Sequence-Selective Topoisomerase II Inhibition by Anthracycline Derivatives in SV40 DNA: Relationship with DNA Binding Affinity and Cytotoxicity

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ABSTRACT: Topoisomerase II mediated double-strand breaks produced by anthracycline analogues were studied in SV40 DNA. The compounds included doxorubicin, daunorubicin, two doxorubicin stereoisomers (4′-epimer and β-anomer), and five chromophore-modified derivatives, with a wide range of cytotoxic activity and DNA binding affinity. Cleavage of ^{32}P-end-labeled DNA fragments was visualized by autoradiography of agarose and polyacrylamide gels. Structure–activity relationships indicated that alterations in the chromophore structure greatly affected drug action on topoisomerase II. In particular, removal of substituents on position 4 of the D ring resulted in more active inducers of cleavage with lower DNA binding affinity. The stereochemistry between the sugar and the chromophore was also essential for activity. All the active anthracyclines induced a single region of prominent cleavage in the entire SV40 DNA, which resulted from a cluster of sites between nucleotides 4237 and 4294. DNA cleavage intensity patterns exhibited differences among analogues and were also dependent upon drug concentration. Intensity at a given site depended on both stimulatory and suppressive effects depending upon drug concentration and DNA sequence. A good correlation was found between cytotoxicity and intensity of topoisomerase II mediated DNA breakage.

In spite of the wide use of anthracycline antibiotics in human cancer chemotherapy, their mechanism of action has continued to be debated over the past several years (Arcamone, 1982). Current evidence strongly suggests that the primary target of these antitumor agents is cellular DNA, to which binding occurs by means of intercalation (Fritzsche & Berg, 1987; Wang et al., 1987). Although strong inhibitions of both DNA and RNA synthesis were shown to be associated with a direct interaction of the drug with DNA or chromatin (Zunino et al., 1980; Phillips & Crothers, 1986), previous studies on structure–activity relationships indicated that DNA intercalation is a necessary but not sufficient condition for optimal antitumor activity of anthracycline antibiotics (Zunino et al., 1979, 1980, 1981, 1986; Quadrifoglio et al., 1982).

In recent years, a different line of investigation discovered a more specific interaction of anthracyclines at the chromatin level: the interference with DNA topoisomerase II (Ross et al., 1979a; Zwelling et al., 1981; Tewey et al., 1984). Topoisomerase II is a major constituent of the nuclear matrix and the chromosome scaffold (Earnshaw et al., 1985; Berrios et al., 1985; Gasser et al., 1986) and is an essential enzyme for many topological interconversions of DNA loops during replication and transcription (Wang, 1985). Topoisomerase II induced DNA breakage (Zwelling et al., 1981; Nelson et al., 1984; Minford et al., 1986) may be a primary lethal event in the mechanism of action of antitumor intercalating agents (Pommier & Kohn, 1989b; Zwelling et al., 1981; Pommier et al., 1985) and demethylepipodophyllotoxins (Ross et al., 1984; Long et al., 1984).

The role of topoisomerase II mediated DNA cleavage in the mechanism of action of anthracyclines has been questioned because the production of protein-associated DNA single-strand breaks by chromophore-modified anthracycline analogues is not quantitatively correlated with cytotoxicity (Zwelling et al., 1982; Capranico et al., 1986). However, this is likely due to the fact that DNA break resealing occurs very slowly with some anthracyclines, such as doxorubicin, because of the prolonged cellular drug retention (Zwelling et al., 1981; Capranico et al., 1989a,b), and that for some drugs, cellular DNA breaks vs drug concentration is a bell-shaped curve (Pommier et al., 1985b; Capranico et al., 1986, 1987).

In the present paper, we report an in vitro study of topoisomerase II mediated DNA breaks produced by several anthracycline derivatives in SV40 DNA. The studied analogues (Figure 1) represent a homologous group of derivatives with limited structural modifications. However, they greatly differ in cytotoxic potency and DNA binding affinity, which provides a means to assess the relationship between drug structure, DNA binding affinity constant, and topoisomerase II inhibition. A detailed analysis of topoisomerase II mediated DNA cleavage patterns induced by the anthracyclines is also presented. A region of prominent cleavage was found in the entire SV40 genome, and its sequence was determined. This investigation also provides a means to assess the relationships between anthracycline cytotoxicity and the ability to induce topoisomerase II mediated DNA breaks.

Materials and Methods

Materials. Anthracycline derivatives were obtained from Farmitalia-Carlo Erba (Milan, Italy). 5-Iminodaunorubicin, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-thenylidene-β-D-glucopyranoside) (VM-26),[1] and *m*-AMSA were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. Stock solutions were made in deionized water at 0.2 mM and kept frozen at −20 °C.

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[1] Abbreviations: IC_{50}, drug concentration required for 50% cell growth inhibition; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; teniposide (VM-26), 4′-demethylepipodophyllotoxin 9-(4,6-*O*-theonylidene-β-D-glucopyranoside); SDS, sodium dodecyl sulfate.

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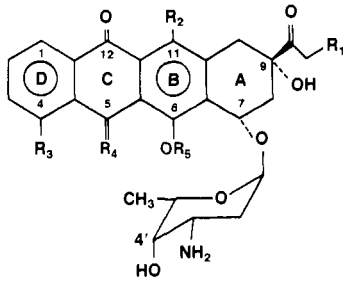
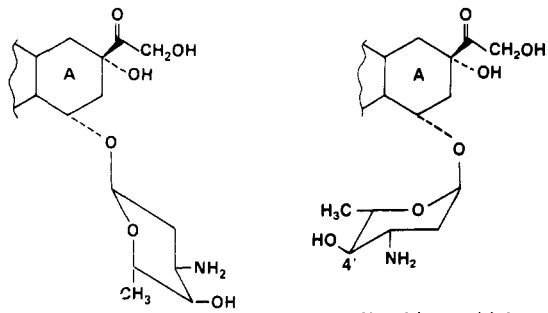


	R_1	R_2	R_3	R_4	R_5
Doxorubicin	OH	OH	OCH_3	O	H
Daunorubicin	H	OH	OCH_3	O	H
4-Demethoxydaunorubicin	H	OH	H	O	H
11-Deoxydaunorubicin	H	H	OCH_3	O	H
4-Demethyl-6-O-methyldoxorubicin	OH	OH	OH	O	CH_3
6-O-methyldaunorubicin	H	OH	OCH_3	O	CH_3
5-Iminodaunorubicin	H	OH	OCH_3	NH	H



β-anomer doxorubicin

4'-epidoxorubicin (Epirubicin)

FIGURE 1: Chemical structures of the anthracycline derivatives studied.

