calculations predict that the C6–N6 bond is shortened and the positive charge is delocalized over the entire adenine molecule.

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REFERENCES

Baker, B. F., & Dervan, P. B. (1989) *J. Am. Chem. Soc. 111*, 2700–2712.
Chidester, C. G., Krueger, W. C., Mizak, S. A., Duchamp, D. J., & Martin, D. G. (1981) *J. Am. Chem. Soc. 103*, 7629–7635.
Dewar, M. J. S., Zoebisch, E. G., Healy, E. F., & Stewart, J. J. P. (1985) *J. Am. Chem. Soc. 107* 3902–3909.
Fujii, T., Saito, T., & Date, T. (1989) *Chem. Pharm. Bull. 37*, 1208–1212.
Gao, X., & Jones, R. A. (1987) *J. Am. Chem. Soc. 109*, 1275–1278.
Hanka, L. J., Dietz, A., Gerpheide, S. A., Kuentzil, S. L., & Martin, D. G. (1978) *J. Antibiot. 31*, 1211–1217.
Hore, P. J. (1983) *J. Magn. Reson. 55*, 283–300.
Hurley, L. H., & Rokem, J. S. (1983) *J. Antibiot. 36*, 383–390.
Hurley, L. H., Reynolds, B. L., Swenson, D. H., & Scahill, T. (1984) *Science 226*, 843–844.
Hurley, L. H., Lee, C.-S., McGovren, J. P., Mitchell, M., Warpehoski, M. A., Kelley, R. C., & Aristoff, P. A. (1988) *Biochemistry 27*, 3886–3892.
Hurley, L. H., Warpehoski, M. A., Lee, C.-S., McGovern, J. P., Scahill, T. A., Kelly, R. C., Mitchell, M. A., Wicnienski, N. A., Gebhard, I., Johnson, P. D., & Bradford, V. S. (1990) *J. Am. Chem. Soc. 112*, 4633–4649.
Lin, C. H., & Hurley, L. H. (1990) *Science* (submitted for publication).
Martin, G. J., Martin, M. L., & Gouesnard, J. P. (1981) *^{15}N-NMR Spectroscopy*, Chapters 6 and 7, pp 75–326, Springer-Verlag, Berlin, Heidelberg, and New York.
McGovren, J. P., Clarke, G. L., Pratt, E. A., & Deckoning, T. F. (1984) *J. Antibiot. 37*, 63–70.
Reynolds, V. L., Molineux, I. J., Kaplan, D., Swenson, D. H., & Hurley, L. H. (1985) *Biochemistry 24*, 6228–6237.
Scahill, T. A., Jensen, R. M., Swenson, D. H., Hatzenbuhler, N. T., Petzold, G, Wierenga, W., & Brahme, N. D. (1990) *Biochemistry 29*, 2852–2860.
Swenson, D. H., Li, L. H., Hurley, L. H., Rokem, J. S., Petzold, G. L., Dayton, B. D., Wallace, T. L., Lin, A. H., & Krueger, W. C. (1982) *Cancer Res. 42*, 2821–2828.
Warpehoski, M. A., & Hurley, L. H. (1988) *Chem. Res. Toxicol. 1*, 315–333.
Zakrzewska, K., Randrianarivelo, M., & Pullman, B. (1987) *Nucleic Acids Res. 15*, 5775–5785.

Articles

Stimulation of Topoisomerase II Mediated DNA Cleavage at Specific Sequence Elements by the 2-Nitroimidazole Ro 15-0216†

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ABSTRACT: The effect of the 2-nitroimidazole Ro 15-0216 upon the interaction between purified topoisomerase II and its DNA substrate was investigated. The cleavage reaction in the presence of this DNA-nonintercalative drug took place with the hallmarks of a regular topoisomerase II mediated cleavage reaction, including covalent linkage of the enzyme to the cleaved DNA. In the presence of Ro 15-0216, topoisomerase II mediated cleavage was extensively stimulated at major cleavage sites of which only one existed in the 4363 base pair pBR322 molecule. The sites stimulated by Ro 15-0216 shared a pronounced sequence homology, indicating that a specific nucleotide sequence is crucial for the action of this drug. The effect of Ro 15-0216 thus differs from that of the clinically important topoisomerase II targeted agents such as mAMSA, VM26, and VP16, which enhance enzyme-mediated cleavage at a multiple number of sites. In contrast to the previous described drugs, Ro 15-0216 did not exert any inhibitory effect on the enzyme's catalytic activity. This observation might be ascribed to the low stability of the cleavage complexes formed in the presence of Ro 15-0216 as compared to the stability of the ones formed in the presence of traditional topoisomerase II targeted drugs.

Eukaryotic topoisomerase II is an essential nuclear enzyme that is involved in central processes concerning nucleic acid metabolism, including replication (Nelson et al., 1986; Brill et al., 1987; Yang et al., 1987), transcription (Brill et al., 1987; Glikin & Blangy, 1986; Rowe et al., 1986), and chromosome segregation (DiNardo et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985). The important physiological properties of the enzyme are fundamentally embodied in its ability to catalyze a double-stranded DNA passage reaction. The catalytic process has been subject to extensive studies, and it has been elucidated that the reaction can be divided into

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discrete steps. These include noncovalent binding of the enzyme to its DNA substrate, transient double-stranded breakage of the DNA backbone, double-stranded DNA passage, religation of the cleaved DNA, and turnover of the enzyme (Wang, 1985; Vosberg, 1985; Maxwell & Gellert, 1986; Osheroff, 1989a). Upon production of double-stranded breaks, a DNA–protein intermediate occurs where one subunit of topoisomerase II is covalently attached to each of the 5′-phosphoryl ends of the cleaved DNA (Sander & Hsieh, 1983; Liu et al., 1983; Rowe et al., 1986; Osheroff & Zechiedrich, 1987; Zechiedrich et al., 1989). Upon addition of a strong protein denaturant, these topoisomerase II–DNA intermediates, designated cleavable complexes, can be isolated and utilized to define the sites of interaction between topoisomerase II and its DNA substrate (Udvardy et al., 1986; Sander et al., 1987). The result of such studies has clearly shown that topoisomerase II is associated with specific preferred sites in DNA (Wang, 1985).

Besides the normal cellular functions, topoisomerase II has been shown to act as the primary intracellular target for various classes of clinically important antineoplastic agents (Glisson & Ross, 1987; Bodley & Liu, 1988; Liu, 1989; D'Arpa & Liu, 1989). It is currently believed that chemotherapeutics directed against topoisomerase II act by shifting the cleavage/religation equilibrium toward cleavage by preventing religation of the broken DNA strands and/or by enhancement of the forward rate of cleavage (Osheroff, 1989b). Characteristically, this effect is exerted at a multiple number of topoisomerase II–DNA interaction sites which show no apparent sequence homology (Tewey et al., 1984; Chen et al., 1984). The interference causes profound physiological disorders, ultimately leading to cell death.

Recently, a number of trypanosidals have been shown to interact with topoisomerase II from the kinetoplast (Shapiro & Englund, 1990). In the present paper we characterize the in vitro effect of a 5-substituted 2-nitroimidazole, Ro 15-0216,[1] upon the interaction of mammalian topoisomerase II with DNA. Ro 15-0216 is a newly developed trypanosidal that has been shown to be active against human and animal pathogenic African trypanosomes (Borowy et al., 1988). Further Ro 15-0216 has proven useful in combination chemotherapy of trypanosomiasis in pigs caused by *Trypanosoma* (*Nannomonas*) *simiae* (Zweygarth & Röttcher, 1987). We demonstrate that Ro 15-0216 targets mammalian DNA topoisomerase II and enhances enzyme-mediated DNA cleavage at specific DNA sequence elements. The observations raise possibilities for design of drugs that target specific regulatory DNA sequences on the genome.

