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Decreased resistance to *N,N*-dimethylated anthracyclines in multidrug-resistant Friend erythroleukemia cells*

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Summary. Doxorubicin-resistant Friend erythroleukemia cells, line F4-6 ADM2^R, were selected by exposure of wild-type F4-6 cells to doxorubicin concentrations of up to 1 μg/ml. In these cells, increased expression of multidrug resistance (MDR) genes was demonstrated by Northern blot analysis. The growth-inhibitory effect of doxorubicin, daunorubicin, *N,N*-dimethyldoxorubicin, *N,N*-dimethyldaunorubicin, morpholinodoxorubicin, and pyrromycin was comparatively investigated in resistant and wild-type cells. The doxorubicin-resistant F4-6 cells showed approx. 200-fold resistance to doxorubicin and about 100-fold resistance to daunorubicin with respect to the drug-sensitive counterpart. A dramatic decrease in resistance was observed for the *N,N*-dimethylated derivatives of doxorubicin and daunorubicin as well as for the *N,N*-dimethylated natural anthracycline pyrromycin and for morpholinodoxorubicin. Uptake studies using [^{14}C]-daunorubicin and [^{14}C]-*N,N*-dimethyldaunorubicin in resistant F4-6 cells showed a decreased accumulation of daunorubicin but no significant reduction in *N,N*-dimethyldaunorubicin accumulation as compared with the wild-type cells. Treatment with verapamil led to increased intracellular levels of daunorubicin in resistant cells, whereas an excess of *N,N*-dimethyldaunorubicin did not have this effect. Thus, the decreased resistance of the doxorubicin-resistant F4-6 cells to the *N*-alkylated anthracyclines may at least in part be due to a reduced affinity of these compounds for the efflux pump. The results indicate that the dimethylation of the amino group of the anthracycline sugar moiety and its incorporation within a morpholinyl ring may overcome MDR by similar mechanisms.

Introduction

The anthracycline antitumor antibiotics doxorubicin and daunorubicin play an important role in the treatment of a variety of neoplastic diseases [58]. The mechanism of their cytostatic action is attributed mainly to DNA intercalation, but other biochemical properties such as the generation of highly reactive free radicals [1, 19, 22], interaction with topoisomerase II [48, 49], and binding to membrane structures [20, 43] have also been discussed in anthracycline-induced cellular damage. The continuous clinical use and the success of anthracycline chemotherapy are limited by dose-related cardiotoxicity as well as by the development of cellular resistance to these agents [30, 31, 58].

Resistance to doxorubicin and daunorubicin has been found to be associated with cross-resistance to various structurally dissimilar drugs such as vinca alkaloids, epipodophyllotoxins, or actinomycin D and to underlie the well-characterized pleiotropic or multidrug resistance (MDR) of tumor cells [9, 12, 28, 33, 57]. Studies on various MDR cell lines have demonstrated a reduced intracellular accumulation of the drugs involved [2, 15, 27, 35, 44] and an enhanced expression of a high-molecular-weight membrane glycoprotein termed P-glycoprotein [11, 35, 54].

This protein has been hypothesized to function as an energy-dependent drug-efflux pump with broad substrate specificity [11]. The P-glycoprotein content in drug-resistant cells has been shown to correlate directly with both the extent of decrease in intracellular accumulation of drugs and the degree of drug resistance observed [29]. Further studies have revealed that overexpression of P-glycoprotein in several MDR cell lines results from amplification of the P-glycoprotein gene [12, 16, 37, 41]. Although additional mechanisms may also contribute to the drug resistance in MDR cells [33], the expression of full-length cDNAs for mouse and human P-glycoprotein genes confers a complete MDR phenotype onto sensitive cells [21, 42, 53]. Moreover, increased expression of MDR gene and P-glycoprotein has also been correlated with the emergence of clinical resistance [4, 38].

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The P-glycoprotein-mediated efflux mechanism in MDR tumor cells is thought to recognize and process a wide spectrum of structurally and functionally unrelated lipophilic drugs. However, studies using anthracycline antitumor antibiotics suggest that the resistance of MDR cells to different anthracyclines may depend on certain structural properties of these compounds. Overcoming of cellular resistance in various MDR cell lines has been achieved using aclacinomycin A as well as several semisynthetic 9-alkyl and morpholinyl anthracycline derivatives [6–8, 23, 40, 46, 55, 56]. The decrease in resistance was accompanied by increased cellular accumulation of these compounds [7, 46].

