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Topostatin, a Novel Inhibitor of Topoisomerases I and II Produced by *Thermomonospora alba* Strain No. 1520

I. Taxonomy, Fermentation, Isolation and Biological Activities

KEITAROU SUZUKI, KAZUHIKO NAGAO, YUTAKA MONNAI, AKEMI YAGI and MASARU UYEDA*

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi, Kumamoto 862-0973, Japan

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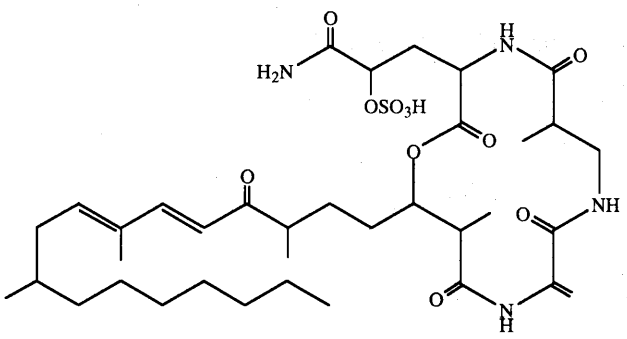
A novel inhibitor of topoisomerases designated as topostatin was isolated from the culture filtrate of *Thermomonospora alba* strain No. 1520. Topostatin inhibited the relaxation of supercoiled pBR322 DNA by calf thymus topoisomerase I, and also inhibited the relaxation of supercoiled pBR322 DNA and decatenation of kinetoplast DNA by human placenta topoisomerase II. Topostatin had neither ability to stabilize the cleavable complex nor ability to intercalate into DNA strands. The inhibitor exhibited growth inhibitory activity against the tumor cells (SNB-75 and SNB-78) of central nervous system, but did not exhibit any antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts and fungi.

In the search for inhibitors of DNA related enzymes, we have screened various actinomycetes and found 4 kinds of DNase inhibitors designated as DNIs[1~3], 6 kinds of DNA methyltransferase inhibitors designated as DMIs[4~7] and 3 kinds of topoisomerase inhibitors[8,9]. These topoisomerase inhibitors designated as 2280-DTI, 2890-DTI and macrostatin are the first reported examples of high molecular inhibitors of microbial origin with inhibitory activity against topoisomerases.

Topoisomerases I and II are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, and the enzymes are involved in producing the necessary topological and conformational changes in DNA which are critical to many cellular processes such as replication, recombination and transcription[10]. Topoisomerase I catalyzes the passage of the DNA strands through a transient single-strand break, while topoisomerase II catalyzes the passage of DNA double strands through a transient double-strand break[11,12]. In addition to their normal cellular functions, both enzymes have recently emerged as important cellular targets for chemical intervention in the development of antitumor drugs[13]. Many topoisomerase inhibitors have been reported and these inhibitors may be classified into two groups. The inhibitors in the first group inhibit the DNA rejoining reaction of topoisomerases by stabilizing a tight topoisomerase-DNA complex termed the "cleavable complex". Representative inhibitors of this group are camptothecin[14], epipodophyllotoxin family[15], terpenoid family[16], adriamycin (doxorubicin)[17], amsacrine[18], ellipticine[19], saintopin[20] and so on. Another group of the inhibitors such as diketopiperazine family[21,22] and CJ-12373[23] inhibit the DNA breaking and rejoining reactions of topoisomerase by direct action on the enzyme molecule without stabilizing the cleavable complex.

As an additional, new topoisomerase inhibitor, we found an inhibitor designated as topostatin (Fig. 1) in the culture filtrate of *Thermomonospora alba* strain No. 1520 isolated from a soil sample. Topostatin is a novel microbial inhibitor which can inhibit both topoisomerases I and II and it has neither ability to stabilize the cleavable complex nor ability to intercalate into DNA strands. This report describes the taxonomy of producing organism, fermentation, purification procedure and biological activities of topostatin. The structure elucidation will be described in the following paper.

Fig. 1. Structure of topostatin.



