SPANDIDOS PUBLICATIONS

Inhibitory action of C22-fatty acids on DNA polymerases and DNA topoisomerases

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Abstract. We reported previously that unsaturated linear-chain fatty acids of the *cis*-configuration with a C18-hydrocarbon chain such as linoleic acid (*cis*-9, 12-octadecadienoic acid, C18:2) could potently inhibit the activity of mammalian DNA polymerases (Biochim Biophys Acta 1308: 256-262, 1996). In this study, we investigated the inhibitory effects of *cis*-type C22-fatty acids including *cis*-7,10,13,16,19-docosapentaenoic acid (DPA, C22:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6) on mammalian DNA polymerases and human DNA topoisomerases. *Cis*-13,16-docosadienoic acid (C22:2) was the strongest inhibitor of both DNA polymerases and topoisomerases of all C22-fatty acids tested. The inhibitory tendency by the fatty acids on DNA polymerases was the same as that of DNA topoisomerases, and the second strongest inhibitor was *cis*-13,16,19-docosatrienoic acid (C22:3). The energy-minimized three-dimensional structures of the fatty acids were calculated and it was found that a length of 19-21 Å and width of more than 7 Å in C22-fatty acid structure were important for enzyme inhibition. The three-dimensional structure of the active site of both DNA polymerases and topoisomerases must have a pocket to join C22:2, and this pocket was 19.41 Å long and 9.58 Å wide.

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Abbreviations: pol, DNA-directed DNA polymerase (E.C.2.7.7.7); Topo, DNA topoisomerase; PUFA, polyunsaturated fatty acids; DMSO, dimethyl sulfoxide; IC_{50}, 50% inhibitory concentration; poly(dA), polydeoxyriboadenylic acid; oligo$(dT)_{12\text{-}18}$, oligo(12-18) deoxyribothymidylic acid; dTTP, 2'-deoxythymidine 5'-triphosphate; dsDNA, double-stranded DNA; BSA, bovine serum albumin

Key words: C22-fatty acid, *cis*-13,16-docosadienoic acid (C22:2), DNA polymerase, DNA topoisomerase, enzyme inhibitor

Introduction

We have studied the *in vitro* and *in vivo* ability of mammalian DNA metabolic enzymes, especially DNA polymerases (pols), to use these selective inhibitors, and established an assay to detect pol inhibitors (1,2). We screened the products of microbial fermentation for those which inhibit pol activity. We found an inhibitor from a bisidiomycete, a well-known fatty acid, linoleic acid (*cis*-9,12-octadecadienoic acid, C18:2) (2). Subsequently, we investigated the effects of commercially available fatty acids on the activity of pols. We found that several fatty acids, particularly long chain fatty acids with a *cis*-configuration, interact with pols and suppress their activity. In this manuscript, we discuss the effects of C22-fatty acids including well-known bioactive compounds such as *cis*-7,10,13,16,19-docosapentaenoic acid (DPA, C22:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6), which are polyunsaturated fatty acids (PUFA), on the activity of DNA metabolic enzymes such as pols and DNA topoisomerases (topos).

Eukaryotic cells reportedly contain three replicative pols, α, δ, and ε, mitochondrial pol γ and at least twelve repair types of pols, ß, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ, and φ (3,4). Pol catalyzes the addition of deoxyribonucleotides to the 3'-hydroxyl terminus of primed double-stranded DNA molecules (3).

Topos are key enzymes that control the topological state of DNA, and topo catalyzes the concerted breaking and rejoining of DNA strands and is involved in producing the necessary topological and conformational changes in DNA (3,5). There are two classes of topos: type I enzymes, which act by transiently nicking one of the two DNA strands, and type II enzymes, which nick both DNA strands, are dependent on ATP, and are involved in many vital cellular processes that influence DNA replication, transcription, recombination, integration and chromosomal segregation (3). In recent years, these enzymes have received special interest because topo inhibitors have emerged as anti-cancer (6) and anti-parasitic agents (7,8). Topos can be inhibited by two distinct mechanisms, and the inhibitors are divided into two classes accordingly: classes I and II. Class I inhibitors stabilize the enzyme-DNA covalent complex and block the subsequent rejoining of the DNA break. Class II inhibitors, also referred

to as catalytic inhibitors, prevent the enzyme and DNA from binding by interacting with either the topo (9,10) or DNA (11).

