Novel triterpenoids inhibit both DNA polymerase and DNA topoisomerase

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As described previously, we found that new triterpenoid compounds, designated fomitellic acids A and B, which selectively inhibit the activities of mammalian DNA polymerases α and β [Mizushina, Tanaka, Kitamura, Tamai, Ikeda, Takemura, Sugawara, Arai, Matsukage, Yoshida and Sakaguchi (1998) Biochem. J. **330**, 1325–1332; Tanaka, Kitamura, Mizushina, Sugawara and Sakaguchi (1998) J. Nat. Prod. **61**, 193–197] and that a known triterpenoid, ursolic acid, is an inhibitor of human DNA topoisomerases I and II (A. Iida, Y. Mizushina and K. Sakaguchi, unpublished work). Here we report that all of these triterpenoids are potent inhibitors of calf DNA polymerase α, rat DNA polymerase β and human DNA topoisomerases I and II, and show moderate inhibitory effects on plant DNA polymerase II and human immunodeficiency virus reverse transcriptase. However, these compounds did not influence the activities of prokaryotic DNA polymerases such as *Escherichia coli* DNA polymerase I or other DNA metabolic enzymes such as human telomerase, T7 RNA polymerase and bovine deoxyribonuclease I. These triterpenoids were not only mammalian DNA polymerase inhibitors but also inhibitors of DNA topoisomerases I and II even though the enzymic characteristics of DNA polymerases and DNA topoisomerases, including their modes of action, amino acid sequences and three-dimensional structures, differed markedly. These triterpenoids did not bind to DNA, suggesting that they act directly on these enzymes. Because the three-dimensional structures of fomitellic acids were shown by computer simulation to be very similar to that of ursolic acid, the DNA-binding sites of both enzymes, which compete for the inhibitors, might be very similar. Fomitellic acid A and ursolic acid prevented the growth of NUGC cancer cells, with LD_{50} values of 38 and 30 μM respectively.

Key words: DNA polymerases α and β, DNA topoisomerases I and II, enzyme inhibitor.

INTRODUCTION

Through our studies of triterpenoid enzyme inhibitors, we obtained results indicative of a three-dimensional structural relationship between DNA topoisomerases and DNA polymerases in mammals, although their amino acid sequences differ markedly. We report here the first in a series of studies.

The triterpenoids designated fomitellic acids (FAs) are novel natural triterpenoid compounds produced by the basidiomycete *Fomitella fraxinea* as inhibitors of mammalian DNA polymerases α and β [1,2]. Another triterpenoid, ursolic acid (UA), found in the higher plants *Tabebuia caraiba* and *Campsis radicans*, was an inhibitor of human DNA topoisomerases I and II (A. Iida, Y. Mizushina and K. Sakaguchi, unpublished work). These observations were made independently at almost the same time. In the tests for FAs, DNA topoisomerases I and II were not used; UA has not yet been tested for DNA polymerase inhibitory activity. DNA polymerase catalyses the addition of deoxyribonucleotides to the 3′-hydroxy terminus of primed double-stranded DNA molecules [3]; DNA topoisomerase catalyses the concerted breaking and rejoining of DNA strands and is involved in producing necessary topological and conformational changes in DNA [3,4]. They are therefore critical to many cellular processes such as DNA replication, repair and recombination, and might act in harmony with each other. The characteristics of these enzymes, including their modes of action, amino acid sequences and three-dimensional structures, differ markedly.

Surprisingly, we found in the present study that the DNA polymerase inhibitors, FAs, were potent inhibitors of DNA topoisomerases I and II, and the DNA topoisomerase inhibitor, UA, was also a DNA polymerase inhibitor. Neither the FAs nor UA inhibited the activities of the many other enzymes related to DNA replication, repair and recombination. A flavone compound from *Psoralea corylifolia* has been reported to inhibit both of these enzyme activities [5]. This compound is an intercalating agent and is thought to bind to the DNA molecule directly, subsequently inhibiting both enzyme activities indirectly. Intercalating agents are generally thought to inhibit these enzyme activities by modifying the three-dimensional structure of the DNA molecule. The triterpenoids used in this study inhibited the enzyme activities, but did not bind to the DNA. These observations suggest that there might be some structural similarities between both enzymes. However, the three-dimensional structures of both triterpenoids were shown by computer simulation to be very similar. The purpose of this study was to investigate the biochemical effects of FAs on DNA topoisomerases I and II and of UA on the DNA polymerases, and to obtain new information about the structure–function relationship between both enzymes. These enzymes have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Information on the structural characteristics of these inhibitors could therefore provide valuable insight into the design of new anti-cancer agents.

MATERIALS AND METHODS

Materials

Fomitellic acid A (FA-A) and fomitellic acid B (FA-B) were purified from the basidiomycete *F. fraxinea* as described previously [2]. UA was purified from the plants *T. caraiba* and *C. radicans* (A. Iida, Y. Mizushina and K. Sakaguchi, unpublished work). Nucleotides, and chemically synthesized template primers such as poly(dA), poly(rA) and oligo$(dT)_{12–18}$ were purchased

Abbreviations used: dsDNA, double-stranded DNA; FA, fomitellic acid; UA, ursolic acid.

