Intracellular Distribution and Pharmacokinetics of Daunorubicin in Anthracycline-sensitive and -resistant HL-60 Cells


Division of Medical Oncology, Department of Medicine, Columbia University, New York, New York 10032, [A. A. H., J. E. G., S. K., V. J. S., M. R., J. L., K. B., R. N. T.], and Toronto General Hospital, Toronto, Ontario, Canada MSG 1L7 [M. A. B.]

ABSTRACT

Anthracycline-sensitive (HL-60) and -resistant (HL-60/AR) cells, which do not overexpress the P-glycoprotein, each transport and distribute daunorubicin (DNR) into distinct intracellular locations, as visualized by digitized video fluorescence microscopy. At pH 7.4, the fluorescence of DNR in HL-60 cells appears distributed diffusely in both the nucleus and cytoplasm. In contrast, HL-60/AR cells show much less fluorescence in the nucleus and cytoplasm; most of the fluorescence localizes first to the Golgi apparatus and is then gradually shifted to the lysosomes and mitochondria.

In pharmacokinetic studies, HL-60/AR cells exposed to different extracellular concentrations of [14C]DNR consistently accumulated less radioactive drug than the parent HL-60 cells. Incubation of HL-60/AR cells with sodium azide and deoxyglucose blocked the efflux of [14C]DNR and also prevented the shift of DNR fluorescence from the Golgi apparatus to the lysosomes/mitochondria. The efflux and the intracellular shift of DNR could also be inhibited by lowering the temperature to 18°C, which stops endosomal membrane fusion.

When DNR was allowed to accumulate in HL-60 or HL-60/AR cells at pH 5 there was an increase in the proportion of drug fluorescence in the membranes of both HL-60 and HL-60/AR cells; a decrease in the amount of drug retained by HL-60, but not by HL-60/AR cells; and a decrease in the cytostatic effects of DNR on both HL-60 and HL-60/AR cells.

These data suggest that DNR resistance is associated with a failure of DNR to pass through membranes and to bind to cytoplasmic and nuclear structures. Instead, most of the drug is taken up by the Golgi apparatus from which it is then shifted to the lysosomes or to mitochondria, or out of the cell.

INTRODUCTION

The intracellular transport of DNR is poorly understood. DNR is thought to cross the cell membrane by Fickian diffusion or by carrier-mediated passive transport; the uptake of the drug is known to be affected by extracellular pH and degree of protonation of DNR. However, once inside the cell, the distribution of DNR to intracellular organelles and compartments has not been defined. Nor is it clear whether DNR efflux from the cell occurs passively or if it is energy dependent. Certain anthracycline-resistant cell lines show decreased accumulation and retention of the drug and increased expression of a hydrophobic membrane glycoprotein (P-glycoprotein). This protein is believed to participate in the active pumping of DNR to the outside of the cell. Other mechanisms of anthracycline resistance have been described, including protection against free radical damage, decreased levels or altered structure of topoisomerase II, and increased drug degradation. Their relationship to DNR efflux or its intracellular organelle disposition is unknown.

The intent of the current study was to analyze the intracellular pharmacokinetics and distribution of DNR during different stages of transport and intracellular processing, and their relationship to cytotoxicity, in an anthracycline-resistant subline (HL-60/AR) of the human myeloid cell line HL-60. The HL-60/AR cells show many of the characteristics of multidrug resistant cell lines, namely, cross-resistance to vincristine and vinblastine, decreased drug accumulation, and increased drug efflux.

In order to elucidate the intracellular destination of DNR, we studied its transport in real time by using DVFM. The anatomic localization of DNR was deduced by comparing fluorescent images obtained with DNR to those obtained with fluorescent probes with known cellular distribution.

Pharmacokinetic studies with [14C]DNR were done in conjunction with the DVFM studies. Experiments were done with and without metabolic inhibitors (sodium azide and deoxyglucose) to determine the contribution of active efflux. The role of exocytosis was investigated by lowering the temperature below 18°C, which blocks fusion of endocytic vesicles. The participation of membrane microfilaments and of the sodium/hydrogen pump was assessed by using blockers such as cytochalasin D, monensin, ouabain, and other metabolic inhibitors.

