Similar Sequence Specificity of Mitoxantrone and VM-26 Stimulation of in Vitro DNA Cleavage by Mammalian DNA Topoisomerase II†

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ABSTRACT: The molecular mechanism of topoisomerase II trapping by antitumor drugs probably involves the formation of a ternary complex DNA–drug–topoisomerase II. Recent studies support the view that a drug molecule might be placed at the DNA cleavage site interacting with the two flanking base pairs and amino acid residues of the enzyme. In this work, the DNA sequence-dependent action of mitoxantrone on topoisomerase II DNA cleavage was investigated in SV40 DNA fragments and short oligonucleotides, in comparison to VM-26, 4-demethoxydaunorubicin, and mAMSA. Mitoxantrone and VM-26 had a much lower degree of selectivity than 4-demethoxydaunorubicin and mAMSA in stimulating DNA cleavage. DNA cleavage patterns stimulated by mitoxantrone and VM-26 were very similar. Mitoxantrone stimulated DNA cleavage at sites that were always stimulated also by VM-26. In contrast, mitoxantrone and 4-demethoxydaunorubicin shared only 7% of cleavage sites, and about 70% of the 4-demethoxydaunorubicin-stimulated sites were also stimulated by VM-26. Unlike what is generally seen with anthracyclines, the structurally related drug, mitoxantrone, stimulated cleavage also at DNA sites observed without drugs. Local base preferences at the cleavage site as determined by statistical analysis showed that mitoxantrone preferentially cleaved the DNA at sites with a cytosine or a thymine at position −1. However, strong DNA cleavage stimulation by mitoxantrone was favored by specific base pairs at the next positions flanking the cleaved bond (positions −2 and +2) and at positions +8 and +9. Effects of base mutations on drug stimulation of DNA cleavage in short DNA oligonucleotides independently showed that a pyrimidine at position −1 is required for mitoxantrone action. Base mutations at position +2 indicate that specific nucleotides were not required at this position for drug action. Nevertheless, a guanine at position +2 could increase mitoxatrone stimulation of DNA cleavage only when a cytosine was present at position −1. In addition, the base mutation analysis indicated that a pyrimidine at position −1 is preferred also by VM-26. Thus, although mitoxantrone is an intercalating agent with a high DNA binding affinity constant comparable to that of 4-demethoxydaunorubicin, the DNA sequence-dependent effects of mitoxantrone on topoisomerase II resemble more closely those of VM-26, which is not an intercalator, and are very different from anthracyclines and mAMSA. These results are consistent with a ternary complex in which VM-26 and mitoxantrone have a similar mode of interaction and may suggest a novel guideline to classify topoisomerase II-trapping antitumor drugs.

DNA topoisomerase II interconverts DNA topoisomers by introducing a double-strand break in a DNA segment and allowing the passage of another DNA duplex through the break before resealing the DNA cleavage (Gellert, 1981; Wang, 1985; Liu, 1989). The two strand breaks are staggered by four base pairs and each 5′ terminus is covalently bound to a tyrosine residue of topoisomerase II (Gellert, 1981; Wang, 1985; Liu, 1989). Antitumor drugs, such as anthracyclines, amsacrine, VM-26, and mitoxantrone, are known to stimulate topoisomerase II-mediated DNA cleavage (Liu, 1989; Pommier & Kohn, 1989), and the effects of drugs of different chemical classes result in specific patterns of DNA cleavage sites in a given DNA fragment (Liu, 1989; Pommier & Kohn, 1989).

A new line of investigation on local base sequence requirements of in vitro DNA cleavage may throw light on the molecular action of topoisomerase II-trapping agents (Capranico et al., 1990a; Pommier et al., 1991a; Jaxel et al., 1991; Capranico & Zunino, 1992). Doxorubicin has been shown to require absolutely an adenine at the 3′ terminus of the DNA break site to stimulate in vitro DNA cleavage by topoisomerase II (Capranico et al., 1990a). This finding suggested that the molecular action of the drug implicates the formation of the ternary complex DNA–doxorubicin–topoisomerase II, in which a drug molecule stabilizes the complex by interacting at the DNA cleavage site with the enzyme and with base pairs immediately adjacent to the break. This hypothesis has received further support from analogous but distinctive local base preferences for topoisomerase II DNA cleavage strongly stimulated by amsacrine and VM-26 (Pommier et al., 1991a).

Thus, it was of interest to evaluate the DNA sequence dependence of the stimulation of topoisomerase II DNA cleavage by mitoxantrone, a high-affinity DNA intercalator with an anthraquinone structure (Figure 1). In this report, we describe the results of a statistical analysis of mitoxantrone-stimulated DNA cleavage sites in the SV40 genome and the effects of nucleotide mutations on mitoxantrone and VM-26 stimulations of DNA cleavage in short DNA oligonucleotides. Strikingly, the present investigation shows that, despite the apparently unrelated chemical structure, the sequence-dependent actions of mitoxantrone and VM-26 are very similar and are different from anthracyclines and mAMSA. The results suggest a new way to classify topoisomerase II-trapping antitumor agents.

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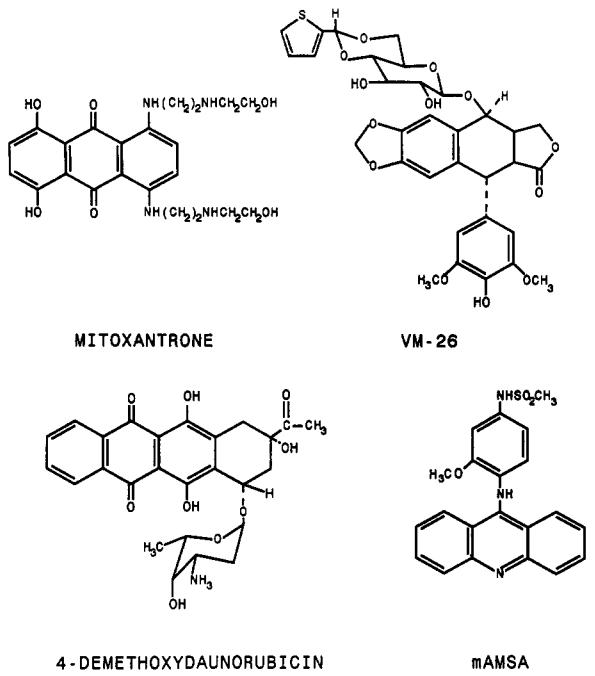

FIGURE 1: Chemical structures of the studied compounds.

EXPERIMENTAL PROCEDURES

Drugs, Enzymes, and Other Materials. Mitoxantrone, VM-26, and 4-demethoxydaunorubicin were obtained from Dr. S. Spinelli (Boehringer Mannheim Italia, Monza), Bristol Italiana (Latina), and Farmitalia-Carlo Erba (Milan), respectively. mAMSA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. Drugs were freshly prepared: mitoxantrone and 4-demethoxydaunorubicin in deionized water; VM-26 and mAMSA in dimethyl sulfoxide and then diluted in deionized water.

DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (De Isabella et al., 1990; Minford et al., 1986) and was stored at −20 °C in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM PMSF, 0.1 mM EDTA, 1 mM β-mercaptoethanol. Topoisomerase II strand passing activity was determined with the P4 DNA unknotting assay, as described (De Isabella et al., 1990). One unit of enzyme activity was defined as the minimum amount of proteins which completely unknotted 0.2 μg of knotted P4 DNA at 37 °C in 30 min.

SV40 DNA, T4 polynucleotide kinase, agarose, and polyacrylamide were purchased from GIBCO-BRL, Life Technologies (Basel, Switzerland). [γ-^{32}P]ATP was purchased from Amersham, Amity s.r.l., Milan, Italy. Calf intestinal phosphatase and restriction endonucleases were purchased from New Englands Biolabs (Taunus, Germany). DNA oligonucleotides were synthesized with a 380B DNA synthesizer (Applied Biosystems, Milan, Italy).

End-Labeling of SV40 DNA Fragments and DNA Oligonucleotides. SV40 DNA fragments were uniquely 5′-end-labeled as already described (Capranico et al., 1990a,b). Briefly, SV40 DNA was restricted 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.

Single-strand oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and then recovered by soaking gel slices in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8, 0.1% SDS, and ethanol precipitation. In each experiment, all the upper strands (as shown in Table III) were ^{32}P-labeled concurrently, in order to obtain similar specific activities, with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol) in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 mM spermidine for 30 min at 37 °C. After extraction with phenol–chloroform, the labeled strand was annealed with a 1.5-fold higher amount of the unlabeled complementary strand in 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA. The mixture was heated at 65 °C for 5 min and slowly chilled at room temperature. After ethanol precipitation, the oligonucleotides were resuspended in distilled water and kept frozen at −20 °C.

Analysis of Topoisomerase II-Mediated DNA Cleavage. DNA cleavage reactions were performed in a volume of 20 μL in 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 and drugs at 37 °C for 20 min; 106 units of topoisomerase II (about 200 ng of proteins) were added at each reaction in the storage buffer. Reactions were stopped by adding SDS and proteinase K (1% and 0.1 mg/mL, respectively) and incubated at 42 °C for an additional 30 min.

DNAs were then ethanol precipitated, resuspended in 2.5 μL of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, heated at 95 °C for 2 min, chilled in ice, and then loaded into an 8% (20%, in the case of DNA oligonucleotides) polyacrylamide gel in TBE buffer containing 7 M urea. Gels were run at 70 W for 2 h, transferred to Whatmann 3MM paper sheets, dried, and autoradiographed with Amersham Hyperfilm MP. Topoisomerase II cleavage sites were located by comparison with purine markers resulting from a Maxam–Gilbert reaction of the same DNA fragment. As topoisomerase II-mediated cleavage generates 3′-OH DNA termini instead of the 3′-P ends generated by Maxam–Gilbert reactions, thus a shift toward higher molecular weights is expected for topoisomerase II cleavage products. This shift is of about 1.5 nucleotides for short oligonucleotides (Tapper & Clayton, 1981). The quantification of cleaved DNA oligonucleotides was done by cutting out gel slices and counting with a MR 300 automatic liquid scintillation system (Kontron).

Statistical Tests. The statistical tests used were as already described (Capranico et al., 1990a; Jaxel et al., 1991; Pommier et al., 1991a). Briefly, (i) the Chi-square one-sample test was used to determine the deviation from the expected base distribution at each position of the aligned sequences. The expected distribution was calculated considering the base frequencies (p) in the whole SV40 DNA ($p = 0.296$ for A and T; $p = 0.204$ for C and G). (ii) Base preferences were determined by calculating the standard deviation (SD) of the expected frequency p:

$$SD = (p[1-p]/n)^{1/2}$$

with n representing the number of sites in the set analyzed. Confidence intervals of 99.9% were calculated ($p \pm 3.3SD$), and base frequencies outside the interval were considered significant. (iii) Probability (P) of deviation from expectation was also calculated as follows. The expected number of sites having a given base at any particular position is pn. Let m be the observed number of sites which have the given base at that position. If $m > pn$, the probability, P, of the chance

