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Inhibition of Topoisomerase II by a Novel Antitumor Cyclic Depsipeptide, BE-22179

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BE-22179, a novel cyclic depsipeptide antibiotic having two 3-hydroxyquinoline moieties, inhibited the DNA-relaxing activity of L1210 topoisomerase II completely at 0.08 μM. This effect was far stronger than that of VP-16. However, it did not show any marked effect on topoisomerase II-mediated DNA cleavage. BE-22179 was ineffective in inhibiting the DNA relaxation by topoisomerase I at concentrations up to 10 μM, but showed DNA-intercalating ability (DNA unwinding) at 30 μM. The structure of BE-22179 is quite novel for a topoisomerase II inhibitor. Echinomycin, a quinoxaline antibiotic structurally related to BE-22179, interfered with DNA relaxation by topoisomerase II, though the effect was not due to inhibition of the catalytic activity of topoisomerase II but to conformational change of DNA based on its intercalation into DNA. Therefore, the potent inhibitory activity on topoisomerase II might not be a common activity of quinoxaline antibiotics, but might rather be specific to BE-22179. BE-22179 prevented DNA synthesis as well as RNA synthesis in L1210 cells and inhibited the growth of the cells. However, it remains unclear to what extent the topoisomerase II inhibition was responsible for the cytotoxicity of BE-22179.

Key words: Topoisomerase II — DNA relaxation — Cyclic depsipeptide — Quinoxaline antibiotic — Antitumor agent

DNA topoisomerase II is an enzyme responsible for the conversion of DNA topology that is essential for the processes of DNA replication and RNA transcription.[1] This enzyme is therefore an attractive intracellular target for anticancer chemotherapy.[2,3] In screening for topoisomerase II inhibitors, we found a new antitumor antibiotic, BE-22179, from the culture broth of streptomyces. This compound showed antitumor activity as well as potent antibacterial activity against gram-positive bacteria[4]: BE-22179 prolonged the survival time of mice bearing L1210 leukemia 2.82-fold over the control when administered i.p. for 10 days at 0.25 mg/kg/day.

The structure of BE-22179 is quite novel for a topoisomerase II inhibitor: it is a cyclic depsipeptide. This structure is similar to that of quinoxaline antitumor antibiotics, such as quinomycins and triostins, which are characterized by a depsipeptide ring attached to two quinoxaline moieties.[5]

Among many quinoxaline antibiotics, the one which came closest to application as a practical antitumor agent is echinomycin (quinomycin A). Its antitumor effect was investigated at the National Cancer Institute of the United States[6] and clinical testing was started.[7] However, further evaluation was halted because of severe toxicity.[6] Echinomycin and other quinoxaline antibiotics have a characteristic bifunctional DNA binding mechanism based on their staple-like stereo structure.[8] Echinomycin inhibits RNA synthesis in cells very potently[9] and this inhibition was considered to be due to its rigid DNA intercalation.[6]

Because of the structural similarities between BE-22179 and echinomycin, we have been interested in whether the inhibition of topoisomerase II by BE-22179 is a common feature of depsipeptides, including echinomycin. In this paper, we describe the biochemical characteristics of BE-22179, focusing on topoisomerase II inhibition and DNA intercalation, in comparison with those of echinomycin.

MATERIALS AND METHODS

Reagents and cells BE-22179 (Fig. 1) was isolated from the culture broth of *Streptomyces*, strain A 22179, as reported previously.[4] Echinomycin (Fig. 1) was a generous gift from Dr. K. Komiyama of the Kitasato Institute. VP-16[4] was obtained from Nippon Kayaku Co., Ltd., Tokyo. [Methyl,1′,2′-^{3}H]thymidine and [5′,6-^{3}H]uridine were purchased from Amersham Japan. L1210 mouse leukemic cells were purchased from Dainippon Pharma-

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[4] The abbreviations used are: VP-16, 4′-demethylepipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside) (etoposide); SDS, sodium dodecyl sulfate; IC_{50}, 50% inhibitory concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