Experimental Procedures

Isolation of DNA Restriction Fragments, End Labeling, and Sequence Analysis. A *Bam*HI–*Hin*dIII DNA fragment was isolated from the central spacer of the rDNA molecule from *Tetrahymena thermophila* and double end labeled at the 5′-ends according to Andersen et al. (1989). Alternatively, the rDNA fragment was 3′ double end labeled with [α-^{32}P]dATP and DNA polymerase I, Klenow fragment (Boehringer). Each of the unlabeled nucleotides dCTP, dGTP, and dTTP was present in a concentration of 100 μM. Plasmid pBR322 was digested with *Sty*I (Biolabs), and the protruding 5′-ends were dephosphorylated by treatment with alkaline phosphatase (Boehringer). The dephosphorylated plasmid was 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). After labeling, the DNA was digested with *Ava*I, resulting in a 4307-bp single end labeled *Ava*I–*Sty*I fragment. Sequencing reactions were performed according to the procedure of Bencini et al. (1984).

Topoisomerase II Mediated DNA Cleavage Reactions. Calf thymus topoisomerase II was purified according to Andersen et al. (1989).

DNA (20 pmol) was incubated with 100 units of purified calf thymus topoisomerase II in a 20-μL reaction volume of 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.2 mM DTT, 2.5% (v/v) glycerol, and 5% (v/v) DMSO. The topoisomerase II mediated cleavage reaction was performed in both the absence and presence of drugs as indicated. Following incubation for 5 min at 30 °C, topoisomerase II mediated cleavage was terminated by addition of SDS to 1%. In control reactions 0.8 M NaCl was added prior to SDS. The samples were digested for 30 min with 250 μg/mL proteinase K (Merck) at 37 °C before addition of 1 volume of deionized formamide, 0.05% bromophenol blue, 0.03% xylene cyanole, and 5 mM EDTA, pH 8.5, and the samples were analyzed on denaturing polyacrylamide gels. When DNA was analyzed on native gels, the formamide was replaced by 30% glycerol. Alternatively, $^1/_5$ volume of 5 mM EDTA, 30% glycerol, and 0.2% bromophenol blue was added, and the samples were analyzed on agarose gels. The topoisomerase II directed drugs employed were mAMSA provided by Parke-Davis, VP16 and VM26 provided by Bristol Meyer, and Ro 15-0216 [molecular weight 369.8; mp approximately 200 °C; solubility 500 mg/mL in water (at room temperature)] provided by Hoffman-La Roche.

Gel Electrophoresis. The products of topoisomerase II mediated cleavage of pBR322 DNA were analyed by electrophoresis on a 1% agarose gel. Electrophoresis was performed in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA at 5 V/cm, and the gel was dried under vacuum before autoradiography.

Products generated from topoisomerase II mediated cleavage of the rDNA fragment were analyzed by electrophoresis on denaturing polyacrylamide gels [6% (w/v) acrylamide/0.3% (w/v) *N*,*N*′-methylenebis(acrylamide) and 8 M urea] at 40 V/cm in TBE (90 mM Tris base, 90 mM boric acid, and 2 mM EDTA, pH 8.3). Alternatively, electrophoresis was performed in native polyacrylamide gels [4% (w/v) acrylamide/0.14% (w/v) *N*,*N*′-methylenebis(acrylamide)] at 15 V/cm in TBE.

Topoisomerase II mediated cleavage products were visualized by autoradiography with Fuji RX films.

Preparation of Interphase Material. Topoisomerase II mediated DNA cleavage products were extracted with 1 volume of phenol–chloroform–isoamyl alcohol (25:24:1), and waterphase material was precipitated with ethanol. Interphase material was washed once with 0.6 M NaCl and ethanol precipitated. Waterphase and interphase material were redissolved, proteinase K treated (250 μg/mL, 30 min at 37 °C), and finally examined on a denaturing polyacrylamide gel.

Elution of DNA Fragments from Native Polyacrylamide Gels. The cleavage products of interest were localized by autoradiography and excised from the gel. DNA was extracted by grinding the gel pieces in 100 μL of 1 mM Tris-HCl (pH 8) and 0.1 mM EDTA followed by heating to 60 °C for 12

[1] Abbreviations: bp, base pair(s); BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; mAMSA, amsacrine [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide]; rDNA, ribosomal DNA; Ro 15-0216, 2-(dimethylamino)-4′-[(1-methyl-2-nitroimidazol-5-yl)methoxy]acetanilide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane; VM26, teniposide [4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside]; VP16, etoposide [demethylepipodophyllotoxin ethylidene-β-D-glucoside].

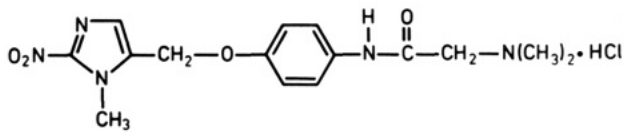

FIGURE 1: Chemical structure of Ro 15-0216.

h. Gel pieces were removed by centrifugation, and the volume of the DNA samples was reduced to approximately 5 μL under reduced pressure. Finally, samples were loaded onto denaturing polyacrylamide gels.

Topoisomerase II Mediated Relaxation of Supercoiled DNA. Relaxation was performed in a 160-μL reaction mixture containing 2.4 μg of pBR322, 10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, 1 mM ATP, 16 units of purified topoisomerase II, and drug as indicated. At different time points, aliquots were taken, and the relaxation was stopped by addition of 4 μL of loading buffer (5% SDS, 30% glycerol, 0.25% bromophenol blue). Samples were run in 1% agarose gels at 8 V/cm for 2 h, and after being stained with 1 μg/mL ethidium bromide for 20 min, the DNA was visualized under UV light.

Quantitation of Topoisomerase II Mediated DNA Cleavage. The level of topoisomerase II mediated cleavage was measured by densitometric scanning of autoradiograms in a CS930 Shimadzu chromatoscanner.

Measurement of the Cleavable Complex Stability. Purified topoisomerase II (500 units) was incubated with the labeled DNA fragment (100 pmol) in the absence or presence of drugs in 100 μL of 10 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.2 mM DTT, 2.5% (v/v) glycerol, and 5% (v/v) DMSO. After 5 min of incubation the reaction was cooled on ice for 4 min, and NaCl was added to 0.4 M. Religation was studied by taking aliquots at different time points and adding SDS to 1%. The samples were finally treated with proteinase K (250 μg/mL, 30 min at 37 °C) and analyzed by electrophoresis on 6% denaturing polyacrylamide gels.

Unwinding Measurements. The unwinding measurements were performed according to Pommier et al. (1985). Relaxed pBR322 plasmid was obtained by reacting supercoiled plasmid with topoisomerase I, followed by extraction with 1 volume of phenol–chloroform–isoamyl alcohol (25:24:1) and ethanol precipitation. Each unwinding reaction (20 μL) contained 300 ng of relaxed pBR322 DNA, 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/mL BSA, and drugs as indicated. Following 5 min of incubation, 20 ng of topoisomerase I was added, and relaxation was performed at 37 °C for 30 min. Finally, the samples were extracted with phenol, ethanol precipitated, and electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide (1 μg/mL), for 10 min, followed by visualization under UV light.