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from Pharmacia (Uppsala, Sweden). [^{3}H]dTTP (43 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Supercoiled pUC19 and pBR322 plasmid DNA were obtained from Toyobo (Osaka, Japan). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan). DNA polymerase α was purified from calf thymus by immunoaffinity column chromatography as described previously [6]. Recombinant rat DNA polymerase β was purified from *Escherichia coli* JMpβ5 as described by Date et al. [7]. DNA polymerase I (plant α-like polymerase) and II (plant β-like polymerase) from cauliflower inflorescence was purified by the methods outlined by Sakaguchi et al. [8]. Purified human placenta DNA topoisomerases I (2 units/μl) and IIα (2 units/μl) were purchased from TopoGen (Columbus, OH, U.S.A.). HIV-1 reverse transcriptase and the Klenow fragment of DNA polymerase I were purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). T4 DNA polymerase, *Taq* DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan). Calf thymus terminal deoxynucleotidyltransferase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA, U.S.A.).

DNA polymerase assays

The activities of DNA polymerases were measured by the methods described previously [9–11]. As substrates for DNA polymerases we used poly(dA)/oligo(dT)$_{12-18}$ and dTTP as template primer DNA and nucleotide substrate respectively. As substrates for HIV-1 reverse transcriptase we used poly(rA)/oligo(dT)$_{12-18}$ and dTTP as template primer and nucleotide substrate respectively. As substrates for terminal deoxynucleotidyl transferase we used oligo(dT)$_{12-18}$ (3′-OH) and dTTP as template primer and nucleotide substrate respectively. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyses the incorporation of 1 nmol of deoxyribonucleotide triphosphates (i.e. dTTP) into the synthetic template primers [i.e. poly(dA)/oligo(dT)$_{12-18}$, A-to-T ratio 2:1] in 60 min at 37 °C under the normal reaction conditions for each enzyme [9,11].

DNA topoisomerase assays

Relaxation activities of DNA topoisomerases were determined by detecting the conversion of supercoiled plasmid DNA into its relaxed form [12,13]. The DNA topoisomerase II reaction was performed in 20 μl reaction mixtures containing 50 mM Tris/HCl buffer, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 200 ng of pBR322 plasmid DNA, 2 μl of inhibitor solution [10% (v/v) DMSO] and 1 unit of DNA topoisomerase II. The reaction mixtures were incubated at 37 °C for 30 min and terminated by adding 2 μl of loading buffer consisting of 5% (v/v) Sarkosyl, 0.0025% Bromophenol Blue and 25% (v/v) glycerol. The mixtures were subjected to 1% (w/v) agarose-gel electrophoresis in TAE (Tris/acetate/EDTA) running buffer. The agarose gels were stained with ethidium bromide; DNA was revealed with a UV transilluminator. Relaxation activity of DNA topoisomerase I was analysed in the same manner as that described above except that the reaction mixtures contained 10 mM Tris/HCl, pH 7.9, 200 ng of pUC19 plasmid DNA, 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% (v/v) glycerol and 1 unit 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 °C.

Other enzyme assays

The activities of T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in each of the standard assays as described by Nakayama and Saneyoshi [14], Soltis and Uhlenbeck [15] and Lu and Sakaguchi [16] respectively. Telomerase activity was determined by using the PCR-based telomeric repeat amplification protocol as described [17], with some modifications [18].

Thermal transition of DNA

Thermal transition profiles of double-stranded to single-stranded DNA with or without each of the FAs and UA 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% (v/v) DMSO. The solution temperature was equilibrated at 78 °C for 10 min, then increased by 1 °C at 2 min intervals for each measurement point. Any change in the absorbance of the compounds themselves at each temperature point was automatically subtracted from that of DNA plus the compounds in the spectrophotometer.

Investigation of cytotoxicity towards cultured cells

For investigation of the effects of FAs and UA *in vivo*, the fibroblast cell line NUGC-3, derived from a human patient with a stomach cancer, was used. The cells were routinely cultured with Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 250 μg/ml Fungizone and 300 μg/ml L-glutamine as standard medium. The cells were routinely cultured at 37 °C in standard medium in a humidified atmosphere of air/CO_2 (19:1). Cytotoxicity of FAs and UA was investigated as follows. High concentrations of the compounds were dissolved in DMSO and stocked. Approximately 2×10^3 cells per well were inoculated in 96-well microplates, then compound stock solution was diluted to various concentrations with standard medium and applied to each well. After incubation for 48 h, the survival rate was determined by assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide [19].

Cell cycle analysis

The cellular DNA content for cell cycle analysis was determined as follows: aliquots of 5×10^6 NUGC-3 cells were inoculated into 75 cm^2 plastic culture flasks, then incubated with medium containing FA-A or UA diluted with ethanol/n-decane solution (49:1, v/v) for 48 h. Cells were then collected by treatment with trypsin and washed with cold PBS three times by centrifugation. Cells were fixed with 10 ml of PBS containing 4% (w/v) paraformaldehyde and washed three times with PBS. The cells were then incubated with 0.25 mg/ml RNase A at 37 °C to improve specific staining. After incubation for 1 h, DNA was stained in the dark at 4 °C with 50 μg/ml propidium iodide for 40 min. Fluoresence intensity was measured by flow cytometry (Beckman Coulter Epics XL System II).

