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Selective inhibition of the activities of both eukaryotic DNA polymerases and DNA topoisomerases by elenic acid

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Abstract

(*R*)-(−)-Elenic acid (*R*-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid) (EA) is a DNA topoisomerase II inhibitor found in an Indonesian sponge, *Plakinastrella* sp. We found and report here that it is a potent inhibitor of calf DNA polymerase α (IC_{50} = 7.7 μM) and rat DNA polymerase β (IC_{50} = 12.9 μM). EA did not bind to DNA directly. EA did not influence the activities of DNA polymerases such as plant DNA polymerases I and II and prokaryotic DNA polymerases such as *Escherichia coli* DNA polymerase I, or other DNA metabolic enzymes such as human immunodeficiency virus (HIV) reverse transcriptase, T7 RNA polymerase and bovine deoxyribonuclease I. Interestingly, EA was also an inhibitor of DNA topoisomerases I and II, although the enzymatic characteristics including modes of action, amino acid sequences and three-dimensional structures were markedly different from those of DNA polymerases. EA could prevent the growth of NUGC-3 cancer cells, and the LD_{50} value was 22.5 μM. The cells were halted at G1 and G2/M phase in the cell cycle. From these results, the action mode of EA is discussed.

Keywords: DNA polymerase; DNA topoisomerase; Elenic acid; Enzyme inhibitor; Cytotoxicity; Apoptosis

1. Introduction

EA is a natural alkylphenol compound produced by an Indonesian sponge, *Plakinastrella* sp., family Plakinidae, and is an inhibitor of DNA topoisomerase II [1]. We chemically synthesized the compound [2]. The purpose of this report is to investigate the biochemical action of EA in detail, and its use as an anti-neoplastic agent. In the early tests for EA, no enzymes except for DNA topoisomerase were used. As described in this report, EA could inhibit not only the activities of DNA topoisomerases I and II but also of DNA polymerases. DNA topoisomerase II catalyzes the concerted breaking and rejoining of DNA strands and is involved in producing the necessary topological and conformational changes in DNA [3,4], and DNA polymerase catalyzes the addition of deoxyribonucleotides to the 3′-hydroxyl terminus of primed double-stranded DNA molecules [3]. Therefore, there are no enzymatic similarities between both the enzymes, although they are critical to many cellular processes such as DNA replication, repair and recombination, and may act in harmony with each other. The characteristics of both the enzymes including their modes of action, amino acid sequences and three-dimensional structures are markedly different from each other.

EA used in this study directly inhibited the enzyme activities, but it did not bind to the DNA. These observations suggested that there may be some structural similarity between both enzymes at the EA-binding site. We previously reported that several inhibitors, long chain fatty acids [5–7], and triterpenoids [8,9], of mammalian DNA

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Abbreviations: EA, (*R*)-(−)-elenic acid (*R*-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid); dTTP, 2′-deoxythymidine-5′-triphosphate; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; HIV-1, human immunodeficiency virus type-1; T_m, melting temperature; dsDNA, double-stranded DNA; EtBr, ethidium bromide.

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polymerase could also inhibit the DNA topoisomerase II activity, and both enzymes have structural homology at the DNA-binding site [10–12]. Therefore, EA was expected to have similar characteristics. The DNA-binding site of long chain fatty acids on the DNA polymerase differed slightly from that of triterpenoids [10,13]. DNA topoisomerases and DNA polymerases have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Therefore, information concerning the structural characteristics of these inhibitors could provide valuable insight for the design of new anti-cancer agents. From this point of view, we investigated the newly-found inhibitor, EA, in detail.

2. Materials and methods

2.1. Materials

EA was chemically synthesized as described previously [2]. Nucleotides, and chemically synthesized template-primers such as poly(dA), poly(rA) and oligo(dT)$_{12–18}$ were purchased from Pharmacia. [^{3}H]dTTP (2′-deoxythymidine-5′-triphosphate) (43 Ci/mmol) was purchased from New England Nuclear Corp. Supercoiled pUC19 plasmid DNA was obtained from Takara (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Wako Chemical Industries. NUGC-3, a human gastric cancer cell line (JCRB0822) [14], was supplied by the Health Science Research Resources Bank.

