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Induction of Mammalian DNA Topoisomerase I and II Mediated DNA Cleavage by Saintopin, a New Antitumor Agent from Fungus

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ABSTRACT: Saintopin is an antitumor antibiotic recently discovered in mechanistically oriented screening using purified calf thymus DNA topoisomerases. Saintopin induced topoisomerase I mediated DNA cleavage comparable to that of camptothecin, and topoisomerase II mediated DNA cleavage equipotent to those of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (VP-16). Treatment of a reaction mixture containing saintopin and topoisomerase I or II with either elevated temperature (65 °C) or higher salt concentration (0.5 M NaCl) resulted in a substantial reduction in DNA cleavage, suggesting that the topoisomerase I and II mediated DNA cleavage induced by saintopin is through the mechanism of stabilizing the reversible enzyme–DNA "cleavable complex". Consistent with the cleavable complex formation with both topoisomerases, saintopin inhibited catalytic activities of both topoisomerase I and topoisomerase II. The DNA cleavage intensity pattern induced by saintopin with topoisomerase I was different from that by camptothecin. A difference in cleavage pattern was also detected between saintopin and *m*-AMSA or VP-16 in topoisomerase II mediated DNA cleavage. DNA unwinding assay using T4 DNA ligase showed that saintopin is a weak DNA intercalator like *m*-AMSA. Thus, saintopin represents a new class of antitumor agent that can induce both mammalian DNA topoisomerase I and mammalian DNA topisomerase II mediated DNA cleavage.

DNA topoisomerases are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, thereby controlling the topological states of DNA. Two major topoisomerases, topoisomerase I and topoisomerase II, have been detected in all eukaryotic cells. Topoisomerase I catalyzes the passage of the DNA strand through a transient single-strand break, while topoisomerase II catalyzes the passage of DNA double strands through a transient double-strand break. These topoisomerases have been known to be involved in many important DNA metabolism reactions including replication, recombination, transcription, and chromosome segregation at mitosis (Wang, 1985). In addition, both topoisomerase I and topoisomerase II have generated extensive clinical interest in chemotherapy. There is now good evidence showing that topoisomerase II is the principal intracellular target for a number of clinically important antitumor drugs (Liu, 1989; D'Arpa & Liu, 1989). Despite their apparent structural diversity, these drugs have the common property of stabilizing a key covalent reaction intermediate of topoisomerase II,

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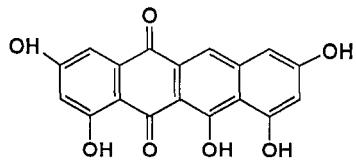

Saintopin

FIGURE 1: Structure of saintopin.

termed the cleavable complex, which upon exposure to denaturant results in the induction of DNA cleavage. Structure–activity studies on acridine derivatives and epipodophyllotoxin congeners have shown a good correlation between cytotoxicity and ability to induce cleavable complexes (Rowe et al., 1986; Yamato et al., 1989; Long, 1987). Furthermore, cells that are resistant to topoisomerase II poisons have been shown to have an altered topoisomerase II activity, indicating that the cytotoxic effects of the drugs are directly mediated by the enzyme (Liu, 1989; D'Arpa & Liu, 1989).

Topoisomerase I can also be trapped as a cleavable complex by a specific topoisomerase I poison, camptothecin (Hsiang et al., 1985). Topoisomerase I mediated DNA cleavage induced by camptothecin differs from that mediated by topoisomerase II poisons in that only a single DNA strand is cleaved. In agreement with the results of topoisomerase II poisons, studies on several new camptothecin derivatives indicate that antitumor activity is correlated with their abilities to induce a cleavable complex with topoisomerase I and DNA (Hsiang et al., 1989a; Jaxel et al., 1989). For topoisomerase II poisons, high levels of the enzyme in proliferating cells and very low levels in quiescent cells appear to explain the selective sensitivity of proliferative tumor cells to the cytotoxic effects of these drugs (D'Arpa & Liu, 1989). In contrast, the intracellular levels of topoisomerase I have been reported to be largely unaffected by growth conditions of culture cells. However, more recent findings by Giovanella et al. (1989) that topoisomerase I is elevated in advanced-stage human colon cancer as compared to normal colon tissues suggest the possibility that topoisomerase I is also an important target of antitumor drugs.

Thus, both topoisomerases now appear to be important targets for the development of new cancer chemotherapeutic drugs. According to this attractive model, we have screened cultures of actinomycetes and fungi for their ability to induce topoisomerase II mediated DNA cleavage in vitro. As previously reported, flavonoids such as genistein and orobol (Yamashita et al., 1990a) and antitumor antibiotics, streptonigrin (Yamashita et al., 1990b), terpentecin, and clerocidin (Kawada et al., 1991), have been identified as topoisomerase II targeting compounds. Recently, we have isolated a new antitumor antibiotic, saintopin, produced by *Paecilomyces* sp. (Figure 1). Saintopin induced potent mammalian topoisomerase II mediated DNA cleavage in vitro and showed antitumor activity against murine leukemia P388 in vivo (Yamashita et al., 1990c). Herein, we report studies in vitro indicating that saintopin is a new class of DNA intercalator which can induce the cleavable complex with both topoisomerase I and topoisomerase II.