RESULTS AND DISCUSSION

As described in the Introduction section, UA is a known triterpenoid isolated from the higher plants *Tabebuia caraiba* and *Campsis radicans*, and has been found to be an inhibitor of human DNA topoisomerases I and II (A. Iida, Y. Mizushina and K. Sakaguchi, unpublished work). Fomitellic acids A to D are

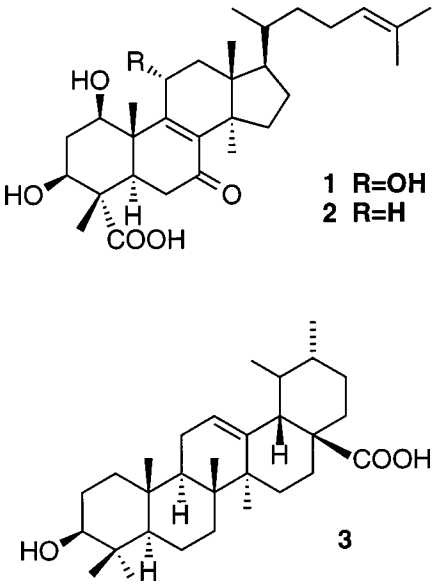

Figure 1 Structures of DNA polymerase and DNA topoisomerase inhibitors

1, FA-A; **2**, FA-B; **3**, UA.

novel natural triterpenoid compounds produced from a basidiomycete, *F. fraxinea*, that inhibit the activities of mammalian DNA polymerases α and β [1,2]. FA-A and FA-B were used in the present study because only these two agents could be isolated in sufficient quantities. The chemical structures of FA-A, FA-B and UA are shown in Figure 1. Previous studies ([1], and A. Iida, Y. Mizushina and K. Sakaguchi, unpublished work) indicated that the inhibitory effects of each of these compounds are highly selective for DNA polymerases or DNA topoisomerases, although the compounds were not tested for inhibitory effects on other enzymes.

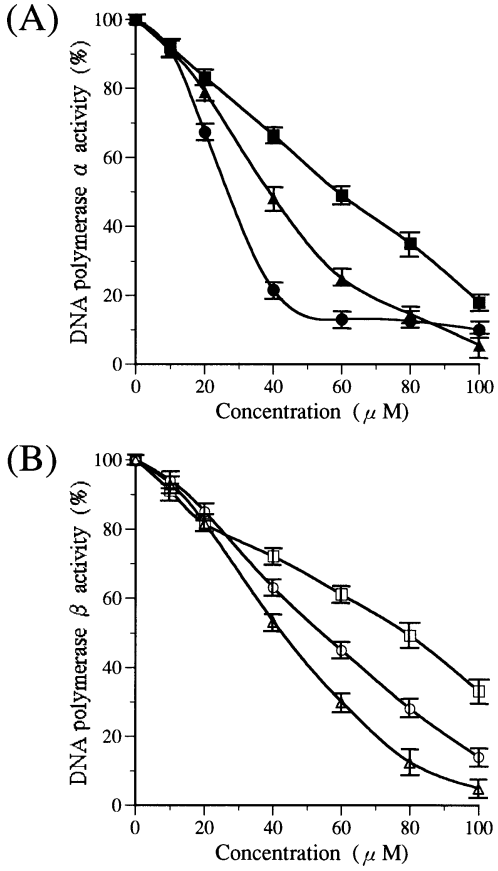

Figure 2 Inhibition of mammalian DNA polymerase activities by FAs and UA

Calf DNA polymerase α (**A**) and rat DNA polymerase β (**B**) (0.05 unit each) were preincubated with the indicated concentrations (0–100 μM) of FA-A (■, □), FA-B (●, ○) or UA (▲, △), and then assayed for these enzyme activities as described previously [9–11]. Enzyme activity in the absence of the compounds was taken as 100%. Results are means ± S.E.M. for three independent experiments.

Effects of UA and FAs on the activities of mammalian DNA polymerases α and β

Figure 2 shows the inhibition dose–response curves of FAs and UA on mammalian DNA polymerases α and β. The inhibition by each of these triterpenoids was dose-dependent: 50% inhibition for DNA polymerase α by FA-A, FA-B and UA was observed at doses of 58, 27 and 38 μM respectively, and more than 80% inhibition was achieved at 97, 42 and 68 μM respectively (Figure 2A). The IC_{50} values of DNA polymerase β with FA-A, FA-B and UA were approx. 79, 53 and 42 μM respectively (Figure 2B). The DNA topoisomerase inhibitor UA was thus shown also to be a potent inhibitor of the DNA polymerases, with an activity comparable to those of FA-A and FA-B. All the triterpenoids showed almost the same dose–response curves with DNA polymerases α and β. The inhibitory doses were almost the same as not only those of aphidicolin and dideoxyTTP, well-known inhibitors of DNA polymerases α and β respectively, but also those of other novel DNA polymerase inhibitors such as sulpholipids [20–23], flavonoids [24], phospholipids [25,26], steroids [27] and fatty acids [9–11,28]. These observations indicated that UA is also a potent inhibitor of mammalian DNA polymerases α and β.

Effects of FA-A and UA on human DNA topoisomerases I and II

The effects of FA-A and UA on the catalytic activities of human DNA topoisomerases I and II were examined by the relaxation assay described in the Materials and methods section. As shown in Figure 3(A), FA-A dose-dependently inhibited DNA topoisomerase I relaxation activity, with complete inhibition at 75 μM (lane 5). In contrast, UA required concentrations higher than 200 μM (Figure 3A, lane 7) for complete inhibition. The IC_{50} values of DNA topoisomerase I with FA-A and UA were 60 and 250 μM respectively (results not shown). FA-A was a much stronger inhibitor of topoisomerase I than UA. DNA topoisomerase II activity was completely inhibited by FA-A and UA at concentrations of 25 μM (Figure 3B, lane 6) and 200 μM (lane 9) respectively. The inhibitory effect of FA-A with topoisomerase II was 8-fold more potent than that of UA; both agents were more effective against DNA topoisomerase II than against DNA topoisomerase I. Because UA inhibited DNA polymerases α and β activities at concentrations lower than 100 μM, UA should be referred to as a DNA polymerase inhibitor rather than a DNA topoisomerase inhibitor.