Materials and Methods

Materials

Topoisomerase I (EC 5.99.1.2) from calf thymus gland, T4 DNA ligase (EC 6.5.1.1) from *Escherichia coli*, *Hin* dIII (EC 3.1.23.21) from *Haemophilus influenzae* Rd, supercoiled pBR322 DNA from *Escherichia coli* HB101 and supercoiled pUC19 DNA from *Escherichia coli* DH5α were purchased from MBI Fermentas. Proteinase K (EC 3.4.21.14) from *Tritirachium album* and salmon sperm DNA were purchased from Boehringer Mannheim GmbH. Topoisomerase II (EC 5.99.1.3) from human placenta and kinetoplast DNA from *Crithidia fasciculata* were purchased from TopoGEN. Camptothecin, etoposide and doxorubicin hydrochloride were obtained from Aldrich, Calbiochem and Sigma, respectively. Test organisms for antimicrobial activity were obtained from the Institute for Fermentation, Osaka (IFO).

Taxonomical Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[24], and WAKSMAN[25]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[26].

Cultural Conditions for Production of Topostatin

A loopful of mature *Thermomonospora alba* strain No. 1520 from yeast malt extract agar slant was inoculated into sterilized S medium composed of glucose 2.0%, starch 3.0%, corn steep liquor 1.0%, soybean flour 1.0%, peptone 0.5%, NaCl 0.3% and $CaCO_3$ 0.5% at pH 7.0. It was cultivated aerobically for 2 days at 28°C and 180 rpm on rotary shaker, termed seed culture. Main culture was inoculated with 4.0% of seed culture in S medium and cultivated for 5 days at 28°C for the production of inhibitor.

DNA Relaxation and Cleavage Assays of Topoisomerase I

Relaxation activity of topoisomerase I was determined by detecting the conversion of supercoiled pBR322 DNA to its relaxed form[27]. Topoisomerase I reaction was performed in 20 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.6 μg BSA, 1 unit topoisomerase I (20 units for DNA cleavage assay) and 0.15 μg supercoiled pBR322 DNA. Enzyme reaction proceeded for 40 minutes at 37°C and terminated by adding 5 μl loading buffer containing 200 mM Tris (pH 7.5), 200 mM boric acid, 5 mM EDTA (pH 7.5), 50% glycerin and 10% bromphenol blue. Fifteen μl of the mixture was subjected to 1.0% agarose gel electrophoresis at 50V for 60 minutes in TBE buffer (100 mM Tris-borate buffer (pH 8.5) containing 2.5 mM EDTA). The agarose gel was stained with ethidium bromide and washed thoroughly with deionized water, and the remaining supercoiled pBR322 DNA on the gel was measured by a densitometer (Atto Co., AE-6900M). One unit of inhibitory activity (IC_{50}) was defined as the amount of inhibitor inhibited 50% of the relaxation of supercoiled pBR322 DNA by 1 unit of topoisomerase I under the above assay conditions.

For DNA cleavage assay[28], the reaction mixture was terminated by the addition of 5 μl of the stop solution containing 5% SDS and 12.5 μg proteinase K, thereafter incubated for an additional 30 minutes at 37°C. Loading buffer was added and the mixture was run into 1.0% agarose gel containing 0.1% SDS and ethidium bromide (0.5 μg/ml) at 50V for 2 hours. After agarose gel electrophoresis, the nicked pBR322 DNA on the gel was measured by a densitometer. The increase in nicked pBR322 DNA (topoisomerase I-mediated DNA cleavage) was estimated as the stabilizing of cleavable complex by an inhibitor.

DNA Relaxation, Cleavage and Decatenation Assays of Topoisomerase II

For relaxation activity of topoisomerase II[29], reaction buffer was supplemented with 0.5 mM ATP and the enzyme reaction was performed under the same conditions for topoisomerase I relaxation assay.

For DNA cleavage activity[30], 10 units topoisomerase II were used. After agarose gel electrophoresis, the increase of linearized pBR322 DNA (topoisomerase II-mediated DNA cleavage) was estimated as the

stabilizing of cleavable complex by an inhibitor.

