J. Biochem. **133**, 541–552 (2003)
DOI: 10.1093/jb/mvg070

Molecular Action Mode of Hippospongic Acid A, an Inhibitor of Gastrulation of Starfish Embryos

Yoshiyuki Mizushina[*,1,2], Chikako Murakami[1], Hirosato Takikawa[3], Nobuyuki Kasai[4], Xianai Xu[1], Kenji Mori[5], Masahiko Oshige[6], Toyofumi Yamaguchi[7], Mineo Saneyoshi[7], Noriko Shimazaki[4], Osamu Koiwai[4], Hiromi Yoshida[1,2], Fumio Sugawara[4,6] and Kengo Sakaguchi[4,6]

[1]Laboratory of Food & Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180; [2]High Technology Research Center, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180; [3]Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Nada-ku, Kobe, Hyogo 657-8501; [4]Department of Applied Biological Science, Tokyo University of Science, Noda, Chiba 278-8510; [5]Department of Chemistry, Faculty of Science, Tokyo University of Science, Shinjuku-ku, Tokyo 162-8601; [6]Frontier Research Center for Genomic & Drug Discovery, Tokyo University of Science, Noda, Chiba 278-8510; and [7]Department of Biological Science, Teikyo University of Science and Technology, Yamanashi 409-0193

Received December 22, 2002; accepted February 5, 2003

Hippospongic acid A (HA-A) is a novel natural triterpene metabolite that exhibits inhibitory activity against the gastrulation of starfish embryos isolated from a marine sponge, *Hippospongia* sp. We succeeded in chemically synthesizing the natural enantiomer and the racemate HA-A. In this study, we examined its action mode *in vitro*. HA-A was a rare compound that could selectively but uniformly inhibit the activities of all the vertebrate DNA polymerases tested such as α, β, δ, ε, η, κ, and λ, in the IC_{50} range of 5.9–17.6 μM, and interestingly also those of human DNA topoisomerases I and II (IC_{50} = 15–25 μM). HA-A exhibited no inhibitory effect on DNA polymerases from insects, plants and prokaryotes, or on many other DNA metabolic enzymes. HA-A was an inhibitor specific to DNA polymerases and DNA topoisomerases from vertebrates, but not selective as to a subclass species among the enzymes. Since DNA polymerase β is the smallest, we used it to analyze the biochemical relationship with HA-A. Biochemical, BIAcore and computer modeling analyses demonstrated that HA-A bound selectively to the N-terminal 8 kDa DNA template-binding domain of DNA polymerase β, and HA-A inhibited the ssDNA binding activity. HA-A could prevent the growth of NUGC-3 cancer cells at both the G1 and G2/M phases, and induce apoptosis in the cells. The LD_{50} value was 9.5 μM, *i.e.* in the same range as for the enzyme inhibition. Therefore, we concluded that one molecular basis of the gastrulation of starfish embryos is a process that requires DNA polymerases and DNA topoisomerases, and subsequently the gastrulation was inhibited by HA-A. We also discussed the *in vivo* role of HA-A.

Key words: cytotoxicity, DNA polymerase, DNA topoisomerase, enzyme inhibitor, gastrulation of starfish embryos, hippospongic acid A.

Abbreviations: HA-A, hippospongic acid A; pol, DNA polymerase (EC 2.7.7.7); topo, DNA topoisomerse; dTTP, 2′-deoxythymidine 5′-triphosphate; HIV-1, human immunodeficiency virus type-1; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

In 1996, hippospongic acid A (HA-A), a natural triterpene metabolite exhibiting inhibitory activity against gastrulation of starfish embryos, was isolated by Ohta *et al.* from a marine sponge, *Hippospongia* sp. (*1*). Gastrulation is, as is well-known, a fundamental process during the embryonic development of multicellular animals. The process involves the transformation of a simple hollow ball of epithelial cells into a multilayered structure with a mesendodermal archenteron produced through the tucking of cells from the exterior into the interior. From the developmental biology point of view, the compound is thought to be of great importance. The inhibitory activity was measured as the inhibition of mass movement of the tissue, but not of each of the cells in the tissue. We need to clarify the effect at the molecular and cellular levels more precisely. Therefore, we tried to synthesize HA-A chemically, and succeeded in producing the natural enantiomer HA-A [*i.e.*, (*R*)-HA-A] and the racemate HA-A [*i.e.*, (±)-HA-A] (*2*, *3*). The purpose of this study was to identify the *in vivo* target molecule of HA-A biochemically, and in general to clarify the biochemical functions of HA-A.

We found no previously reported direct or indirect biochemical information that provided a clue as to the action of HA-A. As shown in this study, HA-A could prevent the growth of human cultured cells, and halt the cell cycle, suggesting that HA-A is a compound that at least influ-

[*]To whom correspondence should be addressed: Tel: +81-78-974-1551 (Ext. 3232), Fax: +81-78-974-5689, E-mail: mizushin@nutr.kobegakuin.ac.jp

ences DNA synthesis. Moreover, we also decided to investigate the molecular target. As reported previously (*4*–*7*), if the sizes and three-dimensional structures of two chemically different agents are quite similar, they can inhibit an enzyme activity in the same manner. Irrespective of the studies on the gastrulation of starfish embryos, we have searched for natural compounds that inhibit eukaryotic DNA polymerase activities selectively, because selective inhibitors of DNA polymerases are useful tools for distinguishing DNA polymerases and for clarifying their biological functions. Subsequently, we found many inhibitors of mammalian DNA polymerases (*4*, *5*, *8*–*17*). Among the compounds, the chemical structure of elenic acid [*R*-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid], which is a natural alkylphenol compound produced by an Indonesian sponge (*Plakinastrella* sp.) and an inhibitor of DNA polymerases, was three-dimensionally similar to that of HA-A, although they differed chemically from each other (*16*). Therefore, we first examined whether or not HA-A could influence the activities of DNA metabolic enzymes. In the sponge, the physiological or embryological role of elenic acid remains unknown.

As described in this report, like elenic acid, HA-A was found to be a potent inhibitor of eukaryotic DNA polymerases, but surprisingly it influenced only the activities of DNA polymerases from vertebrates. Moreover, HA-A could equally influence the activities of all the DNA polymerase species examined, α, β, δ, ε, η, κ, and λ. Then, we found that HA-A could also inhibit the activities of human DNA topoisomerases, although HA-A is sufficiently selective as to DNA polymerases and there are no enzymatic similarities between the two enzymes. These characteristics were quite similar to those of elenic acid, which was originally found as a DNA topoisomerase inhibitor just by chance, and later also as an inhibitor of polymerases (*16*). However, only DNA polymerase α and β were examined in this study on elenic acid, and the discussion was preliminary and lacking in detail (*16*). The effects of HA-A suggest that since both polymerases and topoisomerases are critical for many cellular processes such as DNA replication, repair and recombination, they may act in harmony with each other. Here, we discussed the inhibition of the gastrulation of starfish embryos in relation to the unique inhibitory effect of DNA polymerases and DNA topoisomerases. In the animal kingdom phylogenetic tree, the starfish as well as the vertebrates belong to the deuterostomic branch, therefore, HA-A might show an inhibitory effect selective as to the enzymes from deuterostome species including vertebrates. Interestingly, HA-A had no effect on the polymerases from an insect, *Drosophila melanogaster*, which belongs to the protostomic branch, another branch of the animal kingdom. We discussed the roles of HA-A in developmental biology. This also represents the first report of an inhibitor specific to DNA polymerases and DNA topoisomerases only from vertebrates or probably deuterostomes.

EXPERIMENTAL PROCEDURES

Materials—Both the natural enantiomer [(*R*)-] and racemate [(±)-] hippospongic acid A (HA-A) were chemically synthesized as described previously (*2*, *3*). Elenic acid was chemically synthesized as described previously (*16*). [^{3}H]dTTP (2′-deoxythymidine 5′-triphosphate) (43 Ci/mmol) and chemically synthesized template-primers such as poly(dA), poly(rA), and oligo(dT)$_{12–18}$ were purchased from Amersham Biosciences (Buckinghamshire, UK). Supercoiled pBR322, M13 plasmid DNA and pUC19 plasmid DNA were obtained from Takara (Kyoto). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka). NUGC-3, a human gastric cancer cell line (JCRB0822) (*18*), was supplied by the Health Science Research Resources Bank (Osaka).

Enzymes—DNA polymerase α (pol α) was purified from calf thymus by immuno-affinity column chromatography as described by Tamai *et al.* (*19*). Recombinant rat pol β was purified from *E. coli* JMpβ5 as described by Date *et al.* (*20*). The N-terminal 8 kDa domain of rat pol β (residues 2–87) was overexpressed in *Escherichia coli* strain BL21 harboring the expression plasmid "Lys-87" constructed in our laboratory. Overproduction of the 8 kDa domain and the purification procedure were principally the same as for the intact pol β (*4*). Pol δ was purified from calf thymus (*21*), and pol ε was purified from HeLa cells as described previously (*22*). Recombinant human pol η tagged with His$_6$ at its C-terminal side was expressed in SF9 insect cells using the baculovirus expression system, and was purified from the cells as described previously (*23*). A truncated form of pol κ (*i.e.* hDINB1ΔC) with a 6× His-tag attached at the carboxyl terminus was overproduced using a BAC-to-BAC Baculovirus Expression System kit (GIBCO BRL), and purified as described previously (*24*). Recombinant His-pol λ was overexpressed and purified according to the method described previously (*17*). Fish pol α and δ were purified from cherry salmon (*Oncorhynchus masou*) testis (*25*). Insect pol α was purified from early embryos of the fruit fly (*D. melanogaster*) as described previously (*26*). Pol I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the method outlined by Sakaguchi *et al.* (*27*). The Klenow fragment of pol I and human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (recombinant) were purchased from Worthington Biochemical (Freehold, NJ, USA). Taq DNA polymerase, T4 DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara (Kyoto). Calf thymus terminal deoxynucleotidyl transferase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA, USA). Purified human placenta DNA topoisomerase I and II (topo I and II) (2 units/μl) were purchased from TopoGen (Columbus, OH). Budding yeast (*Saccharomyces cerevisiae*) topo II was purified as described previously (*6*).