MATERIALS AND METHODS

Cells. HL-60 and HL-60/AR cell lines have been previously characterized in our laboratory; the HL-60/AR line is capable of sustained growth in 1.0 μM DNR, more than 20 times the 50% inhibitory dose of the parent line. Studies submitted for publication have shown that HL-60/AR cells do not contain the P-glycoprotein, as determined by nonreactivity with C219 and other monoclonal antibodies directed against P-glycoprotein, and by absence of P-glycoprotein messenger RNA in a dot-blot assay with the MDR-1 probe.

Both HL-60 and HL-60/AR lines were maintained in suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum in 25-cm² flasks at 37°C in 5% CO₂ atmosphere. Cells growing for 1 week or longer in anthracycline-free medium and in the logarithmic phase of growth were used for all experiments.

Received 12/6/88; revised 3/27/89; accepted 5/1/89.

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

Supported by Grants ACS CH-357, CA-31761, and CA-42450, by the National Cancer Institute of Canada.

Present address: Winthrop University Hospital, Division of Oncology/Hematology, Professional Building, 222 Station Plaza North, Mineola, NY 11501. To whom requests for reprints should be addressed.

The abbreviations used are: DNR, daunorubicin; DVFM, digitized video fluorescence microscopy; FD, fluorescein diacetate; PBS, phosphate-buffered saline (Hanks' balanced salt solution); IC₅₀, 50% inhibitory concentration.


4607
Drugs and Chemicals

Daunorubicin was purchased from Ives Laboratories, New York, NY; [14C]DNR (30.9 mCi/mmol) from SRI International, Menlo Park, CA; NBD-ceramide from Avanti Products, Birmingham, AL; and rhodamine-123 from Molecular Probes, Eugene, OR. Other chemicals used were purchased from either Fisher Scientific Co., Springfield, NJ, or Sigma Chemical Co., St. Louis, MO. RPMI-1640 medium with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY) was used for all experiments. The pH was adjusted to pH 5.0, 7.4, or 8.0 with either HCl or NaOH. The medium adjusted to pH 8 also contained 1.0 mM Tris-base to maintain the pH constant through the 1-h incubation.

Growth Inhibition

HL-60 and HL-60/AR cells at 5–10 × 10^5 cells/ml were incubated in the presence of various concentrations of DNR for 1 h at 37°C in 5% CO2 atmosphere. Incubations were carried out at pH 5, 7.4, and 8.0 in a volume of 2 ml. Following the incubation, the cells were washed and resuspended in medium at pH 7.4. They were then seeded in 25-cm2 tissue flasks in a volume of 10 ml at 5–10 × 10^5 cells/ml. At 24-h intervals, 0.1-ml samples were removed from each flask and counted in a Neubauer 0.1-mm hemacytometer. The IC50 for daunorubicin at a particular pH was defined as the drug concentration at which there was a 50% reduction in the cell number at 48 h relative to control cells incubated at the same pH but without the drug. We have previously reported that this assay of anthracycline toxicity correlates closely with clonogenic assays (8).

Accumulation and Retention Studies

Dose. Cells at a concentration of 4 × 10^5 cells/ml were incubated for 1 h in media, pH 7.4, with various concentrations of [14C]DNR at 37°C in 5% CO2 atmosphere. Following incubation, equal aliquots of 2 × 10^6 cells were removed and centrifuged through silicone/mineral oil, solubilized, and the amount of radioactivity was determined (8).

Kinetic Studies. Cells at a concentration of 1 × 10^6 cells/ml were incubated in the presence of 1 µM [14C]DNR in media, pH 5, pH 7.4, and pH 8.0 at 37°C in 5% CO2 atmosphere. At various times (5 s–60 min), the reaction was stopped by the addition of PBS at 4°C. The cells were then washed in PBS at 4°C, solubilized, and the radioactivity was determined.