Materials and Methods

Enzymes, Nucleic Acids, and Chemicals. DNA topoisomerases I and II were isolated from calf thymus gland as described by Halligan et al. (1985) and partially purified with Bio-Rex70, hydroxylapatite, and P-11 phosphocellulose column chromatography, and topoisomerase II was further purified with Mono Q column chromatography as described by Drake et al. (1987). Topoisomerase I and II activities were monitored throughout the purification steps by DNA relaxation assay in the absence (topoisomerase I) or presence (topoisomerase II) of ATP and $MgCl_2$. To rule out contamination by each enzyme, DNA cleavage activities with purified topoisomerases I and II were assayed in the presence of the topoisomerase I specific drug camptothecin or the topoisomerase II specific drug 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA).[1] Thus, the enzymes used in this study were free from contamination of another topoisomerase or endonucleases which was proved by data showing no production of nicked or linear DNA in the relaxation assay. Topoisomerases were kept at −20 °C in a storage buffer containing 50% (v/v) glycerol, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 30 mM potassium phosphate, pH 7.5. One unit of activity was the amount of topoisomerase that relaxes half of the 0.4 μg of supercoiled DNA in the standard conditions described below. Supercoiled pBR322 and pUL402 DNA were purified from *Escherichia coli* as described (Maniatis et al., 1982). Proteinase K was from Sigma Chemical Co. Bacteriophage T4 DNA ligase, S1 nuclease, and restriction endonuclease *Hin*dIII were from Takara Shuzo Co. VP-16[1] was obtained from the National Cancer Institute, and *m*-AMSA was a gift from the Warner–Lambert Co. Camptothecin was isolated from wood of *Camptotheca accuminata* according to the method described previously (Wall et al., 1966). Saintopin was isolated from the culture broth of *Paecilomyces* sp. as reported previously (Yamashita et al., 1990c). Stock solutions of these drugs were dissolved in dimethyl sulfoxide at 50 mM, stored at −20 °C, and diluted in methanol containing 40% dimethyl sulfoxide before use. Solvent used as diluent (dimethylsulfoxide/methanol) did not affect topoisomerse I and II activities over the concentration ranges included in each reaction.

DNA Relaxation and DNA Cleavage Reactions with Topoisomerases. Reaction buffer contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 30 μg/mL bovine serum albumin. In relaxation and cleavage reactions with topoisomerase I, reaction buffer (17 μL), DNA (0.48 μg in 1 μL of Tris–EDTA buffer), drug dissolved in 1 μL of dimethyl sulfoxide/methanol, and 1 μL of topoisomerase I in storage buffer were mixed in this order in ice/water. Reactions were incubated at 37 °C for 30 min and terminated by adding 6× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 15% Ficoll) and analyzed by agarose gel electrophoresis as described below (DNA relaxation assay). For DNA cleavage, reactions were terminated by the addition of 2 μL of a solution containing 5% SDS and 2.5 mg/mL proteinase K and incubated for an additional 30 min at 37 °C. After an appropriate volume of 6× loading buffer was added, samples were run into a 1.2% agarose gel in 89 mM Tris–borate (pH 8.3)/2 mM EDTA buffer containing 0.1% SDS at 2 V/cm overnight. Gels were stained with ethidium bromide and washed in large amounts of water. The DNA band was visualized over UV light and photographed with Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Shimazu scanning densitometer. The increase of nicked DNA was estimated as drug-induced topoisomerase I mediated DNA cleavage.

DNA topoisomerase II reactions were performed in 20 μL of reaction buffer supplemented with 1 mM ATP, 10 mM $MgCl_2$, 0.48 μg of pBR322 DNA, and the indicated amounts

[1] Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VP-16, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); SDS, sodium dodecyl sulfate.

of calf thymus DNA topoisomerase II as described previously (Yamashita et al., 1990b).

DNA Unwinding Measurements. DNA unwinding effects of drugs were assayed according to the method described by Camilloni et al. (1986) with minor modifications. Plasmid DNA was linearized with *Hin*dIII restriction endonuclease and recovered by phenol extraction and ethanol precipitation. Reaction mixtures (200 μL) containing 66 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.7 mM ATP, 0.6 μg of linearized DNA, and drugs were equilibrated at 15 °C for 10 min and then incubated with excess amounts of T4 DNA ligase at 15 °C (controlled within ±0.5 °C) for 60 min. The reactions were stopped by addition of EDTA at 20 mM final concentration. DNA was analyzed by agarose gel electrophoresis after treatment to remove the drugs from the reaction mixture: extraction with phenol and ether and precipitation with ethanol. DNA circle-ligation assay using the nicked circular DNA as substrate was carried out according to the method of Montecucco et al. (1988), except that DNA-bound drugs were removed by the addition of SDS at 1% final concentration.

Comparison of the Major Cleavage Sites. In the DNA cleavage reaction with DNA topoisomerases I and II, linearized pUL402 DNA, which contains the scaffold-associated regions from the *Drosophila* histone gene cluster (Adachi et al., 1989), was used as substrate. Topoisomerase I mediated DNA cleavage patterns (single-strand break) induced by the drugs were detected as a result of a double-strand break in combination with S1 nuclease reactions which can cut the DNA single strand at nicks preinduced by topoisomerase I. After topoisomerase I mediated DNA cleavage in the presence or absence of drugs, the DNA was extracted with phenol and ether and precipitated with ethanol. The obtained DNA (about 0.6 μg) was dissolved in 20 μL of reaction buffer containing 30 mM sodium acetate buffer (pH 4.6), 10 mM NaCl, and 1 mM $ZnSO_4$ and then incubated with 1 unit of S1 nuclease at 37 °C for 60 min. The reactions were stopped by addition of EDTA at 25 mM. DNA fragments produced by the successive reactions with topoisomerase I and S1 nuclease were analyzed by 1.2% agarose gel electrophoresis under the same conditions described for DNA cleavage.

