# A Chemical Strategy To Manipulate the Intracellular Localization of Drugs in Resistant Cancer Cells†

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ABSTRACT: A number of multidrug-resistant (MDR) cancer cells have been shown to have acquired an increased capacity to sequester weakly basic anticancer drugs in their lysosomes relative to drug-sensitive counterparts. In this report we have comparatively evaluated the concentrations of the anticancer agent daunorubicin (DNR) in intracellular compartments of drug-sensitive and MDR HL-60 cell lines, both of which do not express common efflux transporters such as P-glycoprotein at the plasma membrane. Our results suggest that lysosomal sequestration plays a significant role in the emergence of MDR since it effectively limits the drug's ability to interact with target molecules located in the nucleus. Using a series of weakly basic structural isomers with variable basicity, we illustrate that the magnitude of the p$K_a$ value correlates with the degree of lysosomal sequestration. Accordingly, a series of structurally modified forms of DNR with reduced basicity were synthesized, and their intracellular distribution was evaluated. Consistent with model compounds, derivatives of DNR with lowered p$K_a$ values showed visibly reduced lysosomal sequestration in two separate MDR cell lines. Collectively, this work highlights the importance of understanding the intracellular localization of drugs and proposes a rational strategy to manipulate it.

The resistance of tumor cells to anticancer agents continues to be a major cause of treatment failure in patients with cancer. Multidrug resistance (MDR)[1] is a term used to describe a resistance phenotype in which cancer cells become simultaneously resistant to different drugs with no obvious structural similarities or mechanisms of action. The emergence of MDR is multifactorial (*1*). Decreased drug accumulation and/or increased efflux, increased detoxification, increased DNA repair, and altered cell cycle regulation have all been implicated. Of these mechanisms, the overexpression of proteins involved in drug efflux (i.e., P-glycoprotein) at the plasma membrane has received a considerable amount of attention. Consistent with this mechanism, several MDR cell lines have been shown to accumulate significantly lower amounts of drug relative to their drug-sensitive counterparts (*2*). To increase the effectiveness of drugs in MDR cell lines, investigators have evaluated strategies whereby drugs were coadministered with transporter inhibitors. Such inhibitors have been shown to be effective in increasing anticancer drug accumulation in cells (*3*); however, proportional increases in drug activity are not typically observed (*4*−*6*). These findings suggest that alternative intracellular mechanisms play an important role in the MDR phenotype.

Interestingly, a number of MDR cancer cell lines have been shown to be resistant to anticancer agents even though they accumulate similar amounts of drug relative to drug-sensitive lines (*7*). Such MDR lines have also been shown to have an enhanced capacity to sequester weakly basic anticancer agents in their lysosomes, a process that does not appear to occur in drug-sensitive cell lines (*8*, *9*). Similar drug sequestration phenotypes have been documented in more than 20 different MDR cell lines; however, the sites and mechanisms for sequestration have not been fully characterized for the majority of these cell lines (*9*).

The driving force for the accumulation of weakly basic drugs in lysosomes of MDR cells is poorly understood and controversial. What is clear is that the lysosomal sequestration in MDR cells has been shown to occur only when lysosomes are sufficiently acidified. We and others have shown that MDR cells treated with agents that cause lysosomal pH to increase subsequently lose their ability to sequester drugs (*10*, *11*). Moreover, the reason that drug-sensitive cancer cell lines do not sequester weakly basic molecules is presumably due to the fact that they have defective lysosomal acidification (*11*).

Several drug sequestration mechanisms have been postulated that are consistent with the previously stated pH dependency. The most obvious one involves pH partitioning (also referred to as ion trapping). The theoretical basis of this phenomenon in lysosomes of nontransformed cells was initially described by de Duve and colleagues (*12*). Briefly, weak bases that exist to a significant extent in the un-ionized state in the cell cytosol can passively diffuse across the lysosomal lipid bilayers and enter the luminal domain. Once inside the lysosome, the low pH environment favors protonation of the base. In this ionized state, the drug is relatively

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[1] Abbreviations: MDR, multidrug resistance; DNR, daunorubicin; 8-AQ, 8-aminoquinoline; 3-AQ, 3-aminoquinoline; 5-AIQ, 5-aminoisoquinoline; 1-AIQ, 1-aminoisoquinoline; 4-AQ, 4-aminoquinoline; PC, partition coefficient; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MS, mass spectrometry; LC, liquid chromatography; HPLC, high-performance liquid chromatography.

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membrane impermeable and unable to diffuse back out. In addition to this mechanism, others have shown that pH-dependent drug transporter proteins are sometimes associated with lysosomes and can participate in the accumulation of drugs in this organelle (*13*, *14*). Finally, strong ionic associations between a cationic drug molecule with negatively charged molecules associated with lysosomes have also been proposed and would be pH dependent (*15*−*17*). Regardless of the mechanism, the sequestration event represents a potentially important factor in the MDR phenotype since it may serve to limit drug accumulation in other cellular compartments housing drug targets.

