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MACROSTATIN, A NOVEL MACROMOLECULAR INHIBITOR OF TOPOISOMERASES PRODUCED BY *STREPTOMYCES AVERMITILIS* NO. C-127

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A novel inhibitor of topoisomerases designated as Macrostatin† has been isolated from the culture filtrate of *Streptomyces avermitilis* strain No. C-127 and purified by successive chromatography on Dowex, activated charcoal, gel filtration and cellulose. It is an acidic macromolecule having 45 kD molecular weight as determined by gel filtration. Macrostatin inhibited topoisomerase I and II in a noncompetitive manner with $K_i = 3.7$ and 1.3 nM respectively. Macrostatin differed from well-known inhibitors of topoisomerase such as camptothecin, etoposide and doxorubicin which induce topoisomerase-mediated DNA cleavage by stabilizing the cleavable complex or intercalation into DNA strands. Macrostatin had neither ability to stabilize the cleavable complex nor ability to intercalate into DNA strands. It was suggested that Macrostatin inhibits topoisomerase by a direct action on the enzyme.

Keywords: Topoisomerase inhibitor; Macrostatin; *Streptomyces avermitilis*; Enzyme inhibitor

INTRODUCTION

Topoisomerases can adjust the topological state of DNA by breaking and rejoining DNA strands, allowing for alterations in the linking number.[1,2]

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† Macrostatin was originally named as C127-DTI.

Two major categories of topoisomerases are recognized. The type I enzyme catalyses the reactions in which it introduces a transient single strand break, through which opposite (intact) DNA strand can pass.[3–8] Because of this mechanism, the type I topoisomerase characteristically change the linking number of a covalently closed circular DNA.[5,9,10] Alternatively, type II topoisomerase transiently breaks both strands which produces a double strand DNA-protein gate through which an intact section of the duplex can pass.[11,12] Topoisomerase I and II, have been detected in all prokaryotic and eukaryotic cells. They are implicated in events involving DNA, e.g. replication, transcription, mitosis and repair.[2,13] Compounds identified as inhibitors have been instrumental in understanding of mechanism of the reactions catalyzed by topoisomerases as well as being used in chemotherapy.[14,15] All the drugs, referred to as topoisomerase poisons, interfere with the rejoining reaction of topoisomerases by stabilizing a tight topoisomerase-DNA complex termed the "cleavable complex" which is presumed to be the key covalent intermediate. Drugs that have been shown to stabilize the cleavable complex with topoisomerase may be classified into two groups. The first group of drugs intercalate into DNA strands. Representative agents of this group are 4′-(9-acridinyl amino) methanesulfon-*m*-anisidide (*m*-AMSA; amsacrine), ellipticine and adriamycin (doxorubicin) as topoisomerase II poisons and saintopin as topoisomerase I and II poisons.[12,16–18] Another class of drugs stabilizes the cleavable complex without intercalating into DNA strands. This group includes camptothecin as a topoisomerase I poison, and epipodophyllotoxin congeners [demethylepipodophyllotoxin ethylidene-β-D-glucoside (VP-16), 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside (VM-26)] and the terpenticin family (UCT 4B, terpenticin, clerocidin) as topoisomerase II poisons.[19–21]

In order to find new topoisomerase inhibitors, we have screened the cultures of streptomycetes for their ability to inhibit topoisomerases. Two topoisomerase inhibitors designated as 2280-DTI and 2890-DTI were isolated from the culture filtrates of *Micromonospora* sp. strain No. 2280 and *Streptomyces antibioticus* strain No. 2890, respectively. And we described some properties of both inhibitors in a previous paper.[22] Recently, we found another inhibitor in the culture filtrate of *Streptomyces avermitilis* strain No. C-127 which was designated as Macrostatin. Herein, we report studies indicating that Macrostatin is a novel macromolecular substance which can inhibit topoisomerase I and II, and it has neither ability to stabilize the cleavable complex nor ability to intercalate into DNA strands.

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) from *Escherichia coli*, *Bam* HI (EC 3.1.23.6) from *Bacillus amyloliquefaciens* H, *Eco* RI (EC 3.1.23.13) from *Escherichia coli* RY13, *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. 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 and RNA from yeast extract were obtained from Sigma. Proteinase K (EC 3.4.21.14) from *Ttritirachium album* and salmon sperm DNA were purchased from Boehringer Mannheim GmbH. *Alu* I (EC 3.1.23.1) from *Arthrobacter luteus*, *Sca* I (EC 3.1.21.4) from *Streptomyces caespitosus* and *Pst* I (EC 3.1.23.31) from *Providencia stuartii* were purchased from Gibco BRL. 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).