DNA Polymerase Assays—The activities of all DNA polymerases were measured by the methods described previously (*8*, *9*). For DNA polymerases, poly(dA)/oligo(dT)$_{12–18}$ and dTTP were used as the DNA template-primer and nucleotide substrate, respectively. The reaction mixture for DNA polymerase η (24 μM final volume) comprised 40 mM Tris-HCl (pH 8.0), 2.5% (v/v) glycerol, 60 mM KCl, 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 20 μM poly(dA), 5 μM (dT)$_{12–18}$, 10 μM [^{3}H]dTTP (100 cpm/

pmol), and 8 μl of inhibitor (*i.e.*, HA-A)–enzyme mixture solution. The reaction mixture for DNA polymerase κ comprised 40 mM Tris-HCl (pH 8.0), 2.5% (v/v) glycerol, 60 mM KCl, 10 mM DTT, 5 mM $MgCl_2$, 10 μM poly(dA), 5 μM $(dT)_{12–18}$, 10 μM [^{3}H]dTTP (100 cpm/pmol), and 8 μl of inhibitor–enzyme mixture solution. For HIV reverse transcriptase, poly(rA)/oligo$(dT)_{12–18}$, and dTTP were used as the template-primer and dNTP substrate, respectively. The substrates of terminal deoxynucleotidyl transcriptase used were oligo$(dT)_{12–18}$ (3′-OH) and dTTP, as the template-primer and nucleotide substrate, respectively. HA-A was dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Four microliters of each of the sonicated samples was mixed with 16 μl of each enzyme (final, 0.05 unit) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA, followed by standing at 0°C for 10 min. Each of these inhibitor-enzyme mixtures (8 μl) was added to 16 μl of each of the enzyme standard reaction mixtures, and then incubation was carried out at 37°C for 60 min, except in the case of Taq DNA polymerase, which was incubated at 74°C for 60 min. The activity without the inhibitor was considered to be 100%, and the remaining activity with each inhibitor concentration was determined as a percentage of this value. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleotide triphosphate (*i.e.*, dTTP) into the synthetic template-primer [*i.e.*, poly(dA)/oligo$(dT)_{12–18}$, A/T = 2/1] in 60 min at 37°C under the normal reaction conditions for each enzyme (*8*, *9*).

DNA Topoisomerase Assays—The relaxation activity of topo II was determined by detecting the conversion of supercoiled plasmid DNA to its relaxed form (*28*). The topo II reaction was performed in 20 μl of a reaction mixture comprising 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% DMSO), and 2 units of topo II. The reaction mixtures were incubated at 37°C for 30 min, and the reactions 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 (EtBr), and DNA was visualized with a UV transilluminator. The relaxation activity of topo I was analyzed in the same manner as described above except that the reaction mixtures comprised 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 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.

Other Enzyme Assays—The activities of T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured by means of standard assays according to Nakayama and Saneyoshi (*29*), Soltis and Uhlenbeck (*30*), and Lu and Sakaguchi (*31*), respectively.

Gel Mobility Shift Assay—The gel mobility shift assay was carried out as described by Casas-Finet *et al.* (*32*). The binding mixture (final volume, 20 μl) comprised 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 50 μg/ml bovine serum albumin (BSA), 10% DMSO, 2 mM EDTA, M13 plasmid DNA (2.2 nmol; nucleotide, single-strand and singly-primed), and 0.15 nmol of the 8 kDa domain of pol β. Various concentrations of (*R*)-HA-A were added to the binding mixture, followed by incubation at 25°C for 10 min. Samples were run on a 1.0% agarose gel in 0.1 M Tris-acetate buffer, pH 8.3, containing 5 mM EDTA at 50 V for 2 h.

Surface Plasmon Resonance—Binding analyses of the 39 kDa enzyme, the 8 kDa domain fragment of pol β, topo II and (*R*)-NA-A were performed using a Biosensor BIAcore instrument (BIACORE^R X) (BIAcore, Sweden). CM5 research grade sensor chips (BIAcore, Sweden) were used. All buffers were filtered before use. The 8 kDa domain of pol. β (50 μg/ml, 30 μl; *i.e.* 1.87 nmol) in coupling buffer (10 μM sodium acetate, pH 5.0) was injected over a CM5 sensor chip at 20 μl/min to capture the protein on the carboxymethyl dextran matrix of the chip through the NHS/EDC coupling reaction (60 μl of mix) as previously described (*33*). Unreacted *N*-hydroxysuccinimide ester groups were inactivated with 1 M ethanolamine-HCl (pH 8.0). This reaction immobilized about 5,000 response units (RU) of the protein. Binding analysis of (*R*)-HA-A was performed in running buffer [5 mM potassium phosphate buffer (pH 7.0) and 10% DMSO] at a flow rate of 20 μl/min at 25°C. Kinetic parameters were determined using the software BIA evaluation 3.1.

HA-A Docking Modeling—The molecular docking of (*R*)-HA-A and the 8 kDa domain of pol β or topo II was performed using the flexible docking procedure in the Affinity program within the Insight II modeling software (Accelrys Inc., San Diego, CA, USA, 1999). All calculations were conducted with SGI Octane2 (R12000), running with the IRIX 6.5 operating system. The calculations involved the CVFF force-field in the Discovery program, and the Monte Carlo and Simulated Annealing strategy in the Affinity program (*34*). Each energy-minimized final docking position of HA-A was evaluated using the interactive score function in the Ludi module. The Ludi score includes contribution of the loss of translational and rotational entropy of the fragment, the number and quality of hydrogen bonds, and contributions from ionic and lipophilic interactions to the binding energy.

Investigation of Cytotoxicity toward Cultured Cells—A human gastric cancer cell line, NUGC-3, was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C under a humid atmosphere of 5% CO_2/95% air. For the cell viability assay, NUGC-3 cells were plated at 3×10^5 cells into the wells of 96-well microplates with various concentrations of (*R*)- and (±)-HA-A. HA-A was dissolved in phosphate-buffered saline (PBS) at a concentration of 20 mM as a stock solution. The stock solutions were diluted to appropriate final concentrations with growth medium just before use. Cell viability was determined by means of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay as described previously (*35*).

Cell Cycle Analysis—Cells (3×10^5 cells in a 35 mm dish) were collected by trypsinization and washed with ice-cold PBS by centrifugation. The cells were suspended in PBS, fixed with 70% ethanol (v/v), and then stored at –

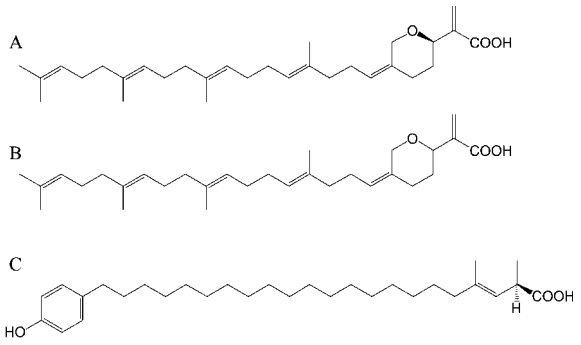

Fig. 1. **Chemical structures of hippospongic acid A (HA-A) and elenic acid.** (A) The natural enantiomer: (*R*)-HA-A. (B) The racemate: (±)-HA-A. (C) Elenic acid.

20°C. The cells were then collected by centrifugation and stained with 4′ 6-diamidino-2-phenylindole (DAPI, 2 μg/m) for at least 20 min at room temperature in the dark. The DNA content of 8,000 stained cells was determined with a cell counter analyzer (Partec, CCA model, Munster, Germany) with Muticycle 3.11 software (Phoenix Flow Systems, San Diego, CA). Cell debris and fixation artifacts were gated out.

Analysis of DNA Fragmentation—Apoptosis was determined by assaying of DNA fragmentation, by means of agarose gel electrophoresis. Total DNA was extracted from 2×10^6 NUGC-3 cells following the method of Sambrook *et al*. (*36*), 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), followed by staining with ethidium bromide. DNA bands were visualized under UV light.

RESULTS

Hippospongic acid A (HA-A), which has been found to be an inhibitor of the gastrulation of starfish embryos (*1*), is a triterpene metabolite isolated from a marine sponge *Hippospongia* sp. (*3*) (Fig. 1, A and B). In 2001, we succeeded in chemically synthesizing it (*2*). The chemical structures of the natural enantiomer HA-A [*i.e*., (*R*)-HA-A] and the racemate HA-A [*i.e*., (±)-HA-A] are shown in Fig. 1, A and B, respectively. (±)-HA-A consists of two stereoisomers, (*R*)-HA-A, which is a natural compound, and (*S*)-HA-A, which is an unnatural type, the (*R*): (*S*) ratio being 1:1. As described under Introduction, we wanted to identify the target enzyme of HA-A, and then to clarify the biological and *in vivo* functions of (*R*)-HA-A and (±)-HA-A using synthetic compounds.

