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Influence of Structural Modifications at the 3′ and 4′ Positions of Doxorubicin on the Drug Ability to Trap Topoisomerase II and to Overcome Multidrug Resistance

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SUMMARY

To better define the role of the amino sugar in the pharmacological and biochemical properties of anthracyclines related to doxorubicin and daunorubicin, we have investigated the effects of various substituents at the 3′- and 4′-positions of the drug on cytotoxic activity and ability to stimulate DNA cleavage mediated by DNA topoisomerase II. The study shows that the nature of the substituent at the 3′-position but not the 4′-position is critical for drug ability to form cleavable complexes. The amino group at the 3′-position is not essential for cytotoxic and topoisomerase II-targeting activities, because it can be replaced by a hydroxyl group without reduction of activity. However, the presence of bulky substituents at this position (i.e., morpholinyl derivatives) totally inhibited the effects on the enzyme, thus supporting previous observations indicating that the cytotoxic potencies of these particular derivatives are not related to topoisomerase II inhibition. This conclusion is also supported by the observation that 3′-morpholinyl and 3′-methoxymorpholinyl derivatives are able to overcome atypical (i.e., topoisomerase II-mediated) multidrug resistance. Because a bulky substituent at the 4′-position did not reduce the ability to stimulate DNA cleavage, these results support a critical role of the 3′-position in the drug interaction with topoisomerase II in the ternary complex. An analysis of patterns of cross-resistance to the studied derivatives in resistant human tumor cell lines expressing different resistance mechanisms indicated that chemical modifications at the 3′-position of the sugar may have a relevant influence on the ability of the drugs to overcome specific mechanisms of resistance.

Like other intercalating agents with antitumor activity, anthracyclines exert their cytotoxic activity by interfering with DNA topoisomerase II function. Despite their apparent structural diversity, their molecular effects have been related to their ability to interfere with the breakage-rejoining action of topoisomerase II. Although the topoisomerase II inhibitors have a common intracellular target, the molecular basis of their variable therapeutic efficacy is still unknown. Similarly to other highly effective antitumor drugs, DOX stabilizes a transient DNA-topoisomerase II complex in which DNA strands are cut and covalently linked to the enzyme subunits (1–4). Investigations on the sequence specificity of DOX stimulation of *in vitro* DNA cleavage have led to a molecular model for drug action on topoisomerase II; drug molecules may be placed at the interface between the DNA cleavage site and the active site of the enzyme, thus forming DNA-drug-enzyme ternary complexes (5, 6).

The efficacy of DOX as an antitumor agent has stimulated many studies aimed at identifying critical substituents required for optimal activity. Previous studies on structure-activity relationships of anthracyclines have shown an important role for the structure and stereochemistry of the amino sugar (daunosamine) in the pharmacological and biochemical activity of anthracyclines related to DNR and DOX (7–10). The basic amino group at C-3′ has been implicated in determining the DNA binding affinity. However, the presence of a basic group at C-3′ is not a strict requirement for cytotoxic activity of anthracyclines (11). The role of substituents at the 3′-position remains unclear, because *N*-acyl derivatives exhibited low affinity for DNA and markedly reduced cytotoxic potency (7, 12), but substitution of the amino group for an hydroxyl group at C-3′ resulted in comparable cytotoxic activity (11).

The influence of selected chemical modifications at different

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ABBREVIATIONS: DOX, doxorubicin; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VM-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside; SDS, sodium dodecyl sulfate; SV40, simian virus 40; MRP, multidrug resistance-related protein; DNR, daunorubicin; 4′-I-DOX, 4′-deoxy-4′-iododoxorubicin; ID_{50}, drug concentration inhibiting cell growth by 50%, compared with drug-free cultured cells; SCLC, small-cell lung cancer.

positions in the amino sugar has been examined with respect to the ability of the drug to trap DNA topoisomerase II (4, 13). In an attempt to better define the molecular pharmacology of anthracyclines, the present study was undertaken to examine the influence of various substituents at the 3′- and 4′-positions of DOX and/or DNR on the ability of the drugs to stimulate enzyme-mediated DNA cleavage. We provide evidence that (a) the 3′-position but not the 4′-position is critical for the ability of the drugs to interfere with topoisomerase II and (b) 3′-deamino-3′-hydroxy derivatives of DOX overcome *mdr1*-mediated but not atypical multidrug resistance.

Experimental Procedures

Materials. Anthracycline derivatives were synthetized at the Chemistry Department of Farmitalia-Carlo Erba (Milan, Italy). Drugs were dissolved in dimethylsulfoxide or deionized water at 0.1 mM, stored at −20° for a few weeks, and diluted in deionized water immediately before use. DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (14, 15) and was stored at −20° in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 1 mM β-mercaptoethanol. SV40 DNA, T4 polynucleotide kinase, agarose, and polyacrylamide were purchased from Bethesda Research Laboratories (Basel, Switzerland). [γ-^{32}P] ATP was purchased from Amersham (Milan, Italy). Calf intestinal phosphatase and restriction endonucleases were purchased from New England Biolabs (Taunus, Germany). Human *mdr1* and murine β-actin probes were as described previously (16).