The identification of anthracycline derivatives that retain activity in resistant cells may provide a novel approach for overcoming clinical MDR. Since aclacinomycin A also has a 9-ethylsubstituent, 9-alkyl substitution as well as the morpholinyl sugar modification of the anthracycline moiety may be key elements in the overcoming of MDR [8].

In an earlier study it was observed that doxorubicin/daunorubicin-resistant P388 murine leukemia cells were less resistant to *N,N*-dimethyldoxorubicin and *N,N*-dimethyldaunorubicin [10]. In the present experiments, we used a phenotypically defined MDR Friend erythroleukemia cell line to study the activity of these compounds and of the natural *N,N*-dimethylated anthracycline, pyrromycin. The results show a dramatically reduced resistance to *N,N*-dimethylated anthracyclines in MDR Friend erythroleukemia cells, which may be due at least in part to an improved cellular accumulation of these compounds. Thus, the dimethylation of the amino group of the anthracycline sugar moiety and its incorporation within a morpholinyl ring may overcome resistance via similar mechanisms.

Materials and methods

Chemicals. The anthracyclines doxorubicin and daunorubicin were kindly provided by Farmitalia-Carlo Erba (Milano, Italy). *N,N*-Dimethyldoxorubicin and *N,N*-dimethyldaunorubicin and morpholinodoxorubicin and pyrromycin were generously donated by Dr. E. M. Acton, National Cancer Institute, NIH (Bethesda, Md., USA), and by Dr. G. Lenaz, Bristol Myers Co. (New York, N. Y., USA), respectively. Verapamil was obtained from Sigma (St. Louis, Mo., USA), and [^{14}C]-daunorubicin (sp. act., 45.0 mCi/mmol), [^{3}H]-inulin (sp. act., 1.8 Ci/mmol), [^{32}P]-deoxycytidine triphosphate (dCTP; sp. act., 3000 Ci/mmol), and [^{14}C]-formaldehyde (sp. act., 17.4 mCi/mmol) were purchased from Amersham Buchler (Braunschweig, FRG). MEM alpha medium containing no nucleosides, fetal calf serum (FCS), and trypan blue solution were obtained from Gibco (Karlsruhe, FRG); *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid (HEPES) buffer, from Serva (Heidelberg, FRG); dibutylphthalate and dinonylphthalate, from Fluka (Buchs, Switzerland); Soluene-350 and Hionic-Fluor, from Packard (Downers Grove, Ill., USA); lambda-DNA and restriction endonuclease Hind III, from Boehringer Mannheim (FRG); and analytical-grade chemicals, from Sigma (St. Louis, Mo., USA), Fluka (Buchs, Switzerland), and Merck (Darmstadt, FRG).

Preparation of ^{14}C-labeled N,N-dimethyldaunorubicin. [methyl-^{14}C]-*N,N*-Dimethyldaunorubicin was synthesized in analogy with the method described by Tong et al. [51] by the reaction of 29 μmol [^{14}C]-formaldehyde with 7 μmol daunorubicin. The crude product was purified by thin-layer chromatography on SiO_2-60 (Merck, Darmstadt, FRG) using the solvent system $CHCl_3/CH_3OH/H_2O$ (80 : 20 : 2, by vol.).

Cell cultures. Friend erythroleukemia cells, line F4-6 [34], were kindly provided by Dr. W. Ostertag, Heinrich-Pette-Institut für Experimentelle Virologie (Hamburg, FRG). Cells were grown in nucleoside-free alpha medium supplemented with 10% FCS. Doxorubicin-resistant cells were derived from drug-sensitive F4-6 cells by continuous exposure to doxorubicin [47]. The drug concentration in the cultures was increased stepwise from 1 ng/ml to 1 μg/ml over a period of 6 months (about 220 generations) and a subclone was isolated. The variant F4-6 ADM2^{R} was used for the experiments in this study. The drug-resistant phenotype has remained stable during growth in the absence of drug for several months. To ascertain its persistence, the cells were treated periodically with doxorubicin at 1 μg/ml for 2 days. Before their use, the resistant cells were grown in drug-free medium for several generations.