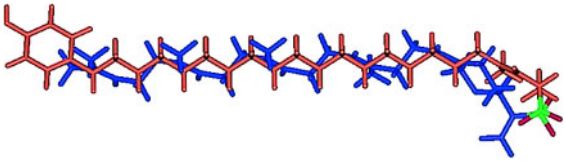

Fig. 2. **Overlaying of (*R*)-HA-A and elenic acid.** The structures of (*R*)-HA-A and elenic acid are indicated in blue and orange, respectively, except that the carbons, oxygens and hydrogens of the carboxyl group are indicated in green, red and white, respectively. The figure was prepared using Insight II (Accelrys).

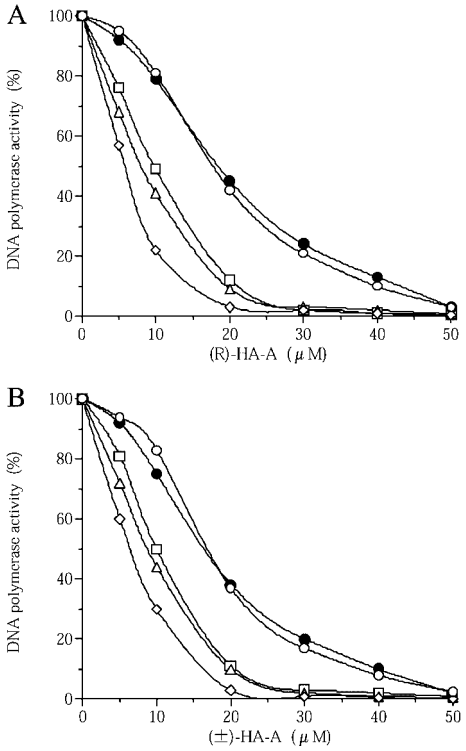

Fig. 3. **DNA polymerase inhibition dose-response curves of HA-A.** (A) (*R*)-HA-A. (B) (±)-HA-A. The enzymes used (0.05 unit each) were calf DNA polymerase α (open squares), rat DNA polymerase β (solid circles), calf DNA polymerase δ (open triangles), human DNA polymerase ε (open diamonds), and human DNA polymerase λ (open circles). The DNA polymerase activities were measured as described under Experimental Procedures. DNA polymerase activity in the absence of these compounds was taken as 100%.

Three-Dimensional Chemical Structure of HA-A—As shown in the later part of this report (Figs. 8 and 9), HA-A showed a potent growth inhibitory effect against the human gastric cancer cell line NUGC-3, the cells being arrested in both the G1 and G2/M phases. The arrest in both the G1 and G2/M phases of the cell cycle was suggested to be caused by the inhibition of DNA synthesis. Therefore, we concentrated our efforts on searching for the molecular target of HA-A among enzymes related to DNA synthesis, especially DNA polymerases and DNA topoisomerases, for the following reasons. As described under Introduction, to search for the *in vivo* target molecule of HA-A biochemically, we compared the three-dimensional structure of HA-A with those of many cytotoxic agents by computer simulation, since we reported previously that two chemically different agents with similar sizes and three-dimensional structures would inhibit an enzyme activity in the same manner (*10*). Among the compounds examined, elenic acid [*R*-2,4-dimethyl-22-(*p*-hydroxyphenyl)-docos-3(*E*)-enoic acid], produced by an Indonesian sponge, *Plakinastrella* sp. (Fig. 1C) (*16*), was three-dimensionally similar to (*R*)-HA-A, although they differed chemically from each other (Fig. 2). Elenic acid was originally found to be an inhibitor of human topo I and II, and then also of mammalian DNA polymerase α and β (pol α and β) (*16*).

Table 1. **IC50 values of hippospongic acid A for the activities of various DNA polymerases and other DNA metabolic enzymes.**

Enzyme	IC_{50} value of hippospongic acid A (μM)	
	(*R*)-HA-A	(±)-HA-A
—Mammalian DNA polymerases—		
Calf DNA polymerase α	10.0	10.0
Rat DNA polymerase β	17.2	16.5
Calf DNA polymerase δ	8.0	8.6
Human DNA polymerase ε	5.9	6.4
Human DNA polymerase η	9.0	9.2
Human DNA polymerase κ	10.4	13.1
Human DNA polymerase λ	17.6	16.9
—Fish DNA polymerases—		
Cherry salmon DNA polymerase α	9.6	9.4
Cherry salmon DNA polymerase δ	8.0	8.0
—Insect DNA polymerase—		
Fruit fly DNA polymerase α	>500	>500
—Plant DNA polymerases—		
Cauliflower DNA polymerase I (α-like)	>500	>500
Cauliflower DNA polymerase II (β-like)	>500	>500
—Prokaryotic DNA polymerases—		
E. coli DNA polymerase I (Klenow fragment)	>500	>500
Taq DNA polymerase	>500	>500
T4 DNA polymerase	>500	>500
—Other DNA metabolic enzymes—		
Calf DNA primase of DNA polymerase α	>500	>500
HIV-1 reverse transcriptase	>500	>500
Human DNA topoisomerase I	25	25
Human DNA topoisomerase II	15	15
yeast DNA topoisomerase II	15	15
T7 RNA polymerase	>500	>500
T4 polynucleotide kinase	>500	>500
Bovine deoxyribonuclease I	>500	>500

(R)- and (±)-hippospongic acid A were incubated with each enzyme (0.05 unit). The enzymatic activity was measured as described with "EXPERIMENTAL PROCEDURES". Enzyme activity in the absence of a compound was taken as 100%.

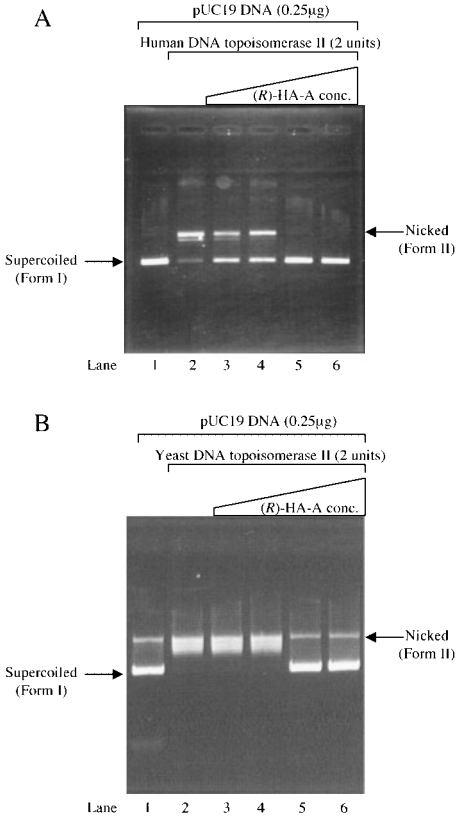

Fig. 4. **Inhibitory effects of (*R*)-HA-A on human DNA topoisomerase I and II.** (A) pUC19 DNA was mixed with human DNA topoisomerase II. (B) pUC19 DNA was mixed with yeast (*Saccharomyces cerevisiae*) DNA topoisomerase II. Supercoiled plasmid DNA was mixed with the enzyme and inhibitor, (*R*)-HA-A dissolved in DMSO. Lanes 1–6, (*R*)-HA-A at concentrations of zero, zero, 5, 10, 20 and 40 μM, respectively; lanes 2–6, 2 units of DNA topoisomerase; lane 1, no enzyme. 0.25 μg of plasmid DNA was added to each of the lanes. Photographs of ethidium bromide-stained gels are shown.

Effects of HA-A on the Activities of DNA Polymerases—At first, we examined the inhibition ability of HA-A as to mammalian DNA polymerases. Figure 3A presents the inhibition dose-response curves. (*R*)-HA-A dose-dependently inhibited the activities of pol α, pol β, pol δ, pol ε, and pol λ, with 50% inhibition being observed at doses of 10.0, 17.2, 8.0, 5.9, and 17.6 μM, respectively (Fig. 1A and Table 1). As expected, (*R*)-HA-A is an agent, like elenic acid, that inhibits DNA polymerase activities. In the inhibition spectrum, interestingly, (*R*)-HA-A inhibited the activity of pol λ, which was recently identified as a new member of the pol β family. The inhibitory effects of pol λ and pol β were of the same extent (Fig. 1A). Although the inhibition by (*R*)-HA-A was slightly stronger toward pol ε among the five polymerases, the compound did not selectively inhibit the activity of a subclass species among the mammalian DNA polymerases (Fig. 1A and Table 1). (*R*)-HA-A also inhibited the activities of fish DNA polymerases (Table 1). Cherry salmon pol α and δ were strongly inhibited by (*R*)-HA-A, and the dose-response curves were almost the same as the inhibition curves for mammalian DNA polymerases (Table 1). On the other hand, (*R*)-HA-A did not influence the activities of pol α from an insect, *Drosophila melanogaster*, and pol I (α like) and II (β like) from a higher plant, cauliflower, and prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* pol I, Taq DNA polymerase and T4 DNA polymerase. When activated DNA was used as the DNA template-primer instead of synthesized DNA [*i.e.*, poly(dA)-oligo$(dT)_{12-18}$], the inhibitory modes of the compounds did not change (data not shown).

The racemate compound [*i.e.*, (±)-HA-A] also exhibited the DNA polymerase inhibitory activity. As shown in Fig. 3B and Table 1, (±)-HA-A also inhibited mammalian DNA polymerase activities, the IC_{50} values being 6.4–16.9 μM. The inhibitory activity of (±)-HA-A was as strong as that of (*R*)-HA-A. (±)-HA-A consists of (*R*)-HA-A and (*S*)-HA-A. This suggested that (*R*)-HA-A and (*S*)-HA-A exhibited the same inhibitory activities toward DNA polymerases. Therefore, (*R*)-HA-A instead of (±)-HA-A was used for the remainder of this study.