Therefore, pol and topo are critical to many cellular processes such as DNA replication, repair and recombination, and may act in harmony. We suggest the three-dimensional site where the enzyme binds to C22-fatty acids using computer modeling and three-dimensional structural analysis. The molecular design method may be useful to create agents for cancer chemotherapy.

Materials and methods

Materials. Saturated C22-fatty acid, *n*-docosanoic acid (behenoic acid, C22:0), the *cis*-configuration of unsaturated C22-fatty acids such as *cis*-13-docosenoic acid (erucic acid, C22:1), *cis*-13,16-docosadienoic acid (C22:2), *cis*-13,16,19-docosatrienoic acid (C22:3), *cis*-7,10,13,16-docosatetraenoic acid (adrenic acid, C22:4), *cis*-7,10,13,16,19-docosapentaenoic acid (DPA, C22:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, C22:6) were purchased from Nu-Chek-Prep Inc. (Minnesota, USA). Nucleotides such as [^{3}H]-2'-deoxythymidine 5'-triphosphate (dTTP) (43 Ci/mmol), and chemically synthesized template-primers such as poly(dA) and oligo(dT)$_{12-18}$ were purchased from Amersham Biosciences (Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Nacalai Tesque Ltd. (Kyoto, Japan).

Enzymes. Pol α was purified from calf thymus by immuno-affinity column chromatography as described by Tamai *et al* (12). Recombinant rat pol ß was purified from *E. coli* JMpß5 as described by Date *et al* (13). Human pol δ and ε were purified from the nuclear fraction of human peripheral blood cancer cells (Molt-4) using the second subunit of pol δ and ε-conjugated affinity column chromatography, respectively (14). Recombinant human His-pol λ was overexpressed and purified according to a method described by Shimazaki *et al* (15). Human recombinant topo I and topo II (2 units/μl each) were purchased from TopoGen, Inc. (Columbus, OH, USA).

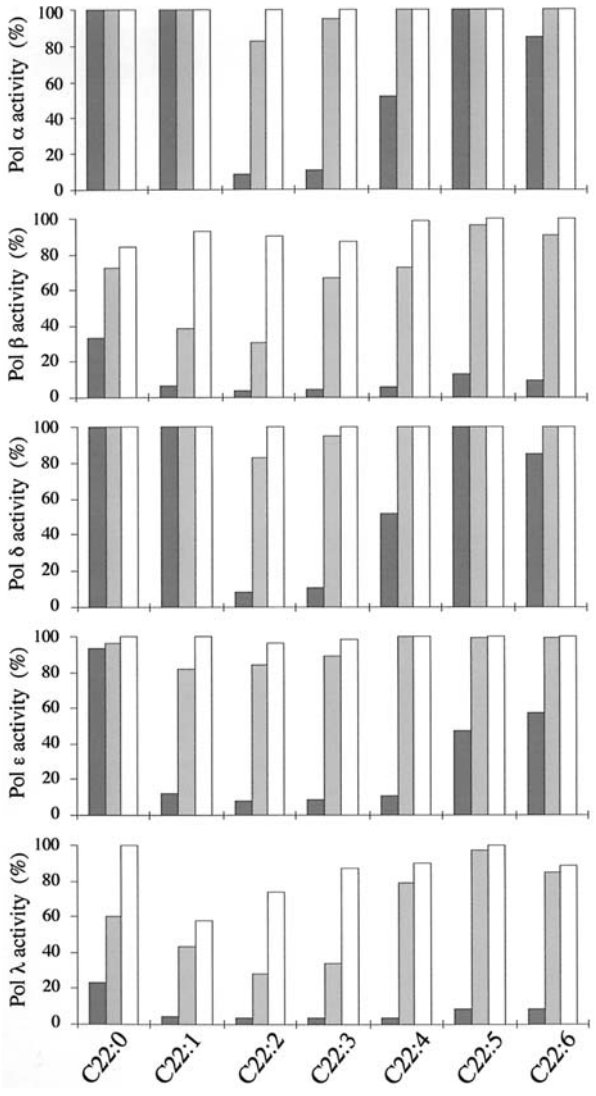

Figure 1. Inhibitory effect of linear-chain C22-fatty acids on mammalian DNA polymerases. The investigated pols are calf pol α, rat pol ß, human pol δ, human pol ε, human pol λ. The dark-gray bars, light-gray bars and white-bars are 100, 10 and 1 μM of C22-fatty acids, respectively. Each pol is 0.05 units. Enzyme activity in the absence of fatty acids was taken as 100%.