Results

Induction of Topoisomerase II Mediated DNA Cleavage by Saintopin. Saintopin induced DNA cleavage in vitro in the assay using calf thymus DNA topoisomerase II and supercoiled pBR322 DNA (Figure 2A). As the concentration of saintopin was increased (from 0.1 μM in lane d to 12.5 μM in lane g), the linear full-length DNA progressively appeared. In the absence of topoisomerase II, saintopin did not induce any changes in the supercoiled structure of pBR322 DNA (data not shown). To estimate the potency of topoisomerase II mediated DNA cleavage, the amount of linearized DNA was measured by scanning negatives with a densitometer (Figure 2B). The DNA cleavage activity of saintopin was stronger than that of VP-16 but slightly less potent than that of *m*-AMSA at a drug concentration range of 0.5–12.5 μM. Contrary to VP-16 and *m*-AMSA, the DNA cleavage activity of saintopin could not be estimated at concentrations higher than 12.5 μM, because of the low solubility of saintopin in the reaction mixture. Topoisomerase II mediated DNA cleavage induced by strong DNA intercalators, anthracycline and ellipticines, has been known to be inhibited at high drug concentrations (Tewey et al., 1984a,b; Capranico et al., 1990). In contrast to the strong intercalators, DNA cleavage induced by saintopin was not suppressed at a relatively higher drug

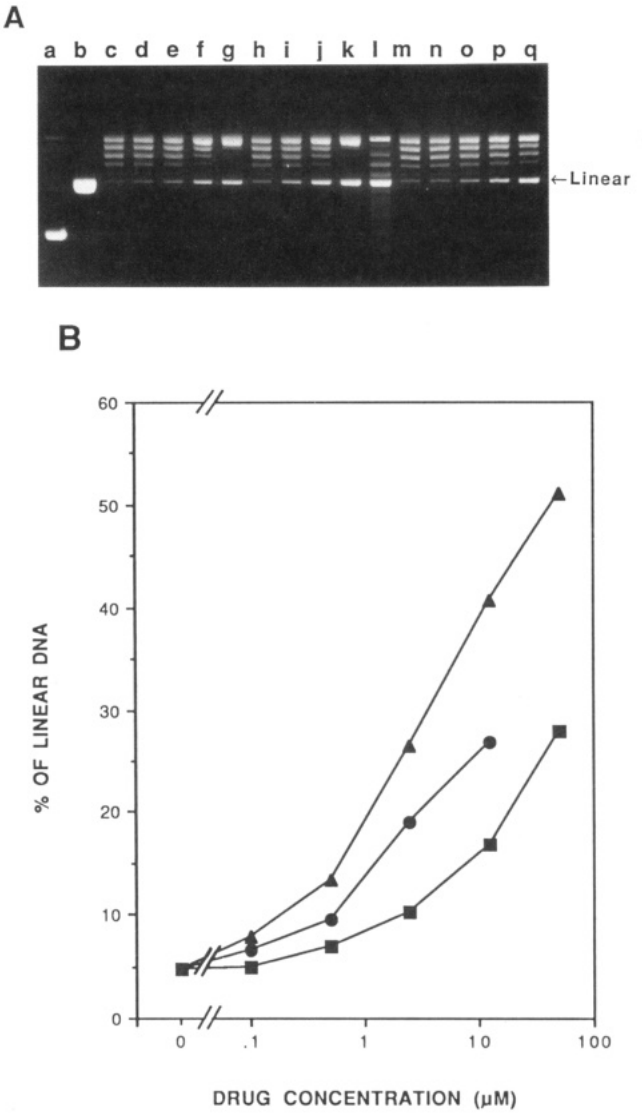

FIGURE 2: Topoisomerase II mediated DNA cleavage by saintopin, *m*-AMSA, and VP-16. In panel A, 0.48 μg of supercoiled pBR322 DNA was treated with 100 units of topoisomerase II in the presence of drugs (lanes d–q) followed by SDS/proteinase K and then analyzed on an agarose gel: lane a, CCC-DNA control; lane b, linear DNA control; lane c, no drug; lanes d–g, saintopin; lanes h–l, *m*-AMSA; lanes m–q, VP-16. Drug concentrations were as follows: lanes d, h, and m, 0.1 μM; lanes e, i, and n, 0.5 μM; lanes f, j, and o, 2.5 μM; lanes g, k, and p, 12.5 μM; lanes l and q, 50 μM. Panel B illustrates the formation of linear DNA as function of drug concentration on the basis of densitometric analysis of lanes c–q from the gel: saintopin (●), *m*-AMSA (▲), VP-16 (■). Assay conditions are described under Materials and Methods.

concentration 12.5 μM, which is consistent with data that show a weak intercalation activity of saintopin as described below.

Induction of Topoisomerase I Mediated DNA Cleavage by Saintopin. In the DNA cleavage assay using purified calf thymus topoisomerase I and plasmid pBR322 DNA, agarose gel electrophoresis was carried out in the presence of ethidium bromide (0.5 μg/mL) to resolve the slower migrating nicked DNA product from covalently closed circular relaxed DNA. With increasing concentrations of saintopin, closed circular DNA was converted to nicked DNA as shown in Figure 3. No nicking was observed in the absence of topoisomerase I. The DNA cleavage activity of saintopin was dose-dependent and slightly less potent than that of camptothecin at drug concentrations up to 12.5 μM (Figure 3B). Thus, it is clear that saintopin can induce both topoisomerase I and II mediated DNA cleavage in vitro with comparable potency to that of the