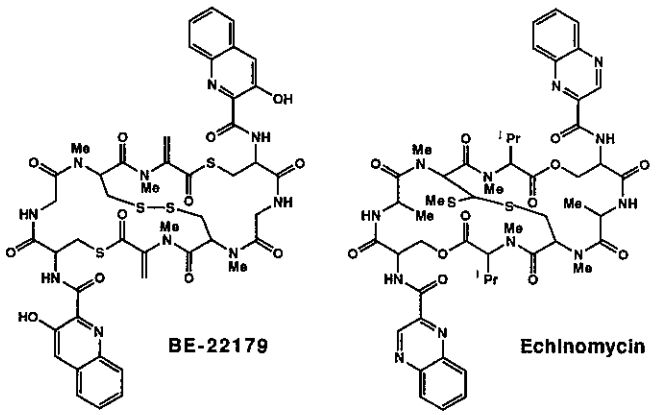

Fig. 1. Structures of BE-22179 and echinomycin.

ceutical Co. Other reagents were obtained from Sigma Chemical Co.

Enzymes Topoisomerases I and II were purified from L1210 cells by hydroxyapatite (Seikagaku Corporation), Mono Q, and Mono S (Pharmacia LKB, Biotechnology) column chromatographies according to the method of Drake *et al.*[10] The purity was 42% for topoisomerase I (Mr 97 kD) and 74% for topoisomerase II (Mr 170/180 kD) as assessed by silver staining in 7.5% SDS polyacrylamide gel. One unit of enzyme was defined as the activity that completely relaxed 0.4 μg of supercoiled pBR322 DNA in the relaxation assay described below.

DNA relaxation by topoisomerase II and topoisomerase I Supercoiled pBR322 DNA (0.4 μg) was incubated with 1 unit or 50 units of topoisomerase II in 20 μl of the reaction buffer [50 m*M* Tris-HCl (pH 7.9), 120 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 0.5 m*M* EDTA, 30 μg/ml bovine serum albumin, and 1 m*M* ATP] in the presence or absence of a test compound at 30°C for 15 min. The reaction was stopped by the addition of 5 μl of 50 m*M* EDTA containing 0.5% SDS and 5 *M* NaCl. In order to remove substances binding DNA, the DNA was extracted twice with phenol:chloroform (4:1) and once with chloroform. After ethanol precipitation, the DNA was subjected to 1.2% agarose gel electrophoresis with 0.5×TBE (90 m*M* Tris, 90 m*M* boric acid, and 4 m*M* EDTA) buffer. The amount of remaining supercoiled DNA in the gel was quantified by a densitometer (flying spot scanner C-9000, Shimadzu Co.).

The DNA relaxation by topoisomerase I was assayed in the same manner as that by topoisomerase II, without ATP.

DNA cleavage mediated by topoisomerase II Supercoiled pBR322 DNA (0.4 μg) was incubated with 50 units of topoisomerase II in the same buffer as used for DNA relaxation assay at 30°C for 15 min. The reaction was stopped by the addition of 3 μl of 5% SDS and the enzyme bound with DNA was digested with 1 mg/ml proteinase K at 45°C for 1 h. DNA was extracted and subjected to gel electrophoresis. Linear DNA generated by double-strand cleavage was quantified by densitometry.

Unwinding of closed circular DNA To discriminate between inhibition of DNA relaxation and unwinding of DNA, the DNA-unwinding assay using relaxed form DNA was performed. Relaxed form of closed circular pBR322 DNA was prepared by incubation with L1210 topoisomerase I followed by extraction with phenol:chloroform and precipitation with ethanol. The relaxed DNA was mixed with the test compound in the same reaction buffer as used for DNA relaxation assay. The reaction was started by adding 25 units of topoisomerase I and terminated by adding 10 m*M* EDTA containing 0.1% SDS and 1 *M* NaCl. DNA was extracted, precipitated and then subjected to gel-electrophoresis without ethidium bromide. Formation of supercoiled DNA in the gel indicates that relaxed DNA is unwound by intercalation of a test compound and is positively supercoiled during the reaction.[11]

Uptake of thymidine and uridine Prior to the addition of radioactive precursor, L1210 cells were exposed to each test compound for 2 h at 37°C. The cells were then incubated with 4 μCi of [^{3}H]thymidine or [^{3}H]uridine for 30 min and harvested on a Millipore SV filter. After washing with 5% trichloroacetic acid and 100% ethanol, the radioactivity of the acid-insoluble fraction was measured.