In this report we have established the importance of lysosomal sequestration on drug resistance in the MDR HL-60 cell line using quantitative assays to determine relevant intracellular concentrations of drugs. We have previously shown that MDR HL-60 cells do not express the common efflux transporters P-glycoprotein and the multidrug resistance associated protein, MRP1, on the plasma membrane (*10*). Using a series of structural isomers with varied p$K_a$ values, we demonstrate that the degree of basicity directly correlates with lysosomal sequestration tendency. Moreover, analogues of the weakly basic anticancer agent daunorubicin (DNR) were synthesized that have reduced basicity and were also shown to have reduced lysosomal sequestration. The potential application of this information into drug design or modification strategies aimed at combating MDR is discussed.

## MATERIALS AND METHODS

The human acute promyelocytic leukemia cell line HL-60 and the doxorubicin-selected drug-resistant MDR HL-60 cell lines were kindly provided by Dr. Yueshang Zhang (Arizona Cancer Center, University of Arizona). The chronic myelogenous leukemia cell line K-562 and the doxorubicin-selected drug-resistant MDR K-562 cell lines were kindly provided by Dr. Jeffrey Kanofsky (Medical Service, Edward Hines, Jr., Department of Veterans Affairs Hospital, IL). All cell lines were grown in RPMI 1640 medium supplemented with 10% bovine calf serum, 0.1% penicillin, 0.1% streptomycin, 10 mM Hepes, and 1 mM sodium pyruvate and maintained at a density of (1−2) × $10^6$ cells/mL at 37 °C in a humidified 5% $CO_2$ atmosphere.

Daunorubicin was purchased from Oakwood Products Inc. (West Columbia, SC). 8-Aminoquinoline (8-AQ), 3-aminoquinoline (3-AQ), 5-aminoisoquinoline (5-AIQ) ,and 1-aminoisoquinoline (1-AIQ) were purchased from Sigma (St. Louis, MO). 4-Aminoquinoline (4-AQ) was purchased from Tyger Scientific (Princeton, NJ). All other reagents and solvents were purchased from Fisher Scientific Inc. (Springfield, NJ).

Analytical thin-layer chromatography (TLC) was performed on precoated silica gel plates ($60F_{254}$) from EM Science (Gibbstown, NJ), and flash chromatography on silica gel with 40−60 μm particle size and 60 Å pore size. TLC spots were detected by UV light and by staining with phosphomolybdic acid (Fisher Scientific Inc., Springfield, NJ). NMR analysis was performed on Bruker Avance 400 and 500 MHz (equipped with a dual C/H cryoprobe) NMR spectrometers. The molecular mass of each synthesized compound was determined using high-resolution ESI-MS performed with Waters Micromass LCT premier at The University of Kansas Mass Spectrometry Lab.

The purity of synthesized daunorubicin analogues was determined by reversed-phase HPLC (Beckman System Gold) using a Phenomenex C-18 column (3 mm i.d. × 150 mm; 5 μm particle size). An isocratic mobile phase comprised of 65% methanol in water containing 0.1% each of acetic acid and triethylamine (pH 4.5) was used at a flow rate of 0.7 mL/min. Compounds were detected using a Beckman Programmable detector (Module 166) at 254 nm. The concentrations of the compounds were determined using a Shimadzu Bio-Mini spectrophotometer.

*Synthesis of N-(Acetylamidomethyl)daunorubicin (**1**).* Acetamide (12 mg, 0.20 mmol) was dissolved in MeOH (500 μL) and further diluted with $CH_2Cl_2$ (500 μL). To this solution was added 15 μL of 37% aqueous formaldehyde solution, and reaction was stirred for 15 min at 25 °C. Daunorubicin hydrochloride (25 mg, 0.04 mmol) in MeOH (250 μL) was added to this reaction, and stirring was continued overnight. The reaction was monitored by analytical thin-layer chromatography ($SiO_2$, 15% MeOH/$CH_2Cl_2$, 1 drop of triethylamine, $R_f$ = 0.5). The crude product was purified by flash chromatography on silica gel (10% MeOH/$CH_2Cl_2$, 5 drops of triethylamine), yielding 17 mg (65%) of **1** as a red solid: $^1$H NMR (400 MHz, $CD_3OD$) δ 7.87 (d, $J$ = 8 Hz, 1H), 7.74 (t, $J$ = 8.0 Hz, 1H), 7.48 (d, $J$ = 8.0 Hz, 1H), 5.35 (s, 1H), 5.01 (s, 1H), 4.20 (m, 3H), 3.93 (s, 3H), 3.67 (s, 1H), 2.94 (q, $J$ = 8 and 16 Hz, 2H), 2.25 (s, 3H), 2.06 (dd, $J$ = 4 and 12 Hz, 1H), 1.7−1.9 (m, 7H), 1.19 (d, $J$ = 8 Hz, 3H); $^{13}$C NMR (500 MHz, $CD_3OD$) δ 211.8, 186.9, 186.6, 172.7, 161.1, 156.0, 154.8, 135.7, 135.0, 134.5, 134.2, 120.2, 119.0, 118.8, 111.0, 110.7, 100.0, 75.8, 70.2, 66.5, 55.6, 50.6, 49.0, 51.0, 35.0, 31.7, 27.3, 22.9, 22.0, 15.6; HRMS (ESI) $[MH]^+$ calculated 599.2241, found 599.2239 (see Supporting Information, Figure 1Sa); degree of purity, HPLC, retention time of 9.6 min, >97% (see Supporting Information, Figure 2Sa).

