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Differential single- versus double-strand DNA breakage produced by doxorubicin and its morpholinyl analogues

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Abstract The morpholinyl analogues of doxorubicin (DOX) have previously been reported to be non-cross-resistant in multidrug resistant (MDR) cells due to a lower affinity for P-glycoprotein relative to the parent compound. In order to further investigate the mechanisms of action of these morpholinyl anthracyclines, we examined their ability to cause DNA single- and double-strand breaks (SSB, DSB) and their interactions with topoisomerases. Alkaline elution curves were determined after 2-h drug treatment at 0.5, 2 and 5 μ*M*, while neutral elution was conducted at 5, 10 and 25 μ*M* in a human ovarian cell line, ES-2. A pulse-field gel electrophoresis assay was used to confirm the neutral elution data under the same conditions. Further, K-SDS precipitation and topoisomerase drug inhibition assays were used to determine the effects of DOX and the morpholinyl analogues on topoisomerase (Topo) I and II. Under deproteinated elution conditions (pH 12.1), DOX, morpholinyl DOX (MRA), methoxymorpholinyl DOX (MMDX) and morpholinyl oxaunomycin (MX2) were equipotent at causing SSB in the human ovarian carcinoma cell line, ES-2. However, neutral elution (pH 9.6) under deproteinated conditions revealed marked differences in the degree of DNA DSB. After 2-h drug exposures at 10 μ*M*, DSBs were 3300 rad equivalents for MX2, 1500 for DOX and 400 for both MRA and MMDX in the ES-2 cell line. Pulse-field data substantiated these differences in DSBs, with breaks easily detected after MX2 and DOX treatment, but not with MRA and MMDX. DOX and MX2 thus cause DNA strand breaks selectively through interaction with Topo II, but not Topo I. In contrast, MRA and MMDX cause DNA breaks through interactions with both topoisomerases with a predominant inhibition of Topo I.

Key words Anthracyclines · DNA strand breakage · Morpholinyl doxorubicin · MX2 · topoisomerases

Introduction

The antineoplastic agent, doxorubicin (DOX), is important clinically, but its use is limited due to cardiotoxicity, myelosuppression and multidrug resistance (MDR). In an attempt to overcome this drug resistance, Acton et al. [1] synthesized a series of DOX derivatives with morpholinyl groups at the 3′ position of the sugar moiety. These DOX analogues are non-cross-resistant in MDR variants in vitro, and have been shown to increase cellular accumulation of drug relative to the parent compound by tritiated and fluorescent assays [2–5]. These data suggest that the addition of the morpholinyl group alters the affinity of DOX for P-glycoprotein, an ATP-dependent efflux pump encoded by the *mdr*1 gene [6–10]. Also, these DOX analogues have been shown to be more lipophilic than DOX [1] and non-cardiotoxic at antitumor doses [2, 3]. Examples of these morpholinyl derivatives include morpholinyl DOX (MRA), methoxymorpholinyl DOX (MMDX), and morpholinyl oxaunomycin (MX2) (Fig. 1).

Previously, we have demonstrated that the MRA and MMDX compounds can be activated by microsomal metabolism and can crosslink DNA [11, 12]. This activation is associated with a potentiation of their cytotoxicity in vivo and in vitro [13, 14]. The closely related but extremely potent cyanomorpholinyl

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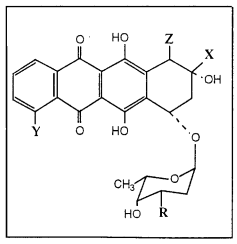

Name	Abbreviation	X	Y	Z	R
1. Doxorubicin	DOX	$-C(=O)-CH_2OH$	$-OCH_3$	-H	$-NH_2$
2. Morpholinyl doxorubicin	MRA	$-C(=O)-CH_2OH$	$-OCH_3$	-H	
3. Morpholinyl oxaunomycin	MX2	$-CH_2CH_3$	-OH	-OH	
4. Methoxymorpholinyl doxorubicin	MMDX	$-C(=O)-CH_2OH$	$-OCH_3$	-H	