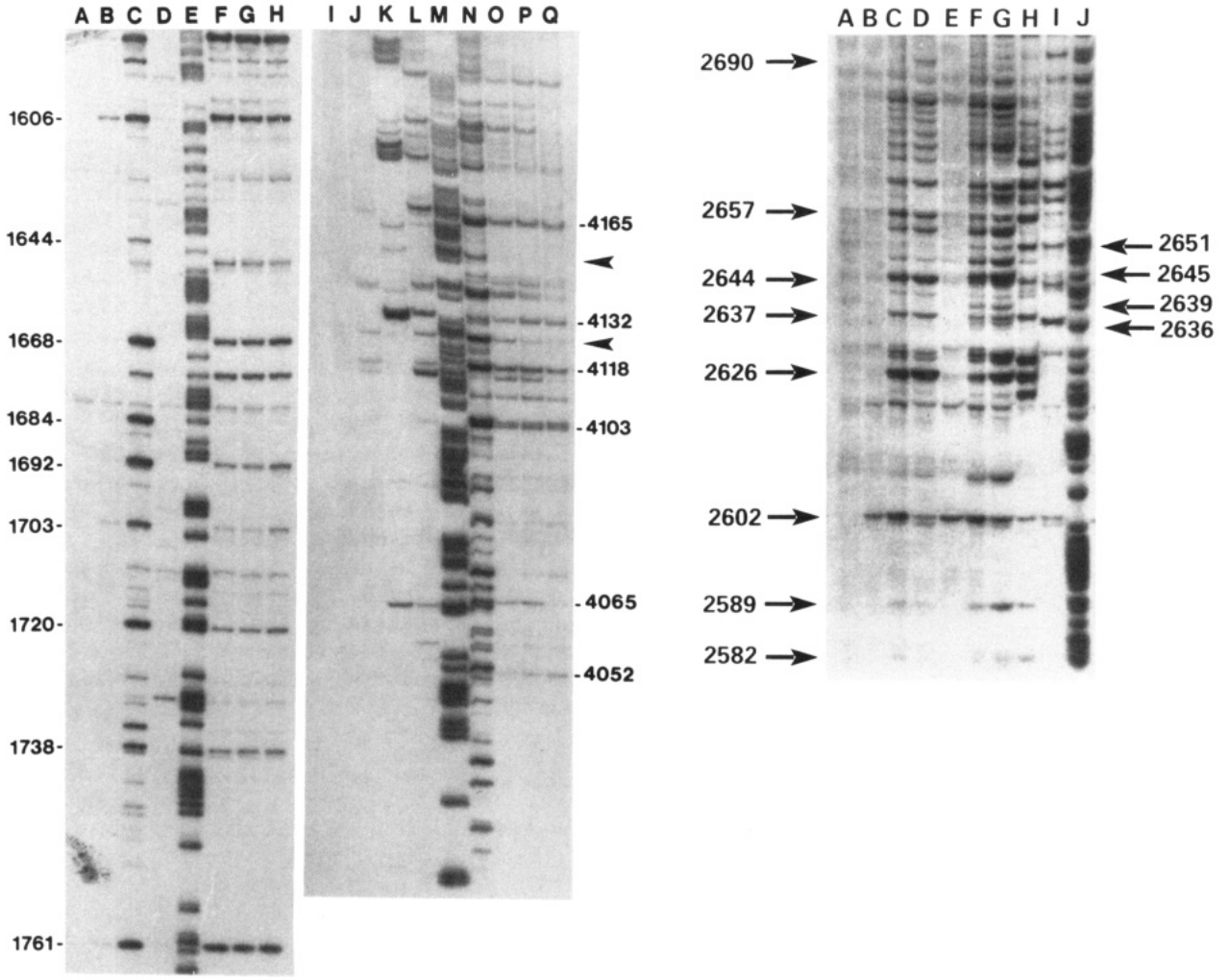

FIGURE 2: DNA cleavage intensity patterns stimulated by mitoxantrone and other compounds. SV40 DNA fragments were reacted with topoisomerase II and drugs for 20 min at 37 °C and then analyzed by 8% polyacrylamide sequencing gels. (left) DNA fragments were uniquely 5′-end-^{32}P-labeled at the *Eco*RI site (lanes A–H) or at a *Hin*dIII site (position 4002) (lanes I–Q). Lanes A and I, control DNA; lanes B and J, topoisomerase II alone; lanes C and N, 10 μM VM-26; lanes D and K, 0.9 μM 4-demethoxydaunorubicin; lane L, 10 μM mAMSA; lanes F–H, 0.11, 0.22, and 1.1 μM mitoxantrone; lanes O–Q, 0.022, 0.22, and 2.2 μM mitoxantrone; lanes E and M, purine molecular weight markers. Numbers indicate cleavage sites. Arrowheads indicate two sites not stimulated by mitoxantrone. (right) The SV40 DNA fragment was uniquely 5′-end-labeled at the *Bam*HI site. Lane A, control DNA; lane B, topoisomerase II alone; lanes C and D, 0.5 and 1 μM mitoxantrone; lanes E–G, 0.1, 1, and 10 μM VM-26; lane H, 10 μM mAMSA; lane I, 0.9 μM 4-demethoxydaunorubicin; lane J, purine molecular weight markers. Numbers on the left indicate cleavage sites stimulated by either mitoxantrone or VM-26. Numbers on the right indicate cleavage sites stimulated by either 4-demethoxydaunorubicin or VM-26 but not stimulated by mitoxantrone.

occurrence of m or more instances was calculated as

$$P = \sum_{i=m}^{n} p_i(1-p)^{(n-i)}(n!)/[i!(n-i)!]$$

If $m < pn$, the chance occurrence of m or fewer instances was calculated as follows:

$$P = \sum_{i=0}^{m} p_i(1-p)^{(n-i)}(n!)/[i!(n-i)!]$$

The opposite values of the logarithm of P, $-\log(P)$, are reported for each base at each position around the cleavage site.

RESULTS

The sequence specificity of mitoxantrone stimulation of topoisomerase II DNA cleavage was studied in SV40 DNA and compared to those of VM-26, mAMSA, and 4-demethoxydaunorubicin (Figure 1). An initial analysis by neutral agarose gel electrophoresis showed that mitoxantrone stimulated strong DNA cleavage around the termination region and from 3840 to 4900 genomic positions, including the major nuclear matrix-attachment region (MAR) of SV40 DNA, in agreement with previous findings on other drugs (Pommier et al., 1991b).

Ninety-three cleavage sites stimulated by mitoxantrone were sequenced in the following SV40 DNA regions: from genomic positions 4010 to 4240 (DNA fragment labeled at the 4002 *Hin*dIII site), and from 2580 to 2665 (DNA fragment labeled at the *Bam*HI site) (Figure 2), which corresponded to regions of strong cleavage stimulation by mitoxantrone, and from genomic positions 1600 to 1765, and from 1800 to 1900 (DNA fragments labeled at the *Eco*RI site) (Figure 2 and not shown), which corresponded to a domain of prominent cleavage (positions 1550–1850) produced by topoisomerase II without drugs (Capranico et al., 1990a). On a total of almost 500 nucleotides analyzed, an average break frequency of approximately one DNA break for every 25 nucleotides (1/25) was observed without drugs. The presence of mitoxantrone (0.1–1 μM) increased the break frequency to 1/6, a value close to that of VM-26 (1/4). 4-Demethoxydaunorubicin and mAMSA increased the break frequency to lower values, 1/18 and

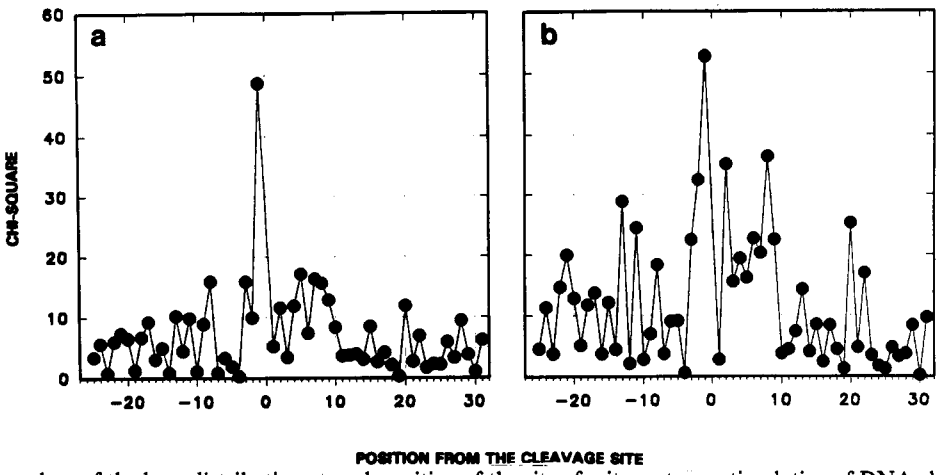

FIGURE 3: Chi-square values of the base distribution at each position of the site of mitoxantrone stimulation of DNA cleavage. Panel a: all sequenced sites (93). Panel b: sites of strong cleavage stimulation (45). The number 0 indicates the cleaved phosphodiester bond observed in sequencing gels. The chi-square values for $p = 0.05$ and 0.01 are 5.99 and 11.34, respectively (3 degrees of freedom).

1/9, respectively. Thus, VM-26 and mitoxantrone were the least sequence-selective studied drugs.

