Pyranicin, a non-classical annonaceous acetogenin, is a potent inhibitor of DNA polymerase, topoisomerase and human cancer cell growth

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Abstract. This report describes the inhibitory activities of the natural and non-natural acetogenins [mucocin (compound 1), jimenezin (compound 2), 19-*epi* jimenezin (compound 3), muconin (compound 4), pyranicin (compound 5), pyragonicin (compound 6), 10-*epi* pyragonicin (compound 7), and a γ-lactone (compound 8)], which were synthesized by us, against DNA polymerase (pol), DNA topoisomerase (topo), and human cancer cell growth. Among the compounds tested, compound 5 was revealed to be the strongest inhibitor of the animal pols and human topos tested, and the IC_{50} values for pols and topos were 2.3-15.8 and 5.0-7.5 μM, respectively. The compound also suppressed human cancer cell (promyelocytic leukemia cell line, HL-60) growth with the same tendency as the inhibition of pols and topos and the LD_{50} value was 9.4 μM. Compound 5 arrested the cells at G2/M and G1 phases, and prevented the incorporation of thymidine into the cells, indicating that it blocks DNA replication by inhibiting the activity of pols and topos. This compound also induced apoptosis of the cells. Based on these results, the action mode of compound 5 is discussed.

Introduction

DNA polymerase (pol) catalyzes the addition of deoxyribonucleotides to the 3'-hydroxyl-terminus of primed double-stranded DNA molecules (1). The human genome encodes 14 pols which conduct cellular DNA synthesis (2). Eukaryotic cells reportedly contain three replicative types: pols α, δ and ε mitochondrial pol γ, and at least twelve repair types: pols ß, δ, ε, ζ, η, θ, ι, κ, λ, μ and σ and REV1 (3).

DNA topoisomerases (topos) are key enzymes that control the topological state of DNA. There are two classes of topos: type I enzymes, which act by transiently nicking one of the two DNA strands, and type II enzymes, which nick both DNA strands and dependent on ATP, are involved in many vital cellular processes that influence DNA replication, transcription, recombination, integration and chromosomal segregation (4).

DNA metabolic enzymes such as pols and topos are not only essential for DNA replication, repair and recombination, but are also involved in cell division. Selective inhibitors of these enzymes are considered as a group of potentially useful anti-cancer and anti-parasitic agents, because some inhibitors suppress human cancer cell proliferation and have cytotoxicity (5-8).

Acetogenins from Annonaceae species are a relatively new class of fatty acid-derived natural products that have a wide range of biological activities such as cytotoxic, antitumor and immunosuppressive effects (9-11). They are characterized by the presence of one to three tetrahydrofuran (THF) rings in the center of a long alkyl chain with a

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Abbreviations: pol, DNA-directed DNA polymerase (E.C. 2.7.7.7); topo, DNA topoisomerase; THF, tetrahydrofuran; THP, tetrahydropyran; dNTP, 2'-deoxyribonucleotide 5'-triphosphate; dTTP, 2'-deoxythymidine 5'-triphosphate; TdT, terminal deoxynucleotidyl transferase; DMSO, dimethyl sulfoxide; IC_{50}, 50% inhibitory concentration; LD_{50}, 50% lethal dose; dsDNA, double-stranded DNA

Key words: acetogenins, pyranicin, enzyme inhibitor, DNA polymerase, DNA topoisomerase, cytotoxicity, cell proliferation, cell cycle arrest, apoptosis

butenolide moiety at the end. Besides such classical types, acetogenins with a tetrahydropyran (THP) ring in the long chain such as mucocin (compound 1), jimenezin (compound 2), muconin (compound 4), pyranicin (compound 5), and pyragonicin (compound 6) have also been discovered (12-15). Recently, we have been interested in the non-classical THP acetogenins because of the powerful antitumor activity, and achieved total synthesis of the natural and non-natural acetogenins (compounds 1-8) (16-24). In connection with our studies on a search for new pol and topo inhibitors and development of new anticancer drugs, we initiated biological studies of these acetogenins. Since the groups of McLaughlin and Mata reported that some acetogenins have cytotoxicity to human cancer cell lines (12-15), the purpose of our studies is to investigate the biochemical action of the compounds in detail and to use the compound as an anti-neoplastic agent.

Herein, we describe the inhibitory activities of chemically synthesized acetogenins against pols, topos and other DNA metabolic enzymes, and cellular proliferation processes such as DNA replication of human cancer cells (HL-60). It was discussed that acetogenins seem to be an ideal model for the development of new anti-cancer drugs.

