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

III. Inhibitory Properties

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A novel inhibitor of topoisomerases designated as topostatin was isolated from the culture filtrate of *Thermomonospora alba* strain No. 1520. The inhibitory activity of topostatin was shown to be pH- and temperature-dependent with a maximum around at pH 6 and 28°C. The stability of topostatin decreased with decreasing pH and rising temperature. Topostatin inhibited topoisomerases I and II in a competitive manner with respect to DNA. The inhibitor also inhibited some restriction endonucleases such as *Sca* I, *Hind* III and *Pst* I, but not *Alu* I, *Bam* HI, *Eco* RI, RNase A, DNase I, DNase II and DNA ligase. Topostatin did not induce the nuclear accumulation of p53 protein by DNA damage in the normal human cells.

In the search for inhibitors of topoisomerases, we have screened various actinomycetes and found 4 kinds of inhibitors designated as 2280-DTI, 2890-DTI, macrostatin and topostatin.[1~4] Among them, topostatin isolated from *Thermomonospora alba* strain No. 1520 is a novel inhibitor of cleavable complex-nonforming type and inhibits both topoisomerases I and II. The structure is a novel 14-membered ring containing peptide and terpenoid. In the previous papers[3,4], taxonomy of the producing organism, fermentation, purification procedure, biological activities, physico-chemical properties and structure elucidation of topostatin were reported. This report describes the inhibitory properties of topostatin.

Materials and Methods

Enzymes and Substrates

Topoisomerase I (EC 5.99.1.2) from calf thymus gland, T4 DNA ligase (EC 6.5.1.1), *Bam* HI (EC 3.1.23.6), *Eco* RI (EC 3.1.23.13), *Hind* III (EC 3.1.23.21), supercoiled pBR322 DNA and supercoiled pUC19 DNA were purchased from MBI Fermentas. DNase I (EC 3.1.21.1) from bovine pancreas, DNase II (EC 3.1.22.1) from porcine spleen, RNase A (EC 3.1.27.5) from bovine pancreas, RNA from yeast extract were obtained from Sigma. Salmon sperm DNA was purchased from Boehringer Mannheim GmbH. *Alu* I (EC 3.1.23.1), *Sca* I (EC 3.1.21.4) and *Pst* I (EC 3.1.23.31) were purchased from Gibco BRL. Topoisomerase II (EC 5.99.1.3) from human placenta and kinetoplast DNA were purchased from Topogen. Camptothecin, etoposide and doxorubicin hydrochloride were obtained from Aldrich, Calbiochem and Sigma, respectively.

For preparation of topoisomerase I from COLO 201 cells, the cells cultured for 5 days were washed with PBS (phosphate buffered saline) and harvested by centrifugation. The cells pellets (1×10^6 cells) were resuspended in 200 μl of the cold lysis buffer (10 mM Tris-HCl, pH 7.5,

1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% 3-[(3-chol-amidopropyl) dimethylammonio]-1-propanesulfonate and 10% glycerin), and kept on ice for 30 minutes. The lysate was centrifuged and the supernatant was used as enzyme solution of the topoisomerase I obtained from COLO 201 cells.

Measurement of Topoisomerases I and II Activities

Relaxation activities of topoisomerases I and II were determined by detecting the conversion of supercoiled pBR322 DNA to its relaxed form.[5,6] Decatenation activity of topoisomerase II was assayed using kinetoplast DNA (kDNA) as a substrate.[7] After enzyme reaction, the incubation mixture was electrophoresed on 1% agarose gel in TBE buffer at 50V for 60 minutes and the remaining supercoiled pBR322 DNA or decatenated kDNA on the gel was measured by a densitometer (Atto Co., AE-6900M). The assay conditions for inhibitory activities and electrophoresis have been described in a previous paper.[3] The inhibitory activities (IC_{50}) were defined as the amount of inhibitor that inhibited 50% of the relaxation of supercoiled pBR322 DNA and decatenation of kDNA by 1 unit of topoisomerases I and II, respectively.