Results

Ro 15-0216 Induces Site-Specific Topoisomerase II Mediated DNA Cleavage. A broad variety of topoisomerase II directed antineoplastics interfere with the breakage and rejoining reaction of the type II topoisomerase, giving rise to enzyme-mediated DNA fragmentation of the genome. We have investigated the effect of the DNA-nonintercalative 5-substituted 2-nitroimidazole Ro 15-0216 (Figure 1) upon the interaction of calf thymus topoisomerase II with DNA. For these studies the entire 4363-bp pBR322 DNA molecule was used as a substrate. Cleaved enzyme–DNA complexes were trapped by addition of SDS, digested with proteinase K, and analyzed by agarose gel electrophoresis (Figure 2A). The results revealed that Ro 15-0216 specifically and extensively

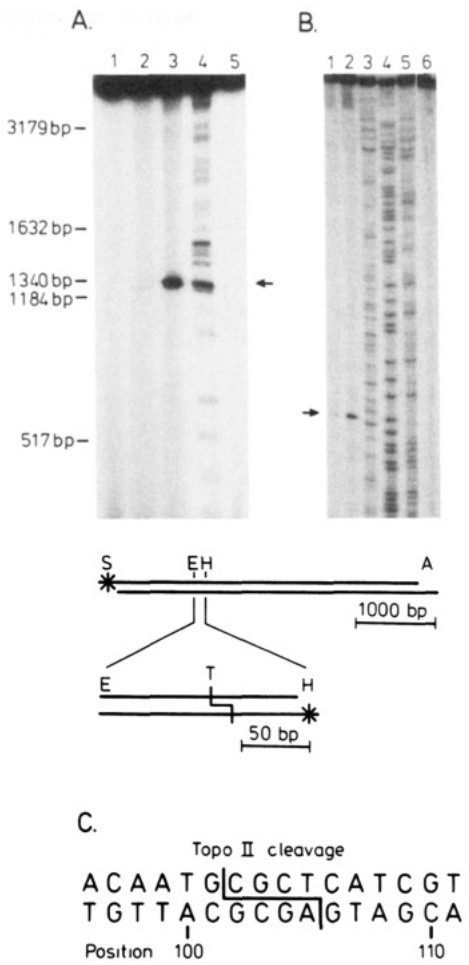

FIGURE 2: Ro 15-0216 stimulates topoisomerase II mediated double-stranded cleavage at a single site in the pBR322 molecule. (A) Calf thymus topoisomerase II mediated cleavage was carried out as described under Experimental Procedures, and reaction products were analyzed on a 1% agarose gel. Lane 1, single end labeled 4307-bp *Ava*I–*Sty*I fragment from plasmid pBR322; lane 2, topoisomerase II mediated cleavage of the pBR322 fragment performed in the absence of drug; lanes 3 and 4, topoisomerase II mediated cleavage of the pBR322 fragment performed in the presence of 1 mM Ro 15-0216 and 100 μM mAMSA, respectively; lane 5, single end labeled pBR322 fragment incubated in the presence of Ro 15-0216 without enzyme. The numbers at the left give the sizes of marker DNA in base pairs. (B) Determination of the nucleotide sequence at the Ro 15-0216 stimulated cleavage site. Topoisomerase II cleavage was performed on a 185-bp *Hin*dIII–*Eco*RV fragment from pBR322 labeled at the protruding 5′-end of the *Hin*dIII site. Lanes 1 and 2, topoisomerase II mediated cleavage of the fragment performed in the absence and presence of 1 mM Ro 15-0216, respectively; lanes 3–5, the TC, AG, and AC reactions of the Maxam–Gilbert degradations, respectively; lane 6 labeled *Hin*dIII–*Eco*RV fragment from pBR322. Arrows denote the position of the Ro 15-0216 stimulated topoisomerase II cleavage site. Schematic representations of the labeled DNA substrates employed are shown. The asterisks (*) denote the labeled 5′-ends, and the cleavage positions of topoisomerase II (T), *Sty*I (S), *Eco*RV (E), *Hin*dIII (H), and *Ava*I (A) are indicated. (C) Nucleotide sequence of the Ro 15-0216 stimulated topoisomerase II mediated cleavage site in pBR322.

stimulated topoisomerase II mediated DNA cleavage at a single site on the plasmid (Figure 2A, lane 3). The effect obtained by Ro 15-0216 was distinct from the effect exerted by the traditional topoisomerase II directed antitumor drug mAMSA. This agent was found to be considerably less specific and induced enzyme-mediated cleavage at multiple DNA sequences (Figure 2A, lane 4).

To determine the nucleotide sequence of the specific DNA cleavage site stimulated by Ro 15-0216, the 185-bp *Hin-*

dIII–*Eco*RV fragment from pBR322 encompassing this site was isolated. The fragment was labeled at the protruding 5′-end of the *Hin*dIII site and employed as substrate for the topoisomerase II mediated DNA cleavage reaction. The cleavage products obtained in the absence and presence of Ro 15-0216 were electrophoresed on a denaturing polyacrylamide gel (Figure 2B, lane 1 and 2, respectively) in parallel with Maxam–Gilbert degradation reactions (Figure 2B, lanes 3–5). A major site of topoisomerase II mediated DNA cleavage appeared (Figure 2B, lane 1), and addition of 1 mM Ro 15-0216 stimulated cleavage at this site (lane 2). The nucleotide sequence of the site is depicted in Figure 2C. Cleavage takes place between the G and C residues at position 101 and 102, respectively. As topoisomerase II cleaves DNA with a 4-bp stagger, the cleavage on the opposite strand is located between A and G at position 105 and 106, respectively (Figure 2C). The site has previously been identified as a major site for topoisomerase II mediated DNA cleavage (Sander & Hsieh, 1983).

Investigations using the extrachromosomal rDNA molecule of the eukaryotic organism *T. thermophila* revealed that a major topoisomerase II cleavage site located near the origin of replication was specifically and extensively induced by the drug. To study the effect of Ro 15-0216 in further detail on this substrate, a 365-bp fragment containing this major cleavage site was labeled at both protruding 5′-ends as shown in Figure 3A (bottom) and employed as substrate for the topoisomerase II mediated cleavage reaction. Cleavage products were obtained as described under Experimental Procedures and analyzed by electrophoresis on a denaturing polyacrylamide gel (Figure 3A). Only one prominent site of topoisomerase II mediated DNA cleavage was observed in the absence of drugs (Figure 3A, lane 3), which on the noncoding strand coincided with an *Sph*I restriction site (lane 2). Due to the double end labeling of the fragment, the specific topoisomerase II mediated DNA cleavage resulted in two bands. The unequal density of the two bands was caused by topoisomerase II mediated single-stranded cleavage taking place at the same sequence (Andersen et al., 1989). Relative differences in the intensities of the two bands resulting from topoisomerase II mediated DNA cleavage reflect differences in the levels of cleavage taking place at each of the two strands, as a *Dra*I digestion of the labeled fragment (Figure 3A, lane 12) demonstrated that the ends were labeled to the same extent. The presence of Ro 15-0216 resulted in an approximate 20-fold increase in the formation of topoisomerase II generated cleavable complexes. As observed with the pBR322 substrate, the stimulatory effect of Ro 15-0216 was specific and limited to the single, major site. The nucleotide sequence of this cleavage site has recently been determined by Andersen et al. (1989). Comparison of the DNA sequences at the Ro 15-0216 induced cleavage site in pBR322 and in the rDNA substrate revealed a high degree of homology:

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                    ↓
rDNA      AGCATG CGTATATCAT
          |  ||| ||   ||| |
pBR322    ACAATG CGCTCATCGT
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The vertical bars denote the positions of sequence identity, and as observed, five out of five positions immediately flanking the cleavage point (indicated with an arrow) are identical. Further, the two nucleotide sequences share an overall homology of 10 out of 16 positions.