Topoisomerase II catalytic activity was also measured by the decatenation of kinetoplast DNA (kDNA)[31]. In 20 μl reaction buffer, 1 unit topoisomerase II was incubated with 0.325 μg catenated kDNA for 40 minutes at 37°C using the same buffer containing 0.5 mM ATP. The enzyme reaction was terminated by addition of 5 μl of the loading buffer and the mixture was electrophoresed on 1% agarose gel in TBE buffer at 50V for 60 minutes. The gel was stained with ethidium bromide and washed thoroughly with deionized water. Decatenated kDNA on the gel was measured by a densitometer. The inhibitory activity (IC_{50}) was defined as the amount of inhibitor causing a decrease in decatenated kDNA concentration by 50%.

DNA Unwinding

DNA unwinding effect of an inhibitor was assayed according to the method described by CAMILLONI *et al.*[32] with minor modifications. pUC19 DNA was linearized with *Hin* dIII restriction endonuclease and recovered by phenol extraction and ethanol precipitation. One hundred μl of the reaction mixture containing 66 mM Tris-HCl (pH 7.5), 5.0 mM $MgCl_2$, 10 mM dithiothreitol, 1.0 mM ATP, 0.9 μg linearized pUC19 DNA and inhibitor solution was equilibrated at 15°C for 20 minutes and then incubated with 15 units T4 DNA ligase at 15°C for 60 minutes. Reaction was stopped by addition of EDTA at final 20 mM. DNA in the reaction mixture was analyzed by 1% agarose gel electrophoresis at 20V for 18 hours after recovery by phenol extraction and ethanol precipitation.

DNA Binding Competition with Ethidium Bromide (EtBr)

DNA intercalation was evaluated by binding an inhibitor to DNA using ethidium bromide as described by HORIGUCHI *et al.*[33] In 200 μl reaction mixture, 5 μM EtBr was mixed with 6.6 μg salmon sperm DNA and the reaction buffer consisted of 50 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, 1 mM EDTA (pH 8.0) and inhibitor. The intensity of fluorescence of the reaction mixture was measured with spectrofluorometer (Hitachi F-4010). Emission wavelength was 575 nm and excitation wavelength was 545 nm or 300 nm for competition between EtBr and inhibitor.

Antimicrobial and Antitumor Activities

Antimicrobial activity was determined by WAKSMAN's agar dilution streak method. Glucose-bouillon agar consisted of glucose 1.0%, Ehrlich meat extract 0.5%, peptone 1.0%, NaCl 0.3% and agar 1.5% was used for bacteria and fungi. Bacteria and fungi were cultivated at 37°C for 18 hours and 28°C for 48 hours, respectively. Minimum inhibitory concentration (MIC) was determined from inhibition of growth.

Antitumor activity was determined by the method of HCC panel (human cancer cell line panel) by YAMORI[34]. The tested human tumor cell lines were central nervous system, breast, lung, stomach, kidney, ovary and melanoma. Tumor cells were incubated for 2 days in 96-well plate containing various concentrations of inhibitor. After incubation at 37°C, cell growth was measured by rhodamine B reagent.

Results and Discussion

Taxonomy of Strain No. 1520

The taxonomic characteristics of the strain No. 1520 were compared with those of type strains listed in "BERGEY's Manual of Systematic Bacteriology, Volume IV"[35]. The strain No. 1520 was cultured in various ISP (International *Streptomyces* Project) media, and taxonomic characteristics were examined and are summarized in Table 1. Aerial mycelia were abundantly developed in yeast malt extract agar, sucrose nitrate agar, inorganic salt starch agar and glycerol asparagine agar. Mycelium produced single, spherical and terminal spores as shown in Fig. 2. Soluble pigments varied in color from yellow to brown. The physiological characteristics and utilization of carbohydrates observed at 28°C for 7 days, revealed close resemblance with *Thermomonospora alba*. The strain was designated as *Thermomonospora alba* strain No. 1520.

Time Course of *Thermomonospora alba* Strain No. 1520 Culture

The time course of topostatin production by the strain in 200 ml Erlenmeyer flask is shown in Fig. 3. pH of the culture broth remained almost at a steady level during incubation. Mycelial growth peaked on 4th day and thereafter gradually declined. Inhibitory activity of topostatin increased rapidly on 4th day and maximized on 5th day and followed by a rapid decline. For an optimum yield of the inhibitor, the culture broth was harvested on 5th day.

