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Sequence Selectivity of Topoisomerase II DNA Cleavage Stimulated by Mitoxantrone Derivatives: Relationships to Drug DNA Binding and Cellular Effects

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SUMMARY

Mitoxantrone, a DNA intercalator, is an effective antitumor drug known to interfere with topoisomerase II function through stimulation of enzyme-mediated DNA cleavage. To clarify the drug structural requirements for stimulation of topoisomerase II DNA cleavage, the cytotoxic activity and molecular effects of mitoxantrone, ametantrone, and a new derivative (BBR2577), bearing a modification on one of the side chains, were examined in relation to their DNA binding affinities and modes of drug-DNA interaction. The results showed a good correlation between cytotoxicity and topoisomerase II DNA cleavage. The modification of one side chain did not influence the cytotoxic potency or the ability of the drug to stimulate DNA cleavage. In contrast, removal of the hydroxyl substituents in the planar aromatic moiety (ametantrone) markedly affected the efficacy of the drug. Ametantrone showed a markedly lower capacity, compared with the other two compounds, to induce cleavable complexes both in intact cells and in SV40 DNA, which suggests a critical role of these substituents in the formation of the ternary topoisomerase II-DNA-drug complex. The poor efficacy of ametantrone is likely due to low stability of the ternary complex. This is possibly related to a different orientation of the drug chromophore intercalated into DNA, compared with those of mitoxantrone and BBR2577. The DNA cleavage efficiencies of the tested drugs at low concentrations correlated with the DNA binding affinity. Identical DNA cleavage patterns were observed with the three compounds, which suggests that all tested drugs share a similar specificity for interaction with sites recognized by the enzyme.

Mitoxantrone, an anthracenedione derivative, is a potent cytotoxic agent with an important role in cancer chemotherapy; it is an effective alternative to anthracyclines (1). Structural similarities to doxorubicin and available evidence suggest that stabilization of the topoisomerase II-DNA cleavable complex is the primary mechanism of drug action (2, 3). The structural requirements and the molecular basis for enzyme inhibition by mitoxantrone remain to be defined. Antitumor DNA-intercalating agents, such as mitoxantrone and doxorubicin, and the nonintercalating etoposide and VM-26 stimulate topoisomerase II-mediated DNA cleavage by inhibiting the DNA religation step of the enzyme catalytic reaction (2, 3).

A molecular model for drug action has been recently proposed, based on the sequence specificities of topoisomerase II cleavage of SV40 DNA in the presence of drugs (4). The model suggests that a ternary DNA-drug-topoisomerase II complex forms in which a drug molecule interacts in an intercalation-like manner with the two bases immediately flanking the DNA cut, thus interfering with enzyme functions (4, 5). This model has also been supported by recent results on the sequence selectivity of mitoxantrone stimulation of topoisomerase II DNA cleavage (6). In this study, both mitoxantrone and VM-26 were shown to require a cytosine or a thymine at position −1 of the DNA site for cleavage stimulation. Moreover, doxorubicin and mitoxantrone showed very different sequence specificities for DNA cleavage stimulation. These results were based on a statistical analysis of cleavage sites in SV40 DNA as well as a base mutation analysis in short DNA oligomers.

Both mitoxantrone and doxorubicin bind to DNA with high affinity and intercalate between adjacent base pairs of the duplex. The alkyl side chains of mitoxantrone appear to interact with anionic groups in the major groove, thereby preventing complete intercalation of the planar aromatic moiety of the drug molecule (7, 8). In contrast, the sugar of the anthracycline

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ABBREVIATIONS: VM-26, teniposide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DTT, dithiothreitol; SSB, single-strand breaks; IC_{50}, drug concentration required for 50% inhibition of cell growth; SV40, simian virus 40.

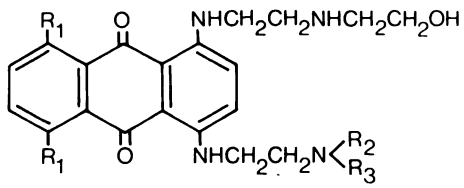
molecule is placed in the minor groove of the double helix and stabilizes DNA-drug interactions (9, 10). Although among anthracycline derivatives DNA binding appears to be a mandatory condition for drug interference with topoisomerase II and antitumor activity, the role of DNA intercalation in the distinctive sequence specificity of these drugs is not yet understood.

In an attempt to clarify the structural requirements for intercalating agents to stimulate topoisomerase II DNA cleavage, the effects of three anthracene-9,10-dione derivatives (Fig. 1) on topoisomerase II-mediated DNA cleavage in SV40 DNA fragments and in human lung cancer cells were compared. Cellular and molecular effects (topoisomerase II DNA cleavage patterns) were also examined in relation to the DNA binding affinity of the drugs and the mode of drug-DNA interaction.