Sequencing analysis of DNA cleavage sites. SV40 DNA fragments were uniquely 5′-end-labeled as described previously (5, 13). Briefly, SV40 DNA was cut with the indicated enzyme, dephosphorylated, and ^{32}P-labeled with T4 kinase. Then, DNA was subjected to a second enzyme digestion to generate uniquely 5′-end-labeled fragments, which were separated by agarose gel electrophoresis and purified by electroelution and ethanol precipitation. DNA cleavage reactions were performed in 20 μl of 10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 15 μg/ml bovine serum albumin, with drugs, at 37° for 20 min. Topoisomerase II (106 units, about 200 ng of protein) was added in storage buffer (14). Reactions were stopped with SDS (1%) and proteinase K (0.1 mg/ml) and were incubated at 42° for 45 min. DNAs were then precipitated with ethanol, resuspended in 2.5 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue, heated at 95° for 2 min, chilled on ice, and then loaded onto a 8% polyacrylamide denaturing gel. Gels were run at 70 W for 2 hr. Autoradiograms of dried gels were carried out using Amersham Hyperfilm.

Cell lines. POGB and POVD cell lines were obtained in our laboratory from lung tumor biopsies of two patients bearing SCLC. At the time of biopsies, patients had not been treated with chemotherapy or radiotherapy. After about 10 passages in RPMI 1640 medium supplemented with 10 nM hydrocortisone, 10 nM 17-β-estradiol, 30 nM sodium selenite, 5 mg/ml insulin, 100 mg/ml transferrin, and 5% FCS (Flow Laboratories), cells were adapted to grow in RPMI 1640 medium supplemented with 10% FCS (CM medium). POGB cells grew loosely attached to the flask and, when needed, they were detached through trypsinization or strong pipetting. POVD cells grew as floating clumps. After 10 passages in CM medium, cells were cultured in the presence of 1 ng/ml DOX; in subsequent passages, the DOX concentration was progressively increased up to 120 ng/ml for POGB cells and 100 ng/ml for POVD cells. Drug-selected cells were then tested for their sensitivity to DOX and were shown to have become resistant to this drug. The established resistant cell variants POGB/DX and POVD/DX were maintained always in the presence of 120 or 100 ng/ml DOX, respectively. One passage before each experiment, resistant cells were cultured in drug-free medium. The morphologies of resistant cells were similar to those of sensitive parental cells. The human leukemic cell line CEM and the vinblastine- and VM-26-resistant sublines CEM/VLB_{100} and CEM/VM1 (17–19) were kindly provided by Dr. W. T. Beck (St. Jude Children's Research Hospital, Memphis, TN). The cell lines were maintained at 37° in minimal essential medium (GIBCO) with Earle's salts, supplemented with 10% FCS and 1% vitamins (GIBCO), and were passaged twice weekly. Vinblastine (100 ng/ml) or VM-26 (66 ng/ml) was added at each passage to CEM/VLB_{100} and CEM/VM1 cells, respectively. Parent and resistant CEM cells grew as floating clumps.

Cytotoxicity test. SCLC cells (10^5/ml) were treated for 1 hr at 37° with drugs at different concentrations. Cells were then washed with phosphate-buffered saline and seeded in drug-free CM medium in 96-well tissue culture plates (10^4 POGB cells/well and 5×10^3 POVD cells/well). Ninety-six hours later cell survival was determined with the MTT assay, as described previously (20).

Immediately after seeding, CEM, CEM/VLB_{100}, and CEM/VM1 cells (2×10^4 cells/ml) were treated for 72 hr at 37° with drugs at different concentrations. Cell survival was then determined by cell counting. IC_{50} values were determined from the dose-effect curves.

Northern blot analysis. Total RNA was prepared by the LiCl-guanidine monothiocyanate method (21) from cells harvested in the logarithmic phase of growth. Total RNA (20 μg) was fractionated on a formaldehyde-containing 1% agarose gel and then transferred to a Hybond nylon membrane. The membrane was then irradiated with UV light, and prehybridization was performed for at least 4 hr at 42°, in 50% formamide, 5× standard saline citrate (i.e., 0.75 M NaCl, 75 mM Na citrate, pH 7.2), 0.2% SDS, 5× Denhardt's solution, 50 mM sodium phosphate, pH 7, 250 μg/ml salmon sperm DNA. DNA probes were ^{32}P-labeled with a random primer kit (specific activity, $2–5 \times 10^8$ cpm/μg of DNA). Hybridization was carried out for 20 hr at 42° in the same buffer containing 10% dextran sulfate.

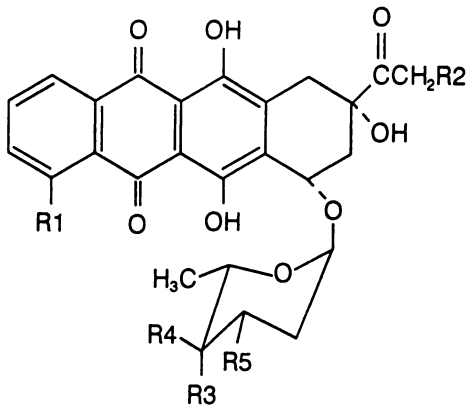
DNA binding studies. DNA binding parameters of anthracycline derivatives were determined by means of the fluorescence quencing method, as described previously (8–10), at the same ionic strength (0.1 M NaCl).