*Synthesis of N-(Trifluoroacetyl)daunorubicin (**2**).* Compound **2** was synthesized according to a previously described procedure (*18*). Daunorubicin hydrochloride (50 mg, 0.088 mmol) was stirred in dry pyridine (600 μL) at −20 °C ($CCl_4$/dry ice bath) for 15 min. Trifluoroacetic anhydride (9 μL, 0.088 mmol) in anhydrous ether (100 μL) was added dropwise over a 2 min period. Reaction was monitored by TLC ($SiO_2$, 4% MeOH/$CH_2Cl_2$, 1 drop of triethylamine, $R_f$ = 0.31). After completion of the reaction, 1 mL of water was added, and stirring was continued for an additional 15 min. The reaction mixture was extracted with ethyl acetate (2 × 5 mL), and the extracts were washed with water (2 × 2 mL). After being dried over $Na_2SO_4$, ethyl acetate solution was filtered and evaporated to dryness. The residue was dissolved in $CHCl_3$, and solid **2** was precipitated by the addition of petroleum ether (52 mg, 95%): $^1$H NMR (400 MHz, $CDCl_3$) δ 13.87 (s, 1H), 13.10 (s, 1H), 7.89 (d, $J$ = 8.0 Hz, 1H), 7.68 (t, $J$ = 8.0 Hz, 1H), 7.30 (d, $J$ = 8.0 Hz, 1H), 6.75 (d, $J$ = 8.4 Hz, 1H), 5.43 (d, $J$ = 3.6 Hz, 1H), 5.12 (q, 1H), 4.16 (m, 2H), 3.9 (s, 3H), 3.6 (s, 1H), 3.1 (d, $J$ = 18.8 Hz, 1H), 2.70 (d, $J$ = 18.8 Hz, 1H), 2.33 (s, 3H), 2.24 (d, $J$ = 16 Hz, 1H), 2.05 (dd, $J$ = 14.8 and 4.4 Hz, 1H), 1.7−1.9 (m, 2H), 1.25 (d, $J$ = 10 Hz, 3H); $^{13}$C NMR (400 MHz, $CDCl_3$) δ 186.9, 186.5, 160.9, 156.6, 156.3, 155.6, 135.7, 135.3, 134.2, 133.7, 120.6, 119.7, 118.4, 117.4,

114.5, 111.4, 111.2, 100.4, 70.3, 68.7, 66.7, 56.5, 46.2, 45.7, 35.0, 33.3, 29.5, 24.8, 16.6; HRMS (ESI) [M$^-$H] calculated 622.1536, found 622.1570 (see Supporting Information, Figure 1Sb); degree of purity, HPLC, retention time of 10.45 min, >98% (see Supporting Information, Figure 2Sb).

*p$K_a$ Determination of* ***1***. The p$K_a$ determination of **1** was carried out using $^1$H NMR as follows. Compound **1** (5 mg, 0.008 mmol) was dissolved in $D_2O$ (0.5 mL), and 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (0.3 mM) was used as an internal standard. The pD of the solution was adjusted by the addition of small amounts (1−5 $\mu$L) of 1% DCl or NaOD and recorded. Using this technique, the $^1$H NMR spectra for **1** were acquired on a 400 MHz Bruker instrument as a function of pD. Spectra were obtained from a total of nine apparent pD values ranging from 2.5 to 10. pD values were converted to pH and plotted against the chemical shift for the methylene protons attached to the 3′-amino group of DNR (*19*). The data were curve fit using Sigma Plot 2001 with the Hill equation (three parameters), and the p$K_a$ value was obtained. See Figure 3S of the Supporting Information for a plot of the data.

*Determination of Octanol−Water Partition Coefficients for Model Compounds.* Experimentally determined p$K_a$ values for the model compounds (Figure 2) have been reported by Perrin (*20*). The partition coefficients for each compound were determined at three pH units above and below the corresponding p$K_a$ value to obtain the intrinsic partition coefficient of the un-ionized ($PC_u$) and ionized ($PC_i$) base, respectively. A shake-flask method previously described by our laboratory was used (*21*). Briefly, each compound was dissolved to a final concentration of 10 $\mu$M in 10 mL of an aqueous buffer containing 5 mM Hepes, 5 mM ammonium acetate, 5 mM KCl, and 154 mM NaCl (300 mOsm; adjusted to the desired pH with 0.1 N NaOH or 0.1 N HCl). 1-Octanol (0.5−1.0 mL) was added, and the two-phase system was shaken (150 oscillations/min) for 30 min at 25 °C. Phases were separated by centrifugation at 1000*g* for 10 min. Aliquots of each phase were appropriately diluted and assayed for compound using a Shimadzu Bio-Mini spectrophotometer. The ratio of $PC_i$ to $PC_u$ was defined as the $\alpha$ value for a given compound. Log *P* values for test compounds were obtained by taking the common logarithm of the corresponding $PC_u$.