Experimental Procedures

Materials. Anthracene-9,10-diones were synthesized in the Chemical Synthesis Division of Boehringer Mannheim Italia (Monza, Italy) (Fig. 1); BBR2577 was synthesized as already described (11). Doxorubicin was obtained from Farmitalia Carlo Erba (Milan, Italy). SV40 DNA, restriction endonucleases, and the 5'-end DNA labeling kit were purchased from Boehringer Mannheim (Mannheim, Germany). [γ-^{32}P] ATP (6000 mCi/mmol) and [2-^{14}C]thymidine (50 mCi/mmol) in an aqueous solution were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Calf thymus DNA and poly(dG·dC) (200–500 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). Calf thymus DNA was dissolved in 0.05 M sodium phosphate buffer, pH 8.0, extracted four times with phenol, and dialyzed against 0.01 M Tris buffer, pH 7.0, containing 1 mM EDTA and known amounts of NaCl to adjust ionic strength to the desired value.

Topoisomerase II was purified from murine leukemia P388 cells by following a modification of published procedures (12). Topoisomerase II activity was detected with the P4 unknotting assay (12). Briefly, nuclear extracts were obtained with 0.4 M NaCl, nucleic acids were removed by precipitation with polymin P, and proteins were precipitated with ammonium sulfate. The ammonium sulfate pellet was resuspended in H buffer (15 mM sodium phosphate buffer, pH 7.2, 10% glycerol, 0.5 mM DTT, 0.01% Triton X-100, 0.1 mM PMSF, 10 mM $Na_2S_2O_5$) and loaded onto a hydroxylapatite column that had been previously equilibrated in W buffer (H buffer containing 100 mM NaCl). The column was then eluted with a linear gradient of potassium phosphate buffer. Fractions containing only topoisomerase II activity were pooled, diluted with P buffer (15 mM sodium phosphate buffer, pH 7.2, 10% glycerol, 0.5 mM DTT, 0.1 mM PMSF, 10 mM $Na_2S_2O_5$, 0.1 mM EDTA), and loaded onto a phosphocellulose column. Proteins were eluted with a linear gradient of NaCl. Fractions with topoisomerase II activity were pooled, dialyzed against storage buffer (20 mM potassium phosphate, pH 7, 0.5 mM PMSF, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 50%, v/v, glycerol), and stored at −20°. The purified topoisomerase II was completely free of topoisomerase I activity, as determined by ATP-independent relaxation of pBR322 DNA.



	R_1	R_2	R_3
MITOXANTRONE	OH	H	CH_2CH_2OH
AMETANTRONE	H	H	CH_2CH_2OH
BBR2577	OH	CH_3	CH_3

Fig. 1. Chemical structures of the anthracene-9,10-dione derivatives studied.

Cell lines and cytotoxicity assay. Murine leukemia P388 cells (13), human epidermoid A431 carcinoma cells (14), and the human small cell lung carcinoma cell line NCI-H187 (15, 16) were cultured in RPMI 1640 medium (ICN Flow, Costa Mesa, CA) containing fetal calf serum (ICN Flow).

Drug treatments were for 1 hr at 37°, with exponentially growing cells. P388 and A431 cells were centrifuged after drug treatments, washed twice, resuspended in drug-free medium, cultured for 72 hr, and then counted. NCI-H187 cells were cultured in drug-free medium for 96 hr, and cell survival was then determined by means of the MTT assay (17). In each experiment, five to seven drug concentrations were used and the IC_{50} value was determined by regression analysis in the linear region of the dose-effect curve.

Self-aggregation studies. Inasmuch as BBR2577 exhibits a nonlinear absorbance-concentration response, it undergoes aggregation phenomena similarly to its congeners mitoxantrone and ametantrone (18). Absorption spectra of mitoxantrone, ametantrone, and BBR2577 were recorded with a wide range of concentrations (5–60 μM) at 0.1 and 0.5 M ionic strength. The molar extinction coefficients of the monomer species were calculated by extrapolation of the apparent extinction coefficients to zero concentration for each compound. The calculations were performed according to the method of Schwartz *et al.* (19) and allowed an evaluation of K_d, the dimerization constant of the drug.

DNA binding studies. The measurements were carried out at 25° in aqueous 0.01 M Tris buffer, pH 7.0, containing 1 mM EDTA and known amounts of NaCl to adjust ionic strength to the desired value. Binding was followed spectrophotometrically in the ligand absorption region (470–800 nm). At a drug concentration sufficiently low to avoid self-aggregation phenomena (about 2×10^{-5} M) and with an ionic strength in the range of 0.1–0.5 M, the presence of a clear isosbestic point at~ 670 nm during titration with DNA allowed an evaluation of the free and DNA-bound drug. To avoid large systematic inaccuracies due to experimental errors in extinction coefficients, the range of bound drug fraction was 0.15–0.85. The data were evaluated according to the method of McGhee and Von Hippel (20), to obtain K_{int} (the intrinsic binding constant) and n (the exclusion parameter). Some of the drug-DNA titrations performed at low ionic strength (0.1 M or less) showed isosbestic regions rather than single points. This fact can be interpreted in terms of a coexistence of two forms of complex, i.e., intercalation and external stacking. No cooperativity appeared to occur. Spectrophotometric measurements were performed with a Perkin-Elmer Lambda 5 apparatus equipped with a Haake F3-C thermostat.

