells.

Drug redistribution into individual cellular compartments using radiolabeled daunorubicin was assessed to understand further the contribution of the lysosomal compartment to the resistance phenotype. Redistribution of anthracyclines was shown to result from a process present primarily in the cytoplasmic, not the nuclear, compartment. When the cells were exposed to daunorubicin and the nuclei isolated, the nuclei from U-A10 cells consistently accumulated significantly less daunorubicin than nuclei from U-937 cells when assayed over a broad concentration range of extracellular daunorubicin (Fig 4A). Other studies in cells with vesicular anthracycline sequestration have suggested that reduced nuclear drug accumulation may result in part from outward drug transport from the nuclear compartment.19 We examined daunorubicin accumulation into freshly isolated nuclei and found little difference in net drug accumulation (Fig 4B). These results suggest that in U-A10 cells the diminished net nuclear daunorubicin accumulation observed in whole cells was due to cytoplasmic sequestration.

To quantify accumulation into the cytoplasmic vesicular compartment, we prepared cytoplasts from parental and drug-resistant cell lines (Figs 5 and 6). Cytoplasts from U-937 cells accumulate daunorubicin in a nonlinear manner; as the cytoplasts are exposed to higher extracellular daunorubicin concentrations, they accumulated proportionally less drug. A similar phenomenon was demonstrated with cytoplasts prepared from the human leukemia cell line K562; when exposed to increasing daunorubicin concentrations, drug was shown to accumulate in a saturable fashion into an ionophore-sensitive compartment.20 The cytoplasmic vesicular compartment in either of these myeloid leukemia cells appears to have a limited capacity for daunorubicin accumulation and can, in effect, become saturated. In the absence of a nucleus, the capacity for anthracycline accumulation into the cytoplast diminishes once the vesicular compartment has become loaded with daunorubicin. In marked contrast, the capacity for daunorubicin accumulation into the cytoplasmic compartment from U-A10 cells is greatly increased and cannot be saturated at external concentrations of up to 1,000 ng/mL. Cytoplasts from U-A10 cells accumulate daunorubicin in a linear manner over a broad concentration range (Fig 6). Because the cytotoxic target of the anthracyclines is topoisomerase II, which resides in the nucleus,
this enhanced cytoplasmic drug accumulation may directly affect the sensitivity of the cells to the anthracyclines. In parental cells, saturation of the vesicular compartment at pharmacologic drug concentrations permits nuclear drug accumulation with resultant cytotoxicity. In resistant cells, the expanded vesicular compartment is not saturable at pharmacologically relevant drug concentrations (<1,000 ng/mL).

The mechanism by which the anthracyclines traverse the lysosomal membrane is unknown; however, substantial data suggest that these agents cross the plasma membrane by passive diffusion.8-30 Although active or facilitated transport into the lysosome cannot be excluded, our studies, which did not detect appreciable amounts of MRP or P-glycoprotein on the vesicles, suggest these transporters may not be involved in an inward vesicular drug transport in U-A10 cells. Once inside the acidic lysosomal environment, the drug will become protonated. The amino group present on the glycone is fixed by immunologic methods. Similar studies using clinical specimens will help determine the relevance of this phenotype in patients with hematologic malignancies who are refractory to treatment with the anthracyclines.

ACKNOWLEDGMENT

We are indebted to Dr Donald W. Kufe for his generous support.

REFERENCES

10. Markwardt D, Center MS: Drug transport mechanisms in...
25. Titus EO: Recent developments in the understanding of the pharmacokinetics and mechanism of action of chloroquine. Ther Drug Monit 11:369, 1989
Vesicular Anthracycline Accumulation in Doxorubicin-Selected U-937 Cells: Participation of Lysosomes

Selwyn J. Hurwitz, Masanori Terashima, Nobuyuki Mizunuma and Christopher A. Slapak