DNA polymerase assays. The reaction mixtures for pols α and ß were described previously (1,2), and those for pols δ and ε were described previously (16). The reaction mixture for pol λ was the same as that for pol ß. For pols, poly(dA)/oligo(dT)$_{12-18}$ and dTTP (2'-deoxythymidine 5'-triphosphate) were used as the template-primer DNA and nucleotide substrate, respectively. C22-fatty acids were dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (final, 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0˚C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each enzyme standard reaction mixture, and incubation was carried out at 37˚C for 60 min. Activity without the inhibitor was considered 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleotide triphosphates (i.e., dTTP) into synthetic template-primers [i.e. poly(dA)/oligo(dT)$_{12-18}$, A/T=2/1] in 60 min at 37˚C under normal reaction conditions for each enzyme (2).

DNA topoisomerase assays. Relaxation activity of topos was determined by detecting the conversion of supercoiled plasmid DNA to its relaxed form. The topo II reaction was performed in 20-μl reaction mixtures containing 50 mM Tris-HCl buffer (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, pBR322 plasmid DNA (200 ng), 2 μl of inhibitor solution (10% DMSO) and 1 unit of topo II. The reaction mixtures were incubated at 37˚C for 30 min and terminated by adding 2 μl of loading buffer consisting of 5% sarkosyl, 0.0025% bromophenol blue and 25% glycerol. The mixtures were subjected to 1% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) running buffer. The agarose gels were stained with ethidium bromide, and DNA was visualized on a UV transilluminator. Zero-D scan (version 1.0, M&S Instruments

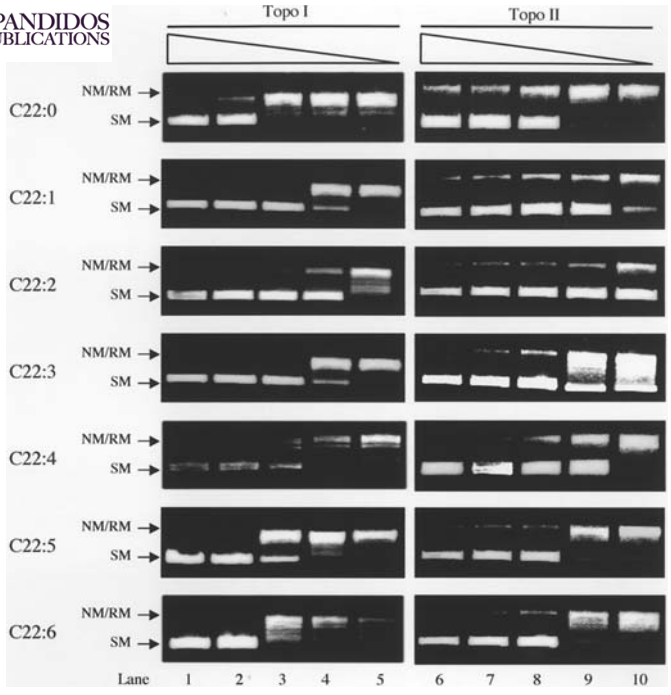

Figure 2. Inhibition of relaxation activity of human DNA topoisomerases by C22-fatty acids. Lanes 1-5 and lanes 6-10 had topo I (2 units) and topo II (2 units) added to the reaction mixture, respectively. Lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9, and 5 and 10 had C-22 fatty acids at concentrations of 25, 5, 1, 0.2, and 0.04 μM, respectively; 200 ng of plasmid DNA was added to each of the lanes. Photographs of ethidium bromide-stained gels are shown.

