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Function of the Anthracycline Amino Group in Cellular Transport and Cytotoxicity

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SUMMARY

Using a number of derivatives of doxorubicin (Adriamycin) and daunomycin, we have examined how substitution of the anthracycline amine affected net cellular accumulation and cytotoxic potency in HL-60 leukemia cells. Octanol/buffer partitioning demonstrated that each of the derivatives had an amino group titratable between pH 5 and 8, with the exception of derivatives containing a cyanomorpholino-substituted amine, which had a significantly lower pK_a value. The steady state cellular drug levels for the Adriamycin and daunomycin series decreased in the following order: *N*,*N*-dimethyl- > morpholino- > parent > cyanomorpholino-. Thus, the net cellular accumulation of an anthracycline was found to be influenced by the basicity of the amino group; drugs with a non-basic amino group exhibited reduced uptake. Soft agar clonogenic assays showed the following order of cytotoxicity for both series: cyanomorpholino- >> parent > morpholino- ≅ *N*,*N*-dimethyl-. The data demonstrate an inverse correlation between uptake and potency; thus, differences in net cellular accumulation do not account for the order of anthracycline potency.

Because of the clinical importance of doxorubicin (Adriamycin) in the treatment of a wide range of neoplastic diseases, the mechanisms through which the anthracyclines exert their cytotoxic activities have been the subject of intense study (1–4). Historically, nuclear DNA has been thought to be the primary target for Adriamycin (5). Proposed mechanisms by which this drug can affect DNA-related functions include interference through intercalation (6, 7), stabilization of enzymatic cleavage complexes (8), and direct damage to DNA or enzymes participating in its biosynthesis by the production of reactive free radicals (9) or alkylating species (10). Thus, it has been suggested that structural features required for an anthracycline to be active include those which result in both drug accumulation in the nuclei and significant affinity for DNA, properties which Adriamycin has been shown to exhibit (6, 11).

Recent findings have indicated that actions at the cell surface may also be involved in the antitumor activity of Adriamycin (see Ref. 12 for review). The strongest evidence for membrane-based activity comes from experiments with polymer-immobilized Adriamycin (13–16), where a nonpenetrating form of the drug was shown to be 2–3 orders of magnitude more potent than an equimolar concentration of free Adriamycin (13). These experiments have demonstrated that an anthracycline can exert its cytotoxic activity at the plasma membrane, but the more difficult questions concerning the molecular mechanism of membrane-induced cytotoxicity, and whether the immobilized and free forms of the drug operate by the same mechanism, remain unanswered. As an approach to this problem, we have been investigating the role of drug structure in the interaction specificity of anthracyclines with phosphatidylcholine vesicles (17, 18), and the role of anthracycline-membrane interactions in the cellular transport of these drugs (18, 19).

Although the exact mechanism by which anthracyclines traverse the plasma membrane is unknown, much of the available data indicate that the cellular accumulation of these agents occurs by passive diffusion of the electroneutral form of the drug molecule through the lipid domains of the biomembrane (19, 20). Assuming that a passive diffusion model is operative, two properties of anthracyclines would be critical determinants of their relative transport rates in a given cell line: (a) the overall membrane affinity and (b) the pK_a of the aminosugar. Prior to the present study, only the first of these properties had been addressed systematically and a general correlation was observed between membrane affinity and cellular uptake for several daunosamine-containing anthracyclines[3] (18). Using the cyanomorpholino-, morpholino-, and *N*,*N*-dimethyl- derivatives of Adriamycin and daunomycin (Fig. 1), we have now examined how substitution of the amine affects the properties

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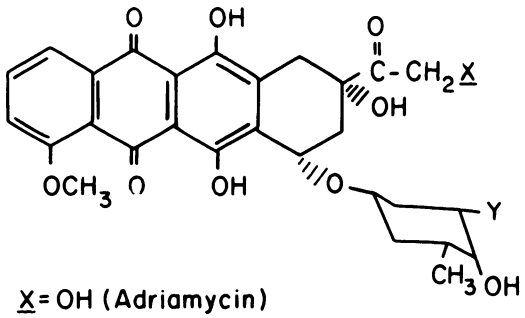


X = OH (Adriamycin)
X = H (Daunomycin)

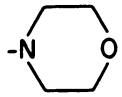
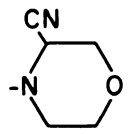
Derivative	Y
Parent	$-NH_2$
N,N-dimethyl-	$-N(CH_3)_2$
Morpholino	
Cyanomorpholino	

Fig. 1. Structures of Adriamycin (*X* = OH) and daunomycin (*Y* = H) derivatives.

of net cellular accumulation and cytotoxic potency in human promyelocytic HL-60 leukemia cells.