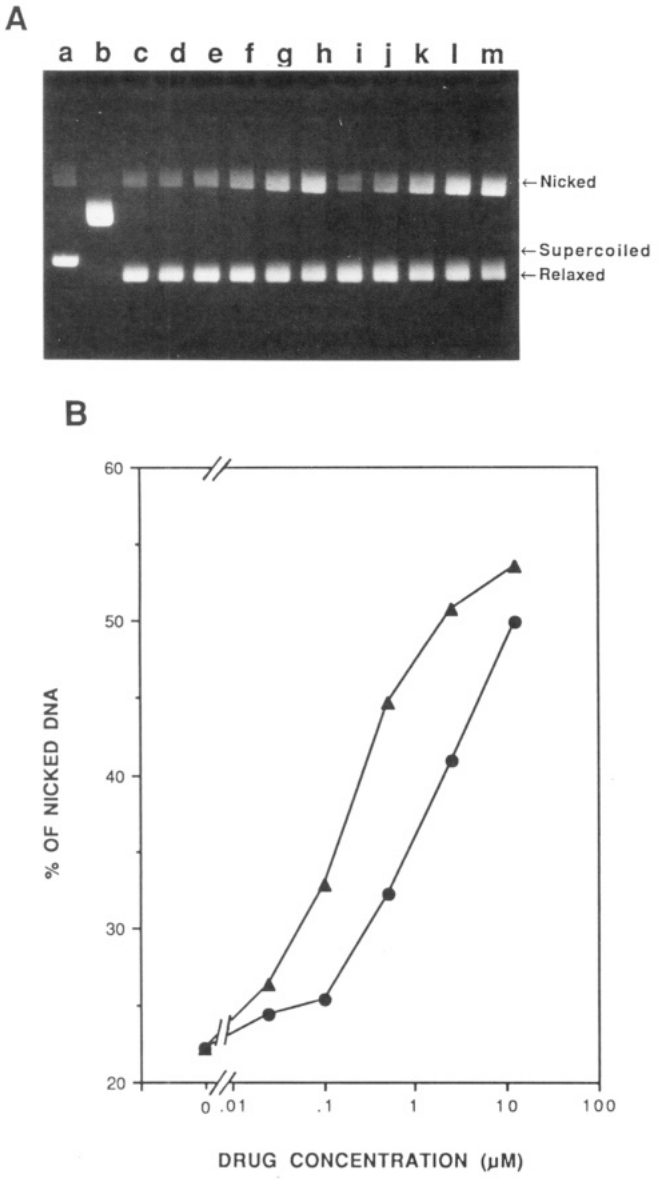

FIGURE 3: Topoisomerase I mediated DNA cleavage by saintopin and camptothecin. In panel A, 0.48 μg of supercoiled pBR322 DNA was treated with 100 units of topoisomerase I in the presence of drugs (lanes d–m) followed by SDS/proteinase K and then analyzed on an agarose gel containing 0.5 μg/mL ethidium bromide: lane a, CCC-DNA control; lane b, linear DNA control; lane c, no drug; lanes d–h, saintopin; lanes i–m, camptothecin. Drug concentrations were as follows: lanes d and i, 0.025 μM; lanes e and j, 0.1 μM; lanes f and k, 0.5 μM; lanes g and l, 2.5 μM; lanes h and m, 12.5 μM. Panel B illustrates the appearance of nicked DNA as function of drug concentration on the basis of densitometric analysis of lanes c–m from the gel: saintopin (●), camptothecin (▲). Assay conditions are described under Materials and Methods.

strongest drugs known as topoisomerase I or II poisons.

Reversibility of Saintopin-Induced DNA Cleavage. The formation of a cleavable complex by antitumor drugs has been shown to be reversed by several treatments for a reaction mixture such as dilution, increased salt concentration, or elevated temperature (Nelson et al., 1984; Chen et al., 1984; Tewey et al., 1984a,b; Hsiang & Liu, 1989). To test whether saintopin-induced DNA cleavage with topoisomerases I and II is reversible, a reaction mixture was briefly heated (65 °C) before termination with SDS and proteinase K. The reversibility of each topoisomerase-mediated DNA cleavage induced by saintopin was analyzed by agarose gel electrophoresis. Camptothecin and VP-16 were included for comparison. As shown in Figure 4A, heat treatment (65 °C) could rapidly reverse saintopin-induced topoisomerase I mediated DNA cleavage in a similar manner to that observed with camptothecin. Within 1 min, DNA cleavage induced by both saintopin and camptothecin was suppressed to a background level; about 20% of nicked DNA was produced in the absence of drug. Saintopin-induced topoisomerase II mediated DNA cleavage was also reversed by heat treatment like that observed with VP-16 as shown in Figure 4B. Although about 40% of the reduction of linear DNA was observed in 1 min, the reversal of topoisomerase II mediated DNA cleavage was relatively slow, comparing the rapid reversal of topoisomerase I mediated DNA cleavage. These results suggest that the mechanism of DNA cleavage induced by saintopin is through the formation of a reversible cleavable complex which has been reported for other known topoisomerase poisons.

Inhibition of the Catalytic Activity of DNA Topoisomerases I and II by Saintopin. It has been shown that cleavable complex formation with antitumor drugs results in inhibition of the catalytic activity of topoisomerase. In the absence of drug (control), 2.5 units of topoisomerase I relaxed supercoiled DNA in a time-dependent manner, and the reaction was completed within 30 min. The presence of saintopin or camptothecin, at 12.5 μM, resulted in a decrease of the velocity of the relaxation as shown in Figure 5A. About 50% of the supercoiled DNA remained unchanged even after 30 min. Thus, saintopin inhibited the relaxation activity of topoisomerase I with the same potency as camptothecin.

The effect of saintopin on the relaxation activity of topoisomerase II was compared with *m*-AMSA which is known to be a weak intercalator. As shown in Figure 5B, in the absence of drug (control), 2.5 units of topoisomerase II relaxed supercoiled DNA in a time-dependent manner, and the reaction was completed within 30 min. Saintopin and *m*-AMSA decreased the velocity of the DNA relaxation, and about 65% of the supercoiled DNA remained unchanged after 30 min. Inhibitory effects of saintopin against both topoisomerase I and topoisomerase II could not be through nonspecific interaction with enzyme, because saintopin did not inhibit other enzymes that act on DNA such as restriction endonuclease (i.e., DNA cutting activity of *Hin*dIII on plasmid pBR322 DNA; data not shown) and T4 DNA ligase used in the assay for the intercalation activity of saintopin as described below. These results indicate that saintopin inhibits the catalytic activity of both topoisomerase I and topoisomerase II due to a specific interaction via a stabilization of cleavable complexes.