*Fluorescence Microscopy.* Cells were incubated with 1 $\mu$M DNR or one of its derivatives for 2 h under normal culture conditions. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4, to remove unincorporated drug and viewed under a Nikon Eclipse 80*i* microscope equipped for epifluorescence. A rhodamine filter set was used to view the fluorescence from compounds. Images were captured using an Orca ER camera (Hamamatsu Corp.) controlled by MetaMorph version 6.2 (Universal Imaging Corp.).

*Cytotoxicity Assay.* Cell sensitivity to daunorubicin and its derivatives was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (*22*). Briefly, cells were seeded in triplicate in a 96-well culture plate at a density of $2 \times 10^4$ cells per well and were treated with selected concentrations of compound and incubated for a period of 72 h. At the end of the incubation period, 10 $\mu$L of a 5 mg/mL solution of MTT in PBS was added to each well, and the plates were returned to the incubator for an additional 4 h. A 100 $\mu$L portion of DMSO was added to each well, and the absorbance was measured at a wavelength of 590 nm in a FluoStar Galaxy microplate reader (BMG Labtechnologies Inc., Durham, NC). $IC_{50}$ was defined as the concentration of drug causing 50% inhibition of cell growth as compared with untreated control.

*DNR Accumulation in Nuclei.* Cells ($125 \times 10^6$) were incubated with 1 $\mu$M DNR for 2 h. Following the incubation period cells were washed twice with ice-cold PBS, and nuclei were isolated using a procedure previously described by our laboratory (*23*). Isolated nuclei were resuspended in 750 $\mu$L of acetonitrile, sonicated for 10 min, and centrifuged at 16000*g* for 5 min. A 500 $\mu$L portion of the supernatant was evaporated to dryness using a DyNA Vap centrifugal evaporator (Labnet International Inc., Woodbridge, NJ). The residue thus obtained was dissolved in the appropriate HPLC mobile phase and analyzed for DNR using LC-MS. The nuclear concentration of DNR was obtained by dividing the amount of DNR extracted by the total nuclear volume (0.023 pL) in the isolated fraction (*23*).

*Determination of Lysosomal Accumulation of Model Compounds.* Cells ($200 \times 10^6$) were incubated with 10 $\mu$M compound for 24 h. Subsequently, intact lysosomes were isolated from the cells using a magnetic chromatographic approach previously described by our laboratory (*24*). Isolated lysosomal fraction was acidified with 0.1% formic acid, vortexed with 600 $\mu$L of acetonitrile for 20 s, and centrifuged at 16000*g* for 5 min. The supernatant was evaporated to dryness, and the residue was analyzed using LC-MS to quantitate the amount of compound extracted. This amount was divided by the total lysosomal volume in the isolated fraction (32.2 nL) to obtain the lysosomal concentration of the compound (*25*). The procedure for determining drug concentrations in cell cytosol has been previously described by our laboratory (*23*). Lysosomal concentrations of compounds were normalized to the respective cytosolic concentrations to account for intrinsic permeability differences between compounds.

*LC-MS Analysis of Compounds.* An integrated Agilent 1100 series capillary liquid chromatography system (comprised of pump, inline degasser, column thermostat, and autosampler) coupled with an API 2000 (Applied Biosciences) triple quadrupole mass spectrometer was employed to quantitate drug extracted from isolated cellular fractions. The mass spectrometer was equipped with a Turbo Ionspray ionization source and operated in the positive mode. Detection was by multiple reaction monitoring (MRM). A Waters Xterra MS $C_{18}$ column (100 × 1 mm, 3.5 $\mu$m particle size) was used. Injection volumes were 5 $\mu$L for all samples.

The mobile phase compositions (organic/aqueous) for the test compounds were as follows: acetonitrile/0.1% formic acid for DNR and 1-AIQ, acetonitrile/10 mM ammonium acetate (pH 8.0) for 3-AQ and 5-AIQ, and methanol/0.1% formic acid for 8-AQ and 4-AQ. Mobile phases A and B contained 2% and 95% organic solvent, respectively. The remaining separation conditions and detection parameters for each compound are shown in Figure 4S and Table 1S of the Supporting Information.