Fig. 1 The chemical structures of DOX and the morpholinyl anthracyclines

derivative (MRA-CN) is capable of crosslinking DNA without microsomal activation [2]. We report here a further study of these native morpholinyl DOX compounds (i.e. no microsomal bioactivation). DNA elution assays were used in order to quantify the degree of single-strand breakage (SSB) and double-strand DNA breakage (DSB) induced after a 2-h treatment in a drug-sensitive human ovarian cell line relative to the parent compound, and the results were confirmed with a pulse-field gel electrophoresis assay. Further, data from K-SDS precipitation assays for protein–DNA complexes and topoisomerase activity inhibition experiments suggest that MRA and MMDX induce DNA strand breaks through interaction with topoisomerase I (Topo I), while the mechanism of action for MX2 appears to be more similar to DOX and related to the inhibition of topoisomerase II (Topo II).

Materials and methods

Drugs

MRA was generously provided by Dr. E. M. Acton (Drug Synthesis and Chemistry Branch, National Cancer Institute). MMDX was obtained from Farmitalia Carlo Erba Laboratories (Milano, Italy). MX2 was provided by the Kirin Company (Japan) and DOX was purchased as a commercial preparation from Adria Laboratories (Columbus, Ohio). Drug stock solutions were prepared in absolute ethanol at a concentration of 1.0 m*M* and stored at $-20\,^{\circ}C$.

Cell culture

The human ovarian carcinoma cell line, ES-2, was established in our laboratory and was grown as a monolayer culture in McCoy's 5A medium supplemented with 10% newborn calf serum, 0.3 mg/l glutamine, 100 U/ml penicillin/ml, and 100 mg/l streptomycin (all from GIBCO Laboratories, Grand Island, N.Y.). ES-2 cells were free of mycoplasma contamination as determined by the GEN-Probe hybridization assay (GEN-Probe, San Diego, Calif).

MTT cytotoxicity assay

The cytotoxicity of DOX and the morpholino anthracyclines was determined using a modified MTT 3-(4,5-dimethylthiazol(-2-yl,1-2,5-diphenyl-tetrazolium bromide assay [15]. ES-2 cells were plated in 96-well microtiter plates (Falcon, Becton Dickinson Co.; Lincoln Park, N.J.) in 200 µl medium. After 24 h, the cells were exposed to drugs at the appropriate dilutions and allowed to incubate for an additional 48 h (approximately two cell divisions). (MTT 20 µl, of MTT 5 mg/ml in phosphate-buffered saline) was added to each well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 3 h, the medium was aspirated and 0.1 *N* HCl-isopropanol solution was added in order to solubilize the formazan crystals. Absorbances were read at 570 nm on a Molecular Devices U.V. Thermomax multiwell spectrophotometer (Molecular Devices, Menlo Park, Calif.). Each drug was tested in quadruplicate and in at least three different experiments. Initial experiments indicated that 48 h was suitable for measuring cytotoxicity, as further drug incubation did not result in enhanced cytotoxicity.

Alkaline elution

The alkaline elution technique employed was modified from the method of Kohn [16]. Briefly, ES-2 cells were labeled for 36–48 h with 0.01 µCi/ml of [methyl-^{14}C]-thymidine and internal standard cells were labeled with 0.1 µCi/ml [methyl-^{3}H]-thymidine (both from Amersham Corporation, Arlington Heights, Il). The cells were chased with cold medium overnight and then exposed to drug for 2 h at 37 °C. In order to calculate radiation equivalents, one flask of ^{14}C-labeled cells was irradiated with 300 cGy using a mark 1, model 25 cesium-137 gamma-irradiating machine (J. L. Shepherd & Associated, Glendale, Calif). Internal control cells labeled with ^{3}H were irradiated with 400 cGy. Approximately 10 000 cpm ^{14}C-labeled cells and 20 000 cpm ^{3}H-labeled cells were loaded onto a smoke stack column with a 0.8 µm vinyl/acrylic copolymer filter (DM Metricel filter, 25 mm; Gelman Sciences, Ann Arbor, Mich). Cells were lysed by adding 2% SDS, 0.02 *M* EDTA at pH 10.0 in the presence and absence of 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, M.) for 30 min; the lysate on the filter was washed with 0.02 *M* EDTA, pH 10.0. The elution buffer consisted of 0.02 *M* EDTA and 0.1% SDS, adjusted to pH 12.1 with tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, N.Y.).