Effects of HA-A on the Activities of Human and Yeast DNA Topoisomerase II, and Other DNA Metabolic Enzymes—Elenic acid is effective in inhibiting human DNA topoisomerase II (topo II) (*16*). Therefore, the effect of

Table 2. **Kinetic analysis of the inhibition by (*R*)-HA-A of the activity of DNA polymerase β, as a function of the DNA template-primer dose and the dNTP substrate concentration.**

Enzyme (0.05 unit)	Substrate	(*R*)-HA-A (μM)	K_m (μM)	V_{max} (pmol/h)	K_i (μM)	Inhibitory mode
Pol β	DNA template-primer[a]	0	6.74			
		10	10.3	111	10.4	Competitive
		20	21.1			
		30	37.8			
	dNTP substrate[b]	0	3.1			
		10	5.9	62.5	11.8	Competitive
		20	9.8			
		30	17.6			

[a]Poly(dA)/oligo(dT)$_{12-18}$. [b]dTTP.

(*R*)-HA-A on the catalytic activity of human topo II was also examined by means of a relaxation assay, as described under Experimental Procedures. As shown in Fig. 4A, (*R*)-HA-A dose-dependently inhibited topo II relaxation activity, complete inhibition occurring at 20 μM (lane 5). Similarly, this compound also dose-dependently inhibited yeast (*Saccharomyces cerevisiae*) topo II relaxation activity, complete inhibition occurring at 20 μM (lane 5 in Fig. 4B). The IC_{50} value of (*R*)-HA-A was 15 μM for both human and yeast topo II. The same inhibitory results were obtained with (±)-HA-A instead of (*R*)-HA-A (data not shown). The inhibitory effect of (*R*)-HA-A on topo II was stronger than that on topo I (Table 1). The IC_{50} values of HA-A for the topoisomerases were approximately 2-fold lower than those for the polymerases (Table 1).

Both (*R*)-HA-A and (±)-HA-A exhibited no inhibitory effect on other DNA-metabolic enzymes such as calf DNA primase of pol α, calf terminal deoxynucleotidyl transferase, HIV-1 reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I (Table 1).

From the above data concerning HA-A and the activities of polymerases, human topo I and II, and other DNA metabolic enzymes, HA-A should be regarded as an inhibitor of the polymerases and topoisomerases from vertebrates. Since HA-A is an inhibitor of the gastrulation of starfish embryos (*1*), and since the starfish is on the deuterostomic branch of the animal kingdom, a broad phylogenetic branch including vertebrates, HA-A may be an inhibitor of deuterostomic DNA polymerases and DNA topoisomerases. Next, we tried to elucidate the mechanism of inhibition of HA-A on the enzymes. To do so, we chose pol β as a representative enzyme at first because it is the smallest among the enzymes, and three-dimensional studies on it are the most advanced.

Mode of Inhibition of DNA Polymerase β by HA-A— The extent of the inhibition of pol β as a function of the DNA template-primer or dNTP substrate concentration was studied (Table 2). For kinetic analysis, poly(dA)/oligo(dT)$_{12-18}$ and dTTP were used as the DNA template-primer and dNTP substrate, respectively. Double reciprocal plots of the results showed that (*R*)-HA-A–induced inhibition of pol β activity was competitive as to both the DNA template-primer and dNTP substrate (Table 2). In the case of the DNA template-primer, the apparent maximum velocity (V_{max}) was unchanged at 111 pmol/h, whereas 153%, 313%, and 561% increases in the Michaelis constant (K_m) were observed in the presence of 10, 20, and 30 μM HA-A, respectively (Table 2). The V_{max} for the dNTP substrate was 62.5 pmol/h, and the K_m for the dNTP substrate increased from 3.1 to 17.6 μM in the presence of 30 μM HA-A (Table 2). The inhibition constant (K_i) values, obtained from Dixon plots, were found to be 10.4 μM and 11.8 μM for the DNA template-primer and dNTP substrate, respectively (Table 2). The inhibition by HA-A with the DNA template-primer was slightly greater than that with the dNTP substrate. When activated DNA and four deoxynucleoside triphosphates were used as the DNA template-primer and dNTP substrates, respectively, the inhibition of pol β by HA-A was competitive as to both the DNA template-primer and dNTP substrate (data not shown).

The inhibition of pol β by ddTTP was non-competitive with activated DNA as the DNA template-primer and competitive with respect to the dNTP substrate (*37*). In contrast, the inhibition of pol β by HA-A was competitive as to the DNA template-primer (Table 2), suggesting that HA-A directly binds to the DNA template-primer binding site of pol β. The ssDNA-binding site is located in the N-terminal 8 kDa domain of pol β (*38*, *39*). We further stud-

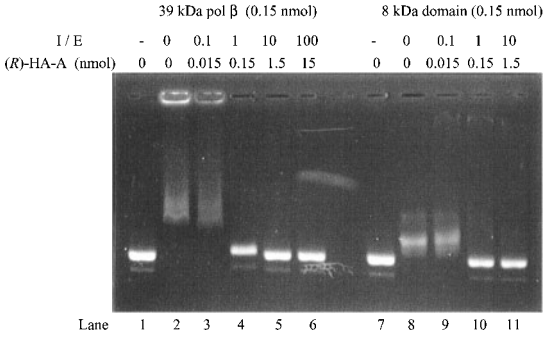

Fig. 5. **Gel mobility shift analysis of binding of the intact 39 kDa pol β and N-terminal 8 kDa domain to ssDNA.** Gel shift analysis of binding between M13 ssDNA and the intact or 8 kDa domain of pol β. M13 plasmid DNA (2.2 nmol; nucleotide, single strand and singly-primed) was mixed with the proteins and (*R*)-HA-A. Lanes 2–6 contained 39 kDa pol β at a concentration of 0.15 nmol; lanes 8–11 contained the 8 kDa domain at a concentration of 0.15 nmol; lanes 1 and 7 contained no enzyme. Lanes 2 and 8, 3 and 9, 4 and 10, 5 and 11, and 6 also contained (*R*)-HA-A: 0, 0.03, 0.15, 0.75, and 3.75 nmol, respectively. Samples were run on a 1.0% agarose gel in 0.1 M Tris-acetate (pH 8.3) containing 5 mM EDTA at 50 V for 2 h. A photograph of an ethidium bromide-stained gel is shown.

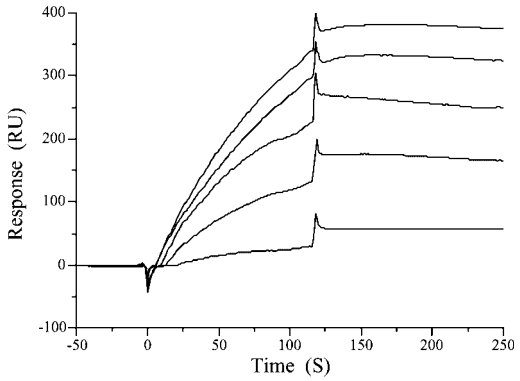

Fig. 6. **BIAcore analysis of binding of (*R*)-HA-A to the immobilized N-terminal 8 kDa domain of pol b.** Binding to (*R*)-HA-A was detected by means of the surface plasmon resonance signal (BIAcore, see Experimental Procedures) and is indicated in response units. Five different concentrations of (*R*)-HA-A (curve 1, 15 μM; curve 2, 12 μM; curve 3, 9 μM; curve 4, 6 μM; and curve 5, 3 μM) were injected over the 8 kDa domain of pol β for 120 s at 20 μl/min, followed by dissociation for 130 s at 20 μl/min. The background resulting from injection of running buffer alone was subtracted from the data before plotting.

ied the interaction between HA-A and the 8 kDa domain of pol β.

Inhibition of the ssDNA Binding Activity of the N-Terminal 8 kDa Domain Fragment of DNA Polymerase β by HA-A—Pol β used in this study has been extensively studied, including on its amino acid sequence, and its secondary and tertiary structures (*40–45*). The enzyme can be divided into two domain fragments of controlled proteolysis: an 8 kDa N-terminal domain fragment and a 31 kDa C-terminal domain fragment (*38*, *39*).

We investigated the interaction among the 8 kDa domain fragment of pol β and (*R*)-HA-A in more detail. The DNA-binding activity of the intact pol β (39 kDa) and the 8 kDa domain fragment was determined by means of a gel mobility shift assay. Figure 5 shows the gel mobility shift assaying of the binding M13 ssDNA to the 39 kDa pol β or the 8 kDa domain binding complex. In the binding assay, M13 ssDNA at 2.2 nmol (nucleotide) was added to 0.15 nmol of both the proteins (lanes 2–6 and lanes 8–11 in Fig. 5). Both the 39 kDa intact pol β and the 8 kDa domain bound to M13 ssDNA were shifted in the gel (lane 2 and 8 in Fig. 5). The molecular ratios of (*R*)-HA-A and the proteins are shown as the inhibitor to enzyme ratio (I/E) in Fig. 5. When the I/E ratio was 1 or more, HA-A interfered with complex formation between M13 ssDNA and pol β or the 8 kDa domain (lanes 4 to 6 and lanes 10–11 in Fig. 5). At a ratio of 0.2 (lanes 3 and 9 in Fig. 5), the binding almost disappeared, suggesting that one molecule of (*R*)-HA-A competes with M13 DNA and subsequently interferes with the binding of ssDNA to one molecule of the intact or 8 kDa domain of pol β. Kinetic analysis indicated that HA-A acted by competing with the DNA template-primer on pol β, and thus HA-A directly binds to the ssDNA binding site of the 8 kDa domain of pol β (Table 2). However, interference with the shift in gel mobility by ddTTP did not occur (data not shown), indicating that the action modes of ddTTP and HA-A on pol β differed. The ddTTP data were in agreement with those obtained in a previous study, indicating that the mode of inhibition of pol β by ddTTP was noncompetitive with the DNA template-primer (*37*).