Measurement of Other Enzyme Activities

Activities of restriction enzymes (*Alu* I, *Bam* HI, *Eco* RI, *Hind* III, *Pst* I and *Sca* I) and nucleases (DNase I, DNase II and RNase A) were determined by measuring the concentration of undigested substrates using either supercoiled pBR322 DNA (0.25 μg) or RNA (1.2 μg) and 4 units each enzyme in 20 μl reaction mixtures.[2] The reaction buffer for *Alu* I, *Eco* RI and *Hind* III consisted of 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol. The buffer for *Bam* HI, *Pst* I and *Sca* I consisted of 5 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 10 mM NaCl and 0.1 mM dithiothreitol. DNase I reacted in 50 mM Tris-HCl (pH 7.5) and 4 mM $MgCl_2$. DNase II reacted in 50 mM acetate (pH 5.7), 10 mM EDTA and 100 mM KCl. For RNase A, 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA were used.

The reaction mixture was incubated at 37°C for 40 minutes and undigested pBR322 DNA or RNA was measured by a densitometer after electrophoresis using 1% agarose gel containing ethidium bromide (0.5 μg/ml).

The assay of T4 DNA ligase was based on ligation of linearized pBR322 DNA which was cleaved by *Hind* III. The reaction mixture (20 μl) consisting of 66 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 0.3 μg linearized pUC19 DNA and 4 units T4 DNA ligase was incubated at 15°C for 40 minutes. Reaction was terminated with 10 μl of 60 mM EDTA. The concentration of remaining linearized pUC19 DNA in the reaction mixture was determined after electrophoresis using agarose gel containing ethidium bromide. The inhibitory activity was defined as the amount of the inhibitor that inhibited 50% of the ligation of linearized pUC19.

Telomerase activity was measured by TRAP-*eze*® Telomerase Detection kit (Intergen Co.).

Assays of Cell Growth and Nuclear Accumulation of p53 Protein

Normal human fibroblasts (100 cells) obtained from a foreskin biopy specimen were cultured in the dish (60 mm diameter) containing 4 ml of Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, penicillin G (100 units/ml) and streptomycin (100 μg/ml) at 37°C for 7 days in a humidified 5% CO_2 incubator. After incubation, the cells were fixed with 50% methanol, stained with Giemsa, and numbers of colonies were counted (colony-forming assay).[8]

Nuclear accumulation of p53 protein was detected by an indirect immunostainig method.[8] Normal human fibroblasts were cultured on coverslips (17 mm diameter) in plastic dishes (30 mm diameter) for 2 days to reach the logarithmic growth stage. For immunostaining, the cells were fixed with 80% methanol for 10 minutes at −10°C, washed vigorously with PBS and stained for 40 minutes at room temperature with a mouse anti-p53 monoclonal antibody (Pab 1801; Ab-2, Oncogene Science) as the first antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG polyclonal antibody (Cappel) as the second antibody.

