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INHIBITION OF TOPOISOMERASES BY FATTY ACIDS

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The inhibitory effects of various fatty acids on topoisomerases were examined, and their structure–activity relationships and mechanism of action were studied. Saturated fatty acids ($C_{6:0}$ to $C_{22:0}$) did not inhibit topoisomerase I, but *cis*-unsaturated fatty acids ($C_{16:1}$ to $C_{22:1}$) with one double bond showed strong inhibition of the enzyme. The inhibitory potency depended on the carbon chain length and the position of the double bond in the fatty acid molecule. The *trans*-isomer, methyl ester and hydroxyl derivative of oleic acid had no or little inhibitory effect on topoisomerases I and II. Among the compounds studied petroselinic acid and vaccenic acid ($C_{18:1}$) with a *cis*-double bond were the potent inhibitors. Petroselinic acid was a topoisomerase inhibitor of the cleavable complex-nonforming type and acted directly on the enzyme molecule in a noncompetitive manner without DNA intercalation.

Keywords: Topoisomerase; Fatty acid; Petroselinic acid; Enzyme inhibitor

INTRODUCTION

Topoisomerases I and II[1,2] are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands and 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.[3] In addition to their normal cellular functions, the enzymes are known as important cellular targets of some antitumor drugs.[4] In the search for new topoisomerase inhibitors, we have screened various microorganisms and found four kinds of novel inhibitors designated as 2280-DTI, 2890-DTI,

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macrostatin and topostatin which are the inhibitors of the cleavable complex-nonforming type.[5-9] During this study, we discovered the inhibition of topoisomerases by unsaturated fatty acids. It is well known that fatty acids inhibit H^+, K^+-ATPase, DNA methyltransferase, carbonyl reductase and so on.[10-12] We have examined the inhibitory activities of various fatty acids against topoisomerases, and elucidated their structure–activity relationships and mechanism of action.

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), *Eco* RI (EC 3.1.23.13), *Hind* III (EC 3.1.23.21) and supercoiled pBR322 DNA were obtained from MBI Fermentas (Vilnius). 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 (Tokyo). Topoisomerase I from wheat germ was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo). Salmon sperm DNA was obtained from Boehringer Mannheim GmbH (Tokyo). *Alu* I (EC 3.1.23.1), *Sca* I (EC 3.1.21.4) and *Pst* I (EC 3.1.23.31) were obtained from Gibco BRL (Tokyo). Topoisomerase II (EC 5.99.1.3) from human placenta and kinetoplast DNA from *Crithidia fasciculata* were obtained from TopoGEN (Columbus). For preparation of topoisomerase I from COLO 201 (human colon carcinoma), HeLa (human cervix carcinoma), A549 (human lung carcinoma), Vero (african monkey kidney) or NIH3T3 (mouse embryo) cells, the cells separately cultured for 5 days were washed with 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-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 10% glycerin), and kept on ice for 30 min. The lysate was centrifuged and the supernatant was used as an enzyme solution of the topoisomerase I obtained from each cell line.

Fatty Acids and Inhibitors

Saturated fatty acids ($C_{6:0}$ to $C_{22:0}$), *cis*-unsaturated fatty acids ($C_{14:1}$ to $C_{22:1}$), linoleic acid, linolenic acid, elaidic acid, ricinoleic acid and

methyloleate were obtained from Funakoshi Co. (Tokyo). *Cis*-unsaturated fatty acids ($C_{20:2}$ to $C_{20:5}$) of twenty carbon atoms were obtained from Cayman Chemical Co. (Ann Arbor). Camptothecin, etoposide and doxorubicin were obtained from Aldrich (Tokyo), Calbiochem (La Jolla) and Sigma, respectively.