Binding of HA-A to DNA Polymerase β or Its 8 kDa Domain Fragment—To confirm the kinetic parameters precisely, the parameters for binding of (*R*)-HA-A were determined using the 8 kDa domain fragment of pol β immobilized on a sensor chip in a BIAcore. Five different concentrations of (*R*)-HA-A (3, 6, 9, 12, and 15 μM) were used for the binding analysis, since the 8 kDa domain (1.87 nmol) was conjugated to the CM5 sensor chip, and

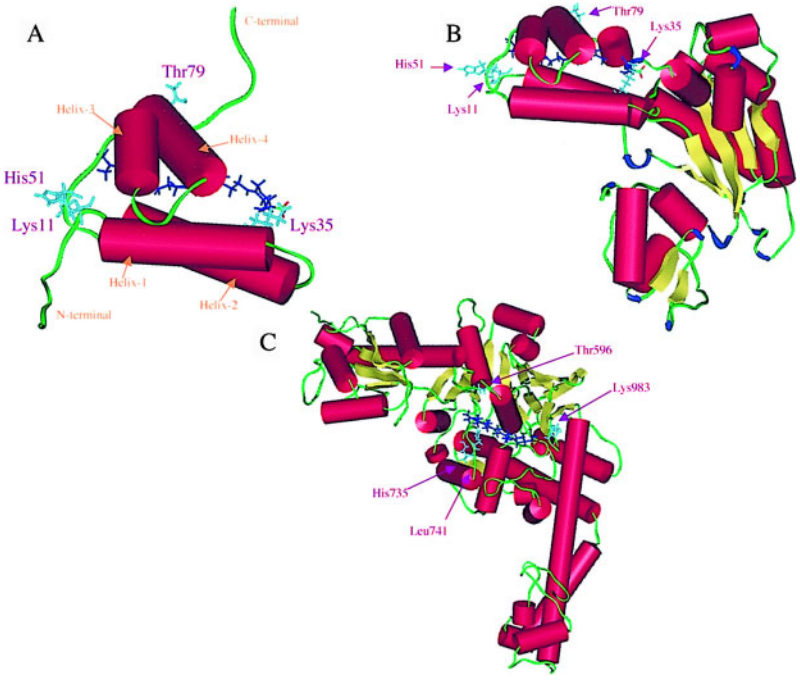

Fig. 7. **Docking simulation of the (*R*)-HA-A interaction interface on the N-terminal 8 kDa domain of pol β and topo II.** (A and B) Interactions between (*R*)-HA-A and the N-terminal 8 kDa domain (residues 1–88) (A) or intact pol β (residues 1–335) (B). Amino acid residues Leu11, Lys35, His51 and Thr79, which bind to linear long-chain fatty acids (C_{18} to C_{24}) such as nervonic acid, are depicted in blue (*4*). The Protein Data Bank codes of the 8 kDa domain and pol β are 1BPD and 1DK3, respectively. (C) Interactions between (*R*)-HA-A and topo II. Amino acid residues Thr596, His735, Leu741 and Lys983, which were determined by evolutionary tracing and the geometrical method, are depicted in blue, and correspond to Thr79, His51, Leu11, and Lys35 in the 8 kDa domain of pol β, respectively (*6*). The Protein Data Bank code of topo II is 1BJT. The Cα-backbone, α-helix and β-sheet of the X-ray crystal structure of these proteins are shown in green, red and yellow, respectively. The structure of (*R*)-HA-A is indicated in blue except that the carbons, oxygens and hydrogens of the carboxyl group are indicated in green, red and white, respectively. These figures were displayed using Insight II (Accelrys).

then (*R*)-HA-A was added to the conjugated protein. (*R*)-HA-A bound to the 8 kDa domain and dissociated from the 8 kDa domain (Fig. 6). The dissociation constant (K_d) of binding of (*R*)-HA-A to the 8 kDa domain was determined to be 228 nM from the data in Fig. 6. This suggested that the binding of HA-A to the 8 kDa domain is almost as tight as the binding of HA-A to the intact 39 kDa pol β (data not shown). Therefore, HA-A must interact with the 8 kDa domain directly

As expected, HA-A must inhibit the DNA-binding activity of the 8 kDa domain of pol β by competing with the DNA template-primer.

Modeling of the HA-A Interaction Interface on the N-Terminal 8 kDa Domain of Pol β—The 8 kDa domain (residues 1–87) of pol β is formed from four α-helices, packed as two antiparallel pairs (Fig. 7A). Two pairs of α-helices cross one another at 50° giving a V-like shape. The 8 kDa domain contains a motif termed the "Helix-hairpin-Helix" (HhH). We reported previously that the linear long-chain fatty acid nervonic acid is a potent inhibitor of mammalian pol β (*8*, *9*). On ^{1}H-^{15}N HMQC NMR analysis, the nervonic acid-binding amino acid residues of the 8 kDa domain were found to be Leu11, Lys35, His51, and Thr79 (*4*). (*R*)-HA-A consists of a carboxyl group and a C_{24}-linear methyl chain, therefore, the three-dimensional structure of (*R*)-HA-A is very similar to that of nervonic acid. The results of computer simulation of the mode of binding between (*R*)-HA-A and the N-terminal 8 kDa domain and intact pol β (39 kDa) are shown in Fig. 7, A and B, respectively. We created a definitive binding set for the Lys35 residue in the ssDNA binding pocket between helix-1 and helix-2 that moved as a 2.5 Å shell around the manually docked ligand during the energy minimization. The number of final docking positions was set to 5, although finally only one promising position was identified. (*R*)-HA-A could be mapped to one face of the 8 kDa domain by docking simulation analysis. In pol β, Lys35 in the Ω-type loop is a hydrophilic amino acid. The carboxyl groups of (*R*)-HA-A may, therefore, show a preference for binding to hydrophilic residue Lys35, and on the other side, the methyl chain may be absorbed to the hydrophobic amino acids including Leu11. On docking simulation, the binding energy between NH_3^+ of Lys35 and the carboxyl group in (*R*)-HA-A was found to be –57.6 kcal/mol on hydrogen bonding, and the binding force consisted of the coulomb force (–60.6 kcal/mol) and van der Waals forces (2.97 kcal/mol) (data not shown). The energy of binding between the methyl chain of (*R*)-HA-A and hydrophobic amino acids (*i.e.*, phenyl moiety of Leu11 and Thr79) was –1.1 kcal/mol. On the 8 kDa domain, (*R*)-HA-A was smoothly intercalated into the pocket between helix-1, 2, and helix-3, 4, and the residues around the Lys35 site appeared to be important for (*R*)-HA-A binding.

Only X-ray crystal structure analyses of topo II from yeast have been reported so far (*46*, *47*). Using this information, we performed similar docking model simulation for the enzyme and nervonic acid that could be inhibited the topo II activity (*6*). Computer modeling analysis, evolutionary tracing and the geometrical method were performed with Insight II/Binding Site Analysis (Accelrys), and revealed that the nervonic acid interaction interface

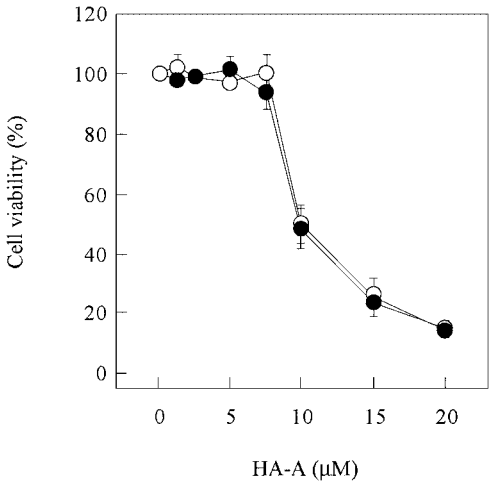

Fig. 8. **Effect of HA-A on the proliferation of NUGC-3 cells.** Dose-responsive curves of growth inhibition of NUGC-3 cells incubated with (*R*)-HA-A (solid circles) and (±)-HA-A (open circles). Cell proliferation was determined by MTT assaying. Data are shown as means ± SEM for four independent experiments.

in both pol β and topo II has a group of four amino acids in common forming a pocket, which binds to the nervonic acid molecule. The set of four amino acid residues located at analogous positions in the three-dimensional structure of topo II consisted of Thr596, His735, Leu741, and Lys983, corresponding to Thr79, His51, Leu11 and Lys35 in the 8 kDa domain of pol β, respectively. The results of computer simulation of the mode of superimposition of yeast topo II and (*R*)-HA-A are shown in Fig. 7C. Yeast topo II as well as the 8 kDa domain of pol β had an agent-inserted pocket, which is specific to each agent. Lys35 in the pocket of the 8 kDa domain of pol β corresponds to Lys983 in that of yeast topo II. The carboxyl groups of (*R*)-HA-A may show a preference for binding to hydrophilic residue Lys983, and on the other side, the methyl chain may be absorbed to hydrophobic amino acids (*i.e.*, Thr596 and Leu741). Since (*R*)-HA-A is effective only on deuterosomic enzymes, docking simulation using yeast topo II may be meaning less. However, the structure of the pocket must be similar but slightly, to an extent that (*R*)-HA-A does not quite fit, different from that in the deuterosomic topo II. (*R*)-HA-A might be able to be superimposed on the slightly-distorted pocket. Therefore, based on the simulation data for the (*R*)-HA-A inserted pocket of the 8 kDa domain of pol β, we simulated a docking model of the pocket on the surface of yeast topo II. Like the 8 kDa domain of pol β, (*R*)-HA-A might also inhibit ssDNA binding activity by competing with the DNA template by binding to residue Lys983 of topo II.