Materials and methods

Materials. Eight chemically synthesized acetogenins (i.e., compounds 1-8; Fig. 1) were employed (16-24). Nucleotides and chemically synthesized DNA template-primers, such as poly(dA), poly(rA), and oligo(dT)$_{12-18}$, and radioisotope reagents, such as [^{3}H]-dTTP (2'-deoxythymidine 5'-triphosphate) (43 Ci/mmol), [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine and L-[4,5-^{3}H]-leucine, were purchased from GE Healthcare Bio-Science Corp. (Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Nacalai Tesque, Ltd. (Kyoto, Japan). HL-60, a human promyelocytic leukemia cell line (IFO 050022), was supplied by the Health Science Research Resources Bank (Osaka, Japan).

Enzymes. Pol α was purified from the calf thymus by immunoaffinity column chromatography as described by Tamai *et al* (25). Recombinant rat pol ß was purified from *E. coli* JMpß5 as described by Date *et al* (26). The human pol γ catalytic gene was cloned into pFastBac, and histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (Life Technologies, MD, USA) and purified using ProBoundresin (Invitrogen, Tokyo, Japan) (27). Human pols δ, and ε were purified by the nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pols δ and ε-conjugated affinity column chroma-tography, respectively (28). Recombinant human pols η and ι tagged with His6 at their C-terminal were expressed in SF9 insect cells using the Baculovirus Expression System, and were purified as described previously (29,30). A truncated form of pol κ (i.e., hDINB1DC) with 6X His-tags attached at the C-terminal was overproduced using the BAC-to-BAC Baculovirus Expression System kit (Gibco-BRL) and purified as described by Ohashi *et al* (31). Recombinant human His-pol λ was overexpressed and purified according to a method described by Shimazaki *et al* (32). Fish pol δ was purified from the testis of cherry salmon (*Oncorhynchus masou*) (33).

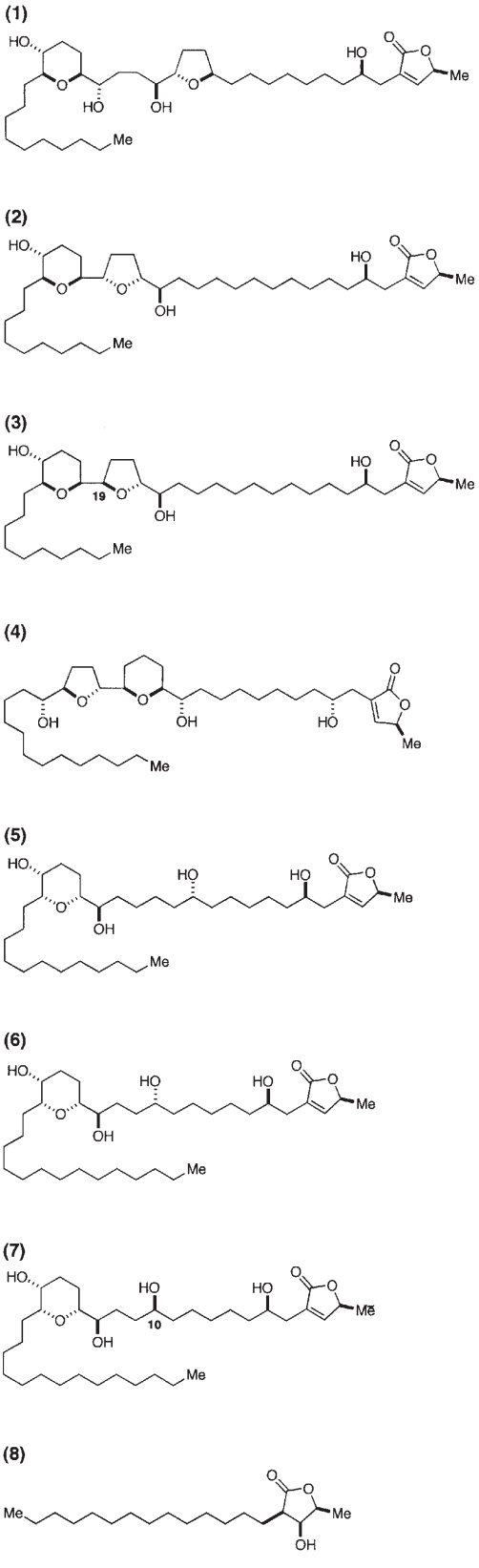

Figure 1. Chemical structures of acetogenins. (1) Compound 1, mucocin; (2) compound 2, jimenezin; (3) compound 3, 19-*epi* jimenezin; (4) compound 4, muconin; (5) compound 5, pyranicin; (6) compound 6, pyragonicin; (7) compound 7, 10-*epi* pyragonicin; and (8) compound 8, 3-hydroxy-4-methyl-2-tetradecyl-4-butanolide.