DNA Relaxation and DNA Cleavage Reactions with Topoisomerase I and II

Inhibition of topoisomerase I activity was determined by monitoring the relaxation of supercoiled pBR322 DNA.[23] Unless otherwise stated, 20 µl of reaction mixture contained 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 and indicated amount of inhibitor. After preincubation at 37°C for 5 min, 0.15 µg supercoiled pBR322 DNA was added to the reaction mixture. Enzyme reaction proceeded for 40 min at 37°C and was terminated by adding 5 µl loading buffer containing 0.2 M Tris (pH 7.5), 0.2 M boric acid, 5 mM EDTA (pH 7.5), 50% glycerin and 10% bromphenol blue. Reaction products were electrophoresed using 1% agarose gel in TBE buffer (0.1 M Tris-borate buffer containing 2.5 mM EDTA, pH 8.5) at 50 V for 60 min. The gel was stained with ethidium bromide and washed in large amounts of water. Supercoiled pBR322 DNA were quantitated by densitometer (Atto Co., AE-6900M). Inhibition (%IH) was calculated from the following formula: $\%IH = (I - C/B - C) \times 100$ where C, B and I are concentrations of

remaining supercoiled pBR322 in the reaction mixture (*C*: control) without enzyme (*B*: blank) and with inhibitor (*I*: inhibitor), respectively. Fifty percent inhibition was obtained graphically from these values. One unit of inhibitor was defined as the amount that inhibited 50% of the relaxation of supercoiled pBR322 DNA by 1 unit of topoisomerase I under the above assay conditions.

For the DNA cleavage reaction,[18,19] topoisomerase I (20 units) was used and the enzyme reaction 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 min at 37°C. Loading buffer was added and the mixture was run into 1% agarose gel containing 1% SDS and ethidium bromide (0.5 µg/ml) at 50 V for 2 h. The increase of nicked DNA was estimated as topoisomerase I-mediated DNA cleavage induced by inhibitor.

For relaxation activity of topoisomerase II,[11,25] the reaction buffer was supplemented with 0.5 mM ATP and the enzyme reaction was carried out under the same conditions for topoisomerase I. For DNA cleavage activity, 0.03 µg supercoiled pBR322 DNA and 10 units topoisomerase II were used.[24,26] After agarose gel electrophoresis, the increase of linearized pBR322 DNA was estimated as topoisomerase II-mediated DNA cleavage by inhibitor.

Topoisomerase II catalytic activity was also measured by ATP-dependent decatenation of kinetoplast DNA (kDNA).[11,25] In 20 µl reaction buffer, 1 unit topoisomerase II was incubated with 0.325 µg catenated kDNA for 40 min at 37°C using a reaction buffer consisted of 50 mM Tris-HCl buffer (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM ATP, 0.5 mM dithiothreitol and 0.6 µg BSA. The enzyme reaction was terminated by addition of 5 µl of the loading buffer and the mixture was subjected to electrophoresis on 1% agarose gel in TBE buffer at 50 V for 60 min. The gel was stained with ethidium bromide and washed thoroughly with water. Decatenated kDNA on the gel was measured by the densitometer. The inhibitory activity (IC_{50}) was defined as the amount of inhibitor causing a decrease of decatenated kDNA concentration by 50%. Fifty percent inhibition was obtained as previously described in the relaxation assay of topoisomerase I.

Measurement of Other Enzyme Activities

Activities of restriction enzymes (*Alu* I,[27] *Bam* HI,[28] *Eco* RI,[29] *Hin* dIII,[30] *Pst* I[31] and *Sca* I[32]) and nucleases (DNase I,[33] DNase II[34] and RNase A[35]) were determined by measuring the concentration of undigested substrate

using either supercoiled pBR322 DNA (0.25 μg) or RNA (1.2 μg) and 4 units of each enzyme) in 20 μl reaction mixtures. The reaction buffer for *Alu* I, *Eco* RI and *Hin* dIII 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 was reacted in 50 mM Tris-HCl (pH 7.5) and 4 mM $MgCl_2$. DNase II was 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 min 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 *Hin* dIII.[36] 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 of T4 DNA ligase were incubated at 15°C for 40 min. 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.

Competition of DNA Binding with Ethidium Bromide (EtBr)

DNA intercalation was evaluated by binding of DNA with inhibitor using ethidium bromide as described by Horiguchi *et al.*[37] 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 · 2Na (pH 8.0) and inhibitor. The intensity of fluorescence of the reaction mixture was measured with a spectrofluorometer (Hitachi F-4010). Emission wavelength was 575 nm and excitation wavelength was 545 or 300 nm for competition between EtBr and inhibitor.