Intercalation Activity of Saintopin. Most of the antitumor drugs which can induce the cleavable complex are DNA intercalators such as *m*-AMSA, adriamycin, and ellipticines. To investigate whether saintopin intercalates into DNA, an unwinding assay was performed using linearized pBR322 DNA and T4 DNA ligase (Camilloni et al., 1986). In this assay, weak intercalator, *m*-AMSA, and a strong intercalator, adriamycin, were included as control. Saintopin produced a concentration-dependent DNA band shift, indicating change in the DNA linking number (Figure 6, lanes d–h), which is similar to the effect of *m*-AMSA (Figure 6, lanes i–m). Adriamycin produced positively supercoiled DNA at concentrations greater than 0.5 μM (Figure 6, lanes n–q). In the presence of adriamycin at 12.5 μM, substrate linear DNA remained unchanged (lane r), indicating that strong intercalation activity of adriamycin causes inhibition of T4 DNA ligase as reported previously (Montecucco et al., 1988). Since saintopin was precipitated in a reaction mixture at concentrations more than 12.5 μM, the DNA unwinding effect at higher concentrations could not be estimated. In addition, the

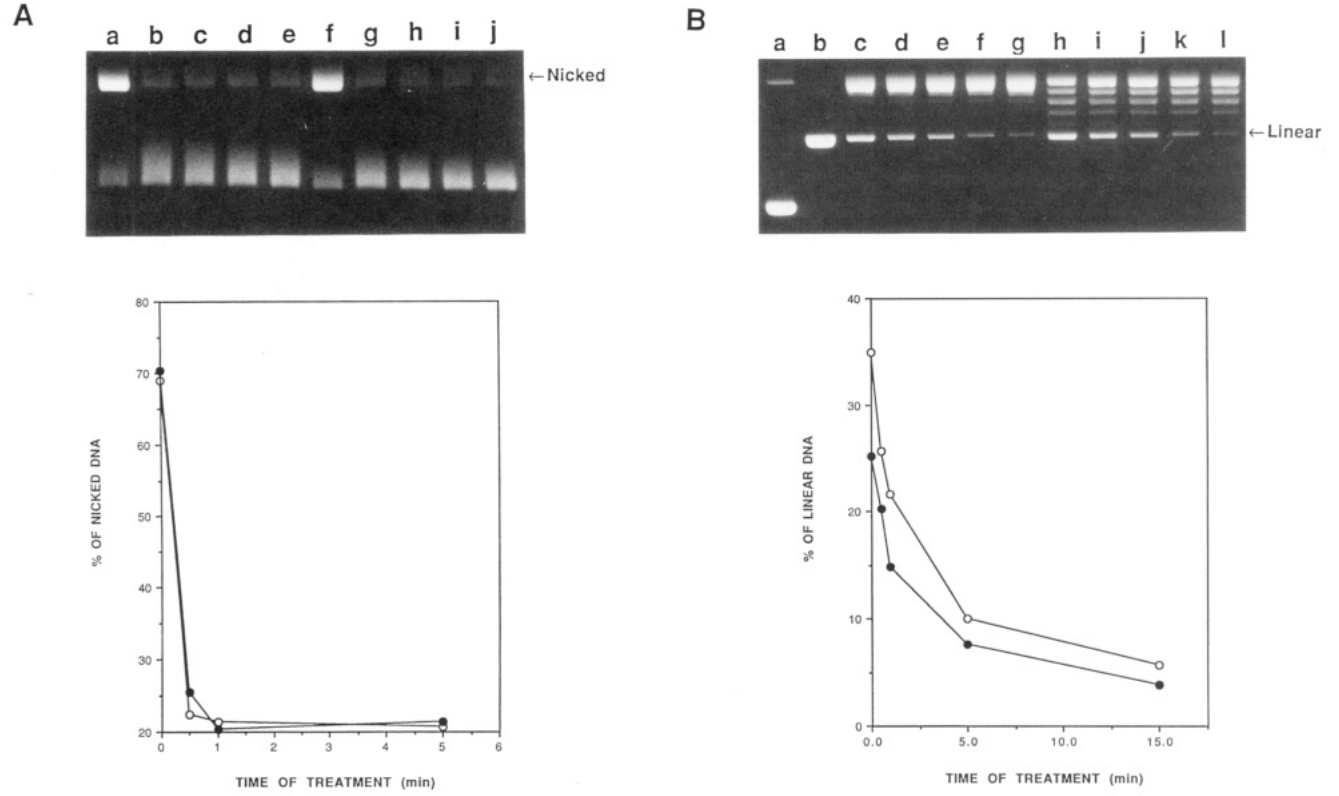

FIGURE 4: Rapid reversal of saintopin-induced DNA cleavage following a brief heat treatment. (A) Topoisomerase I mediated DNA cleavage. A large reaction mixture (120 μL) containing saintopin or camptothecin (12.5 and 2.5 μM, respectively) was incubated with 200 units of topoisomerase I at 37 °C for 30 min. The reaction mixture was then heated to 65 °C, and aliquots (20 μL) were withdrawn at various times after the treatment. SDS/proteinase K treatments were done as described under Material and Methods: lanes a–e, samples containing saintopin withdrawn at 0, 0.5, 1, 5, and 15 min after heat treatment; lanes f–j, same as lanes a–e, respectively, except that camptothecin was used. In the bottom panel, the amount of nicked DNA was quantified by densitometric analysis of lanes a–d and f–i from the gel: saintopin (○), camptothecin (●). (B) Topoisomerase II mediated DNA cleavage. A large reaction mixture (120 μL) containing saintopin or VP-16 (12.5 and 50 μM, respectively) was incubated with 200 units of topoisomerase II at 37 °C for 30 min. Heat treatment was the same as topoisomerase I mediated DNA cleavage described above: lane a, CCC-DNA control; lane B, linear DNA control; lanes c–g, samples containing saintopin withdrawn at 0, 0.5, 1, 5, and 15 min after heat treatment; lanes h–l same as lanes c–g, respectively, except that VP-16 was used. In the bottom panel, the amount of linear DNA was quantified by densitometric analysis of lanes c–l from the gel: saintopin (○), VP-16 (●).

other unwinding assay using nicked pBR322 DNA instead of linear DNA (Montecucco et al., 1988) revealed that saintopin intercalates into DNA with potentials similar to that of *m*-AMSA (data not shown).