Retention. Cells at a concentration of 4 × 10^5 cells/ml were incubated at 37°C in 5% CO2 atmosphere for 1 h with 1 µM [14C]DNR at pH 5, 7.4, or 8. These cells were washed at 4°C and resuspended in drug-free media, pH 7.4, for 30 min at 37°C in 5% CO2 atmosphere. At the end of this period, the amount of radioactive drug remaining in the cells was determined and expressed as a percentage of the initial 1-h net drug accumulation. In parallel studies, cells were preincubated at 37°C for 15 min with sodium azide (15 mM) plus deoxyglucose (50 mM), monensin (10 µM), ouabain (1 µM), or cyclohexalin D (5 µg/ml) prior to the addition of 1 µM [14C]DNR. After a 1-h incubation with DNR at 37°C in 5% CO2 atmosphere, the cells were washed and resuspended in media containing the agent but without DNR, and further incubated for 30 min. To reverse the effect of sodium azide/deoxyglucose, the cells were washed and resuspended in drug-free media to which 20 mM glucose was added. The retention of [14C]DNR at 37, 18, and 4°C was then measured.

DVFM Studies. The apparatus and the methodology used have been previously described (12). DVFM images obtained by Ploem epillumination fluorescence microscopy by using a 100-W mercury lamp at a magnification of ×1200 are electronically amplified by an ISIT (intensified-silicon-intensified-target) television camera: the video signal is digitized in real time by a dedicated image processor unit and transferred to the main memory of an IBM PC-AT computer. Fifteen consecutive images are averaged, and the results are photographed from the video screen. The DVFM offers an advantage over fluorescence photomicroscopy in that the intracellular distribution and efflux of anthracycline fluorescence can be immediately visualized in cells such as HL-60/AR which have a rapid redistribution of DNR.

Incubations with DNR. Cells were exposed to 0.1–10 µM DNR in media at pH 5, 7.4, or 8.0 for 1 h at 37°C in 5% CO2 atmosphere. They were centrifuged and resuspended in 10 µl PBS at 4°C. The fluorescence was studied under DVFM with the use of excitation and barrier filters of 530–560 nm and 580 nm, respectively.

Intracellular Distribution of Fluorescent Probes. Fluorescent probes known to stain specific intracellular structures or organelles were used to aid in defining the anatomic distribution of DNR. FD stains the nucleus/cytoplasm (13), acridine orange stains lysosomes of living cells (14), rhodamine-123 stains mitochondria (15), and NBD-ceramide preferentially stains the Golgi apparatus (10). Cells were incubated with FD, 5 µg/ml, rhodamine-123, 5 µg/ml, acridine orange, 1 µg/ml, or NBD-ceramide, 10 µg/ml, in PBS for 1 h at 37°C at pH 7.4. Cells were centrifuged and resuspended in 10 µl PBS at 4°C. DVFM images were obtained by using excitation and barrier filters of 530–560 nm and 580 nm for rhodamine-123 and acridine orange, and 390–490 nm and 515 nm for FD and NBD-ceramide. Because there is significant overlap of fluorescence between DNR and the various probes, colocalization studies could not be done except in the case of NBD-ceramide and DNR.

RESULTS

Intracellular Distribution of Fluorescent Probes. The staining patterns of HL-60 and HL-60/AR cells with different probes are shown in Fig. 1. The fluorescence of fluorescein is present diffusely throughout the cell (Fig. 1, A and B). The fluorescence of acridine orange is distributed mainly in the cytoplasm, appearing granular or punctate (Fig. 1, C and D). The pattern obtained with rhodamine-123 as shown in Fig. 1, E and F is punctate, similar to that of acridine orange, except that there are larger fluorescent aggregates at the cell surface. The fluorescence of NBD-ceramide is notable for bright uptake of the stain into the perinuclear region corresponding to the Golgi apparatus (Fig. 1, G and H).

Intracellular Distribution of DNR in HL-60 and HL-60/AR Cells at pH 7.4 and 37°C. Fluorescence was not detected in HL-60 cells or HL-60/AR cells exposed to <0.4 µM DNR. HL-60 cells exposed to 0.5–2.5 µM DNR for 5–60 min show a diffuse pattern of fluorescence (Fig. 2A) similar to the cytoplasmic and nuclear fluorescence of FD. At higher concentrations (>3.0 µM DNR for 60 min), HL-60 cells show evidence of nuclear denaturation, with bright nuclear fluorescence, staining of the chromatin (Fig. 2B), and visualization of the nuclear outline by phase microscopy (data not shown).