Antimicrobial Spectrum

Antimicrobial activity was determined by the agar dilution streak method.[38] Bouillon agar consisted of 0.5% Ehrlich meat extract, 1% peptone, 0.5% NaCl and 1.5% agar was used for common bacteria. Yeast and fungi were cultivated in trypticase soy agar supplemented with 1% glucose. Bacteria and fungi were cultivated at 37°C for 18 h and 28°C for 48 h, respectively. Minimum inhibitory concentration (MIC) was determined from inhibition of growth.

Cultural Conditions for Production of Macrostatin

Streptomyces avermitilis strain No. C-127 was grown aerobically with 50 ml of S medium in 200 ml-Erlenmeyer flask at 28°C on a rotary shaker (180 rpm) for 2 days. Later on, 200 ml inoculum of seed culture was transferred to 5 l of production medium of the same composition and cultivated additionally for 4 days in a jar fermentor. S medium was composed of 2% glucose, 3% starch, 1% soybean flour, 1% corn steep liquor, 0.5% peptone, 0.3% NaCl and 0.5% $CaCO_3$, prepared in tap water at pH 7.0.

Isolation and Purification of Macrostatin

Mycelial and cellular residues of culture broth were removed by centrifugation at $6,000 \times g$ and 5°C for 10 min. The culture filtrate was precipitated with HCl at pH 3 and left overnight at 5°C. The resulting precipitate was collected by centrifugation at $12,000 \times g$ for 15 min, dissolved in 0.02 N NaOH and then dialyzed against water for 48 h. The dialysate was applied on Dowex 50 W × 2 column (2.5 × 4.9 cm, Dow Chemical) and eluted with 0.1 N NaOH. The eluate was extracted with equal volume of 1-butanol. The aqueous layer was concentrated and treated with activated charcoal column (1.8 × 10 cm, Wako Chemicals) equilibrated at pH 3. The fraction passing through was subjected to filtration through Sephadex G-75 column (1.6 × 60 cm, Pharmacia Fine Chemicals). The column was equilibrated and eluted with 0.05 N NaOH-KCl buffer (pH 12). The eluate was dialyzed against water and filtered through Sephadex G-100 column (1.8 × 90 cm, Pharmacia Fine Chemicals). The eluate was concentrated *in vacuo* and dissolved in a mixture of 20% MeOH and 1% AcOH. It was finally applied on a cellulose column (1.8 × 16 cm, Merck) and eluted with the above mentioned solvent. The accumulated active fractions were concentrated and dialyzed against water. The dialysate was lyophilized and designated as purified Macrostatin. The inhibitor obtained from one liter of the culture filtrate was 4.0 mg.

RESULTS AND DISCUSSION

Results of Screening Tests

Among 358 strains of actinomycetes tested, cultures filtrates of 5 strains were found to be the potent inhibitors of both topoisomerase I and II. The inhibitor produced by strain No. C-127 showed strong inhibition, and it was stable to heat treatment and organic solvents.

Cultural Characteristics of Strain No. C-127

Strain No. C-127 was cultured in various ISP (International *Streptomyces* Project) media and the characteristics are summarized in Table I.[39,40] Aerial

TABLE I Cultural characteristics of strain No. C-127

Yeast-malt ext. agar (ISP No. 2)	Growth	Abundant
	Aerial mycelium	Abundant, greyish brown
	Reverse side of colony	Dark greyish brown
	Soluble pigment	Brown
Oatmeal agar (ISP No. 3)	Growth	Abundant
	Aerial mycelium	Abundant, greyish brown
	Reverse side of colony	Greyish reddish brown
	Soluble pigment	Greyish reddish brown
Sucrose-nitrate agar (Czapek's soln. agar)	Growth	Abundant
	Aerial mycelium	Abundant, greyish white
	Reverse side of colony	Greenish grey
	Soluble pigment	None
Inorganic salts-starch agar (ISP No. 4)	Growth	Abundant
	Aerial mycelium	Abundant, greyish brown
	Reverse side of colony	Moderate brown
	Soluble pigment	Brownish black
Glycerol-asparagine agar (ISP No. 5)	Growth	Moderate
	Aerial mycelium	Abundant, light brownish grey
	Reverse side of colony	Brownish black
	Soluble pigment	Brownish black
Peptone-yeast ext. iron agar (ISP No. 6)	Growth	Moderate
	Aerial mycelium	None
	Reverse side of colony	Colorless
	Soluble pigment	Dark brown
Tyrosine agar (ISP No. 7)	Growth	Abundant
	Aerial mycelium	Abundant, greyish brown
	Reverse side of colony	Brownish black
	Soluble pigment	Deep brown
Nutrient agar	Growth	Moderate
	Aerial mycelium	None
	Reverse side of colony	Colorless
	Soluble pigment	None
Formation of melanoid pigment		Positive
Liquefaction of gelatin		Negative
Coagulation of milk		Negative
Peptonization of milk		Positive
Hydrolysis of starch		Positive
Decomposition of cellulose		Neutral
Utilization of carbon sources:	Positive	D-glucose, L-arabinose, sucrose, D-xylose, inositol, D-mannitol, D-fructose, rhamnose, raffinose, salicin, starch
	Negative	cellulose