Results

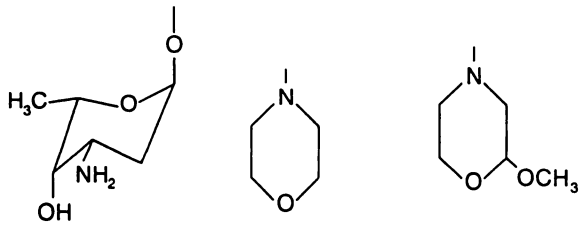
Stimulation of topoisomerase II DNA cleavage. The effect of various substituents at the 3′- and 4′-positions of the anthracycline molecule (Fig. 1) on the stimulation activity of topoisomerase II DNA cleavage was investigated with the experiments shown in Figs. 2 and 3. Drug stimulation of DNA cleavage was determined by incubating 5′-^{32}P-labeled SV40 DNA fragments with topoisomerase II and different concentrations of the analogs and analyzing DNA cleavage intensity patterns with polyacrylamide denaturing gels. Drug analogs were always compared with either DOX or 4-demethoxy-DNR, which have been shown to stimulate identical DNA cleavage intensity patterns (5).

3′-Morpholinyl and 3′-methoxymorpholinyl analogs of DOX did not stimulate enzyme-mediated DNA cleavage; however, they suppressed DNA cleavage at 1 and 10 μM (see 4880 site in Fig. 2). In contrast, 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX stimulated DNA cleavage at the same sites and with similar relative intensities, compared with the parent drug (Fig. 2). Because the 3′-deamino-3′-hydroxy-4′-morpholinyl analog stimulated topoisomerase II DNA cleavage, the chemical nature of the morpholinyl group *per se* may not explain the inability of the 3′-morpholinyl derivatives to interfere with topoisomerase II function. Thus, the relative position of the substitution (3′ versus 4′) appeared to be the critical structural feature of the drug for retention of stimulation activity of DNA cleavage.

We then investigated the 3′-deamino-3′-hydroxy derivatives of DOX shown in Fig. 1. 3′-Deamino-3′-hydroxy-4′-amino-DOX and 3′-deamino-3′-hydroxy-4′-epi-DOX stimulated DNA cleavage to similar extents, compared with the parent



Compound	R1	R2	R3	R4	R5
Doxorubicin (DOX)	OCH_3	OH	OH	H	NH_2
Daunorubicin (DNR)	OCH_3	H	OH	H	NH_2
3'deamino-3'hydroxy -4'epiDOX	OCH_3	OH	H	OH	OH
4demethoxy-3'deamino-3'hydroxy-4'epiDOX	H	OH	H	OH	OH
3'deamino-3'hydroxy-4'aminoDOX	OCH_3	OH	NH_2	H	OH
3'morpholinylDOX	OCH_3	OH	OH	H	(b)
3'methoxy morpholinylDOX	OCH_3	OH	OH	H	(c)
3'deamino-3'hydroxy-4'morpholinylDOX	OCH_3	OH	(b)	H	OH
4'daunosaminylDNR	OCH_3	H	(a)	H	NH_2
4'I-DOX	OCH_3	OH	I	H	NH_2



(a) Daunosaminyl (b) Morpholinyl (c) Methoxymorpholinyl

Fig. 1. Chemical structures of the studied anthracycline derivatives.

drug (Fig. 2). 4-Demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX was the most potent analog in stimulating DNA cleavage, because it was effective even at 0.1 μM. Because 4′-epimerization has been shown to have no effect on cleavage stimulation by anthracyclines (13), these observations indicated that the amino group at the 3′-position is not necessary for the drug effect on topoisomerase II. Indeed, lack of the amino group in the sugar increased the stimulation activity of anthracyclines for DNA cleavage (Fig. 2). 4′-Morpholinyl-DOX, 3′-deamino-3′-hydroxy-4′-epi-DOX, and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX even at 10 μM stimulated DNA cleavage, whereas global suppression of DNA cleavage was observed at 10 μM for DOX, 3′-morpholinyl analogs, and 3′-deamino-3′-hydroxy-4′-amino-DOX (Fig. 2 and data not shown; see also Ref. 13). A lack of suppressive effect of the 3′-hydroxy derivatives may be rationalized in terms of reduced DNA binding affinity (Table 1), causing a different biphasic response.

To further evaluate the role of the 4′-position, we investigated 4′-*O*-daunosaminyl-DNR and 4′-I-DOX, which have an additional amino sugar and an iodine atom, respectively, at the 4′-position (Fig. 1). Both of these analogs stimulated cleavage of SV40 DNA in the presence of topoisomerase II (Fig. 3). 4′-I-DOX has been shown to stimulate protein-associated DNA breaks in living tumor cells (22).

