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

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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/or 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. Inclusion 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 cytotoxic 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 (1). 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 (2, 3). 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 (2, 3).

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 (4). Other mechanisms of anthracycline resistance have been described, including protection against free radical damage (5), decreased levels or altered structure of topoisomerase II (6), and increased drug degradation (7). 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 relation 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 cells, namely, cross-resistance to vincristine and vinblastine, decreased drug accumulation, and increased drug efflux (8). The decreased drug accumulation, the increased drug efflux and resistance can be modulated in HL-60/AR cells by coinubating the cells in the presence of both DNR and verapamil (8). These cells have recently been shown not to overexpress the P-glycoprotein, although they possess other altered membrane glycoproteins (8).

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 (9), monensin (10), or ouabain (11). Because the degree of protonation of DNR affects its ability to pass through membranes, we analyzed the accumulation, distribution, retention, and cytostatic effects of DNR at extracellular pH 5.0, 7.4, and 8.0.

MATERIALS AND METHODS

Cells. HL-60 and HL-60/AR cell lines have been previously characterized in our laboratory (8); 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.


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3 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.
Drugs and Chemicals

Daunorubicin was purchased from Ives Laboratories, New York, NY; \(^{14} \text{C}\)-Daunorubicin (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 \( \times 10^5 \) cells/ml were incubated in the presence of various concentrations of DNR for 1 h at 37°C in 5% \( \text{CO}_2 \) atmosphere. Incubations were carried out at pH 5.4, 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-cm\(^2\) tissue flasks in a volume of 10 ml at 5–10 \( \times 10^5 \) cells/ml.

Accumulation and Retention Studies

Dose. Cells at a concentration of 4 \( \times 10^5 \) cells/ml were incubated for 1 h in media, pH 7.4, with various concentrations of \(^{14} \text{C}\)-Daunorubicin at 37°C in 5% \( \text{CO}_2 \) atmosphere. Following incubation, equal aliquots of 2 \( \times 10^5 \) cells were removed and centrifuged through silicon/mineral oil, solubilized, and the amount of radioactivity was determined (8).

Kinetic Studies. Cells at a concentration of 1 \( \times 10^5 \) cells/ml were incubated in the presence of 1 \( \mu \text{M} \) \(^{14} \text{C}\)-Daunorubicin in media, pH 5, pH 7.4, and pH 8.0 at 37°C in 5% \( \text{CO}_2 \) 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 radioactive content was determined.

Retention. Cells at a concentration of 4 \( \times 10^5 \) cells/ml were incubated at 37°C in 5% \( \text{CO}_2 \) atmosphere for 1 h with 1 \( \mu \text{M} \) \(^{14} \text{C}\)-Daunorubicin at pH 5, 7.4, or 8.0. These were washed at 4°C and resuspended in drug-free media, pH 7.4, for 30 min at 37°C in 5% \( \text{CO}_2 \) 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 \( \mu \text{M} \)), ouabain (1 \( \mu \text{M} \)), or cytochalasin D (5 \( \mu \text{g/ml} \)) prior to the addition of 1 \( \mu \text{M} \) \(^{14} \text{C}\)-Daunorubicin. After a 1-h incubation with DNR at 37°C in 5% \( \text{CO}_2 \) 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 40 mM glucose was added. The retention of \(^{14} \text{C}\)-Daunorubicin 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 \( \times 1200 \) are electronically amplified by an 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 \( \mu \text{M} \) DNR in media at pH 5, 7.4, or 8.0 for 1 h at 37°C in 5% \( \text{CO}_2 \) atmosphere. They were centrifuged and resuspended in 10 \( \mu \text{l} \) 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.

Results

Incubations with 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 \( \mu \text{g/ml} \), rhodamine-123, 5 \( \mu \text{g/ml} \), acridine orange, 1 \( \mu \text{g/ml} \), or NBD-ceramide, 10 \( \mu \text{g/ml} \), in PBS for 1 h at 37°C at pH 7.4. Cells were centrifuged and resuspended in 10 \( \mu \text{l} \) 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.

Accumulation of \(^{14} \text{C}\)-Daunorubicin

HL-60/AR cells exposed to 0.5–10 \( \mu \text{M} \) DNR for 5–10 min acquire a peripheral and perinuclear fluorescence similar to the plasma membrane/Golgi apparatus fluorescence 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 (HL-60/AR cells) (Fig. 2A). The fluorescence of the Golgi apparatus becomes more pronounced 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).