A Manostat cassette pump (New York, N.Y.) was employed to provide an elution rate of 2 ml/h and 4-ml elution fractions were collected using an LKB SuperRac fraction collector (LKB-Produkter, Bromma, Sweden). Ecolite scintillation cocktail (ICN Biomedicals, Irvine, Calif.) was added to each fraction, as well as to the 1 *N* HCl-treated filter. Dual radioactivity was counted using an LS-8000 counter (Beckman Instruments, Palo Alto, Calif.). Log-fractions of ^{14}C retained against ^{3}H retained were plotted using an Excel program on a Macintosh computer.

Neutral elution

The alkaline elution procedure was followed with the following modifications. ^{14}C-labeled ES-2 cells were irradiated with 3000 cGy and ^{3}H-labeled cells with 5000 cGy. The elution buffer consisted of 0.02 *M* EDTA and 0.1% SDS, adjusted to pH 9.6 with tetrapropylammonium hydroxide for the detection of DSBS.

Asymmetric field inversion gel electrophoresis (AFIGE)

A modified AFIGE technique was followed for the quantification of DNA DSBs induced in ES-2 cells [17]. ES-2 cells were labeled with [methyl-^{14}C]-thymidine for 24 h followed by 2-h drug incubations. Cells were then cast in a 1% insert agarose (Seakem), and lysed in a 1% sodium lauryl sarkosine, 0.5 *M* EDTA and 1 mg/ml proteinase K solution at 50 °C overnight. The cells were treated with 100 μg/ml DNase-free ribonuclease A for 4 h at 37 °C. The 0.8% (w/v) agarose gel in 0.5 × Tris-borate/boric acid (TBE) buffer was run at 900s forward pulse time at 1.25 V/cm and 75 s backward pulse time at − 2.5 V/cm for a total electrophoresis time of 33 h. The agarose plugs were then removed from the gel, treated with 10 *N* HCl and melted on a hotplate. Ecolite scintillation cocktail was added to each and read in a Beckman LS-8000 counter. Quantification of DNA DSBs was determined by calculating the percentage of DNA released from the agarose plugs using an Excel program on a Macintosh computer.

K-SDS precipitation assay

The topoisomerase assay of Rowe et al. [18] was used with some modification. Briefly, ES-2 cells were labeled with 1 μCi/ml of [methyl-^{3}H]-thymidine for 24 h. Cells were then washed once in PBS and exposed to the appropriate drug dilution (10 μ*M* for each cytotoxin tested) for 1 h at 37 °C in an atmosphere containing 5% CO_2. The medium was subsequently aspirated and cells lysed with a 1.25% SDS, 5 m*M* EDTA (pH 8.0) supplemented with 0.4 mg/ml salmon sperm DNA. After shearing the DNA, cell lysates were transferred to 1.5-ml Eppendorf tubes containing 200 μl 325 m*M* KCl. Each tube was maintained at 65 °C for 5 min and then cooled on ice for an additional 10 min. Tubes were then spun at 10 000 *g* for 2 min at room temperature in an Eppendorf centrifuge. The supernatants were aspirated and pellets washed twice with 10 m*M* Tris-HCl (pH 8.0), 100 m*M* KCl, 1 m*M* EDTA, supplemented with 0.1 mg/ml salmon sperm DNA. The suspension was cooled on ice and spun at 10 000 *g*. The pellet was resuspended in 500 μl H_2O and maintained at 65 °C until the pellet was dissolved. After the suspension was transferred to a scintillation vial, 10 ml Ecolite cocktail was added to each and was counted in a Beckman LS-8000 counter. The percentage of specifically precipitated DNA was calculated using an Excel program on a Macintosh computer as follows:

$$\%\ \text{precipitated} = \frac{\text{Counts}_{\text{tested}} - \text{Counts}_{\text{control}}}{\text{Counts}_{\text{plated}} - \text{Counts}_{\text{control}}} \times 100$$