Enzyme Reactions

Relaxation activities of topoisomerases I and II were measured by detecting the conversion of supercoiled pBR322 DNA to its relaxed form.[2,13] Decatenation activity of topoisomerase II was measured by detecting the conversion of catenated kinetoplast DNA (kDNA) to minicircle monomers.[2,13] DNA cleavage activities of topoisomerases I and II were determined by measuring the increase of nicked and linearized pBR322 DNA induced by inhibitors, respectively.[14,15] 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 supercoiled pBR322 DNA or RNA after enzyme reactions.[5,6] The assay of T4 DNA ligase was based on ligation of linearized pBR322 DNA which was cleaved by *Hind* III.[5,6] Telomerase activity was measured by TRAP-*eze*® Telomerase Detection kit (Intergen Co., Purchase). After each enzyme reaction, the incubation mixture was subjected to gel electrophoresis and DNA or RNA on the gel was measured by a densitometer with transilluminator (Atto Co., AE-6900M). The assay conditions for inhibitory activity and electrophoresis have been described in previous papers.[5,6] The inhibitory activity (IC_{50}) was defined as the amount of inhibitor that reduced each enzyme activity by 50%.

Other Methods

DNA intercalation was evaluated by binding of DNA with inhibitor using ethidium bromide (EtBr) as described in previous papers.[5,6] The intensity of fluorescence of EtBr was measured with a spectrofluorometer (Hitachi F-4010). CD (circular dichroism) spectral change was obtained by subtraction of the CD of reagent from the CD of DNA with reagent. CD spectra was recorded in 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM KCl, 1 mM $MgCl_2$ and salmon sperm DNA (25 μg/ml) by a spectropolarimeter (JASCO J-720).

RESULTS AND DISCUSSION

Inhibition of Topoisomerase I by Saturated Fatty Acids

The inhibitory effects of some saturated fatty acids ($C_{6:0}$ to $C_{22:0}$) on topoisomerase I were examined. Arachidic acid ($C_{20:0}$) showed weak inhibition (IC_{50}: 950 μM), and the other saturated fatty acids had no inhibitory effect even at a concentration of 2000 μM (data not shown).

Inhibition of Topoisomerase I by *Cis*-Unsaturated Fatty Acids

Cis-unsaturated fatty acids containing one double bond in the molecule inhibited topoisomerase I as shown in Table I. The inhibitory potency increased with increase in carbon chain length and reached a maximum at twenty carbon atoms ($C_{20:1}$). The results suggested that the existence of the double bond in a fatty acid molecule is essential to inhibit topoisomerase I and that carbon chain length affected its inhibitory potency. The inhibitory effect of the number of double bond on the enzyme was examined for *cis*-unsaturated fatty acids of C_{18} and C_{20}. As shown in Table II, the inhibitory

TABLE I Inhibitory effects of *cis*-unsaturated fatty acids containing one double bond on topoisomerase I

Fatty acid	*No. of carbon atoms and double bonds*	*Position of double bond*	*Inhibition*[a] (IC_{50}, μM)
Myristoleic acid	14 : 1	9	232
Palmitoleic acid	16 : 1	9	47
Oleic acid	18 : 1	9	31
Eicosanoic acid	20 : 1	11	18
Docosenoic acid	22 : 1	13	45

[a]Values represent the means obtained from two independent experiments.

TABLE II Inhibitory effects of the number of double bond in *cis*-unsaturated fatty acids on topoisomerase I

Fatty acid	*No. of carbon atoms and double bonds*	*Position of double bonds*	*Inhibition*[a] (IC_{50}, μM)
Oleic acid	18 : 1	9	31
Linoleic acid	18 : 2	9, 12	31
Linolenic acid	18 : 3	9, 12, 15	35
Eicosanoic acid	20 : 1	11	18
Eicosadienoic acid	20 : 2	11, 14	16
Eicosatrienoic acid	20 : 3	8, 11, 14	20
Eicosatetraenoic acid	20 : 4	5, 8, 11, 14	24
Eicosapentaenoic acid	20 : 5	5, 8, 11, 14, 17	43

[a]Values represent the means obtained from two independent experiments.

activity was not altered up to three double bonds, and the inhibition rather decreased around five double bonds.