Cytotoxicity studies. Dose response curves for doxorubicin-sensitive and -resistant cell lines were obtained by growing cells in increasing levels of drugs and counting those that were capable of excluding trypan blue [32, 47]. Cells were incubated for 72 h in nucleoside-free alpha medium supplemented with 10% FCS in the presence and absence of the test substances. To maintain exponential growth during this period, the cells were seeded at 0.6×10^5/ml. After 48 and 72 h, cells were collected from the plates and the trypan-blue-excluding cells were counted. IC_{50} values were calculated from the dose-response curves as the concentration of drug required to reduce cell viability to 50% of control growth. The IC_{50} value recorded during each experiment was expressed as the mean value for the calculations carried out after 48 and 72 h.

Northern blot analysis of RNA. Total cellular RNA from drug-sensitive and -resistant F4-6 cells was isolated by the method of Chomczynski and Sacchi [5]. Approximately 10×10^6 cells were used, yielding 150–200 μg RNA; 30 μg RNA was denaturated by glyoxalation, electrophoresed through a 1% agarose gel, and transferred to Biodine A membrane (PALL, Portsmouth, UK) by the capillary blotting technique [50] using 20 × SSC solution (1 × SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate). RNA was immobilized by baking the membrane at 80° C for 1 h. cDNA probes for hybridization were labeled with [^{32}P]-dCTP by random priming [13] to a specific activity of $2–4 \times 10^8$ cpm/μg using the Multiprime DNA labeling system from Amersham (Braunschweig, FRG). Membrane hybridization was carried out after 4 h prehybridization as described by Braun et al. [3]. Nonhybridized radioactivity was removed by incubation in 2 × SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min followed by 4 washing steps at 50° C in 2 × SSC and 0.1% SDS for 15 min each. Hybridization with MDR1 cDNA did not change qualitatively when the membrane was washed more stringently (final washing in 0.2 × SSC and 0.1% SDS at 60° C). The membrane was placed on X-ray film (Fuji Photo Film, Japan) with an intensifying screen to produce an autoradiogram. The membrane was sequentially hybridized with the cDNA probes. The following probes were used for hybridization: human MDR1 probe pHDR5A, 1.38 kb [38] (generously provided by Drs. M. M. Gottesman and I. Pastan, National Cancer Institute, NIH, Bethesda, Md., USA); human β-actin cDNA probe, 1.2 kb (kindly provided by Dr. T. Braun, Department of Toxicology, University of Hamburg, Hamburg, FRG); and human *c-myc*, 3rd exon, 1.8 kb (purchased from Oncor, Gaithersburg, Md., USA).

Drug-uptake studies. Anthracycline accumulation was measured under conditions similar to those previously used to determine amino acid uptake in F4-6 cells [39]. Drug-sensitive and -resistant F4-6 cells (5×10^6/ml) were incubated at 37° C in nucleoside-free alpha medium and 20 mM HEPES buffer in the presence of various concentrations of [^{14}C]-daunorubicin or [^{14}C]-*N,N*-dimethyldaunorubicin and [^{3}H]-inulin (ca. 2 μCi/ml) as a marker of extracellular fluid. At several time points up to 40 min, 100- to 200-μl samples were collected by centrifugation through a dibutylphthalate-dinonylphthalate (3 : 1, v/v) oil mixture. Pellets were solubilized in 250 μl Soluene-350 by incubation at 70° C for 4 h. After solubilization, 5 ml Hionic-Fluor scintillation fluid was added and radioactivity was measured in a liquid scintillation counter. The amount of anthracycline taken up by the cells was calculated using internal standards.

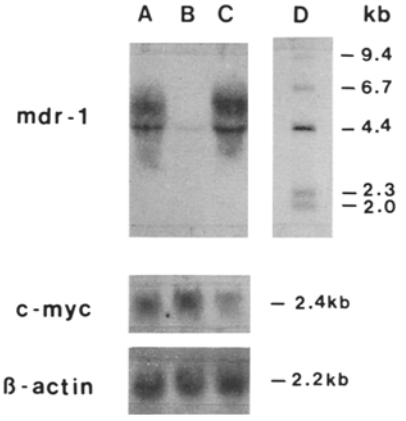

Fig. 1. Northern blot analysis of the RNA fraction isolated from doxorubicin-sensitive and -resistant F4-6 cells. Amounts of 30 µg glyoxalated RNA were dissolved by electrophoresis on 1% agarose gel, transferred onto a nylon membrane, and hybridized as described in Materials and methods. Sequential hybridization of the same blot was performed using the cDNA probes. *A*, F4-6 ADM2^R; *B*, wild-type F4-6; *C*, F4-6 ADM1^R; *D*, lambda-DNA-Hind III marker