Topoisomerase I/II drug screening

The induction of cleavage complexes by Topo I and II was studied using drug screening assay kits purchased from TopoGEN (Columbus, Ohio). Briefly, purified human DNA Topo I (10 units) was incubated with 0.25 μg of supercoiled *pHOT* plasmid (form I DNA) with a specific cleavage site for Topo I in a buffer containing 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA and 100 m*M* NaCl. Drugs were added to the mixture to final concentrations of 0.1, 0.5, 0.75 and 1 μ*M* and incubated at 37 °C for 30 min in a Perkin Elmer 9600 DNA thermal cycler (Norwalk, Ct.). Camptothecin (CPT) was used as a positive control drug for the inhibition of Topo I activity. The reaction mixture (20 μl) was stopped by the addition of 10% SDS and proteinase K (50 μ g/ml) followed by extraction with chloroform and isoamyl alcohol (24: 1 v/v).

For the Topo II assays, 4 units of human Topo II was incubated with a supercoiled DNA substrate (*pRYG* DNA) containing a single Topo II cleavage site in the presence and absence of drug for 30 min at 37 °C. VM-26, a known Topo II inhibitor, was used as a positive control. The buffer for the Topo II assays consisted of 30 m*M* Tris-HCl (pH 7.6), 3 m*M* ATP, 15 m*M* mercaptoethanol, 8 m*M* $MgCl_2$ and 60 m*M* NaCl. The assay was extracted as described above and the samples were then analyzed by agarose (1%) gel electrophoresis at 1.5 V/cm in 4 × Tris-acetate/EDTA (TAE) buffer. DNA was visualized by ethidium bromide staining.

Results

The in vitro cytotoxicity data for DOX and its morpholinyl derivatives in the ES-2 cell line are summarized in Table 1. MMDX was the most potent, followed by MRA and DOX, while MX2 was only half as potent as the parent compound. The three morpholinyl drugs were non-cross-resistant in our DOX-selected MDR variants [5].

In order to further investigate the mechanism(s) of action for these compounds, DNA elution studies were conducted and the alkaline elution profiles of ES-2 cells are shown in Fig. 2. After 2-h drug incubations, protein-associated DNA SSBS appeared to peak at 2 μ*M* for DOX, MMDX and MX2, while MRA continued to show a linear increase in DNA SSBs at 5 μ*M*. With the exception of MRA, DOX and its morpholinyl derivatives appeared to reach a maximum number of SSBs at approximately 200 rad equivalents (Table 2). The neutral elution profiles under deproteinated conditions are shown in Fig. 3. Interestingly, MX2 induced the greatest number of protein-associated DNA DSBs in the ES-2 cell line, followed by DOX. MRA and MMDX induced only a fraction of the DSBs as compared to the parent compound or MX2. MX2 at 25 μ*M* surpassed the 5000 cGy control in its ability to cleave DNA. DOX, on the other hand, reached its maximum DSBs at 10 μ*M*, producing approximately 1500 rad equivalents (Table 3). MRA and MMDX both reached a plateau at 10 μ*M* with approximately 300 rad equivalents.

To verify the results found with neutral elution, we employed the technique of AFIGE or pulse-field gel electrophoresis. The same drug concentrations were used in ES-2 cells for 2-h drug incubations in order to determine the percentage of DNA released under deproteinated conditions. The pulse-field gel profiles substantiated our elution data (Fig. 4), demonstrating that MX2 was clearly the most potent in causing DNA DSBs. DOX-induced DSBs were significant, while strand breaks in ES-2 cells treated with MRA and MMDX were hardly detectable in this assay. In these experiments, however, all compounds appeared to have induced the maximum number of DSBs at 5 μ*M*, with 18% DNA released from the agarose plugs in cells treated with MX2 (Table 4). DOX resulted in approximately 14% DNA released, while both MRA and MMDX released only 1.8 and 1.6%, respectively. Although this assay substantiated our results in terms of the total amount of DNA released post-drug treatment,

Table 1 Cytotoxicity of DOX and the morpholinyl anthracyclines in the ES-2 cell line after 48 h drug exposure. Each IC_{50} value represents the mean of at least four experiments ± SD