Trading Inc., Tokyo, Japan) was used for densitometric quantitation of the plasmid DNA products. The relaxation activity of topo I was analyzed in the same manner as described above except that reaction mixtures contained 10 mM Tris-HCl (pH 7.9), pUC19 plasmid DNA (200 ng), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol and 1 unit of topo I. One unit was defined as the amount of enzyme capable of relaxing 0.25 μg of DNA in 15 min at 37˚C.

Computational analysis of C22-fatty acids. A compound model was constructed and simple-minimized. Compound models were simulated with force field parameters based on the consistent valence force field (CVFF). Group-based cut-offs, 0.95 nm for van der Waals and 0.95 nm for Coulomb interactions, were introduced. The temperature was set at 298 K. Calculations based on simulation images were carried out using Insight II (Accelrys Inc., San Diego, CA, USA).

Results

Inhibitory effects of C22-fatty acids on the activity of mammalian DNA polymerases. First, we tested whether linear-chain C22-fatty acids have the ability to inhibit representative nuclear pols such as calf pol α, rat pol ß, human pol δ, human pol ε and human pol λ. Pols α, δ and ε are replicative pols, and pols ß and λ are repair-related pols (3,4). One, 10 and 100 μM of fatty acids with a 22-hydrocarbon chain were tested (Fig. 1). Inhibition by these C22-fatty acids was dose-dependent, and the inhibitory effect had the same tendency among the fatty acids. In the IC_{50} values and the relative effect on pols α - λ by 100 μM of compounds, *cis*-13,16-docosadienoic acid (C22:2) was the strongest inhibitor of all C22-fatty acids tested, and *cis*-13,16,19-docosatrienoic acid (C22:3) was the second strongest. Since the inhibitory effect of saturated C22-fatty acid, *n*-docosanoic acid (C22:0), was weaker than that of C22-PUFAs, the *cis*-configuration of double bonds must be important for pol inhibition. In the order of pol effect by C22-fatty acids, the inhibition of pols ß and λ was stronger than that of pols α, δ and ε, because the IC_{50} values of *cis*-13,16-docosadienoic acid (C22:2) for pols α, ß, δ, ε and λ were 47, 6.0, 46, 47 and 3.0 μM, respectively. These results suggested that C22-fatty acids, especially *cis*-13,16-docosadienoic acid (C22:2), were more potent inhibitors for repair-related pols than replicative pols.

Inhibitory effects of C22-fatty acids on the activity of human DNA topoisomerases. Next, we also investigated the inhibitory activity of human topos I and II instead of mammalian pols. Pols and topos are DNA metabolic enzymes, and both inhibitors could be anti-cancer agents. As shown in Fig. 2, C22-fatty acids dose-dependently inhibited the activity of topo I and topo II. The inhibitory effect of topo I had the same tendency as that of topo II, and in the order of their effect, C22-fatty acids ranked as follows: C22:2 > C22:3 > C22:1 > C22:4 > C22:5 > C22:6 > C22:0. This ranking was almost the same as the inhibitory effect for mammalian pols (Fig. 1), suggesting that pols and topos might have the same inhibitory mechanism, although their modes of action, amino acid sequences and 3D structures are markedly different (3,5). The IC_{50} values of *cis*-13,16-docosadienoic acid (C22:2) for topos I and II were 0.1 and 0.03 μM, respectively, suggesting that the effect of topo II was stronger than that of topo I. The inhibition of topo II by C22-fatty acids was approximately 200-fold, 1,500-fold stronger than that of replicative pols such as pols α, δ and ε, and repair-related pols such as pols ß and λ, respectively.

Double reciprocal plots of the results indicated that the inhibition of both calf pol α, rat pol ß and human topos I and II by *cis*-13,16-docosadienoic acid (C22:2) was through competition with the substrate DNA (data not shown), suggesting that C22-fatty acids might compete with the DNA to bind to the catalytic site of the pols and topos.

C22-fatty acids did not inhibit the activity of other DNA-metabolic enzymes such as the primase of calf pol α, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase or bovine deoxyribonuclease I (data not shown). The results suggested that these compounds could selectively inhibit the activity of mammalian pols and human topos.