In contrast to the specific effect exerted by Ro 15-0216, traditional topoisomerase II targeted agents led to extensive fragmentation of the DNA substrate. This is demonstrated

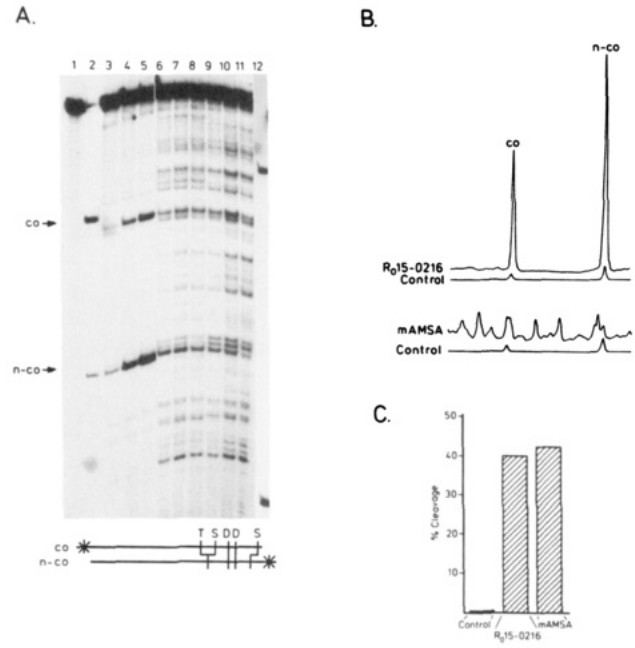

FIGURE 3: The effect of Ro 15-0216 upon topoisomerase II mediated DNA cleavage differs significantly from the effect of VM26 and mAMSA. (A) Topoisomerase II mediated cleavage of the 5′ double end labeled rDNA fragment from *T. thermophila* was carried out as described under Experimental Procedures, and reaction products were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. Lane 1, untreated double end labeled rDNA fragment; lane 2, marker DNA, labeled DNA fragment digested with *Sph*I; lane 3, topoisomerase II mediated cleavage pattern in the absence of drugs; lanes 4 and 5, effect of adding 0.1 and 1 mM Ro 15-0216, respectively, to the cleavage reaction; lanes 6–8, effect of VM26 (lane 6, 10 μM; lane 7, 50 μM; lane 8, 100 μM); lanes 9–11, effect of mAMSA (lane 9, 1 μM; lane 10, 10 μM; lane 11, 100 μM); lane 12, labeled rDNA fragment digested with *Dra*I. The bottom diagram shows the double end labeled rDNA fragment employed as substrate in this experiment. The asterisks (*) denote the labeled 5′-ends of the coding (co) and noncoding (n-co) DNA strands, and the cleavage positions of topoisomerase II (T), *Sph*I (S), and *Dra*I (D) are indicated. The location of the cleavage sites relative to the axis of symmetry of the palindromic rDNA molecule was position 1010 for topoisomerase II and *Sph*I on the noncoding strand, while cleavages were mapped to positions 1014 (topoisomerase II) and 1006 (*Sph*I), respectively, on the coding strand. A second *Sph*I cleavage site is present close to the end of the fragment, resulting in partial *Sph*I cleavage at this position (Andersen et al., 1989). Due to their small size, these *Sph*I cleavage products migrated out of the gel. The positions for *Dra*I cleavages are 949 and 965, respectively. (B) Densitometric scanning of the cleavage products presented in panel A. Control, DNA from panel A (lane 3); Ro 15-0216, DNA from panel A (lane 5); mAMSA, DNA from panel A (lane 11). (C) Histogram demonstrating the total amount of topoisomerase II mediated cleavage in the lanes presented in panel B, as determined by scanning densitometry.

in Figure 3A, which shows the effect of VM26 (lanes 6–8) and mAMSA (lanes 9–11). In the presence of either of these drugs, cleavage sites appeared at about every 20 bp on the employed DNA substrate. These sites may represent novel sites of interaction or sites poorly utilized by topoisomerase II in the absence of drugs. The distinct difference between the effect of Ro 15-0216 and that of the well-described antitumor agents is further illustrated in Figure 3B, which shows the result of densitometric scanning of the cleavage products in Figure 3A, lanes 3, 5, and 11. In addition, this scanning established that the overall amount of topoisomerase II mediated cleavage of the rDNA fragment employed was almost identical in the presence of either Ro 15-0216 (1 mM) or mAMSA (100 μM), as shown in Figure 3C.

To elucidate the effect of the Ro 15-0216 concentration on the topoisomerase II mediated cleavage level, cleavage reac-

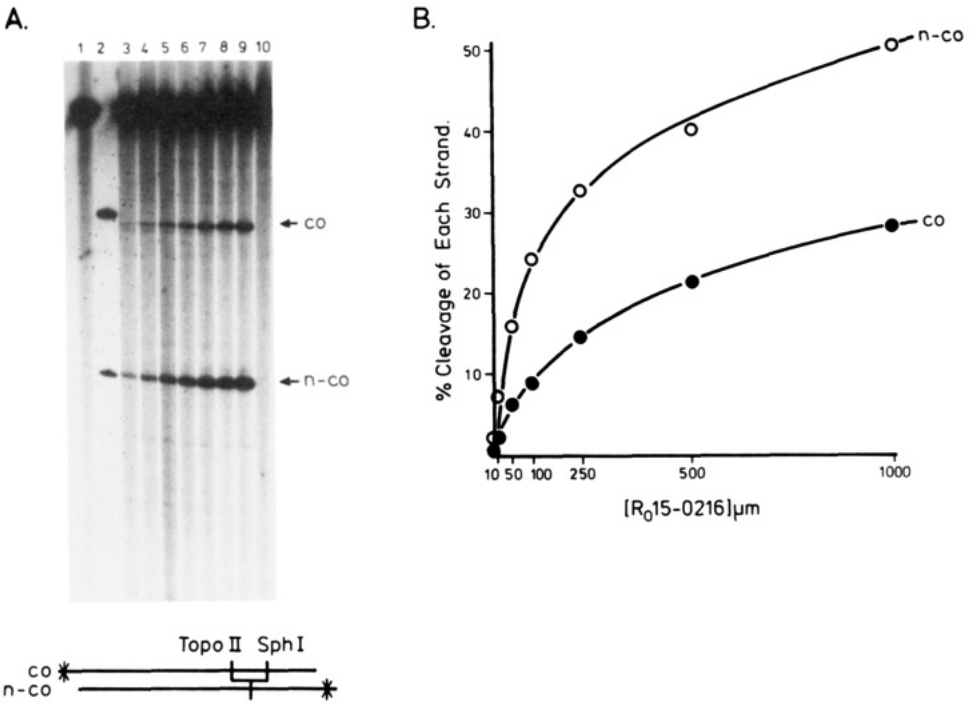

FIGURE 4: Ro 15-0216 induces topoisomerase II mediated DNA cleavage in a concentration-dependent manner. (A) Topoisomerase II mediated cleavage of the 5′ double end labeled rDNA fragment (shown in the bottom diagram) was carried out as described under Experimental Procedures. Reaction products were analyzed on a 6% denaturing polyacrylamide gel and visualized by autoradiography. Lane 1, untreated double end labeled rDNA fragment; lane 2, *Sph*I digest of the rDNA fragment; lane 3, topoisomerase II generated cleavage pattern in the absence of drugs; lanes 4–9, cleavage products formed in the presence of 10 (lane 4), 50 (lane 5), 100 (lane 6), 250 (lane 7), 500 (lane 8), and 1000 (lane 9) μM Ro 15-0216; lane 10, control without topoisomerase II but containing 1 mM Ro 15-0216. (B) Plot of percent cleavage on each strand versus the concentration of Ro 15-0216, as obtained from densitometric scanning of the topoisomerase mediated cleavages shown in panel A.