2.2. Enzymes

DNA polymerase was purified from the calf thymus by immuno-affinity column chromatography as described previously [15]. Recombinant rat DNA polymerase β was purified from *E. coli* JMpβ5 as described by Date *et al*. [16]. DNA polymerases I (plant α-like polymerase) and II (plant β-like polymerase) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi *et al*. [17]. Purified human placenta DNA topoisomerases I and II (2 units/μL) were purchased from TopoGen Inc. (Columbus, OH). The Klenow fragment of DNA polymerase I and HIV-1 reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ). Taq DNA polymerase, T4 DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara. Calf thymus terminal deoxynucleotidyl transferase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems.

2.3. DNA polymerase assays

Activities of DNA polymerases were measured by the methods described in previous reports [5–7]. The substrates of DNA polymerases used were poly(dA)/oligo(dT)$_{12–18}$ and dTTP as template-primer DNA and nucleotide substrate, respectively. The substrates of terminal deoxynucleotidyl transferase used were oligo(dT)$_{12–18}$ (3′-OH) and dTTP as primer and nucleotide substrate, respectively. The substrates of HIV-1 reverse transcriptase used were poly(rA)/oligo(dT)$_{12–18}$ and dTTP as template-primer and nucleotide substrate, respectively. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleotide triphosphate into the synthetic template-primers (i.e. poly(dA)/oligo(dT)$_{12–18}$, A/T = 2/1) in 60 min at 37° under the normal reaction conditions for each of the enzymes [6,7].

2.4. DNA topoisomerase assays

Relaxation activity of DNA topoisomerase II was determined by detecting the conversion of supercoiled plasmid DNA to its relaxed form [18]. DNA topoisomerase II reaction was performed in 20 μL of reaction mixture containing 50 mM Tris–HCl buffer (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, supercoiled pUC19 DNA (0.25 μg), 2 μL of inhibitor solution (10% dimethylsulfoxide (DMSO)) and 2 units of DNA topoisomerase II. The reaction mixtures were incubated at 37° 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 gel was stained with ethidium bromide (EtBr) and DNA was visualized on a UV transilluminator. Relaxation activity of DNA topoisomerase I was analyzed in the same manner as described except that the reaction mixtures contained 10 mM Tris–HCl (pH 7.9), pBR322 DNA (0.25 μg), 1 mM EDTA, 150 mM NaCl, 0.1 % bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol and 2 units of DNA topoisomerase I. One unit was defined as the amount of enzyme capable of relaxing 0.25 μg of DNA in 15 min at 37°.

2.5. Other enzyme assays

The activities of T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in each of the standard assays according to Nakayama and Saneyoshi [19], Soltis and Uhlenbeck [20], and Lu and Sakaguchi [21], respectively.

2.6. Thermal transition of DNA

Thermal transition profiles of double-stranded to single-stranded DNA with or without EA were determined with a spectrophotometer (U3210, Hitachi, Tokyo) equipped with a thermoelectric cell holder. Calf thymus DNA (6 μg/mL)

was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% DMSO. The solution temperature was equilibrated at 75° for 10 min, and then increased by 1° at 2 min intervals for each measurement point. Any change in the absorbance of the compound itself at each temperature point was automatically subtracted from that of DNA plus the compound in the spectrophotometer.

2.7. Investigation of cytotoxicity on cultured cells

For investigation of the effects of EA in cultured cells, a human gastric cancer cell line NUGC-3, derived from a patient with cancer was used. The cells were routinely cultured in Eagle's MEM (modified Eagle's medium) medium supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 unit/mL penicillin, and 1.6 mg/mL $NaHCO_3$. The cells were routinely cultured at 37° in standard medium in a humidified atmosphere of 5% CO_2–95% air. The cytotoxicity of EA was investigated as follows. High concentrations (10 mM) of the compounds were dissolved in DMSO and stocked. Approximately 1×10^4 cells per well were inoculated in 96-well micro-plates, then the compound stock solution was diluted to various concentrations, and applied to each well. After incubation for 48 hr, the survival rate was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [22].

2.8. Cell cycle analysis

Cellular DNA content for cell cycle analysis was determined as follows: aliquots of 3×10^5 NUGC-3 cells were inoculated into a 35 mm dish, and incubated with medium containing EA for 48 hr [23]. Then, the cells were collected by trypsinization and washed with cold PBS three times by centrifugation. The cells were fixed with 10 mL of PBS containing 70% (v/v) ethanol, and stored at 4°. DNA was stained with DAPI staining solution for at least 10 min at room temperature in the dark. Fluorescence intensity was measured by flow cytometry (Patec cell counter analyzer, CCA). Cell cycle distribution was analyzed with the MULTICYCLE software program (version 2.5; Phoenix Flow Systems, San Diego, CA).