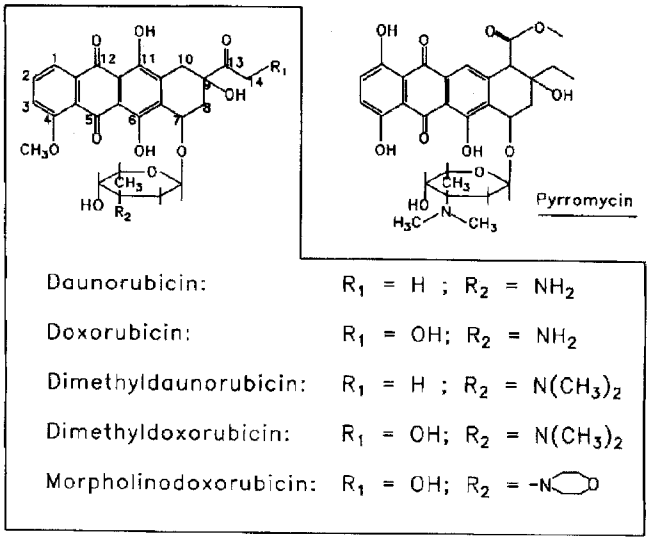

Fig. 2. Structures of the anthracyclines studied

Determination of partition coefficients. Partition coefficients were obtained by measuring the fluorescence of drug solutions in PBS before and after extraction with *n*-octanol as described by Twentyman et al. [52].

Results

Characterization of doxorubicin-resistant F4-6 cells by Northern blot analysis

The expression of the MDR genes was studied in doxorubicin-sensitive F4-6 cells and in two doxorubicin-resistant F4-6 cell variants selected independently using Northern blot analysis. Levels of *c-myc* and β-actin mRNA were also analyzed comparatively. β-Actin was used as an internal control for equivalent amounts of RNA loaded.

As shown in Fig. 1, wild-type doxorubicin-sensitive F4-6 cells expressed MDR transcripts of approx. 4.5 kb at very low levels. This position corresponds to the size of the MDR1b mRNA [25]. In comparison, in the two independently selected doxorubicin-resistant cell lines F4-6 ADM1^R and F4-6 ADM2^R, transcripts ranging from 4.5 to 6 kb reacted very strongly with the MDR1 cDNA probe. The combined message obviously resulted from the cross-reaction of the human MDR1 cDNA probe with the mouse MDR1a and MDR1b mRNAs and from the heterogeneity in the size of MDR1a transcripts [25, 26]. The expression of *c-myc* was reduced in doxorubicin-resistant variants as compared with the wild-type sensitive cells at roughly identical levels of β-actin mRNA.

These results show that independent exposure of F4-6 cells to increasing concentrations of doxorubicin leads to an overexpression of the MDR gene products and confirm the MDR phenotype of the F4-6 ADM2^R clone used in the following experiments.

Cell-growth inhibition by and cytotoxicity of anthracyclines in doxorubicin-sensitive and -resistant F4-6 cells

The effect of doxorubicin on cell viability in F4-6 and F4-6 ADR2^R cells was compared with that of daunorubicin, *N,N*-dimethyldoxorubicin, and *N,N*-dimethyldaunorubicin as well as with that of two other *N*-alkylated anthracyclines, morpholinodoxorubicin and pyrromycin. Morpholinodoxorubicin has been shown to circumvent resistance in MDR cells [8, 46]. Pyrromycin is a monosaccharide anthracycline of the *N,N*-dimethylated group of natural anthracyclines. It differs from the *N,N*-dimethyl derivatives of doxorubicin and daunorubicin by its aglycone structure (Fig. 2).