tions were performed in the presence of increasing concentrations of Ro 15-0216 (Figure 4A). A plot of the values obtained by densitometric scanning of the topoisomerase II mediated cleavage products versus the concentration of Ro 15-0216 showed a concentration-dependent stimulation of topoisomerase II mediated cleavage, reaching a maximum level at 1 mM drug (Figure 4B). The double end labeling of the DNA fragment employed made it possible to estimate the percentage of topoisomerase II mediated cleavage on both DNA strands as each represented 50% of the labeled DNA substrate. At the optimal Ro 15-0216 concentration, 52% of the DNA substrate was cleaved on the noncoding strand, while 28% was cleaved on the coding strand, demonstrating a DNA strand preference. Relative to the control, Ro 15-0216 stimulated DNA cleavage to the same extent on the two complementary DNA strands, giving rise to an ~20-fold stimulation on both strands. Excision of the individual bands from the polyacrylamide gel (Figure 4A, lane 9) followed by scintillation counting confirmed the results obtained by scanning (not shown).

The formation of covalent binding of the topoisomerase II subunits to the broken DNA strands is a characteristic of the enzyme. To demonstrate that this hallmark is retained in the presence of Ro 15-0216, we investigated topoisomerase II mediated cleavage on the rDNA fragment by trapping the abortive protein–DNA intermediate with SDS. The cleaved DNA products were subjected to phenol extraction, separating the material into interphases and waterphases as described under Experimental Procedures. DNA molecules covalently linked to protein accumulated in the interphase whereas free DNA substrate was contained in the waterphase. DNA located in either of the phases was precipitated with ethanol, proteinase K digested, and subjected to electrophoresis in a denaturing polyacrylamide gel (Figure 5A). The interphase material obtained in the absence or presence of 1 mM Ro 15-0216 is shown in lane 1 and 2, respectively. Consistent with the results presented in Figures 3A and 4A, a pronounced increase in the amount of topoisomerase II mediated cleaved material was observed in the interphase when Ro 15-0216 was present. In contrast, the waterphase material held no cleavage products, but only uncleaved DNA fragments (Figure 5A, lanes 3 and 4). These results demonstrate a covalent nature of the topoisomerase II–DNA complexes. We investigated these complexes further by labeling the rDNA fragment at the 3′-ends, resulting in covalent binding of topoisomerase II to the labeled DNA strand. Topoisomerase II mediated cleavage was performed on the fragment in the presence of Ro 15-0216, and the reaction products were subjected to phenol extraction. The interphase material was isolated, and half was proteinase K treated, whereas the other half was left untreated (Figure 5B, lane 1 and 2, respectively). Electrophoresis of the two samples showed that the cleavage products in the untreated sample were retained in the slot (lane 2), demonstrating a covalent linkage of topoisomerase II to the 5′-end of the DNA in the presence of Ro 15-0216. The band located at the arrow in lane 1 and 2 of both panels A and B of Figure 5 represents the uncleaved strand of a topoisomerase II mediated single stranded cleaved molecule. As can be seen from Figure 5B, the mobility of these molecules is independent of proteinase K treatment, demonstrating that no protein is attached.

Ro 15-0216 Induces an Equal Formation of both Single- and Double-Stranded Topoisomerase II Mediated DNA Cleavages. Traditional topoisomerase II directed chemotherapeutics give rise to both single- and double-stranded enzyme-mediated DNA cleavage (Liu, 1989). To characterize the influence of Ro 15-0216 upon the relative amount of single- to double-stranded cleavage, experiments were performed in the absence and presence of Ro 15-0216 (1 mM), employing the 5′ double end labeled rDNA fragment. The covalent

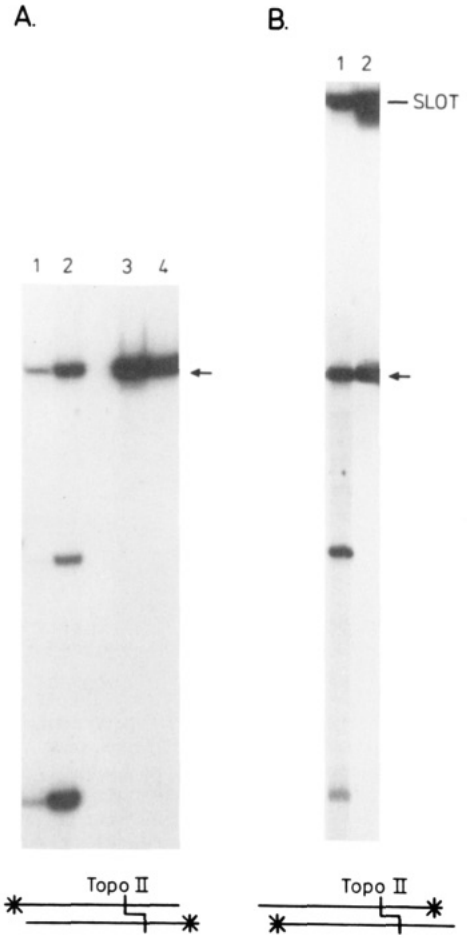

FIGURE 5: Topoisomerase II mediated DNA cleavage products formed in the presence of Ro 15-0216 are protein bound. (A) Topoisomerase II mediated cleavages were generated on the 5′ end labeled rDNA fragment in the absence and presence of 1 mM Ro 15-0216. Cleavage products were enriched in phenol interphases, as described under Experimental Procedures and analyzed on a 6% denaturing polyacrylamide gel. Lane 1, interphase material obtained in the absence of drugs; lane 2, interphase mateial obtained in the presence of 1 mM Ro 15-0216; lanes 3 and 4, waterphase material obtained in the absence and presence of Ro 15-0216, respectively. (B) Topoisomerase II mediated DNA cleavages were introduced on the 3′ double end labeled rDNA fragment in the presence of 1 mM Ro 15-0216. Lanes 1 and 2, Ro 15-0216 stimulated topoisomerase II mediated cleavage products treated with and without proteinase K, respectively. The band observed at the arrow in lanes 1 and 2 of both panels A and B represents the intact noncleaved DNA strand resulting from topoisomerase II mediated single-stranded DNA cleavage. The position of the slots is indicated in the figure. The 5′ and 3′ double end labeled rDNA substrates are shown below panels A and B, respectively. *Dra*I digestion of the 3′ end labeled rDNA fragment demonstrated that the two ends were labeled to the same extent (not shown).