2.9. Analysis of DNA fragmentation

Apoptosis was determined by assay of DNA fragmentation, by means of agarose electrophoresis. Total DNAs were extracted from 2×10^6 NUGC-3 cells following the method of Sambrook *et al*. [24] and 5 μg aliquots were separated by 1.4% (w/v) agarose gel electrophoresis in 40 mM Tris–5 mM sodium acetate–1 mM EDTA (pH 7.8) and stained with ethidium bromide. DNA bands were visualized under UV light.

3. Results

As described in Section 1, EA (Fig. 1) is a known alkylphenol isolated from the Indonesian sponge *Plakinastrella* sp., and has been found to be an inhibitor of human DNA topoisomerase II [1]. The chemical structure of EA is shown in Fig. 1. We describe here that EA was a potent inhibitor of DNA polymerases α and β, although the enzymatic characteristics of the DNA polymerases were markedly different from those of DNA topoisomerase II.

3.1. Effects of EA on various DNA polymerases and other DNA metabolic enzymes

As shown in Table 1, EA significantly inhibited the activities of both calf DNA polymerase α and rat DNA polymerase β. The inhibition by the compound was dose-dependent, with 50% inhibition for DNA polymerases α and β observed at doses of 7.7 and 12.9 μM, respectively, and almost complete inhibition (more than 80%) was achieved at 15 and 17 μM, respectively. In the kinetic analysis, the extent of inhibition as a function of DNA template or nucleotide substrate concentrations was studied. Lineweaver–Burk plots of the results show that the inhibition of DNA polymerase α activity was non-competitive for both the DNA template-primer (the K_m was unchanged at 13.0 μM) and the nucleotide substrate (the K_m was unchanged at 1.65 μM), and Dixon plots of the results show that the K_i values for the DNA template-primer and nucleotide substrate were 1.9 and 2.9 μM, respectively. On the other hand, the inhibition of DNA polymerase β activity was competitive for both the DNA template-primer (the V_{max} was unchanged at 111 pmol/hr) and the nucleotide substrate (the V_{max} was unchanged at 62.5 pmol/hr), and the K_i values for the DNA template-primer and nucleotide substrate were 7.5 and 10.3 μM, respectively. These K_i values suggested that the affinity of EA is stronger at the DNA template-primer-binding site than at the nucleotide substrate-binding site of both DNA polymerases α and β. The DNA topoisomerase II inhibitor, EA, was thus shown to also be a potent inhibitor of the DNA polymerases. The inhibitory doses were stronger than those of aphidicolin [25], and dideoxyTTP [26], well-known DNA polymerases α and β inhibitors, respectively.

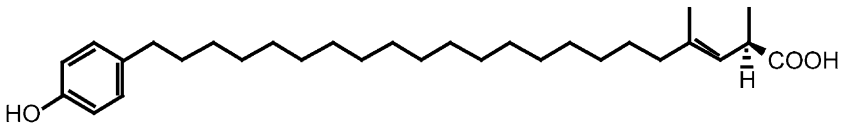

Fig. 1. Structure of (*R*)-(−)-elenic acid (*R*-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid).

Table 1
IC_{50} values of EA on the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} values of EA (μM)
Calf DNA polymerase α	7.7 ± 0.3
Rat DNA polymerase β	12.9 ± 0.7
Plant DNA polymerase I (α-like)	>250
Plant DNA polymerase II (β-like)	>250
E. coli DNA polymerase I (Klenow fragment)	>250
Taq DNA polymerase	>250
T4 DNA polymerase	>250
Calf terminal deoxynucleotidyl transferase	>250
Human DNA topoisomerase I	1.5 ± 0.2
Human DNA topoisomerase II	1.5 ± 0.2
HIV-1 reverse transcriptase	>250
T7 RNA polymerase	>250
T4 polynucleotide kinase	>250
Bovine deoxyribonuclease I	>250

EA was incubated with each enzyme. The enzymatic activity was measured as described in Section 2. Enzyme activity in the absence of the compounds was taken as 100%. Results are presented as mean ± SD of three independent experiments.