When the carboxyl group of C22-fatty acid was chemically modified resulting in a methyl-ester, ethyl-ester, alcohol, or completely removed, C22-hydrocarbon (i.e., docosane) did not inhibit the activity of pols and topos (data not shown), showing the importance of the free carboxyl group for inhibition. The inhibitory effect of the *trans*-configuration of C22-fatty acid such as *trans*-13-docosenoic acid (brassidic acid, *trans*-C22:1) was significantly weaker than that of *cis*-type. These results suggested that *cis*-type unsaturated C22-fatty acids such as *cis*-13,16-docosadienoic acid (C22:2) with free carboxylate were potent inhibitors of these enzymes.

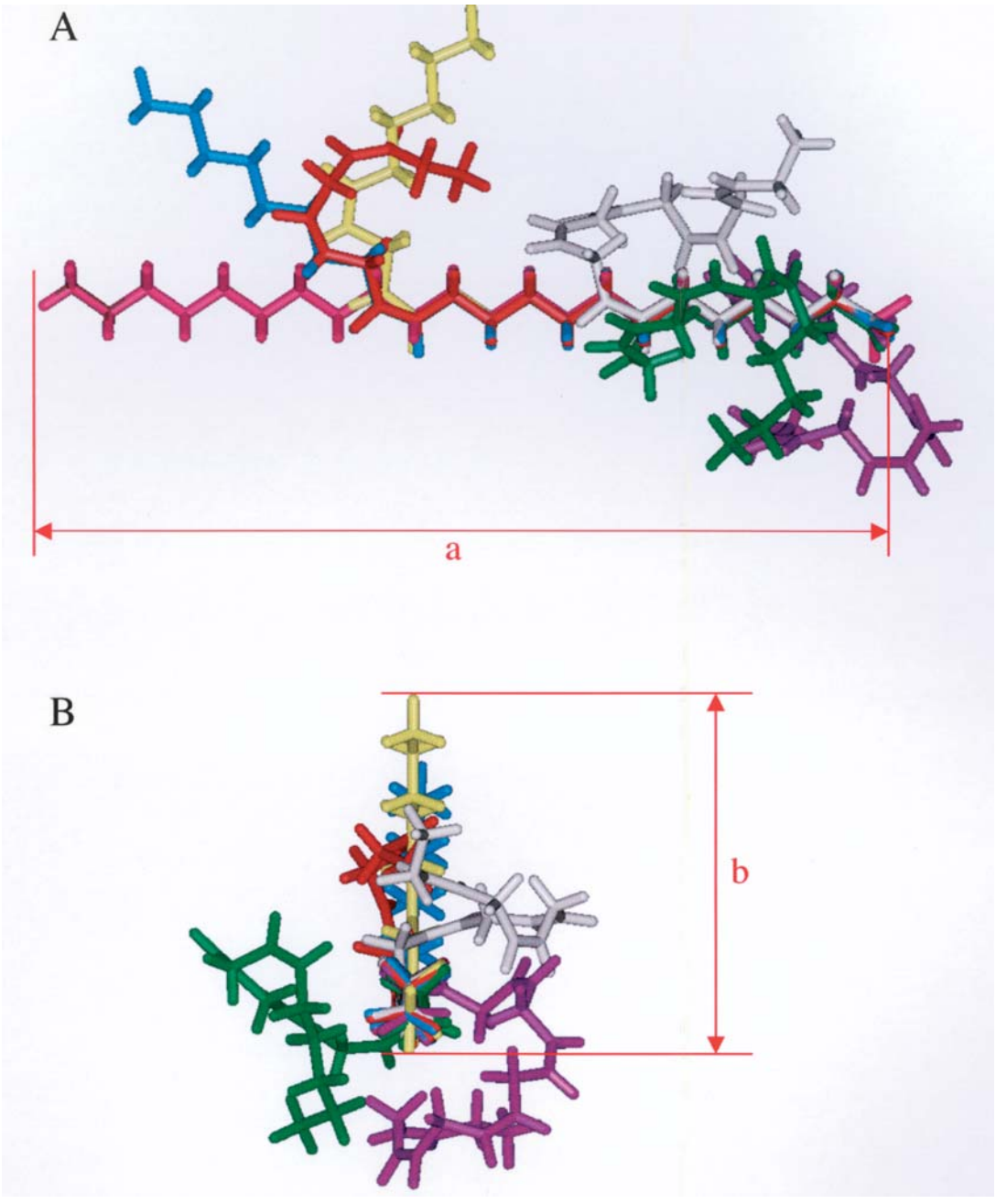

Figure 3. Computer graphics of the overlay of linear-chain C22-fatty acids. (A) The colors of C22-fatty acids are as below: *n*-docosanoic acid (C22:0, violet), the *cis*-configuration of unsaturated C22-fatty acids such as *cis*-13-docosenoic acid (C22:1, light-blue), *cis*-13,16-docosadienoic acid (C22:2, yellow), *cis*-13,16,19-docosatrienoic acid (C22:3, red), *cis*-7,10,13,16-docosatetraenoic acid (C22:4, green), *cis*-7,10,13,16,19-docosapentaenoic acid (C22:5, light-gray) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (C22:6, blue). Both carbon atoms in the carboxyl group and the hydrocarbon chain of their fatty acids were joined. (B) The right side of A is shown. The length (a) and width (b) of the fatty acid molecular structure are shown. Prepared using Insight II (Accelrys, San Diego, CA, USA).

Inhibition mechanism of C22-fatty acids on DNA polymerases and topoisomerases. To determine whether the inhibitor resulted in binding to DNA or the enzyme, the interaction of C22-fatty acids with double-stranded DNA (dsDNA) was investigated based on the thermal transition of dsDNA with or without C22-fatty acids. The Tm of dsDNA with an excess amount of C22-fatty acids (100 μM) was measured using