## RESULTS

*Intracellular Distribution and Activity of Daunorubicin.* In order for a drug to elicit a pharmacological response, it

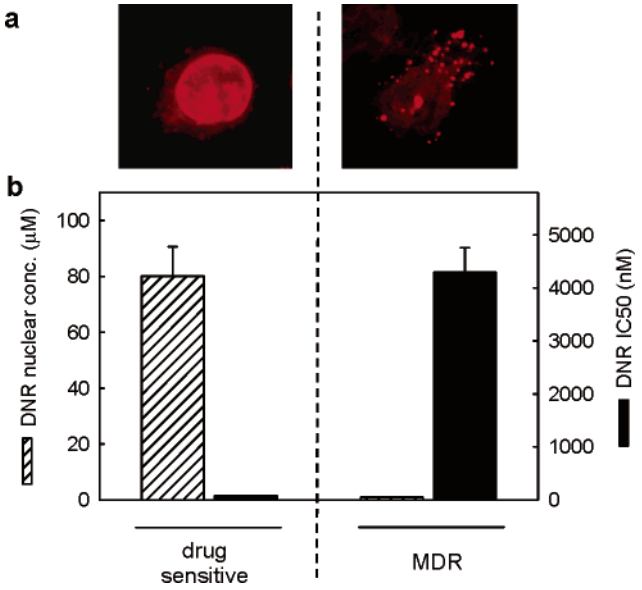



FIGURE 1: Intracellular localization and activity of DNR in drug-sensitive (left side of dashed line) and MDR (right side of dashed line) HL-60 cell lines. (a) Fluorescence micrographs of cells incubated with drug, indicating differential intracellular distributions. (b) Daunorubicin concentration in the nuclei of cells ($\mu$M) and activity of the drug in cells represented as $IC_{50}$ for cytotoxicity (nM).

must be sufficiently concentrated in the local environment immediately surrounding the target molecule. For the anticancer agent daunorubicin, the proposed target molecules (i.e., DNA and topoisomerase II) are localized within the cell nucleus. Correspondingly, the sequestration of DNR in lysosomes would be expected to decrease the amount of drug available to interact with its nuclear targets. Since DNR is a fluorescent molecule, intracellular distribution differences between drug-sensitive and MDR HL-60 cells can be visualized using a fluorescence microscope (Figure 1a). From these micrographs it is apparent that DNR is predominantly contained within the nucleus in the drug-sensitive cell. However, the accumulation of the drug in the nuclear compartment of MDR cells is visibly reduced, presumably due to the pronounced sequestration in perinuclear vesicles that we have previously shown to be lysosomes (*10*). Despite the fact that the MDR phenotype is considered multifactorial, it is possible that the reduced effectiveness of DNR could be rationally explained by the reduced nuclear concentration of the drug in the MDR cell line relative to the drug-sensitive counterpart. To evaluate this possibility, we quantitatively evaluated the concentration of DNR in isolated nuclei from MDR and drug-sensitive HL-60 cells following incubations with identical concentrations of daunorubicin. In Figure 1b we show that the nuclear concentrations of DNR are approximately 85 times higher in drug-sensitive cells than is observed with the MDR cell line. Also in Figure 1b, we show the corresponding $IC_{50}$ values for DNR in these cell lines. Interestingly, we observed a 56-fold reduction in drug sensitivity in the MDR cell line compared to the drug-sensitive cell line, which is comparable to the fold reduction in nuclear concentration. These findings are consistent with the important role that lysosomes play in the MDR phenotype in this cell line.

*Substrate Specificity for Lysosomal Sequestration.* We have previously evaluated how permeability characteristics

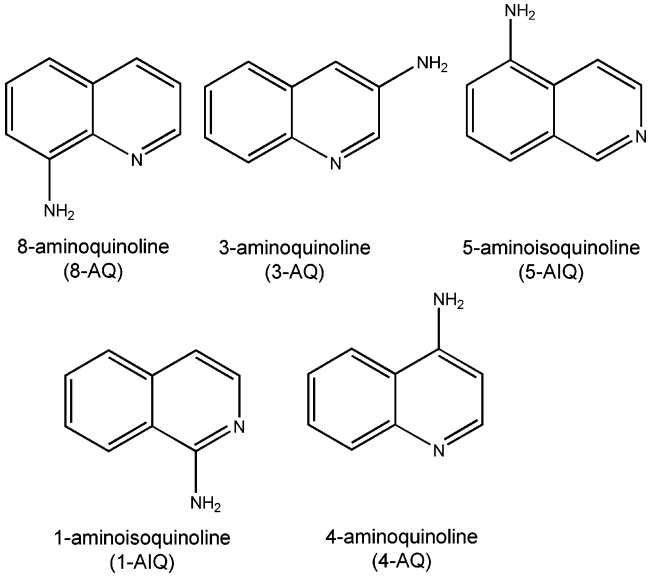



FIGURE 2: Structures of model weakly basic amines.

