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Correlation between the Formation of Cleavable Complex with Topoisomerase I and Growth-Inhibitory Activity for Saintopin-Type Antibiotics

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SUMMARY

New saintopin-type antibiotics (e.g., saintopin, saintopin E, UCE1022, UCE6) with a naphthacene-dione structure have been discovered through our mechanistically oriented screening using purified mammalian DNA topoisomerases. Saintopin is a dual inducer of topoisomerase I- and topoisomerase II-mediated DNA cleavages in a cell-free system using purified enzymes, whereas others induced topoisomerase I- but not topoisomerase II-mediated DNA cleavage. The order of topoisomerase I-mediated DNA cleavage activity at lower concentrations (<1 μM) was UCE6 > saintopin > saintopin E > UCE1022. The DNA cleavage-intensity patterns induced by these antibiotics with topoisomerase I were identical, indicating that saintopin-type antibiotics have a similar DNA sequence selectivity in stabilization of the cleavable complex with topoisomerase I. Increases in protein/DNA complexes were observed in saintopin-type antibiotic-treated HeLa S3 cells using the potassium/sodium dodecyl sulfate precipitation method. Brief heating of these drugs-treated cells at 65° for 10 min resulted in a rapid reduction in the number of protein/DNA complexes. Immunoblot analysis using antibody against human topoisomerase I or II revealed that the protein linked to DNA in saintopin-type antibiotic-treated cells is most likely topoisomerase I. These results suggest that saintopin-type antibiotics interfere with topoisomerase I in cells by trapping reversible topoisomerase I/DNA cleavable complexes. The formation of topoisomerase I/DNA complexes by saintopin-type antibiotics correlates well with their growth-inhibitory activities, suggesting that topoisomerase I can be the principal target of these antibiotics.

Topological problems of DNA may arise in the course of cellular processes such as DNA replication, transcription, recombination, repair, chromosome segregation, and maintenance of chromosome structure. During these events, torsional strain of double-strand DNA leads to supercoiling, which inevitably interferes with biological functions. DNA topoisomerases are the enzymes that resolve such problems by catalyzing the concerted breakage and rejoining of DNA strands (1). Two major topoisomerases, topoisomerase I and topoisomerase II, have been identified in all eukaryotic cells; the former type catalyzes the passage of the DNA strand through a transient single-strand break, whereas the latter catalyzes the passage of DNA double strands through a transient double-strand break (1).

In addition to their cellular function, both topoisomerase I and topoisomerase II have generated extensive clinical interest in chemotherapy. There is good evidence that topoisomerases are the principal intracellular targets for a number of clinically important antitumor drugs (2, 3). These drugs, referred to as topoisomerase poisons, include synthetic intercalators (e.g., mAMSA, mitoxantrone), antibiotics from microbes (e.g., anthracyclines, actinomycin D), and derivatives of plant metabolites [e.g., camptothecin derivatives such as CPT-11 and topotecan and epipodophyllotoxin derivatives such as VP-16 (etoposide) and VM-26 (teniposide)] (4–10). These drugs interfere with the breakage-rejoining reaction of topoisomerases by stabilizing key reaction intermediates of topoisomerases ("cleavable complex"), which can be detected as DNA strand breaks on exposure to a strong protein denaturant such as SDS or alkaline (4–6, 11). A number of studies have shown that the clinical efficacies of these drugs correlate with their abilities to induce topoisomerase-mediated DNA cleavage (12–14).

According to this attractive model, we screened microbial cultures, plant extracts, and synthetic compounds for their ability to induce topoisomerase-mediated DNA cleavage in a purified enzyme assay system. As a result of this screening, we found that antitumor indolocarbazole derivatives and blue pigment bulgarein induced topoisomerase I-mediated DNA cleavage (15, 16) and that flavonoids (e.g., genistein and orobol), plant naphthoquinones (e.g., plumbagin and shikonin), and antitumor antibiotics (e.g., streptonigrin and mem-

ABBREVIATIONS: mAMSA, 4′-(9-acridinylamino)methanesulfon-manisidide; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline/0.1% Tween 20; SDS, sodium dodecyl sulfate.

bers of the terpentecin family) induced topoisomerase II-mediated DNA cleavage (17–20). Recently, we further isolated saintopin-type antibiotics, members of a new chemical family with the naphthacene-dione structure that induce the cleavable complex with topoisomerase I (21–23). These compounds include saintopin, saintopin E, and UCE1022, which are produced by fungi; and UCE6, which is produced by actinomycetes. Among these compounds, only saintopin has been identified as the dual inducer of the cleavable complex with both topoisomerase I and topoisomerase II (21).

In the current study, we examined a relationship between the formation of cleavable complex with topoisomerases and growth-inhibitory activity for saintopin-type antibiotics. We report that 1) the order of topoisomerase I-mediated DNA cleavage activity at a lower concentration (<1 μM) is UCE6 $>$ saintopin $>$ saintopin E $>$ UCE1022; 2) these compounds have similar DNA sequence selectivity in stabilization cleavable complex with topoisomerase I; 3) saintopin-type antibiotics stimulate covalent linking of topoisomerase I but not topoisomerase II to chromosomal DNA, as revealed by immunoblot analysis in intact cells; and 4) the levels of topoisomerase I-mediated DNA cleavage induced by these compounds in purified system correlate with the levels of protein/DNA complexes in cultured cells and growth-inhibitory activities against HeLa S3 cells. These data indicate that topoisomerase I is a principal intracellular target of saintopin-type antibiotics and that the drug-induced cleavable complex is responsible for their anticellular activities.

Materials and Methods

Enzymes, nucleic acids, and chemicals. DNA topoisomerases I and II were isolated from calf thymus gland as described previously (16). 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 poison camptothecin or the topoisomerase II-specific poison mAMSA. Thus, the enzymes used in this study was free from contamination of another topoisomerase or endonucleases, which was proved with data that showed no production of nicked or linear DNA in the assay. Topoisomerases were kept at $-20°$ in a storage buffer [30 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.1 mM EDTA, 50% (v/v) glycerol]. One unit of activity was the amount of topoisomerase that relaxed half of the 0.4 μg of supercoiled pUL402 DNA containing the scaffold-associated region from the *Drosophila* histone gene cluster (24). Saintopin, UCE1022, and UCE6 were isolated from cultured broth as reported previously (22, 23, 25). Camptothecin was purchased from Sigma Chemical (St. Louis, MO). mAMSA was a gift from Warner-Lambert (Ann Arbor, MI). Stock solutions of these drugs were dissolved in dimethylsulfoxide, stored at $-20°$, and diluted in methanol containing 20% (v/v) dimethylsulfoxide before use. All other reagents used were of biochemical reagent grade.