HL-60/AR cells exposed to 0.5–10 µM DNR for 5–10 min acquire a peripheral and perinuclear fluorescence similar to the plasma membrane/Golgi apparatus fluorescence distribution of NBD-ceramide (Fig. 2C); the fluorescence is fainter than that seen in sensitive cells. Colocalization studies with NBD-ceramide and DNR confirm that DNR distributes in the Golgi apparatus of HL-60/AR cells. With longer incubation, the fluorescence of the Golgi apparatus fades and a punctate pattern of fluorescence appears, similar to the lysosomal/mitochondrial fluorescence observed with acridine orange and rhodamine-123, respectively. In addition to the punctate fluorescence, weak diffuse fluorescence is observed; this late diffuse fluorescence is reduced by incubating HL-60/AR cells in drug-free media at 37°C (but not at 18°C) for 30 min (Fig. 2, G and H).

To assess whether differences in DNR metabolism between HL-60 and HL-60/AR cells might affect its distribution, we incubated HL-60/AR cells in DNR (1 µM) for 1 h at 37°C, washed the cells, and then extracted DNR from the cells by sonication. A second suspension of HL-60 cells was then exposed to the extracted DNR. These cells exhibited a diffuse pattern of distribution (data not shown).

Cellular Accumulation of [14C]DNR. HL-60/AR cells accumulate less [14C]DNR compared with HL-60 cells at pH 7.4
Fig. 1. Intracellular distribution of various fluorescent probes: FD, nucleus/cytoplasm; acridine orange, lysosomes; rhodamine-123, mitochondria; NBD-ceramide, Golgi apparatus. HL-60 and HL-60/AR cells were incubated for 1 h at 37°C with the different probes. DVFM images were obtained by epillumination fluorescence microscopy. The photographs represent computer-enhanced images derived by averaging 15 consecutive frames. HL-60 cells: A, FD; C, acridine orange; E, rhodamine-123; G, NBD-ceramide. HL-60/AR cells: B, FD; D, acridine orange; F, rhodamine-123; H, NBD-ceramide.
Fig. 2. Intracellular distribution of DNR into HL-60 and HL-60/AR cells. Each photograph represents DVFM images following the averaging of 15 frames as described in “Materials and Methods.” HL-60 cells: A, 1-h incubation with 2 μM DNR at 37°C; B, 1-h incubation with 10 μM DNR at 37°C. HL-60/AR cells: C, 5-min incubation with 10 μM DNR at 37°C; D, 1-h incubation with 10 μM DNR at 37°C; E, 1-h incubation with 10 μM DNR in the presence of sodium azide and deoxyglucose at 37°C; F, 1-h incubation with 10 μM DNR in the presence of sodium azide and deoxyglucose, at 37°C followed by a 30-min “washout” in media plus 20 mM glucose at 37°C; G, 1-h incubation with 10 μM DNR at 37°C followed by a 30 min washout in media at 18°C; H, 1-h incubation with 10 μM DNR at 37°C followed by a 30 min washout in media at 37°C.
or lowering their temperature is without effect. Fluorescence is blocked if the temperature of the cells is lowered to 18 or 4°C prior to the addition of glucose (data not shown) (Fig. 3).

Cells were incubated in the presence of DNR in media, pH 5, pH 7.4, or pH 8. At various times, the reaction was stopped by the addition of PBS at 4°C. Points, mean of quintuplicate experiments, each done in triplicate; bars, S.D.

In the parent HL-60 cells, retention of \([^{14}C]DNR\) in the presence of 1 \(\mu M\) DNR for 1 h at 37°C. They were then washed, resuspended in drug-free media, and incubated at 37, 18, or 4°C for 30 min. Another group of cells was preincubated with 15 \(mM\) sodium azide/deoxyglucose and 50 \(mM\) deoxyglucose at 37°C with \([^{14}C]DNR\). Following incubation, the cells were washed and resuspended in drug-free media which contained either 15 \(mM\) sodium azide and 50 \(mM\) deoxyglucose or 20 \(mM\) glucose and incubated at 37, 18, or 4°C. Columns, percentage of the labeled drug retained at 30 min relative to the net accumulation at 1 h. Experiments were performed in triplicate on at least three separate occasions.

Preincubation of HL-60/AR cells at 37°C with DNR for at least 30 min results in lysosomal/mitochondrial pattern of cellular drug distribution. The subsequent lowering of the temperature has no effect on the fluorescence distribution pattern of DNR.