Table 1: Physicochemical Properties of DNR and Model Amines

| compound[a] | log $P^{b,c}$ | $\alpha^{b,c}$ | p$K_a{}^b$ |
|---|---|---|---|
| DNR | 2.03 ± 0.03 | 0.0210 ± 0.0030 | 8.4 |
| 8-AQ | 1.55 ± 0.09 | 0.0078 ± 0.0022 | 4.0 |
| 3-AQ | 1.48 ± 0.05 | 0.0034 ± 0.0008 | 5.0 |
| 5-AIQ | 1.26 ± 0.05 | 0.0077 ± 0.0006 | 6.0 |
| 1-AIQ | 1.41 ± 0.07 | 0.0075 ± 0.0008 | 7.4 |
| 4-AQ | 1.65 ± 0.01 | 0.0034 ± 0.0007 | 9.0 |

[a] For structures see Figures 2 and 4. [b] Experimentally determined values. [c] Values are the means of three determinations ± SD.

of weakly basic compounds influenced their intracellular distribution behavior (*21*). In this work we identified a unique permeability parameter associated with amines, referred to as α, which predictably influenced intracellular distribution behavior. The α value for an amine represents the ratio of the partition coefficients in its ionized form over its value in the un-ionized state. We had evaluated a series of weakly basic compounds that all had similar p$K_a$ values near neutrality but had varied α values (ranging from 0 to 1). Weakly basic molecules with low α (near 0) were shown to specifically accumulate into lysosomes; conversely, those with high α (near 1) specifically accumulated in mitochondria.

The focus of the present work is to evaluate the influence of p$K_a$ on weak base sequestration in lysosomes. To successfully isolate the influence of p$K_a$, compounds employed in this evaluation need to have similar structure, similar lipophilicity (log *P*), similar and low α, and substantially different p$K_a$ values. The structures of a series of amine-containing structural isomers utilized in this work are shown in Figure 2. Table 1 contains experimentally determined physicochemical information on these compounds. As can be seen, this group of molecules fits the aforementioned criteria quite nicely. The experimentally determined ratio of drug concentrations in lysosomes versus the cell cytosol for each of the model compounds is shown in Figure 3. These data illustrate a strong correlation between the p$K_a$ of the molecule and the magnitude of lysosomal sequestration.

*Intracellular Distribution and Activity of Structurally Modified Forms of DNR.* Two structural derivatives of DNR

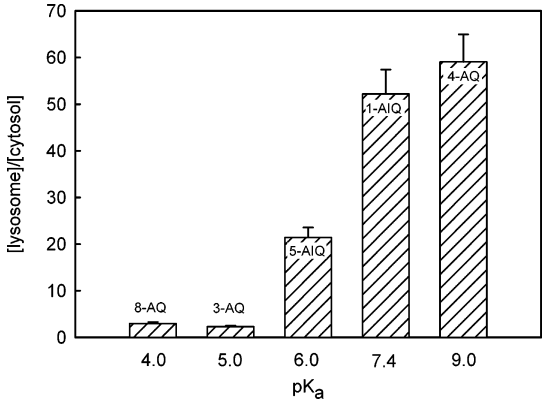
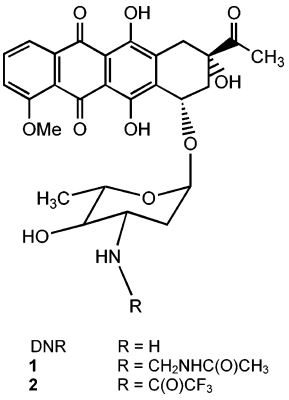



FIGURE 3: Lysosome to cytosol concentration ratios as a function of p$K_a$ value for model weakly basic amines in the MDR HL-60 cell line.




| | |
|---|---|
| DNR | R = H |
| **1** | R = $CH_2NHC(O)CH_3$ |
| **2** | R = $C(O)CF_3$ |

FIGURE 4: Structures of DNR and two synthesized analogues (**1** and **2**) with lowered p$K_a$ values (see Table 2).

Table 2: p$K_a$ and $IC_{50}$ Values for DNR and Its Analogues in HL-60 Cells

| | | $IC_{50}$ | |
|---|---|---|---|
| compound[a] | p$K_a$[b] | sensitive cells | MDR cells |
| DNR | 8.4 | 76.6 ± 4.3[c] | 4292 ± 471[c] |
| **1** | 6.9 | 680 ± 170[c] | 3100 ± 550[c] |
| **2** | n/a[d] | >10000 | >10000 |

[a] For structures see Figure 4. [b] Experimentally determined values. [c] Values are the means of three determinations ± SD. [d] Compound is not considered to be a basic molecule.

were synthesized in order to evaluate if modifications of its p$K_a$ value could lead to a reduction in lysosomal sequestration capacity in MDR cancer cells. The modifications were intended to lower the p$K_a$ value of DNR (p$K_a$ 8.4) with minimum perturbation to the overall structure. The structures and experimentally determined p$K_a$ values for DNR and the two synthesized analogues (**1** and **2**) are presented in Figure 4 and in Table 2. The intracellular distribution of these molecules in both HL-60 and K-562 cells is shown in Figure 5. Compound **1**, which has a slightly reduced p$K_a$ of 6.9 (see Figure 3S of Supporting Information), is still sequestered into lysosomes of MDR cells; however, the degree of which appears to be reduced. The acylation of the 3′-amine of DNR to create **2** abolishes the basic nature of the parent amine and also halts lysosomal sequestration in MDR cells.