Drug	IC_{50} (n*M*)[1]	Potency ratio[a]
DOX	78 ± 7.0	1.0
MRA	29 ± 5.0	2.7
MMDX	2.7 ± 0.3	29
MX2	170 ± 19	0.5

[a] Relative to the IC_{50} for DOX

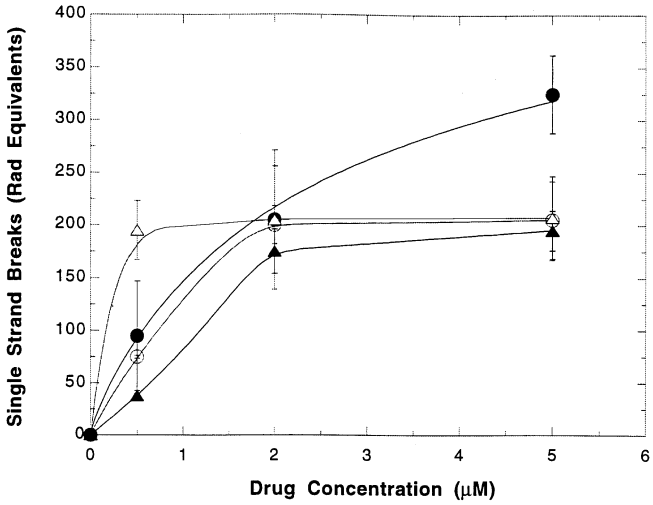

Fig. 2 The alkaline elution (pH 12.1) profiles of ES-2 cells treated with DOX (○), MRA (○), MMDX (△) or MX2 (△) under deproteinated conditions

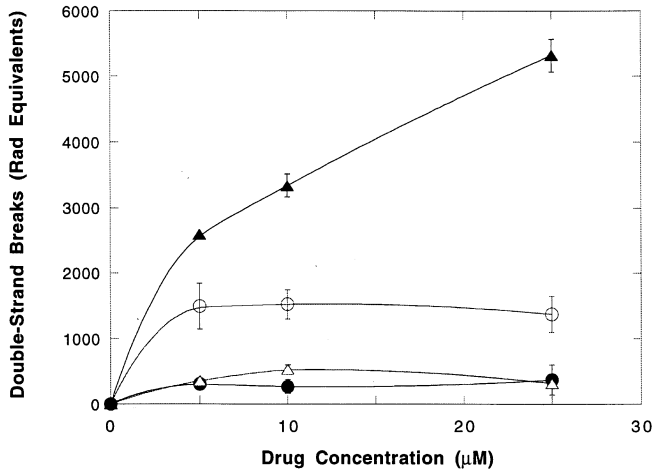

Fig. 3 The neutral elution (pH 9.6) profiles of ES-2 cells treated with DOX (○), MRA (○), MMDX (△) or MX2 (△) under deproteinated conditions

Table 3 DNA double strand breaks induced in the ES-2 cell line after 2-h exposures to DOX and the morpholinyl anthracyclines. The data are expressed as rad equivalents. Each value represents the mean of at least two experiments ± SD

	Drug			
Concentration (μ*M*)	DOX	MRA	MMDX	MX2
5	1490 ± 350	306 ± 31	355 ± 32	2590 ± 19
10	1520 ± 220	270 ± 110	529 ± 71	3340 ± 180
25	1370 ± 270	370 ± 230	315 ± 76	5320 ± 250

Table 2 DNA single strand breaks induced in the ES-2 cell line after 2-h exposures to DOX and the morpholinyl anthracyclines. The data are expressed as rad equivalents. Each value represents the mean of at least two experiments ± SD

	Drug			
Concentration (μ*M*)	DOX	MRA	MMDX	MX2
0.5	78 ± 1.4	92 ± 52	200 ± 28	39 ± 2.1
2.0	200 ± 18	210 ± 51	210 ± 66	170 ± 21
5.0	220 ± 37	340 ± 37	220 ± 40	200 ± 19

the neutral elution technique provided a better method of quantitating the degree of DNA DSB produced by these compounds because of its ability to measure DNA DSB over a time course (five fractions over 10 h). In this way, one could detect subtle differences after various stages of DNA elution impossible using the AFIGE technique.