mycelia were abundantly developed in yeast-malt extract agar, oat meal agar, sucrose-nitrate agar, inorganic salts-starch agar and tyrosine agar. Mycelium produced spiral chains of spores. Soluble pigments were grey in series. Melanoid pigments were observed in peptone-yeast extract agar and tyrosine agar. From the physiological characteristics and utilization of carbohydrates, the strain was identified as *Streptomyces avermitilis*.[41]

Time Course of Strain No. C-127

The time course of Macrostatin production by *Streptomyces avermitilis* strain No. C-127 in a jar fermentor is shown in Figure 1. The pH of the culture medium invariably remained between 7.0 to 7.5. Mycelial growth gradually peaked on the third day and thereafter declined steadily. Inhibitory activity of Macrostatin increased rapidly on the second day, peaked on the fifth day and, then followed a gradual decline. For an optimum yield of the inhibitor, the culture broth was harvested on the fifth day.

Physicochemical Properties of Macrostatin

The physicochemical properties of Macrostatin are summarized in Table II. Macrostatin was obtained as a white amorphous powder, soluble in water but insoluble in acidic water and organic solvents. The molecular weight

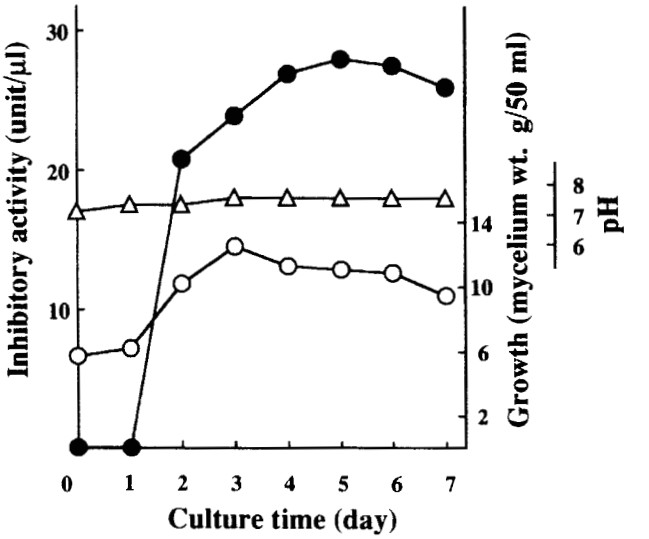

FIGURE 1 Time course of *Streptomyces avermitilis* Strain No. C-127 culture. ●, inhibitory activity; ○, growth; △, pH.

TABLE II Some properties of Macrostatin

Appearance	white powder
Solubility	
Soluble	water
Insoluble	acidic water, methanol, chloroform
Molecular weight	45,000
UV $\lambda_{max}^{H_2O}(E_{1cm}^{1\%})$	225 nm (371.5)
Colour reaction	
Positive	sulfuric acid, azure A, ninhydrin, hydroxamic acid (225 residues of carboxyl group), Rydone-Smith, bromcresol green
Negative	Dittmer, phenol-sulfuric acid, orcinol, Molisch, Elson-Morgan, Ehrlich
Stability (remaining activity)	
(100°C, pH 7.5, 15 min)	100%
(28°C, pH 3.0, 60 min)	100%
(28°C, pH 12.0, 60 min)	18%
Effect on inhibition	
Temp. (pH 7.5, 40 min)	independent (0–60°C)
pH (37°C, 40 min)	dependent (< pH 6.0)

was found to be 45 kDa by Sephadex G-75 gel filtration in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.1 M KCl. The UV spectrum of Macrostatin exhibited maximum absorption at 225 nm in water. Macrostatin gave a positive reaction in tests with sulfuric acid (detection of carbohydrate), bromcresol green (acidic group), ninhydrin (amino group), Rydone-Smith (peptide bond), azure A (sulfur) and hydroxamic acid (carboxyl group) reactions. Dittmer (inorganic phosphorus) and phenol-sulfuric acid (glycoside) reactions were negative. Quantitative estimations revealed that 1 mol Macrostatin contained approximately 225 mol of carboxylic acid.[42] The amino acid content determined by the PICO-TAG method[43] appeared to be 5 mol lysine, 2 mol tyrosine, 1 mol serine and 1 mol threonine per 1 mol Macrostatin. The inhibitor was considered to be an acidic carbohydrate containing some amino acids and carboxylic acids but having no glycoside groups. Further investigations are required for complete structural elucidation. Macrostatin was stable at acidic pH and on heat treatment whereas it was unstable at alkaline pH. The inhibitory activity of Macrostatin was independent of temperature whereas the activity was shown to be pH-dependent and decreased below pH 6.0 and with completely lost at pH 5.0.