The distribution of DNR and its analogues in drug-sensitive cell lines is also shown in Figure 5. The observed intracellular distribution of the compounds in the sensitive cells provides qualitative information regarding the DNA

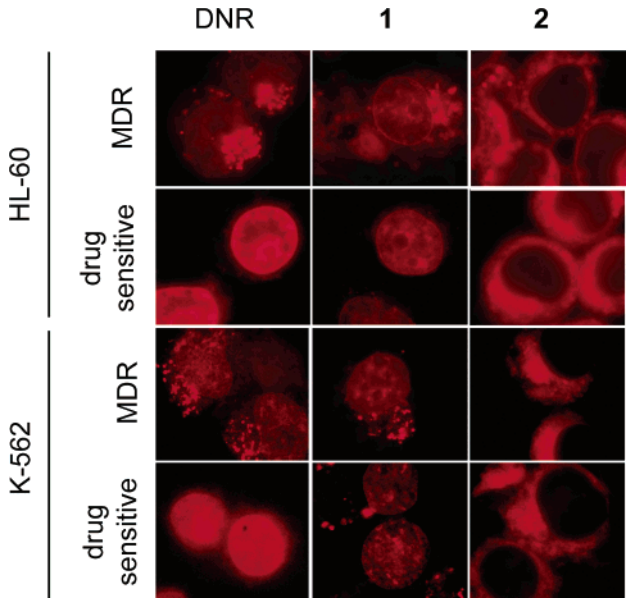



FIGURE 5: Fluorescence micrographs illustrating the intracellular distribution of DNR and its analogues with reduced basicity (**1** and **2**) in drug-sensitive and MDR cancer cell lines.

binding affinity of compounds. It is well-known that fluorescent compounds with high DNA binding affinity show preferential distribution in the nuclear compartment when incubated with cells (*9*). Accordingly, DNR is known to have high affinity to DNA and shows strong localization in the nuclear compartment of sensitive cells. Alternatively, **2** is not associated with the nuclear compartment in the drug-sensitive cell lines to any visible extent, which suggests a loss of DNA binding affinity. Compound **1**, on the other hand, appears to have retained some capacity to bind DNA; however, the degree of which appears to be slightly reduced relative to DNR.

The $IC_{50}$ values for DNR and its analogues with HL-60 cell lines are listed in Table 2. The lack of nuclear accumulation of **2** is consistent with its loss of activity in both cell lines. Reduced activity is also evident for **1** in the drug-sensitive cell line. Alternatively, **1** has about the same $IC_{50}$ as DNR in the MDR cell line.

## DISCUSSION

The results presented here demonstrate that lysosomal sequestration represents an important aspect of MDR in the human leukemic cell line HL-60. A successful strategy to combat this MDR phenotype requires an in-depth understanding of the mechanism as well as the substrate specificity for sequestration.

Considering the pH dependency of sequestration, one approach to limit it would be to manipulate the intracellular pH associated with the MDR cell line. The addition of chemical agents to these cell lines which raise lysosomal pH has previously been shown to limit lysosomal sequestration and improve the activity of weakly basic drugs (*26*). Despite the fact that this approach has proven effective in cultured cell lines, the concentrations of these agents that would be required to raise lysosomal pH in an in vivo situation are unknown and would potentially lead to systemic toxicity.

The modification of existing drugs or rational design of new drugs that are not substrates for lysosomal sequestration

offers a more practical and attractive approach in overcoming this particular resistance mechanism. Despite the appealing nature of this approach, no previous attempts to synthesize drugs with reduced compartmentalization have been reported. This is presumably due to lack of suitable structure−localization relationships to guide the synthesis of such analogues. We have recently developed a quantitative approach to investigate the lysosomal sequestration capacity in the MDR HL-60 cell line (*21*). This approach allowed for quantitative structure−transport relationship studies to assess the contribution of physical and structural properties of drugs on lysosomal sequestration. Using this method, we had previously shown that a molecular permeability parameter (referred to as α; see Results section) of weakly basic molecules could be used to predict lysosomal sequestration tendency (*21*). We established that weakly basic molecules with sufficient membrane permeability in their ionized state (α near 1) were no longer sequestered into lysosomes. Such compounds were, however, shown to be specifically sequestered into mitochondria due to the net negative membrane potential associated with this organelle. This finding could be very useful for the design of drugs that have mitochondrial targets; however, the fact that these agents were still compartmentalized would limit the usefulness of this approach on conventional anticancer agents that need to distribute into the nuclear compartment to elicit their cytotoxicity.

In this work we systematically evaluated the influence of p$K_a$ on lysosomal sequestration. The model compounds used in this study (Figure 2) all had similar permeability characteristics to DNR (log *P* near 2 and α values near 0; see Table 1); therefore, they are all expected to be substrates for lysosomal but not mitochondrial sequestration. The amine-containing structural isomers used in this study had dramatically different p$K_a$ values resulting from the relative positioning of the heterocyclic amine to the exocyclic amine. These properties made this set of compounds extremely ideal for this evaluation. In Figure 3, we had shown that compounds with p$K_a$ values near and above neutrality achieved concentrations in lysosomes over 50 times greater than that in the cytosol. Lower p$K_a$ compounds had progressively reduced lysosomal sequestration, and compounds with p$K_a$ values 5 and below were not concentrated in lysosomes to any detectable extent.