Human Cancer Cell Growth Inhibitory Properties of HA-A—From a different viewpoint, we also examined the cellular effect of HA-A. HA-A is a potent vertebrate DNA polymerase and DNA topoisomerase inhibitor. Therefore, HA-A could be a cytotoxic agent. We confirmed that (*R*)-HA-A and (±)-HA-A could be anti-cancer agents.

As shown in Fig. 8, both (*R*)-HA-A and (±)-HA-A showed a potent growth inhibitory effect against the human gastric cancer cell line NUGC-3. The concentra-

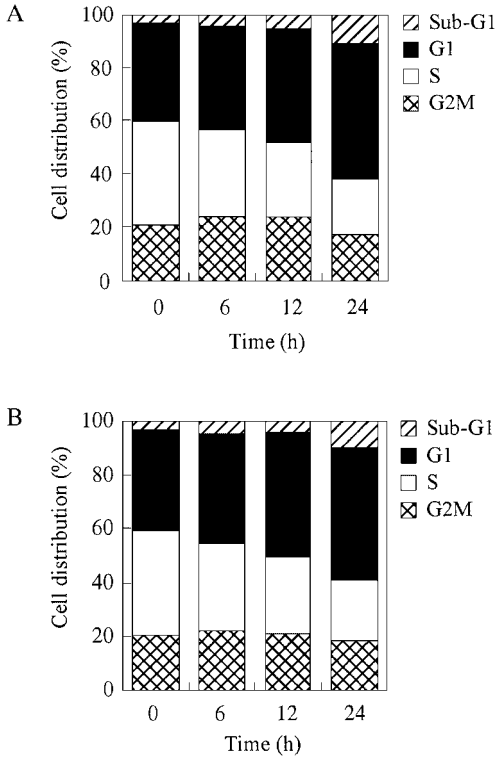

Fig. 9. **Effect of cell cycle distribution by HA-A.** NUGC-3 cells were incubated with 10 μM (*R*)-HA-A (A) and (±)-HA-A (B) for the indicated times. The cell cycle distribution was calculated as the percentage of cells that contained Sub-G1, G1, S, and G2/M DNA, as determined with a flow cytometer and DAPI staining.

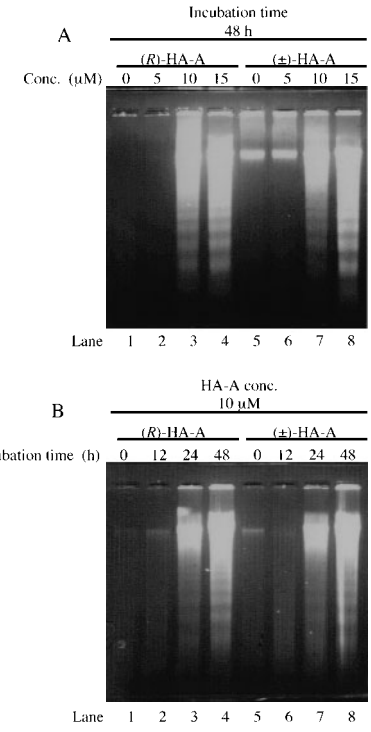

Fig. 10. **Agarose gel electrophoresis of DNA fragments.** (A) NUGC-3 cells were incubated with the indicated concentrations of (*R*)-HA-A and (±)-HA-A for 48 h. Lanes 1–4, (*R*)-HA-A at concentrations of zero, 5, 10, and 15 μM, respectively. Lanes 5–8, (±)-HA-A at concentrations of zero, 5, 10, and 15 μM, respectively. (B) The cells were incubated with 10 μM (*R*)-HA-A and (±)-HA-A. Lanes 1–4, (*R*)-HA-A at concentrations of zero, 12, 24, and 48 h, respectively. Lanes 5–8, (±)-HA-A at concentrations of zero, 12, 24, and 48 h, respectively. DNA fragments were analyzed by 1.5% agarose gel electrophoresis and then stained with ethidium bromide.

tions of (*R*)-HA-A and (±)-HA-A required for LD_{50} were 9.5 μM and 10.0 μM, respectively. The IC_{50} values of HA-A were 5.9 to 17.6 μM for mammalian DNA polymerases, and the IC_{50} values of HA-A were 15 to 25 μM for human DNA topoisomerases (Table 1), therefore, the LD_{50} value of (*R*)-HA-A was almost the same as the IC_{50} value *in vitro* for DNA polymerases rather than that for DNA topoisomerases, suggesting that the cell growth inhibition resulted in the inhibition of the DNA polymerase activities.

To examine this suggestion in more detail, we investigated the effects on the cell cycle of NUGC-3 cells by flow cytometry. Since the inhibition curves *in vitro* and *in vivo* with (*R*)-HA-A showed parallel dose-dependent reductions (Figs. 3A, 4 and 8), the cell growth inhibition seems to occur with inhibition of either DNA polymerases or DNA topoisomerases. The cell cycle distribution was analyzed with the MULTICYCLE software program (version 3.11; Phoenix Flow Systems, San Diego, CA). Cell debris and fixation artifacts were gated out. The ratio of the four phases (*i.e.*, sub-G1, G1, S and G2/M) in the cell cycle is shown in Fig. 9. As shown in Fig. 9A, the cells were arrested in both the G1 and G2/M phases (G1 phase cells were increased by 5.8%, S phase cells were decreased by 10.5% and G2/M phase cells were increased by 3.8%) by 10 μM (*R*)-HA-A on 12 h incubation, and then the peak at the G2/M phase had decreased by 3.7% on 24 h incubation. The arrest in both the G1 and G2 phases in the cell cycle was suggested to be the cause of inhibition of the activities of both DNA polymerases and DNA topoisomerases by (*R*)-HA-A, respectively. The cells arrested in the G2/M phase might be dead due to (*R*)-HA-A, therefore, the sub-G1 peak was increased at 24 h. The effects on both cancer cell growth and cell cycle of (±)-HA-A showed the same tendency as in the case of (*R*)-HA-A (Fig. 9B).

To determine whether or not the decrease in cell number caused by HA-A was due to apoptosis, DNA fragmentation in NUGC-3 cells was analyzed by agarose gel electrophoresis. DNA ladder formation was observed in NUGC-3 cells treated with more than 10 μM (*R*)-HA-A or (±)-HA-A for 48 h (Fig. 10A). Such ladders were not evident for the initial 12 h but were apparent at 24 h and thereafter (Fig. 10B). The same results were 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). HA-A is suggested to be a strong apoptosis-inducer. The effects of (*R*)-HA-A and (±)-HA-A must involve the combination of growth arrest and cell death.

DISCUSSION

The purpose of this study was to elucidate the molecular action mode of hippospongic acid A (HA-A), which was found to selectively inhibit the gastrulation of starfish

embryos from a marine sponge, *Hippospongia* sp., in 1996 (*1*). Gastrulation is a fundamental process that occurs during the embryonic development of multicellular animals. An inhibitor selective as to this event is potentially useful for studying one of the most important embryological events, gastrulation, biochemically. Therefore, we attempted to clarify the molecular action mode of HA-A, and subsequently to understand the molecular basis of gastrulation more precisely. Unfortunately, however, there were no clues to help us investigate the molecular target of HA-A when we started the study. We started by using a new method we developed (*6*, *7*). As described under Introduction briefly, if the sizes and three-dimensional structures of two chemical compounds are very similar, even if they differ chemically from each other, their action modes are often the same (*4*–*7*). On computer modeling analysis of many compounds, we found, and show in Fig. 1, that the size and three-dimensional structure of HA-A were quite similar to those of elenic acid produced by an Indonesian sponge, *Plakinastrella* sp (Fig. 2). Biochemical studies of elenic acid have progressed well in our laboratory. Elenic acid was found to be an agent that inhibits the activity of topo II on the screening of anti-neoplastic medicines from the sponge, and the biological role of elenic acid in the sponge remains unknown (*48*). Elenic acid is a potent inhibitor of both DNA polymerases and DNA topoisomerases from only vertebrates (*16*). Therefore, we started our studies on HA-A to determine whether or not HA-A could be the inhibitor of polymerases and topoisomerases.

As expected, HA-A could inhibit the activities of DNA polymerases and DNA topoisomerases from only deuterostomic animals including vertebrates. Interestingly, although the selectivity of HA-A as to polymerases and topoisomerases is very high, all of the DNA polymerases examined such as α, β, δ, ε, ξ, κ, and λ, were uniformly inhibited in the IC_{50} range of 5.9–17.6 μM HA-A, and also both human topo I and II were influenced with 15–25 μM HA-A (IC_{50}). HA-A was not selective as to a subclass species among the polymerases and topoisomerases. The mode of action of HA-A , therefore, are very similar to or almost the same as those of elenic acid. Since vertebrates and starfish phylogenetically belong to the deuterostomic branch of the animal kingdom, and since HA-A is a kind of toxin for starfish development, HA-A might be an inhibitor of these enzymes from deuterostomes.