Table 2
Effects of poly(rC) and BSA on the inhibition of DNA polymerase activities by EA

Compounds added to the reaction mixture	Rat DNA polymerase β (%)	Calf DNA polymerase α (%)
Without the compounds		
None (control)	100 ± 2.2	100 ± 2.3
50 μM poly(rC)	100 ± 2.4	100 ± 2.5
200 μg/mL BSA	100 ± 3.4	100 ± 3.3
10 μM EA		
10 μM EA	70.5 ± 2.5	36.8 ± 1.5
10 μM EA + 50 μM poly(rC)	67.8 ± 2.2	34.9 ± 1.2
10 μM EA + 200 μg/mL BSA	68.0 ± 2.3	38.3 ± 1.8
20 μM EA		
20 μM EA	4.5 ± 0.3	9.2 ± 0.8
20 μM EA + 50 μM poly(rC)	4.4 ± 0.2	8.4 ± 0.6
20 μM EA + 200 μg/mL BSA	4.1 ± 0.2	9.1 ± 0.7

50 μM poly(rC) or 200 μg/mL BSA was added to the reaction mixture. In the absence of the compounds, DNA polymerase activity was taken as 100%. Results are presented as mean ± SD of three independent experiments.

EA consists of *p*-hydroxyphenol and long chain (carbon number of 22) fatty acid. Although C_{22}-fatty acid, docosanoic acid, also inhibits the activities of DNA polymerases, it was a 2-fold weaker DNA polymerase inhibitor than EA [7]. The *p*-hydroxyphenol group of EA may stimulate the inhibition of the DNA polymerase activities. The compound also strongly inhibited the activities of human DNA topoisomerases I and II. EA had little effect on the activities of higher plant (cauliflower) DNA polymerases I (α-like polymerase) and II (β-like polymerase), calf thymus terminal deoxynucleotidyl transferase, HIV-1 reverse transcriptase, prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* DNA polymerase I, Taq DNA polymerase and T4 DNA polymerase, and DNA metabolic enzymes such as T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I (Table 1). These observations indicated that EA is also a potent inhibitor of mammalian DNA polymerases α and β, and is not necessarily a DNA topoisomerase-specific inhibitor as reported earlier.

3.2. Effects of reaction conditions on DNA polymerase inhibition

We tested whether an excess amount of a substrate analog, poly(rC) (50 μM), or a protein, BSA (200 μg/mL), could prevent the DNA polymerase inhibitory effects of EA. If EA binds to DNA polymerases α and β by non-specific adhesion, the addition of the DNA and/or the protein would reduce the inhibitory activity. Poly(rC) and BSA showed no influence and/or no binding on the effects of the compound, suggesting that the binding to both DNA polymerases α and β occurs selectively or to bind to a specific site on DNA polymerases α and β (Table 2).

3.3. Effects of EA on the activities of human DNA topoisomerases I and II

The effect of EA on the catalytic activities of human DNA topoisomerases I and II was examined by the relaxation assay as described in Section 2. As shown in Fig. 2A, EA dose-dependently inhibited DNA topoisomerase I relaxation activity, and complete inhibition occurred at 2 μM (lane 5). Similarly, this compound also dose-dependently inhibited DNA topoisomerase II relaxation activity, and complete inhibition occurred at 2 μM (lane 5 in Fig. 2B). The IC_{50} value of EA was 1.5 μM for both topoisomerases. Therefore, EA should be referred to as a DNA topoisomerase inhibitor rather than a DNA polymerase inhibitor. EA is a kind of C_{22}-fatty acid with *p*-hydroxyphenol. Interestingly, C_{22}-fatty acid, docosanoic acid, also inhibits the activity of DNA topoisomerase II, although the inhibition was 10-fold weaker than EA (data not shown). Since we found and reported previously that long chain fatty acids (C_{18}–C_{24}) could strongly inhibit the activities of both DNA polymerases and DNA topoisomerase [11], it is suggested that the inhibition of these enzymes occurs by the C_{22}-fatty acid site, and that the *p*-hydroxyphenol group of EA enhances the inhibition of both the enzyme activities.

3.4. Influence of EA on the hyperchromicity of double-stranded DNA

To determine whether EA binds to DNA, the melting temperature (T_m) of double-stranded DNA (dsDNA) in the presence of 15 μM of the compound was measured using a spectrophotometer equipped with a thermoelectric cell holder (Fig. 3). As described in Section 2, calf thymus dsDNA at 6 μg/mL was dissolved in 0.1 M sodium