The relative effectiveness of these substances in reducing cell viability is shown in Table 1. The F4-6 ADM2^R cells exposed to doxorubicin at concentrations of up to 1 µg/ml showed about 200-fold resistance to doxorubicin as compared with the wild-type F4-6 cells. The data indicate cross-resistance to daunorubicin; however, the resistance of these cells to daunorubicin proved to be lower than that to doxorubicin. In contrast, none of the N-alkylated anthracyclines investigated induced similar levels of resistance in F4-6 ADM2^R cells. These results clearly show that not only the morpholinyl substitution of the amino sugar but also the dimethylation of the amino group of the anthracycline sugar moiety is effective in reducing doxorubicin/daunorubicin resistance in MDR F4-6 cells.

Accumulation of [^{14}C]-daunorubicin and [^{14}C]-dimethyldaunorubicin in doxorubicin-sensitive and -resistant F4-6 cells

To obtain further information on the mechanisms involved in the reduced resistance of the doxorubicin/daunorubicin-resistant F4-6 cells to the *N*-alkylated anthracycline derivatives studied, we compared the uptake and accumulation of

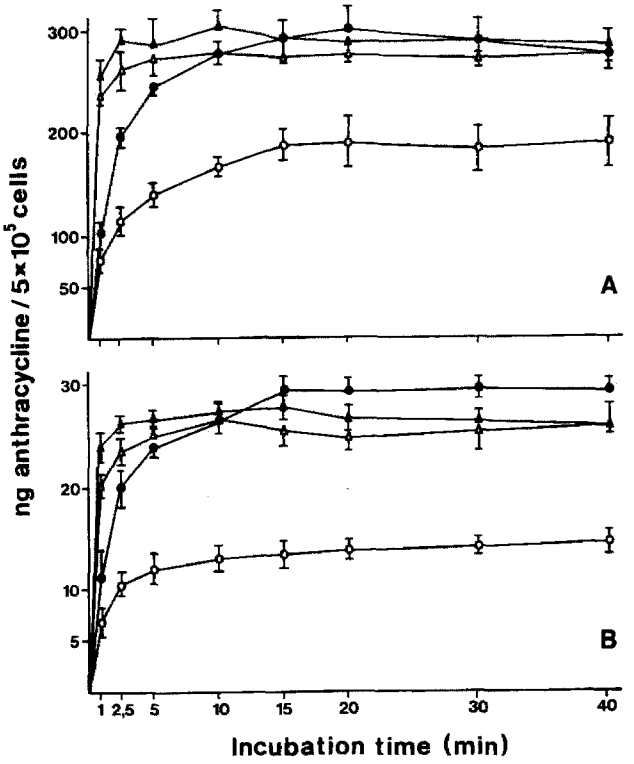

Fig. 3 A, B. Time course of daunorubicin and *N,N*-dimethyldaunorubicin accumulation in doxorubicin-sensitive and -resistant F4-6 cells at drug concentrations of **A** 3.5 μg/ml and **B** 350 ng/ml. Anthracycline accumulation was measured using ^{14}C-labeled compounds as described in Materials and methods. Daunorubicin accumulation: ●, wild-type F4-6; ○, F4-6 ADM2^{R}. *N,N*-Dimethyldaunorubicin accumulation: ▲, wild-type F4-6; △, F4-6 ADM2^{R}. Data points represent mean values ± SE for 3 experiments

^{14}C-labeled daunorubicin and *N,N*-dimethyldaunorubicin in sensitive and resistant F4-6 cells.

Figure 3 shows the kinetics of the cellular uptake of these substances at two different concentrations: 350 ng/ml and 3.5 μg/ml. In wild-type F4-6 cells, a steady-state level of daunorubicin accumulation was reached within 15 min. Probably due to its higher lipophilicity, dimethyldaunorubicin was accumulated more rapidly, within 2.5 min. In F4-6 ADM2^{R} cells, a reduced accumulation of daunorubicin was observed. This decrease seemed to be accentuated at the lower concentration of 350 ng/ml, whereby the maximal accumulation was 40%–50% of the level in the parent F4-6 cells. In the case of dimethyldaunorubicin, no significant difference in drug accumulation was observed between the sensitive and the resistant F4-6 cells.

To exclude the possibility that the P-glycoprotein efflux pump in the resistant cells might be saturated by the rapid influx of the more lipophilic *N,N*-dimethylated derivate, we also investigated drug accumulation at a 20-fold lower concentration of 17.5 ng/ml. As shown in Fig. 4, at this low concentration, too, the accumulation of daunorubicin was reduced in F4-6 ADM2^{R} cells, whereas that of dimethyldaunorubicin was not affected.