Saintopin E isolation. During the high performance liquid chromatographic analysis of the culture broth extract of saintopin-producing strain (25), we found the presence of a novel topoisomerase I-targeting compound, saintopin E, which has UV spectra similar to those of saintopin. The isolation procedure was as follow: saintopin E was extracted with an equal volume of acetone from the culture broth of saintopin-producing strain (15 liters). After filtration, the acetone extract was diluted with water (30 liters) and then applied to a Diaion HP-20 column (2 liters) (Mitsubishi Chemical Industries, Tokyo, Japan). The column was washed with water/methanol (8:2 v/v) and eluted with water/methanol (4:6 v/v). The eluate was diluted with an equal volume of water and then applied to a Diaion HP-20SS column (1 liter) (Mitsubishi). The column was eluted stepwise from water/methanol (6:4 v/v) to water/methanol (4:6 v/v). The active fractions were concentrated and extracted with ethyl acetate at pH 2.5. The organic layer was concentrated and further purified by high performance liquid chromatography (YMC-Pack ODS SH-343-15, ϕ 20 $\times$ 250 mm; YMC, Tokyo, Japan) with water/methanol (3:7 v/v) containing 5 mM ammonium acetate at a flow rate of 10 ml/min. To remove the salt, the active eluate was absorbed on Diaion HP-20SS column (0.5 liter) and washed with water. Saintopin E was eluted with methanol and then evaporated to dryness; 11.2 mg of saintopin E was obtained as bluish-purple powder. The structure of saintopin E was determined by NMR and mass spectrometric analyses (Fig. 1).

Preparation of ^{32}P-end-labeled DNA. Unique 5′-end-labeled probe was prepared as follows. Supercoiled pUL402 DNA (5 pmol) was cut with *Ava*I, and its termini were labeled with [γ-^{32}P]dATP (50 μCi) and T4 polynucleotide kinase (20 units). After *Nru*I digestion, the 5′-end-labeled *Ava*I/*Nru*I fragment (5193 base-pairs, labeled at *Ava*I site) was gel isolated.

Topoisomerase I-mediated DNA cleavage assay. In cleavage reactions, topoisomerase I reactions were performed in 20 μl of reaction buffer (50 mM Tris·HCl, pH 7.5, 100 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin) with 0.4 μg of supercoiled pUL402 DNA (in 1 μl of Tris-EDTA buffer), drug (1 μl), and 20 units of topoisomerase I (1 μl). Reactions were incubated at 37° for 30 min and terminated by the addition of 2 μl of a solution containing 5% SDS and 2.5 mg/ml proteinase K. After an additional incubation at 37° for 30 min, reactions were stopped by the addition of 3.5 μl of 6$\times$ loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, and 15% Ficoll). The samples were run overnight at 2 V/cm onto a 1.2% agarose gel in buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA, 0.1% SDS) containing 0.5 μg/ml ethidium bromide. 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 665 positive/negative film. The amount of

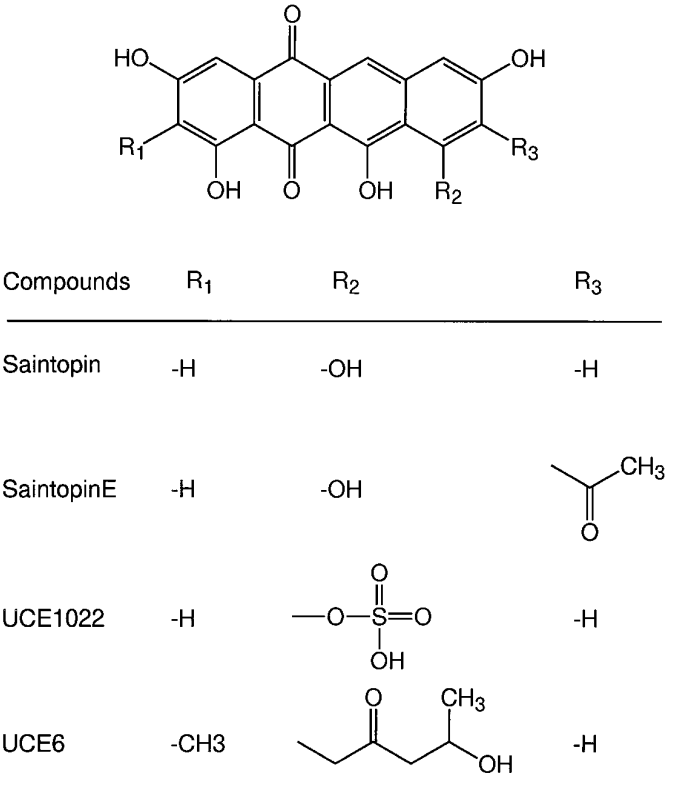

Fig. 1. Structures of saintopin-type antibiotics.

DNA was quantified by scanning the negatives with a Shimazu scanning densitometer (CS-930; Kyoto, Japan). The increase in nicked DNA was estimated as drug-induced topoisomerase I-mediated DNA cleavage.

Comparison of the major cleavage sites. In this experiment, ^{32}P-end-labeled pUL402 DNA was used as substrate for the DNA cleavage reaction with DNA topoisomerase I; 0.04 μg of 5′-end-labeled DNA and 5 units of topoisomerase I were used in the DNA cleavage assay. After the DNA cleavage reaction in the presence or absence of drugs, the DNA was extracted with 1-butanol, phenol, and ether and then precipitated with ethanol. The obtained DNA was dissolved in 10 μl of alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, 0.025% bromcresol green) and run overnight at 2.5 V/cm onto a 1.5% alkaline agarose gel in 30 mM NaOH/1 mM EDTA buffer containing 0.1% SDS. Gels were dried on 3-mm paper sheets and autoradiographed for 12 hr.

Cells and cell culture. HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, MD). HeLa S3 cells were cultured in Eagle's minimal essential medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% (v/v) heat-inactivated (56° for 30 min) fetal bovine serum, 2 mM L-glutamine, 0.1% (w/v) sodium bicarbonate, 50 μg/ml penicillin, and 85 μg/ml streptomycin. Cultures were incubated at 37° in a water-saturated atmosphere containing 5% CO_2.