SV40 DNA, *Ban*I, *Hpa*II, *Acc*I, and *Eco*RI restriction endonucleases, T4 polynucleotide kinase, and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Calf intestine phosphatase and *Xho*II, *Bcl*I, *Apa*I, and *Pfl*MI restriction enzymes were purchased from New England Biolabs (Beverly, MA), and [γ-^{32}P]ATP was from New England Nuclear Research Products (Boston, MA). Topoisomerase II was purified from mouse leukemia L1210 cell nuclei as described previously (Minford et al., 1986).

DNA Cleavage of End-Labeled SV40 DNA. SV40 DNA was uniquely 5'-end-labeled at the *Ban*I or *Acc*I sites as described previously (Pommier et al., 1989). Briefly, SV40 DNA was first linearized with *Ban*I or *Acc*I enzymes; then the 5'-DNA termini were dephosphorylated with calf alkaline phosphatase and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled fragments were then subjected to a second enzyme digestion with *Hpa*II or *Eco*RI in the case of *Ban*I or *Acc*I labeling, respectively. This procedure generates DNA fragments uniquely 5'-end-labeled that can be used to map unequivocally the DNA cleavage sites induced by the drugs. The DNA was purified by phenol–chloroform extraction and ethanol precipitation between each step and at the end of the labeling procedure. The whole restriction mixtures could be used in the topoisomerase reactions because the small fragments were 52 and 154 nucleotides, respectively, and did not interfere in the interpretation of the position of the DNA cleavage sites in agarose gels. End-labeled DNA fragments were reacted with 60 ng of purified topoisomerase II with and without various concentrations of anthracycline derivatives. The reactions were performed in 0.01 M Tris-HCl, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, and 15 μg/mL bovine serum albumin for 20 min at 37 °C, unless otherwise indicated. Purified topoisomerase II was added to each 20-μL reaction volume in 3 μL of storage buffer [40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.4]. Reactions were stopped by adding SDS,[1] EDTA, and proteinase K (final concentrations 1%, 20 mM, and 0.5 mg/mL, respectively) and were incubated for an additional 60 min at 42 °C. Samples were then loaded into 1.2% agarose gels in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8) containing 0.1% SDS in order to remove DNA-bound drug molecules which otherwise retard the electrophoretic migration of DNA fragments. Agarose gels were run at 2 V/cm overnight and then dried. Dried gels were autoradiographed with Kodak XAR-5 films.

In order to evaluate the sequences of cleavage sites induced by anthracyclines, SV40 DNA was first cut with *Xho*II or *Bcl*I restriction endonucleases, 5'-end-labeled as above, and then digested with *Pfl*MI or *Apa*I endonucleases, respectively. The DNA fragment of interest was then isolated and purified by preparative agarose gel electrophoresis, followed by electroelution and ethanol precipitation. DNA sequencing gels were made in 6% polyacrylamide [29:1 acrylamide:bis(acrylamide) ratio] and 7 M urea in TBE buffer. After proteinase K treatment, DNA was ethanol precipitated and resuspended in 2.5 μL of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) and heated at 90 °C for 1–2 min before being loaded into sequencing gels. Electrophoresis was at 1500 V (60 W) for 2–3 h; gels were then dried on a 3MM paper sheet and autoradiographed as above.

Mapping of DNA Breaks. The genomic localization of drug-induced topoisomerase II mediated DNA breaks was determined as described previously (Pommier et al., 1987a, 1989a). Briefly, autoradiography films were scanned with a DU-8B Beckman spectrophotometer set at 555 nm. The densitometer was connected to a computer in order to store, graph, and analyze the data. ^{32}P-End-labeled *Hin*dIII–*Eco*RI-digested λ DNA was usually run as a DNA marker in four lanes per gel to check the uniformity of DNA migration throughout the gel. Regression lines of the logarithm of the fragment size (in base pairs) versus the migration distance of each fragment from a reference line were determined for the DNA markers. Regression coefficients were consistently near 0.99. Each autoradiography lane was analyzed by using the same reference line, and the size of each DNA fragment induced by topoisomerase II was computed (Pommier et al., 1989a). Fragment size determination was usually within 50 bp for a given fragment analyzed in different gels. A final correction was made to determine the genomic position of DNA breaks by taking into account the position of the labeled nucleotide relative to the conventional zero position of the SV40 genome (Fiers et al., 1978).

Quantification of Drug-Induced Cleavage. DNA cleavage at the major cleavage site of SV40 DNA (site V) induced by the studied derivatives was quantified by means of densitometer scanning (DU-8B Beckman spectrophotometer) of the gels shown in Figures 2 and 3. The area of the peak was corrected by considering the background level in each lane, in order to normalize for small differences in sample loadings. DNA cleavage was expressed in arbitrary units, relative to that induced by 1 μM doxorubicin.

Cytotoxicity. Cytotoxic activity of 1-h treatment of anthracyclines was determined on murine P388 leukemia cells