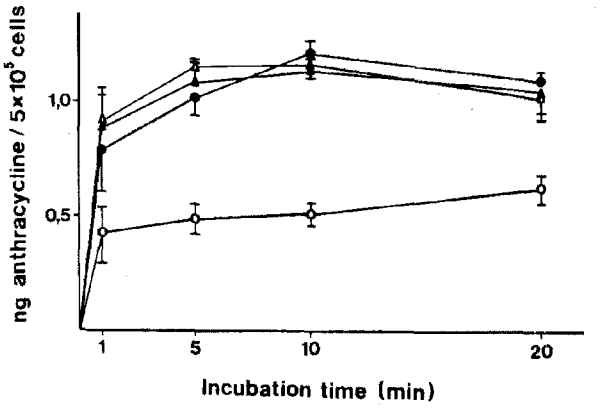

Fig. 4. Daunorubicin and *N,N*-dimethyldaunorubicin accumulation in doxorubicin-sensitive and -resistant F4-6 cells at a low drug concentration (17.5 ng/ml). Anthracycline accumulation was measured using ^{14}C-labeled compounds as described in Materials and methods. Daunorubicin accumulation: ●, wild-type F4-6; ○, F4-6 ADM2^{R}. *N,N*-Dimethyldaunorubicin accumulation: ▲, wild-type F4-6; △, F4-6 ADM2^{R}. Data points represent mean values ± SE for 3 experiments

Effect of N,N-dimethyldaunorubicin and verapamil on daunorubicin accumulation in drug-resistant F4-6 cells

The calcium antagonist verapamil and a variety of other lipophilic agents have been shown to inhibit the P-glycoprotein-mediated efflux of drugs involved in MDR, leading to improved cellular drug accumulation and to reversal of drug resistance [6, 7, 24]. Moreover, *N*-acetyldaunorubicin has been reported to increase the steady-state uptake of daunorubicin in resistant Ehrlich ascites tumor cells [45]. Therefore, the question arises as to whether the increased accumulation of *N,N*-dimethyldaunorubicin relative to daunorubicin observed in F4-6 ADM2^{R} cells might have been due to an inhibitory action of the dimethyl derivate on the drug-efflux mechanism in resistant cells.

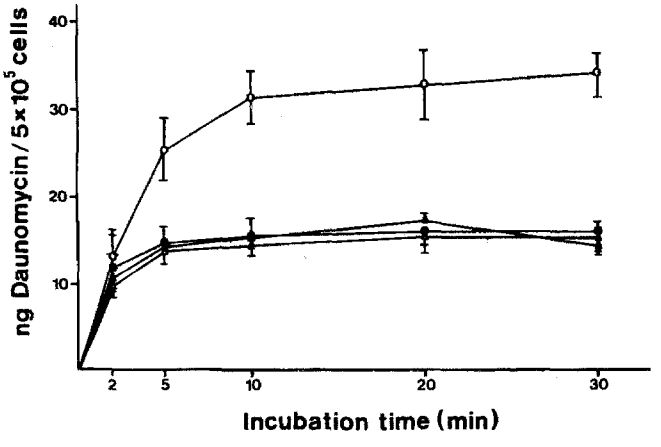

Fig. 5. Effect of *N,N*-dimethyldaunorubicin and verapamil on the accumulation of daunorubicin in F4-6 ADM2^{R} cells. Daunorubicin accumulation was measured at a concentration of 0.5 μg/ml using [^{14}C]-daunorubicin as described in Materials and methods. ●, Daunorubicin accumulation, control; △, *N,N*-dimethyldaunorubicin, 1 μg/ml; ▲, *N,N*-dimethyldaunorubicin, 5 μg/ml; ○, verapamil, 5 μg/ml. Data points represent mean values ± SE for 3 experiments

To investigate this possibility, we studied the effect of *N,N*-dimethyldaunorubicin on daunorubicin accumulation in F4-6 ADM2^R cells (Fig. 5). Daunorubicin accumulation was measured at a concentration of 0.5 μg/ml. The effect of *N,N*-dimethyldaunorubicin was tested at concentrations of 1 μg/ml and 5 μg/ml. Verapamil (5 μg/ml) was also investigated as a positive control. The results show an increased accumulation of daunorubicin in the presence of verapamil. However, the accumulation of daunorubicin was not affected by the presence of *N,N*-dimethyldaunorubicin. These results indicate that *N,N*-dimethyldaunorubicin exerts no intrinsic inhibitory activity on the drug-efflux mechanism in F4-6 ADM2^R cells under the experimental conditions used. Accordingly, the increased accumulation of *N,N*-dimethyldaunorubicin in resistant cells (Fig. 3) cannot be explained by an inhibition of the efflux pump.