Cytotoxicity toward cultured cells L1210 cells (3×10^3/ml) were cultured with test compounds in a 96-well plate for 3 days. The number of living cells was measured by the MTT method.[12]

RESULTS

Inhibitory effect on DNA-relaxing activity of topoisomerase II The effect of BE-22179 on the catalytic activity of 1 unit of topoisomerase II was evaluated by assay of the relaxation of pBR322 supercoiled DNA. BE-22179 inhibited the DNA relaxation completely at 0.08 μM (Fig. 2A). Echinomycin and VP-16 inhibited the reaction completely at 10 and 50 μM, respectively.

In this assay, DNA intercalating agents, without inhibiting topoisomerases, can show "false" inhibition by inducing conformational change of DNA.[13] To clarify whether the inhibitory effects obtained here were due to suppression of the catalytic activity of topoisomerase II, a mode of decline of inhibition upon adding topoisomerase II was investigated (Fig. 2B) and the results are summarized in terms of the IC_{50} values against 1 and 50 units topoisomerase II in Table I. The inhibitory effects of BE-22179 and VP-16 were significantly weakened

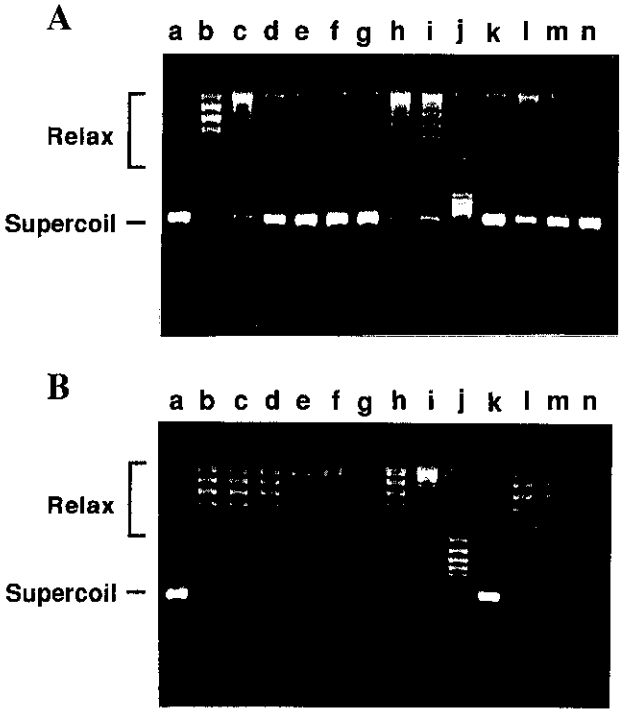

Fig. 2. Effect of BE-22179 on the DNA-relaxing activity of topoisomerase II. Supercoiled pBR322 DNA was incubated with 1 unit (A) or 50 units (B) of L1210 topoisomerase II in the presence or absence of a test compound at 30°C for 15 min. After removal of the test compound, DNA was subjected to electrophoresis in 1.2% agarose gel with 0.5×TBE buffer. Lane a; control DNA without topoisomerase II; lane b; control DNA plus topoisomerase II; lanes c–g, 0.016, 0.08, 0.4, 2.0 and 10 μM BE-22179, respectively, plus topoisomerase II; lanes h–k, 0.08, 0.4, 2.0 and 10 μM echinomycin, respectively, plus topoisomerase II; lanes l–n, 10, 50 and 250 μM VP-16, respectively, plus topoisomerase II.