DNA cleavage intensity patterns were identical among all of the studied analogs (Figs. 2 and 3). Weak cleavage sites were, however, more easily detected with the most potent derivatives (see sites from 4779 to 4814 in Fig. 2). The sequence specificity of DNA cleavage stimulation by the studied derivatives was thus the same as that reported for the parent drugs (5).

Cytotoxic potency of the anthracycline derivatives in human SCLC cell lines. All of the studied analogs were cytotoxic against human SCLC cells, although differences in potency could be noted. 3′-Deamino-3′-hydroxy-4′-epi-DOX was 3–4-fold less potent than DOX, and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX was about as potent as the parent drug (Table 2). The increased potency of 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX, compared with 3′-deamino-3′-hydroxy-4′-epi-DOX, showed that removing the methoxy group at the 4-position in the planar ring system enhanced drug cytotoxic activity, in agreement with previous findings (23, 24). Because 4′-epimerization did not affect DOX cytotoxicity (8), removal of the 3′-amino group appears to be responsible for the reduced cell-killing activity. No precise correlation was found between the cytotoxic potency and DNA-cleaving activity, because these analogs were more active than DOX in stimulating topoisomerase II DNA cleavage (Fig. 2). Therefore, it may be possible that cellular pharmacokinetics of these analogs were somewhat altered by the chemical modification at the 3′-position, compared with DOX.

3′-Deamino-3′-hydroxy-4′-amino-DOX and 4′-*O*-daunosaminyl-DNR were somewhat less potent than DOX in SCLC cell lines (Table 2). However, they have been shown to be 2–3 times more potent than and as potent as the parent drug, respectively, in a sensitive CEM human leukemia cell line (25).

3′-Morpholinyl analogs were 3–6-fold more potent than DOX in the two sensitive SCLC cell lines, POGB and POVD (Table 2), in agreement with previous findings showing marked cytotoxic activity of 3′-morpholinyl-anthracyclines (26–28). In contrast, 4′-morpholinyl-DOX was similar to DOX, or slightly less potent than the parent drug (Table 2). Consistently, 4′-morpholinyl-DOX was 2.5-fold less potent, whereas 3′-morpholinyl analogs were about 4.5-fold more potent than DOX in an unrelated SCLC cell line, NCI-H187 (data not shown).

Cell-killing activity of drugs in human multidrug-resistant tumor cell lines with different mechanisms of resistance. The cytotoxic activities of the studied derivatives were also determined in the multidrug-resistant variants of the POGB and POVD SCLC lines (Table 2) and in two multidrug-resistant lines derived from a CEM human leukemia line (16–18) (Table 3). Both POVD/DX and POGB/DX lines were obtained from the corresponding sensitive lines by DOX selection (see Experimental Procedures for details).[1] It is likely that different mechanisms of drug resistance have been activated in these two DOX-selected SCLC sublines. Overexpression of the *mdr1* gene was found in POVD/DX cells, compared with the

[1] M. Binaschi, R. Supino, G. Capranico, and F. Zunino. Multidrug resistance in small cell lung cancer cell lines. Manuscript in preparation.