Materials and Methods

Chemicals. Adriamycin, daunomycin, *N,N*-dimethyladriamycin and *N,N*-dimethyldaunomycin were the gift of Dr. Leonard Kedda of the Division of Cancer Treatment, National Cancer Institute. 3′-Deamino-3′-(4-morpholinyl)adriamycin, 3′-deamino-3′-(4-morpholinyl)daunomycin, 3′-deamino-3′-(3-cyano-4-morpholinyl)adriamycin, and 3′-deamino-3′-(3-cyano-4-morpholinyl)daunomycin were generously provided by Dr. Edward M. Acton of SRI International (Menlo Park, CA). All of the above anthracyclines were in the hydrochloride form except for the cyanomorpholino- derivatives, which were in the free base form. The anthracyclines were used without further purification, since thin layer chromatography analysis on silica gel, using a solvent system of chloroform/methanol/water (40:10:1, v/v), showed their purity to be greater than 99%.

Preparation of drug stock solutions. Stock solutions of the anthracyclines were prepared in methanol or ethanol and stored in the dark at −20°. The following absorption wavelengths and extinction coefficients for methanol solutions were used to calibrate drug stocks: Adriamycin, 480 nm, 12,200 M^{-1} cm^{-1} (21); daunomycin, 478 nm, 12,100 M^{-1} cm^{-1} (22); *N,N*-dimethyldaunomycin, 478 nm, 12,500 M^{-1} cm^{-1} (23); 3′-deamino-3′-(4-morpholinyl)adriamycin, 479 nm, 12,700 M^{-1} cm^{-1} (24); 3′-deamino-3′-(4-morpholinyl)daunomycin, 479 nm, 11,800 M^{-1} cm^{-1} (22); 3′-deamino-3′-(3-cyano-4-morpholinyl)adriamycin, 479 nm, 12,400 M^{-1} cm^{-1} (24); and 3′-deamino-3′-(3-cyano-4-morpholinyl)daunomycin, 479 nm, 12,300 M^{-1} cm^{-1} (24). The same extinction coefficient was used for both *N,N*-dimethyladriamycin and Adriamycin.

Fluorescence instrumentation. All fluorescence measurements were obtained using an SLM 4800 subnanosecond spectrofluorometer interfaced to a Hewlett-Packard 9825 data processor. Steady state fluorescence intensity measurements were made in the ratio mode without polarizers, and these measurements were corrected for background fluorescence or scatter from solvents by subtraction of the signal from a blank. Measurements on the anthracycline derivatives were conducted using an excitation wavelength of 470 nm, slit widths of 4 nm, and two 500-nm short pass filters (Melles Griot) in the excitation beam to prevent transmission of stray light from the excitation monochromator. A 550-nm long pass filter (Schott) was employed for the emission channel(s) to isolate fluorescence from scattered light. The fluorescence spectral characteristics of the anthracyclines in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.4) were found to be invariant with modifications in the aminosugar portion of the molecule. All experiments were conducted in 1-cm quartz cuvettes located in a thermostated cuvette compartment. The background fluorescence and scatter was always less than 1% of the total intensity. 1-Octanol/buffer partition coefficient measurements were conducted as described previously (18). Citrate, phosphate, and borate buffers at a concentration of 10 mM were used to span the pH 4–10 range studied.