Intercalation geometry. Chiroptical measurements were carried out with poly(dG·dC) in the visible region corresponding to the transitions of the DNA-drug complex only, because the drugs themselves are not optically active. The experiments were performed using a Jasco J-500 A spectropolarimeter interfaced to a J-500 N computing station. The appropriate mode was used for linear and circular dichroism studies. Four to eight scans were accumulated for each measurement.

Alkaline elution assay for DNA SSB. NCI-H187 cells were labeled with 0.1 μCi/ml [^{14}C]thymidine for 90 hr at 37°. The labeled nucleoside precursor was removed 24 hr before drug treatment, by centrifugation and resuspension of cells in fresh medium. The filter elution procedures were essentially as reported by Kohn *et al.* (21) and described extensively elsewhere (13, 22).

DNA cleavage of end-labeled SV40 DNA. SV40 DNA was double-end or uniquely 5'-end labeled at sites of various restriction enzymes as described previously (23). Briefly, SV40 was first linearized with a restriction enzyme, dephosphorylated with calf alkaline phosphatase, and then labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. DNA was purified by phenol-chloroform extraction and ethanol precipitation. In the case of uniquely 5'-end-labeled DNA, fragments

were digested first with *Xho*II and then with *Taq*I restriction enzymes. After further purification steps, fragments of interest were isolated and purified by agarose gel electrophoresis, electroelution, and ethanol precipitation. The DNA fragment was reacted with about 120 ng of topoisomerase II, with or without various concentrations of drugs, in 0.04 M Tris·HCl, pH 7.5, 0.08 M KCl, 0.01 M $MgCl_2$, 0.005 M DTT, 1 mM ATP, 15 μg/ml bovine serum albumin, for 30 min at 37°. Topoisomerase II was added to each 20-μl reaction volume in 2 μl of storage buffer. Reactions were stopped by addition of SDS and proteinase K (Merck, Darmstadt, Germany) (final concentrations of 1% and 0.5 mg/ml, respectively), and samples were incubated for an additional 60 min at 42°. Samples were then fractioned by electrophoresis through a 1% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) containing 0.1% SDS. Agarose gels were dried and autoradiographed with Amersham Hyperfilm-MP films. For sequencing gels after proteinase K treatment, DNA was precipitated with ethanol, resuspended in 2.5 μl of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue), and heated at 90° for 1–2 min before being loaded onto an 8% polyacrylamide sequencing gel [29:1, acrylamide:bis(acrylamide) ratio; 7 M urea in TBE buffer]. Electrophoresis was performed at 80 W for about 2 hr; gels were then dried and autoradiographed as described above.

DNA cleavage stimulated by anthracenediones at selected cleavage sites of SV40 DNA fragments was quantified by means of densitometric scanning (LKB Ultrascan XL laser densitometer). The area of the peak was corrected by considering the background level in each lane, to normalize for small differences in sample loadings. DNA cleavage was expressed in arbitrary units, relative to that induced by topoisomerase II without drugs.

Results

Cytotoxic activity of the studied anthracenedione derivatives. The chemical structures of the studied anthracenedione derivatives are shown in Fig. 1. Mitoxantrone and ametantrone are characterized by two symmetrical side chains and differ in the planar aromatic moiety by the presence or absence of two hydroxyl groups at positions 1 and 4. BBR2577 differs from the other two compounds by a modification in one side chain.

Cytotoxic activity of the studied anthracenediones was examined in two human tumor cell lines (NCI-H187 and A431) and in P388 murine leukemia cells (Table 1). IC_{50} values showed that ametantrone was the least potent of the studied compounds (60–500-fold less potent). BBR2577 was about 3-fold more cytotoxic than mitoxantrone in all three cell lines. NCI-H187 cells were apparently less chemosensitive to these agents; this effect might be related, at least in part, to the different cytotoxic test (MTT assay) used for this cell line.

DNA binding and self-aggregation by BBR2577. Like mitoxantrone and ametantrone (18, 24), BBR2577 also exhibited a remarkable tendency to self-aggregate in aqueous solutions. The phenomenon was more prominent with increasing ionic strength (Table 2). BBR2577 behaved similarly to ametantrone and aggregated slightly less than did mitoxantrone. A possible reason for this behavior lies in the asymmetric substitution of this test compound, which renders stacking somewhat less stable.