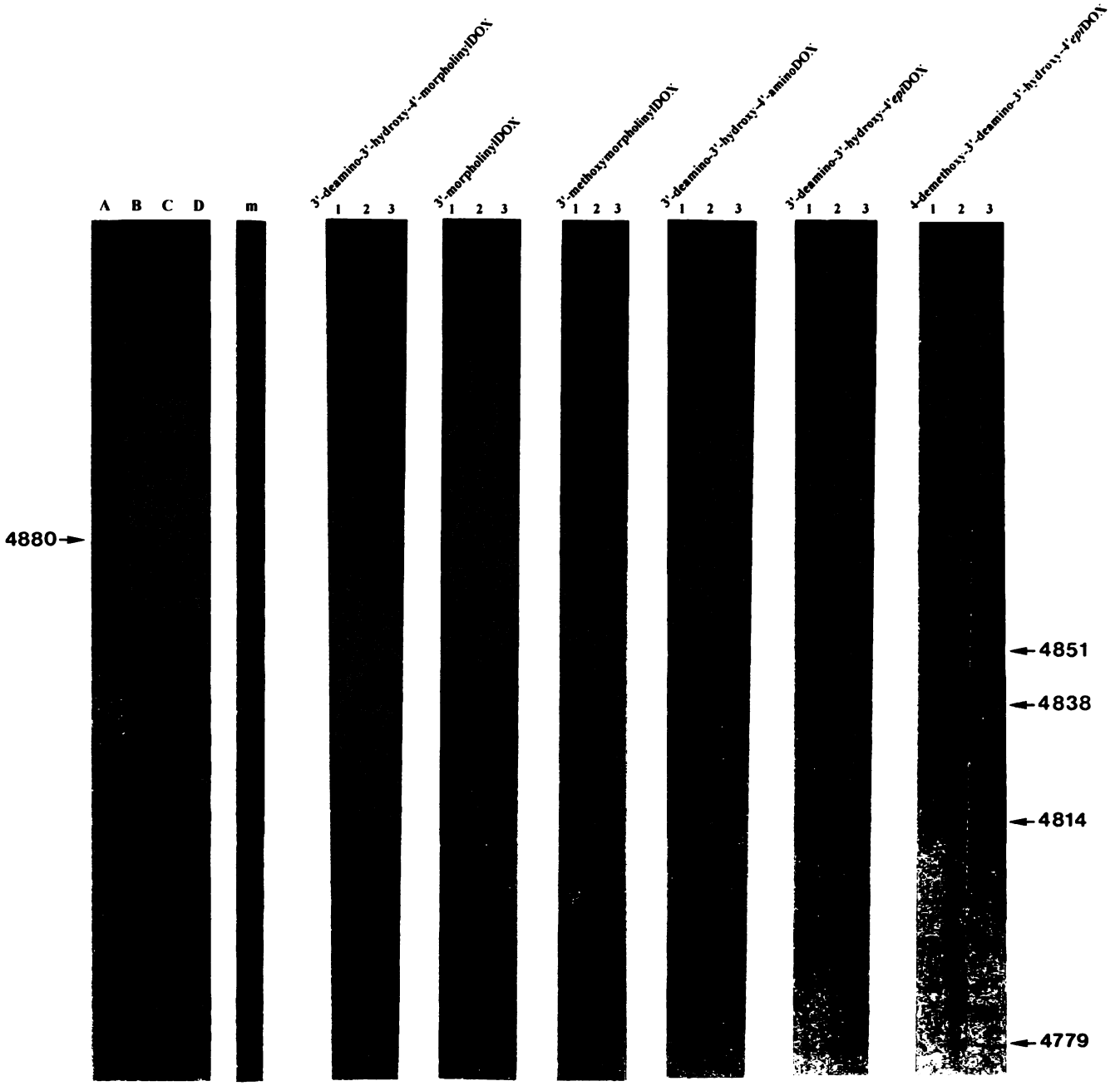


Fig. 2. Topoisomerase II DNA cleavage stimulated by the studied DOX derivatives in SV40 DNA. SV40 DNA ^{32}P-labeled at the *TaqI* site was incubated for 20 min at 37° with purified murine topoisomerase II and different concentrations of drugs. Cleavage reactions were stopped with 1% SDS and 0.1 mg/ml proteinase K, incubated at 42° for 45 min, precipitated with ethanol, and then analyzed on 8% polyacrylamide sequencing gels. *Lane A*, control DNA; *lane B*, topoisomerase II alone; *lane C*, with 1 μM DOX; *lane D*, with 1 μM 4-demethoxy-DNR; *lane m*, purine molecular weight markers; *lanes 1*, *2*, and *3*, 0.1, 1, and 10 μM indicated drug, respectively. *Arrows*, some cleavage sites; *numbers*, genomic position in SV40 DNA.

sensitive parental POVD cells (Fig. 4). In these experiments human colon cancer LoVo and LoVo/DX cells were used as a control system for P-glycoprotein overexpression (29). POVD/DX exhibited a pattern of cross-resistance typical of the multidrug-resistant phenotype, because it included vincristine, etoposide, and taxol but not cisplatin, melphalan, or 5-fluorouracil. In contrast, *mdr1* gene expression was not detected in POGB/DX cells, which instead exhibited amplification (about 50-fold) and overexpression of the MRP gene.[2] The phenotypes of multidrug-resistant CEM sublines have been shown to be distinct (17–19). CEM/VLB$_{100}$ cells had a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene, whereas CEM/VM1 cells showed an atypical multidrug-resistant phenotype and did not overexpress the *mdr1* gene but instead had a mutated topoisomerase IIα gene coding for a drug-resistant protein (17–19).

The studied 3′-deamino-3′-hydroxy-4′-epi derivatives could overcome the drug resistance of POGB/DX, POVD/DX, and CEM/VLB cells but not that of CEM/VM1 cells (Tables 2 and 3). These observations suggested that an amino group in the sugar moiety of DOX might be critical for drug transport by P-glycoprotein. 3′-Morpholinyl derivatives were as active in all of the drug-resistant variant lines as in the corresponding

[2] M. Binaschi, R. Supino, R. A. Gambetta, G. Giaccone, E. Prosperi, G. Capranico, and F. Zunino. MRP gene overexpression and amplification in a human doxorubicin-resistant SCLC cell line.