Clonogenicity of HL-60 cells. HL-60 human acute promyelocytic leukemia cells were the gift of Dr. Robert C. Gallo, National Cancer Institute. HL-60 cells were maintained at 37° as logarithmically growing cultures in Roswell Park Memorial Institute Medium 1640 containing 20% heat-inactivated fetal bovine serum (Grand Island Biological Company) in a humidified atmosphere of 95% air, 5% CO_2. The surviving fraction of HL-60 cells exposed for 2 hr to various concentrations of anthracycline was determined by plating 300 viable cells in a medium containing Roswell Park Memorial Institute Medium 1640, 20% heat-inactivated fetal bovine serum, 10% Giant Cell Tumor-conditioned medium (Grand Island Biological Company), and 0.35% Bacto-agar (Difco). After 11 days of growth in a humidified atmosphere of 95% air, 5% CO_2, the plates were overlayed with a 0.1% solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride hydrate (Aldrich Chemical Company) and colony formation was assessed using a dissection microscope. Cloning efficiency of untreated cells was routinely measured to be 70–85% with this system.

Drug uptake. The association of the anthracyclines with HL-60 cells was measured by the methods described by Johnston and Glazer (25), with some minor modifications. Drug stocks were prepared as 100 μM solutions and 30-μl aliquots were added to siliconized glass culture tubes. The final concentration of methanol in each tube was 0.3%. A 1-ml aliquot of HL-60 cells concentrated to 4×10^6 cells/ml was added to each tube at various intervals and incubated at 37° in a shaking water bath. At various times thereafter, 6 volumes of cold Dulbecco's phosphate-buffered saline (Grand Island Biological Company) were added to each of the tubes, which were then immersed in an ice water bath and centrifuged at 500 × *g* at 4°. The supernatants were removed and the cell pellets were extracted in 3 ml of 0.3 N HCl in 50% methanol for 30 min at 25°. Insoluble materials were then removed by centrifugation and the anthracycline concentrations in the supernatants were measured fluorometrically. The samples were diluted 6-fold prior to the removal of the supernatant, and errors due to residual anthracycline in the extracellular space were minimal. The initial time point in these uptake experiments was 15 sec; this methodology did not allow for accurate measurements at shorter exposure times. Because of the lack of data at shorter exposure times, we did not attempt to evaluate cell membrane association of the analogues by extrapolating back to zero time. Fluorescence values for cell-associated anthracyclines were adjusted for nonspecific association with the glass tubes (typically less than 5% of total drug). Using this protocol, we are able to account for greater than 99% of the total anthracycline added in each of the possible compartments (cell pellet, supernatant, or glass tube).

Results

In an effort to confirm the expected low pK_a for the cyanomorpholino- derivative, relative to the other anthracyclines

employed, the pH dependencies of 1-octanol/buffer partition coefficients were examined for the daunomycin derivatives (data not shown). Each of the analogues, except for the non-basic cyanomorpholino- derivative, demonstrated an amino group titratable between pH 5 and 8. The resolution of this method did not allow the determination of absolute pK_a values for the parent, morpholino-, and *N,N*-dimethyl- derivatives, but it was clear that the cyanomorpholino- derivative had a markedly lower pK_a value, since its partition ratio between the two phases was independent of pH over the range 4–10.

The data in Figure 2 demonstrate that the total amount of cell-associated drug was greater for the parent molecule than for the cyanomorpholino- derivative. The data indicate that the steady state drug levels at 40 min were greater for Adriamycin and daunomycin than for their respective cyanomorpholino- derivatives. The steady state levels of drugs associated with HL-60 cells had the following order for both the Adriamycin and daunomycin series: *N,N*-dimethyl- > morpholino- > parent > cyanomorpholino-. As visualized by fluorescence microscopy, HL-60 cells exposed to the parent, *N,N*-dimethyl-, and morpholino- derivatives all showed the typical anthracycline nuclear fluorescence, as well as granular fluorescence in the cytoplasm. In contrast, cells exposed to the cyanomorpholino- derivative showed only a diffuse, low intensity fluorescence (data not shown).