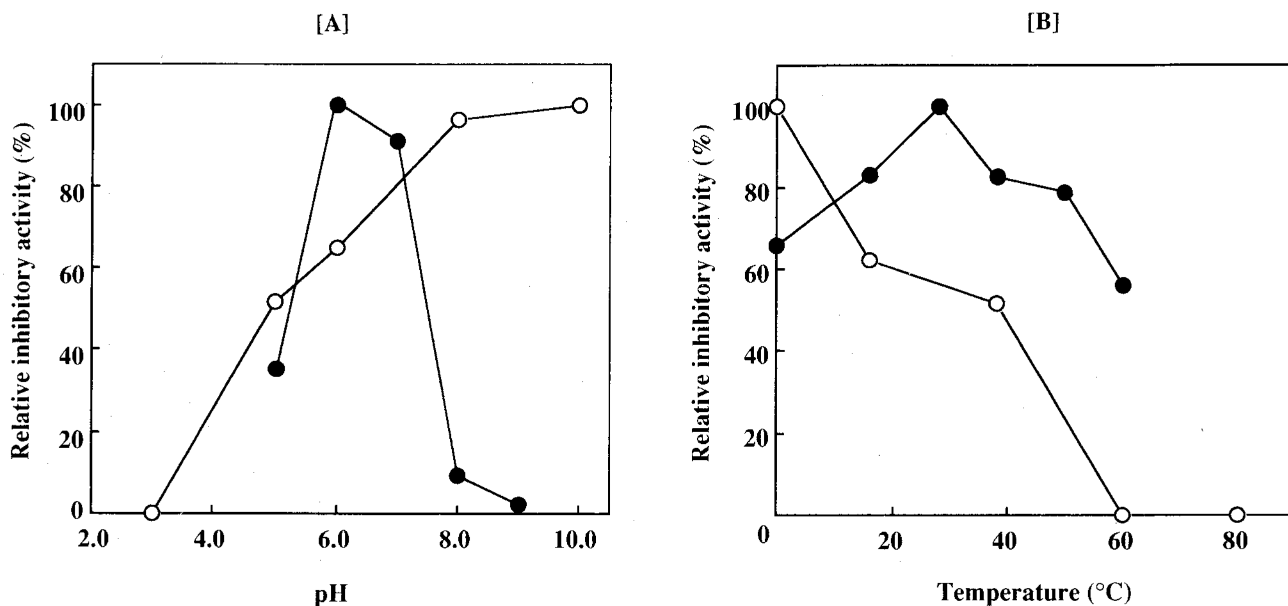
Results and Discussion

Effects of pH and Temperature on Inhibitory Activity and Stability of Topostatin

The relaxation activities of topoisomerase I were measured by the standard assay method replaced with the buffers (acetate, Tris-HCl and glycine-NaOH buffers) of various pHs with and without topostatin, and the effect of pH on the inhibitory activity of topostatin was examined. As shown in Fig. 1[A], the inhibitory activity was found to be pH-dependent with the maximum at pH 6.0. And also, the stability of topostatin was examined by storage in the buffers over the pH 3~10 range. After

Fig. 1. Effects of pH and temperature on the inhibitory activity and stability of topostatin.

[A] Effect of pH on the inhibitory activity and stability.
[B] Effect of temperature on the inhibitory activity and stability.



●: Inhibitory activity. The inhibitory activities at each pH and temperature were assayed by the standard assay method for topoisomerase I except for pH and temperature, respectively. The activities at pH 6 and 28°C were expressed as 100, respectively.

○: Stability. After treatment at each pH and temperature for 60 minutes, residual inhibitory activities were assayed by the standard assay method for topoisomerase I. The stability (residual activity) at pH 8 and 0°C was expressed as 100, respectively.

incubation at 28°C for 60 minutes in each buffer, the residual inhibitory activity of topostatin was measured using aliquots of each incubation mixture. Topostatin was fairly stable at pH 8 ~ 10 by retaining 90 ~ 100% of the inhibitory activity against topoisomerase I, but the inactivation of topostatin was coincident with decreasing pH.

Similarly as in the above section, the effects of temperature on the inhibitory activity and the stability of topostatin were examined and shown in Fig. 1[B]. Although the inhibitory activity was shown to be temperature-dependent with a maximum at 28°C, the activity was almost constant at 20 to 50 °C. On the other hand, the stability of topostatin decreased with rising temperature and completely lost at 60°C.

These results suggest that the condition at pH 6 and 28°C is favorable for the inhibition by topostatin, but it is unsuitable for storage of the inhibitor.

Inhibitory Spectrum of Topostatin

The effects of topostatin on various DNA related enzymes were examined and summarized in Table 1. For comparison, camptothecin[9] and doxorubicin[10] were also examined as specific inhibitors against topoisomerases I and II, respectively. Reactions were performed as described in Materials and Methods. Topostatin inhibited the relaxation activities of topoisomerases I from calf thymus gland and COLO 201 cells, and IC_{50} values of topostatin and camptothecin were almost the same concentration (15 ~ 20 μM)). And also, topostatin inhibited the relaxation and decatenation activities of topoisomerase II from human placenta at the same concentration (4 μM). The inhibitions were about 4-fold potent than those against topoisomerase I. Camptothecin and doxorubicin did not inhibit topoisomerases II and I at extreme concentrations such as 100 μM, respectively.