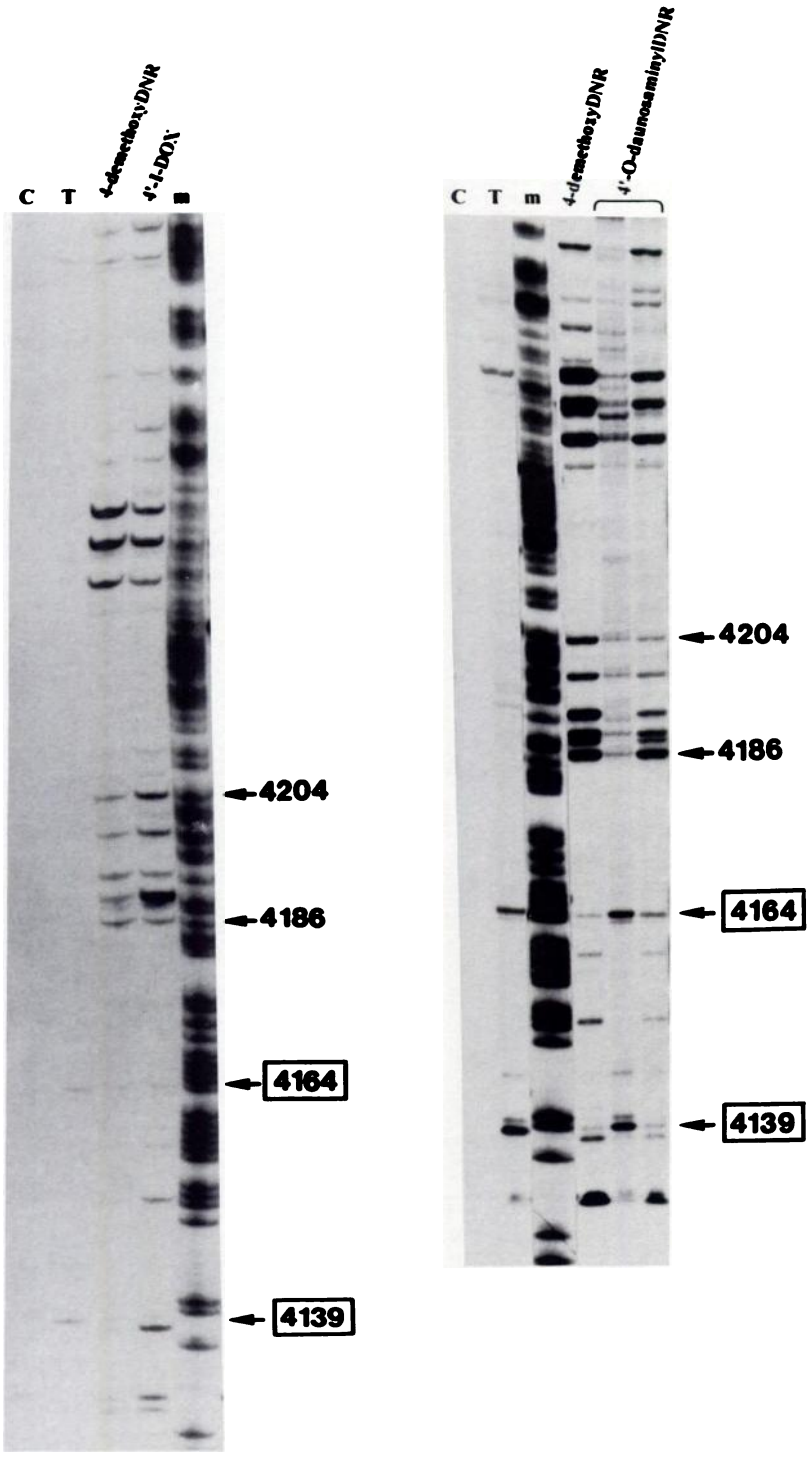


Fig. 3. Topoisomerase II DNA cleavage stimulated by the studied DOX derivatives in SV40 DNA. SV40 DNA was ^{32}P-labeled at a *Xho*II site. See legend to Fig. 2 for additional details. *Lane C*, control DNA; *lane T*, topoisomerase II alone; *lane m*, purine molecular markers. 4-Demethoxy-DNR and 4′-I-DOX were used at 1 μM. 4′-*O*-Daunosaminyl-DNR was used at 0.1 and 1 μM in the *left* and *right lanes*, respectively. *Arrows*, cleavage sites; *numbers*, genomic position in SV40 DNA. *Boxed numbers*, sites not stimulated by drugs.

parent cell lines. In contrast, CEM/VM1 but not CEM/VLB cells were fully cross-resistant to 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX, supporting the idea that this derivative was directed against topoisomerase II also in living tumor cells.

The patterns of cross-resistance of these variant lines were not similar (Tables 2 and 3). Some observations can be made. (a) 3′-Morpholinyl derivatives were the only drugs showing no cross-resistance in the CEM/VM1 cell line, whereas all other drugs showed cross-resistance at similar or higher levels, compared with DOX (Table 3). (b) Only 4′-*O*-daunosaminyl-DNR and 3′-deamino-3′-hydroxy-4′-amino-DOX showed cross-resistance fully in CEM/VLB cells and at the highest level among the studied drugs in POVD/DX cells (Tables 2 and 3). Both of these analogs retained an amino group in the sugar moiety, and these two lines were drug resistant due to overexpression of the P-glycoprotein gene. (c) POGB/DX cells showed a specific pattern of cross-resistance; these cells were cross-resistant only to 3′-deamino-3′-hydroxy-4′-morpholinyl-DOX and 4′-*O*-daunosaminyl-DNR. This finding was consistent with a different mechanism of resistance developed by POGB/DX cells.