The results described above indicate that these triterpenoids

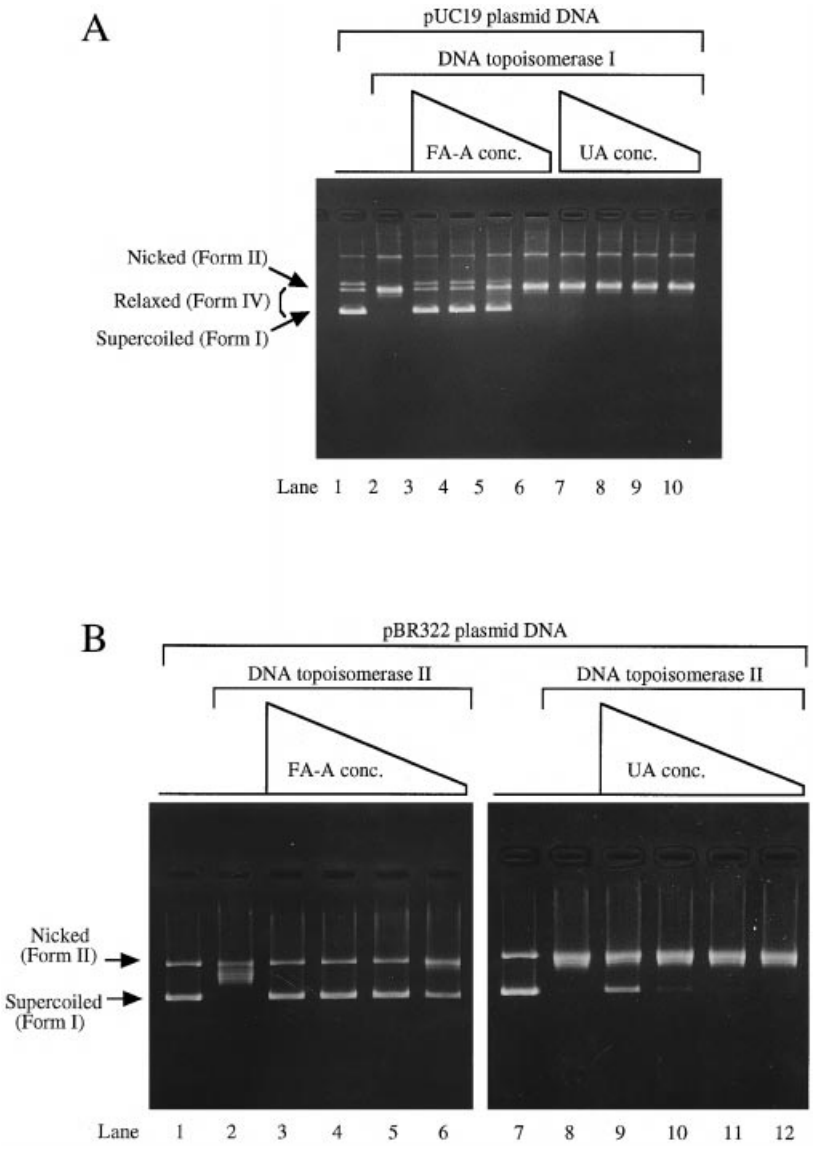

Figure 3 Inhibitory effects of FA-A and UA on human DNA topoisomerase I and II

Photographs of gels stained with ethidium bromide are shown. (**A**) pUC19 DNA was mixed with DNA topoisomerase I and the inhibitor FA-A or UA. Lanes 3–6, FA-A at concentrations of 200, 100, 75 and 50 μM respectively; lanes 7–10, UA at concentrations of 200, 100, 75 and 50 μM respectively. Lanes 2–10, 1 unit of DNA topoisomerase I; lane 1, no enzyme. Each lane contained 200 ng of pUC19 plasmid DNA. (**B**) pBR322 DNA was mixed with DNA topoisomerase II and the inhibitor FA-A or UA. Lanes 1–6, FA-A at concentrations of 0, 0, 200, 100, 50 and 25 μM respectively; lanes 7–12, UA at concentrations of 0, 0, 200, 100, 50 and 25 μM respectively. Lanes 2–6 and 8–12, 1 unit of DNA topoisomerase II; lanes 1 and 7, no enzyme. Each lane contained 200 ng of pBR322 plasmid DNA. These experiments were reproduced three times.

are not necessarily DNA polymerase-specific or DNA topoisomerase-specific inhibitors as reported previously.

Effects of FAs and UA on various DNA polymerases and other DNA metabolic enzymes

As shown in Table 1, FA-A, FA-B and UA at 100 μM significantly inhibited the activities of both calf DNA polymerase α and rat DNA polymerase β. FA-A and FA-B also strongly inhibited the activities of human DNA topoisomerases I and II, although UA showed only a slight inhibitory effect (Table 1). Activities of higher-plant (cauliflower) DNA polymerase II (β-like polymerase) and HIV-1 reverse transcriptase were moderately inhibited by all of the triterpenoids at this concentration (Table 1). The triterpenoids at this concentration showed little effect on the activities of DNA polymerase I (α-like polymerase) from cauliflower, calf thymus terminal deoxynucleotidyltransferase, human telomerase, prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase and *Taq* DNA polymerase, and DNA metabolic enzymes such as T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I (Table 1). The inhibition patterns were almost the same with each of the three triterpenoids except towards DNA topoisomerases I and II (Table 1). All the triterpenoids used here should therefore be referred to as mammalian DNA polymerase-specific and/or human DNA topoisomerase-specific inhibitors.