Inhibition of Topoisomerases I and II by Isomers of Oleic Acid

The inhibitory effects of isomers of oleic acid ($C_{18:1}$) on topoisomerases I and II were examined and are shown in Table III. The inhibitory potency depended on the position of the double bond in the fatty acid molecule, and petroselinic acid and vaccenic acid ($C_{18:1}$) with a *cis*-double bond were the potent inhibitors. The *trans*-isomer (elaidic acid) and the methyl ester (methyloleate) of oleic acid did not inhibit topoisomerases I and II, and the hydroxyl derivative (ricinoleic acid) of oleic acid showed weak inhibition against the enzymes. The *cis*-form unsaturated fatty acid has a molecular shape different from the *trans*-form and the former may be well fitted to the molecular structure of topoisomerase with regard to steric interaction. The carboxyl group in the fatty acid molecule may be important in the interaction with the topoisomerase molecule. Also, the unsaturated fatty acid inhibited topoisomerases I and II at almost the same concentration, suggesting that it acted on a similar structural site in both enzyme molecules.

K_i Values of Petroselinic Acid against Topoisomerases I and II

The type of inhibition was determined by a Lineweaver–Burk plot[16] of substrate concentrations against the rate of relaxation of supercoiled pBR322 DNA by topoisomerases I and II in the presence or absence of petroselinic acid. As shown in Figure 1(A) and (B), relaxations of pBR322 DNA by the enzymes were noncompetitively inhibited by petroselinic acid, and K_i values were 23 and 38 μM, respectively. The K_m values of topoisomerases I and II were 10 and 17 nM, respectively. Petroselinic acid showed almost the same

TABLE III Inhibitory effects of isomers of oleic acid on topoisomerases I and II

Fatty acid	*No. of carbon atoms and double bonds*	*Position of double bond*	*Inhibition* (IC_{50}, μM)[a]	
			Topo I	*Topo II*
Petroselinic acid	18:1	6-*cis*	13	18
Vaccenic acid	18:1	11-*cis*	17	21
Oleic acid	18:1	9-*cis*	31	40
Elaidic acid	18:1	9-*trans*	>1000	>1000
Methyloleate	19:1	9-*cis*	>1000	>1000
Ricinoleic acid[b]	18:1	9-*cis*	275	265

[a]Values represent the means obtained from two independent experiments. [b]12-Hydroxy-*cis*-9-octadecenoic acid.

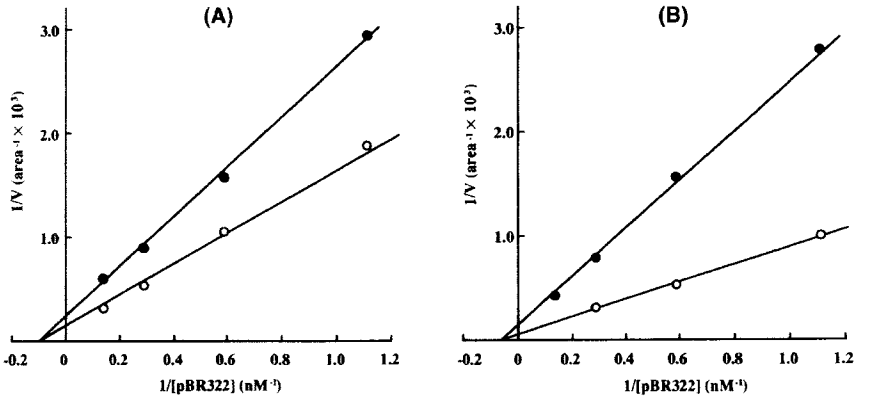

FIGURE 1 Lineweaver–Burk plots of substrate (supercoiled pBR322 DNA) concentrations against rate of relaxation by topoisomerase I (A) and topoisomerase II (B) with (●) and without (○) petroselinic acid. The K_i values of petroselinic acid against topoisomerases I and II were 23 and 38 μM, respectively. The K_m values of topoisomerases I and II were 10 and 17 nM, respectively.

inhibitory potency against both the enzymes. From these results, petroselinic acid was considered to bind strongly to a different site from the binding site of the substrate DNA on both the enzyme molecules.