Major DNA Cleavage Sites Induced by Topoisomerases I and II in the Presence of Saintopin: Comparison with Other Topoisomerase Poisons. Mapping studies of the topoisomerase II mediated DNA cleavage sites have revealed that different DNA cleavage patterns are observed when drugs from the different chemical classes are compared while drugs from the same chemical classes show similar cleavage patterns (Tewey et al., 1984b; Capranico et al., 1990). The major cleavage sites induced by saintopin were compared with other topoisomerase poisons using a DNA cleavage assay with linearized plasmid pUL402 DNA. Topoisomerase I mediated DNA cleavage sites were identified in combination with a S1 nuclease digestion at nicks as described under Materials and Methods. As shown in Figure 7A, S1 nuclease treatment enables the single-strand breaks induced by saintopin and camptothecin (lanes g and h) to be detected as the production of smaller DNA fragments (lanes c and e). Comparing the major fragments produced by saintopin (lanes c and d) with those produced by camptothecin (lanes e and f), two major DNA fragments produced by saintopin (indicated by long arrows) were not present in the lanes of camptothecin. However, some DNA fragments produced by saintopin (indicated by short arrows) were present in the lanes of camptothecin with stronger intensities. Thus, topoisomerase I mediated DNA cleavage was different between saintopin and camptothecin in their site specificity.

In the case of the topoisomerase II mediated DNA cleavage shown in Figure 7B, the pattern of DNA cleavage produced by saintopin (lanes c and d) was distinctly different from those of *m*-AMSA (lanes e and f) and VP-16 (lanes g and h). Three major DNA fragments observed with saintopin (indicated by arrows) were absent in the lanes of *m*-AMSA and VP-16, indicating a difference in cleavage sites between saintopin and other topoisomerase II poisons.

Discussion

DNA topoisomerase I and topoisomerase II are now viewed as important cellular targets of a number of antitumor drugs, which include camptothecin and its derivatives as topoisomerase I poisons and the acridines, ellipticines, anthracyclines, and epipodophyllotoxins as topoisomerase II poisons. These drugs, which act selectively on either DNA topoisomerase I or DNA topoisomerase II, have been used to establish a relationship between drug-induced cleavable complex formation and cytotoxicity. In this study, we present data showing that saintopin is a new class of antitumor drug, which induces both topoisomerase I and topoisomerase II mediated DNA cleavage. The cleavage activities of saintopin were dose-dependent and nearly as potent as those of camptothecin and *m*-AMSA or VP-16, respectively (Figures 2 and 3). Trask et al. (1988) and Wassermann et al. (1990) reported that actinomycin D could induce topoisomerase I as well as topo-

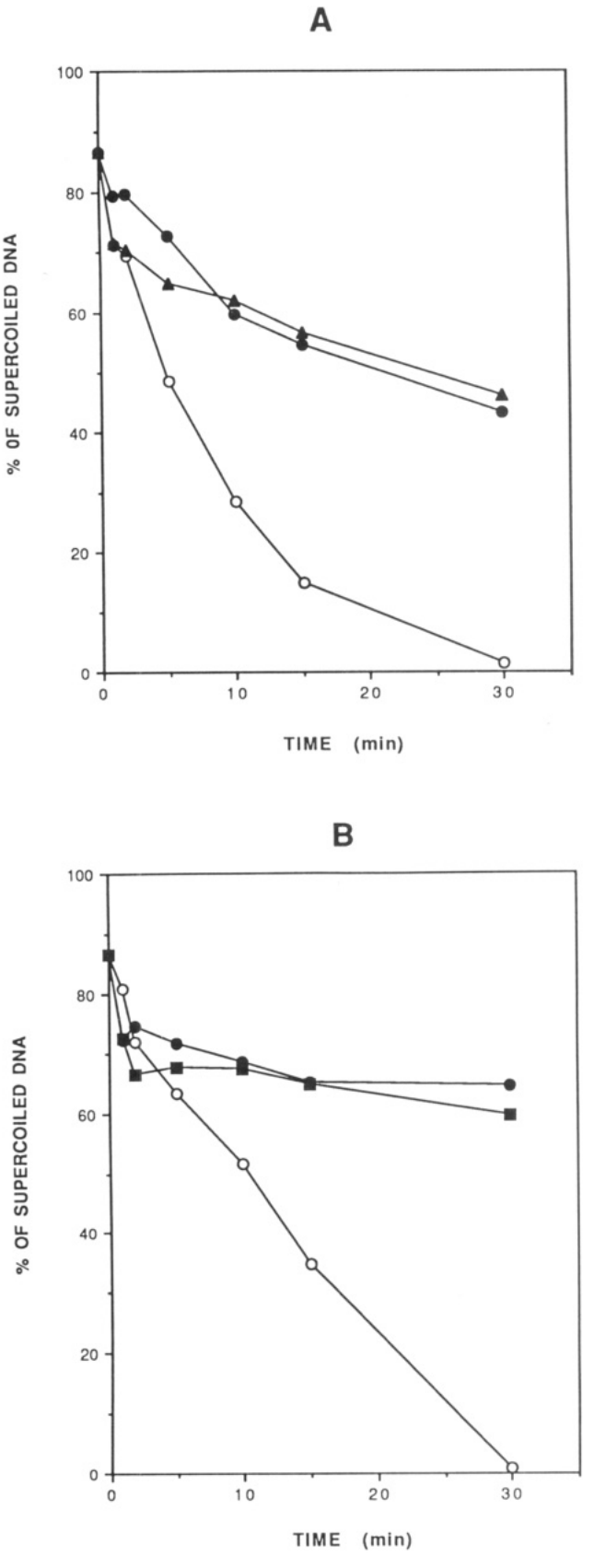

FIGURE 5: Inhibition of DNA topoisomerase I (panel A) and topoisomerase II (panel B) catalytic activity of saintopin and other topoisomerase inhibitors. Supercoiled pBR322 DNA was reacted with 2.5 units of either topoisomerase I or topoisomerase II in the absence (○, control) or presence of drugs at a concentration of 12.5 μM. The amount of supercoiled DNA was quantified by densitometric analysis from the gel: saintopin (●), camptothecin (▲) in panel A, *m*-AMSA (■) in panel B.