Table I. Inhibitory Effect of BE-22179 against 1 and 50 Units of Topoisomerase II

Compound	IC_{50}[a] (μM)	
	1 unit	50 units
BE-22179	0.034	8.0
Echinomycin	4.7	4.7
VP-16	9.2	>250

a) IC_{50} Values were estimated by densitometric determination of supercoiled DNA in Fig. 2 A and B.

when 50 units of topoisomerase II was added. On the other hand, echinomycin inhibited the relaxation by 50 units of topoisomerase II at almost the same concentration as in the case of 1 unit of topoisomerase II. These results indicate that the inhibitory effect of BE-22179

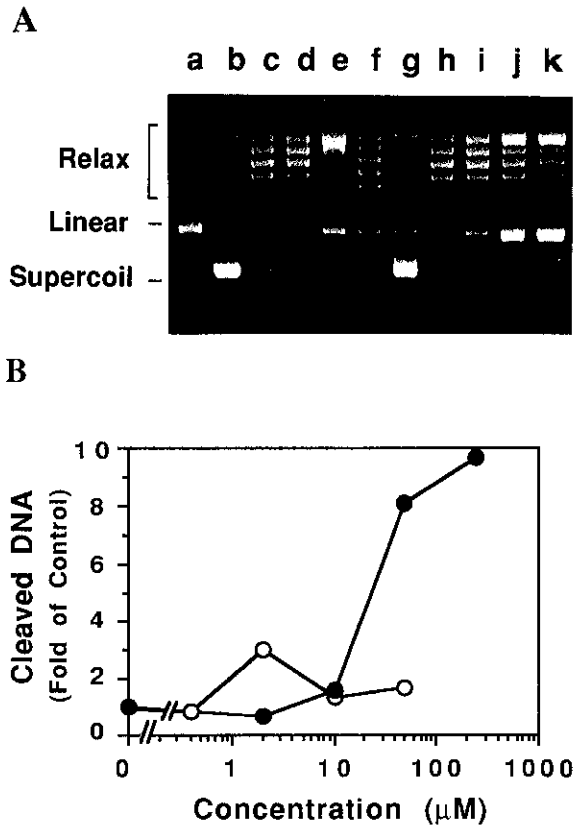

Fig. 3. Effect of BE-22179 on topoisomerase II-mediated DNA cleavage. DNA was subjected to electrophoresis in 1.2% agarose gel with 0.5×TBE buffer. (A) lane a, marker DNA of linear form; lane b, control DNA without topoisomerase II; lane c, control DNA plus topoisomerase II; lanes d, e, f and g, 0.4, 2.0, 10 and 50 μM BE-22179 plus topoisomerase II; lanes h, i, j and k, 2, 10, 50 and 250 μM VP-16 with topoisomerase II. (B) The cleaved DNA (linear form) in Fig. 3A was quantified by a densitometer. The ordinate scale indicates the intensity of topoisomerase II-mediated DNA cleavage expressed relative to the control (fold). (○), BE-22179; (●), VP-16.

greatly depended on the amount of topoisomerase II, while that of echinomycin was not affected by the amount of topoisomerase II. This finding also suggests that BE-22179 interacts with enzyme itself.

Effect on topoisomerase II-mediated DNA cleavage To elucidate the inhibitory mechanism of BE-22179 on topoisomerase II, the effect of BE-22179 on topoisomerase II-mediated DNA cleavage was measured. VP-16 showed dose-dependent enhancement of topoisomerase II-mediated cleavage; VP-16 increased the cleaved DNA by 10-fold over the control at 250 μM. On the other hand, BE-22179 induced little cleavage in spite of its potent inhibition of DNA relaxation: the concentration-

response curve of BE-22179 was bell-shaped and the concentration exhibiting maximal response (3-fold over the control) was 2.0 μM (Fig. 3). These results indicate that the stabilization of a cleavable complex is not the main mechanism of the inhibitory effect of BE-22179.