Similar DNA Cleavage Intensity Patterns Stimulated by Mitoxantrone and VM-26. The relative intensities of cleavage sites stimulated by mitoxantrone and VM-26 were strikingly similar to each other, while being different from those stimulated by 4-demethoxydaunorubicin and mAMSA (Figure 2). The similarity between mitoxantrone and VM-26 was somewhat dependent on the DNA region analyzed, as it was more convincing around the termination (Figure 2, right) and from positions 1600 to 1760 than in the MAR (Figure 2 (left); compare also lanes C–D and F–G between positions 2657 and 2690, Figure 2 (right)). Nevertheless, all the sites of mitoxantrone-stimulated DNA cleavage were also sites of cleavage stimulation by VM-26, while only 7% and 51% of the mitoxantrone sites were also stimulated by 4-demethoxydaunorubicin and mAMSA, respectively. Some VM-26 sites were not or were very weakly stimulated by mitoxantrone (sites 2651, 2645, 2639, 2636, 1644, 1684, and arrowhead, Figure 2). Interestingly, many of these VM-26 sites were stimulated by 4-demethoxydaunorubicin. Therefore, the mitoxantrone-stimulated sites constituted a subset of the VM-26-stimulated sites, and this might be explained, at least partially, by the very low sequence selectivity of VM-26. However, in contrast to mitoxantrone, a significant number of mAMSA and 4-demethoxydaunorubicin sites (about 20% and 30%, respectively) were not stimulated by VM-26 in the studied DNA fragments. Moreover, the sequence specificity differences between mitoxantrone and 4-demethoxydaunorubicin were also underlined by the observation that mitoxantrone stimulated DNA cleavage at sites observed without drugs, referred to as "no-drug sites" (sites 1606, 1668, 1761, 4118, and 2602, Figure 2), while anthracyclines did not (Capranico et al., 1990a).

Adjacent Base Requirements of Mitoxantrone To Stimulate Topoisomerase II DNA Cleavage. DNA sequences of 93 mitoxantrone-stimulated DNA cleavage sites were aligned at the point of the observed phosphodiester bond cleavage in the 5′ to 3′ orientation, and the bases at the 5′ and 3′ sides of the break point were ordered with negative and positive numbers, as follows:

−3	−2	−1	+1	+2	+3	+4	+5	+6	+7
5′ N–p–	N–p–	N	N–p–	N–p–	N–p–	N–p–	N–p–	N–p–	N 3′

(the cleaved bond is between bases −1 and +1; N represents any base; p represents phosphate group).

Chi-square analysis of base distributions at each position relative to the cleavage site showed that the most significant deviation from the global SV40 DNA base distribution was at position −1 (Figure 3). In the case of the 45 strongest sites, the analysis showed the existence of a region of nonrandom base distribution from positions −3 to +9, with only position +1 having a Chi-square value not statistically significant (Figure 3b). Positions −1, +8, +2, and −2 were the most biased ones; however, more distant positions also showed high Chi-square values (Figure 3b). The Chi-square curve did not show any obvious symmetry at the cleavage site (Figure 3), in contrast to those reported for the sets of "no-drug sites", VM-26, and doxorubicin sites (Capranico et al., 1990a; Pommier et al., 1991a).

At the most biased position −1, 83% of all mitoxantrone sites had a pyrimidine (42%, cytosine, and 41%, thymine; data not shown), in agreement with the base distribution observed for the "no-drug sites" set (Capranico et al., 1990a); almost identical fractions (42%, cytosine, and 42%, thymine) were observed for the strongest cleavage sites (Table I). At the dyadic position +5, purines were present in 69% of the strongest sites (Table I) and in 70% of all sites (not shown). Base preferences, as determined by probability analysis (see Experimental Procedures), showed that only at position −1 the frequencies of all the four bases were biased, with pyrimidines and purines being preferred and excluded, respectively, in both sets of all and the strongest sites (Table I and not shown). In the case of the set of all 93 sites, the highest $-\log(P)$ values were found at position −1 for the exclusion of A (6.074) and preference of C (5.705). At the same position, thymines were preferred ($-\log(P) = 1.872$) and guanines were excluded ($-\log(P) = 2.757$). At the dyadic position +5, purines were preferred ($-\log(P)$ being 1.641 and 1.765 for A and G, respectively) and cytosines were excluded ($-\log(P) = 2.757$). These results then suggested that a pyrimidine, and particularly a cytosine, is required at the 3′ terminus of the break site for mitoxantrone stimulation of DNA cleavage.

Base Requirements around the Cleavage Site for Strong Stimulation of DNA Cleavage. In the case of the set of 45 strongest sites, the dyadic positions −2 and +6 and positions +2, +8 and −8, in addition to position −1, showed the strongest base preferences (Table I). They were the following: (i) at position −2, cytosines and adenines were excluded and preferred, respectively; at the dyadic position +6, guanines and thymines were excluded and preferred, respectively; (ii)

Table I: Statistical Analysis of the Base Distributions at the Site of Mitoxantrone-Stimulated DNA Cleavage (45 Strongest Sites)

	base frequency[a] (%)				base probability[b] (−log(*P*))				
position	A	C	G	T	A	C	G	T	preferences[c]
−8	24	13	13	49	0.55	0.79	0.79	2.30	T
−7	36	13	20	31	0.63	0.79	0.25	0.33	
−6	40	13	13	34	1.05	0.79	0.79	0.46	
−5	20	18	31	31	0.99	0.38	1.22	0.33	
−4	31	18	22	29	0.33	0.38	0.36	0.27	
−3	42	7	11	40	1.31	1.96	1.10	1.05	(no C)
−2	47	0*	26	27	1.94	4.46	0.72	0.39	no C, (A)
−1	11	42*	5	42	2.52	3.14	2.57	1.31	C, no R
+1	31	16	18	36	0.33	0.56	0.38	0.63	
+2	20	31	38	11	0.99	1.22	2.26	2.52	no T, G
+3	20	36	16	29	0.99	1.88	0.56	0.27	(C)
+4	27	7	20	47	0.39	1.96	0.25	1.94	(no C), (T)
+5	38	9	31	22	0.82	1.48	1.22	0.75	
+6	24	20	7	49	0.55	0.25	1.96	2.30	T, (no G)
+7	36	13	7	44	0.63	0.79	1.96	1.61	(no G), (T)
+8	25	40	2	33	0.55	2.68	3.36	0.46	no G, C
+9	40	7	11	42	1.05	1.96	1.10	1.31	(no C)
+10	31	13	20	36	0.33	0.79	0.25	0.63	
+11	33	18	13	36	0.46	0.38	0.79	0.63	
+12	33	13	29	24	0.46	0.79	0.95	0.55	

[a] An asterisk indicates a value outside the 99.9% confidence intervals (0.6–40.2%, for G and C; 7.1–52.1%, for A and T. See Experimental Procedures for details). [b] *P* is the probability of observing that deviation or more, as either deficiency (underlined values) or excess (not underlined values) relative to the expected frequency of each individual base (0.204 for G and C; 0.296 for A and T). [c] Preferences are considered when the −log(*P*) value is more than or equal to 2.0 ($P = 0.01$); a lower preference (in parentheses) is considered when the −log(*P*) value is more than or equal to 1.6 ($P = 0.025$).