Intracellular Distribution of DNR

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 \( \mu \text{M} \) DNR. HL-60 cells exposed to 0.5–2.5 \( \mu \text{M} \) DNR for 5–60 min show a diffuse pattern of fluorescence (Fig. 2A) similar to the cytoplasmic and nuclear fluorescence of DNR. At higher concentrations (>3.0 \( \mu \text{M} \) 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 \( \mu \text{M} \) 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 \( \mu \text{M} \)) 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 \(^{14} \text{C}\)-Daunorubicin

HL-60/AR cells accumulate less \(^{14} \text{C}\)-Daunorubicin 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.
In the parent HL-60 cells, retention of \(^{14}\text{C}\)DNR is not affected by sodium azide/deoxyglucose, or by changing the temperature of the efflux medium to 18 or 4°C each has an equal partial inhibitory effect on the efflux of \(^{14}\text{C}\)DNR.

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 \(^{14}\text{C}\)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 \(^{14}\text{C}\)DNR.

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In the parent HL-60 cells, retention of \(^{14}\text{C}\)DNR is not affected by sodium azide/deoxyglucose, or by changing the temperature of the efflux medium (Fig. 5). Incubation of HL-60 cells with sodium azide/deoxyglucose or lowering their temperature is without effect.
31 ± 2%, respectively. These data indicate that there is no
effect on drug retention following incubation with cytochalasin
D, monensin, or ouabain in both HL-60 and HL-60/AR cells.

Kinetics of $[^{14}C]$DNR and DNR Fluorescence at pH 5 and 8.
In both HL-60 and HL-60/AR cells, the initial uptake of $[^{14}C]$DNR is slowed at pH 5, increases at pH 7.4, and is maximal
at pH 8 (Fig. 4). At the end of a 1-h incubation at pH 5, both
HL-60 and HL-60/AR cells accumulate equal amounts of
DNR, which is less than that accumulated by HL-60 cells at
pH 7.4. Also, at pH 5.0 in HL-60 cells as well as HL-60/AR
cells, the diffuse fluorescence of DNR is decreased, whereas the
membrane and lysosomal/mitochondrial fluorescence remain
the same. As a result, HL-60 cells assume a slightly punctate
pattern, while the HL-60/AR cells become even more punctate
(Fig. 7). Also at pH 5, there is decreased retention of $[^{14}C]$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 fluores-
cence (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 IC$_{50}$ for DNR
(1-h exposure) in both HL-60 and HL-60/AR cells. The 200-
fold difference in IC$_{50}$ for DNR between the two cell lines is
therefore preserved (Table 1). At pH 8, the IC$_{50}$ of DNR for
HL-60 and HL-60/AR cells is only mildly increased; the differ-
ce between HL-60 and HL-60/AR cells is maintained. Cor-
relation 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 IC$_{50}$ for DNR. In contrast, HL-60/AR
cells incubated at pH 5 at which they exhibit the least cyto-
plasmic/nuclear fluorescence (Fig. 7), have the highest IC$_{50}$
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 accumu-
lates 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 differ-
ences 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 chro-
omes 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, anthra-
cycline fluorescence is not readily quenched during its interac-
tion 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. How-
ever, the subsequent shift of DNR fluorescence from the Golgi
apparatus to lysosomes/mitochondria, and efflux of $[^{14}C]$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 mech-
nisms.

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 appa-
ratus.

These processes proceed independently of cytoskeletal move-
ment or the activity of the sodium/hydrogen pump since neither

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![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.](image-url)
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 mitochondrial pattern 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/mitochondrial 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 (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

### Table 1
Comparison of IC50 values of DNR in HL-60 and HL-60/AR cells at various extracellular pH values

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<th>pH</th>
<th>IC50 (μM)</th>
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<tr>
<td>5</td>
<td>2.9 ± 0.9</td>
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<tr>
<td>7.4</td>
<td>0.11 ± 0.02</td>
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<tr>
<td>8</td>
<td>0.076 ± 0.016</td>
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<td>21 ± 10</td>
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* Mean ± SD of at least four separate experiments.

Fig. 8. Retention of [14C]DNR in HL-60 and HL-60/AR cells incubated with DNR at different pHs. Cells were incubated at 37°C for 1 h with 1 μM [14C]DNR at pH 5, pH 7.4, or pH 8. They were washed and resuspended in drug-free media, pH 7.4, for 30 min at 37°C. Columns, percentage of the labeled drug retained at 30 min relative to the net accumulation at 1 h.
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 anthracyclines 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 M, 160,000, and M, 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 tunicamycin, an inhibitor of N-linked glycan synthesis. Hypoglycosylation of membrane proteins may increase their hydrophobicity 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 membranes and its transport into the Golgi apparatus from which it is either exocytosed out of the cell or translocated into lysosomes/mitochondria is associated with drug resistance. The techniques used in the current study should help elucidate the transport pathways of DNR and other fluorescent chemotherapeutic agents in other anthracycline-resistant cell lines and in cells obtained from patients with clinically drug-resistant disease.

REFERENCES

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


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