Purification Procedure of Topostatin

Flow diagram of the purification procedure of topostatin is shown in Fig. 4. Mycelial and cellular

Table 1. Cultural characteristics of strain No. 1520.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast malt extract agar (ISP No. 2)	Abundant	Excellent, light grayish brown	Brown
Oatmeal agar (ISP No. 3)	Moderate	Moderate, pinkish gray	Light yellow
Sucrose-nitrate agar (Czapek's soln. agar)	Abundant	Scant, brownish pink	Brown
Inorganic salts-starch agar (ISP No. 4)	Abundant	Moderate, light brownish gray	Light brown
Glycerol-asparagine agar (ISP No. 5)	Abundant	Scant, light brownish gray	Light brown
Peptone-yeast ext. iron agar (ISP No. 6)	Moderate	Moderate, light yellowish brown	Yellowish
Tyrosine agar (ISP No. 7)	Moderate	Moderate, light grayish brown	Light yellow
Nutrient agar	Moderate	Scant, white patches	None

Temperature range for growth (°C)		28 ~ 37
Optimum temperature (°C)		28
Formation of melanoid pigment		negative
Liquefaction of gelatin		negative
Coagulation of milk		negative
Peptonization of milk		negative
Hydrolysis of starch		positive
Decomposition of cellulose		negative
Carbon utilization	: positive	L-glucose, L-arabinose, sucrose, D-xylose, D-mannitol, D-fructose, rhamnose, raffinose, salicin, starch
	: negative	inositol, cellulose

Fig. 2. Scanning electron micrograph of *Thermomonospora alba* strain No. 1520 grown on yeast malt extract agar for 2 weeks at 28°C.

Bar represents 10 μm.

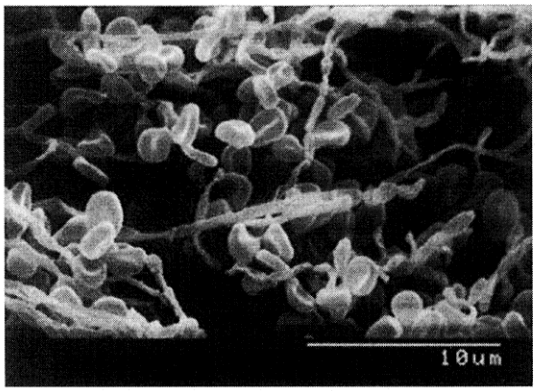
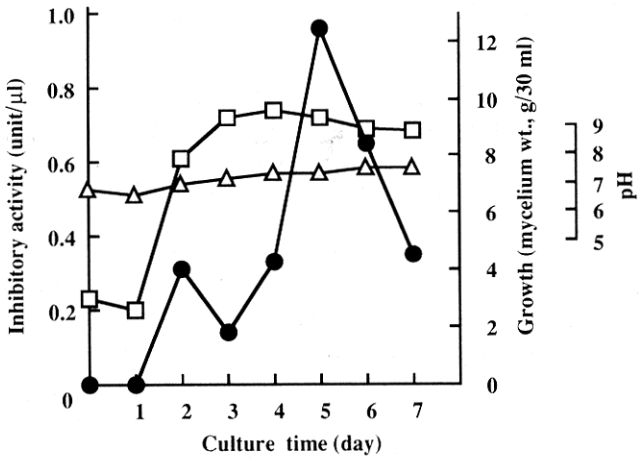


Fig. 3. Time course of *Thermomonospora alba* strain No. 1520 culture.