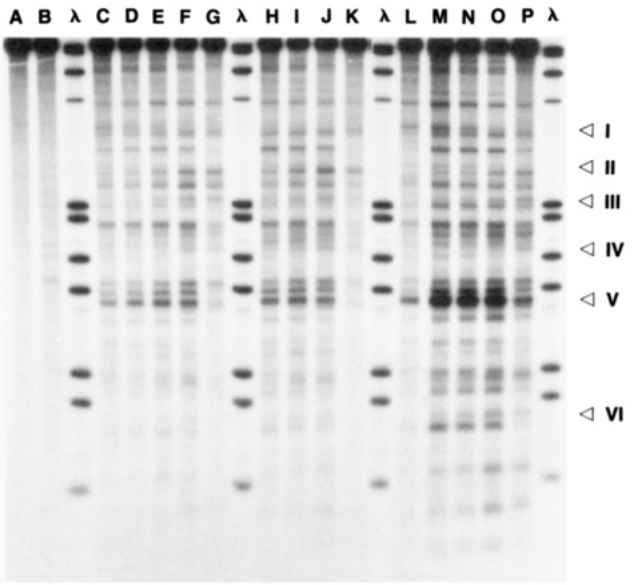


FIGURE 2: DNA cleavage induced by L1210 topoisomerase II in the presence of anthracycline derivatives. *Ban*I–*Hpa*II SV40 DNA fragment 5′-end-labeled at the *Ban*I site was incubated with 60 ng of topoisomerase II and drugs at 37 °C for 20 min. Reactions were stopped with SDS (1%) and EDTA (20 mM) and were digested with proteinase K (0.5 mg/mL) for 1 h at 42 °C. Samples were then loaded into a 1.2% agarose gel, and autoradiography was performed. Lanes λ: λ *Hin*dIII–*Eco*RI markers (size in bp is, from bottom to top, 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, and 5148). Open triangles, marked with Roman numerals, indicate regions of differences in sequence specificity of DNA breakage between drugs or as a function of drug concentration.

by means of an in vitro cell growth inhibition assay (Capranico et al., 1986, 1987).

Results

Induction of Topoisomerase II Mediated DNA Cleavage by Anthracyclines. Figures 2–4 show the relative potency of the anthracycline derivatives in inducing topoisomerase II mediated double-strand breaks in SV40 DNA. First, doxorubicin, 4′-*epi*-doxorubicin (epirubicin), and 4-demethoxydaunorubicin (idarubicin), which are currently used in human cancer chemotherapy, were compared in the *Ban*I–*Hpa*II DNA fragment at drug concentrations ranging from 0.2 to 5 μM (Figure 2). 4-Demethoxydaunorubicin was more effective in inducing DNA cleavage than doxorubicin and 4′-*epi*-doxorubicin, which were equally active. No DNA cleavage was induced by the drugs in the absence of purified topoisomerase II. DNA cleavage was suppressed at the highest concentration (5 μM) used of each drug and was maximum at intermediate drug concentrations (1–2 μM) (Figures 2 and 4). This result is in agreement with the bell-shaped curve for cellular DNA breaks as a function of anthracycline concentration measured by alkaline elution (Capranico et al., 1986, 1987). The patterns of DNA cleavage were similar, although not identical, for the three derivatives and were very different from those induced by topoisomerase II alone (Figure 2, lane B) or in the presence of *m*-AMSA or teniposide (Pommier et al., 1989a; Fesen & Pommier, 1989; see also Figure 8). The pattern of DNA cleavage was concentration dependent: cleavage at region V (Figure 2) occurred at the lowest drug concentration and markedly decreased at the highest drug

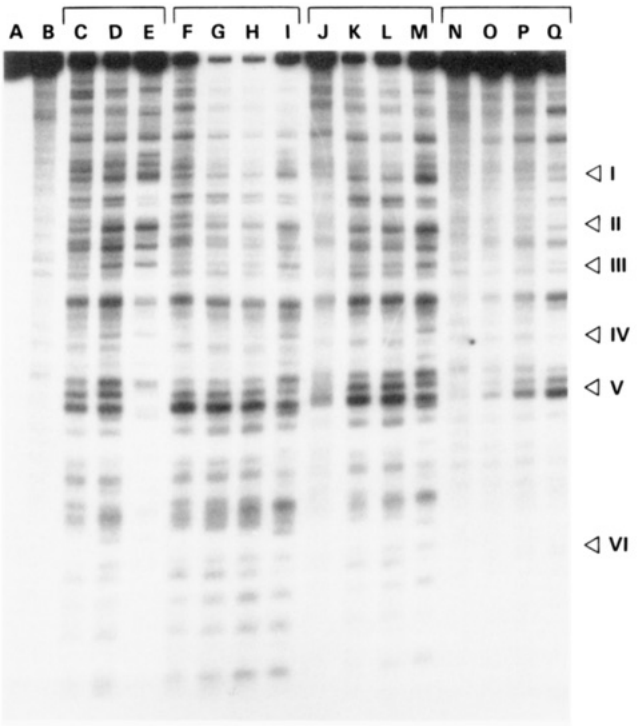


FIGURE 3: DNA cleavage induced by L1210 topoisomerase II in the presence of anthracycline derivatives. *Acc*I–*Eco*RI SV40 DNA fragment 5′-end-labeled at the *Acc*I site was used, and reactions were performed as described in the legend of Figure 2. Open triangles, marked with Roman numerals, indicate regions of differences in sequence specificity of DNA breakage between drugs or as a function of drug concentration.

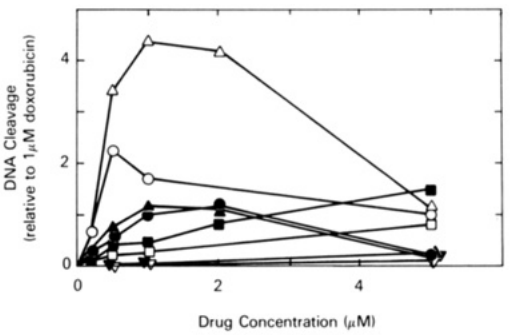


FIGURE 4: Topoisomerase II induced DNA cleavage by anthracyclines. DNA cleavage was determined at the major cleavage site (site V, Figures 2 and 3) by means of densitometer scanning. See Materials and Methods for details. (●) Doxorubicin; (▲) 4′-*epi*-doxorubicin; (■) 4-demethyl-6-*O*-methyldoxorubicin; (▼) doxorubicin β-anomer; (○) daunorubicin; (△) 4-demethoxydaunorubicin; (□) 11-deoxydaunorubicin; (▽) 6-*O*-methyldaunorubicin.

concentrations, whereas cleavage at region II occurred at intermediate drug concentration and persisted at the highest drug concentration. Similarly, cleavage at region I occurred at a site further away from the labeled nucleotide at 0.2 μM drug concentration (lane L) and then at a site closer to the labeled end at higher concentrations (lanes M–P). These findings indicate a sequence-selective action of anthracyclines which changed for each drug as a function of concentration.