Discussion

Previous studies from this laboratory demonstrated the importance of the natural amino sugar (daunosamine) as a critical

TABLE 1

Binding parameters for the interaction of anthracycline derivatives with calf thymus DNA

K_{app} is the apparent binding constant; n is the apparent binding sites/nucleotide.

	K_{app} ($\times 10^6$)	n
	M^{-1}	
DNR	4.8	0.160
DOX	6.5	0.179
3′-Deamino-3′-hydroxy-4′epi-DOX	0.77	0.062
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	1.0	0.072
3′-Deamino-3′-hydroxy-4′amino-DOX	2.4	0.178
4′-I-DOX	6.4	0.100
4′-*O*-Daunosaminyl-DNR	2.7	0.283

determinant of anthracycline activity (8). In agreement with the view that DNA topoisomerase II is the primary target of drug action (4), we now provide evidence that the sugar moiety is also important for drug stimulation of topoisomerase II-mediated DNA cleavage. The 3′-*N*-substituted anthracyclines exhibited a reduced ability to stimulate topoisomerase II-mediated cleavage (30). The inability of 3′-morpholinyl and 3′-methoxymorpholinyl, but not 4′-morpholinyl, derivatives of DOX to stimulate enzyme-mediated DNA cleavage is consistent with these observations (see also Ref. 31) and suggests that a bulky substituent at the 3′-position is a steric hindrance for the formation of the ternary complex (drug-enzyme-DNA). Previous studies have emphasized the role of the amino group in the stabilization of the intercalation complex (32), and a free protonated amino group has been implicated in electrostatic interactions in the minor groove (33–35). Indeed, *N*-acetyl derivatives of DNR and DOX exhibited low DNA binding activity and markedly reduced cytotoxic and antitumor potencies. In contrast, because 3′-morpholinyl and 3′-methoxymorpholinyl derivatives are still very potent cytotoxic agents, it is evident that these compounds may exert cytotoxic activity by other mechanisms that are independent of topoisomerase II inhibition. 3′-Methoxymorpholinyl-DOX has a potential for covalent binding to DNA (36), and the formation of a drug-DNA adduct by active 3′-morpholinyl-DOX metabolites has been reported (27). 3′-Morpholinyl-DOX itself was found to retain DNA-binding ability and to stimulate topoisomerase I-induced DNA cleavage (31). Further investigations at a cellular level are required to better understand the mechanism of action of these derivatives.

The present study provides further evidence that an amino group at the 3′-position is not required to stimulate topoisomerase II-mediated DNA cleavage, because substitution of a hydroxyl group for the amino group resulted in compounds (i.e., 3′-deamino-3′-hydroxy-4′-epi-DOX and 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-DOX) with activity comparable or superior to that of the corresponding 3′-aminoanthracyclines. This finding is consistent with previous observations that 3′-hydroxy derivatives retained cytotoxic and antitumor activities (11, 25). In contrast to 3′-morpholinyl-DOX, the compound with this substituent at the 4′-position has been found to have the same ability to trap topoisomerase II as exhibited by the parent compound, DOX. Similarly, different substitutions at the 4′-position (i.e., 4′-*O*-daunosaminyl-DNR and 4′-I-DOX) resulted in agents effective as topoisomerase II inhibitors. Overall, these results indicated that the presence of a bulky substituent at the 3′- but not the 4′-position prevents drug stimulation of topoisomerase II cleavage. As expected, the removal of the amino group in the sugar moiety caused an appreciable reduction of drug affinity for DNA. However, this reduction was accompanied by an appreciable increase in drug ability to trap DNA topoisomerase. A lack of precise correlation between the DNA binding affinity and the cellular and molecular effects of anthracyclines supports the view that the specific mode of DNA interaction is a more critical determinant for drug activity than is the strength of binding (4).

All of the tested 3′-hydroxy derivatives (Table 2) showed marked activity in the cytotoxicity assay. Using SCLC cell lines, the pattern of cross-resistance indicated that, with the exception of 3′-deamino-3′-hydroxy-4′-amino-DOX, all 3′-hydroxy derivatives overcame multidrug-resistance mediated by *mdr1* gene overexpression (i.e., POVD/DX cell line). It is possible that the presence of a free amino group is an important determinant for drug recognition by P-glycoprotein, because 4′-*O*-daunosaminyl-DNR also displays partial cross-resistance in this cell system. Alternatively, by removal of the sugar amino group the drug becomes more lipophilic, and this may favor cellular drug uptake by passive diffusion, thus counteracting the P-glycoprotein-dependent increased drug efflux in resistant cells. Similar results were obtained in the CEM/VLB cell line, with a typical multidrug resistant phenotype (Table 3).