△: pH, □: growth, ●: inhibitory activity of topostatin.



residues of culture broth were removed by centrifugation at 4,000 × *g* and 5°C for 15 minutes. The supernatant was extracted with 2 volumes of ethyl acetate at pH 4.0 and dried *in vacuo*. The crude extract was dissolved in 50% MeOH and applied to a column of Diaion HP-10 (2 × 16 cm, Mitsubishi Chemical Industries) deaerated and equilibrated with 50% methanol. The fractions containing topostatin (Active fraction I) were eluted with 75% MeOH, pooled and concentrated *in vacuo*. The concentrated solution was applied to a column of Silica gel 60 (1.5 × 15 cm, Merck) equilibrated with $CHCl_3$ and eluted stepwisely with mixtures of $CHCl_3$-EtOH (7:3, 6:4 and 5:5). The eluate (Active fraction II) were pooled and applied to a column of Bondapak C_{18} (1.9 × 17 cm, Waters), and topostatin was eluted with linear concentrations of 35~100% MeOH. The eluate (Active fraction III) was concentrated and further purification was afforded by column chromatographies on Silica gel 60 and Bondapak C_{18} eluting with $CHCl_3$-EtOH (6:4) and 75% MeOH, respectively. Finally, active fractions of Bondapak C_{18} column were pooled and dried *in vacuo*, termed purified topostatin. About 5.6 mg of the inhibitor were finally obtained from 1.0 liter of the culture filtrate.

Fig. 4. Purification procedure of topostatin.

Culture filtrate (1.0 liter)
↓ extraction with AcOEt at pH 4
Crude solution
↓ column chromatography on Diaion HP-10
Active fraction I
↓ column chromatography on Silica gel 60
Active fraction II
↓ column chromatography on ODS
Active fraction III
↓ column chromatographies on Silica gel and ODS
Purified topostatin (5.6 mg)

Biological Activities of Topostatin

The effects of topostatin on the catalytic activities of topoisomerases I and II were examined by the relaxation and decatenation assays. Camptothecin and doxorubicin were used as specific inhibitors against topoisomerases I and II, respectively. As shown in Fig. 5 (A), in the presence of increasing topostatin, topoisomerase I relaxation activity was inhibited and its IC_{50} was 13 ng/μl. And also, topostatin inhibited the relaxation and decatenation activities of topoisomerase II at almost same concentration (3.0 ng/μl) as shown in Fig. 5 (B) and (C). Inhibition of topostatin against topoisomerase II was 4-fold potent than that against topoisomerase I. Camptothecin and doxorubicin did not inhibit topoisomerases II and I at extreme doses such as 100 ng/μl, respectively.

Some topoisomerase inhibitors such as doxorubicin, amsacrine and ellipticine are DNA intercalators. To determine whether topostatin has any ability to intercalate into DNA strands, at first, DNA unwinding assay was performed using linearized pUC19 DNA and T4 DNA ligase. In this assay, doxorubicin, a strong intercalator, was used as control under the same conditions. As shown in Fig. 6, doxorubicin produced a concentration-dependent DNA band shift by DNA intercalation and led to the formation of supercoiled

Fig. 5. Inhibitions of topoisomerases I and II by topostatin, camptothecin and doxorubicin.

●: Topostatin, ○: camptothecin, △: doxorubicin.