Since 4-demethoxydaunorubicin presents two structural modifications (at R_1 and R_3, Figure 1) with respect to doxorubicin, 4-demethoxydaunorubicin and also 11-deoxydau-

norubicin were compared with daunorubicin and doxorubicin (Figure 3). 4-Demethoxydaunorubicin was again the most active agent, and 11-deoxydaunorubicin was less active than the two parent compounds. Daunorubicin was slightly more active than doxorubicin. Suppression of DNA cleavage was observed at a 5 μM concentration of each drug, except for 11-deoxydaunorubicin, which inhibited DNA cleavage only at higher concentrations (40 μM; results not shown). These observations show that removing the methoxy group in the C-4 position increases the ability of the anthracycline to interfere with topoisomerase II or decreases the ability to suppress DNA cleavage, whereas removing the hydroxyl group in the C-11 position has the opposite effect.

The effectiveness of the other derivatives in inducing topoisomerase II mediated DNA cleavage was then analyzed in comparison to doxorubicin (results not shown). 4-Demethyl-6-*O*-methyldoxorubicin was as active as doxorubicin at low concentrations and was more active than doxorubicin at higher concentrations, as no suppression of DNA cleavage was observed. The β-anomer of doxorubicin was much less active, and 6-*O*-methyldaunorubicin was the least active derivative. The drug ability to induce DNA cleavage in the presence of topoisomerase II was quantified by measuring the optical density of the major cleavage site (site V, Figures 2 and 3; and see below) at each drug concentration studied. The results (Figure 4) showed that the studied analogues may be ordered from the most to the least effective in inducing topoisomerase II mediated double-strand breaks in SV40 DNA as follows: 4-demethoxydaunorubicin $\gg$ daunorubicin $>$ doxorubicin = 4′-*epi*-doxorubicin = 4-demthyl-6-*O*-methyldoxorubicin $>$ 11-deoxydaunorubicin $>$ doxorubicin β-anomer $>$ 6-*O*-methyldaunorubicin.

Localization of Anthracycline-Induced Double-Strand Breaks in SV40 DNA. In order to extend the mapping of the topoisomerase II mediated DNA cleavage sites, the DNA cleavage patterns induced by different concentrations of doxorubicin and 4-demethoxydaunorubicin were also studied in *Acc*I–*Eco*RI DNA. They were compared with those induced by 5-iminodaunorubicin (Figure 1), a known inhibitor of topoisomerase II (Tewey et al., 1984) (Figure 5). 5-Iminodaunorubicin-induced DNA cleavage increased steadily with drug concentration (lanes C–F); no suppression could be detected even at 10 μM (data not shown). At both 0.5 and 1 μM, 4-demethoxydaunorubicin was more effective than doxorubicin, which was more active than 5-iminodaunorubicin. For the three drugs, all the cleavage sites were at similar locations, but their relative intensities were different, thus suggesting some difference in sequence selectivity between the three anthracycline derivatives. This is particularly clear when positions VIII–X are compared. In the case of 5-iminodaunorubicin (Figure 5, lanes C–F), two prominent bands are at positions VIII and IX, while in the case of 4-demethoxydaunorubicin (lanes L–P), cleavage is relatively weak at position IX and is strong at positions VIII and X. In addition, DNA cleavage at position VII is common for doxorubicin and 4-demethoxydaunorubicin and is not seen with 5-iminodaunorubicin. Furthermore, the DNA cleavage patterns were affected by drug concentration. In the case of 4-demethoxydaunorubicin (Figure 5, lanes L–P), cleavage at positions VII and XI was only induced at high drug concentrations, while that at positions VI and IX was suppressed in these conditions. A similar dependence upon drug concentration was observed also in the case of doxorubicin (lanes G–K).

DNA cleavage sites induced by topoisomerase II in the presence of anthracyclines were, then, mapped in the SV40

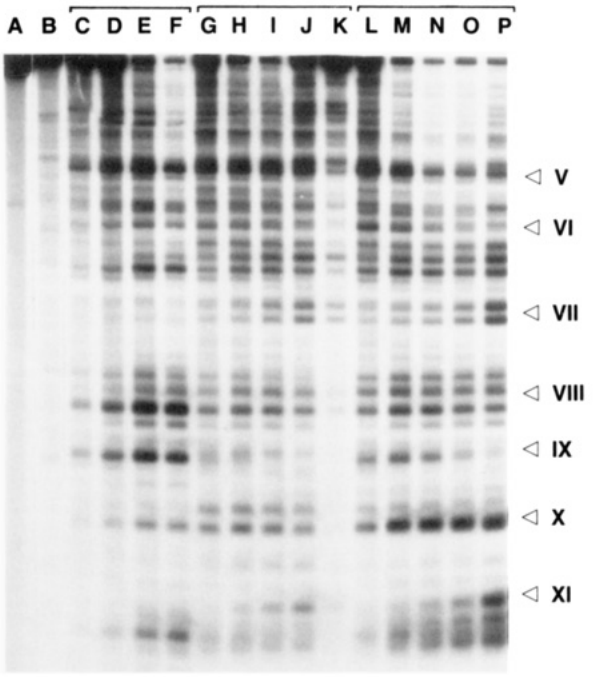


FIGURE 5: Topoisomerase II mediated DNA cleavage patterns induced by anthracycline derivatives. *Acc*I–*Eco*RI SV40 DNA fragment 5′-end-labeled at the *Acc*I site was used, and reactions were performed as described in the legend of Figure 2. Open triangles, marked with Roman numerals, indicate regions of differences in sequence specificity of DNA breakage between drugs or as a function of drug concentration.

genome by means of computer analysis from the autoradiographs shown in Figures 2 and 5. For all the anthracycline derivatives, a major cleavage site (site V, Figures 2, 3, and 5) was observed around position 4240 of the SV40 genome (Figure 6). Two other peaks were detected to the left of the 4240 site, at positions 4095 and 4150 (Figure 6). In addition, the DNA cleavage patterns induced by 1 μM doxorubicin, 4′-*epi*-doxorubicin, and 4-demethoxydaunorubicin were globally similar (Figure 6A) Interestingly, 4-demethoxydaunorubicin (upper curve) increased DNA cleavage disproportionally more at the 4240 site than at sites 4095 and 4150, when compared with the other two derivatives. Thus, 4-demethoxydaunorubicin is more selective for the major cutting site than doxorubicin or 4′-*epi*-doxorubicin. Figure 6B shows the DNA cleavage patterns induced by 1 μM 5-iminodaunorubicin, 0.2 μM doxorubicin, and 0.2 μM 4-demethoxydaunorubicin in *Acc*I–*Eco*RI DNA. Different drug concentrations were examined so as to achieve conditions where no depletion of the full-size DNA fragment was observed (see Figure 5), thereby allowing an accurate comparison of the sequence-specific action of the drugs. As for the other derivatives, although most of the cleavage sites were common to the three drugs, the curves exhibited differences. This is particularly clear for regions VII–X (Figure 6B).