Table II: Statistical Analysis of the Base Distributions at the 25 Cleavage Sites Stimulated by VM-26 and Not Stimulated by Mitoxantrone

	base frequency[a] (%)				base probability[b] (−log(P))				
position	A	C	G	T	A	C	G	T	preferences[c]
−8	16	20	16	48	1.01	0.22	0.44	1.40	
−7	28	16	28	28	0.28	0.40	0.63	0.28	
−6	28	8	12	52	0.28	1.04	0.66	1.81	(T)
−5	20	20	8	52	0.69	0.22	1.04	1.81	(T)
−4	28	36	12	24	0.28	1.28	0.66	0.45	
−3	44	0	12	44	1.05	2.48	0.66	1.05	no C
−2	8	20	16	56	2.00	0.22	0.40	2.28	T, no A
−1	24	48*	12	16	0.45	2.73	0.66	1.01	C
+1	32	4	16	48	0.33	1.61	0.40	1.40	(no C)
+2	20	12	12	56	0.69	0.66	0.66	2.28	T
+3	24	36	12	28	0.45	1.28	0.66	0.28	
+4	32	28	12	28	0.33	0.63	0.66	0.28	
+5	16	12	16	56	1.01	0.66	0.40	2.28	T
+6	16	40	28	16	1.01	1.70	0.63	1.01	(C)
+7	40	20	0	40	0.75	0.22	2.48	0.75	no G
+8	16	24	20	40	1.01	0.39	0.22	0.75	
+9	16	28	28	28	1.01	0.63	0.63	0.28	
+10	28	16	16	40	0.28	0.40	0.40	0.75	
+11	12	8	24	56	1.44	0.39	1.04	2.28	T
+12	36	36	4	24	0.51	1.28	1.61	0.45	(no G)

[a] An asterisk indicates a value outside the 99.9% confidence intervals (0–47%, for G and C; 0.5–59.7%, for A and T. See Experimental Procedures for details). [b] *P* is the probability of observing that deviation or more, either as deficiency (underlined values) or as excess (not underlined values) relative to the expected frequency of each individual base (0.204 for G and C; 0.296 for A and T). [c] Preferences are considered when the −log(*P*) value is more than or equal to 2.0 ($P = 0.01$); a lower preference (in parentheses) is considered when the −log(*P*) value is more than or equal to 1.6 ($P = 0.025$).

guanines and cytosines were preferred at positions +2 and +3, respectively; (iii) preferences for thymines and cytosines were noted at positions −8 and +8, respectively. Other base preferences and exclusions were noted from −3 to +9 positions (Table I) and, in addition, adenines were preferred at more distant positions (−13, +13, +20, and +22), and thymines were preferred at position −11 (not shown). It has to be noted that base preferences were symmetrical from −3 to +7 positions, but not at positions further distant from the cleavage site. Interestingly, a cluster of preferred bases was observed from +6 to +9 positions, which corresponded to the strand cleavage site opposite to the observed one.

Base Sequence at the Site of DNA Cleavage Stimulated by VM-26 but Not Stimulated by Mitoxantrone. As the mitoxantrone-stimulated sites constituted a subset of the VM-26-stimulated sites, some sites were thus found that were stimulated by VM-26 but not by mitoxantrone (see for example sites 1644, 1684, 2651, 2645, 2639, and 2636, Figure 2). Twenty-five of such cleavage sites were then collected, and to better characterize the sequence specificity of mitoxantrone action, an analysis of their base sequences was carried out (Table II). Between the two sets of mitoxantrone-stimulated and -unstimulated sites, opposite base preferences were noted at positions −2 and +2 (Tables I and II). At position −2, an adenine was preferred for the stimulated sites and excluded for the unstimulated sites, a thymine being preferred in this last case. At position +2, a thymine was preferred for the unstimulated sites and excluded for the stimulated sites, a guanine being preferred in this case. These observations suggested that positions −2 and +2 may influence the stability

Table III: Mitoxantrone and VM-26 Stimulation of DNA Cleavage in 42-bp Oligonucleotides

A. Base Sequence[a] of Oligonucleotide I

Oligo I

4265

4263 | 4269

5' | | | 3'

4244-GATTATAACTGTTATGCCTA**C**T**T**ATAAAGGTTACAGAATATT-4285

B. Effects of Base Mutations on the Fraction of Cleaved DNA at the 4265 Site[b]

	base[d] at			drug x-fold increase[c]		
				mitoxantrone		
oligo	4264	4266	no drug (%)	0.5 μM	1 μM	VM-26 (10 μM)
I	C	T	0.38 ± 0.02	2.03 ± 0.26	2.23 ± 0.31	9.87 ± 3.72
II	*a*	T	0.10 ± 0.01	0.25 ± 0.25	0.20 ± 0.20	3.25 ± 0.25
III	*t*	T	0.18 ± 0.08	4.00 ± 1.70	4.35 ± 3.15	5.40 ± 1.60
IV	*g*	T	0.28 ± 0.12	0.95 ± 0.65	0.20 ± 0.20	1.95 ± 0.35
V	*g*	*g*	0.15 ± 0.05	1.05 ± 0.05	0.98 ± 0.18	4.18 ± 1.83
VI	C	*g*	0.45 ± 0.15	5.50 ± 0.10	4.80 ± 0.80	9.55 ± 1.85

[a] The base sequence of oligonucleotide I was identical to SV40 DNA from 4244 to 4285 positions. Bases in bold and underlined were mutated in oligonucleotides II to VI. [b] The percent of DNA molecules cleaved at position 4265 was calculated by cutting out the corresponding gel slices and those corresponding to the uncleaved DNA. Radioactivity was counted by liquid scintillation in a Kontron MR 300 counter. The values are means ± SE of two to three independent experiments. [c] The drug x-fold increase values are relative to the corresponding percentage of cleaved DNA without drugs. [d] Mutated bases are in italics and lowercase characters.

of drug interactions in the ternary complex. Moreover, other positions showed important differences. (i) Position −1: a cytosine, but not a thymine, was preferred in the case of unstimulated sites. Moreover, this position was less biased for the unstimulated than for the stimulated sites (Tables I and II). (ii) Position +5: in the unstimulated sites, a thymine was preferred in contrast to in the stimulated sites. This last preference corresponded to an adenine at position −1 on the other strand, resembling the doxorubicin base requirement (Capranico et al., 1990a). Indeed, many of these sites were stimulated by 4-demethoxydaunorubicin (Figure 2). (iii) Position +6: at the dyadic position of the position −2, a thymine and a cytosine were preferred for the stimulated and unstimulated sites, respectively. Other positions showed only minor or no differences (Tables I and II). Therefore, altogether these results indicate that the sequence 5′-AYNG-3′ (Y, pyrimidine; N, any base) are required at positions from −2 to +2 for strong cleavage stimulation by mitoxantrone. In addition, adenines are highly excluded from position −1.