Studies on the correlation between lipophilicity and decrease in drug resistance

The structural properties of the anthracycline derivatives shown to decrease resistance in doxorubicin-resistant F4-6 cells suggest a higher lipophilicity of these substances relative to doxorubicin (Table 1, Fig. 2). Therefore, we investigated the possibility of a correlation between the lipophilic character of the anthracyclines studied and their ability to overcome resistance in F4-6 ADM2^R cells. The lipid solubility of the compounds was characterized by determining the partition coefficients between the water phase and *n*-octanol. The results are summarized in Table 2.

The most lipophilic substances, pyrromycin, morpholinodoxorubicin, and dimethyldaunorubicin, indeed showed the highest cytotoxic activity in doxorubicin-resistant F4-6 cells (Table 1). However, a tight correlation between cytotoxic activity and lipophilicity could not be established; for instance, although it was less lipophilic than daunorubicin, *N,N*-dimethyldoxorubicin exerted significantly higher cytotoxicity in doxorubicin-resistant cells than did daunorubicin.

Table 1. Sensitivity of wild-type and doxorubicin-resistant F4-6 cells to anthracycline derivatives

Compound	IC_{50} value[a]		IC_{50} resistant
	F4-6	F4-6 ADM2^R	IC_{50} sensitive
Doxorubicin	9.17 ± 1.75	2127.1 ± 114.2	232
N,N-Dimethyl-doxorubicin	10.8 ± 1.17	223.3 ± 14.7	20.7
Daunorubicin	6.81 ± 0.51	877.8 ± 113.5	129
N,N-Dimethyl-daunorubicin	8.36 ± 0.94	46.5 ± 5.2	5.6
Morpholino-doxorubicin	9.27 ± 0.86	57.1 ± 9.3	6.2
Pyrromycin	18.6 ± 5.43	94.5 ± 22.8	5.1

[a] Concentration of drug (nM) resulting in a 50% loss of cell viability as determined from the dose-response curves and described in Materials and methods. Data represent mean values ± SE for at least 4 independent experiments

Table 2. Lipophilicity of the anthracycline derivatives studied

Compound	Partition coefficient (*n*-octanol/water)
Doxorubicin	0.67 ± 0.14
N,N-Dimethyldoxorubicin	0.75 ± 0.01
Daunorubicin	4.84 ± 0.34
N,N-Dimethyldaunorubicin	6.03 ± 0.58
Morpholinodoxorubicin	31.2 ± 3.3
Pyrromycin	112.2 ± 32.1

Partition coefficients between *n*-octanol and drug solutions in PBS were estimated according to Twentyman et al. [52]. Data represent mean values ± SE for at least 3 independent determinations

Discussion

The mechanisms and the possible prevention of MDR of tumor cells to anthracycline antitumor antibiotics have been the subject of intensive research. Different strategies to overcome such resistance have been investigated, such as the use of antibodies to P-glycoprotein [18] or of chemosensitizers, which may bind to P-glycoprotein and inhibit its pump function [17].

A different approach for the prevention of MDR consists of the identification of structural analogues of the drugs involved, which retain activity in resistant cells. Studies using various anthracycline derivatives have suggested that the 9-alkyl substitution and the incorporation of the amino group of the anthracycline sugar moiety into a morpholinyl ring are important structural modifications required for activity against MDR cells [6–8, 40, 46, 52, 55, 56].

In the present experiments, structure-activity relationships were studied using *N*-alkylated anthracyclines in a resistant Friend erythroleukemia cell line, F4-6 ADM2^R, selected for doxorubicin resistance. The MDR phenotype of these cells was characterized on the basis of their expression of the MDR gene products (Fig. 1) as well as their increased daunorubicin accumulation in the presence of verapamil (Fig. 5). In addition, a decreased expression of *c-myc* mRNA was also demonstrated in the MDR cell lines investigated (Fig. 1). This observation is consistent with previous results and with the suggestion that acquisition of the MDR phenotype is associated with the down-regulation of *c-myc* expression in certain cell lines [14, 36].