Once the self-aggregation properties were established, the DNA binding parameters could be evaluated from spectroscopic measurements (Table 2). The intercalation of compound BBR2577 into DNA was deduced from linear dichroism experiments (data not shown). The same mode of interaction was confirmed for mitoxantrone and ametantrone under our experimental conditions. The exclusion parameter n was very similar in all cases and indicated the involvement of two or three base pairs with each drug molecule. A cooperativity factor of 1 was always used, which pointed to the absence of neighboring interactions between drug molecules in the complex. For all the compounds, the affinity for DNA decreased with increasing salt concentration, which confirmed the involvement of electrostatic interactions in the complex. This was in agreement with observations on mitoxantrone and ametantrone (18, 24) and with the presence of positively charged groups in the side chains of the studied drugs. Under physiologic conditions of ionic strength, mitoxantrone exhibited the highest affinity for calf thymus DNA. At a salt concentration of 0.5 M, BBR2577 behaved more like mitoxantrone than like ametantrone. Our data on mitoxantrone and ametantrone are in fairly good agreement with quantitative DNA binding parameters reported under similar experimental conditions (18, 24).

Conformational properties of the drug-DNA complex. Electronic transitions of drug bound to DNA are optically active as a result of the asymmetric induction by the nucleic acid. The induced rotational strength is related to the structural relationship between DNA bases and drug chromophore (25). Recent theoretical developments (25) on the circular dichroism of poly(dG-dC) and its complexes with small molecules produce a sound basis for interpreting the induced rotational strength in terms of molecular geometry. The induced circular dichroism spectra of BBR2577, mitoxantrone, and ametantrone interacting with poly(dG·dC) are presented in Fig. 2. Clearly, whereas the former two compounds exhibited positive and similar dichroism, the latter showed dichroic bands that were almost the mirror image of the others (the wavelength shift is related to the absence of the 1,4-dihydroxy groups in ametantrone). According to the described theoretical work (25), BBR2577 and

TABLE 1

Cytotoxic effects of the studied anthracenedione derivatives

Cells were exposed to drugs for 1 hr at 37°. Drug cytotoxicity was determined with the MTT assay in NCI-H187 cells and with the cell-counting method in the A431 and P388 cell lines. See Experimental Procedures for details.

Drug	IC_{50}		
	NCI-H187	A431	P388
		μM	
Mitoxantrone	2.2 ± 0.8	0.4 ± 0.2	0.13 ± 0.03
Ametantrone	130 ± 4	63	11
BBR2577	0.9 ± 0.4	0.11	0.035 ± 0.01

TABLE 2

DNA-binding and self-aggregation parameters for mitoxantrone, ametantrone, and BBR2577

Values are mean ± standard deviation of four independent determinations at different drug concentrations.

Compound	Ionic strength	DNA binding affinity constant, $K_{int} \times 10^{-5}$ [a]	Exclusion parameter, n [b]	Self-aggregation constant, $K_d \times 10^{-4}$ [c]
	M	M^{-1}	base pairs	M^{-1}
Mitoxantrone	0.1	9.22 ± 1.12	2.6 ± 0.2	3.0 ± 0.3
	0.5	1.78 ± 0.23	2.9 ± 0.2	4.4 ± 0.4
Ametantrone	0.1	4.46 ± 0.51	2.6 ± 0.2	0.69 ± 0.4
	0.5	0.30 ± 0.06	2.7 ± 0.2	ND [d]
BBR2577	0.1	5.09 ± 0.34	2.2 ± 0.2	0.71 ± 0.4
	0.5	1.00 ± 0.18	3.0 ± 0.2	2.1 ± 0.2

[a] K_{int}, intrinsic constant for the binding of the first ligand to DNA.
[b] n, number of adjacent base pairs forming the binding site of the drug.
[c] K_d, dimerization constant for the drug.
[d] ND, not done.