Effects of Metabolic Inhibition on \([^{14}C]DNR\) Retention. In HL-60/AR cells preincubated with DNR in the presence of sodium azide/deoxyglucose, the efflux of \([^{14}C]DNR\) out of the cell is blocked (Fig. 6). Removal of sodium azide/deoxyglucose and addition of 20 \(mM\) glucose releases the block and allows efflux to occur at 37°C but not at 18 or 4°C. We also studied the effects of sodium azide/deoxyglucose or temperature on the efflux of DNR in HL-60/AR cells which had been incubated with \([^{14}C]DNR\) in the absence of metabolic inhibitors. As discussed above, such cells demonstrate a lysosomal/mitochondrial DNR fluorescence pattern. In these cells, either addition of sodium azide/deoxyglucose or lowering the temperature of the efflux medium to 18 or 4°C each has an equal partial inhibitory effect on the efflux of \([^{14}C]DNR\).

In the parent HL-60 cells, retention of \([^{14}C]DNR\) is not affected by sodium azide/deoxyglucose, or by changing the temperature of the efflux medium (Fig. 5). We also studied the effects of sodium azide/deoxyglucose or temperature on the efflux of DNR in HL-60/AR cells which had been incubated with \([^{14}C]DNR\) in the absence of metabolic inhibitors. As discussed above, such cells demonstrate a lysosomal/mitochondrial DNR fluorescence pattern. In these cells, either addition of sodium azide/deoxyglucose or lowering the temperature of the efflux medium to 18 or 4°C each has an equal partial inhibitory effect on the efflux of \([^{14}C]DNR\).

Lack of Effect of Cytochalasin D, Monensin, or Ouabain. The percentage of \([^{14}C]DNR\) retained in HL-60 cells and HL-60/AR cells incubated with cytochalasin D, monensin, or ouabain was 70 ± 1% (SD), 80 ± 1%, 81 ± 6%, and 34 ± 4%, 34 ± 4%, respectively. We also studied the effects of sodium azide/deoxyglucose or temperature on the efflux of DNR in HL-60/AR cells which had been incubated with \([^{14}C]DNR\) in the absence of metabolic inhibitors. As discussed above, such cells demonstrate a lysosomal/mitochondrial DNR fluorescence pattern. In these cells, either addition of sodium azide/deoxyglucose or lowering the temperature of the efflux medium to 18 or 4°C each has an equal partial inhibitory effect on the efflux of \([^{14}C]DNR\).
cells, the diffuse fluorescence of DNR is decreased, whereas the effect on drug retention following incubation with cytochalasin cells incubated at pH 5 at which they exhibit the least cyto-
ence between HL-60 and HL-60/AR cells is maintained. Cor relation was observed between the pattern of intracellular DNR distribution, while the HL-60/AR cells become even more punctate (Fig. 7). Also at pH 5, there is decreased retention of [14C]DNR by HL-60 cells comparable to that of HL-60/AR cells (Fig. 8). Exposure to DNR, pH 8, further increases the net accumulation of DNR at 1 h (Fig. 4). However, the pattern of DNR fluorescence (data not shown), retention (Fig. 8), and DNR-mediated growth inhibition (Table 1) are not changed.

Growth-inhibition Studies. When extracellular pH is lowered, there is a significant and parallel increase in the IC50 for DNR (1-h exposure) in both HL-60 and HL-60/AR cells. The 200-fold difference in IC50 for DNR between the two cell lines is therefore preserved (Table 1). At pH 8, the IC50 of DNR for HL-60 and HL-60/AR cells is only mildly increased; the differ change between HL-60 and HL-60/AR cells is maintained. Correlation was observed between the pattern of intracellular DNR fluorescence distribution and DNR-mediated growth inhibition of HL-60 and HL-60/AR cells. At pH 8 and pH 7.4, HL-60 cells have a diffuse pattern of intracellular DNR distribution, the cells have the lowest IC50 for DNR. In contrast, HL-60/AR cells incubated at pH 5 at which they exhibit the least cytoplasmic/nuclear fluorescence (Fig. 7), have the highest IC50 for DNR.