Table 1. Inhibitory spectrum of topostatin.

Enzyme	Inhibitory activity (IC_{50}, μM)[a]		
	Topostatin	Camptothecin	Doxorubicin
Topo I[b] from calf thymus gland	17	17	>100
Topo I[b] from COLO 201 cell	15	20	>100
Topo II[b] from human placenta	4	>100	1
Topo II[c] from human placenta	4	>100	1
Alu I	>100	>100	24
Bam HI	>100	>100	>100
Eco RI	>100	>100	>100
Hind III	17	>100	96
Pst I	19	>100	>100
Sca I	7	>100	25
RNase A	>100	>100	>100
DNase I	>100	>100	>100
DNase II	>100	>100	>100
T4 ligase	>100	>100	73
Telomerase	>100	—	—

a) concentration required to give 50% inhibition.
b) relaxation activity. c) decatenation activity.

Among restriction endonucleases tested, *Sca* I, *Hind* III and *Pst* I were fairly affected at the concentration of 7, 17 and 19 μM, respectively, whereas other enzymes such as, *Alu* I, *Bam* HI, *Eco* RI, RNase A, DNase I, DNase II, T4 DNA ligase and telomerase were not inhibited even at higher concentration of topostatin (100 μM). Inhibitory effects of topostatin on both topoisomerases I and II may be nonspecific interaction against the enzyme molecules because it also inhibited the other enzymes which act on DNA.

Inhibitory Manner of Topostatin on Topoisomerases I and II

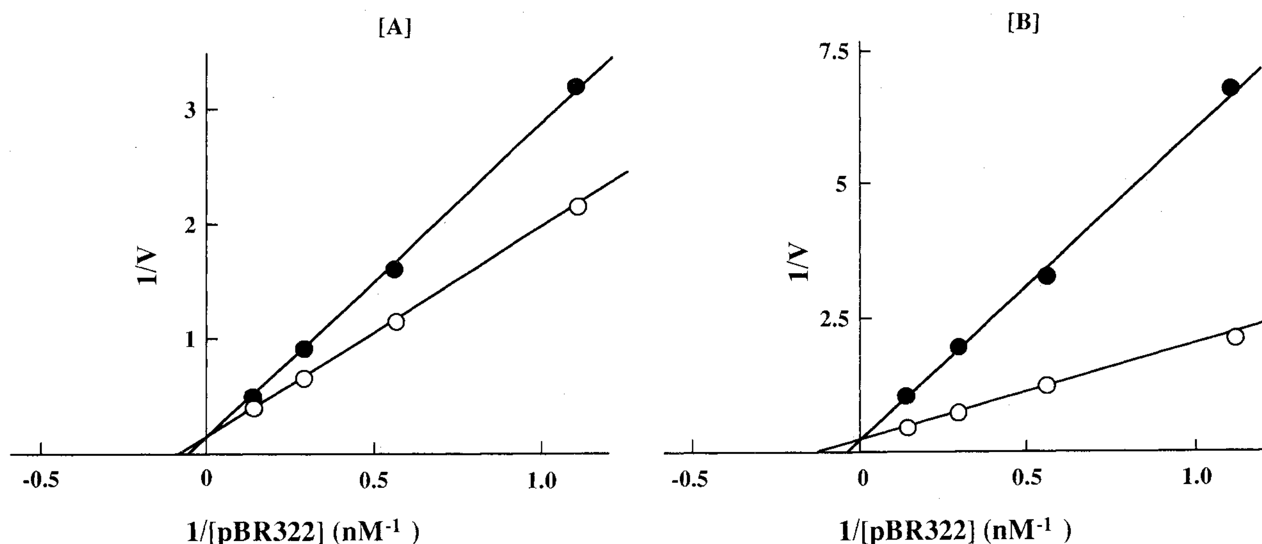
The type of inhibition was determined by Lineweaver-Burk plots[11] of substrate concentrations against the rate of relaxation of supercoiled pBR322 DNA by topoisomerase I from calf thymus gland and topoisomerase II from human placenta in the presence or absence of topostatin. As shown in Fig. 2 [A] and [B], topostatin inhibited the relaxation activities of topoisomerases I and II competitively with respect to pBR322 DNA exhibiting *Ki* values of 28.4 μM and 1.1 μM, respectively. The Michaelis constants (*Km* values) of topoisomerase I and II were 8.3 nM and 5.3 nM, respectively. In view of inhibitory potency (*Ki*/*Km*) against DNA relaxation by topoisomerases I and II, topostatin was 16-fold potent against topoisomerase II than topoisomerase I. From these results, topostatin was highly potent inhibitor against topoisomerase II and the inhibitor was considered to bind strongly to the binding site of the substrate DNA on the enzyme molecule.