Generally, replicative DNA polymerases and DNA topoisomerases are known to be present in proliferating cells (*49*, *50*). Starfish gastrulation, for which cells in the tissues efficiently proliferate, must start with a large amount of polymerases and topoisomerases, and the development is characterized by the exponential cell division accompanying DNA synthesis (*51*). The HA-A effect might just reflect the inhibition of replicative DNA polymerases and DNA topoisomerases, and subsequently of the cell proliferation in during gastrulation. If so, HA-A must more effectively inhibit the first or earlier cleavage, because the cells proliferate more efficiently. As reported previously, a novel compound that inhibits the first cleavage of fertilized sea urchin eggs, kohamaic acid A, was an inhibitor of replicative DNA polymerases such as α, δ, and ε from vertebrates, but did not influence the activities of repair DNA polymerases such as β [Mizushina and Sakaguchi, in preparation]. Kohamaic acid A was also found and extracted from a marine sponge, *Ircinia* sp. (*52*). Kohamaic acid A inhibited cell growth by blocking the primary step of DNA replication at the G1 phase, which occurs through an influence on replicative DNA polymerases. Kohamaic acid A had no remarkable influence on gastrulation [Mizushina and Sakaguchi, in preparation]. On the other hand, HA-A, and also elenic acid, could arrest the cells at both the G1 and G2/M phases, and inhibited all the deuterostomic polymerase activities examined to the same extent (*16*). The most effective stage of development with HA-A was gastrulation rather than the first cleavage. The action of HA-A during gastrulation should be considered from more precisely.

The fact that HA-A uniformly inhibited the activities of polymerases and topoisomerases only from deuterostomes is also of interest. Elenic acid may be a similar agent to HA-A in the sponge. Kohamaic acid A was also uniformly effective on replicative polymerases only from vertebrates. Therefore, we have considered the agents from two different viewpoints here. HA-A and elenic acid could be useful inhibitors for investigating the three-dimensional structures of the agent-binding sites on polymerases and topoisomerases. In addition, we could also take into consideration the role of each of the three agents including kohamaic acid A in development.

At first, there was useful information for studying the three-dimensional structure of the agent-binding site. We reported previously that linear long-chain fatty acids (C_{18} to C_{24}) such as nervonic acid and bile acid could strongly inhibit the activities of both DNA polymerases and DNA topoisomerase (*4*–*7*). The inhibition occurred through binding between both the alkyl chain site and the carboxyl group of the agents, and the agent-inserted pocket in the enzymes. Each of HA-A, elenic acid and kohamaic acid A also has an alkyl site and a carboxyl group. They inhibited the ssDNA binding activity by competing with the DNA template-primer by intercalating into a pocket containing a Lys-residue in the 8 kDa domain of pol β and/or topo II. Other polymerases and topoisomerases likely have a similar pocket on the surface, which is slightly different from those in the polymerases from protostomes. Kohamaic acid A might recognize the three-dimensional difference between pockets in the replicative and repair polymerases.

Aside from the mode of inhibition of starfish gastrulation, one of the original purposes of this study was to screen useful agents as tools and molecular probes for distinguishing DNA polymerases, and for clarifying their biological and *in vivo* functions. Nevertheless, HA-A was unexpectedly a natural compound that uniformly inhibited the activities of pol α, β, δ, ε, ξ, κ, and λ, and of topo I and II to the same extent. All of HA-A, elenic acid and kohamaic acid A are natural compounds extracted from marine sponges, and their *in vivo* roles, although they are effective only on deuterostomic enzymes, remain unknown. To explain these results satisfactorily, we would like to propose the hypothesis that HA-A, and perhaps also elenic acid and kohamaic acid A, is not merely a toxic inhibitor but a natural suppressor that slows down or halts cell proliferation at unique developmental stages by uniformly inhibiting the key enzymes. There are no

enzymatic similarities between the polymerases and topoisomerases, including their modes of action, amino acid sequences and three-dimensional structures, that are markedly different from each other, but they are critical for many cellular processes such as DNA replication, repair and recombination, and may act in harmony with each other. A subclass of polymerases must also act together in harmony. If a polymerase species was only inhibited at a developmental stage, the physiological balance of polymerases and topoisomerases involved in DNA synthesis at that stage would be unstable, and the cells might be more liable to be killed. To control the proliferation of the cell population in a tissue uniformly, a suppressor must be secreted out of the master cells, and penetrate into the neighboring cells. Natural compounds are better than signal-transduction proteins for controling the cell population.

This work was partly supported by a Grant-in-Aid for Kobe Gakuin University Joint Research (B) (Y.M., C.M. and H.Y.), and by a Sasakawa Scientific Research Grant (to X.X.) from the Japan Science Society. Y.M. acknowledges the Grant-in-Aid from the Tokyo Biochemical Research Foundation and Grant-in-Aid 14780466 for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan. We are grateful for the donations of calf DNA polymerase α, rat DNA polymerase β, human DNA polymerase ε, human DNA polymerase η, and human DNA polymerase κ by Dr. S. Yoshida and Dr. M. Takemura of Nagoya University School of Medicine (Nagoya), Dr. A. Matsukage of Japan Women's University (Tokyo), Dr. S. Linn and Dr. H. Asahara of University of California, Berkeley (California, USA), Dr. F. Hanaoka and Dr. C. Masutani of Osaka University (Osaka), and Dr. H. Ohmori and Mr. E. Ohashi of Kyoto University (Kyoto), respectively.

REFERENCES

1. Ohta, S., Uno, M., Tokumasu, M., Hiraga, Y., and Ikegami, S. (1996) Hippospongic acid A: An unusual triterpenoic acid from a marine sponge, Hippospongia sp., which inhibits gastrulation of starfish embryos. *Tetrahedron Lett.* **37**, 7765–7766
2. Ichihashi, M., Takikawa, H., and Mori, K. (2001) Synthesis of (*R*)-(+)-hippospongic acid A, a triterpene isolated from the marine sponge, *Hippospongia* sp. *Biosci. Biotechnol. Biochem.* **65**, 2569–2572
3. Takikawa, H., Koizumi, J., Kato, Y., and Mori, K. (1999) Synthesis of (±)-hippospongic acid A, a triterpene isolated from the marine sponge *Hippospongia* sp. *J. Chem. Soc. Perkin Trans* **1**, 2271–2275
4. Mizushina, Y., Ohkubo, T., Date, T., Yamaguchi, T., Saneyoshi, M., Sugawara, F., and Sakaguchi, K. (1999) Mode analysis of a fatty acid molecule binding to the N-terminal 8-kDa domain of DNA polymerase β: A 1:1 complex, and binding surface. *J. Biol. Chem.* **274**, 25599–25607
5. Mizushina, Y., Ohkubo, T., Sugawara, F., and Sakaguchi, K. (2000) Structure of lithocholic acid binding to the N-terminal 8-kDa domain of DNA polymerase β. *Biochemistry* **39**, 12606–12013
6. Mizushina, Y., Sugawara, F., Iida, A., and Sakaguchi, K. (2000) Structural homology between DNA binding sites of DNA polymerase β and DNA topoisomerase II. *J. Mol. Biol.* **304**, 385–395
7. Mizushina, Y., Kasai, N., Sugawara, F., Iida, A., Yoshida, H., and Sakaguchi, K. (2001) Three-dimensional structural model analysis of the binding site of lithocholic acid, an inhibitor of DNA polymerase β and DNA topoisomerase II. *J. Biochem. (Tokyo)* **130**, 657–664
8. Mizushina, Y., Tanaka, N., Yagi, H., Kurosawa, T., Onoue, M., Seto, H., Horie, T., Aoyagi, N., Yamaoka, M., Matsukage, A., Yoshida, S., and Sakaguchi, K. (1996) Fatty acids selectively inhibit eukaryotic DNA polymerase activities in vitro. *Biochim. Biophys Acta* **1308**, 256–262
9. Mizushina, Y., Yoshida, S., Matsukage, A., and Sakaguchi, K. (1997) The inhibitory action of fatty acids on DNA polymerase β. *Biochim. Biophys Acta* **1336**, 509–521
10. Mizushina, Y., Tanaka, N., Kitamura, A., Tamai, K., Ikeda, M., Takemura, M., Sugawara, F., Arai, T., Matsukage, A., Yoshida S., and Sakaguchi, K. (1998) The inhibitory effect of novel triterpenoid compounds, fomitellic acids, on DNA polymerase β. *Biochem. J.* **330**, 1325–1332
11. Mizushina, Y., Hanashima, L., Yamaguchi, T., Takemura, M., Sugawara, F., Saneyoshi, M., Matsukage, A., Yoshida, S., and Sakaguchi, K. (1998) A mushroom fruiting body-inducing substance inhibits activities of replicative DNA polymerases. *Biochem. Biophys. Res. Commun.* **249**, 17–22
12. Mizushina, Y., Takahashi, N., Ogawa, A., Tsurugaya, K., Koshino, H., Takemura, M., Yoshida, S., Matsukage, A., Sugawara, F., and Sakaguchi, K. (1999) The cyanogenic glucoside, prunasin (D-mandelonitrile-β-D-glucoside), is a novel inhibitor of DNA polymerase β. *J. Biochem.* **126**, 430–436
13. Mizushina, Y., Kobayashi, S., Kuramochi, K., Nagata, S., Sugawara, F., and Sakaguchi, K. (2000) Epolactaene, a novel neuritogenic compound in human neuroblastoma cells, selectively inhibits the activities of mammalian DNA polymerases, and human DNA topoisomerase II. *Biochem. Biophys. Res. Commun.* **273**, 784–788
14. Mizushina, Y., Kamisuki, S., Mizuno, T., Takemura, M., Asahara, H., Linn, S., Yamaguchi, T., Matsukage, A., Hanaoka, F., Yoshida, S., Saneyoshi, M., Sugawara, F., and Sakaguchi, K. (2000) Dehydroaltenusin, a mammalian DNA polymerase α inhibitor. *J. Biol. Chem.* **275**, 33957–33961
15. Murakami, C., Takemura, M., Sugiyama, Y., Kamisuki, S., Asahara, H., Kawasaki, M., Ishidoh, T., Linn, S., Yoshida, S., Sugawara, F., Yoshida, H., Sakaguchi, K., and Mizushina, Y. (2002) Vitamin A-related compounds, all-trans retinal and retinoic acids, selectively inhibit activities of mammalian replicative DNA polymerases. *Biochim. Biophys Acta* **1574**, 85–92
16. Mizushina, Y., Murakami, C., Ohta, K., Takikawa, H., Mori, K., Yoshida, H., Sugawara, F., and Sakaguchi, K. (2002) Selective inhibition of the activities of both eukaryotic DNA polymerases and DNA topoisomerases by elenic acid. *Biochem. Pharmacol.* **63**, 399–407
17. Mizushina, Y., Kamisuki, S., Kasai, N., Shimazaki, N., Takemura, M., Asahara, H., Linn, S., Yoshida, S., Matsukage, A., Koiwai, O., Sugawara, F., Yoshida, H., and Sakaguchi, K. (2002) A plant phytotoxin, solanapyrone A, is an inhibitor of DNA polymerase β and λ. *J. Biol. Chem.* **277**, 630–638
18. Akiyama, S., Amo, H., Watanabe, T., Matsuyama, M., Sakamoto, J., Imaizumi, M., Ichihashi, H., Kondo, T., and Takagi, H. (1988) Characteristics of three human gastric cancer cell lines, NU-GC-2, NU-GC-3 and NU-GC-4. *Jpn. J. Surg.* **18**, 438–446
19. Tamai, K., Kojima, K., Hanaichi, T., Masaki, S., Suzuki, M., Umekawa, H., and Yoshida, S. (1988) Structural study of immunoaffinity-purified DNA polymerase alpha-DNA primase complex from calf thymus. *Biochim. Biophys Acta* **950**, 263–273
20. Date, T., Yamaguchi, M., Hirose, F., Nishimoto, Y., Tanihara, K., and Matsukage, A. (1988) Expression of active rat DNA polymerase β in *Escherichia coli*. *Biochemistry* **27**, 2983–2990
21. Shoji-Kawaguchi, M., Izuta, S., Tamiya-Koizumi, K., Suzuki, M., and Yoshida, S. (1995) Selective inhibition of DNA polymerase ε by phosphatidylinositol. *J. Biochem. (Tokyo)* **117**, 1095–1099
22. Chui, G.S. and Linn, S. (1995) Purification of mammalian polymerases: DNA polymerase ε. *Methods Enzymol.* **262**, 93–98