In agreement with our results, the net cellular accumulation of the *N,N*-dimethyl- derivatives of both Adriamycin and daunomycin has previously been shown to exceed that of the parent compounds in P388 leukemia cells (26). Similarly, HT-29 cells demonstrated greater uptake of the morpholino- derivative of daunomycin than the parent drug (25). The total cellular equilibrium concentration of drug is also influenced by efflux. In most cell types this is considered to be an active, energy-dependent process. In our experiments conducted with HL-60 cells, the relative amounts of accumulation of the various analogues were not altered when uptake measurements were carried out in glucose-free medium containing 10 mM sodium azide, suggesting that differences in drug efflux did not represent major factors in these observations (27, 28).

The data in Table 1 summarize the cytotoxicity of the various anthracycline congeners on HL-60 leukemia cells as measured by a soft-agar clonogenic assay; cells were exposed to various drug concentrations for 2 hr. The cyanomorpholino- derivative of Adriamycin was the most potent analogue, with no surviving colonies being evident with drug concentrations as low as 1×10^{-8} M. This derivative was at least 100 times more potent than Adriamycin in this study, whereas the *N,N*-dimethyl- and the morpholino- derivatives of Adriamycin demonstrated reduced potency relative to the parent drug. For the daunomycin series, the cyanomorpholino- derivative was also found to be the most potent, being at least 10 times more active than daunomycin, wheres the morpholino- and *N,N*-dimethyl- derivatives exhibited approximately a 10-fold reduction in potency relative to the parent drug. The sample of 3′-deamino-3′-(4-morpholinyl)adriamycin used in this study contained a trace amount (0.3%) of its cyanomorpholino- derivative which, because of its intense potency, most likely contributed to some of the observed cytotoxicity. We have also demonstrated that the cyanomorpholino- derivatives of daunomycin and Adriamycin are 2 and 3 orders of magnitude more potent, respectively, than are the parent compounds in terms of inhibition of HL-60 cell