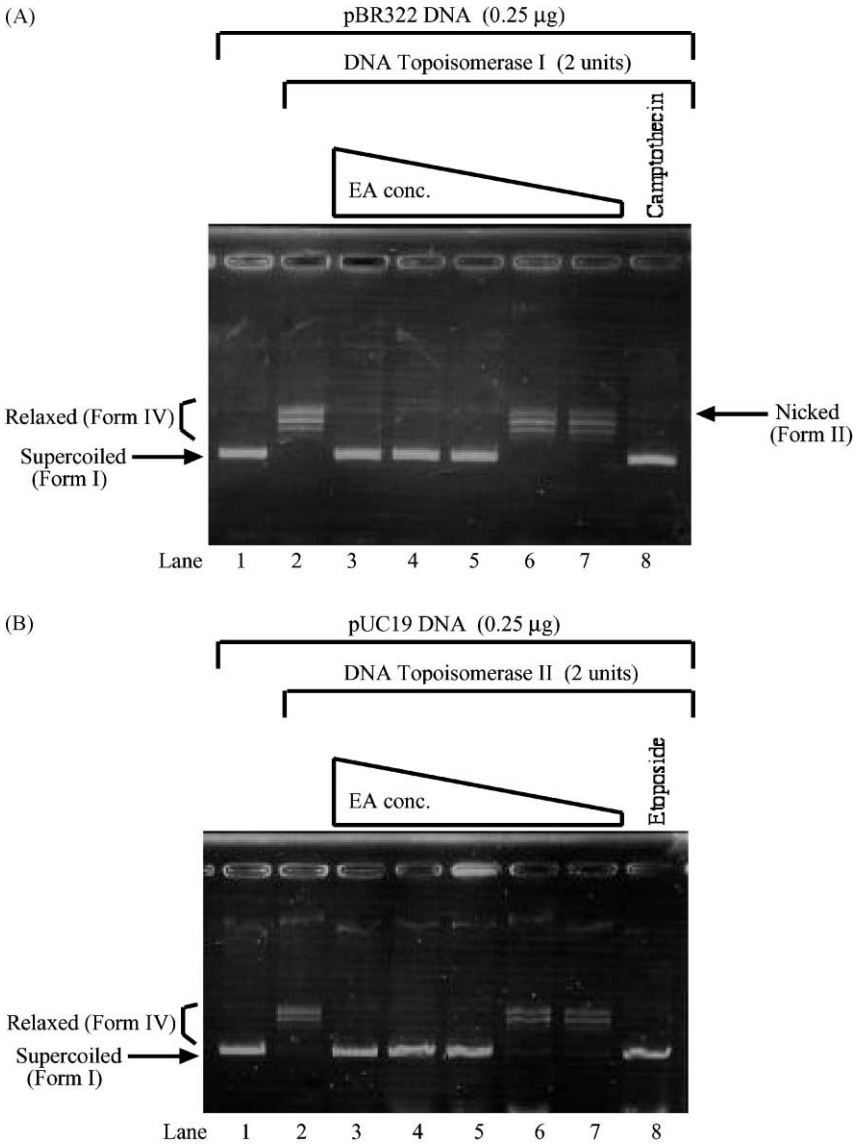

Fig. 2. Inhibitory effects of EA on human DNA topoisomerases I and II. (A) pBR322 DNA was mixed with DNA topoisomerase I; (B) pUC19 DNA was mixed with DNA topoisomerase II. Supercoiled plasmid DNA was mixed with the enzyme and inhibitor, EA dissolved in DMSO. Lanes 1–7, EA at concentrations of 0, 0, 10, 5, 2, 1 and 0.5 μM, respectively; lane 8, 100 μM of a known inhibitor (A: camptothecin, B: etoposide), lanes 2–8, 2 unit of DNA topoisomerase I; lane 1, no enzyme; 0.25 μg of plasmid DNA was added in each of the lanes. Photographs of ethidium bromide-stained gels are shown. All experiments were performed three times.

phosphate buffer (pH 7.0) containing 1% DMSO. At this concentration of EA, the thermal transition of T_m was not observed, whereas 15 μM EtBr, a typical intercalating agent, caused the thermal transition (Fig. 3). The compound at 100 μM also showed no thermal transition of T_m (data not shown). Thus, none of EA bound to the dsDNA, suggesting that it must inhibit the enzyme activities by interacting with the enzymes directly.

3.5. Effects of EA on cultured mammalian cells

The DNA topoisomerases and DNA polymerases have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. EA could, therefore, be useful as a new anti-cancer chemotherapy agent. We tested the cytotoxic effect of EA against a human stomach cancer cell line, NUGC-3, *in vitro*.