Effects of Base Mutations on Mitoxantrone and VM-26 Stimulation of DNA Cleavage in Short DNA Oligonucleotides. The importance of the base identity at positions −1 and +2 for drug stimulation of DNA cleavage was further independently evaluated by a base mutation analysis in 42-base-pair oligonucleotides (Table III). Oligonucleotide I corresponded to the SV40 DNA sequence from genomic positions 4244 to 4285, containing at the center a site (4265) of prominent topoisomerase II DNA cleavage, as determined in larger DNA fragments (Capranico et al., 1990a). This site was also the main topoisomerase II DNA cleavage site in oligonucleotides and was stimulated by mitoxantrone and VM-26 (Figure 4). Cleavage patterns stimulated by mitoxantrone and VM-26 were very similar also in oligonucleotides.

Effects of mutations of the 4264 base pair were first studied (Figure 4, top panels). The 4264 base in the upper strand corresponded to position −1 of the 4265 cleavage site. Topoisomerase II without drugs promoted cleavage mainly at sites 4265, 4269, and 4263, and this cleavage pattern was unchanged in oligonucleotides III and IV (Figure 4, upper panels). In contrast, only weak cleavages were observed in oligonucleotide II (Figure 4 and Table III), showing that an adenine at position −1 markedly reduced the cleavage by the enzyme alone at the 4265 site, in agreement with previous findings (Capranico et al., 1990a).

Stimulation by mitoxantrone was observed at sites 4265, 4269, 4263, 4264, and 4259 in oligonucleotide I (Figure 4). With either a cytosine or a thymine at 4264, mitoxantrone stimulated DNA cleavage at site 4265, whereas it did not stimulate cleavage if a purine was present at that position (Figure 4 and Table III). On the other hand, the weaker stimulation by mitoxantrone of DNA cleavage at sites 4263, 4264, and 4259 was not changed in the mutated oligonucleotides (Figure 4 and data not shown). These results independently suggested that mitoxantrone requires a cytosine or a thymine at position −1 to stimulate topoisomerase II DNA cleavage. The reduction of cleavage at the 4269 site was observed in oligonucleotides II, III, and IV with and without drugs (Figure 4). Therefore, the effect of the 4264 mutation on that site probably was not specific for mitoxantrone. Replacing the 4264 cytosine with a purine reduced 3–5-fold the stimulation of DNA cleavage by VM-26 at position 4265 (Table III). However, VM-26 stimulation was never abolished and a thymine reduced the DNA cleavage about 2-fold. This showed that mitoxantrone and VM-26 preferred either one of the two pyrimidines, although VM-26 may more strongly stimulate cleavage if a cytosine is present at position −1 (Table III).

Effects of mutations of the 4266 base pair, which corresponded to the +2 position of the 4265 site in the upper strand, was then studied (Figure 4, lower panels), as the statistical analysis showed that a guanine at position +2 may markedly favor mitoxantrone stimulation of topoisomerase II DNA cleavage (Tables I and II). In the particular experiment shown in Figure 4 (lower panels), topoisomerase II induced more intense cleavages. Mitoxantrone did not stimulate the cleavage at site 4265 if guanines were present at positions −1 and +2 (oligonucleotide V), while it stimulated the cleavage at that site if a cytosine was at position −1 and a guanine at position +2 (oligonucleotide VI) (Figure 4 and Table III). Moreover, a comparison between oligonucleotides I and VI showed that mitoxantrone stimulation was stronger in oligonucleotide VI than I, while VM-26 stimulation was similar in the two