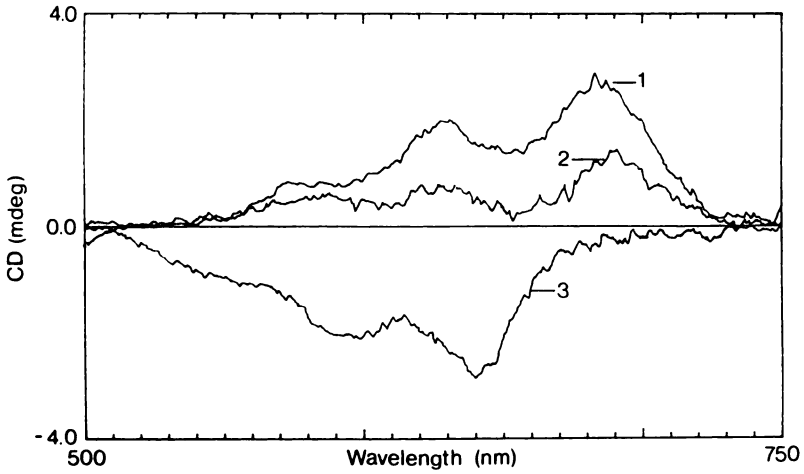


Fig. 2. Induced circular dichroism (*CD*) in the ligand absorption region for the complex of BBR2577 (*trace 2*), mitoxantrone (*trace 1*), and ametantrone (*trace 3*) with poly(dG·dC) in 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.0, at 22°. The DNA concentration was on the order of 0.5 mM (base residues) and the nucleotide to ligand ratio was 12–15.

mitoxantrone are probably intercalated into poly(dG·dC) with their planar systems almost perpendicular to the longest dimension of the base pair, whereas ametantrone might be placed parallel to it in the intercalation pocket. NMR investigations on the intercalation of mitoxantrone into a CG-containing duplex oligonucleotide have confirmed the circular dichroism data (8).

DNA SSB in a human small cell lung carcinoma cell line. DNA SSB produced by the three anthracenediones were determined after a 1-hr drug exposure in human NCI-H187 cells (Fig. 3). Drug-stimulated DNA breaks were protein associated, because proteolytic treatments of cell lysates on the filter were needed to detect DNA cleavage (data not shown). The number of DNA SSB stimulated by mitoxantrone and BBR2577 were comparable and dose dependent up to about 1 μM. A suppression of cellular DNA breaks was observed for BBR2577 at high drug concentrations (Fig. 3), whereas a plateau was observed from 1 to 100 μM for mitoxantrone. Ametantrone stimulated no SSB up to 2 μM; SSB were detected at very high concentrations (Fig. 3). The relationship between cytotoxic effects and DNA SSB is shown in Fig. 4. A similar quantitative relation between DNA SSB and cytotoxicity was

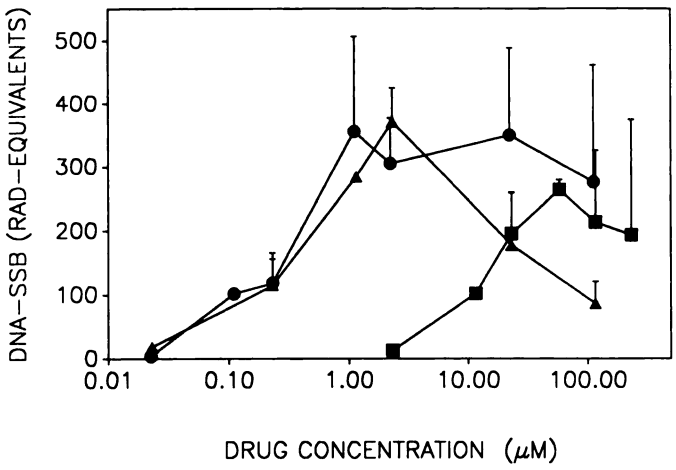


Fig. 3. DNA SSB induced by anthracenedione derivatives in NCI-H187 cells. Cells were exposed to drugs for 1 hr at 37°, lysed on the filter in the presence of proteinase K, and eluted at pH 12.15. See Experimental Procedures for details. *Standard deviation bars* are shown when values were derived from two to four independent determinations. *Points without standard deviation bars* are single determinations. ●, Mitoxantrone; ■, ametantrone; ▲, BBR2577.

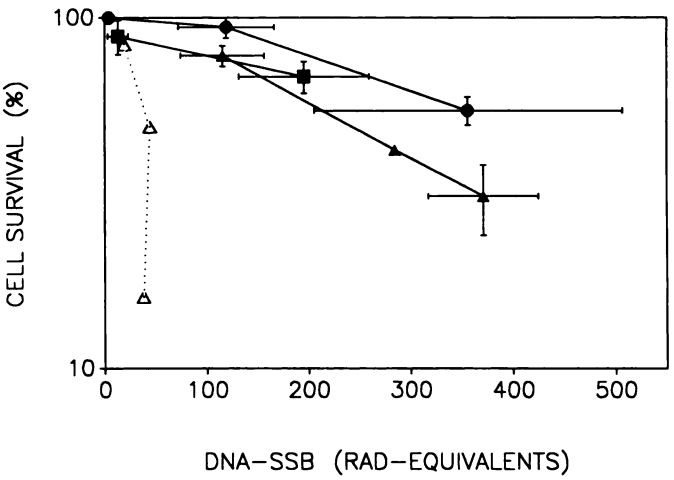


Fig. 4. Relationship between cell survival and DNA SSB for mitoxantrone (●), ametantrone (■), BBR2577 (▲), and doxorubicin (△) after 1-hr treatments of NCI-H187 cells. *Standard deviation bars* are shown when values were derived from two to four independent determinations. *Points without standard deviation bars* are single determinations.

observed for these derivatives. The results were compared with those obtained on effects of doxorubicin (Fig. 4). Although both doxorubicin and mitoxantrone produced persistent DNA damage (22, 26, 27), doxorubicin stimulated many fewer DNA breaks than did the anthracenediones at equitoxic drug concentrations (Fig. 4). The quantitative relations between DNA SSB and cytotoxicity of the anthracenediones examined in this study resembled more closely those observed with etoposide (28).