DISCUSSION

The present study extends our previous observation that the distribution of DNR fluorescence is markedly different in HL-60 and HL-60/AR cells (12). Comparison of the patterns of DNR fluorescence with those of characterized cellular probes suggests that uptake of DNR into the cytoplasm and nucleus, as is seen in anthracycline-sensitive cells, is associated with enhanced retention and increased cytotoxicity. In resistant HL-60/AR cells, DNR is diverted from the nucleus, and accumulates into membranes and the Golgi apparatus. This is followed by a shift to lysosomes or mitochondria, and is accompanied by reduced net drug accumulation and cytotoxic resistance.

It is likely that these fluorescent patterns reflect actual differences in in vivo localization and binding of drug to nuclear structures and cellular organelles of sensitive and resistant cells. In a previous study, the fluorescence intensity of intracellular DNR paralleled measurements of uptake of radioactive drug (8). Anthracycline fluorescence is only partially quenched after binding to intact nuclear DNA and is a function of GC:AT base composition (16); both DNR and doxorubicin can stain chromosomes brightly (17) or in a banded pattern (18). The lack of nuclear staining by DNR in HL-60/AR cells is probably due to diminished access of the drug to nuclear targets. Also, anthracycline fluorescence is not readily quenched during its interaction with cellular membranes (19, 20). Minor degrees of quenching due to alterations in such membranes would not account for the striking differences between HL-60 and HL-60/AR fluorescent phenotypes.

Incubation of HL-60/AR cells with DNR during continuous metabolic inhibition by sodium azide/deoxyglucose does not prevent uptake of fluorescence into the Golgi apparatus. However, the subsequent shift of DNR fluorescence from the Golgi apparatus to lysosomes/mitochondria, and efflux of [14C]DNR both appear to be energy dependent. It is noteworthy that DNR fluorescence does not localize to the nucleus of metabolically inhibited HL-60/AR cells, suggesting that drug distribution and drug efflux are mediated by distinct biochemical mechanisms.

Lowering the temperature to 18 or 4°C arrests the glucose-dependent intracellular shift and efflux of DNR in HL-60/AR cells. The complete inhibition of the intracellular shift and efflux of DNR at 18 or 4°C, temperatures at which the fusion of vesicles is blocked (21), suggests that exocytosis is involved in the transport of DNR out of the cell from the Golgi apparatus.

These processes proceed independently of cytoskeletal movement or the activity of the sodium/hydrogen pump since neither

Fig. 7. Intracellular distribution of DNR in HL-60 cells at pH 5. Each photograph represents DVF images following the averaging of 15 frames as described in "Materials and Methods." A, 1-h incubation of HL-60 cells with 10 μM DNR at 37°C; B, 1-h incubation of HL-60/AR cells with 10 μM DNR at 37°C.
cytochalasin D (9), nor monensin (10), nor ouabain (11) affects the distribution or efflux of drug.

In HL-60/AR cells where DNR is arrested in the Golgi apparatus by sodium azide/deoxyglucose treatment, DNR efflux is completely inhibited at a temperature of 18°C even if glucose is restored. In contrast, in cells where the DNR fluorescence shift has been allowed to proceed from the Golgi apparatus to lysosomes/mitochondria, DNR efflux can no longer be completely inhibited by sodium azide/deoxyglucose or by lowering the temperature to 18°C. Thus, the lysosomal/mitochondrial pattern of DNR fluorescence denotes the functional redistribution of DNR into an intermediate exchange compartment from which efflux can occur without energy expenditure and without fusion of endocytic vesicles. This intermediate exchange compartment seems to be associated with the faint diffuse cytoplasmic fluorescence seen in addition to the lysosomal/mitochondrial punctate fluorescence of DNR in HL-60/AR cells. The lysosomal/mitochondrial pattern without the accompanying faint diffuse cytoplasmic fluorescence may represent a slow-exchange compartment for DNR, since it persists even after prolonged incubation in drug-free medium at 37°C.

Cellular toxins such as DNR, which possess an affinity for membranes, may be exocytosed as part of a general mechanism by which secretory proteins or recycled membranes are transported from the Golgi apparatus to the external cell surface (21). Alternatively, the Golgi vesicles can fuse with other vesicles and become intracellular, membrane-limited, storage reservoirs for the secretory proteins and recycled membrane components (22). DNR may be similarly transported via endocytic vesicles from the Golgi apparatus to the lysosomal/mitochondria or to the cell membrane. The speculation that DNR may be exocytosed is supported by the observations in anthracycline-resistant Ehrlich ascites tumor cell lines in which increased endocytic activity has been demonstrated (23).