As shown in Fig. 4, EA showed a potent growth inhibitory effect against this cancer cell line. The concentration of EA required for the LD_{50} was 22.5 μM. The IC_{50} values of EA were 7.7 μM for DNA polymerase α, 12.9 μM for DNA polymerase β and 1.5 μM for both DNA topoisomerases I and II (Table 1). The LD_{50} value was obviously 2-fold higher than the IC_{50} values *in vitro* for DNA polymerases α and β and 10-fold higher than DNA topoisomerases I and II. The inhibition curves of the enzymes and cell growth showed parallel dose-dependent reductions. At 12.5 μM of EA, 79.8% of DNA polymerase activity and more than 90% of DNA topoisomerase activity in the NUGC-3 cell extract were inhibited. EA was suggested to inhibit the

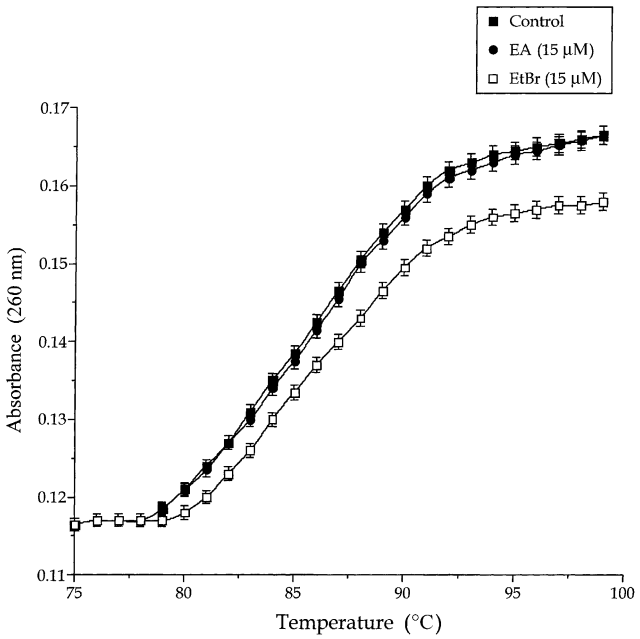

Fig. 3. Effects of EA on the thermal transition of double-stranded DNA. None (control, ■), EA (●) and ethidium bromide (□) (15 μM each) were incubated with 6 μg/mL of calf thymus double-stranded DNA in 0.1 M Na-phosphate buffer (pH 7.0). Data are shown as means ± SEM for three independent experiments.

activities of DNA polymerases and DNA topoisomerases in the intact cell. These observations suggested that the cell growth inhibition occurs in a manner dependent on the enzyme inhibition, and that inhibition of both of the enzymes influences cell growth *in vivo*.

To confirm this suggestion in detail, we examined the compound effect on the cell cycle of NUGC-3 cells by flow cytometry. As shown in Fig. 5A and B, the cells were arrested in G1 phase (increase of 3.9%) and G2/M phases (increase of 5.4%) and decreased in the rate of S phase by 12.5 μM EA with incubation for 12 hr. After 24 and 48 hr

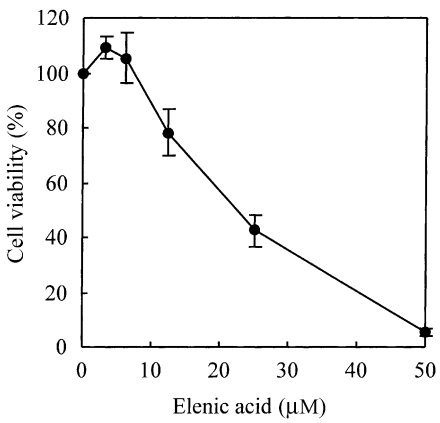

Fig. 4. Effects of EA on proliferation of NUGC-3 cancer cells. Dose–response curves of growth inhibition of the human stomach cancer cell line NUGC-3. The assays were carried out under the conditions described in Section 2 with EA at the indicated concentrations. Survival rate was determined by MTT assay [23]. Data are shown as means ± SEM for three independent experiments.