Stabilization of Topoisomerases I and II-Cleavable Complexes by Petroselinic Acid

Topoisomerase inhibitors of the cleavable complex-forming type such as camptothecin and etoposide stabilize the cleavable complex (topoisomerase–DNA reaction intermediate) and inhibit the DNA rejoining reaction of topoisomerase, which is the inhibitory mechanism of the inhibitors.[17,18] Therefore, the inhibitors induce nicked DNA or linearized DNA in the cleavage assay. To determine whether petroselinic acid is an inhibitor of the cleavable complex-forming type or not, cleavage assays were carried out. Camptothecin and etoposide were used as specific inhibitors against topoisomerases I and II, respectively. As shown in Figure 2(A), camptothecin induced nicked DNA with increasing concentrations. Unlike camptothecin, petroselinic acid did not induce nicked DNA.

The results of stabilization of the topoisomerase II-cleavable complex are shown in Figure 2(B). Etoposide induced linearized DNA, but petroselinic acid failed to linearize DNA even at 1000 μM, an extreme concentration. These results suggested that petroselinic acid is an inhibitor of the cleavable complex-nonforming type which does not inhibit topoisomerase by stabilizing

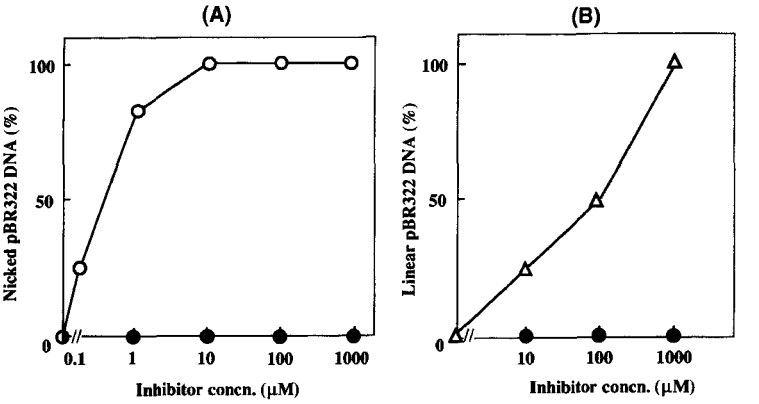

FIGURE 2 Stabilization of topoisomerases I (A) and II (B)-cleavable complexes by petroselinic acid (●), camptothecin (○) as control of topoisomerase I-cleavable complex and etoposide (△) as control of topoisomerase II-cleavable complex.

the cleavable complex. Petroselinic acid may act directly on the molecules of topoisomerases I and II in an earlier step than the formation of the enzyme–DNA complexes (cleavable complexes) and so inhibit DNA breaking and rejoining reactions by the enzymes.

DNA Intercalation by Petroselinic Acid

Some topoisomerase inhibitors such as doxorubicin and amsacrine are DNA intercalators.[19,20] To determine whether petroselinic acid has the ability to intercalate into DNA strands, initially an ethidium bromide competition assay was carried out using salmon sperm DNA. Camptothecin and doxorubicin, being a nonintercalator and an intercalator, respectively, were used as controls at the same concentration. As shown in Figure 3, doxorubicin competed with ethidium bromide for DNA and showed a decrease in the intensity of fluorescence. On the other hand, petroselinic acid did not decrease the intensity of fluorescence and therefore, it is considered that the fatty acid is not an intercalator.

In order to confirm this view, the CD spectral change of DNA following the addition of petroselinic acid was measured since the CD spectrum is sensitive to the conformational changes of DNA brought about by intercalators.[21] As shown in Figure 4, the spectra of DNA changed considerably with an increase in the concentrations of doxorubicin and ethidium bromide, respectively. On the other hand, the spectral change by petroselinic acid did not occur even at extreme concentration (50 μM). This finding was

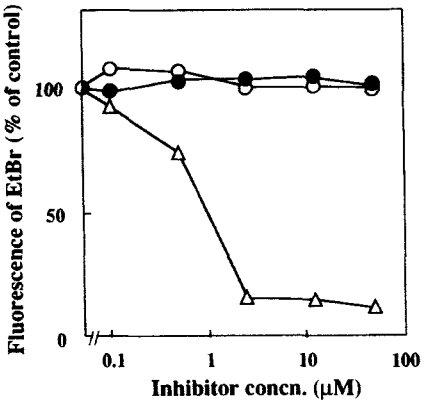

FIGURE 3 Effects of petroselinic acid (●), camptothecin (○) and doxorubicin (△) on DNA binding competition with ethidium bromide.