Inhibition of Topoisomerase Relaxation and Decatenation Activities

Inhibitory activities of Macrostatin against topoisomerase I relaxation, topoisomerase II relaxation and decatenation were measured as shown in Figure 2. In the presence of increasing Macrostatin, all the activities were

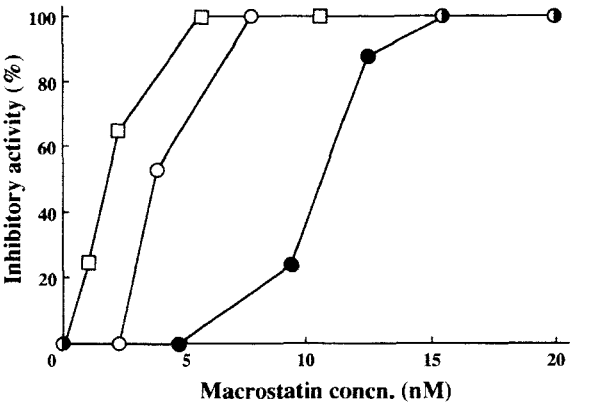

FIGURE 2 Inhibitory activities of Macrostatin against topoisomerase I relaxation (●); topoisomerase II relaxation (○) and topoisomerase II decatenation (□).

antagonized dose-dependently as evidenced by the appearance of supercoiled pBR322 DNA and decatenated kDNA. The inhibitory concentration (IC_{50}) of Macrostatin against topoisomerase I was 10.7 nM whereas it was 3.7 and 2.0 nM against topoisomerase II relaxation and decatenation activities, respectively. Macrostatin showed the strongest inhibition against topoisomerase II decatenation activity. The inhibition was double than that against topoisomerase II relaxation activity and 5-fold higher than that against topoisomerase I relaxation activity.

K_i Values of Macrostatin against Topoisomerases

The type of inhibition was determined by a Lineweaver-Burk plot[43] of the reciprocal of substrate concentration against the rate of relaxation of supercoiled pBR322 DNA by topoisomerase I and topoisomerase II in the presence or absence of Macrostatin. As shown in Figures 3(A) and (B), relaxation of pBR322 DNA by topoisomerase I and II was noncompetitively inhibited by Macrostatin, and the K_i values were 3.7 and 1.3 nM, respectively. The K_m values of topoisomerase I and II were 9.0 and 15.6 nM, respectively. Macrostatin was 3-fold more potent against topoisomerase II. From these results, Macrostatin was a highly potent inhibitor against topoisomerase II and the inhibitor was considered to bind strongly to a different site from the binding site of the substrate DNA on the enzyme molecule.

Topoisomerase I and II-mediated DNA Cleavage by Macrostatin

Topoisomerases adjust the topological state of DNA by breaking and rejoining DNA strands, resulting in alterations in DNA linking number.

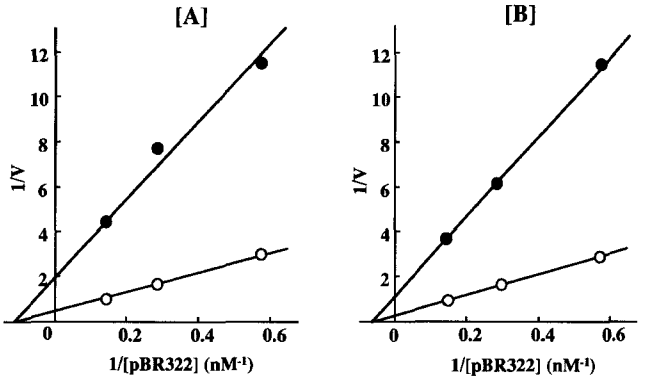

FIGURE 3 Lineweaver–Burk reciprocal plots of substrate (supercoiled pBR322 DNA) concentration against rate of relaxation by topoisomerase I [A] and relaxation by topoisomerase II [B] with (●) and without (○) Macrostatin. The K_i values of Macrostatin against topoisomerase I and II were 3.7 and 1.3 nM, respectively.