a spectrophotometer equipped with a thermoelectric cell holder. In the concentration range used, no thermal transition of Tm was observed, whereas ethidium bromide as a positive control, a typical intercalating compound, produced a clear thermal transition. These results indicated that C22-fatty acids did not intercalate to dsDNA as a substrate, and the compounds might directly bind to the enzyme and inhibit its activity.

To determine the effects of a non-ionic detergent on the binding of C22-fatty acids to pols and topos, Nonidet P-40 (NP-40) was added to the reaction mixture at a concentration of 0.1%. In the absence of C22-fatty acids, the activity of pols and topos was not affected by the addition of NP-40, and we designated the activity in these cases as 100%. The inhibitory effect of C22-fatty acids at 100 μM was largely reversed by the addition of 0.1% NP-40 to the reaction mixture.

The molecular length and width of the three-dimensional structure of C22-fatty acids.

Fatty acid	Length (Å)	Width (Å)
C22:0	28.86	-
C22:1	27.15	6.82
C22:2	19.41	9.58
C22:3	20.73	5.64
C22:4	9.18	6.18
C22:5	11.94	6.09
C22:6	5.77	5.10

Energy-minimized three-dimensional compounds were prepared (Fig. 3) using Insight II (Accelrys, San Diego, CA, USA).

These results suggested that C22-fatty acids could bind to and interact with the hydrophobic region of the protein of pols and topos. We also tested whether an excess amount of a substrate DNA analog, poly(rC) (100 μg/ml), or a protein, BSA (200 μg/ml), could prevent the inhibitory effects of C22-fatty acids. If C22-fatty acids bind to enzymes by non-specific adhesion, the addition of nucleic acid and/or protein would be expected to reduce inhibitory activity. The fact that neither poly(rC) nor BSA influenced the inhibitory effects of C22-fatty acids suggests that the compound occurs selectively or binds to a specific site on the enzymes and not to the nucleic acid.