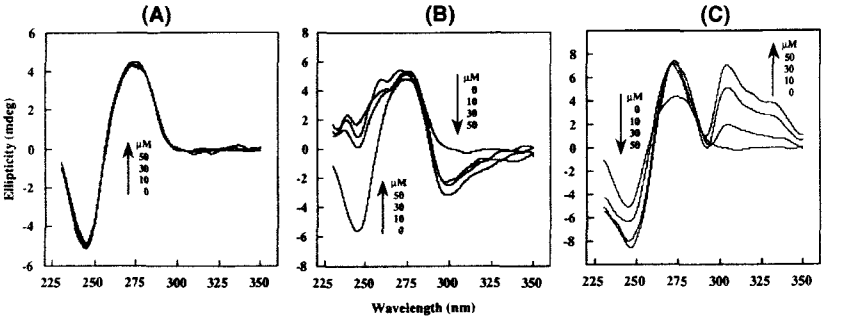

FIGURE 4 CD spectral changes of DNA by the addition of petroselinic acid (A), doxorubicin (B) and ethidium bromide (C). The concentrations of reagents were 0, 10, 30 and 50 μM.

in good agreement with the result from the ethidium bromide competition assay with petroselinic acid. From these results, it is clear that petroselinic acid does not intercalate into DNA.

Inhibitory Spectrum

The effects of petroselinic acid on various DNA-related enzymes were examined and are summarized in Table IV. For comparison, camptothecin[17] and doxorubicin[19] were also examined as specific inhibitors against topoisomerases I and II, respectively. Petroselinic acid inhibited topoisomerase Is from wheat germ, calf thymus gland and NIH3T3 cells, and topoisomerase II, and weakly inhibited topoisomerase Is from carcinoma cells

TABLE IV Inhibitory spectra

Enzyme	*Inhibition* (IC_{50}, μM)[c]		
	Petroselinic acid	*Camptothecin*	*Doxorubicin*
Topoisomerase I from wheat germ[a]	2	3	> 100
Topoisomerase I from calf thymus gland[a]	13	17	> 100
Topoisomerase I from NIH 3T3 cell[a]	14	27	> 100
Topoisomerase I from Vero cell[a]	36	4	> 100
Topoisomerase I from A549 cell[a]	41	12	> 100
Topoisomerase I from COLO 201 cell[a]	141	17	> 100
Topoisomerase I from HeLa cell[a]	170	9	> 100
Topoisomerase II from human placenta[a]	17	> 100	1
Topoisomerase II from human placenta[b]	18	> 100	1
Pst I from *Providencia stuartii*	12	> 100	> 100
Bam HI from *Bacillus amyloliquefaciens*	15	> 100	> 100
Sca I from *Streptomyces caespitosus*	59	> 100	25
Alu I from *Arthrobacter luteus*	> 1000	> 100	24
Eco RI from *Escherichia coli*	> 1000	> 100	> 100
Hin dIII from *Haemophilus influenzae*	> 1000	> 100	96
RNase A from bovine pancreas	> 1000	> 100	> 100
DNase I from bovine pancreas	> 1000	> 100	> 100
DNase II from porcine spleen	> 1000	> 100	> 100
T4 ligase from *Escherichia coli*	> 1000	> 100	73
Telomerase from COLO201 cell	> 100	> 100	—

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

such as A549, COLO201 and HeLa. Petroselinic acid showed different inhibitory properties from those of other inhibitors.

It would be interesting to establish the correlation between structure and inhibitory activity of fatty acids since various fatty acids exist in living cells and these structures are ceaselessly altered by oxidation, reduction, esterification and epoxidation, and they exhibit various physiological effects. Our data suggests that carbon chain length, a *cis*-double bond and carboxyl group in the fatty acid molecule play important roles in the process of topoisomerase inhibition, and that fatty acids directly act on the enzyme molecule in a noncompetitive manner without DNA intercalation.

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