Therefore, DOX, MX2, MRA and MMDX all cause protein-associated DNA SSBs to the same degree, but MX2 and DOX are more potent at causing DNA DSBs. These results suggest that MRA and MMDX may cause DNA strand breaks through interaction with Topo I, and that both MX2 and DOX interact with Topo II. K-SDS assays for the quantification of covalently linked Topo I- and II-DNA precipitates were consistent with this hypothesis. MRA and MMDX precipitated significant amounts of DNA linked to protein in whole ES-2 cell preparations relative to CPT, a compound known to interact with Topo I (Fig. 5). Likewise, MX2 and DOX precipitated significant amounts of DNA relative to VP16 used as a positive control.

Further, topoisomerase drug screening assays also suggested that MRA and MMDX interact with Topo I. MRA and MMDX stimulated the formation of cleavable complexes in a dose-dependent fashion, resulting in an increase in the amount of nicked, open circular form of DNA. Also, these drugs inhibited the conversion of supercoiled (form I) *pHOT* DNA to relaxed DNA topoisomers normally seen after Topo I incubation (Fig. 6), while incubation with DOX and MX2 had no effect at the same drug concentrations. Treatment with CPT resulted in an increase in the amount of open circular DNA from relaxed topoisomers at 0.1 m*M*.

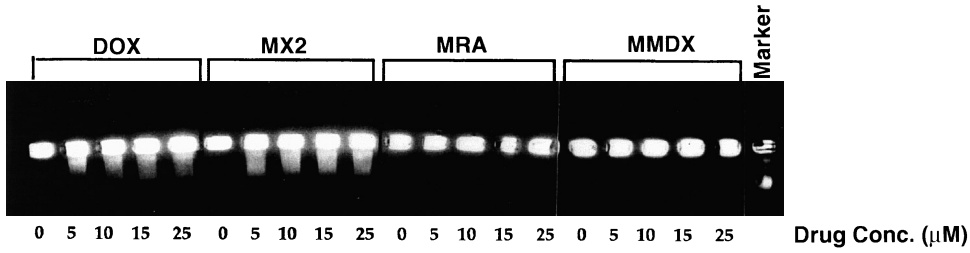

Fig. 4 Pulse-field gel electrophoresis analysis of ES-2 cells treated for 2 h with DOX or the morpholinyl anthracyclines

Table 4 DNA double strand breaks induced in the ES-2 cell line after 2-H exposures to DOX and the morpholinyl anthracyclines analyzed by pulse field gel electrophoresis. The data are expressed as the percentage of labeled DNA released from the loading well into the gel. Each value represents the mean ± SD of at least three experiments

	Drug			
Concentration (μM)	DOX	MRA	MMDX	MX2
5	14.2 ± 0.5	1.8 ± 0.4	1.5 ± 0.2	17.8 ± 1.6
10	11.8 ± 8.4	1.7 ± 0.7	1.4 ± 0.1	14.2 ± 1.9
25	12.0 ± 0.6	1.2 ± 0.5	1.9 ± 0.7	15.3 ± 2.5

In the Topo II assays, DOX and MX2 inhibited the complete conversion of form I DNA to relaxed topoisomers, resulting in an increase in linear DNA stimulated by the formation of cleavable complexes when incubated with the purified human enzyme in the presence of supercoiled *pRYG* DNA (Fig. 7). VM-26, another known Topo II inhibitor was used as a positive control. Treatment with this compound resulted in cleavable complex formation in the form of linear DNA at a concentration of 0.1 μM. At lower concentrations ($\sim$ 0.1 μM), treatment with DOX and the morpholinyl anthracyclines resulted in the formation of cleavable complexes, whereas higher concentrations (0.5 to 1.0 μM) prevented the enzyme from binding to DNA resulting in supercoiled form I DNA.