The pattern of cross-resistance was somewhat different in the POGB/DX cell line. In this system, only compounds with a bulky substituent at the 4′-position showed cross-resistance. This subline exhibited a multidrug resistant phenotype with reduced intracellular drug accumulation, without *mdr1* expression.[2] It is possible that other transport systems (MRP gene

TABLE 2

Cytotoxic activities and cross-resistance of anthracycline derivatives in human SCLC cell lines

POVD/DX cells showed a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene (Fig. 4); POGB/DX cells did not express the *mdr1* gene but had amplification and overexpression of the MRP gene.[2] IC_{50} values were determined from dose-response curves after 1-hr exposure to the drug. The shapes of the dose-response curves were similar for all tested compounds. In parentheses is the resistance index, calculated as the ratio of the IC_{50} values in DOX-resistant and parental cell lines.

Drug	IC_{50}			
	POGB	POGB/DX	POVD	POVD/DX
	$\mu g/ml$			
DOX	0.30	1.90 (6.3)	0.30	4.80 (16)
3′-Deamino-3′-Hydroxy-4′epi-DOX	1.22	2.00 (1.6)	1.00	2.10 (2.1)
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	0.22	0.25 (1.1)	0.41	0.25 (0.6)
3′-Deamino-3′-hydroxy-4′-amino-DOX	0.80	1.30 (1.6)	0.62	3.20 (5.2)
3′-Morpholinyl-DOX	0.12	0.14 (1.2)	0.05	0.17 (3.4)
3′-Methoxymorpholinyl-DOX	0.10	0.18 (1.8)	0.05	0.06 (1.2)
3′-Deamino-3′-hydroxy-4′-morpholinyl-DOX	0.38	1.55 (4.1)	0.70	1.20 (1.7)
4′-*O*-Daunosaminyl-DNR	0.88	3.95 (4.5)	0.76	3.65 (4.8)

TABLE 3

Cross-resistance to the studied anthracycline derivatives of two human multidrug-resistant CEM cell lines

CEM/VLB$_{100}$ cells showed a classical multidrug-resistant phenotype and overexpressed the *mdr1* gene, whereas CEM/VM1 cells showed an atypical multidrug-resistant phenotype and had a mutated topoisomerase IIα gene (16-18). Drug treatments were for 72 hr at 37°; drugs were then washed out, and cell survival was evaluated by cell counting. ID$_{50}$ values were 3.3, 72.3, and 28.7 ng/ml for 3′-deamino-3′-hydroxy-4′-amino-DOX and 6.1, 270, and 15.5 ng/ml for 4′-daunosaminyl-DNR in the parent CEM, CEM/VLB, and CEM/VM1 cell lines, respectively. ID$_{50}$ values for the other drugs were reported previously (24).

Drug	Resistance index relative to sensitive CEM line	
	CEM/VLB	CEM/VM1
DOX	31	5.7
4-Demethoxy-DNR	2.8	4.8
3′-Deamino-3′-hydroxy-4′epi-DOX	3.9	13.2
4-Demethoxy-3′-deamino-3′-hydroxy-4′epi-DOX	0.9	8.1
3′-Deamino-3′-hydroxy-4′-amino-DOX	22	8.7
3′-Morpholinyl-DOX	2.4	2.0
3′-Methoxymorpholinyl-DOX	1.0	1.1
3′-Deamino-3′-hydroxy-4′-morpholinyl-DOX	1.2	5.6
4′-*O*-Daunosaminyl-DNR	44	2.5
4′-I-DOX	1.2	2.9

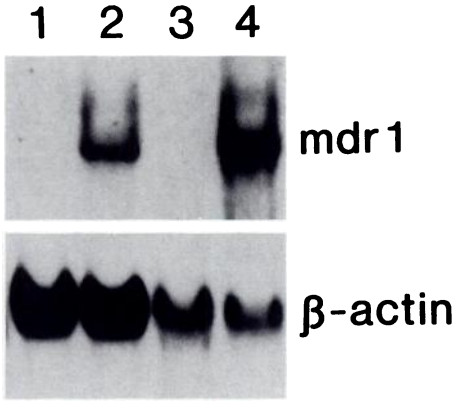


Fig. 4. Overexpression of the *mdr1* gene in DOX-resistant POVD/DX cells. Twenty micrograms of total RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with the indicated human probes. *Lane 1*, POVD; *lane 2*, POVD/DX; *lane 3*, LoVo; *lane 4*, LoVo/DX.

product?) are involved, conferring a different cross-resistance pattern. Among the compounds examined, only 3′-morpholinyl and 3′-methoxymorpholinyl derivatives were found to be able to overcome resistance mediated by topoisomerase II gene mutations (i.e., in CEM/VM1 cells). This finding is in agreement with the hypothesis that these derivatives differ from conventional anthracyclines, with the natural amino sugar, in their mechanism of action (27). In CEM/VM1 cells all 3′-hydroxy derivatives were found to be cross-resistant, with a resistance index comparable or superior to that of DOX. Again, this observation is consistent with the conclusion that 3′-hydroxy derivatives exert cytotoxic activity through inhibition of topoisomerase II function. The inability of these compounds to overcome topoisomerase II-mediated resistance is expected on the basis of the identical sequence specificities of DNA cleavage stimulation, compared with DOX (5), suggesting a similar structural basis of drug interaction with topoisomerase II in the ternary complex.

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