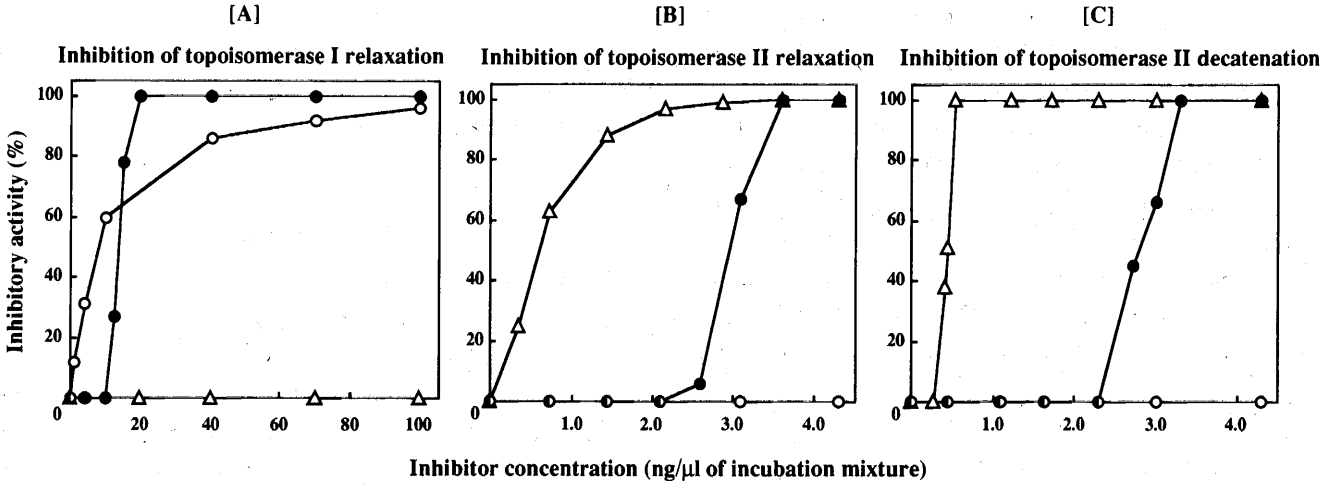
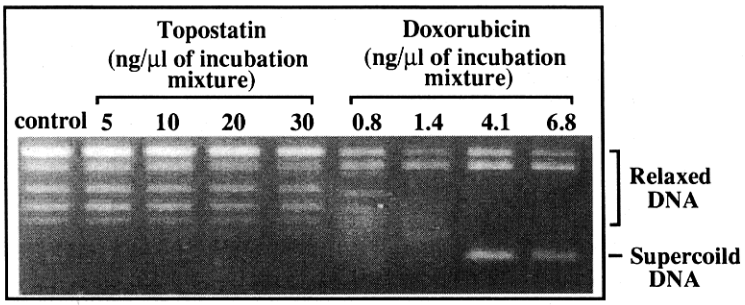


Fig. 6. Unwinding of DNA by topostatin and doxorubicin.



DNA. On the other hand, topostatin did not lead to the formation of supercoiled DNA even at the concentration of 30 ng/μl.

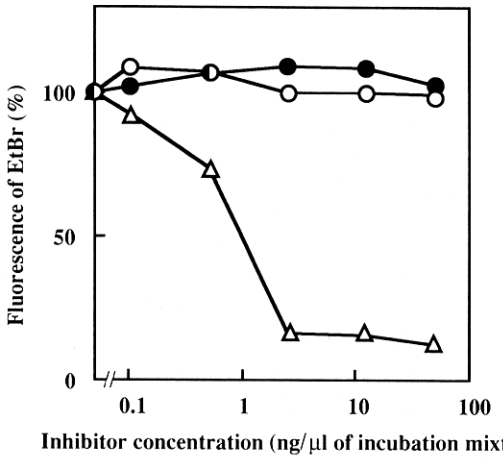
In order to confirm the above fact, ethidium bromide competition assay was carried out using salmon sperm DNA. Camptothecin and doxorubicin were used as control of a nonintercalator and an intercalator, respectively. As shown in Fig. 7, doxorubicin competed with ethidium bromide for DNA and showed decrease in the intensity of fluorescence. On the other hand, topostatin did not decrease the intensity of fluorescence at 40 ng/μl, therefore, it was implied that topostatin could not compete with ethidium bromide for DNA. The finding was in good agreement with the result of DNA unwinding assay of topostatin. From these results, it was made clear that topostatin has no ability to intercalate into DNA strands.

To determine whether topostatin stabilizes topoisomerase-cleavable complex, the cleavage assays were carried out. Camptothecin and etoposide inducing the cleavable complex were used as specific inhibitors against topoisomerases I and II, respectively. As shown in Fig. 8 [A], camptothecin stabilized the cleavable complex with topoisomerase I and induced the nicked DNA with increasing concentrations. Unlike camptothecin, topostatin could not induce the nicked DNA. And also, the stabilization of topoisomerase II-cleavable complex by topostatin was examined. As shown in Fig. 8 [B], etoposide induced the linearized DNA, but topostatin failed to linearize DNA even at 1,000 ng/μl, an extreme concentration. These results suggested that topostatin has not the ability to stabilize the cleavable complexes of topoisomerases I and II. This inhibitor is thought to inhibit the DNA breaking and rejoining reactions of topoisomerases by direct action on the enzyme molecules.

The antimicrobial activity of topostatin was tested by

Fig. 7. Effects of topostatin, camptothecin and doxorubicin on DNA binding competition with ethidium bromide.