Flavones are weak inhibitors of both DNA polymerase and

Table 1 Effects of FAs and UA on the activities of various DNA polymerases and other DNA metabolic enzymes

FA-A, FA-B and UA (each at 100 μM) were incubated with each enzyme (0.05 unit). The enzymic activity was measured as described previously [9–17]. Enzyme activity in the absence of the compounds was taken as zero. Each result is the mean for three separate experiments with an S.D. of less than 5%.

Enzyme	Inhibitory activity (%)		
	FA-A	FA-B	UA
Calf DNA polymerase α	82	90	92
Rat DNA polymerase β	67	95	86
Plant DNA polymerase I (α-like)	0	0	0
Plant DNA polymerase II (β-like)	22	48	9
E. coli DNA polymerase I (Klenow fragment)	0	0	0
T4 DNA polymerase	0	0	0
Taq DNA polymerase	0	7	0
Calf terminal deoxynucleotidyltransferase	0	0	0
Human telomerase	0	0	0
HIV-1 reverse transcriptase	21	29	28
T7 RNA polymerase	0	0	0
T4 polynucleotide kinase	0	0	0
Bovine deoxyribonuclease I	0	0	0
Human DNA topoisomerase I	85	85	20
Human DNA topoisomerase II	95	95	20

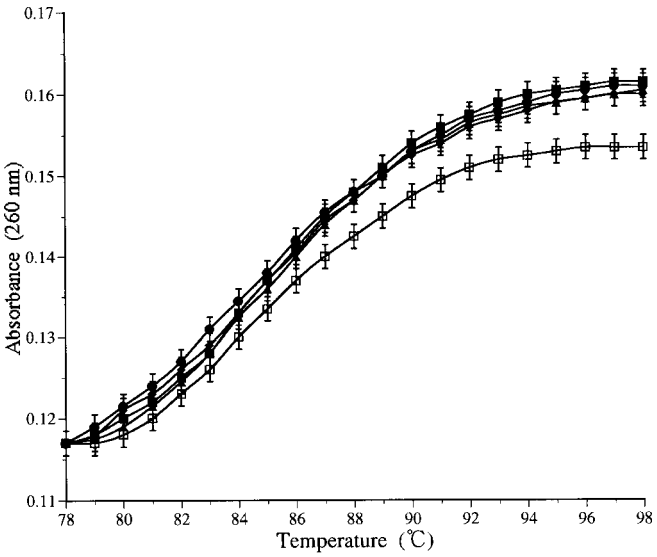

Figure 4 Effects of FAs and UA on the thermal transition of double-stranded DNA

No addition (control, ■), FA-A (●), FA-B (▲), UA (◆) and ethidium bromide (□) (15 μM of each) were incubated with 6 μg/ml calf thymus double-stranded DNA in 0.1 M sodium phosphate buffer, pH 7.0. Results are means ± S.E.M. for three independent experiments.

topoisomerase II. Indeed, Sun et al. reported that one of natural flavone compounds, daidzein, from *P. corylifolia* inhibited not only the activity of simian virus 40 DNA polymerase but also that of DNA topoisomerase II, although the effects were weak [5]. Daidzein, one of the known flavones, has slight inhibitory effects on both enzymes at high concentrations (nearly 400 μM) [5]. We emphasize that the flavone first intercalates into the DNA molecule as a template primer and subsequently inhibits both activities indirectly through the induction of a conformational change in the DNA. In this study we therefore examined whether the triterpenoids inhibited the activities through intercalation.

Influence of FAs and UA on the hyperchromicity of double-stranded DNA

To determine whether FA-A, FA-B or UA bind to DNA, the melting temperature (T_m) of double-stranded DNA (dsDNA) in the presence of each triterpenoid at 15 μM was measured in a spectrophotometer equipped with a thermoelectric cell holder (Figure 4). As described in the Materials and methods section, calf thymus dsDNA at 6 μg/ml was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, containing 1% (v/v) DMSO. At this concentration of each triterpenoid, the thermal transition of T_m was not observed, whereas 15 μM ethidium bromide, a typical intercalating agent, caused the thermal transition (Figure 4). Each compound at 100 μM also showed no thermal transition of T_m (results not shown). Thus none of the triterpenoids bound to the dsDNA, suggesting that they must inhibit the enzyme activities by interacting with the enzymes directly. As described previously, the kinetics of inhibition by FA-A and FA-B showed that they inhibited the activity of mammalian DNA polymerase β by competing with the DNA template primer [1]. Kinetic studies supported the possibility that the triterpenoids interact directly with the enzymes.

Several anti-cancer agents in clinical use have been shown to be potent inhibitors of DNA topoisomerases. For example, adriamycin (doxorubicin), amsacrine (m-AMSA), ellipticine, saintopin, streptonigrin and terpentecin have been demonstrated to show significant activity as inhibitors of DNA topoisomerase II. The plant alkaloid camptothecin and its synthetic derivatives such as CRT-11 and topotecans are extensively studied DNA topoisomerase I inhibitors. All of these agents inhibit the rejoining reaction of topoisomerases by stabilizing a tight topoisomerase–DNA complex termed the 'cleavable complex'. However, there have been no previous reports of inhibitors that inhibit the activities of DNA topoisomerases themselves before binding to the DNA molecule. Thus FA-A, FA-B and UA are markedly different from the other DNA topoisomerase inhibitors reported previously.