Drug stimulation of SV40 DNA cleavage induced by topoisomerase II. The relative drug activity in stimulating topoisomerase II-mediated double-strand breaks in SV40 DNA was initially studied by neutral agarose gel electrophoresis. *Eco*RI-digested, double-end ^{32}P-labeled SV40 DNA was used in these experiments, and drug concentrations ranged from 0.02 to 20 μM (Fig. 5). No DNA cleavage was induced by drugs in the absence of topoisomerase II (data not shown). Ametantrone was less effective in stimulating DNA cleavage than were mitoxantrone and BBR2577, whose activities were similar. DNA cleavage was dose dependent, with a maximum stimulation at drug concentrations ranging from 0.2 to 2 μM; cleavage suppression was observed at higher drug concentrations for all three derivatives, thus resembling the effects of other DNA-

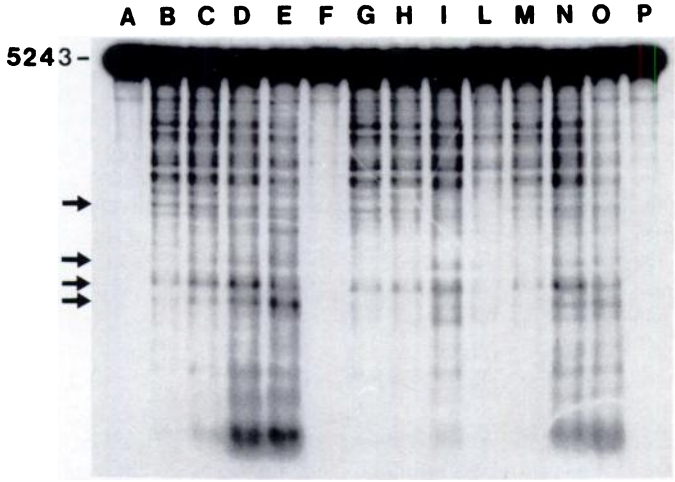


Fig. 5. DNA cleavage induced by topoisomerase II in the presence of anthracenedione derivatives. Double-end ^{32}P-labeled SV40 DNA (*lane A*, control) was incubated at 37° with 120 ng of topoisomerase II without drug (*lane B*) or with 0.02, 0.2, 2, or 20 μM mitoxantrone (*lanes C-F*), ametantrone (*lanes G-L*), or BBR2577 (*lanes M-P*), respectively. The reactions were stopped with SDS (1% final concentration) and samples were digested with proteinase K (0.5 mg/ml) for 1 hr at 42°. Samples were then electrophoresed in a 1% agarose gel, and autoradiography was performed. *5243*, full-length SV40 DNA. *Arrows*, selected cleavage sites used to measure drug stimulation of DNA cleavage (see Fig. 6).

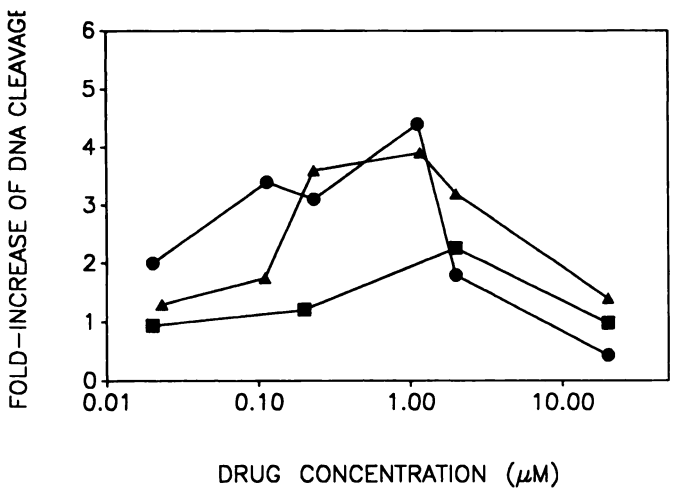


Fig. 6. Quantification of drug-stimulated cleavage of SV40 DNA. DNA cleavage at selected sites (see *arrows* in Fig. 5) induced by anthracenedione derivatives was quantified by means of densitometric scanning of autoradiograms of agarose gels. DNA cleavage was expressed in arbitrary units, relative to that induced by topoisomerase alone. *Points* are mean values of DNA cleavage increase deduced from two to four experiments (standard deviation, ±20%). ●, Mitoxantrone; ■, ametantrone; ▲, BBR2577.

binding topoisomerase II inhibitors (29–31). As proposed for anthracyclines and other intercalating compounds (29, 31), the suppression of topoisomerase II-mediated DNA cleavage may be related to the high DNA binding affinity of these drugs.