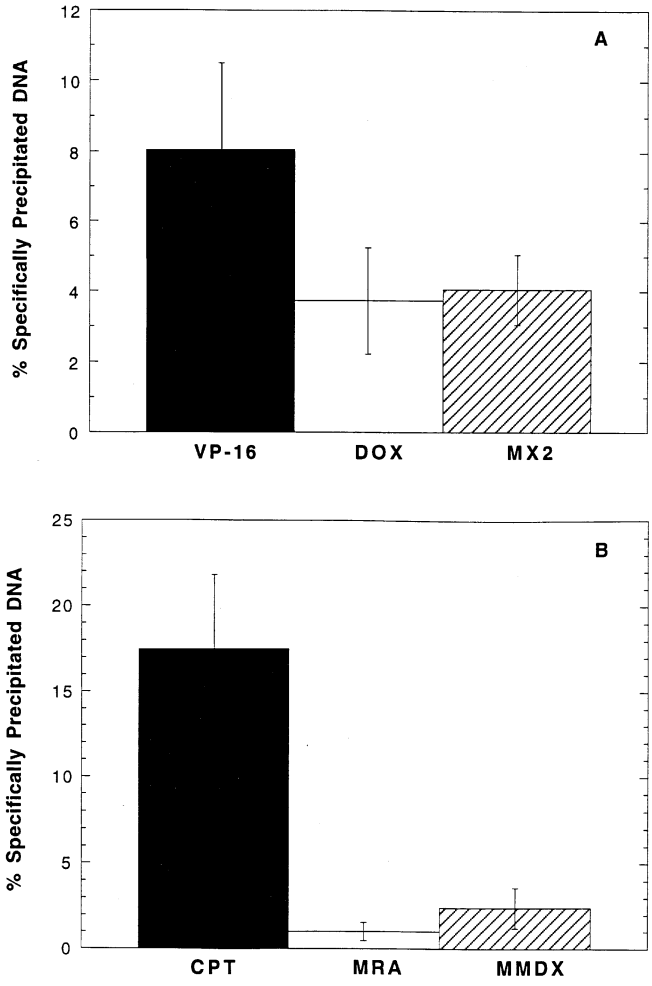

Fig. 5A, B Formation of stabilized cleavable complexes in ES-2 cells exposed to 10 μM of the appropriate drug for 1 h at 37°C as described in the Materials and methods. **A** VP-16, DOX and MX2; or **B** CPT, MRA and MMDX. These data are expressed as the mean of four independent experiments calculated as the percentage of DNA specifically precipitated

Discussion

Extensive studies of the effects of DOX on DNA have revealed that it binds to DNA via intercalation [19], subsequently poisoning Topo II [20] and causing protein-associated DNA strand breakage [21]. Several DOX analogues have been synthesized to overcome its cardiotoxic and myelosuppressive effects upon clinical administration. Some analogues, especially those with substitutions at the 3′ amino position of the daunosamine sugar, also show a decreased affinity for the drug transport pump, P-glycoprotein, improving intracellular drug accumulation and in vitro cytotoxicity in MDR models [22].

In the present study, the DNA SSB produced by MMDX and MRA differed significantly from that of the parent compound and MX2. MMDX induced

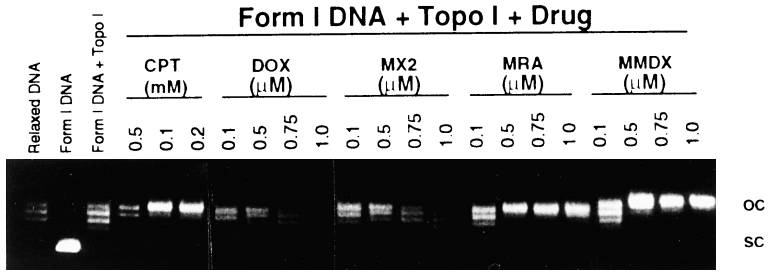

Fig. 6 *pHOT* plasmid (form I DNA) exposed to human topoisomerase I ± DOX or the morpholinyl anthracyclines (0.1, 0.5, 0.75 and 1.0 μ*M*). CPT was used as a positive control for Topo I inhibition (0.05 to 0.2 m*M*)