23. Masutani, C., Kusumoto, R., Iwai, S., and Hanaoka, F. (2000) Mechanisms of accurate translesion synthesis by human DNA polymerase η. *EMBO J.* **19**, 3100–3109
24. Ohashi, E., Ogi, T., Kusumoto, R., Iwai, S., Masutani, C., Hanaoka, F., and Ohmori, H. (2000) Error-prone bypass of certain DNA lesions by the human DNA polymerase κ. *Genes Dev.* **14**, 1589–1594
25. Tomikawa, A., Seno, M., Sato-Kiyotaki, K., Ohtsuki, C., Hirai, T., Yamaguchi, T., Kawaguchi, T., Yoshida, S., and Saneyoshi, M. (1998) Synthetic nucleosides and nucleotides. *Nucl. Nucl.* **17**, 487–501
26. Aoyagi, N., Matsuoka, S., Furunobu, A., Matsukage, A., and Sakaguchi, K. (1994) Drosophila DNA polymerase δ: Purification and characterization. *J. Biol. Chem.* **269**, 6045–6050
27. Sakaguchi, K., Hotta, Y., and Stern, H. (1980) Chromatin-associated DNA polymerase activity in meiotic cells of lily and mouse. *Cell Struct. Funct.* **5**, 323–334
28. Spitzner, J.R., Chung, I.K., and Muller, M.T. (1990) Eukaryotic topoisomerase II preferentially cleaves alternating purine-pyrimidine repeats. *Nucleic Acids Res.* **18**, 1–11
29. Nakayama, C. and Saneyoshi, M. (1985) Inhibitory effects of 9-β-D-xylofuranosyladenine 5′-triphosphate on DNA-dependent RNA polymerase I and II from cherry salmon (*Oncorhynchus masou*). *J. Biochem.* **97**, 1385–1389
30. Soltis, D.A. and Uhlenbeck, O.C. (1982) Isolation and characterization of two mutant forms of T4 polynucleotide kinase. *J. Biol. Chem.* **257**, 11332–11339
31. Lu, B.C. and Sakaguchi, K. (1991) An endo-exonuclease from meiotic tissues of the basidiomycete *Coprinus cinereus*. Its purification and characterization. *J. Biol. Chem.* **266**, 21060–21066
32. Casas-Finet, J.R., Kumar, A., Morris, G., Wilson, S.H., and Karpel, R.L. (1991) Spectroscopic studies of the structural domains of mammalian DNA β-polymerase. *J. Biol. Chem.* **266**, 19618–19625
33. Olson, M.W., Dallmann, H.G., and McHenry, C.S. (1995) The chi psi complex functions by increasing the affinity of tau and gamma for delta: delta' to a physiologically relevant range. *J. Biol. Chem.* **270**, 29570–29577
34. Kurinov, I.V., Myers, D.E., Irvin, J.D., and Uckun, F.M. (1999) X-ray crystallographic analysis of the structural basis for the interactions of pokeweed antiviral protein with its active site inhibitor and ribosomal RNA substrate analogs. *Protein Sci.* **8**, 1765–1772
35. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63
36. Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning. A Laboratory Manual, 2nd Ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
37. Izuta, S., Saneyoshi, M., Sakurai, T., Suzuki, M., Kojima, K., and Yoshida, S. (1991) The 5′-triphosphates of 3′-azido-3′-deoxythymidine and 2′, 3′-dideoxynucleosides inhibit DNA polymerase gamma by different mechanisms. *Biochem. Biophys. Res. Commun.* **179**, 776–783
38. Kumar, A., Widen, S.G., Williams, K.R., Kedar, P., Karpel, R.L., and Wilson, S.H. (1990) Studies of the domain structure of mammalian DNA polymerase β: Identification of a discrete template binding domain. *J. Biol. Chem.* **265**, 2124–2131
39. Kumar, A., Abbotts, J., Karawya, E.M., and Wilson, S.H. (1990) Identification and properties of the catalytic domain of mammalian DNA polymerase β. *Biochemistry* **29**, 7156–7159
40. Davies II, J.F., Almassy, R.J., Hostomska, Z., Ferre, R.A., and Hostomsky, Z. (1994) 2.3 _ crystal structure of the catalytic domain of DNA polymerase β. *Cell* **76**, 1123–1133
41. Pelletier, H., Sawaya, M.R., Kumar, A., Wilson, S.H., and Kraut, J. (1994) Structures of ternary complexes of rat DNA polymerase β, a DNA template-primer, and ddCTP. *Science* **264**, 1891–1903
42. Sawaya, M.R., Pelletier, H., Kumar, A., Wilson, S.H., and Kraut, J. (1994) Crystal structure of rat DNA polymerase β: evidence for a common polymerase mechanism. *Science* **264**, 1930–1935
43. Pelletier, H., Sawaya, M.R., Wolfle, W., Wilson, S.H., and Kraut, J. (1996) Crystal structures of human DNA polymerase β complexed with DNA: implications for catalytic mechanism, processivity, and fidelity. *Biochemistry* **35**, 12742–12761
44. Pelletier, H., Sawaya, M.R., Wolfle, W., Wilson, S.H., and Kraut, J. (1996) A structural basis for metal ion mutagenicity and nucleotide selectivity in human DNA polymerase β. *Biochemistry* **35**, 12762–12777
45. Pelletier, H., and Sawaya, M.R. (1996) Characterization of the metal ion binding helix-hairpin-helix motifs in human DNA polymerase β by X-ray structural analysis. *Biochemistry* **35**, 12778–12787
46. Berger, J.M., Gamblin, S.J., Harrison, S.C., and Wang, J.C. (1996) Structure and mechanism of DNA topoisomerase II. *Nature* **379**, 225–232
47. Fass, D., Bogden, C.E., and Berger, J.M. (1999) Quaternary changes in topoisomerase II may direct orthogonal movement of two DNA strands. *Nat. Struct. Biol.* **6**, 322–326
48. Juagdan, E.G., Kalidindi, R.S., and Scheuer, P.J. (1995) Elenic acid, an inhibitor of topoisomerase II, from a sponge, *Plakinastrella* sp. *Tetrahedron Lett.* **36**, 2905–2908
49. Wang, J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635–691
50. Sarngadharan, M.G., Robert-Guroff, M., and Gallo, R.C. (1978) DNA polymerases of normal and neoplastic mammalian cells. *Biochim. Biophys Acta* **516**, 419–487
51. Fansler, B. and Loeb, L.A. (1969) Sea urchin nuclear DNA polymerase. II: Changing localization during early development. *Exp. Cell Res.* **57**, 305–310
52. Kokubo, S., Yogi, K., Uddin, M.J., Inuzuka, T., Suenaga, K., Ueda, K., and Uemura, D. (2001) Kohamaic acid A and B, novel cytotoxic sesterterpenic acids, from the marine sponge *Ircinia* sp. *Chem. Lett.* 176–177