DNR has a p$K_a$ value of 8.4, and consistent with high p$K_a$ compounds evaluated in Figure 3, it is extensively sequestered into lysosomes of the MDR cell lines (see Figure 5). Two derivatives of DNR were synthesized to explore if the relationship observed with the model compounds could be translated to this drug. The first derivative, **1**, is an N-Mannich derivative of daunorubicin. In studies with N-Mannich additions to other amines it has been shown that such additions typically reduce the p$K_a$ of the parent amine by several units (*27*). We found the new p$K_a$ to be 6.9. Although the p$K_a$ value was reduced, **1** was still noticeably sequestered in lysosomes (see Figure 5). This may be attributed to DNR formed by the hydrolysis of **1**; however, Cogan and co-workers have shown that a similar Mannich derivative of doxorubicin had a half-life for hydrolysis of greater than 50 h (*28*). Considering that in Figure 5 cells were incubated with **1** for only 2 h, hydrolysis of **1** is expected to be negligible. Alternatively, lysosomal sequestration of **1** can be explained by its p$K_a$ value of 6.9, which is consistent with the results presented in Figure 3 where a complete loss of lysosomal sequestration was only observed with compounds with p$K_a$ values 5 or less. In a subsequent derivative, the 3′-amine of DNR was acylated to form **2**, which is no longer considered to be basic and would be analogous to the low p$K_a$ model compounds in Figure 3. As anticipated, **2** was not visibly sequestered in lysosomes in any of the cell lines evaluated.

The derivatives of DNR illustrated here were synthesized to illustrate a proof of principle for the concept. When modifying existing drugs, it is important to consider the effect that the derivatization has on the binding to target molecules. The energetics of DNR binding to DNA has been thoroughly examined by Chaires and colleagues (*29*). From this work we anticipated that derivatives of the 3′-amino group of DNR would have reduced capacity to bind DNA and therefore potentially reduced activity. There are essentially two reasons that may explain the decreased interaction. First, the positive charge associated with the 3′-amino group has been shown to be important for electrostatic interactions with negatively charged DNA. Therefore, derivatives of DNR with lower p$K_a$ values would be less ionized at nucleoplasmic pH relative to DNR and would be predicted to have reduced DNA binding affinity. Second, steric effects of groups attached to the primary amine would be expected to contribute to the reduced binding affinity. This is because the carbohydrate region of DNR (daunosamine moiety) is thought to snugly fit into the minor groove of DNA; therefore, the addition of moieties here would be expected to interfere with binding.

From evaluations presented in Figure 5 it is evident that **2** did not bind extensively to DNA and was not localized to the nuclear compartment in either drug-sensitive or MDR cell lines. Thus, abolishment of basicity is very effective at reducing lysosomal sequestration; however, the molecule's ability to bind to DNA is severely impaired. This qualitative observation is consistent with higher $IC_{50}$ values associated with this molecule relative to DNR in both drug-sensitive and MDR HL-60 cell lines (see Table 2).

Surprisingly, **1** had retained significant DNA binding capacity. This is most readily apparent in drug-sensitive cells, which show predominant nuclear accumulation. These results suggest that the derivatization of the primary amine itself does not totally preclude DNA binding but rather the presence or lack thereof of the positive charge seems to have a stronger influence on DNA binding affinity.

Cogan and co-workers have previously synthesized and evaluated a number of N-Mannich derivatives of DNR and doxorubicin and evaluated their activity in both drug-sensitive and MDR cancer cells (*28*). The authors proposed that the reason for improved activity of N-Mannich base derivatives was due to breakdown of the analogue to produce formaldehyde in the vicinity of DNA, which would promote covalent coupling of the drug with DNA. Our results presented here suggest that the improved activity may also be due, in part, to the altered p$K_a$ value and reduced lysosomal sequestration in the MDR cell line.

The enhanced intracellular compartmentalization of drugs has been observed in over 20 different MDR cancer cell lines, and it is currently recognized as an important contributor to the drug resistance phenotype (*8*, *9*). Collectively, the work presented here establishes quantitatively the importance of

lysosomal sequestration of drugs in MDR and offers a rational strategy to overcome it. It is clear from this work that simple alterations of p$K_a$ values using chemical approaches may result in reduced lysosomal sequestration; however, the impact of the substitution on the drug's ability to bind target molecules needs to be carefully considered. The most direct way to utilize this information would be in the initial design efforts of a drug where such chemical attributes can be deliberately incorporated and optimized. Moreover, one of the essential steps in drug development is the process of narrowing down a large array of potential drug candidates into a small subset that will endure more extensive and expensive evaluations. The information contained in this work may allow for rational selection of drugs that will be expected to have the least probability for lysosomal sequestration and thus the greatest chances for sustained activity in MDR cancer cells.

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## SUPPORTING INFORMATION AVAILABLE

High-resolution mass spectra for **1** and **2**, HPLC chromatograms for **1** and **2**, p$K_a$ determination of **1**, and LC-MS conditions for model compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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