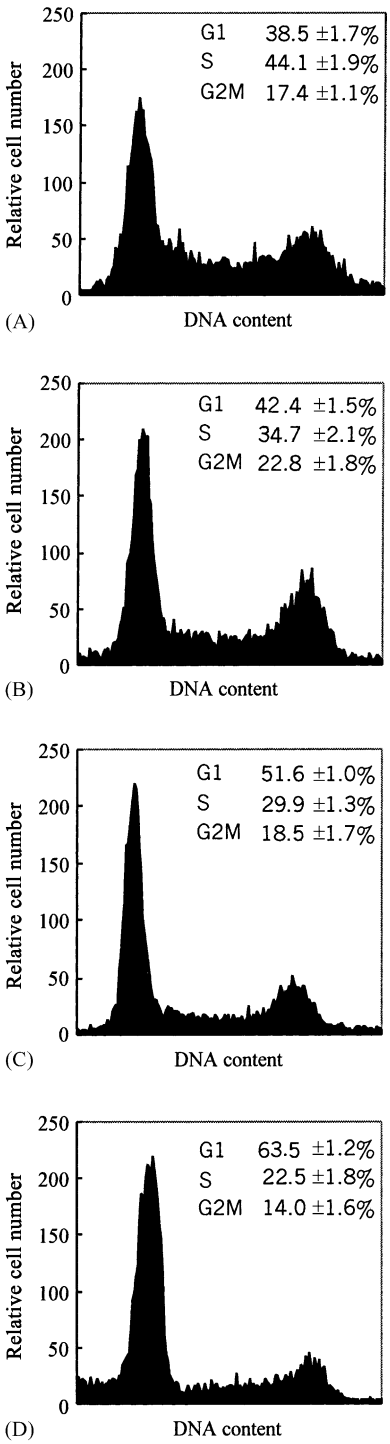

Fig. 5. Flow cytometric analysis of cell cycle perturbation by EA. Panels A–D, the cells were incubated with 12.5 μM EA for 0, 12, 24 and 48 hr, respectively. DNA was stained with DAPI solution. Fluorescence intensity was measured by flow cytometry. Results are presented as mean ± SD of six independent experiments.

treatment with EA, the G2/M block was partially released and G1 phase was increased (Fig. 5C and D). Aphidicolin, which is a DNA polymerase α inhibitor, and etoposide (VP-16), which is a DNA topoisomerase II inhibitor, block at G1/S and G2/M of the cell cycle, respectively [27,28]. EA arrested G1 phase in the cell cycle and could inhibit the

activity of DNA polymerase in the cell, although the enzymatic inhibitory activities of DNA topoisomerases by EA were stronger than those of DNA polymerases (Table 1). EA must inhibit cell growth mainly by blocking the primary step of DNA replication and cell division, by acting not only on the DNA polymerases but also on the DNA topoisomerases. Furthermore, EA may influence the second step of cell replication by DNA polymerases after 24 and 48 hr exposure to EA.

3.6. Effect of EA on apoptotic cell death

To examine whether the decrease in cell numbers by EA (Fig. 4) was due to apoptosis, DNA fragmentation was analyzed by electrophoresis. DNA ladder formation was observed in NUGC-3 cells treated with 0–12.5 μM EA for 48 hr (lanes 1–3 in Fig. 6). Such ladders were not evident for the initial 12 hr but were apparent at 24 hr and thereafter (lanes 4–7 in Fig. 6). These results were also obtained using Ball-1 cells (human acute lymphoblastoid leukemia cell line) as a non-adherent cell line instead of NUGC-3 cells as an adherent cell line (data not shown). EA is a strong apoptosis-inducer. The effect of EA must occur in the combination of growth arrest and cell death.

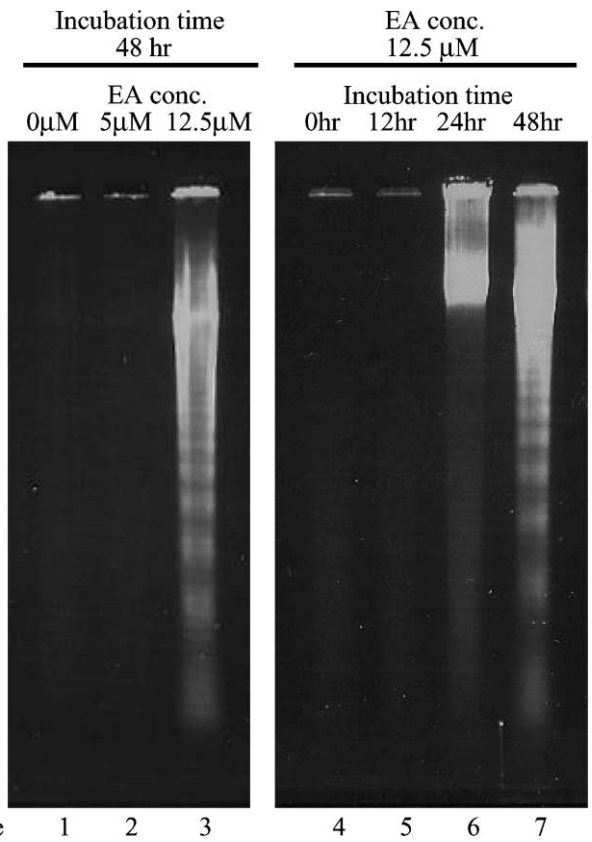

Fig. 6. DNA fragmentation of EA-treated cells on agarose gel electrophoresis. Lanes 1–3, NUGC-3 cells were incubated for 48 hr with 0, 5 and 12.5 μM EA, respectively. Lanes 4–7, the cells were incubated with 12.5 μM EA for 0, 12, 24 and 48 hr, respectively. Total DNA was then extracted and analyzed by 1.4% agarose gel electrophoresis.