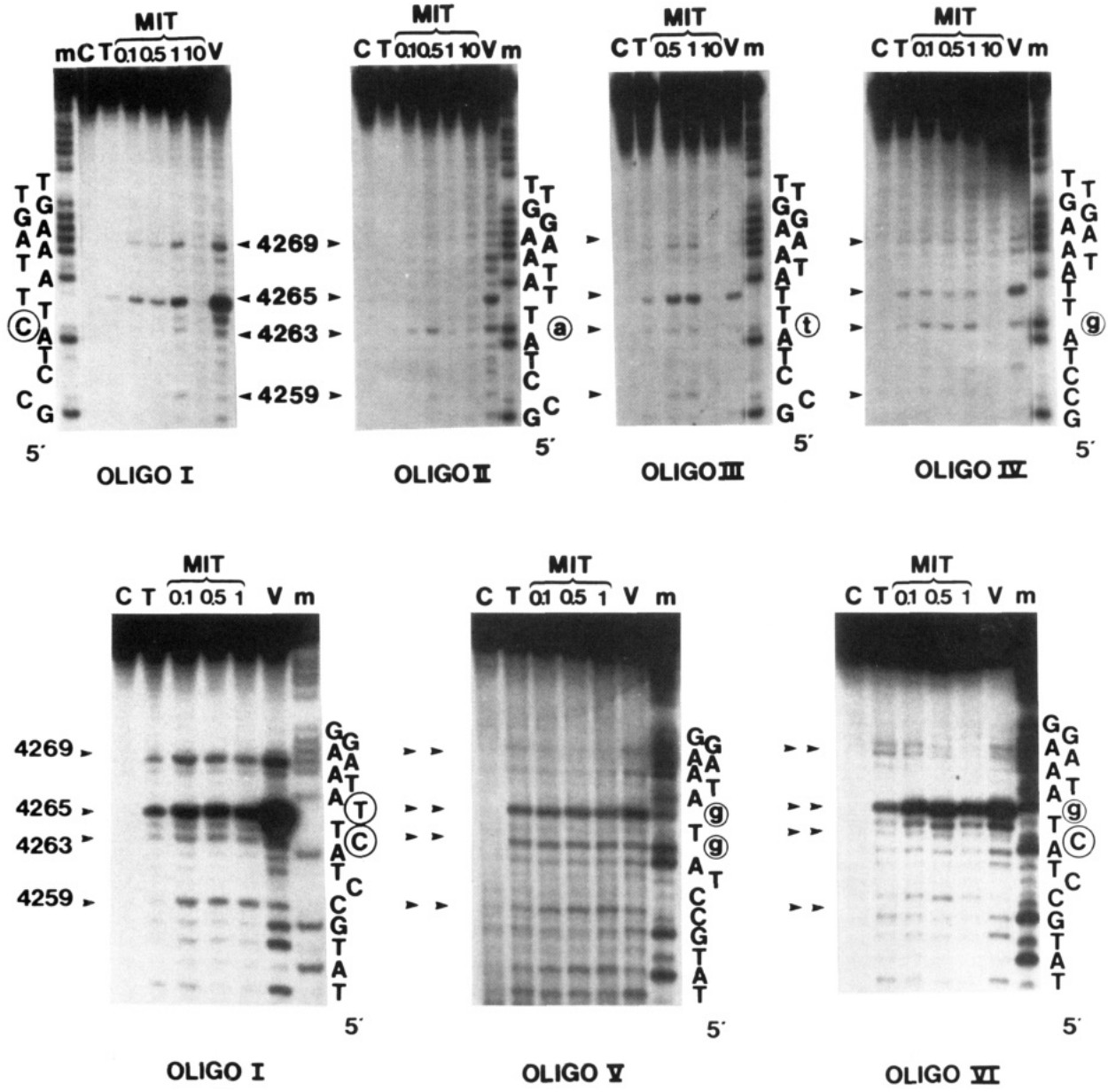

FIGURE 4: Base mutation analysis of topoisomerase II DNA cleavage stimulated by mitoxantrone and VM-26 in 42-bp oligonucleotides (see Table III). In each experiment, the upper strand were 5′-labeled concurrently to obtain similar specific activities and, then, annealed to the complementary unlabeled strands. The oligonucleotides were reacted with topoisomerase II and drugs for 10 min at 37 °C and analyzed by 20% polyacrylamide sequencing gels. Top panels: effects of base mutation at 4264 position. Bottom panels: effects of base mutations at 4266 position. Lanes C, control DNA; lanes T, topoisomerase II alone; lanes V, 10 μM VM-26; lanes MIT, mitoxantrone at the indicated concentrations (μM); lanes m, purine molecular weight markers. Numbers and arrows indicate cleavage sites. The DNA sequence of each oligonucleotide is shown at one side of the m lanes; the circle indicates the bases at positions 4264 and 4266 which were mutated (lowercase characters) in oligo II, III, IV, V, and VI.

oligonucleotides (Table III). These results further indicated that the base at position −1 is crucial for cleavage stimulation by mitoxantrone and VM-26 and that the base at position +2 may increase mitoxantrone stimulation only when the drug-required base is present at position −1. A comparison between oligonucleotides IV and V indicated that a +2 guanine may also favor the VM-26 stimulation of DNA cleavage at least in this case (Table III).

DISCUSSION

Drug Sequence Specificity of in Vitro Topoisomerase II DNA Cleavage. Early investigations showed that different topoisomerase II-trapping drugs produce distinct, drug-specific patterns of in vitro DNA cleavage sites (Tewey et al., 1984a,b; Chow & Ross, 1987). The present study provides evidence that mitoxantrone and VM-26, two structurally unrelated drugs, induce similar patterns of topoisomerase II DNA cleavage in SV40 DNA fragments. These results are not conflicting, as this is the first paper that reports on a direct and detailed comparison of the sequence selectivities of these two drugs. Both the statistical analysis of SV40 DNA cleavage sites and the effects of base mutations on DNA cleavage in short oligonucleotides indicated that mitoxantrone-specific requirements are at position −1 for cytosines and thymines. Therefore, mitoxantrone is so far the only topoisomerase II-trapping drug that shows a clear preference for two nucleotides (C and T) at position −1 (or +1), even for strong cleavage stimulation (Table IV). This observation is consistent with the low sequence selectivity of mitoxantrone and the relatively low values of −log(P) (Table I) in comparison to other drugs

Table IV: Summary of the Sequence Specificities of Topoisomerase II-Trapping Antitumor Drugs

drug	base requirements[a] at position −1	+1	reference
doxorubicin, 4-demethoxydaunorubicin	A		Capranico et al. (1990a)
mAMSA		A	Pommier et al. (1991a)
VM-26, VP-16	C (T)		Pommier et al. (1991a)
mitoxantrone	C/T		this work
ellipticine	T		Fossé et al. (1991)

[a] At the site of topoisomerase II DNA cleavage without drugs, pyrimidines and adenines were preferred at positions −1 and +1, respectively. The secondary base requirement of the drug is in parentheses. Abbreviations: A, adenine; C, cytosine; T, thymine.

(Capranico et al., 1990a; Pommier et al., 1991a). VM-26 was shown to highly prefer a cytosine, and secondarily a thymine, at position −1 for strong stimulation of DNA cleavage (Pommier et al., 1991a). This conclusion is further supported by the present results on the effects of base mutations on VM-26-stimulated DNA cleavage in short oligonucleotides. Thus, mitoxantrone and VM-26 share the primary requirements of local bases for DNA cleavage stimulation, which are different from those of mAMSA and 4-demethoxydaunorubicin (Table IV).

A high preference for pyrimidines at position −1 has been also observed for "no-drug sites", which are cleavage sites observed without drugs (Capranico et al., 1990a). Consistently, both mitoxantrone and VM-26 stimulated DNA cleavage also at these sites, whereas a previous investigation showed that doxorubicin and 4-demethoxydaunorubicin did not stimulate DNA cleavage at "no-drug sites" (Capranico et al., 1990a). Indeed, the sequence specificities of mitoxantrone and anthracyclines are completely different. Many of the VM-26 sites not stimulated by mitoxantrone were instead stimulated by 4-demethoxydaunorubicin and had an adenine at position −1 of the strand opposite to the observed cleaved strand (Table II). Therefore, the different sequence specificities of mitoxantrone and 4-demethoxydaunorubicin are not related to the high DNA affinity constants of these agents. Their stimulation of topoisomerase II DNA cleavage may thus rely on specific features of the interaction with the DNA and/or the enzyme in the ternary complex.

Sequence Specificity of Drug Action as a Novel Basis To Classify Topoisomerase II-Trapping Antitumor Drugs. The high similarity of mitoxantrone and VM-26 actions on topoisomerase II further supports the hypothesis that intercalating and nonintercalating agents may bind to the same site in the ternary complex. Then, mitoxantrone and VM-26 might interact in the ternary complex in a similar manner. These interactions may be diverse from those of 4-demethoxydaunorubicin and mAMSA, which have different sequence selectivities (Table IV). Further investigations may probably establish that other structurally unrelated compounds share specific base requirements, thus suggesting similar interactions in the ternary complex. This information could then provide a new way to classify topoisomerase II-trapping antitumor drugs, not related to the chemical structure (Table IV).

A drug-resistant variant of topoisomerase II may provide further knowledge of the mechanism of drug action. An HL60/AMSA topoisomerase II has been shown to be resistant to mitoxantrone while retaining sensitivity to etoposide (Zwelling et al., 1991). As a point mutation of the topoisomerase II gene has been shown to be associated with this drug-resistant enzyme (Hinds et al., 1991), amino acid substitutions might affect mitoxantrone but not etoposide stimulation of DNA cleavage. It could then be interesting to study the sequence specificity of drug-stimulated DNA cleavage by the mutated HL60/AMSA topoisomerase II or other enzymes with a differential sensitivity to drugs.