FA-A, FA-B and UA are structurally similar triterpenoids. For example, they each have a carbon atom number of 30; they consist of terpenoid rings as the hydrophobic region and have a carboxy group as the hydrophilic region. Moreover, the three-dimensional structures of FA-A, FA-B and UA, as determined by computer simulation, were very similar (Figure 5). As shown in Figure 5, when FA-A (mid-grey) and UA (black) were superposed at terpenoid rings, the carboxy group (light grey) of both compounds was found to lie at almost the same position, indicating that the distances between the hydrophobic and hydrophilic sites in each compound were three-dimensionally the same. If each of the triterpenoids binds to the DNA polymerase or the DNA topoisomerase directly, and subsequently inhibits the activity, the structural similarity between the triterpenoids might be significant. The 39 kDa of DNA polymerase β was proteolytically separated into two fragments consisting of the template-primer-DNA-binding domain (8 kDa) and the catalytic domain (31 kDa) [29,30]. FA-A and FA-B bound tightly to the 8 kDa fragment [1]. The DNA-binding site on the DNA polymerases might be three-dimensionally very similar to those of the DNA topoisomerases, and each of the triterpenoids might competitively bind to the DNA-binding site. The amino acid sequences of both enzymes show almost no similarity to each other. Because the hydrophobic and the hydrophilic regions in

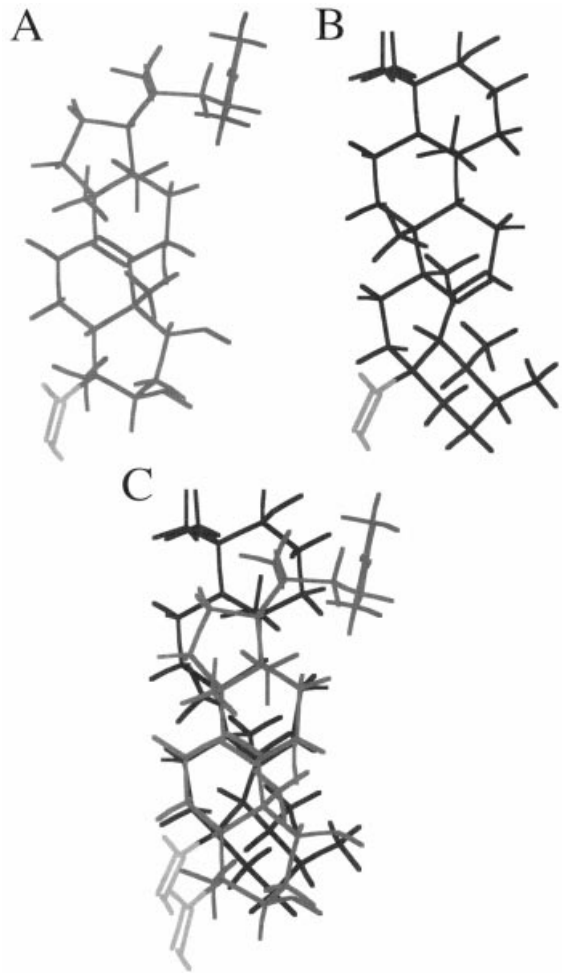

Figure 5 Overlay of FA-A and UA

(**A**) FA-A; (**B**) UA; (**C**) overlay of FA-A (mid-grey) and UA (black). The carboxy ends of the compounds are shown in light grey. The Figure was prepared using MolGraph (Daikin Ind. Ltd).