Potassium/SDS precipitation method for protein/DNA complexes. To determine the amount of the covalent protein/DNA complexes in cells, the potassium/SDS precipitation method described by Rowe *et al*. (12) was modified as follows. The DNA in logarithmically growing cells (2×10^6 cells/ml) was labeled by the addition of [*methyl*-^{3}H]thymidine into the medium to a final concentration of 0.9 μCi/ml. After overnight incubation, cells were washed three times in PBS (137 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4). The trypsinized cells were resuspended in fresh medium to a final concentration of 10^5 cells/ml. Cells were aliquoted (1 ml each) onto a 24-well microtiter plate (Falcon) and incubated for an additional 2 hr at 37°. The cells were then treated with various concentrations of drugs (5 μl) for 30 min. The medium was removed from each well, and cells were lysed by the addition of 1 ml of prewarmed (65°) lysis solution (1.25% SDS, 5 mM EDTA, 0.4 mg/ml salmon sperm DNA). The cellular DNA in the lysate was sheared by pipetting it up and down 30 times with C20 micropipette tips (Gilson) and the addition of 250 μl of 325 mM KCl. After vigorous vortexing, the sample was cooled on ice for 10 min and centrifuged for 15 min at 4°. The pellet was resuspended in 1 ml of a prewarmed (65°) wash solution (10 mM Tris·HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA) and then incubated at 65° for 10 min with occasional mixing. The suspension was cooled on ice for 10 min and then recentrifuged for 10 min at 4°. The pellet was washed again before resuspension in 200 μl of H_2O (65°). The suspension was then combined with 4 ml of a scintillation liquid (Clear-sol I; Nacalai Tesque, Kyoto, Japan), and the radioactivity was determined.

Immunoblot analysis of whole-cell lysates. HeLa S3 cells (1.5×10^5 cells/well) were preincubated at 37° for 17 hr and then treated with various concentrations of drugs for 30 min. The medium was removed, and cells were suspended in 100 μl of 2× SDS sample buffer (125 mM Tris·HCl, pH 6.8, 6% SDS, 10% β-mercaptoethanol, 20% glycerol). After being heated in a boiling water bath for 10 min, boiled lysates were separated electrophoretically on a 7.5% SDS-polyacrylamide gel and electroblotted onto Immunobilon PVDF (Millipore, Bedford, MA). The filter was incubated overnight at 4° in PBST containing 5% (w/v) skim milk and then hybridized with polyclonal antibody to human topoisomerase I from serum from a patient with scleroderma or rabbit anti-human topoisomerase II antibody (TopoGEN, Columbus, OH) for 1 hr at room temperature. After being washed three times in PBST with shaking, peroxidase-conjugated rabbit anti-human IgA, IgG, IgM, κ, λ, or swine anti-rabbit immunoglobulins (DAKOPATTS, Glostrup, Denmark) was added for 1 hr at room temperature. After being washed five times in PBST with shaking, topoisomerase bands were detected using ECLTM Western blotting detection reagents (Amersham International, Buckinghamshire, UK). Band intensities were quantified by scanning the film with a Shimazu scanning densitometer (CS-930). The decrease in the intensity of the topoisomerase I (molecular mass, 100 kDa) band or topoisomerase II (molecular mass, 170 kDa) band in lysates of drug-treated cells relative to the bands in untreated control cells was expressed in percentage of enzyme depletion.

Growth-inhibitory activity. The growth-inhibitory effects were measured according to the method described by Mirabelli *et al*. (26) with minor modifications. HeLa S3 cells (1.5×10^5 cells/well) were preincubated at 37° for 24 hr in 96-well microtiter plates (Nunc, Roskilde, Denmark) and then treated with different dilutions of drugs for 3 days. After the cells were washed in PBS, they were fixed with methanol at room temperature for 10 min. The methanol was then removed, and 100 μl of Giemsa stain solution [Giemsa solution/PBS (1:10); Merck] was added to each well. After a 5-min incubation, the cells were washed in 0.9% (w/v) NaCl solution and then solubilized with 200 μl of 0.1 N HCl. Absorbances were measured with a microplate reader (Corona Electric, Ibaragi, Japan) at a sample wavelength of 630 nm and a reference wavelength of 415 nm. The growth-inhibitory activity of drugs was expressed by the IC_{50} value [concentration of drug required to reduce the absorbance to 50% of that in control cultures (drug-free)].

DNA (un)winding measurement. DNA (un)winding effects were assayed according to the method described by Camilloni *et al*. (27) with minor modifications. 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 pUL402 DNA linearized with *Hin*dIII restriction endonuclease, and drug (5 μl) were equilibrated at 15° for 15 min and followed by incubation with an excess amount of T4 DNA ligase (200 units) at 15° for 60 min. The reactions were stopped by the addition of EDTA at a final concentration of 20 mM. DNAs were extracted with 1-butanol, phenol, and ether and then precipitated with ethanol to remove the drug. Two-dimensional electrophoresis was performed as described previously (16).

Results and Discussion

Topoisomerase I-mediated DNA cleavage by saintopin-type antibiotics. Saintopin-type antibiotics (Fig. 1) were discovered in the course of screening for topoisomerase I-targeting antitumor drugs (21–23). To gain better understanding of the interaction with topoisomerase I, we compared the topoisomerase I-mediated DNA cleavage activities of these compounds by using purified calf thymus topoisomerase I and supercoiled plasmid pUL402 DNA. In the DNA cleavage assay, topoisomerase I-mediated DNA cleavage is represented by a conversion of closed circular DNA into slower migrating nicked DNA in an agarose gel containing 0.5 μg/ml ethidium bromide.

As shown in Fig. 2A, saintopin-type antibiotics produced the nicked DNA in a manner similar to that of camptothecin. In the absence of topoisomerase I, these compounds did not induce any DNA band changes in a gel (data not shown). To estimate the potency of topoisomerase I-mediated DNA cleavage, the amounts of nicked DNA were measured by scanning the negatives with densitometer. As shown in Fig. 2B, UCE6 showed the most potent topoisomerase I-mediated DNA cleavage activity, comparable to that of camptothecin. The DNA cleavage activity of UCE6 reached saturation at 1 μM, and the maximal amount of nicked DNA was ~50% of substrate DNA. The saturation of the DNA cleavage activity was supposed to be due to the low solubility of UCE6 in the reaction mixture. Saintopin showed the intermediate DNA

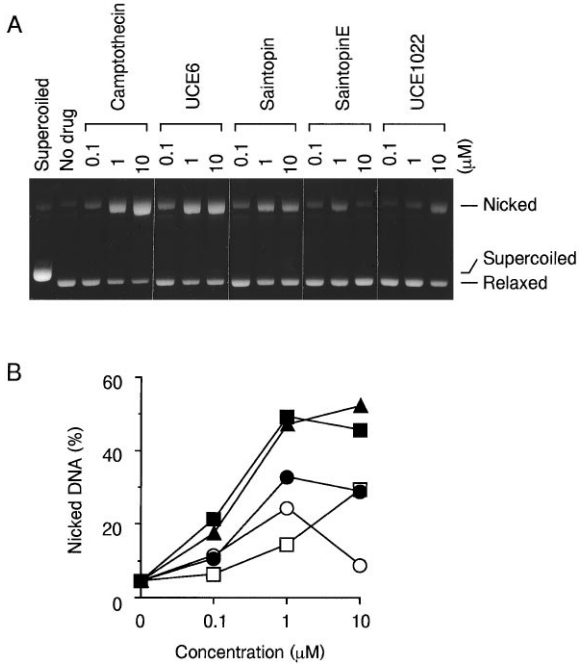

Fig. 2. Topoisomerase I-mediated DNA cleavage activities induced by saintopin-type antibiotics. A, supercoiled pUL402 DNA (0.4 μg) was incubated with 20 units of topoisomerase I in the presence of the drugs at various concentrations, followed by SDS/proteinase K treatment, and then analyzed on an agarose gel containing 0.5 μg/ml ethidium bromide. B, Percentage of nicked DNA induced by drugs in the presence of topoisomerase I was determined by scanning the photograph negatives with a densitometer as described in Materials and Methods. ▲, Camptothecin; ■, UCE6; ●, saintopin; ○, saintopin E; □, UCE1022.