Sequencing of the Major Topoisomerase II Cleavage Site Induced by Anthracyclines in SV40 DNA. The prominent cleavage region detected by agarose gel electrophoresis near base pair 4240 was sequenced by using a short DNA fragment (458 bp) which was 5′-end-labeled at the *Xho*II restriction site (nucleotide 4100 of the Watson strand). This DNA fragment

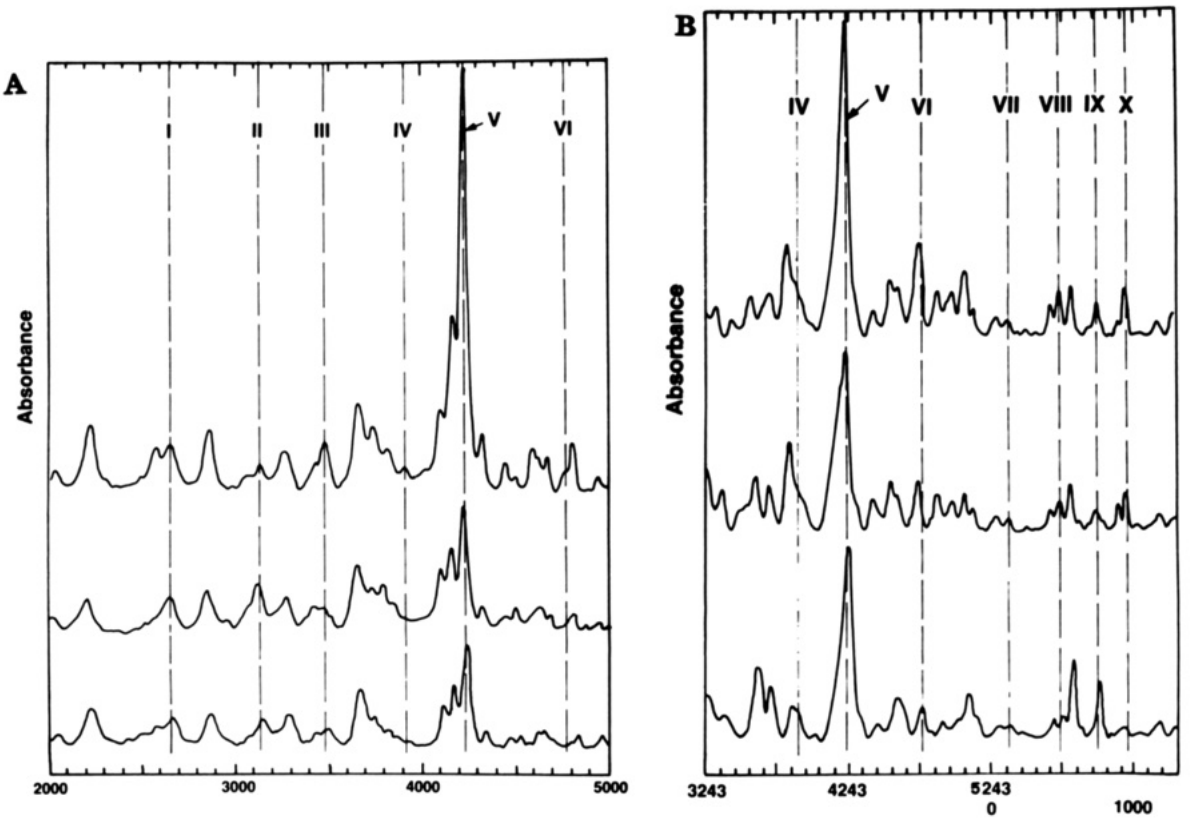


FIGURE 6: Mapping of anthracycline-induced DNA cleavage in the SV40 genome. (A) The lower, middle, and upper curves correspond to the computerized densitometer scannings of lanes E (1 μM doxorubicin), I (1 μM 4′-*epi*-doxorubicin), and N (1 μM 4-demethoxydaunorubicin), respectively, of the autoradiograph shown in Figure 2. (B) The lower, middle, and upper curves correspond to the densitometer scannings of lanes D (1 μM 5-iminodaunorubicin), G (0.2 μM doxorubicin), and L (0.2 μM 4-demethoxydaunorubicin), respectively, of the autoradiograph shown in Figure 4. Roman numerals and dashed lines correspond to the Roman numerals and open triangles in Figures 2–4.