Drug-stimulated DNA cleavage was quantified by densitometric analysis of the cleavage bands indicated in Fig. 5. Drug-induced changes in DNA cleavage were averaged over all the selected bands for each drug level, and the mean values deduced from at least two independent experiments are reported in Fig. 6. BBR2577 and mitoxantrone stimulated similar levels of DNA cleavage, although the parent drug was the most active compound at up to 0.1 μM. Ametantrone was remarkably less active than the other two compounds. Cleavage suppression at 20 μM drug concentration was almost complete for all three drugs. The relative potency of the three compounds in stimulating cleavage of SV40 DNA resembled that observed for stimulation of DNA cleavage in NCI-H187 cells. Nevertheless, alkaline elution experiments revealed somewhat different behavior between BBR2577 and mitoxantrone at high drug concentrations. These discrepancies could reflect differences in cellular drug uptake and/or efflux.

Sequencing analysis of cleavage sites stimulated by anthracenediones. Topoisomerase II-trapping antitumor drugs from different chemical families are known to stimulate DNA cleavage at specific sites in a given DNA fragment (32). The patterns of DNA cleavage sites stimulated by the anthracenedione derivatives were therefore compared, to investigate the effects of the structural modifications of the drug molecules on the DNA sequence specificity of anthracenedione action. A uniquely 5′-end-labeled *Xho*II-*Taq*I SV40 DNA fragment, corresponding to a region of major DNA cleavage by topoisomerase II in the SV40 genome, was incubated with the enzyme and different concentrations of the three drugs and was then analyzed by denaturing sequencing gels (Fig. 7). In agreement with agarose gel experiments (Fig. 5), DNA cleavage was found to be concentration dependent, with evidence of cleavage suppression at high drug concentrations. Again, ametantrone was the least active drug. The studied compounds stimulated DNA cleavage at identical sites with the same relative intensity, resulting in identical DNA cleavage patterns. These observations were confirmed by additional experiments using different SV40 DNA fragments (data not shown). Thus, the studied structural modifications did not influence the sequence specificity of DNA cleavage of the parent compound.

It should be noted that some cleavage sites induced by the enzyme without drugs (sites 4234, 4205, and 4164 in Fig. 7) were further stimulated by the anthracenedione derivatives. This finding contrasts with previous observations on doxorubicin-stimulated DNA cleavage sites (4). In addition, other sites seen with topoisomerase II without drugs were not stimulated by the studied compounds (sites 4244 and 4126 in Fig. 7). Similarly, cleavage suppression by drugs appeared to be sequence selective, because DNA cleavage at sites 4164 and 4205 disappeared almost completely in the presence of 2 μM mitoxantrone, whereas cleavage at site 4234 persisted (Fig. 7). These observations suggest a certain degree of structural heterogeneity of topoisomerase II-DNA complexes.

Discussion

The synthesis of new mitoxantrone derivatives bearing various substitutions in the chromophore and side chains allows a comparison of biochemical and biologic effects brought about by the different substituents. In the present study, a detailed evaluation of cell-killing effects, topoisomerase II DNA cleavage, and DNA-drug interactions of mitoxantrone and two analogues emphasizes some structural requirements for optimal activity. An interesting observation that emerged from the present results is the close similarity of BBR2577 and mitoxantrone. Considering that the nature of the side-chain substituents plays a major role in modulating drug activity (33), it is possible to conclude that just one hydroxyethylaminoethylamino side chain is sufficient to grant to the hydroxyanthracenedione moiety marked antiproliferative properties and activity