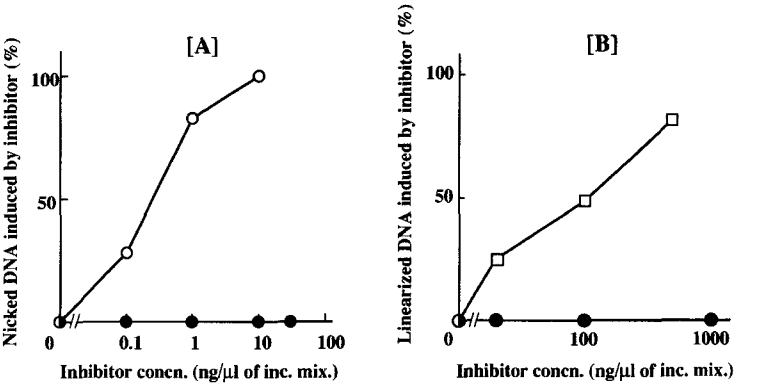

FIGURE 4 Topoisomerase I [A] and II [B]-mediated DNA cleavage by Macrostatin (●); camptothecin (○) as control of topoisomerase I-mediated cleavage and etoposide (□) as control of topoisomerase II-mediated cleavage.

Camptothecin and etoposide, topoisomerase inhibitors, stabilize the cleavable complex (enzyme-DNA reaction intermediate) and inhibit DNA rejoining reaction of topoisomerases,[7,45,46] which is the mechanism of its enzyme inhibition.[20] Therefore, these inhibitors induce the nicked DNA or linearized DNA in the cleavage assays described in Materials and Methods. To determine whether Macrostatin stabilizes the complex, these assays were carried out. As shown in Figure 4(A), camptothecin induced nicked DNA with increasing concentrations and the activity of camptothecin reached the maximum at 10 ng/μl in the incubation mixture. Unlike camptothecin, Macrostatin could not induce nicked DNA.

The stabilization of the Topoisomerase II-cleavable complex by etoposide[12] and Macrostatin was examined using human placental topoisomerase II. As shown in Figure 4(B), etoposide induced the linearized DNA and 83% linearization of DNA was observed with 500 ng of etoposide. Macrostatin failed to linearize DNA even at 1,000 ng, an extreme concentration. These data suggested that Macrostatin does not inhibit topoisomerase by stabilizing the cleavable complex.

The inhibition mechanism of Macrostatin differed from that of camptothecin and etoposide. Macrostatin may directly act on the topoisomerase molecule in the earlier step before the formation of the cleavable complex and inhibits DNA breaking and rejoining reactions by the enzyme.

DNA Binding Competition with Ethidium Bromide

To determine whether Macrostatin has the ability to intercalate into DNA strands, EtBr-competition assay was carried out using salmon sperm DNA. Camptothecin and doxorubicin, being nonintercalator and intercalator, respectively, were used as controls at the same concentrations. As shown in Figure 5, doxorubicin competed with EtBr for DNA and produced an 84% decrease in the fluorescence of EtBr at a concentration of 1 ng/μl in the incubation mixture. Since camptothecin did not intercalate into DNA, the

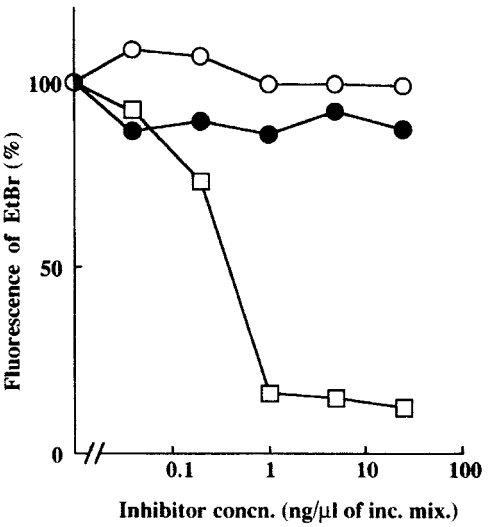

FIGURE 5 Effects of Macrostatin (●), camptothecin (○) and doxorubicin (□) on DNA binding competition with ethidium bromide.

intensity of fluorescence did not decrease at all. Similarly, Macrostatin did not decrease the fluorescence of EtBr at 25 ng. Consequently it is clear that Macrostatin has no ability to intercalate into DNA strands.

Antimicrobial Activity

The antimicrobial activity of Macrostatin was tested by the agar dilution streak method.[38] 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 *Aspergillus niger*), no significant antimicrobial activity could be detected.