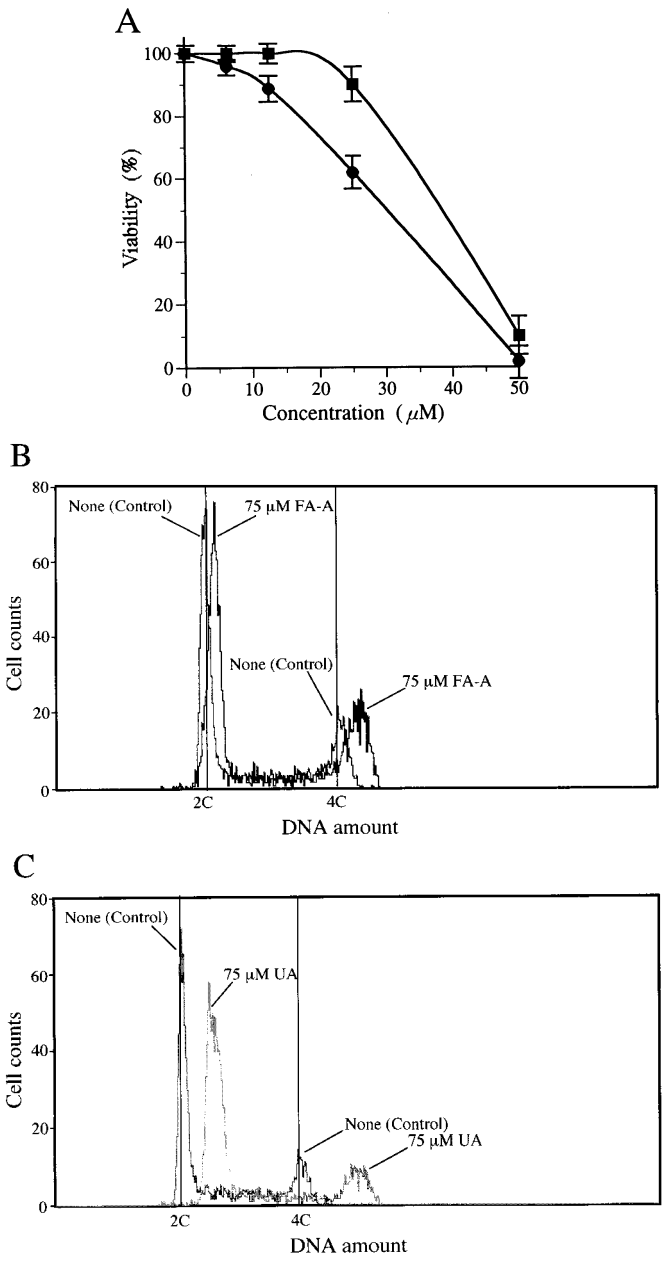

Figure 6 Effects of FAs and UA on proliferation of NUGC-3 cancer cells

(**A**) Dose–response curves of the human stomach cancer cell line NUGC-3 growth inhibition. The assays were performed under the conditions described in the Materials and methods section with the test compounds FA-A (■) and UA (●) at the indicated concentrations. Survival rate was determined by assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide [19]. Results are means ± S.E.M. for three independent experiments. (**B**, **C**) Flow cytometric analysis of cell cycle perturbation by FA-A (**B**) and UA (**C**). Cells of a human stomach cancer cell line, NUGC-3, were incubated with 75 μM FA-A or 75 μM UA for 48 h. DNA was stained with 50 μg/ml propidium iodide for 40 min in the dark at 4 °C. Fluorescence intensity was measured by flow cytometry. All experiments were performed four times.

the triterpenoids are thought to bind to the hydrophobic sheet and the hydrophilic amino acids respectively in the DNA-binding site of each enzyme, the three-dimensional structures might be similar in both of the DNA-binding sites. Further direct evidence should be obtained, for example by using NMR analysis and plasmon surface resonance (BIAcore) analysis. We have performed such analyses of DNA polymerase β with a terpenoid (Y. Mizushina and K. Sakaguchi, unpublished work). However, it is still too difficult to obtain sufficient amounts of DNA topoisomerase proteins for such analyses. However, we suggest, although the evidence is indirect, that DNA polymerase β and the DNA topoisomerase II have a common binding site for triterpenoids on the surface of the DNA.

We reported previously the results of an amino acid analysis of the binding sites of the other DNA polymerase inhibitors, unsaturated long-chain fatty acids, and demonstrated by NMR analysis the changes in the three-dimensional structure of the 8 kDa domain of DNA polymerase β before and after binding of the fatty acid [28]. The DNA-binding site bound to the fatty acid molecule as a 1:1 complex; a fatty acid molecule could bind to this site more strongly than a DNA molecule (DNA template primer) [28]. The hydrophobic (methyl end) and the hydrophilic regions (carboxy end) of the fatty acids bind to the hydrophobic sheet and the hydrophilic amino acids respectively in the DNA-binding site of DNA polymerase β and subsequently interfere with binding of the template primer [28]. The similarities in the modes of inhibition by these triterpenoids and fatty acids [1,11] should be examined in more detail in future studies.

Effects of FAs and UA on cultured mammalian cells

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

As shown in Figure 6(A), each of the triterpenoids showed similarly potent effects of growth inhibition against this cancer cell line. The LD_{50} values of FA-A and UA were 38 and 30 μM respectively (Figure 6A). As shown in Figures 2 and 3, the IC_{50} values of FA-A and UA were approx. 58 and 38 μM for DNA polymerase α, 79 and 42 μM for DNA polymerase β and 15 and 150 μM for DNA topoisomerase II respectively. The LD_{50} values were obviously smaller than the IC_{50} values *in vitro* for DNA polymerase α and β and DNA topoisomerase II. The inhibition curves *in vitro* and *in vivo* showed parallel dose-dependent decreases (Figures 2 and 6A). These observations suggested that the inhibition of cell growth occurs in a manner dependent on the enzyme inhibition, and that the inhibition of both of the enzymes influences cell growth *in vivo*.

To confirm this suggestion in more detail, we examined the effects of triterpenoids on the cell cycle of NUGC-3 cells by flow cytometry. As shown in Figures 6(B) and 6(C), the cells were arrested in the middle of S-phase and the region at greater than 4*C* by incubation for 48 h with 75 μM FA-A (Figure 6B) or 75 μM UA (Figure 6C). The effect was dependent on the incubation time (results not shown). The triterpenoids must inhibit cell growth mainly by blocking the primary step of DNA replication, by acting not only on the DNA polymerases but also on the DNA topoisomerases. In this experiment, a significant number of cells were found to be in the region at greater than 4*C* after G_2/M even after 72 h of incubation (results not shown). The accumulation of the region at greater than 4*C* might be explained by the induction of endoreduplication by the triterpenoids in a significant number of cells, and halting of the cell cycle at the second middle S-phase. These results indicate that inhibition of the DNA polymerases and/or the DNA topoisomerases leads primarily to cell growth inhibition *in vivo*.