enzyme–DNA intermediates were trapped by SDS, proteinase K treated, and subsequently subjected to electrophoresis on a native polyacrylamide gel (Figure 6A). Fragments containing a single-stranded break comigrated in this gel system with the uncleaved DNA fragment (position a) while fragments produced by a double-stranded cleavage migrated to the positions designated b (222 bp) and c (143 bp). Densitometric scanning of the gel revealed that the level of double-stranded breaks was increased ~11-fold in the presence of Ro 15-0216 (compare lanes 2 and 3), thus representing half of the observed increase (~20-fold) seen on the denaturing gels in Figures 3 and 4. To determine the amount of Ro 15-0216 induced single-stranded cleavages, the DNA material located in position a was excised from the native gel, eluted, and subsequently applied to a denaturing polyacrylamide gel

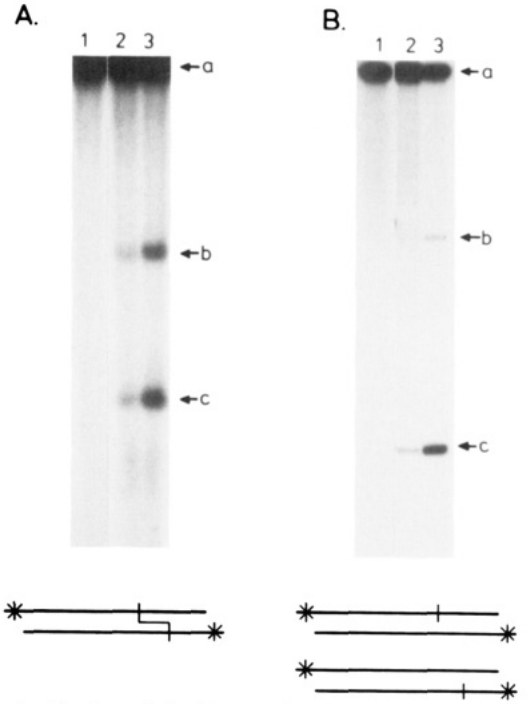

FIGURE 6: Single- and double-stranded topoisomerase II mediated DNA cleavages were equally induced by Ro 15-0216. (A) 5′ double end labeled rDNA fragment was incubated with topoisomerase II, and cleavage products were analyzed on a 4% native polyacrylamide gel. Lane 1, untreated fragment; lanes 2 and 3, double-stranded enzyme-mediated cleavages introduced in the absence or presence of 1 mM Ro 15-0216, respectively. (B) The band at position a in lanes 1–3 on the native gel shown in (A) was excised. DNA was eluted and analyzed for single-stranded cleavage on a 6% denaturing polyacrylamide gel. Lane 1, untreated DNA fragment; lanes 2 and 3, single-stranded topoisomerase II mediated DNA cleavage in the absence and presence of 1 mM Ro 15-0216, respectively. The structures of the double- and single-stranded cleavage products are schematically illustrated in the bottom parts of panels A and B, respectively.

(Figure 6B). As observed for double-stranded cleavages, also single-stranded DNA breaks were significantly enhanced by Ro 15-0216, and densitometric scanning established an ~10-fold stimulation on both strands (Figure 6B, lanes 2 and 3).

Complexes Formed in the Presence of Ro 15-0216 Are Easily Reversible. It has been established that the traditional topoisomerase II targeted agents stabilize topoisomerase II–DNA cleavable complexes (Glisson & Ross, 1987; Liu, 1989). We have investigated the stability of the complexes formed in the presence of Ro 15-0216. Topoisomerase II was incubated with the 5′ double end labeled rDNA substrate as described under Experimental Procedures. After 5 min of incubation the ionic strength in the reaction mixture was increased by raising the NaCl concentration to 400 mM, and the temperature was lowered to 4 °C. The residual amount of cleavable complexes was monitored at different time points followed by analysis on a denaturing polyacrylamide gel (Figure 7A). The results showed that NaCl treatment of the cleavable complexes efficiently reversed the DNA cleavages obtained in the presence of Ro 15-0216 (lanes 1–8). In contrast, the complexes generated in the presence of mAMSA (lanes 9–16) exerted a considerably higher degree of stability. Thus, densitometric scanning of the gel presented in Figure 7A demonstrated ~70-fold lower salt stability of the complexes formed in the presence of Ro 15-0216 as compared to the stability with mAMSA (Figure 7B).

The relaxation activity of topoisomerase II was relatively unaffected even at the concentration of Ro 15-0216 optimal

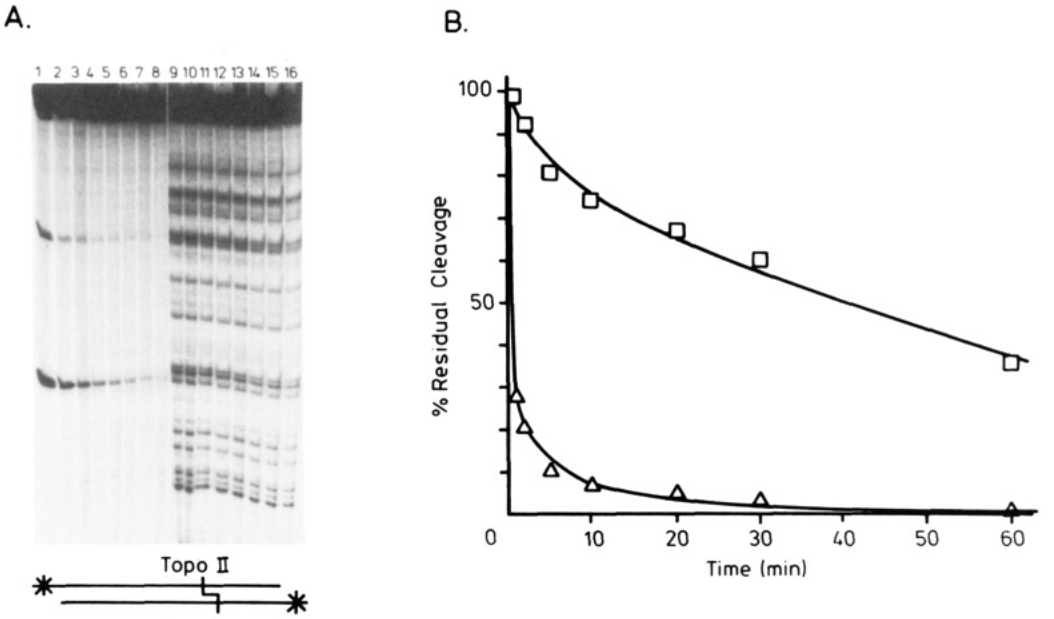

FIGURE 7: (A) Cleavable complexes formed in the presence of Ro 15-0216 are reversible. Cleavable complexes were obtained on the 5′-double end labeled rDNA fragment (bottom diagram) in the presence of either 1 mM Ro 15-0216 or 20 μM mAMSA. The stability of the complexes was measured by monitoring the rate of religation at a high ionic strength (0.4 M NaCl) in a time course. The stability was examined in a reaction containing 1 mM Ro 15-0216. Samples were taken at 0 min (lane 1), 0.5 min (lane 2), 2.5 min (lane 3), 5 min (lane 4), 10 min (lane 5), 20 min (lane 6), 30 min (lane 7), and 60 min (lane 8). The same time points were employed in the time course shown in lanes 9–16, where 20 μM mAMSA was added. (B) Graphic representation of the cleavage products obtained by densitometric scanning of the results presented in (A). Ro 15-0216 (△); mAMSA (□).