Nuclear Accumulation of p53 Protein by Topostatin

The topoisomerase inhibitors of cleavable complex-forming type such as camptothecin and doxorubicin show strong cytotoxicity against normal human cells because these inhibitors cause DNA damage (cleavage) in the process of topoisomerase inhibition. And it is also known that DNA damage caused by these inhibitors induces the nuclear accumulation of p53 protein which blocks the cell cycle at G1/S phase.[12]

Topostatin is neither cleavable complex-forming

Fig. 2. Lineweaver-Burk plots of substrate (supercoiled pBR322 DNA) concentration against rate of relaxation by topoisomerase I [A] and topoisomerase II [B] with (●) and without (○) topostatin.



The *Ki* values of topostatin against topoisomerases I and II were 28.4 μM and 1.1 μM, respectively. The *Km* values of topoisomerase I and II were 8.3 nM and 5.3 nM, respectively.

Table 2. Nuclear accumulation of p53 protein in normal human fibroblasts by topostatin.

Inhibitor	Optimum concentration for accumulation of p53 protein (μM) a)	Fluorescence intensity of nuclei stained with the antibody b)	Growth inhibition (IC_{50}, μM) c)
None		–	
Topostatin	250.0	±	80.0000
Camptothecin	10.0	+++	0.0012
Doxorubicin	0.2	+++	0.0046

a) Normal human fibroblasts were grown on coverslips for 2 days in the presence of various concentration of inhibitors. After fixing with methanol, cells were stained for p53 protein by an indirect immunofluorescence method.
b) Intensity of nuclear fluorescence at the optimum concentration was determined by inspection and evaluated in three grades (–, negative; ±, negligible; +++, very strong).
c) Concentration required to give 50% growth inhibition was determined by a colony-forming assay as described in the text.

type inhibitor nor DNA intercalator as described in the previous paper.[3] To determine whether topostatin causes DNA damage in the normal human cells, nuclear accumulation of p53 protein was examined using the normal human fibroblasts. As shown in Table 2, the optimum concentration of accumulation by topostatin was 250 μM, and at the concentration, nuclei of the cells were barely stained with the antibody. In the case of camptothecin and doxorubicin, all nuclei were strongly stained at low concentration. These results suggest that

topostatin does not cause DNA damage and not induce the nuclear accumulation of p53 protein in the normal human cells. And also, further investigation using sensitive cells for topostatin will be necessary to clarify the accumulation by the inhibitor.

Topostatin is completely different from the antitumor drugs causing DNA damage such as camptothecin and doxorubicin so far clinically used. Topostatin is topoisomerase inhibitor having no cytotoxicity against normal human cells. Regarding to the growth inhibition against human tumor cells by topostatin, we also reported in the previous paper.[3] Topostatin shows a specific inhibition against the growth of SNB-75 and SNB-78 which are the tumor cells of central nervous system. In the future, the inhibitor may be a useful tool for the investigation and therapy for brain cancer.

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