cleavage activity that was one half of that of UCE6, and the maximal amount of nicked DNA was ~30% of substrate DNA at a concentration of 1 μM. UCE1022 and saintopin E showed weak DNA cleavage activity. The DNA cleavage activity of UCE1022 was dose dependent, and the maximum amount of nicked DNA was ~30% of substrate DNA at a drug concentration of 10 μM. On the other hand, the DNA cleavage activity of saintopin E was reached a maximum at a concentration of 1 μM (the maximal amount of nicked DNA was ~25% of substrate DNA) and was suppressed at >10 μM. This result is similar to the bell-shaped curve for DNA breaks versus drug concentration as a function of DNA (un)winding topoisomerase poisons (7, 16, 28–30). However, as described in the following section, no (un)winding activity was detected for saintopin E. On the basis of these observations, the possible explanations for the self-inhibition of topoisomerase I-mediated DNA cleavage activity by saintopin E were 1) that saintopin E interacts with DNA in some non-DNA (un)winding mode that is undetectable with the methodology used; or 2) that saintopin E interacts directly with topoisomerase I, rather than with DNA, and denatures the tertiary structure of the enzyme, resulting in inhibition of the catalytic activity of topoisomerase I in a nonspecific manner.

Major DNA cleavage sites induced by topoisomerase I in the presence of saintopin-type antibiotics. Antitumor topoisomerase poisons display various clinical activities. Previous studies have suggested that this variability could be related to DNA sequence selectivity of topoisomerase-mediated DNA cleavage sites, which differ for each class of drug (31). The major DNA cleavage sites induced by topoisomerase I in the presence of saintopin-type antibiotics were therefore mapped using a DNA cleavage assay with a uniquely 5′-end-labeled pUL402 *Ava*I/*Nru*I fragment (labeled at the *Ava*I site).

As shown in Fig. 3, saintopin-type antibiotics stimulated DNA cleavage at identical sites with the same relative intensity, resulting in identical DNA cleavage patterns. These observations were confirmed by additional experiments with different pUL402 DNA fragments (data not shown). Thus, the difference in the side chains of the saintopin chromophore did not influence the sequence selectivity of the drug-induced DNA cleavage sites; saintopin-type antibiotics and camptothecin, two chemically unrelated drugs, also induced similar topoisomerase I-mediated DNA cleavage patterns. This result is consist with previous findings that a majority of the cleavage sites induced by saintopin are subsets of camptothecin sites (32). Similar cleavage patterns induced by different chemical classes have been reported for topoisomerase II-targeting drugs mitoxantrone and VM-26 (33). Further investigations were needed to clarify whether similar sequence selectivity of topoisomerase-targeting drugs actions may contribute to their cytotoxic profiles.

Protein/DNA complex formations in intact cells by saintopin-type antibiotics. Topoisomerase-targeting antitumor drugs stabilize covalent topoisomerase/DNA complexes that are intermediates in the catalytic cycle of these enzymes (2). From these mechanisms of drugs action, cells that are treated with topoisomerase poisons accumulate protein/DNA complexes. In addition, topoisomerases/DNA complexes in cultured cells have been shown to be rapidly reversed by elevated temperature (34, 35). To test whether saintopin-type antibiotics induce reversible protein/DNA

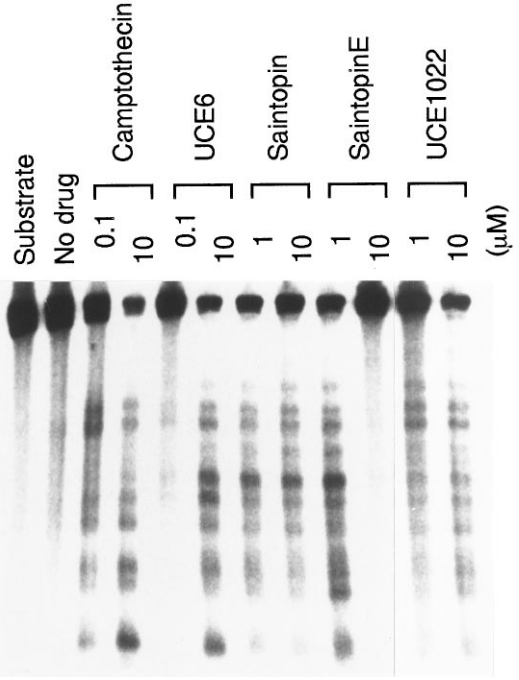

Fig. 3. Comparison of DNA cleavage intensity patterns induced by saintopin-type antibiotics with topoisomerase I. In this assay, unique ^{32}P-end-labeled pUL402 DNA *Ava*I/*Nru*I fragments (labeled at *Ava*I site) were used. Topoisomerase I-mediated DNA cleavage assay was performed as described in Materials and Methods. Single-strand DNA fragments were analyzed with an alkaline agarose gel.

complexes, the levels of protein/DNA complexes in drug-treated cells were measured using the potassium/SDS precipitation method.

As shown in Table 1, saintopin-type antibiotics induced potassium/SDS precipitates in HeLa S3 cells, and these precipitates were abolished with prior proteinase K treatment of the cell lysate, indicating that saintopin-type antibiotics induce protein/DNA complexes in intact cells. In addition, brief heating (65° for 10 min) of drug-treated cells before lysis with SDS reduced the amount of the protein/DNA complexes to the background level. Based on these combined results, it is considered that the protein/DNA complexes in saintopin-type antibiotics-treated cells are due to topoisomerase-mediated cleavable complexes.

Furthermore, in this assay, UCE6, which induced the highest potency of DNA cleavage by topoisomerase I in purified enzyme assay system, also induced the highest level of protein/DNA complexes in cultured cells, whereas saintopin, which demonstrated intermediate topoisomerase I-mediated DNA cleavage that was one half of that of UCE6, induced an intermediate level of protein/DNA complex that was ~2-3-fold less than that of UCE6. Saintopin E and UCE1022, which have weak effects in topoisomerase I-mediated DNA cleavage, induced correspondingly lower levels of protein/DNA complexes in HeLa S3 cells. On the basis of these results, the levels of protein/DNA complexes in cultured cells induced by saintopin-type antibiotics correlate with the drug-induced topoisomerase I-mediated DNA cleavage activity in the purified assay system, suggesting that topoisomerase I is responsible for saintopin-type antibiotic-induced protein/DNA complex formations.