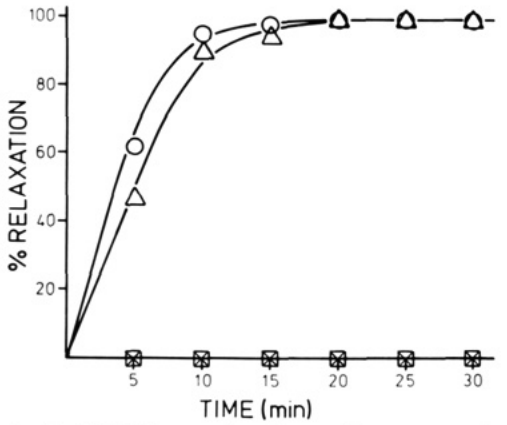

FIGURE 8: Ro 15-0216 exerts virtually no effect upon topoisomerase II mediated DNA relaxation. The catalytic activity of topoisomerase II was investigated by measurement of ATP-dependent relaxation of negatively supercoiled pBR322 DNA, as described under Experimental Procedures. Samples were collected at the indicated time points and electrophoresed in a 1% agarose gel. Densitometric scanning was performed, and the percentage of relaxation was plotted against the time of incubation. The catalytic activity was followed in the absence (○) or presence of either 1 mM Ro 15-0216 (△), 20 μM VP16 (×), or 20 μM mAMSA (□).

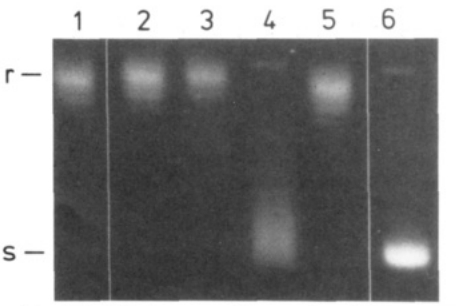

FIGURE 9: Ro 15-0216 does not intercalate DNA. Unwinding measurements were performed as described under Experimental Procedures. Lane 1, relaxed pBR322 plasmid employed as substrate; lane 2, DNA subjected to topoisomerase I relaxation without drug. DNA was treated with 1 mM Ro 15-0216 (lane 3), 20 μM mAMSA (lane 4), 50 μM VP16 (lane 5), or 0.3 μM ethidium bromide (lane 6) before topoisomerase I relaxation. The position of relaxed (r) or supercoiled (s) DNA is indicated at the left.

for cleavage (1 mM) (Figure 8). In contrast, addition of low concentrations of mAMSA (20 μM) or VP16 (20 μM) caused an absolute inhibition of the activity. The difference in the effect of Ro 15-0216 and the traditional topoisomerase II directed agents upon the catalytic activity might be explained by the observed difference in stability of the enzyme–DNA complexes formed in the presence of these drugs, suggesting that they have different modes of action.

Ro 15-0216 Does Not Intercalate DNA. Topoisomerase II directed agents have been divided into DNA-intercalative and -nonintercalative compounds (Liu, 1989). To assess whether Ro 15-0216 intercalated DNA, an unwinding assay was applied. Relaxed pBR322 plasmid was in this assay incubated with different drugs followed by relaxation with topoisomerase I (Pommier et al., 1985). This was possible as none of the compounds examined had any inhibitory effect upon topoisomerase I relaxation at the concentrations employed (data not shown). Following relaxation, the reaction mixtures were extracted with phenol, ethanol precipitated, and electrophoresed on a 1% agarose gel. No unwinding effect was observed with Ro 15-0216 even at relative high concentrations of the drug (<1 mM) (Figure 9, lane 3). A similar result was obtained when the nonintercalating compound VP16 (50 μM) was employed (Figure 9, lane 5) (Chen et al., 1984). In contrast, mAMSA, which belongs to the group of DNA intercalators (Waring, 1981), was demonstrated to intercalate DNA extensively at a concentration of 10 μM (Figure 9, lane 4). We thus conclude that Ro 15-0216 belongs to the group of nonintercalating drugs.

Discussion

The presented results demonstrate a pronounced influence of the 2-nitroimidazole Ro 15-0216 upon purified calf thymus topoisomerase II as it selectively stimulates enzyme-mediated

DNA breakage at specific nucleotide sequences. Only a single cleavage site is stimulated by the drug on the entire pBR322 DNA molecule. This site, located at position 101, has previously been characterized as a major recognition sequence for *Drosophila* topoisomerase II (Sander & Hsieh, 1983). The unique sequence-specific enhancement of topoisomerase II mediated DNA cleavage caused by Ro 15-0216 differs from the effect of the traditional and well-described chemotherapeutic agents which enhance enzyme-mediated cleavage at a multiple number of DNA sequences (Chen et al., 1984; Tewey et al., 1984; Rowe et al., 1986).

Previous studies have elucidated that a major site for topoisomerase II mediated DNA cleavage is located close to the origin of replication in the central nontranscribed spacer of the *T. thermophila* rRNA genes (Andersen et al., 1989). In our study, Ro 15-0216 was shown to increase the level of topoisomerase II mediated DNA cleavage at this site about 20-fold, and in contrast to VM26 and mAMSA, Ro 15-0216 only stimulated cleavage at this major site. The observation demonstrates a highly sequence-specific influence of the compound upon the enzyme–DNA interaction and underlines that Ro 15-0216 holds a preference for interacting with topoisomerase II at specific DNA sequence elements. The facts that the stimulated sequences from pBR322 and rDNA share 100% homology of the five base pairs immediately flanking the cleavage point and that computer analyses reveal that the DNA motif containing these five base pairs only is present once in either of the two substrates examined here further stress this specificity.

Topoisomerase II has recently been demonstrated to introduce both single- and double-stranded DNA cleavages into the nucleic acid backbone (Muller et al., 1988; Zechiedrich et al., 1989; Lee et al., 1989). From our studies we were able to discern between these two types of breaks and to demonstrate that the cleavage enhancement observed in the presence of Ro 15-0216 originated from an equal stimulation of both single- and double-stranded cleavage. We have previously reported that topoisomerase II during single-stranded cleavage discriminates between the two complementary DNA strands, thereby displaying strand specificity in its interaction with DNA (Andersen et al., 1989). The enzyme retained its strand preference in the presence of Ro 15-0216. When these observations are considered together with the fact that the drug-stimulated cleavage products are protein linked, it is evident that the cleavage reaction performed by topoisomerase II in the presence of Ro 15-0216 shares the characteristics of the one taking place in the absence of this compound.

It is generally believed that topoisomerase II directed chemotherapeutics prevent religation of the cleaved DNA strands by stabilizing the covalent enzyme–DNA cleavable complexes (Glisson & Ross, 1987; Liu, 1989; Osheroff, 1989a,b; Robinson & Osheroff, 1990). Our studies revealed that the cleavable complexes formed in the presence of Ro 15-0216 were easily reversible and ~70-fold less stable than those generated in the presence of mAMSA. Further, Ro 15-0216 was found to exert virtually no inhibitory effect upon topoisomerase II mediated DNA relaxation. This might be explained by the low stability of the cleavable complexes formed in the presence of the drug. The observations stress a different mode of action of Ro 15-0216 as compared to traditional topoisomerase II directed chemotherapeutics.