The intracellular distribution of DNR may also be a function of drug hydrophobicity and whether it enters the cell by endocytosis or by diffusion. We examined the effect of altering extracellular pH, which is known to affect membrane permeability of DNR (24) on its intracellular distribution and pharmacokinetics. We found that lowering the extracellular pH, which favors the ionic form of DNR and affects its membrane permeability (3), caused the drug to distribute in a lysosomal/mitochondrial pattern in HL-60-sensitive cells, and was associated with enhanced drug efflux and resistance. Raising the pH to 8.0 favored the nonionized lipophilic form of DNR and its Fickian diffusion through the plasma membrane, and was associated with increased drug accumulation.

The increased efflux of DNR from HL-60/AR cells seems not to be mediated by any active transport protein that pumps drug out of the cell. The distinctive feature of active carrier transport, namely saturation with the substrate, is not observed in this system. In HL-60 and HL-60/AR cells exposed to a 5-log range (0.01–100 μm) of DNR, there is only a 40–75% difference in net drug accumulation (Fig. 3). If an active carrier transport mechanism were operating, there should be greater differences between sensitive and resistant cells at the lower concentrations.

Moreover, the degree of drug resistance maintained in the HL-60/AR line is far greater than that predicted by the differences in net drug accumulation. The amount of DNR needed to cause 50% growth inhibition of HL-60 and HL-60/AR cells is 17 and 2446 pmol/cell, respectively. This strongly indicates that in these cells, the intracellular distribution of DNR is a better correlate of drug resistance than either efflux magnitude or net drug accumulation.

Further evidence to support this hypothesis stems from studies of growth inhibition, distribution, accumulation, and retention performed at different extracellular pHs. Lowering the extracellular pH to 5 results in a 50-fold increase in the IC50 in both HL-60 and HL-60/AR cells. In parallel studies, there is a marked shift of DNR fluorescence toward the membranous pattern in both HL-60 and HL-60/AR cells. There appears to be a total absence of diffuse fluorescence in HL-60/AR cells; in contrast, HL-60 parent cells do maintain a residual cytoplasmic/nuclear fluorescence. Thus, at pH 5, HL-60 and HL-60/AR cells preserve their differences in terms of sensitivity to DNR and in terms of cytoplasmic/nuclear distribution. In contrast, the pharmacokinetics of DNR at pH 5 correlates poorly with resistance as it is nearly identical in HL-60 and HL-60/AR cells. The HL-60 cells acquire the HL-60/AR phenotype in the way they accumulate and retain DNR; whereas the HL-60/AR cells accumulate and retain DNR similarly at pH 5 and pH 7.4. Furthermore, increasing the accumulation of DNR into HL-60 and HL-60/AR cells by increasing extracellular pH fails to perturb the IC50, the distribution and retention differences between the HL-60 and HL-60/AR cells.

It may be that acquisition of resistance involves a change in membrane structure which alters intracellular partitioning of DNR, increasing the proportion of drug which is associated with cellular membranes. Duarte-Karim and Hildebrand (25) have shown that in vitro partitioning of anthracyclines between hydrophobic and hydrophilic organic solvent compartments is affected by the presence of substances such as cardiolipin which bind to the drug. The P-glycoprotein present in the membranes of some resistant cells is thought to bind to DNR (4) and may thus increase its partitioning into the membrane phase.

Willingham et al. (17) have previously found that P-glycoprotein containing KB cells distribute DNR in a different pattern as compared to the parental cell line. In our hands, parental KB cells distribute DNR in the nuclear fluorescence pattern only in the presence of a concentration of DNR that is lethal to the parent cell. Parental KB cells exposed to lower concentrations distribute DNR in a pattern similar to the one.
described in resistant cells. We have also previously shown that 
HL-60/AR cells distribute DNR in a punctate pattern rather 
than the diffuse-appearing pattern found in viable parental HL-
60 cells. Furthermore, agents such as verapamil cause anthra-
cyclines to redistribute from the membrane compartments to 
the nuclear/cytoplasmic compartments in HL-60/AR cells (12).