●: Topostatin, ○: camptothecin, △: doxorubicin.



the agar dilution streak method. Since the MIC (minimum inhibitory concentration) of the inhibitor was greater than 100 μg/ml against Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*), yeast (*Saccharomyces cerevisiae* and *Candida albicans*) and fungi (*Penicillium chrysogenum*, *Aspergillus oryzae*, and *A. niger*), no significant antimicrobial activity could be detected.

As shown in Table 2, topostatin showed growth inhibition against human tumor cells. Topostatin exhibited the strong inhibition against the growth of SNB-75 and SNB-78 cells, weak inhibition against BSY-1 and MDA-MB-231 cells. Topostatin did not inhibit the growth of other tumor cells at concentrations up to 100 μM. Further investigation will be necessary to clar-

Fig. 8. Topoisomerases I and II-mediated DNA cleavage by topostatin, camptothecin and etoposide.

●: Topostatin, ○: camptothecin, □: etoposide.

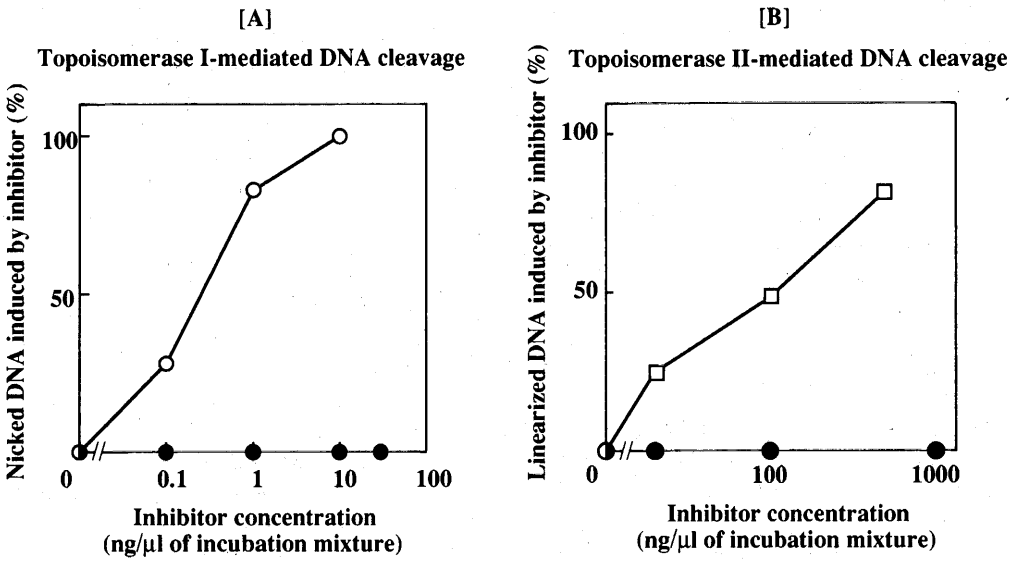


Table 2. Growth inhibition of human tumor cells by topostatin.

Tissue	Cell	IC_{50} (μM)
Central nervous system	SNB-75	0.4
	SNB-78	7
	U251, SF-268, SF295, SF539	>100
Breast	BSY-1	59
	MDA-MB-231	64
	HBC-4, HBC-5, MCF-7	>100
Lung	NCI-H23, NCI-H226,	>100
	NCI-H460, A549,	>100
Stomach	DMS114, DMS273	>100
	ST-4, MKN1, MKN7,	>100
Kidney	MKN28, MKN45, MKN74	>100
	RXF-631L, ACHN	>100
Colon	HCC2998, KM-12, HT-29,	>100
	WIDR, HCT-15, HCT-116	>100
Ovary	OVCAR-3, OVCAR-4,	>100
	OVCAR-5, OVCAR-8, SK-OV-3	>100
Melanoma	LOX-IMVI	>100

ify the antitumor activity of the inhibitor.

The properties of topostatin are completely different from those of other topoisomerase inhibitors so far reported, and the inhibitor may be a useful compound for exploring various aspects of cancer therapy.

Acknowledgment

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