A recent study by Hsiang et al. (1989) has demonstrated that the intercalative antitumor drug amonafide enhances topoisomerase II mediated DNA cleavage by the calf thymus enzyme in a highly sequence-dependent manner as observed for Ro 15-0216. Cleavage was induced at a single major site located at about position 1830 on the pBR322 DNA molecule (Hsiang et al., 1989). Ro 15-0216 did not induce enzyme-mediated DNA cleavage at this position, demonstrating that this drug and amonafide share no homology regarding their DNA sequence preference. The two drugs hold clinical potential as they might be valuable prototypes for design and development of drugs that selectively target the enzyme at specific regulatory DNA elements on the genome. The identification of drugs that target topoisomerase II at specific sequences furthermore represents a valuable tool for molecular studies of the interaction between topoisomerase II and DNA.

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References

Andersen, A. H., Christiansen, K., Zechiedrich, E. L., Jensen, P. S., Osheroff, N., & Westergaard, O. (1989) *Biochemistry 28*, 6237–6244.

Bencini, D. A., O'Donovan, G. A., & Wild, J. R. (1984) *BioTechniques 4*, 4–5.

Bodley, A. L., & Liu, L. F. (1988) *Bio/Technology 6*, 1315–1319.

Borowy, N. K., Nelson, R. T., & Hirumi, H. (1988) *Ann. Trop. Med. Parasitol. 82*, 13–19.

Brill, S. J., DiNardo, S., Voelkel-Meimann, K., & Sternglanz, R. (1987) *Nature 326*, 414–416.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 13560–13566.

D'Arpa, P., & Liu, L. F. (1989) *Biochim. Biophys. Acta 989*, 163–177.

DiNardo, S., Voelkel, K., & Sternglanz, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2616–2620.

Glikin, G. C., & Blangy, D. (1986) *EMBO J. 5*, 151–155.

Glisson, B. S., & Ross, W. E. (1987) *Pharmacol. Ther. 32*, 89–106.

Holm, C., Goto, T. Wang, J. C., & Botstein, D. (1985) *Cell 41*, 553–563.

Hsiang, Y.-H., Jiang, J. B., & Liu, L. F. (1989) *Mol. Pharmacol. 36*, 371–376.

Lee, M. P., Sander, M., & Liu, L. F. (1989) *J. Biol. Chem. 264*, 13510–13518.

Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351–375.

Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., & Chen, G. L. (1983) *J. Biol. Chem. 258*, 15365–15370.

Maxwell, A., & Gellert, M. (1986) *Adv. Protein Chem. 38*, 69–107.

Muller, M. T., Spitzner, J. R., DiDonato, J. A., Mehta, V. B., Tsutsui, K., & Tsutsui, K. (1988) *Biochemistry 27*, 8369–8379.

Nelson, W. G., Liu, L. F., & Coffey, D. S. (1986) *Nature 322*, 187–189.

Osheroff, N. (1989a) *Pharmacol. Ther. 41*, 223–241.

Osheroff, N. (1989b) *Biochemistry 28*, 6157–6160.

Osheroff, N., & Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303–4309.

Pommier, Y., Minford, J. K., Schwartz, R. E., Zwelling, L. A., & Kohn, K. W. (1985) *Biochemistry 24*, 6410–6416.

Robinson, M. J., & Osheroff, N. (1990) *Biochemistry 29*, 2511–2515.

Rowe, T. C., Wang, J. C., & Liu, L. F. (1986) *Mol. Cell. Biol. 6*, 985–992.

Sander, M., & Hsieh, T. (1983) *J. Biol. Chem. 258*, 8421–8428.
Sander, M., Hsieh, T., Udvardy, A., & Schedl, P. (1987) *J. Mol. Biol. 194*, 219–229.
Shapiro, T. A., & Englund, P. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 950–954.
Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 9182–9187.
Udvardy, A., Schedl, P., Sander, M., & Hsieh, T. (1986) *J. Mol. Biol. 191*, 231–246.
Uemura, T., & Yanagida, M. (1984) *EMBO J. 3*, 1737–1744.
Vosberg, H.-P. (1985) *Curr. Top. Microbiol. Immunol. 114*, 19–102.
Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665–697.
Waring, M. J. (1981) *Annu. Rev. Biochem. 50*, 159–192.
Yang, L., Wold, M. S., Li, J. J., Kelly, T. J., & Liu, L. F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 950–954.
Zechiedrich, E. L., Christiansen, K., Andersen, A. H., Westergaard, O., & Osheroff, N. (1989) *Biochemistry 28*, 6229–6236.
Zweygarth, E., & Röttcher, D. (1987) *Trop. Med. Parasitol. 38*, 175–176.

Slalom Chromatography: Size-Dependent Separation of DNA Molecules by a Hydrodynamic Phenomenon†

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ABSTRACT: Slalom chromatography, a size-dependent DNA fractionation method based on a new principle [Hirabayashi, J., & Kasai, K. (1989) *Anal. Biochem. 178*, 336–341], was systematically studied in detail. In this method, larger DNA fragments are eluted much later than smaller ones from columns packed with spherical microbeads. Elution of a series of DNA fragments was systematically examined by using columns packed with polymer-based packings of different diameter and different pore size for high-performance gel permeation chromatography. Packings of smaller diameter proved to be superior for resolving the smaller size range of DNA, while the reverse was the case for larger DNAs. Application of a faster flow rate led to larger retardation of every DNA fragment, while at the lowest flow rate applied (0.067 cm/min), all the fragments were eluted almost at the void volume. When the column temperature was lowered, retardation of DNA became larger. On the other hand, differences in the chemical nature and the pore size of packings, or in the hydrophobicity of the eluting solvent, had little effect on DNA retardation. Size-dependent fractionation of DNA was also achieved even on columns packed with nonporous packings having anionic groups (cation exchangers). In conclusion, these results confirmed the previous conclusion that slalom chromatography is not based on an adsorption or equilibrium phenomenon but should be attributed to a hydrodynamic phenomenon.

High-performance liquid chromatography is useful for both analysis and separation of various materials (from small organic compounds to macromolecules, e.g., proteins, nucleic acids, polysaccharides). This method became feasible only when column packings tolerant of high pressure were developed, and these are usually made of silica or synthetic organic polymers. The method is considerably superior to conventional low-pressure chromatography using open columns in speed, accuracy, reproducibility, and quantitative precision. Various modes of high-performance liquid chromatography are now used. For the separation of biological macromolecules, gel permeation chromatography is one of the most commonly used modes. In this mode, larger molecules are eluted faster than smaller molecules, because the former have difficulty in permeating into the pores of the packing materials. Though it is a powerful tool for protein research, its application to DNA has been avoided because DNA molecules have been believed to be too large for conventional gel filtration materials. Even small DNA fragments have Stokes radii considerably larger than proteins of similar molecular weight.

Recently, we found that double-stranded DNA molecules can be separated according to their size by using a system for high-performance gel permeation chromatography, though the order of elution was opposite to that expected for gel permeation chromatography (Hirabayashi & Kasai, 1989); that is, larger fragments are eluted later than smaller ones. We proposed to name this new mode of separation "slalom chromatography". In the previous paper, we provided evidence that the separation is based on a hydrodynamic phenomenon; the separation significantly depends on the flow rate and particle size of packings, but does not depend on the pore size or the chemical nature of the packings (silica or synthetic polymer). A tentative model of DNA separation in slalom chromatography is illustrated in Figure 1. The existence of open spaces between closely packed spherical particles and also

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