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REFERENCES

1 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
2 Tanaka, N., Kitamura, A., Mizushina, Y., Sugawara, F. and Sakaguchi, K. (1998) Fomitellic acids, triterpenoid inhibitors of eukaryotic DNA polymerases from a basidiomycete, *Fomitella fraxinea*. J. Nat. Prod. **61**, 193–197
3 Kornberg, A. and Baker, T. A. (1992) DNA Replication, 2nd edn, pp. 197–225, W. H. Freeman, New York
4 Wang, J. C. (1985) DNA topoisomerases. Annu. Rev. Biochem. **54**, 665–697
5 Sun, N. J., Woo, S. H., Cassady, J. M. and Snapka, R. M. (1998) DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. J. Nat. Prod. **61**, 362–366
6 Tamai, K., Kojima, K., Hanaichi, T., Masaki, S., Suzuki, M., Umekawa, H. and Yoshida, S. (1988) Structural study of immunoaffinity-purified DNA polymerase α-DNA primase complex from calf thymus. Biochim. Biophys. Acta **950**, 263–273
7 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
8 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
9 Mizushina, Y., Tanaka, N., Yagi, H., Kurosawa, T., Onoue, M., Seto, H., Horie, T., Aoyagi, N., Yamaoka, M., Matsukage, A. et al. (1996) Fatty acids selectively inhibit eukaryotic DNA polymerase activities in vitro. Biochim. Biophys. Acta **1308**, 256–262
10 Mizushina, Y., Yagi, H., Tanaka, N., Kurosawa, T., Seto, H., Katsumi, K., Onoue, M., Ishida, H., Iseki, A., Nara, T. et al. (1996) Screening of inhibitor of eukaryotic DNA polymerases produced by microorganisms. J. Antibiot. (Tokyo) **49**, 491–492
11 Mizushina, Y., Yoshida, S., Matsukage, A. and Sakaguchi, K. The inhibitory action of fatty acids on DNA polymerase β. (1997) Biochim. Biophys. Acta **1336**, 509–521
12 Lui, L. F. and Miller, K. G. (1981) Eukaryotic DNA topoisomerases: two forms of type I DNA topoisomerases from HeLa cell nuclei. Proc. Natl. Acad. Sci. U.S.A. **78**, 3487–3491
13 Ferro, A. M. and Olivera, B. M. (1984) Poly(ADP-ribosylation) of DNA topoisomerase I from calf thymus. J. Biol. Chem. **259**, 547–554
14 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. (Tokyo) **97**, 1385–1389
15 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
16 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
17 Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L. and Shay, J. W. (1994) Specific association of human telomerase activity with immortal cells and cancer. Science **266**, 2011–2015
18 Ueno, T., Takahashi, H., Oda, M., Mizunuma, M., Yokoyama, A., Goto, Y., Mizushina, Y., Sakaguchi, K. and Hayashi, H. (2000) Inhibition of human telomerase by rubromycins: implication for spiroketal group as an active moiety. Biochemistry, **39**, 5996–6002
19 Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. **65**, 55–63
20 Kobayashi, J., Mikami, S., Shigemori, H., Takao, T., Shimonishi, Y., Izuta, S. and Yoshida, S. (1995) Flavocristamides A and B, new DNA polymerase α inhibitors from a marine bacterium *Flavobacterium sp.* Tetrahedron **51**, 10487–10490
21 Mizushina, Y., Watanabe, I., Ohta, K., Takemura, M., Sahara, H., Takahashi, N., Gasa, S., Sugawara, F., Matsukage, A., Yoshida, S. and Sakaguchi, K. (1998) Studies on inhibitors of mammalian DNA polymerase α and β: sulfolipids from a pteridophyte, *Athyrium niponicum*. Biochem. Pharmacol. **55**, 537–541
22 Ohta, K., Mizushina, Y., Hirata, N., Takemura, M., Sugawara, F., Matsukage, A., Yoshida, S. and Sakaguchi, K. (1998) Sulfoquinovosyldiacylglycerol, KM043, a new potent inhibitor of eukaryotic DNA polymerases and HIV-reverse transcriptase type 1 from a marine red alga, *Gigartina tenella*. Chem. Pharm. Bull. **46**, 684–686
23 Ohta, K., Mizushina, Y., Hirata, N., Takemura, M., Sugawara, F., Matsukage, A., Yoshida, S. and Sakaguchi, K. (1999) Action of a new mammalian DNA polymerase inhibitor, sulfoquinovosyldiacylglycerol. Biol. Pharm. Bull. **22**, 111–116
24 Ono, K., Nakane, H. and Fukushima, M. (1988) Differential inhibition of various deoxyribonucleic and ribonucleic acid polymerases by suramin. Eur. J. Biochim. **172**, 349–353
25 Murofushi, K. M., Shiota, M., Kaji, K., Yoshida, S. and Murofushi, H. (1992) Inhibition of eukaryotic DNA polymerase α with a novel lysophosphatidic acid (PHYLPA) isolated from myxoamoebae of *Physarum polycephalum*. J. Biol. Chem. **267**, 21512–21517
26 Murofushi, K. M., Kobayashi, S., Onimura, K., Matsumoto, M., Shiota, M., Shoji, M. and Murofushi, H. (1995) Selective inhibition of DNA polymerase-α family with chemically synthesized derivatives of PHYLPA, a unique *Physarum* lysophosphatidic acid. Biochim. Biophys. Acta **1258**, 57–60
27 Mizushina, Y., Takahashi, N., Hanashima, L., Koshino, H., Esumi, Y., Uzawa, J., Sugawara, F. and Sakaguchi, K. (1999) Lucidenic acid O and lactone, new terpene inhibitors of eukaryotic DNA polymerases from a basidiomycete, *Ganoderma lucidum*. Bioorg. Med. Chem. **7**, 2047–2052
28 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
29 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
30 Kumar, A., Widen, S. G., Wiliams, 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

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