DNA-unwinding activity Quinoxaline antibiotics, including echinomycin, are known to intercalate into DNA via the two quinoxaline moieties.[5] BE-22179 has two 3-hydroxyquinoline moieties which are expected to intercalate into DNA. The intercalating ability of BE-22179 was evaluated by an unwinding assay using relaxed circular DNA. BE-22179 appeared to intercalate into DNA at 30 μM and more (Fig. 4). Echinomycin and actinomycin D unwound the DNA at 3 and 1 μM, respectively. When the DNA-unwinding activity was measured with 50 units of topoisomerase II instead of topoisomerase I, relaxed DNA was also converted to supercoiled form by the addition of 3 μM echinomycin and 1 μM actinomycin D, whereas the DNA remained relaxed after the addition of BE-22179 (data not shown). This finding confirms that the DNA-unwinding assay using topoisomerase I was performed properly, that is, the tested compounds did not interfere with topoisomerase I but unwound the DNA.

Considering these results together with the data of Fig. 2, the inhibition of DNA relaxation by echinomycin seems to be due to the unwinding of substrate DNA, not to the inhibition of topoisomerase II, because the concentration of echinomycin showing DNA-unwinding activity was very close to that inhibiting DNA relaxation of topoisomerase II.

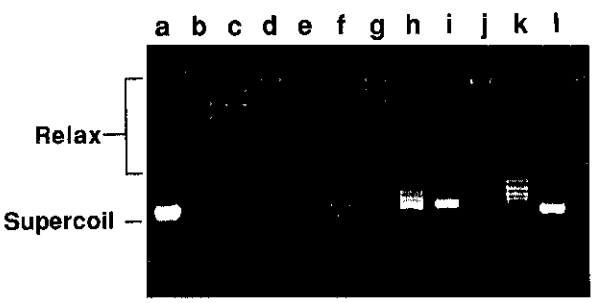

Fig. 4. Effect of BE-22179 on DNA unwinding. Relaxed pBR322 DNA was incubated with a test compound in the same reaction buffer as for the relaxation assay at room temperature for 30 min. The reaction was started by adding 25 units of topoisomerase I. After the removal of the test compound, DNA was subjected to 1.2% agarose gel electrophoresis. Lane a, marker DNA of supercoiled form; lane b, control DNA without topoisomerase I; c, control DNA plus topoisomerase I; lanes d, e and f, 10, 30 and 100 μM BE-22179 plus topoisomerase I; lanes g, h and i, 1, 3 and 10 μM echinomycin plus topoisomerase I; lanes j, k and l, 0.3, 1 and 3 μM actinomycin D plus topoisomerase I.

Effect on DNA-relaxing activity of topoisomerase I BE-22179 did not inhibit the DNA-relaxing activity of 1 unit of topoisomerase I at concentrations up to 10 μM (Fig. 5). The DNA incubated with 50 μM BE-22179 remained supercoiled, but, as shown in Fig. 4, the effect was probably due not to topoisomerase I inhibition but to conformational change of closed circular DNA. Camptothecin, a topoisomerase I inhibitor, completely inhibited topoisomerase I at 10 μM.

Effect on nucleic acid synthesis in cells To study the effect of BE-22179 on DNA and RNA syntheses in intact cells, incorporation of radioactive precursors, uridine and thymidine, into macromolecules was investigated. Consistent with the report of Gause *et al.*,[9] echinomycin inhibited predominantly the uptake of uridine rather than that of thymidine (Table II). BE-22179 inhibited the uptake of thymidine as well as uridine. By comparing the ratio of IC_{50}s against uridine uptake and thymidine uptake (B/A in Table II), it was shown that the selective inhibitory effect of BE-22179 against RNA synthesis was not so marked as that of echinomycin.

Cytotoxicity L1210 cells were incubated with BE-22179 for 3 days and the number of live cells was estimated by the MTT method. BE-22179 suppressed the growth of L1210 cells by 50% at 24 nM (Table III).