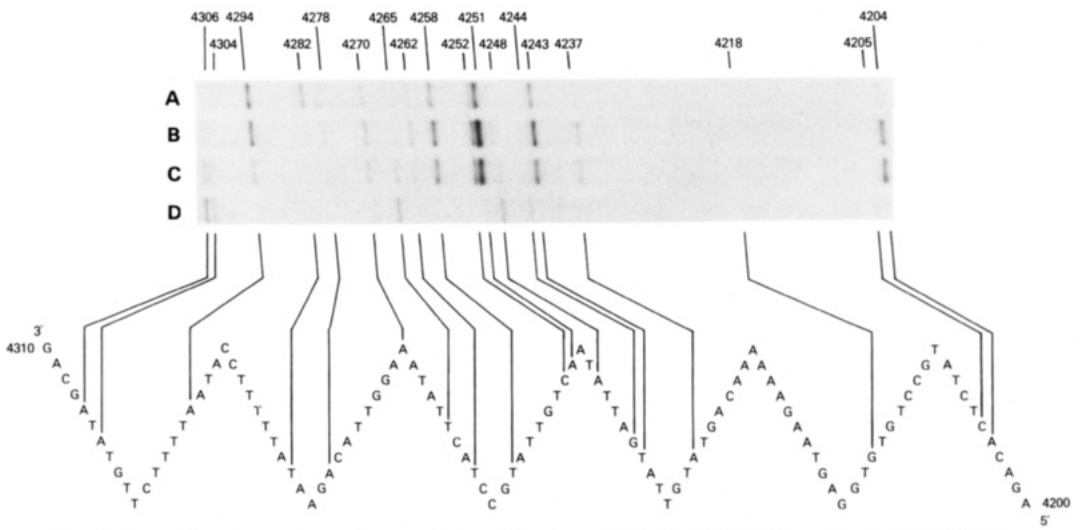


FIGURE 7: Sequencing of the region of prominent cleavage induced by doxorubicin in SV40 DNA. *Xho*II–*Pfl*MI DNA was reacted with 70 ng of L1210 topoisomerase II without (lane D) or with 0.125, 0.5, and 2 μM doxorubicin (lanes C, B, and A, respectively) for 20 min at 37 °C. Reactions were stopped as described in the legend of Figure 2. Samples were run on a 6% polyacrylamide gel with 7 M urea in TBE buffer. Nucleotide positions of DNA cutting sites are shown at the bottom of the figure (Fiers et al., 1978) and were determined by using purine markers run in the same gel.

was incubated with topoisomerase II in the presence of different concentrations of doxorubicin, and the DNA cleavage sites were determined by means of a denaturing 6% polyacrylamide gel (Figure 7). The prominent cleavage band seen in agarose gels appeared actually to be constituted of several cleavage sites between positions 4237 and 4294. The most intense of these sites was at nucleotide 4251. Moreover, the three major cleavage sites induced by the enzyme in the absence of drug at positions 4248, 4265, and 4306 were actually suppressed by doxorubicin. Thus, anthracyclines induce topoisomerase II mediated DNA cleavage at specific sites, which are different from those induced by topoisomerase II alone.

Doxorubicin Suppression of m-AMSA- and VM-26-Induced DNA Cleavage. In order to evaluate the suppression effects of anthracyclines upon topoisomerase II mediated DNA cleavage, a *Bcl*I–*Apa*I fragment (512 bp) of SV40 DNA was incubated with a range of doxorubicin concentrations in the presence of 10 μM *m*-AMSA or VM-26 and analyzed in a sequencing gel (Figure 8). The results show that doxorubicin can suppress cleavage induced by itself, by *m*-AMSA or VM-

Table I: Relationship between Cytotoxicity, DNA Binding Affinity Constant, and Topoisomerase II Induced DNA Cleavage for Anthracycline Derivatives

derivative	cytotoxicity[a]		DNA binding affinity constant[b]		Topo II mediated DNA breaks[c]
	ID_{50} (μM)	rel value[d]	$K_{app} \times 10^{-6}$ (M^{-1})	rel value[e]	
doxorubicin (DOX)	1.03	1	4.2	1	1
4′-*epi*-DOX	0.86	1.2	2.2	0.524	1.17
4-demethyl-6-*O*-methyl-DOX	0.93	1.1	0.20	0.048	0.44
β-anomer of DOX	94.8	0.011	0.015[f]	0.008	0.02
daunorubicin (DNR)	0.53	1.9	4.8	1.14	2.24
4-demethoxy-DNR	0.12	8.6	2.4	0.571	4.38
11-deoxy-DNR	5.84	0.18	0.57	0.136	0.21
6-*O*-methyl-DNR	43.3	0.024	0.035[g]	0.003	0.01

[a] Cytotoxicity was determined by the cell growth inhibition assay after 1-h drug exposure of P388 leukemia cells. The IC_{50} was determined from dose–response curves of at least three independent experiments; standard deviations were less than 15%. [b] DNA binding affinity constants were determined at 20 °C in 0.01 M Tris-HCl, pH 7.0, 0.5 mM Na_2EDTA, and 0.1 M NaCl (Zunino et al., 1979, 1980, 1986; Quadrifoglio et al., 1982). The number of binding sites were similar among all the studied derivatives (range of n_{app} between 0.15 and 0.2). [c] DNA cleavage activity was calculated at 1 μM each derivative as described under Materials and Methods (see also Figure 4) and expressed relative to 1 μM doxorubicin. [d] Calculated by dividing the IC_{50} of doxorubicin by that of the corresponding drug. [e] Calculated by dividing the K_{app} of each drug by that of doxorubicin determined under the same conditions (see also footnotes *e* and *f*). [f] K_{app} reported from Britt et al. (1986) and determined at 30 °C in 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl, pH 7.0; the corresponding value for doxorubicin was 1.8×10^6. [g] K_{app} determined at 0.01 M NaCl instead of 0.1 M NaCl; the corresponding value for doxorubicin was 11.6×10^6 (Quadrifoglio et al., 1982; Zunino et al., 1977).

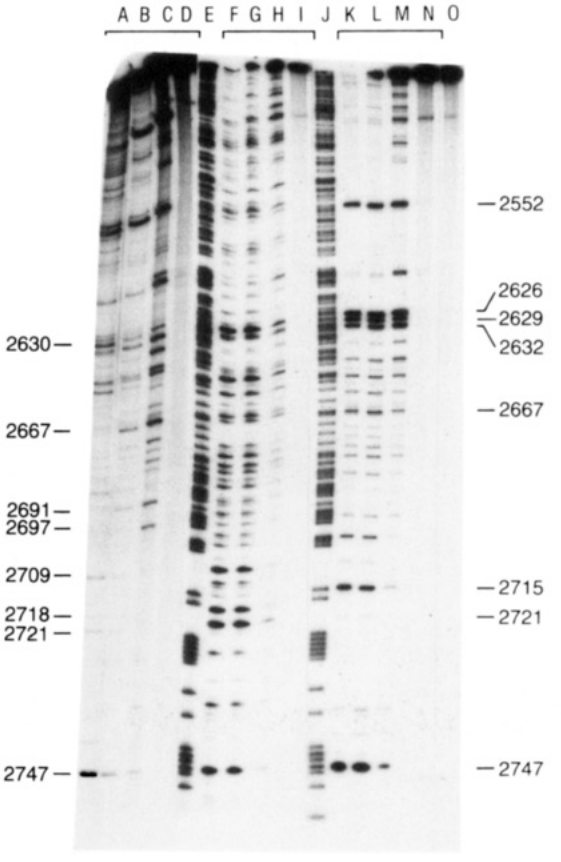
26, and by topoisomerase II alone. Concentrations of doxorubicin as low as 0.1–1 μM stimulated DNA cleavage at certain sites (lanes B and C, Figure 8) while reducing the DNA cleavage at sites induced by topoisomerase II in the absence (lanes A–C, Figure 8) or in the presence of VM-26 or *m*-AMSA (lanes F–H and K–M, Figure 8, respectively). Doxorubicin concentrations above 10 μM suppressed completely topoisomerase II mediated DNA cleavage at any sites regardless of the presence of other drugs (Figure 8 and results not shown).