Specific covalent complexes between topoisomerase I and chromosomal DNA in intact cells by saintopin-type antibiotics. The possibility that topoisomerase I was responsible for saintopin-type antibiotic-induced protein/DNA complexes was further supported by the following experiment. Proteins in whole-cell lysates from drug-treated and control cells were separated by SDS-polyacrylamide gel, electrotransferred, and immunoblotted with either topoisomerase I-specific (Fig. 4A) or topoisomerase II-specific (Fig. 4B) antibodies.

As shown in Fig. 4A, the intensities of 100-kDa molecular mass topoisomerase I protein bands were decreased after treatment of HeLa S3 cells with saintopin-type antibiotics in a similar manner with camptothecin. The decrease in topoisomerase I molecules in the gel can be explained by the covalent linking of the topoisomerase I molecules to chromosomal DNA, which are too large to enter the SDS-polyacrylamide gel (34). To estimate the intracellular topoisomerase depletion, the protein bands (Fig. 4, A and B, *top*) were measured by scanning densitometry and expressed as enzyme depletion relative to the control (Fig. 4, A and B, *bottom*). As shown in Fig. 4A (*bottom*), UCE6 showed the most potent reduction of the topoisomerase I protein band, comparable to that of camptothecin. Almost all of the topoisomerase

TABLE 1

Induction of reversible protein/DNA complexes in HeLa S3 cells by saintopin-type antibiotics

Protein/DNA complexes in drug-treated cells were measured by using the potassium/SDS precipitation method as described in Materals and Methods. The total trichloroacetic acid-precipitable cpm for each sample average was ~4.5 × 10^4.

Compound	Radioactivity precipitated by K/SDS procedure with increasing drug concentrations				
	0 μM	0.01 μM	0.1 μM	1 μM	10 μM
			%		
Camptothecin	2.1	5.0	10.8 (2.4)[a]	23.6 (3.5)[a] (4.9)[b]	34.5 (3.8)[a] (5.3)[b]
UCE6	2.1	4.5	14.9 (3.0)[a]	33.3 (4.0)[a] (5.3)[b]	31.2 (4.2)[a] (5.5)[b]
Saintopin	2.1	2.2	3.3 (3.0)[a]	8.6 (2.1)[a] (2.1)[b]	17.0 (3.0)[a] (4.2)[b]
Saintopin E	2.1	2.7	2.9 (3.0)[a]	4.3 (1.7)[a] (1.8)[b]	7.2 (2.3)[a] (2.8)[b]
UCE1022	2.1	2.1	2.1 (2.0)[a]	3.2 (2.0)[a] (1.6)[b]	13.9 (2.5)[a] (3.0)[b]

[a] Cell lysates were treated with proteinase K (400 μg/ml) at 37° for 2 hr.
[b] Drugs-treated cells were heated to 65° for 10 min before SDS lysis.

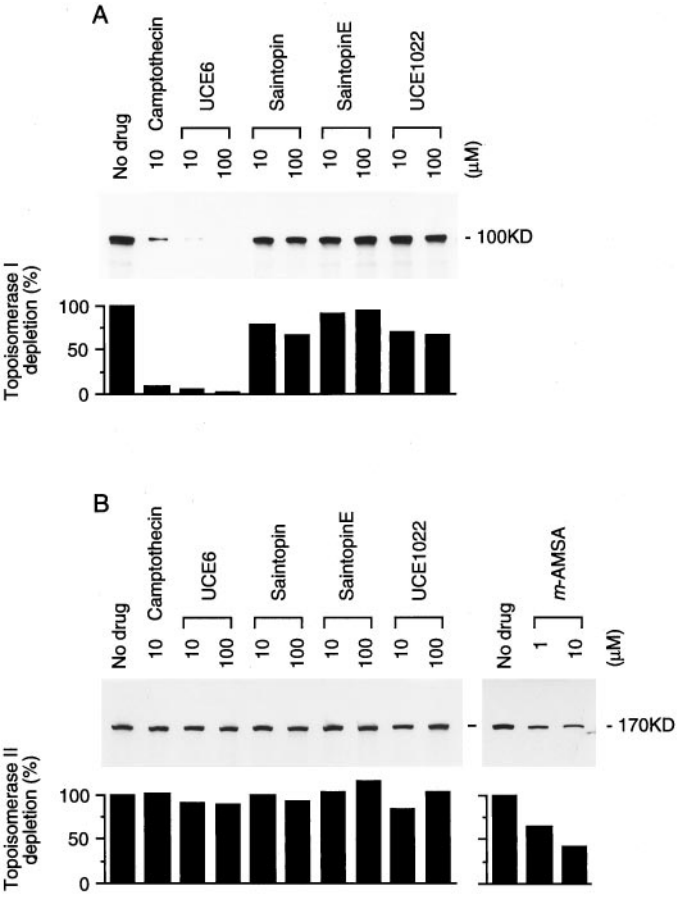

Fig. 4. Immunoblot analysis of lysates of HeLa S3 cells treated with saintopin-type antibiotics. Immunoblot analysis of whole-cell lysates was performed as described in Materials and Methods. A, *Top*, immunoblot probed with antibody against topoisomerase I. *Bottom*, quantification of topoisomerase I protein levels. The topoisomerase I bands (molecular mass, 100 kDa) were measured by scanning densitometry, and the topoisomerase I depletion caused by covalent DNA linkage was expressed as a percentage of the control level. B, *Top*, immunoblot probed with antibody against topoisomerase II. *Bottom*, quantification of topoisomerase II protein levels. The topoisomerase II bands (molecular mass, 170 kDa) were measured by scanning densitometry, and the topoisomerase II depletion were expressed as a percentage of the control level.