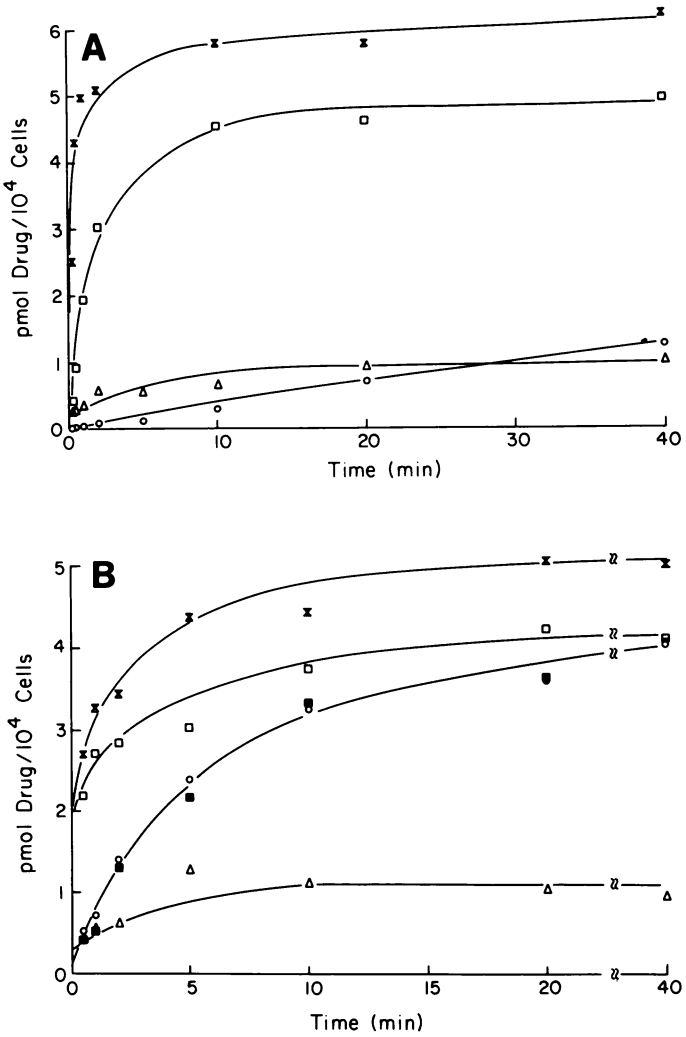


Fig. 2. Time-dependent association of anthracyclines with HL-60 leukemia cells. A shows the cellular association of the Adriamycin series and B depicts the association of the daunomycin series. The derivatives are represented by the following symbols in both panels: *N,N*-dimethyl- (X̄), morpholino- (□), parent (○), and cyanomorpholino- (△). These plots represent the amount of drug (in pmol of drug/10^4 cells) which was extracted in acidified methanol from 4×10^6 cells exposed to 3×10^{-6} M drug, and subsequently quantified by fluorescence measurements. The accumulation of daunomycin by HL-60 cells was also studied by measurement of radiolabeled drug associated with the cell pellet (■); results from both techniques were in close agreement (B). Fluorescence quantitation of the amount of cyanomorpholinodaunomycin in both the supernatant and the cell extract showed a total drug recovery of 99%; these results indicate that metabolism to nonfluorescent species and inefficient drug extraction from cells were not major determinants in the cyanomorpholinodaunomycin uptake profile. Experiments carried out at lower drug concentrations (2×10^{-7} M) revealed patterns of uptake qualitatively identical to those shown above. Each data point in chart 2 represents the average of duplicate samples using the same cell preparation for each series of compounds. The same trends in the data were observed during repeat studies with a day-to-day uncertainty of less than 10%.

DNA and RNA biosynthesis; similar results were recently reported by Acton *et al.* (24) with L1210 cells.

Discussion

Extensive experimental and clinical studies have been conducted on anthracycline analogues modified at the aminosugar,

TABLE 1

The surviving fractions of clone HL-60 leukemia cells treated with various anthracycline derivatives

Survival was determined by a soft agar clonogenic assay done in triplicate for each drug concentration as described in Materials and Methods. Cloning efficiency for untreated cells was routinely 70–85% with this system.

Derivative	Cloning efficiency at drug concentrations of				
	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-10} M
Adriamycin series					
Parent	4	43			
N,N-Dimethyl-	38				
Morpholino-[a]	9	71			
Cyanomorpholino-	0	0	0	25	70
Daunomycin series					
Parent	0	36			
N,N-Dimethyl-	36	91			
Morpholino-	37	95			
Cyanomorpholino-	0	0	4	71	86

[a] The sample of 3′-deamino-3′-(4-morpholinyl)adriamycin used in this study contained a trace amount (0.3%) of the corresponding cyanomorpholino- derivative which most likely contributed to some of the observed cytotoxicity.

but little emphasis has been placed on how such structural changes alter the pK_a of these drugs in solution. Two factors are primarily responsible for limiting these types of physical studies: (a) spectrophotometric determinations of the pK_a of the amino group are complicated by the presence of other dissociation equilibria, as well as by the insensitivity of the electronic spectrum of the aromatic chromophore to the amino group of the sugar moiety which is several atoms removed (29), and (b) spectroscopic or titrimetric studies requiring in excess of 5 μM drug are complicated by anthracycline self-association in solution (30–33). Spectrophotometric investigations of monomeric Adriamycin in aqueous solution showed a pK_a of 8.2 for the amino group (29). A pK_a of 7.6 (37°, 0.15 M ionic strength) was obtained for the Adriamycin amine by studying its efflux from loaded erythrocytes into a drug-free medium (34). Although the amino group pK_a values of the *N,N*-dimethyl- and morpholino- derivatives of Adriamycin and daunomycin have not been reported, it is likely that their pK_a values are higher than those of the parent molecules because of the substitution of electron-releasing groups (35). The cyanomorpholino- derivatives also contain a tertiary amine but, with an adjacent α-nitrile group, the basicity of these analogues is thought to be decreased by approximately 6 pH units, resulting in a pK_a of approximately 2 (24). Accordingly, the pK_a values for the amino groups of the anthracycline derivatives tentatively have the following order: *N,N*-dimethyl- and morpholino- > parent >> cyanomorpholino-. We have estimated the pK_a values by octanol/buffer partitioning and our results are consistent with this prediction.