Cytotoxicity and DNA Binding Affinity of Anthracyclines. The cytotoxic activity on murine P388 leukemia cells and DNA binding affinity constants of the various anthracycline derivatives are shown in Table I in comparison with those of doxorubicin. Drug IC_{50} is reported (Table I) as dose–response curves were similarly shaped. 4-Demethoxydaunorubicin was the most cytotoxic compound; doxorubicin, 4′-*epi*-doxorubicin, and 4-demethyl-6-*O*-methyldoxorubicin were equally cytotoxic; daunorubicin was slightly more active than doxorubicin; 11-deoxydaunorubicin was less cytotoxic than doxorubicin, and 6-*O*-methyldaunorubicin and the β-anomer of doxorubicin were almost inactive. In order to compare these results with the DNA cleavage activity, the intensity of DNA cleavage induced by a 1 μM sample of each derivative (Figure 4) at the major cleavage site has been reported in Table I. These results show that the cytotoxic potency of anthracyclines correlated well with the induction of topoisomerase II mediated DNA breaks (Table I).

DNA binding affinity constants were very different among the studied drugs, ranging from 4.8×10^6 M^{-1} for daunorubicin to 1.5×10^4 M^{-1} for 6-*O*-methyldaunorubicin (Table I), which has been reported not to intercalate into DNA (Quadrifoglio et al., 1982). As expected (Capranico et al., 1986, 1989b), the DNA binding affinity constants were not quantitatively correlated with cytotoxicity or DNA cleavage activity.

Discussion

The molecular basis of the induction of topoisomerase II mediated DNA cleavage by antitumor DNA intercalators is still unknown. Anthracyclines appear to be highly DNA sequence-selective when compared to *m*-AMSA or demethylepipodophyllotoxins since they induce a prominent cleavage in a 60 bp region of SV40 DNA [Figures 2–4 and Pommier et al. (1989a) and Fesen and Pommier, (1989)]. The cleavage



A-D: 0, 0.1, 1 and 10 μM Doxorubicin
F-I: VM-26 (10 μM) + Doxorubicin
K-N: *m*-AMSA (10 μM) + Doxorubicin
O :Control (Bcl I/Apa I SV40 DNA)

FIGURE 8: Effects of doxorubicin upon VM-26- and *m*-AMSA-induced DNA cleavage. *Bcl*I–*Apa*I DNA (lane O, control DNA) was reacted for 20 min at 37 °C with 70 ng of topoisomerase II without doxorubicin (lanes A, F, and K) or with 0.1 (lanes B, G, and L), 1 (lanes C, H, and M) and 10 μM (lanes D, I, and N) doxorubicin; in addition, 10 μM VM-26 was present in the samples of lanes F–I and 10 μM *m*-AMSA in lanes K–N. Lanes E and J are purine markers. Samples were processed as described in Figure 7. Lanes A–D are from a more exposed autoradiography than lanes E–O. Nucleotide positions of some DNA cutting sites are indicated.

sites induced by anthracyclines never corresponded to an enhancement of sites of enzyme-mediated DNA cleavage in the absence of drug (Figures 7 and 8). It is remarkable that the

DNA cleavage sites were located at similar positions for all the anthracycline derivatives studied. However, derivatives not only differed by their potency but also exhibited some differences in DNA cleavage patterns. In fact, for a given derivative, the DNA cleavage pattern varied markedly with drug concentration.

DNA cleavage induced by anthracyclines at any site is the result of a balance between induction and suppression of topoisomerase II mediated DNA cleavage (Figures 7 and 8). In addition, for a given drug, different sites of cleavage exhibited different concentration dependence, which indicates that the balance between induction and suppression of cleavage is DNA sequence-specific (Figures 2, 3, and 5).

Induction of topoisomerase II mediated cleavage requires DNA binding of the anthracyclines because the two drugs with the lowest DNA binding affinity, the β-anomer of doxorubicin and 6-*O*-methyldaunorubicin, were inactive, while the strong DNA binders, doxorubicin, daunorubicin, 4-demethoxydaunorubicin, and 4′-*epi*-doxorubicin, were the most active inducers of topoisomerase II cleavage (Table I). However, DNA binding of the drugs is not the only determinant for activity since there is no quantitative correlation between DNA binding affinity constants and potency in inducing topoisomerase II mediated DNA breaks (Table I). Therefore, the ability of the drug to intercalate seems required but is not sufficient for the induction of topoisomerase II DNA cleavage. This conclusion is in good agreement with previous findings for anthracycline-induced cellular DNA breaks (Capranico et al., 1986, 1989b) and for amsacrine derivatives (Pommier et al., 1987a,b; Rowe et al., 1986).

Suppression of topoisomerase II mediated DNA cleavage results from drug intercalation. The two anthracycline derivatives, 4-demethyl-6-*O*-methyldoxorubicin and 5-iminodaunorubicin, which showed no suppression of DNA cleavage at the highest drug concentration used (10 μM), had the lowest DNA binding activity among the active compounds (Table I; Tong et al., 1979). Moreover, doxorubicin suppressed DNA cleavage induced by structurally unrelated drugs (*m*-AMSA and VM-26, Figure 8), thus suggesting that anthracycline intercalation may prevent the interference of any drug with topoisomerase II and DNA. Therefore, DNA intercalators can have two different types of effects upon topoisomerase II mediated DNA breaks: (1) induction of DNA breaks which occurs at low levels of DNA intercalation, and (2) suppression of the enzyme-induced cleavage which is a direct function of drug intercalation. These two effects can be dissociated (Pommier & Kohn, 1989b). Some drugs exhibit both induction of DNA breaks at low concentrations and suppression of these breaks at higher concentrations: such is the case for several of the anthracyclines studied (present results; Tewey et al., 1984; Capranico et al., 1986, 1987) and of ellipticines (Pommier et al., 1987a; Tewey et al., 1984). Some drugs with low DNA binding affinities exhibit only the induction of DNA cleavage: such is the case of two of the anthracyclines studied (5-iminodaunorubicin and 4-demethyl-6-*O*-methyldoxorubicin) and of amsacrine (Pommier et al., 1987a,b). Some drugs exhibit only the suppression of topoisomerase II mediated DNA cleavage: such is the case of ethidium bromide (Tewey et al., 1984) and ditercalinium, a bifunctional intercalator (Markovits et al., 1986), and 9-aminoacridine (Pommier et al., 1987a).