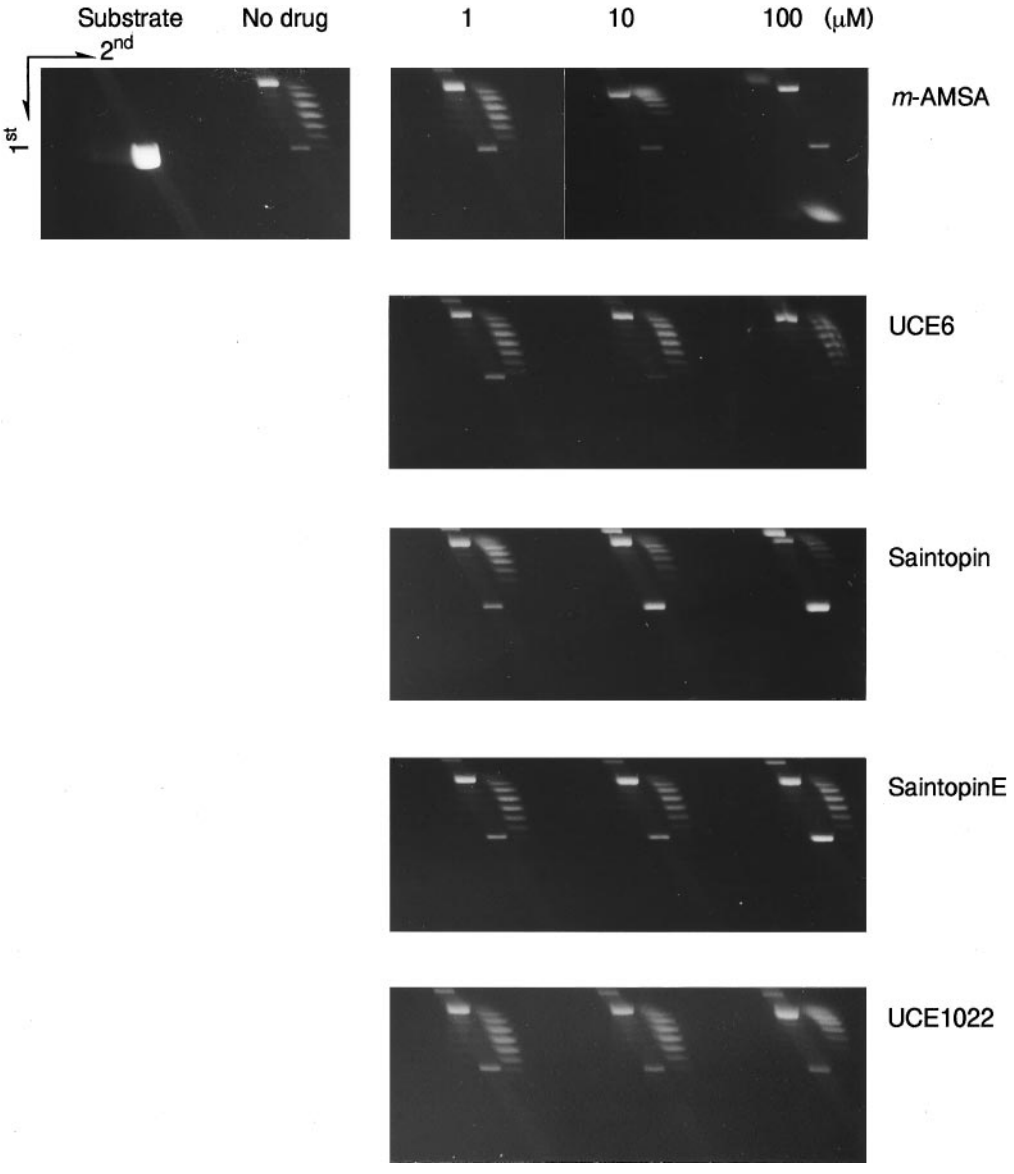

Fig. 5. Unwinding of DNA by saintopin-type antibiotics. (Un)winding measurements were performed as described in Materials and Methods. In this assay, linearized plasmid DNA was incubated with T4 DNA ligase in the presence of the drugs at various concentrations. Drug-induced DNA (un)winding was analyzed by two-dimensional gel electrophoresis. *Arrows*, directions of first and second runs.

I band was depleted by UCE6 treatment at a concentration of 100 μM. Treatment of HeLa S3 cells with saintopin or UCE1022 (100 μM) caused a ~30% reduction in the topoisomerase I band. Saintopin E, which has the lowest potency of protein/DNA complex formation in cultured cells, showed marginal topoisomerase I band depletion at the test concentration. These reductions in topoisomerase I bands were consistent with the levels of protein/DNA complex formations in saintopin-type antibiotic-treated cells (see Table 1). Although the intensities of 170-kDa molecular mass topoisomerase II protein bands did not reduce after treatment with saintopin-type antibiotics, ~60% of topoisomerase II bands were reduced by treatment with 10 μM mAMSA (Fig. 4B). These results indicate that saintopin, a dual inducer of the cleavable complex with both topoisomerase I and topoisomerase II that has a potency similar to that of camptothecin and mAMSA in a purified system (21), stimulates the covalent complex between topoisomerase I and chromosomal DNA at the cellular level specifically.

Recently, Taniguchi *et al.* (36) reported that saintopin-resistant KB cell lines KB/STP-1 and KB/STP-2 showed a markedly higher cross-resistance to camptothecin analogs than KB cells but only weak cross-resistance to VP-16. They mentioned that development of resistance to saintopin in KB cells seemed to be associated with preferential changes in the topoisomerase I-mediated pathway rather than in that mediated by topoisomerase II. These data are consistent with our results that topoisomerase I is the intracellular target of saintopin-type antibiotics. Further studies are needed to clarify why saintopin stabilizes topoisomerase I/DNA complexes specifically in HeLa S3 cells.

Effects of saintopin-type antibiotics on a DNA (un)winding assay. Drugs stabilizing cleavable complexes with topoisomerases can be classified into four groups: 1) intercalator (e.g., *m*-AMSA, anthracyclines, mitoxantrone, and actinomycin D) (5–7, 9), 2) DNA winding agents that alter the DNA structure in a manner leading to tightening of the helical twist (e.g., bulgarein and streptonigrin) (16, 37), 3) DNA minor groove-binding agents (e.g., bisbenzimides) (30), and 4) non-DNA-interactive agents (e.g., camptothecin derivatives, epipodophyllotoxin derivatives, and members of the terpentecin family) (4, 11, 19). In a previous section, we

TABLE 2
Correlation between the topoisomerase I-mediated activity and growth-inhibitory activity for saintopin-type antibiotics

Compound	Topoisomerase I-mediated DNA cleavage activity[a]	Protein/DNA complex formation in HeLa S3 cells[b]	Effect on DNA[c]	Growth inhibition (IC_{50})[d]
Camptothecin	0.12	0.081	No activity	0.095
UCE6	0.073	0.033	No activity	0.09
Saintopin	0.25	1.4	Unwinding	1.20
Saintopin E	0.4	>10	No activity	>10
UCE1022	2.1	4.0	Unwinding	>10

[a] The concentrations of drugs (μM) that produce nicked DNA at a yield of 20% of substrate DNA in cell-free assay (purified enzyme assay system) (see Fig. 2).
[b] The concentrations of drugs (μM) that precipitate 10% of total trichloroacetic acid-precipitable cpm in cell based assay (potassium/SDS precipitation method) (see Table 1).
[c] The DNA-binding properties were measured using DNA (un)winding assay as described in Materials and Methods. Unwinding, unwinding property; No activity, no (un)winging property at concentration of ≤100 μM (see legend to Fig. 5 and Ref. 11).
[d] The growth-inhibitory activities against HeLa S3 cells were expressed by the IC_{50}, the concentrations of drugs (μM) required for 50% inhibition of cell growth over 72 hr of exposure. Cell growth was quantified by absorbance measurement (at 630 nm) in the microtiter plate assay as described in Materials and Methods.

presented data showing that saintopin E suppresses the topoisomerase I-mediated DNA cleavage activity at a higher drug concentration. The bell-shaped concentration-response curves were reported in the case of DNA-binding topoisomerase poisons such as anthracyclines, mitoxantrone derivatives, bulgarein, and bisbenzimides (7, 16, 30, 38). To determine whether the self-inhibition of topoisomerase-mediated DNA cleavage activity at a higher saintopin E concentration could be due to the effects of the drugs on DNA, the DNA-binding activities were measured using a DNA (un)winding assay. In this assay, DNA (un)winding activity of drugs was monitored by the changes in linking number of DNA on two-dimensional agarose gel electrophoresis.