Using the cyanomorpholino-, morpholino-, and *N,N*-dimethyl- derivatives of Adriamycin and daunomycin, we have examined the function of the anthracycline amine in cellular transport. The net cellular accumulation of the anthracyclines was observed to be greatly enhanced by the presence of an ionizable amino group. Assuming the prediction of higher pK_a values for the *N,N*-dimethyl- and morpholino- derivatives is correct, a qualitative correlation exists between the pK_a value of the drugs and cellular accumulation.

As previously indicated, overall membrane binding and the pK_a of the amino group are two properties of an anthracycline that are important in determining transportability by a passive diffusion mechanism. Increasing the hydrophobicity of the aglycone portion of an anthracycline through structural modification, which should not significantly affect the pK_a of the amino group, has been shown to significantly enhance the affinity of anthracyclines for neutral as well as for negatively charged membranes[4] (18). In general, membrane affinity correlates with cellular accumulation for Adriamycin, daunomycin, and other daunosamine-containing anthracyclines (18).

The dependence of net cellular accumulation on the anthracycline aminosugar substitution has been demonstrated in this report, with the presence of a nonbasic amino group resulting in reduced uptake. The levels of the cellular uptake of the amine-modified anthracyclines do not correlate with previously measured affinities for either electroneutral or negatively charged membranes[4] (18). Thus, it appears that the net cellular accumulation is also determined by the basicity of the amino group and not simply by membrane affinity. Net cellular accumulation by a passive diffusion process is controlled by a concentration gradient, and only unbound drug participates in this gradient. It has previously been shown that the parent, *N,N*-dimethyl-, and morpholino- derivatives of Adriamycin and daunomycin exhibited greater DNA affinities than did their unchanged cyanomorpholino- analogues (23, 24). Since charged species are considered to be more effective than uncharged species at binding intracellular sites such as DNA (36), it is not surprising that the anthracyclines with basic amino groups were accumulated by HL-60 cells to high levels.

Acton *et al.* (24) have demonstrated that the cytotoxic potencies of the cyanomorpholino- derivatives of Adriamycin, daunomycin, 5-iminoadriamycin, Adriamycinol, daunomycinol, and 5-iminoadriamycinol all significantly exceeded those of their corresponding morpholino- derivatives against L1210 leukemia cells *in vitro* and *in vivo*. In this report, the superior potency of the cyanomorpholino- derivatives of Adriamycin and daunomycin against HL-60 cells has been demonstrated, even though these congeners displayed relatively low levels of cellular uptake. These findings suggest that the cyanomorpholino- derivatives must have higher intrinsic potencies, if an intracellular mechanism accounts for anthracycline activity. Consistent with this hypothesis, it has recently been shown that the reactive cyanomorpholino-containing drug resulted in extensive physical damage to DNA (37–39), which may account for the superior activity of cyanomorpholino-Adriamycin. Alternatively, these results could be explained by postulating that the cell surface, rather than intracellular sites, is a sensitive target for anthracycline action since the congeners that are taken up the least are the most active.

In addition to enhancing the cytotoxic properties of an anthracycline, the presence of the cyanomorpholino- moiety brings added benefits. The unfavorable cytotoxicity of Adriamycin and daunomycin to cardiac tissue has limited their usefulness as antitumor agents in the treatment of human cancer. Several mechanisms have been proposed for the observed cardiotoxicity of the anthracyclines, including free radical damage (40–42), direct membrane effects (43), and inhibition of ubiquinone-requiring enzymes (44). Mitochondria have been postulated as the primary site of cardiac damage for each of these mechanisms, the mitochondrion and sarcoplasmic reticulum representing two subcellular sites in which cardiac tissue damage is prominent (1). It is evident that damage to

[4] T. G. Burke, A. C. Sartorelli, and T. R. Tritton, manuscript in preparation.

mitochondria require that the drug gain access to the cell interior. Following this reasoning, one predicts that the cyanomorpholino- derivative might have a reduced cardiotoxicity relative to Adriamycin, since the former has reduced uptake at comparable cytotoxic concentrations. This, in fact, has been shown to be the case in a fetal mouse heart culture system (45). The improved antitumor activity relative to cardiotoxicity for 3′-deamino-3′-(3-cyano-4-morpholinyl)adriamycin provides evidence for different fundamental mechanisms for these biological activities, which are differentially affected by substitution of the amino group.

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