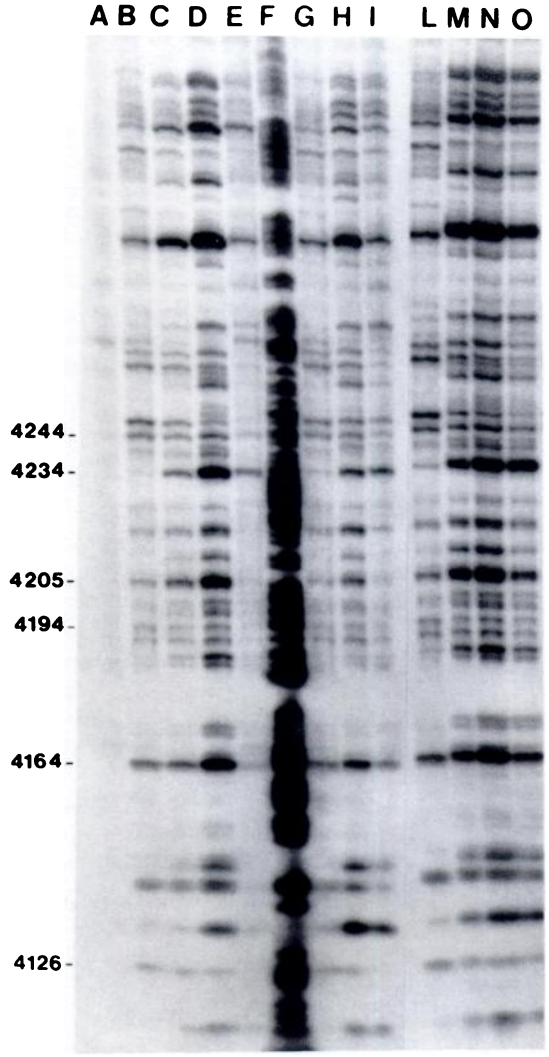


Fig. 7. Topoisomerase II-mediated DNA cleavage sites stimulated by anthracenediones. A *Xho*II-*Taq*I SV40 DNA fragment, 5′-end labeled at the *Xho*II site, was used (*lane A*, control DNA), and reactions were performed as described in the legend to Fig. 5. *Lane B*, topoisomerase II without drugs; *lanes C-E*, 0.02, 0.2, and 2 μM mitoxantrone, respectively; *lane F*, purine markers; *lanes G-I*, 0.2, 2, and 20 μM ametantrone, respectively; *lanes L-O*, 0.02, 0.2, 2, and 20 μM BBR2577, respectively. Nucleotide positions of some DNA cleavage sites are indicated.

against topoisomerase II. The remarkably reduced efficiency of ametantrone, in comparison with the other tested compounds, in producing topoisomerase II-mediated DNA cleavage points to a critical relevance of the hydroxyl substituents in enzyme inhibition. These substituents in the aromatic moiety may thus affect the stability of the ternary topoisomerase II-DNA-drug complex and hence modify the biologic response. Relevant to this point is the observation that the geometry of the binary drug-DNA complex of ametantrone is different from that of the other two analogues bearing the hydroxyl groups. In addition, dramatic differences were observed in the kinetics of mitoxantrone versus ametantrone binding to DNA (34). These results document a good correlation between drug cytotoxicity and induction of DNA cleavage in the cultured cells and with purified topoisomerase II, in agreement with previous reports on different inhibitors (23).

BBR2577 had a somewhat lower DNA affinity constant than did mitoxantrone and was as effective as the parent drug in stimulating topoisomerase II DNA cleavage. However, at low drug concentrations mitoxantrone was the most active compound in the presence of the purified enzyme. DNA cleavage induced by intercalating drugs is the result of a balance between induction and suppression of topoisomerase II-mediated cleavage (23). Therefore, to evaluate the effects of drug structural modifications on the drug stimulation of DNA cleavage, it is probably more appropriate to compare drug-induced DNA cleavage at low drug concentrations. Under these conditions the suppressive effects of the drugs on DNA cleavage are less prominent. The DNA cleavage efficiency of the tested agents at low drug concentrations appears to be reasonably well correlated with the intrinsic DNA binding affinity (Table 2; Fig. 6). For a set of structurally related compounds it is conceivable that the forces that stabilize the binary drug-DNA complex (stacking, hydrogen bonding, and electrostatic interactions) also operate in the ternary enzyme-drug-DNA complex. Accordingly, the K_{int} value for the binding of the drug to DNA alone would give a useful indication of the stability of the cleavable complex for a set of congeners exhibiting similar drug-DNA interaction forces.

Mitoxantrone and VM-26 stimulated similar intensity patterns of topoisomerase II DNA cleavage in SV40 DNA fragments (6) and showed a similar preference for pyrimidines at position −1 of the cleavage site. Identical DNA cleavage patterns have now been found for all the tested drugs, suggesting that the studied compounds have the same sequence specificity for DNA cleavage stimulation. These results suggest that the type of molecular interaction of mitoxantrone in the ternary complex is not altered by the structural modifications of the two derivatives. Unlike what is generally seen with anthracyclines (23), anthracenediones stimulated cleavage also at DNA sites induced by the enzyme without drugs, as occurred also for stimulation induced by VM-26 (35). This finding is reminiscent of the observation that the relationships between SSB and cytotoxicity of anthracenediones resemble more closely those of epipodophyllotoxins (28) than those of doxorubicin. However, the pharmacologic role of the sequence specificity of anthracenedione stimulation of DNA cleavage remains to be established.

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