As shown in Fig. 5, saintopin and UCE1022 induced slight DNA band shifts to the negatively supercoiled form. The DNA unwinding activities observed for saintopin and UCE1022 were less than those of weak intercalator *m*-AMSA. In contrast, saintopin E and UCE6 did not modify the DNA topoisomer distribution at concentrations of 0.1–100 μM, indicating that these compounds did not display detectable (un)winding properties at pharmacologically relevant drug concentrations (≤100 μM). The absence of DNA (un)winding activity for saintopin E was further confirmed by another measurement using a relaxed form of DNA and an excessive amount of topoisomerase II (see Ref. 16) (data not shown). On the basis of these results, we consider the self-inhibition of topoisomerase-mediated DNA cleavage activity at a higher concentration of saintopin E to be due to mechanism(s) other than DNA (un)winding mode.

Correlation between topoisomerase I-mediated activity and growth-inhibitory activity. The effect of topoisomerase I-mediated DNA cleavage activity in the purified system, the protein/DNA complex formation in cultured cells, on DNA and growth-inhibitory activity against HeLa S3 cells of the saintopin-type antibiotics is summarized in Table 2 in comparison with camptothecin. UCE6, with the most potent activity on the formation of topoisomerase I/DNA cleavable complex in both the purified system and cultured cells, showed the most potent growth inhibition against HeLa S3 cells, with an IC_{50} value of 0.09 μM, which was comparable to that of camptothecin. In contrast, saintopin E and UCE1022, with the lowest topoisomerase I-mediated activity, showed the least growth inhibition, and saintopin was intermediate in terms of topoisomerase I-mediated activity and growth inhibition. As expected, the effect on DNA were not quantitatively correlated with growth-inhibitory or topoisomerase I-mediated DNA cleavage activity. The good correlation between topoisomerase I-mediated activity and growth-inhibitory activity suggests that topoisomerase I is a principal target of saintopin-type antibiotics and the drug-induced cleavable complex is responsible for their anticellular activities.

DNA topoisomerases are now viewed as important therapeutic targets in cancer chemotherapy. A number of clinically useful antitumor drugs (e.g., mAMSA, adriamycin, VP-16, and VM-26) have been shown to induce cleavable complexes with topoisomerase II; however, clinically, camptothecin derivatives are only topoisomerase I-targeting antitumor drugs. Giovanella *et al*. (39) reported a correlation between the expression level of topoisomerase I and the extent of malignancy of human colon cancer. Furthermore, Husain *et al*. (40) showed a distinct increase of topoisomerase I in colorectal and prostate tumors compared with their normal tissue counterparts. Therefore, the identification of new drugs that induce cleavable complexes with topoisomerase I is considered a promising approach to the search for clinically effective antitumor drugs. In an attempt to find new topoisomerase I-targeting antitumor drugs, saintopin-type antibiotics were discovered in our mechanistically oriented screening using purified enzymes. We report that these antibiotics act selectively on topoisomerase I at the cellular level. Among them, UCE6 showed the most potent topoisomerase I-mediated activity and growth-inhibitory activity against HeLa S3 cells (Table 2) and other various tumor cell lines, comparable to the action of camptothecin *in vitro*.[1]

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References

1. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **54:** 665–697 (1985).
2. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58:**351–375 (1989).
3. Muggia, F. M., and H. A. Burris. Clinical development of topoisomerase-interactive drugs. *Adv. Pharmacol.* **29B:**1–31 (1994).
4. Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259:**13560–13566 (1984).
5. Nelson, E. M., K. M. Tewey, and L. F. Liu. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. *Proc. Natl. Acad. Sci. USA* **81:**1361–1365 (1984).
6. Tewey, K. M., T. C. Rowe, L. Yang, B. D. Halligan, and L. F. Liu. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science (Washington D. C.)* **226:**466–468 (1984).

[1] N. Fujii and H. Nakano, unpublished observations.