4. Discussion

EA, which is *R*-(−)-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid, was found originally as a DNA topoisomerase II inhibitor from an Indonesian sponge, *Plakinastrella* sp. As described here, EA was not only an inhibitor of DNA topoisomerases I and II, but also a potent inhibitor of DNA polymerases α and β. Interestingly, the enzymatic characteristics of the enzyme species including modes of action, amino acid sequences and three-dimensional structures were markedly different from each other. Like EA, some long chain fatty acids and triterpenoids could inhibit both the activities of DNA topoisomerases and DNA polymerases by binding to the enzymes directly [11,12]. DNA topoisomerases and DNA polymerases have structural homology between the DNA-binding sites [11] and the DNA-binding site of a C_{24}-fatty acid (nervonic acid) on the DNA polymerase differed slightly from that of triterpenoids [10,13]. Therefore, EA was expected to have similar characteristics.

We reported previously the results of amino acid analysis of the binding site of C_{24}-fatty acid on DNA polymerase β, and demonstrated by NMR analysis the changes in the three-dimensional structure of DNA polymerase β before and after binding of the fatty acid [10]. The DNA-binding site bound to the fatty acid molecule as a 1:1 complex, and a fatty acid molecule could bind to this site more strongly than a DNA molecule (DNA template-primer) [10]. The hydrophobic (methyl chain) and the hydrophilic regions (carboxyl group) of the fatty acids bind to the hydrophobic sheet and the hydrophilic amino acids in the DNA-binding site of the DNA polymerase β, respectively, and subsequently interfere with template-primer binding [10]. Since EA consists of C_{22}-fatty acid, it may also bind to the DNA-binding site, and the similarities in the modes of inhibition by *p*-hydroxyphenol and EA should be examined in future studies.

We have been engaged in analyzing the structure and function of DNA polymerase and DNA topoisomerase using EA from two different viewpoints. This agent could be a useful inhibitor not only to investigate the three-dimensional structure of the EA-binding site on DNA polymerase and DNA topoisomerase. In addition, we also investigated whether the agent could be a useful key drug to develop a design strategy for cancer chemotherapy agents. Since EA could prevent the growth of NUGC-3 cancer cells, the cells were halted at G1 and G2/M phase in the cell cycle, the agent should also be considered as the lead compound of a group of potentially useful cancer chemotherapy agents.

The DNA topoisomerase inhibitors such as adriamycin, amsacrine, ellipticine, saintopin, streptonigrin and terpentecin are intercalating agents, and were thought to bind to the DNA molecule directly, and subsequently to inhibit both activities indirectly. They inhibited the rejoining reaction of topoisomerases by stabilizing a tight topoisomerase–DNA

complex termed the ''cleavable complex''. To determine whether EA binds to DNA, the T_m of dsDNA was measured, and none of the compound was found to bind to the dsDNA. Thus, EA must inhibit the enzyme activities by interacting with the enzymes directly. DNA topoisomerase inhibitors were categorized into two classes, ''suppressors'', which are believed to interact directly with the enzyme and ''poisons'', which stimulate DNA cleavage and DNA intercalation [29]. EA may be considered as a ''suppressor'' of DNA topoisomerase functions rather than as a conventional poison, since EA does not stabilize topoisomerase–DNA covalent complexes such as the already described agents. Although, in general, suppressors have not been thought to be useful as anti-cancer agents *in vivo* [29], EA could be an anti-cancer agent because the compound could inhibit the activities not only of DNA topoisomerases, but also of DNA polymerases (Table 1 and Fig. 2). In fact, novel sulfolipids, which show almost the same molecular action mode as those of long chain fatty acids [30,31], were potent as anti-cancer agents *in vivo* [32]. The sulfolipids are thought to be in the category of ''suppressor'' compounds. EA could also be considered as a possible candidate as an anti-cancer agent.

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