Because the strength of DNA intercalation of the anthracyclines is not well correlated with induction of topoisomerase II mediated DNA cleavage (Table I), it is interesting to analyze further the structure–activity relationships among these derivatives. The most striking result is that removal of the C-4 methyl group of the D ring (Figure 1) greatly enhanced topoisomerase II inhibition, as the 4-demethoxy and 4-demethyl analogues, while having similar or lower DNA binding constants than doxorubicin and daunorubicin, were more active than the parent compounds (Figures 2 and 3; Capranico et al., 1986, 1989b). In addition, removing the hydroxyl group in C-11 or methylation in C-6 in the B ring markedly decreased both DNA binding affinity and DNA breakage activities (Figure 3 and Table I). Therefore, the anthracycline chromophore appears to be involved in the molecular interactions of the drug with topoisomerase II–DNA complexes (Capranico et al., 1986). Since modifications in the chromophore moiety of the anthracycline molecule are expected to affect the mode of drug intercalation into the DNA (Quadrifoglio et al., 1982; Wang et al., 1987), it seems likely that the intercalation geometry plays a crucial role for DNA–drug–topoisomerase II interactions. Current modeling of anthracycline intercalation indicates that the drug chromophore lays between two adjacent base pairs while the sugar and the A ring are situated in the minor groove of the double helix (Quigley et al., 1980; Wang et al., 1987). Possibly, alterations in the geometry of intercalation by the chromophore may affect the interactions of the A ring and its sugar with topoisomerase II. Interestingly, epimerization of the sugar had marginal effects when at the 4′-position and markedly decreased cytotoxicity and DNA binding when at the 1′-position (β-anomer of doxorubicin) (Table I). The β-anomer of doxorubicin also appeared to be weak in inducing DNA breaks, suggesting that a precise orientation of the sugar relative to the chromophore is required for DNA binding as well as for induction of topoisomerase II DNA cleavage (Table I).

Among the analogues whose DNA cleavage activity has been analyzed previously in cultured cells (doxorubicin, 4-demethoxydaunorubicin, 11-deoxydaunorubicin, and 4-demethyl-6-*O*-methyldoxorubicin) (Capranico et al., 1986, 1987), a good agreement is found between the relative effectiveness of the drugs in inducing protein-linked DNA breaks in cells and isolated nuclei and their relative activities in inducing DNA breaks with purified topoisomerase II. The results reported in this paper demonstrate a good correlation between the cytotoxicity of the anthracycline analogues and the induction of DNA double-strand breaks in the presence of purified topoisomerase II (Table I). However, a discrepancy is apparent between DNA cleavage and cytotoxicity, as cytotoxicity curves are not biphasic. It has to be pointed out that the time courses of cellular DNA cleavage after drug removal from the culture medium can be very different at different drug concentrations. DNA single-strand breaks induced by 4-demethoxydaunorubicin in cultured P388 leukemia cells resealed after drug removal at low drug concentrations, while cleavage persisted and even increased at high drug concentrations (Capranico et al., 1989a). Similar results have been found with an ellipticine derivative (Pierson et al., 1988). Thus, the simple correlation between cytotoxicity and cellular DNA cleavage determined at a fixed time point is not appropriate in the case of strong DNA binders.

Previous reports have already documented a correlation between cytotoxicity and DNA cleavage for acridine derivatives (Pommier et al., 1987a; Covey et al., 1988) and epipodophyllotoxin congeners (Ross et al., 1984; Long et al., 1984). Therefore, it is likely that topoisomerase II is the cellular target of different classes of antitumor drugs, including anthracycline antibiotics. In agreement with this possibility is the present finding that the drugs currently used in human

cancer chemotherapy [doxorubicin (adriamycin), 4′-*epi*-doxorubicin (epirubicin), daunorubicin (daunomycin), and 4-demethoxydaunorubicin (idarubicin)] were the most potent inducers of topoisomerase II DNA cleavage. Attempts to find a precise correlation between cytotoxicity and anthracycline-induced single-stranded cleavage of cellular DNA were in the past years not successful (Zwelling et al., 1982; Capranico et al., 1986). This can be explained by (1) differences in cellular pharmacokinetics (Zwelling et al., 1982; Capranico et al., 1986, 1987, 1989a), (2) differential production of DNA single- and double-strand breaks (Pommier et al., 1985a; Zwelling et al., 1982), and (3) bell-shaped curves of DNA breaks vs drug concentration, such as in the case of some anthracycline or ellipticine analogues (Capranico et al., 1986, 1987, 1989a; Pierson et al., 1988). Indeed, when selected anthracycline derivatives, with identical cellular pharmacokinetics, similar DNA affinity constants, and similar ratios of single- and double-strand DNA breaks, were studied, a good correlation was also observed between drug-induced cellular DNA cleavage and cell killing effects (Capranico et al., 1989b).

Registry No. Doxorubicin, 23214-92-8; daunorubicin, 20830-81-3; 4-demethoxydaunorubicin, 58957-92-9; 11-deoxydaunorubicin, 84325-15-5; 4-demethyl-6-*O*-methyldoxorubicin, 97777-78-1; 6-*O*-methyldaunorubicin, 78008-04-5; 5-iminodaunorubicin, 72983-78-9; β-anomer doxorubicin, 57819-79-1; 4′-*epi*-doxorubicin, 56420-45-2; DNA topoisomerase, 80449-01-0.

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