Inhibitory Spectrum

The effects of Macrostatin on various enzymes were examined and are summarized in Table III. Reactions were performed as described in Materials and Methods. All of the restriction endonucleases tested were significantly inhibited by low concentrations of Macrostatin. Other enzymes such as RNase, DNase I, DNase II and T4 DNA ligase were not inhibited even at 400 nM of Macrostatin. Inhibitory effects of Macrostatin against both topoisomerase I and II might be through nonspecific interaction against the enzyme molecules because it also inhibited the other enzymes which act on DNA.

TABLE III Inhibitory spectrum of Macrostatin

Enzyme	IC_{50} (nM)[a]
Topoisomerase I[b]	10.7
Topoisomerase II[b]	3.8
Topoisomerase II[c]	2.0
Eco RI	2.0
Bam HI	8.9
Pst I	3.6
Sca I	1.3
Hin dIII	3.6
Alu I	3.6
RNase A	> 400.0
DNase I	> 400.0
DNase II	> 400.0
T4 ligase	> 400.0

[a]Values represent the means obtained from two independent experiments.
[b]Relaxation activity.
[c]Decatenation activity.

TABLE IV Comparison of Macrostatin with other inhibitors

Inhibitor	*Molecular weight*	*Origin*	*Inhibition*			*Stabilization of cleavable complex*	*Intercalation into DNA strands*
			Topo I		*Topo II*		
Macrostatin	45,000	*Streptomyces* sp.	+	<	+	−	−
2890-DTI	370,000	*Streptomyces* sp.	+	>	+	−	−
2280-DTI	3,000,000	*Micromonospora* sp.	+		−	−	−
Camptothecin	348	plant	+		−	+	−
Etoposide	589	plant	−		+	+	−
Doxorubicin	544	*Streptomyces* sp.	−		+	+	+
Amsacrine	393	synthetic	−		+	+	+

+: positive, −: negative.

Comparison with Other Inhibitors

Some properties of topoisomerase inhibitors are summarized in Table IV. Previously, we reported two inhibitors named 2280-DTI and 2890-DTI having 3,000 and 370 kDa molecular weight, respectively. Macrostatin has a molecular weight of 45 kDa. 2280-DTI inhibited only topoisomerase I. 2890-DTI was a more potent inhibitor of topoisomerase I than topoisomerase II. In contrast, Macrostatin was more effective against topoisomerase II than topoisomerase I.

The reported inhibitors of topoisomerase such as camptothecin and etoposide inhibit the DNA rejoining reaction of the enzyme by stabilizing the cleavable complex, and those such as doxorubicin and amsacrine show stabilization of the cleavable complex and intercalation into DNA strands. In contrast, Macrostatin, 2280-DTI and 2890-DTI directly acted on topoisomerase molecule and inhibited the DNA breaking and rejoining reactions of the enzyme.

From these properties, it has been shown that Macrostatin is different from the other topoisomerases inhibitors reported so far.

References

[1] Vosberg, H.P. (1985) *Curr. Top. Microbiol. Immunol.*, **114**, 19–101.
[2] Wang, J.C. (1985) *Ann. Rev. Biochem.*, **54**, 655–697.
[3] Liu, L.F. (1980) In *ICN-UCLA Symposia on Molecular and Cellular Biology* (Alberts, B.M. and Fox, C.F. (eds)), Vol. 19, pp. 817–831. Academic Press; New York.
[4] Tse, Y.C. and Wang, J.C. (1980) *Cells*, **22**, 269–276.
[5] Brown, P.O. and Cozzarelli, N.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 843–847.
[6] Depew, R.E., Liu, L.F. and Wang, J.C. (1978) *J. Biol. Chem.*, **253**, 511–518.
[7] Liu, L.F. and Wang, J.C. (1979) *J. Biol. Chem.*, **254**, 11082–11088.
[8] Champoux, J.J. (1978) *Annu. Rev. Biochem.*, **47**, 449–479.
[9] Pulleyblank, D.E., Shure, M., Tang, D., Vinograd, J. and Vosberg, H.P. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4280–4284.