Three-dimensional structure modeling of linear-chain C22-fatty acids. *Cis*-13,16-docosadienoic acid (C22:2) was the strongest pol and topo inhibitor among the linear-chain C22-fatty acids tested. Therefore, we carried out three-dimensional structure modeling analysis of the linear-chain C22-fatty acids, and speculated the enzyme model with the fatty acid. The energy-minimized three-dimensional linear-chain fatty acids by computer modeling are shown in Fig. 3. The hydrocarbon chain in the saturated and *trans*-unsaturated C22-fatty acid molecule was linear because of no or little width. Di- and more unsaturated C22-fatty acids of the *cis*-configuration lost molecular length and formed width because the hydrocarbon chains in their fatty acids fold up at the double bonds position in the *cis*-configuration. The molecular lengths and widths of linear-chain C22-fatty acids are compared in Table I. The hydrocarbon chain in the saturated and *trans*-unsaturated fatty acid molecules was linear, the longest length being 28.86 Å (Table I), and very weakly inhibited the activity of pols and topos (Figs. 1 and 2). Mono- and more unsaturated C22-fatty acids of *cis*-configuration reduced molecular length, and more than four unsaturated fatty acids formed a ball-shaped curve. *Cis*-13,16-docosadienoic acid (C22:2) and *cis*-13,16,19-docosatrienoic acid (C22:3) strongly inhibited the activity of topos and pols, and a length of approximately 19-21 Å fatty acid was required for inhibition. Furthermore, the molecular widths in C22-fatty acids are compared in Fig. 3B. *Cis*-13,16-docosadienoic acid (C22:2), which was the strongest inhibitor among C22-fatty acids, formed a V-shaped curve, and the molecular width was the largest (9.58 Å) (Fig. 3B and Table I). The molecular width (>7 Å) in the fatty acids also appeared to be important for enzyme inhibition.

From the results above, the inhibitory effects of C22-fatty acids on the activity of pols and topos could occur by binding between the enzyme protein and the fatty acid. The substrate DNA binding sites of the enzymes had a pocket which can bind to the fatty acid. If their fatty acid-binding site structures were three-dimensionally similar, the substrate DNA binding site of the topos would also have a pocket which can bind to *cis*-13,16-docosadienoic acid (C22:2), like pols. The size of the pocket must have sufficient length and width to join to the *cis*-13,16-docosadienoic acid (C22:2) molecule (i.e., length of 19.41 Å and width of 9.58 Å). The pocket must consist of hydrophilic amino acids to bind to the substrate DNA and a hydrophobic polypeptide sheet to bind to the hydrocarbon chain of fatty acid. Moreover, the carboxyl group of *cis*-13,16-docosadienoic acid (C22:2) must bind to the hydrophilic amino acid in the pocket in competition with substrate DNA, because the free carboxyl group was important for enzyme inhibition. These are predictable for future X-ray crystal and NMR analyses of the complex of enzymes and fatty acids.

Discussion

PUFAs have been documented to inhibit or even prevent cancer. Epidemiological evidence strongly links fish oil [rich in DHA (C22:6)] with low incidences of several types of cancer (17-22). The inhibitory effects of PUFAs including C22-fatty acids such as DPA (C22:5) and DHA on cancer development and progression are supported by studies using cultured cells and animal models (23-30). In this study, PUFAs such as unsaturated C22-fatty acids were investigated for the inhibitory activity of DNA metabolic enzymes such as pols and topos, and *cis*-13,16-docosadienoic acid (C22:2) was a stronger inhibitor than DPA and DHA. Therefore, this fatty acid could be an anti-cancer fatty acid among PUFAs.

Several anti-cancer agents in clinical use have been shown to be potent inhibitors of topos. For example, adriamycin (doxorubicin), amsacrine (m-AMSA) and ellipticine have been demonstrated to show significant activity as inhibitors of topo II (31). The plant alkaloid camptothecin and its synthetic derivatives such as CPT-11 are extensively studied topo I inhibitors (32). All of these agents inhibit the rejoining reaction of topos by stabilizing a covalent topo-DNA complex termed the 'cleavable complex'. Thus, no C22-fatty acids such as *cis*-13,16-docosadienoic acid (C22:2) bound to the dsDNA, suggesting that it must inhibit enzyme activity by interacting with the enzymes directly, and that C22-fatty acids were of the non-cleavable complex type of topo II inhibitors.

As described in the Introduction, both pols and topos are attractive targets for cancer chemotherapy (3,6-8); therefore, clarifying the molecular mechanism of their inhibition should provide clues to create an ideal artificial cancer chemotherapy agent. Our studies may provide insight into the mechanism of this effect, because *cis*-configurated di-unsaturated linear-chain C22-fatty acids, *cis*-13,16-docosadienoic acid (C22:2), strongly inhibited the activity of human topos, and because the inhibition mode of topos by C22-fatty acids was almost the same as that of pols. Therefore, the three-dimensional structural information

of linear-chain C22-fatty acids concerning inhibition described in this report may facilitate the computer design of new molecular probes capable of functioning as anti-neoplastic agents.

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