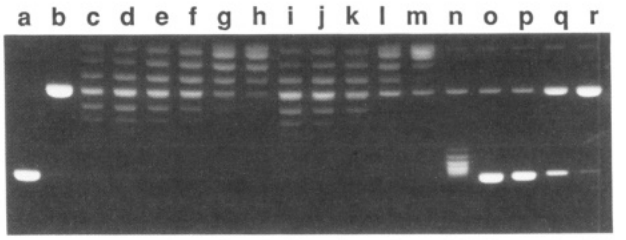

FIGURE 6: Unwinding of DNA by saintopin, *m*-AMSA, and adriamycin. Unwinding measurements were done as described under Materials and Methods. Lane a, CCC-DNA control; lane b, linear DNA control; lane c, no drug; lanes d–h, saintopin; lanes i–m, *m*-AMSA; lanes n–r, adriamycin. Drug concentrations were as follows: lanes d, i, and n, 0.5 μM; lanes e, j, and o, 1.5 μM; lanes f, k, and p, 2.5 μM; lanes g, l, and q, 7.5 μM; lanes h, m, and r, 12.5 μM.

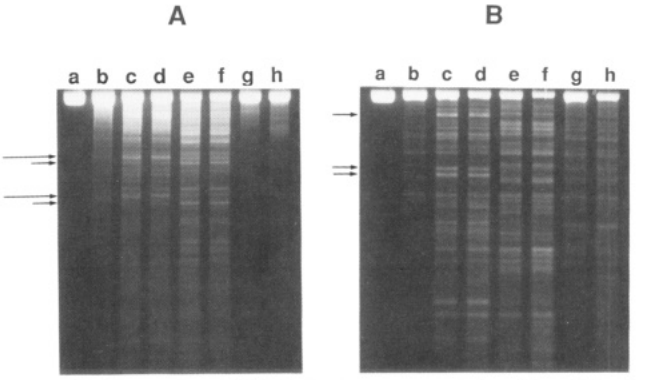

FIGURE 7: Comparison of DNA cleavage patterns induced by saintopin and other antitumor drugs. Plasmid pUL402 DNA linearized with *Hin*dIII was used in this assay. In panel A, topoisomerase I mediated DNA cleavage assay in combination with S1 nuclease digestion was done under the conditions described under Materials and Methods. Lane a, DNA alone; lane b, DNA alone plus topoisomerase I/S1 nuclease; lane c, 2.5 μM saintopin plus topoisomerase I/S1 nuclease; lane d, 12.5 μM saintopin plus topoisomerase I/S1 nuclease; lanes e and f, same as lanes c and d, respectively, except that camptothecin was used; lane g, 12.5 μM plus topoisomerase I without S1 nuclease digestion; lane h, 12.5 μM camptothecin plus topoisomerase I without S1 nuclease digestion. Long arrows indicate the DNA fragments produced by saintopin but not by camptothecin. Arrows indicate the fragments produced by both saintopin and camptothecin. In panel B, topoisomerase II mediated DNA cleavage assay was done under the conditions described under Materials and Methods. Lane a, DNA alone; lane b, no drug; lanes c and d, saintopin; lanes e and f, *m*-AMSA; lanes g and h, VP-16. Drug concentrations were as follows: lanes c, e, and g, 2.5 μM; lanes d, f, and h, 12.5 μM. Arrows indicate the major fragments produced by saintopin but not by *m*-AMSA and VP-16.

isomerase II mediated DNA cleavage in vitro. However, we could not detect any significant DNA cleavage activity of actinomycin D with topoisomerase I and also topoisomerase II when assayed under the conditions used in our studies, indicating that the enzyme-mediated DNA cleavage activity of actinomycin D is marginal. Thus, saintopin represents a new class of topoisomerase poison that induces potent DNA cleavage with both topoisomerases I and II.

The cleavable complex induced by saintopin has the following characteristics which are essentially comparable to those of previously known topoisomerase poisons: (1) The topoisomerase I mediated DNA cleavage induced by saintopin or camptothecin is reversed efficiently by heat treatment, while the topoisomerase II mediated DNA cleavage induced by saintopin or VP-16 is reversed but its efficiency is less than that of topoisomerase I (see Figure 4). In addition to heat treatment, exposure of a reaction mixture to high salt concentration (0.5 M NaCl) resulted in a complete reversal of topoisomerase I mediated DNA cleavage induced by saintopin

and camptothecin, and a partial reversal of topoisomerase II mediated DNA cleavage induced by saintopin and VP-16 (data not shown). The slightly resistant properties against heat and salt treatments of topoisomerase II mediated DNA cleavage indicate that the drug-induced topoisomerase II–DNA complexes are more stable than the complex with topoisomerase I. This is consistent with previous studies in which the camptothecin-induced topoisomerase I–DNA complexes are less stable to mild detergent than the *m*-AMSA-induced topoisomerase II–DNA complexes (Covey et al., 1989). (2) DNA unwinding assay shows that saintopin is a DNA intercalator with a comparable unwinding potency to that of *m*-AMSA (Figure 6). Previous studies with anthracyclines or ellipticine derivatives indicated that topoisomerase II mediated DNA cleavage by these strong intercalators was suppressed at higher drug concentrations (5 μM) due to the change of DNA conformation which blocks topoisomerase II access to the DNA (Tewey et al., 1984a,b; Capranico et al., 1990; Fosse et al., 1990). In the case of saintopin, however, topoisomerase II mediated DNA cleavage increased in a dose-dependent manner, and no suppression of DNA cleavage was observed at a concentration range up to 12.5 μM. Thus, saintopin in categorized into a class of drugs with low DNA binding affinity such as *m*-AMSA and 5-iminodaunorubicin (Pommier et al., 1987; Capranico et al., 1990), which exhibit induction of DNA cleavage without suppression at higher drug concentrations. (3) The distinct DNA cleavage pattern generated by saintopin is consistent with previous studies in which drugs of different classes showed strictly different cleavage patterns. Recent studies have demonstrated that amonafide (Hsiang et al., 1989b) and Ro 15-0216 (Sorensen et al., 1990) enhanced topoisomerase II mediated DNA cleavage in a highly sequence-dependent manner, differing from *m*-AMSA and epipodophyllotoxin which enhance topoisomerase II mediated DNA cleavage at a variety of DNA sequences. As shown in Figure 7, saintopin induces DNA cleavage at multiple sites with both topoisomerase I and topoisomerase II.