[10] Nash, H.A., Mizuuchi, K., Enquist, L.W. and Weisberg, R.A. (1981) *Cold Spring Harbor Symp. Quant. Biol.*, **45**, Part 1: 417–428.
[11] Muller, M.T., Spitzner, J.R., DiDanato, J.A., Metha, V.B., Tsutsui, K. and Tsutsui, K. (1988) *Biochemistry*, **27**, 8369–8379.
[12] Yamashita, Y., Kawada, S., Fujii, N. and Nakano, H. (1991) *Biochemistry*, **30**, 5838–5845.
[13] Mattern, M.R., Paone, R.F. and Days, R.R. (1982) *Biochim. Biophys. Acta*, **697**, 6–13 III.
[14] Liu, L.F. (1989) *Annu. Rev. Biochem.*, **58**, 351–375.
[15] Drlica, K. and Franco, J.R. (1988) *Biochemistry*, **27**, 2253–2259.
[16] Nelson, E.M., Tewey, K.M. and Liu, L.F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1361–1365.
[17] Tewey, K.M., Chen, G.L., Nelson, E.M. and Liu, L.F. (1984) *J. Biol. Chem.*, **259**, 9182–9187.
[18] Tewey, K.M., Row, T.C., Yang, L., Halligan, B.D. and Liu, L.F. (1984) *Science*, **226**, 466–468.
[19] Chen, G.L., Yang, L., Rowe, T.C., Halligan, B.D., Tewey, K.M. and Liu, L.F. (1984) *J. Biol. Chem.*, **259**, 13560–13566.
[20] Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F. (1985) *J. Biol. Chem.*, **260**, 14873–14878.
[21] Kawada, S., Yamashita, Y., Fujii, N. and Nakano, H. (1991) *Cancer Res.*, **51**, 2922–2925.
[22] Suzuki, K., Siddique, T., Nishimura, H., Sekimoto, J. and Uyeda, M. (1998) *J. Enz. Inhib.*, **13**, 41–55.
[23] Liu, L.F. and Miller, K.G. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3487–3491.
[24] Fujii, N., Yamashita, Y., Saitoh, Y. and Nakano, H. (1993) *Biochemistry*, **268**, 13160–13165.
[25] Spitzner, J.R. and Muller, M.T. (1988) *Nuclei Acid Res.*, **16**, 5533–5556.
[26] Yamashita, Y., Kawada, S., Fujii, N. and Nakano, H. (1990) *Cancer Res.*, **50**, 5841–5844.
[27] Roberts, R.J., Myers, P.A., Morrison, A. and Murray, K. (1976) *J. Mol. Biol.*, **102**, 157.
[28] Georg, J., Blakesley, R.W. and Chirikjian, J.G. (1980) *J. Biol. Chem.*, **255**, 6521.
[29] Green, P.J., Betlach, M.C., Goodman, H.M. and Boyer, H.W. (1974) *Mol. Biol.*, **7**, 87.
[30] Old, R., Murray, K. and Roizes, G. (1975) *J. Mol. Biol.*, **92**, 331–339.
[31] Smith, D.L., Blattner, F.R. and Davis, J. (1976) *Nucleic Acids Res.*, **3**, 343.
[32] Kojima, H., Takahasi, H. and Saito, H. (1985) *Biochem. J.*, **231**, 229.
[33] Kunitz, M. (1950) *J. Gen. Physiol.*, **33**, 349.
[34] Kunitz, M. (1950) *J. Gen. Physiol.*, **33**, 365.
[35] Richards, F.M. and Wyckoff, H.W. (1971) In *The Enzymes* (Boyer, P. D. (ed.)) Vol. 4, p. 647. Academic Press; New York.
[36] Weiss, B., Jacquemin-Sablon, A., Live, T.R., Fareed, G.C. and Richardson, C.C. (1968) *J. Biol. Chem.*, **243**, 4543–4555.
[37] Horiguchi, T., Hayashi, K., Tsubotani, S., Iinuma, S., Harada, S. and Tanida, S. (1994) *J. Antibiotics*, **47**, 545–555.
[38] Waksman, S.A. and Reilly, H.C. (1945) *Ind. Eng. Chem.*, **17**, 566.
[39] Shiring, E.B. and Gottlieb, (1966) *Int. J. Syst. Bacteriol.*, **16**, 313–340.
[40] Williams, S.T., Sharp, M.E. and Holt, G.J. (1989) *Bergey's Manual of Systematic Bateriology*, Vol. 4. Williams and Wilkins Co.; Baltimore.
[41] Burg, R.W., Miller, B.M., Baker, E.E., Birnbaum, J., Currie, S.A., Hartman, R., Kong, Y., Monaghan, R.L., Olson, G., Putter, I., Tunac, J.B., Wallick, H., Stapley, E.O., Oiwa, R. and Omura, S. (1979) *Antimicrob. Agents Chemother.*, **15**, 361–367.
[42] Kasai, Y., Tanimura, T. and Tamura, Z. (1975) *Anal. Chem.*, **47**, 34–37.
[43] Shoji, S., Ichikawa, M., Yamaoka, T., Funakoshi, T. and Kubota, Y. (1986) *J. Chromatogr.*, **354**, 463–470.
[44] Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.*, **56**, 658.
[45] Champoux, J.J. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3800–3804.
[46] Trask, D.K. and Muller, M.T. (1983) *Nucleic Acid Res.*, **11**, 2779–2800.