Structure–activity relationship studies among different chemical classes may provide insight into specific drug structural features required for trapping topoisomerase II at specific DNA sites (Capranico & Zunino, 1992). This may ultimately lead to the design of new compounds that can direct topoisomerase II to cleave DNA strands at highly specific sites on the human genome. A recent paper on in vivo topoisomerase II DNA cleavage detected by using VM-26 suggested that the chromatin structure might strongly influence the base sequence determinants of topoisomerase II DNA cleavage established in vitro (Käs & Laemmli, 1992). In particular, the drug-specific base requirements might be overruled by the local chromatin structure. VM-26 is the least sequence-selective drug; thus, it would be of interest to define the in vivo sequence specificities of more selective topoisomerase II-trapping drugs. The role of the sequence selectivity of antitumor topoisomerase II inhibitors in drug clinical efficacy remains to be established.

Strong Stimulation of DNA Cleavage by Mitoxantrone. The present analysis of sites stimulated and unstimulated by mitoxantrone suggests a high heterogeneity of DNA–topoisomerase II–drug complexes. Different enzyme–DNA complexes can be distinguished by the presence of mitoxantrone. The results indicate that the sequence 5′-AYNG-3′ (Y, pyrimidine; N, any base) is required from positions −2 to +2 in order for mitoxantrone to strongly stimulate DNA cleavage. An adenine at position −1 in the strand opposite to the observed cleaved strand may thus prevent cleavage stimulation by mitoxantrone (Table II), suggesting that two mitoxantrone molecules have to interact at the two strand sites to stimulate cleavage, at least in these instances. Furthermore, preferred bases were also observed at positions +8 and +9 in the set of strong cleavage sites. In particular, from positions +6 to +9 the sequence 5′-TTCT-3′ was preferred (Table I). The same sequence was not observed in the set of the VM-26 sites not stimulated by mitoxantrone (Table II). This finding could indicate that, for strong mitoxantrone stimulation of DNA cleavage, a specific enzyme–DNA interaction may be necessary to provide additional stabilization to the ternary complex.

Effects of DNA Sequence on in Vitro Topoisomerase II DNA Cleavage. DNA sequence probably governs at several levels the site of in vitro topoisomerase II DNA cleavage in linear DNA molecules. (i) Long-range effects have been described: topoisomerase II appears to bind preferentially to DNA cross-overs and bent regions (Zechiedrich & Osheroff, 1990; Howard et al., 1991). Major DNA cleavage without drugs has been shown to occur at the MAR of SV40 DNA (Capranico et al., 1990a,b; Pommier et al., 1991b). Moreover, MAR-related sequences can trigger the polymerization of topoisomerase II along a DNA fragment (Adachi et al., 1989). Thus, long-range sequence effects may determine at a regional level the DNA binding and cleavage by topoisomerase II. (ii) DNA sequence effects are also known in the intermediate range. DNA cleavage consensus sequences spanning about 15 nucleotides have been found (Sander & Hsieh, 1985; Spitzner & Muller, 1988; Capranico et al., 1990a), and alternating purine/pyrimidine repeats are preferentially cleaved by eukaryotic topoisomerase II (Spitzner et al., 1990).

Therefore, the base sequence at the cleavage site may locally determine the phosphodiester bond cleaved by the enzyme. Moreover, the observation that cleavage sites are identical in short oligonucleotides and in larger DNA fragments (Capranico et al., 1993; and present study) indicates that the local DNA sequence is sufficient for the local recognition of cleavage sites by topoisomerase II. (iii) While long- and intermediate-range DNA sequence effects are enzyme specific, drug stimulation of DNA cleavage is instead dependent on the DNA sequence immediately adjacent to the cleaved phosphodiester bond. This observation suggests that drug action on topoisomerase II occurs locally at the DNA cleavage site.

REFERENCES

Adachi, Y., Kas, E., & Laemmli, U. K. (1989) *EMBO J. 8*, 3997.

Capranico, G., & Zunino, F. (1992) *Eur. J. Cancer 28A*, 2055.

Capranico, G., Kohn, K. W., & Pommier, Y. (1990a) *Nucleic Acids Res. 18*, 6611.

Capranico, G., Zunino, F., Kohn, K. W., & Pommier, Y. (1990b) *Biochemistry 29*, 562.

Capranico, G., Tinelli, S., Zunino, F., Kohn, K. W., & Pommier, Y. (1993) *Biochemistry 32*, 145.

Chow, K. C., & Ross, W. E. (1987) *Mol. Cell. Biol. 7*, 3119.

De Isabella, P., Capranico, G., Binaschi, M., Tinelli, S., & Zunino, F. (1990) *Mol. Pharmacol. 37*, 11.

Fossé, P., René, B., Le Bret, M., Paoletti, C., & Saucier, J.-M. (1991) *Nucleic Acids Res. 19*, 2861.

Gellert, M. (1981) *Annu. Rev. Biochem. 50*, 879.

Hinds, M., Deisseroth, K., Mayes, J., Altschuler, E., Jansen, R., Ledley, F. D., & Zwelling, L. A. (1991) *Cancer Res. 51*, 4729.

Howard, M. T., Lee, M. P., Hsieh, T. S., & Griffith, J. D. (1991) *J. Mol. Biol. 217*, 53.

Jaxel, C., Capranico, G., Kerrigan, D., Kohn, K. W., & Pommier, Y. (1991) *J. Biol. Chem. 266*, 20418.

Kās, E., & Laemmli, U. K. (1992) *EMBO J. 11*, 705.

Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351.

Minford, J., Pommier, Y., Filipski, J., Kohn, K. W., Kerrigan, D., Mattern, M., Michaels, S., Schwartz, R., & Zwelling, L. A. (1986) *Biochemistry 25*, 9.

Pommier, Y., & Kohn, K. W. (1989) in *Developments in Cancer Chemotherapy* (Glazer, R. I., Ed.) pp 175–195, CRC Press, Inc., Boca Raton, FL.

Pommier, Y., Capranico, G., Orr, A., & Kohn, K. W. (1991a) *Nucleic Acids Res. 19*, 5973.

Pommier, Y., Capranico, G., Orr, A., & Kohn, K. W. (1991b) *J. Mol. Biol. 222*, 909.

Sander, M., & Hsieh, T. S. (1985) *Nucleic Acids Res. 13*, 1057.

Spitzner, J. R., & Muller, M. T. (1988) *Nucleic Acids Res. 16*, 5533.

Spitzner, J. R., Chung, I. K., & Muller, M. T. (1990) *Nucleic Acids Res. 18*, 1.

Tapper, D. P., & Clayton, D. A. (1981) *Nucleic Acids Res. 9*, 6787.

Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984a) *J. Biol. Chem. 259*, 9182.

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984b) *Science 226*, 466.

Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665.

Zechiedrich, E. L., & Osheroff, N. (1990) *EMBO J. 9*, 4555.

Zwelling, L. A., Mayes, J., Hinds, M., Chan, D., Altschuler, E., Carroll, B., Parker, E., Deisseroth, K., Radcliffe, A., Seligman, M., Li, L., & Farquhar, D. (1991) *Biochemistry 30*, 4048.