The results show that the *N,N*-dimethyl derivatives of doxorubicin and daunorubicin overcome resistance in MDR Friend erythroleukemia cells. Therefore, the function of dimethylation of the amino group of the anthracycline sugar moiety in the reduction of doxorubicin/daunorubicin resistance may be similar to that of its incorporation within a morpholinyl ring. In addition, our results show that the resistant phenotype of F4-6 ADM2^R cells did not include resistance to pyrromycin, an *N,N*-dimethylated natural anthracycline that also bears a 9-alkyl substitution. The results obtained using *N,N*-dimethyldoxorubicin and *N,N*-dimethyldaunorubicin suggest that the dimethylation of the amino group of the amino sugar in addition to/instead of the 9-alkyl substitution suggested for aclacinomycin A [6, 8] may also account for the decrease in resistance induced by pyrromycin and related compounds.

Improved cellular drug accumulation was observed for anthracyclines that retain activity in resistant cells [7, 46, 56]. It has been proposed that these derivatives may be less efficient substrates for the P-glycoprotein efflux pump [8]. The results of the present uptake studies using [^{14}C]-daunorubicin and [^{14}C]-*N,N*-dimethyldaunorubicin are consistent with this hypothesis. Our results also demonstrate the absence of an accumulation deficiency for *N,N*-dimethyldaunorubicin in F4-6 ADM2^{R} cells. Accumulation of daunorubicin was about 50% lower in resistant F4-6 cells than in the wild-type cells. For *N,N*-dimethyldaunorubicin, no significant reduction in the drug accumulation in resistant cells as compared with sensitive cells was observed. This indicates that the P-glycoprotein efflux pump is incapable of efficiently reducing the accumulation of the *N,N*-dimethyl derivate.

Decreased resistance to the *N,N*-dimethyl analogues of doxorubicin and daunorubicin has previously been demonstrated in doxorubicin/daunorubicin-resistant P388 leukemia cells [10]. However, this phenomenon was not attributable to a differential accumulation of dimethylated compounds between resistant and wild-type cell lines. In contrast to the results obtained in our cell line, resistant P388 cells used by Egorin et al. [10] showed a reduction in their accumulation of the dimethylated anthracyclines similar to that observed for the parent compounds.

It is noteworthy that in contrast to the in vitro data [10], in vivo experiments in doxorubicin-resistant P388 cells have demonstrated complete cross-resistance to *N,N*-dimethylated derivatives of doxorubicin and daunorubicin [28]. In view of the discrepancy between the results obtained in vivo and those obtained in vitro, we consider it necessary to investigate the resistance of MDR phenotypic cells in vivo to complement our in vitro studies.

In daunorubicin-resistant Ehrlich ascites tumor cells, *N*-acetyldaunorubicin has been shown to increase daunorubicin accumulation via inhibition of the drug-efflux mechanism [45]. The experiments shown in Fig. 5 provide no evidence that the increased accumulation of *N,N*-dimethyldaunorubicin in drug-resistant F4-6 cells was due to an inhibitory action on the P-glycoprotein pump.

Increased lipophilicity via 9-alkyl and morpholinyl substitution, modified basicity via morpholinyl substitution, and steric factors have been suggested to be involved in the overcoming of MDR by anthracycline derivatives [8]. These factors may play a role in the decreased resistance of the F4-6 ADM2^{R} cells to the *N,N*-dimethylated derivatives as well. All anthracyclines investigated that reduced the viability of F4-6 ADM2^{R} cells more effectively than did doxorubicin also exhibited higher lipid solubility. However, a tight correlation between lipophilicity and prevention of resistance could not be established (Tables 1, 2). Accordingly, the increased lipophilicity alone cannot explain the reversal of resistance in F4-6 ADM2^{R} cells. Therefore, the present results point to the importance of the *N*-alkylation of the amino sugar of the anthracycline moiety, which may reduce the drug's affinity for the P-glycoprotein multidrug transporter more specifically, for example, by steric prevention or altered basicity. Further studies using various *N*-alkylated anthracycline derivatives may provide insight into the mechanisms that overcome resistance to anthracycline antitumor antibiotics in MDR tumor cells.

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