We previously reported that HL-60/AR cells show increased 
expression of two surface glycoproteins: the M₄, 160,000, and 
M₅, 120,000 glycoproteins (8). Our current studies demonstrate 
that the expression of these two glycoproteins may reflect 
decreased glycosylation of existing HL-60/AR glycoproteins, 
as they are inducible in HL-60 cells by treatment with tunica-
mycin, an inhibitor of N-linked glycan synthesis. Hypoglyco-
sylation of membrane proteins may increase their hydrophobic-
ity and may increase their affinity for DNR, causing a change 
in drug distribution. This possibility is being investigated in our 
laboratory.

In summary, the present study demonstrates that DNR is 
transported via different pathways in HL-60 and HL-60/AR 
cells. Passage of DNR across the cell membrane followed by 
distribution in cytoplasmic/nuclear compartments is associated 
with drug sensitivity. The retention of DNR within cell mem-
branes and its transport into the Golgi apparatus from which it 
is either exocytosed out of the cell or translocated into lys-
somes/mitochondria is associated with drug resistance. The 
techniques used in the current study should help elucidate the 
transport pathways of DNR and other fluorescent chemother-
apeutic agents in other anthracycline-resistant cell lines and in 
cells obtained from patients with clinically drug-resistant dis-
ease.

REFERENCES

2. Skovsgaard, T. Transport and binding of daunorubicin, Adriamycin, and 
222, 1977.
3. Daño, K. Active outward transport of daunomycin in resistant Ehrlich ascites 
5. Batist, G., Tulpule, A., Sinha, B. K., Kartki, A. G., Myers, C. E., and Cowen, 
K. H. Overexpression of a novel glutathione transferase in multidrug-resis-
topoisomerases as a basis for antineoplastic drug action. In: V. DeVita, S. 
Hellman, and S. Rosenberg (eds.), Important Advances in Oncology 1988, 
7. Vasanthakumar, G., and Ahmed, N. K. Contribution of drug transport and 
reductases to daunorubicin resistance in human myelocytic cells. Cancer 
8. Bhalla, K., Hindenburg, A., Taub, R. N., and Grant, S. Isolation and 
characterization of an anthracycline-resistant human leukemic cell line. Can-
11. Lawrence, T. S. Reduction of doxorubicin cytotoxicity by ouabain: correlation 
with topoisomerase-induced DNA strand breakage in human and hamster 
R. N. Effect of verapamil and other agents on the distribution of anthra-
13. Takasigi, M. An improved fluorochromic cytotoxic test. Transplantation 
14. Kielland, M. D., Steinman, R. M., and Cohn, Z. A. Intralysosomal accumu-
lation of polyamines: fusion of pinocytic and phagocytic vacuoles with 
15. Collins, J. M., and Foster, K. A. Differentiation of promyelocytic (HL-60) 
cells into mature granulocytes: mitochondrial-specific rhodamine-123 fluo-
Interaction of anthracyclines with DNA in chromosomes. Chromosoma 
17. Williamsam, M. C., Cornwell, M. M., Cardarelli, C. O., Gottesman, M. M., 
and Pastan, I. Single cell analysis of daunomycin uptake and efflux in 
multidrug-resistant and -sensitive KB cells: effects of verapamil and other 
18. Lin, C. C., and Van deSande, J. H. Differential fluorescence staining of 
human chromosomes with daunomycin and Adriamycin—the D-bands. Sci-
ence (Wash. DC), 190: 61–63, 1975.
anthracyclines bound to unilamellar phosphatidylcholine vesicles. Biochem-
of daunomycin to membrane domain structures by fluorescence resonance 
22. Pastan, I., and Willingham, M. D. Receptor-mediated endocytosis: coated 
23. Sehested, M., Skovsgaard, T., vanDuers, B., and Winther-Nielson, H. In-
crease in non-specific adsorptive endocytosis in anthracycline and Vinca 
alkaloid-resistant Ehrlich ascites tumor cell lines. J. Natl. Cancer Inst., 78: 
24. Goldman, I. D. Pharmacokinetics of Antineoplastic Agents at the Cellular 
Level. In: B. Chabner (ed.), Pharmacologic Principles of Cancer Treatment, 
pp. 15–44.
25. Duarte-Karim, R. M., and Hildebrand, J. Affinity of Adriamycin to phos-
Intracellular Distribution and Pharmacokinetics of Daunorubicin in Anthracycline-sensitive and -resistant HL-60 Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/16/4607

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.