7. Capranico, G., F. Zunino, K. W. Kohn, and Y. Pommier. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA binding affinity and cytotoxicity. *Biochemistry* **29:**562–569 (1990).
8. Kaneda, N., H. Nagata, T. Furuta, and T. Yokokura. Metabolism and pharmacokinetics of the camptothecin analog CPT-11 in the mouse. *Cancer Res.* **50:**1715–1720 (1990).
9. Wassermann, K., J. Markovits, C. Jaxel, G. Capranico, K. W. Kohn, and Y. Pommier. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol. Pharmacol.* **38:**38–45 (1990).
10. Kingsbury, W. D., J. C. Boehm, D. R. Jakas, K. G. Holden, S. M. Hecht, G. Gallagher, M. J. Caranfa, L. F. Mccabe, L. F. Faucette, R. K. Johnson, and R. P. Hertzberg. Synthesis of water-soluble (aminoalkyl) camptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J. Med. Chem.* **34:**98–107 (1991).
11. Hsiang, Y. H., R. Hertzberg, S. Hecht, and L. F. Liu. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.* **260:**14873–14878 (1985).
12. Rowe, T. C., G. L. Chen, Y. H. Hsiang, and L. F. Liu. DNA damage by antitumor acridines mediated by mammalian DNA topoisomerase II. *Cancer Res.* **46:**2021–2026 (1986).
13. Jaxel, C., K. W. Kohn, M. C. Wani, M. E. Wall, and Y. Pommier. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. *Cancer Res.* **49:**1465–1469 (1989).
14. Yamato, M., Y. Takeuchi, K. Hashigaki, Y. Ikeda, M.-r. Chang, K. Takeuchi, M. Matsushima, T. Tsuruo, T. Tashiro, S. Tsukagoshi, Y. Yamashita, and H. Nakano. Synthesis and antitumor activity of fused tetracyclic quinoline derivatives. 1. J. *Med. Chem.* **32:**1295–1300 (1989).
15. Yamashita, Y., N. Fujii, C. Murakata, T. Ashizawa, M. Okabe, and H. Nakano. Induction of mammalian DNA topoisomerase I mediated DNA cleavage by antitumor indrocarbazole derivatives. *Biochemistry* **31:**12069–12075 (1992).
16. Fujii, N., Y. Yamashita, Y. Saitoh, and H. Nakano. Induction of mammalian DNA topoisomerase I-mediated DNA cleavage and DNA winding by bulgarein. *J. Biol. Chem.* **268:**13160–13165 (1993).
17. Yamashita, Y., S. Kawada, and H. Nakano. Induction of mammalian topoisomerase II dependent DNA cleavage by nonintercalative flavonoids, genistein and orobol. *Biochem. Pharmacol.* **39:**737–744 (1990).
18. Yamashita, Y., S. Kawada, N. Fujii, and H. Nakano. Induction of mammalian topoisomerase II dependent DNA cleavage by antitumor antibiotic streptonigrin. *Cancer Res.* **50:**5841–5844 (1990).
19. Kawada, S., Y. Yamashita, N. Fujii, and H. Nakano. Induction of a heat-stable topoisomerase II-DNA complex by nonintercalative terpenoides, terpentecin and clerocidin. *Cancer Res.* **51:**2922–2925 (1991).
20. Fujii, N., Y. Yamashita, Y. Arima, M. Nagashima, and H. Nakano. Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. *Antimicrob. Agents Chemother.* **36:**2589–2594 (1992).
21. Yamashita, Y., S. Kawada, N. Fujii, and H. Nakano. Induction of mammalian DNA topoisomerase I and II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. *Biochemistry* **30:**5838–5845 (1991).
22. Fujii, N., Y. Yamashita, S. Chiba, Y. Uosaki, Y. Saitoh, Y. Tuji, and H. Nakano. UCE6, a new antitumor antibiotic with topoisomerase I mediated DNA cleavage activity, from actinomycetes. *J. Antibiotics* **46:**1173–1174 (1993).
23. Fujii, N., Y. Yamashita, K. Ando, T. Agatsuma, Y. Saitoh, K. Gomi, Y. Nishiie, and H. Nakano. UCE1022, a new antitumor antibiotic with topoisomerase I mediated DNA cleavage activity, from *Paecilimyces*. *J. Antibiotics* **47:**949–950 (1994).
24. Adachi, Y., E. Kas, and U. K. Laemmli. Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO J.* **8:**3997–4006 (1989).
25. Yamashita, Y., Y. Saitoh, K. Ando, K. Takahashi, H. Ohno, and H. Nakano. Saintopin, a new antitumor antibiotic with topoisomerase II dependent DNA cleavage activity, from *Paecilimyces*. *J. Antibiot.* **43:**1344–1346 (1990).
26. Kirabelli, C. K., H. Bartus, J. O. L. Bartus, R. Johnson, S. M. Mong, C. P. Sung, and S. T. Crooke. Application of a tissue culture microtiter test for the detection of cytotoxic agents from natural products. *J. Antibiot.* **38:**758–766 (1985).
27. Camilloni, G., F. D. Seta, R. Negri, A. G. Ficca, and E. D. Mauro. Structure of RNA polymerase II promoters: conformational alterations and template properties of circularized *Saccharomyces cerevisiae GAL1-GAL10* divergent promoters. *EMBO J.* **5:**763–771 (1986).
28. Pommier, Y., R. E. Schwartz, L. A. Zwelling, and K. W. Kohn. Effects of DNA intercalating agents on topoisomerase II induced DNA strand cleavage in isolated mammalian cell nuclei. *Biochemistry* **24:**6406–6410 (1985).
29. Capranico, G., A. Riva, S. Tinelli, T. Dasdia, and F. Zunino. Markedly reduced level of anthracycline-induced DNA strand breaks in resistant P388 leukemia cells and isolated nuclei. *Cancer Res.* **47:**3752–3756 (1987).
30. Chen, A. Y., C. Yu, B. Gatto, and L. F. Liu. DNA minor groove-binding ligands: a different class of mammalian DNA topoisomerase I inhibitors. *Proc. Natl. Acad. Sci. USA* **90:**8131–8135 (1993).
31. Pommier, Y., A. Orr, K. W. Kohn, and J.-F. Riou. Differential effects of amsacrine and epipodophyllotoxins on topoisomerase II cleavage in the human c-*myc* protooncogene. *Cancer Res.* **52:**3125–3130 (1987).
32. Leteurtre, F., A. Fujimori, A. Tanizawa, A. Chhabra, A. Mazumder, G. Kohlhagen, H. Nakano, and Y. Pommier. Saintopin, a dual inhibitor of topoisomerases I and II, as a probe for drug-enzyme interactions. *J. Biol. Chem.* **269:**28702–28707 (1994).
33. Capranico, G., P. D. Isabella, S. Tinelli, M. Bigioni, and F. Zunino. Similar sequence specificity of mitoxantrone and VM-26 stimulation of in vitro DNA cleavage by mammalian DNA topoisomerase II. *Biochemistry* **32:**3038–3046 (1993).
34. Hsiang, Y. H., and L. F. Liu. Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res.* **48:**1722–1726 (1988).
35. Hsiang, Y. H., and L. F. Liu. Evidence for the reversibility of cellular DNA lesion induced by mammalian topoisomerase II poisons. *J. Biol. Chem.* **264:**9713–9715 (1989).
36. Taniguchi, K., K. Kohno, K. Kawakami, M. Wada, T. Kanematsu, and M. Kuwano. Drug-induced down-regulation of topoisomerase I in human epidermoid cancer cells resistant to saintopin and camptothecin. *Cancer Res.* **56:**2348–2354 (1996).
37. Capranico, G., M. Palumbo, S. Tinelli, and F. Zunino. Unique sequence specificity of topoisomerase II DNA cleavage stimulation and DNA binding mode of streptonigrin. *J. Biol. Chem.* **269:**25004–25009 (1994).
38. Isabella, P. D., G. Capranico, M. Palumbo, C. Sissi, A. P. Krapcho, and F. Zunino. Sequence selectivity of topoisomerase II DNA cleavage stimulated by mitoxantrone derivatives: relationships to drug DNA binding and cellular effects. *Mol. Pharmacol.* **43:**715–721 (1993).
39. Giovanella, B. C., J. S. Stehlin, M. E. Wall, M. C. Wani, A. W. Nicholas, L. F. Liu, R. Silber, and M. Potmesil. DNA topoisomerase I-targeted chemotherapy of human colon cancer in xenografts. *Science (Washington D. C.)* **246:**1046–1048 (1989).
40. Husain, I., J. L. Mohler, H. F. Seigler, and J. M. Besterman. Elevation of topoisomerase I messenger RNA, protein, and catalytic activity in human tumors: demonstration of tumor-type specificity and implications for cancer chemotherapy. *Cancer Res.* **54:**537–546 (1994).

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