As previously reported, saintopin shows cytotoxicity in vitro, as well as antitumor activity in vivo (Yamashita et al., 1990c). Both topoisomerase I and topoisomerase II mediated DNA cleavage can be induced by saintopin, and so it is interesting to see which topoisomerase is the principal target of saintopin in tumor cells. Comparing the results of topoisomerase I and II mediated DNA cleavage assays in vitro, saintopin shows comparable DNA cleavage activity with both topoisomerases (see Figures 2 and 3). In order to clarify the principal target of saintopin at the cellular level, it will be necessary to study the cytotoxic effects on mammalian cells which are resistant to saintopin.

The data described in this paper should be useful for further studies in the following aspects: (i) With respect to therapeutic application, the possible synergy between the drugs targeting topoisomerase I and topoisomerase II has been suggested. Giovanella et al. (1989) showed that human colon cancer with low sensitivity to the antitumor drugs acting on topoisomerase II is highly sensitive to camptothecin derivatives due to an elevated cellular topoisomerase I activity. Thus, a drug like saintopin which acts on topoisomerse I as well as topoisomerase II is likely to exhibit cytotoxic effects on a variety tumor cells altered in either topoisomerase I or topoisomerase II activities. (ii) Reaction mechanisms of DNA topoisomerases I and II share several common features; both enzymes catalyze DNA strand breakage and rejoining, and transiently form enzyme–DNA covalent links through a similar phosphotyrosine linkage. The data presented here suggest that the structures around the active site of each enzyme have some similarity to permit saintopin to interact with both enzymes by a common mechanism. At present, little is known about how topoisomerase-active drugs form cleavable complexes between enzyme and DNA. Thereby, further comparative studies on the saintopin-induced cleavable complexes with topoisomerases I and II should greatly facilitate determination of the precise molecular mechanism of cleavable complex formation.

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Registry No. Saintopin, 131190-63-1; topoisomerase, 80449-01-0.

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Avian Nuclear Matrix Proteins Bind Very Tightly to Cellular DNA of the β-Globin Gene Enhancer in a Tissue-Specific Fashion†

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ABSTRACT: We have previously shown that a cloned 480 bp DNA fragment that spans the 3′-enhancer region of the avian β-globin gene cluster can become very tightly, perhaps covalently, bound to protein in avian nuclear matrices in vitro [Zenk et al. (1990) *Biochemistry 29*, 5221-5226]. This binding was not tissue-specific and was probably not mediated by topoisomerase enzymes. In the present study, we have examined avian nuclear matrices (or scaffolds) for the presence of very tight cellular DNA-protein complexes in the region of the β-globin gene enhancer and of several other avian genes. Nuclear matrices were prepared by both high- and low-salt methods, and protein-DNA complexes were isolated by SDS/K^+ precipitation after restriction enzyme digestion. In adult reticulocytes, up to 30% of the intact 3800 bp *Hin*dIII-*Eco*RI fragment that encompasses the β-globin enhancer element may be very tightly bound to nuclear matrix protein. In adult avian thymus nuclei, the β-globin enhancer is neither matrix-associated nor tightly bound to protein. In contrast, a 5.0-kb *Hin*dIII fragment of the malic enzyme gene is very tightly bound to nuclear matrix-associated protein in thymus cells, but not reticulocytes. The malic enzyme gene is active in thymus cells, and not in reticulocytes. These results suggest that certain regions of avian cellular DNA are very tightly, perhaps covalently, attached to nuclear matrix-associated proteins. Attachment follows a tissue-specific pattern that is associated with transcriptional activity.

Eukaryotic chromatin is organized into large loops of DNA that are associated with histones and a wide variety non-histone proteins, which together are attached to a salt-resistant structure collectively referred to as the nuclear matrix or scaffold (Berezney & Coffey, 1974; Cook & Brazell, 1975; Benyajati & Worcel, 1976). Evidence suggests that both replication (Pardoll et al., 1980; Blow & Laskey, 1988) and transcription (Jackson et al., 1981; Ciejek et al., 1983; Robinson et al., 1985; Keppel, 1986; Buttyan & Olsson, 1986; Roberge et al., 1988) occur at the interface of DNA with the nuclear matrix. Regions of DNA, capable of mediating matrix attachment [matrix attachment regions (MARs)],[1] have been shown to correspond to enhancer regions in several genes (Cockerill & Garrard, 1987; Loc et al., 1990; Mielke et al., 1990) and autonomously replicating sequences in yeast and humans (Amati & Gasser, 1988; Sykes et al., 1988). It appears that the sequence ATATTT is necessary for matrix attachment (Cockerill & Garrard, 1986; Mielke et al., 1990). This sequence is the core of the consensus sequence for the enzyme topoisomerase II, itself a major constituent of the nuclear matrix (NM) (Berrios et al., 1985; Gasser et al., 1986), and recent evidence has suggested that topoisomerase II may be involved in DNA binding to NMs (Cockerill & Garrard, 1986; Sperry et al., 1989; Adachi et al., 1989; Pommier et al., 1990; Mielke et al., 1990). However, this conclusion has not been universally accepted. We have recently shown that a 480 bp fragment that spans the avian β-globin gene enhancer can bind in vitro to NMs prepared from a variety of cell types and that a fraction of this matrix-associated DNA becomes very tightly, perhaps covalently, bound to non-topoisomerase NM-associated proteins (Zenk et al., 1990). These results were somewhat surprising as the β-globin enhancer region contains

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[1] Abbrevations: Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; MAR, matrix attachment region; NM, nuclear matrix.

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