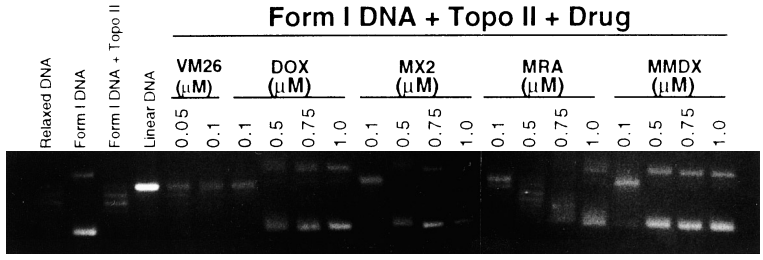

Fig. 7 Supercoiled *pRYG* DNA exposed to human Topo II ± DOX or the morpholinyl anthracyclines (0.1, 0.5, 0.75 and 1.0 μ*M*). VM26 was used as a positive control for Topo II inhibition (0.05 and 0.1 μ*M*)

higher levels of SSB than DOX at lower drug concentrations (Tables 1, 2; Fig. 2). Specifically, MMDX induced 2.5 times higher SSB than DOX at 0.5 μ*M*, which was reflected in MTT assays where MMDX was 29 times more potent than the parent compound. Likewise, MRA induced higher amounts of SSB at the higher concentration of 5.0 μ*M*, and was 2.7 times more potent than DOX after 48 h drug incubation in our cytotoxicity assays.

The MRA and MMDX compounds failed to induce any significant DSB in our neutral elution and pulse-field electrophoresis assays. In contrast, DSBs induced by MX2 and DOX were readily detectable by both techniques. This difference in SSBs and DSBs indicates that MRA and MMDX may work primarily through Topo I since the dominant DNA lesion produced was an SSB. Indeed, our drug inhibition topoisomerase assays provide direct evidence that MRA and MMDX cleavage is associated with Topo I, and that MX2 cleavage is associated with Topo II.

Wasserman et al. have previously reported on the effects of MRA on purified mouse leukemia L1210 DNA Topo I [23]. MRA treatment (1–2 μ*M*) resulted in *fok*I DNA cleavage at position 4955 as well as two novel areas at positions 4975 and 5007 relative to the cleavage pattern usually observed post-CPT exposure. MRA also suppressed cleavage at position 4997 in a manner similar to CPT, with total suppression of Topo I-mediated cleavage at higher concentrations in a dose-dependent manner. In similar assays with purified Topo II, MRA failed to have any effect, while DOX stimulated Topo II cleavage and failed to induce Topo I-associated strand breaks. The cyanomorpholinyl derivative also failed to have any inhibitory effects on this enzyme in the study by Wasserman et al., but stimulated Topo II-mediated cleavage in a manner similar to DOX under the same conditions. The presence of the α-cyano group on the morpholinyl moiety results in a completely different mechanism of action. MRA-CN causes DNA–DNA crosslinks in the ES-2 cell line quite like the bioactivated MRA and MMDX compound upon incubation with human liver microsomes in the presence of NADPH [24]. Further, Capranico et al. found that the morpholinyl group must be at the 3′ position in order to form cleavable complexes since derivatives with the moiety at the 4′ position of DOX failed to stimulate DNA cleavage and trap topoisomerase [25].

There are other fundamental differences between DOX and the morpholino anthracyclines. Data suggest that morpholinyl substitution has a profound effect on ribosomal gene transcription [26]. In contrast to studies with DOX, MRA has been reported to have potent inhibitory effects on ribosomal RNA transcription, while the Topo I inhibitor CPT has been shown to inhibit the synthesis of the 45 S rRNA precursor [27]. These effects have been observed in another antineoplastic agent, actinomycin-D, which induces Topo I-associated DNA strand breaks [23] and has been shown to inhibit ribosomal gene transcription [25]. Thus, there may be a significant correlation between the

effects on Topo I and the inhibitory effects on rRNA transcription.

Finally, since the double-strand lesion is generally considered to be the most lethal, one might expect that MX2 would be the most potent compound in our cytotoxicity testing, since it was the most potent in causing DNA DSBS. Yet, MX2 was only half as potent as DOX in vitro. Although the reasons for this discrepancy are not known, possible explanations include differential repair of MX2 DNA damage and different specificity for DNA sequences or topology. These experiments provide further evidence that minor alterations either on the morpholinyl moiety or on the anthracycline profoundly affect the interaction of these compounds with DNA and topoisomerases.

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