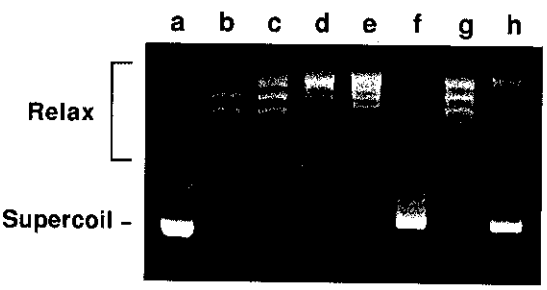

Fig. 5. Effect of BE-22179 on the DNA relaxing activity of topoisomerase I. Supercoiled pBR322 DNA was incubated with 1 unit of topoisomerase I at 30°C for 15 min. Lane a, control DNA without topoisomerase I; lane b, control DNA plus topoisomerase I; lanes c, d, e and f, 0.2, 3, 10 and 50 μM BE-22179 plus topoisomerase I; lanes g and h, 2 and 10 μM camptothecin plus topoisomerase I.

Table II. Inhibitory Effect of BE-22179 on Uptake of Uridine and Thymidine into L1210 Cells

Compound	IC_{50} (μM)		(B)/(A)
	Uridine (A)	Thymidine (B)	
BE-22179	0.078	0.43	5.5
Echinomycin	0.0072	1.4	194

Table III. Inhibitory Effect of BE-22179 on the Growth of L1210 Cells

Compound	IC_{50} (nM)
BE-22179	24
Echinomycin	0.56
VP-16	130

DISCUSSION

BE-22179 potently inhibited the DNA-relaxing activity of topoisomerase II: IC_{50} of BE-22179 for the topoisomerase II-mediated DNA relaxation was approximately 270-fold lower than that of VP-16 (Table I). From the results obtained in the DNA-unwinding assay (Fig. 4), this compound appeared to be an intercalator, like a number of quinoxaline antibiotics. However, the fact that BE-22179 inhibited topoisomerase II at far lower concentration than that showing DNA-unwinding activity indicates that the anti-topoisomerase II activity of BE-22179 is independent of conformational change of DNA. On the other hand, echinomycin inhibited the DNA relaxation activity of topoisomerase II at a concentration very close to that unwinding the DNA (2–10 μM). Therefore the apparent inhibition of DNA relaxation by echinomycin was probably due to conformational change of DNA caused by intercalation. This view is supported by the observation that the IC_{50} of echinomycin against DNA relaxation was constant (4.7 μM) no matter how much topoisomerase II was added to the reaction mixture.

In spite of the potent inhibition of topoisomerase II-mediated DNA relaxation, BE-22179 induced double-stranded DNA cleavage at only 3 times the control level, and its concentration-response curve was bell-shaped (Fig. 3). Such a bell-shaped response has often been observed with DNA-intercalative topoisomerase II inhibitors,[14, 15] but their kinetic mode of inhibition has not been explained. The finding that IC_{50} value of BE-22179 on topoisomerase II-mediated relaxation declined 235-fold on increasing the amount of topoisomerase II from 1 to 50 units (Table I) suggests that BE-22179 interacts with the topoisomerase II molecule directly or prevents the binding of topoisomerase II to its reactive site on DNA. Further investigation is necessary to elucidate its inhibitory mechanism.

The inhibitory effect of BE-22179 on DNA relaxation by topoisomerases was specific to topoisomerase II. Since BE-22179 changed the conformation of DNA at 30 μM, precise evaluation of the inhibitory effect against topoisomerase I was difficult. However, BE-22179 was at least 100-fold more inhibitory towards topoisomere II than topoisomerase I.

The inhibition of uridine uptake by BE-22179 was not as specific as that by echinomycin. BE-22179 seems to have some other activity than inhibition of RNA synthesis which is considered to be the main antitumor mechanism of quinoxaline antibiotics. Although the contribution of topoisomerase II inhibition to the inhibition of nucleic acid syntheses and the cytotoxicity to intact cells by BE-22179 remains to be elucidated, our discovery may represent a new biochemical potential of certain cyclic depsipeptide anticancer antibiotics.

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