Fruit fly pols α, δ, and ε were purified from early embryos of *Drosophila melanogaster* as described by Aoyagi *et al* (34,35). Pols I (α-like) and II (ß-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi *et al* (36). Calf thymus terminal deoxynucleotidyl transferase (TdT) and bovine pancreas deoxyribonuclease I were obtained from Stratagene Cloning Systems (La Jolla, CA, USA). The Klenow fragment of pol I from *E. coli* was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). *Taq* pol, T4 pol, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan). Purified human placenta topos I and II were purchased from TopoGen, Inc. (Columbus, OH).

DNA polymerase assays. The reaction mixtures for pol α, pol ß, plant pols and prokaryotic pols were described previously (37,38), and those for pol γ, and pols δ and ε were as described by Umeda *et al* (27) and Ogawa *et al* (39), respectively. The reaction mixtures for pols η, ι and κ were the same as that for pol α, and the reaction mixture for pol λ was the same as that for pol ß. For pols, poly(dA)/oligo$(dT)_{12-18}$ (A/T = 2/1) and dTTP were used as the DNA template-primer and nucleotide (i.e., 2'-deoxyribonucleotide 5'-triphosphates, dNTP) substrate, respectively. For TdT, oligo$(dT)_{12-18}$ (3'-OH) and dTTP were used as the DNA template-primer and nucleotide substrate, respectively.

Chemically synthesized acetogenins were dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA and kept at 0˚C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures and incubation was carried out at 37˚C for 60 min, except for *Taq* pol, which was incubated at 74˚C for 60 min. Activity without the inhibitor was considered 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into synthetic DNA template-primers in 60 min at 37˚C under the normal reaction conditions for each enzyme (37,38).

Other enzyme assays. The primase activity of pol α the activities of T7 RNA polymerase, human topos I and II, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in standard assays according to the manufacturer's specifications as described by Tamiya-Koizumi *et al* (40), Nakayama *et al* (41), Spitzner *et al* (42), Soltis *et al* (43) and Lu and Sakaguchi (44), respectively.

Investigation of cytotoxicity on cultured cells. To investigate the effects of acetogenins (i.e., compounds 1-8) on cultured cells, a human cancer cell line HL-60, derived from a cancer patient, was used. The cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 unit/ml penicillin and 1.6 μg/ml $NaHCO_3$. The cells were cultured at 37˚C in standard medium in a humidified atmosphere of 5% CO_2-95% air. The cytotoxicity of the compound was investigated as follows. High concentrations (10 mM) of the compounds were dissolved in DMSO and stocked. Approximately $1x10^4$ cells per well were inoculated in 96-well micro-plates, and then the compound stock solution was diluted to various concentrations and applied to each well. After incubation for 24 h, the survival rate was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (45).

Cell cycle analysis. Cellular DNA content for cell cycle analysis was determined as follows: aliquots of $3x10^5$ HL-60 cells were harvested into a 35-mm dish, and incubated with medium containing compound 5 for various times. The cells were then washed with ice-cold PBS three times by centrifugation, fixed with 70% (v/v) ethanol and stored at -20˚C. DNA was stained with DAPI staining solution for at least 10 min at room temperature in the dark. Fluorescence intensity was measured by FACSCanto flow cytometer in combination with FACSDiVa software (BD, Becton-Dickinson and Company, NJ, USA).

Measurement of inhibition of synthesis of DNA, RNA and protein. The effect of compound 5 on DNA, RNA and protein synthesis was examined independently. HL-60 cells ($1x10^4$) were inoculated into 96-well micro-plates and preincubated without the compound for several hours. Medium containing compound 5 diluted with 0.5% DMSO solution was then applied to the cells, and this time point was taken as 0 h. At -0.5 h, as probes for DNA, RNA or protein synthesis, [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine or L-[4,5-^{3}H]-leucine (final concentration, 3, 4 and 4 μCi, respectively) was added. At specified time points, incubation was stopped and cell lysate was prepared to measure the incorporated radioactivity as described for the cytotoxicity assay.

Analysis of DNA fragmentation. Apoptosis was determined by an assay of DNA fragmentation by means of agarose electrophoresis. Total DNAs were extracted from $6x10^5$ HL-60 cells, following the method of Sambrook *et al* (46), and 5 μg aliquots were separated by 1.5% (w/v) agarose gel electrophoresis in 40 mM Tris -5 mM sodium acetate - 1 mM EDTA (pH 7.8) and stained with ethidium bromide. DNA bands were visualized under UV light.

Results

Effects of acetogenins on mammalian DNA polymerases α, ß and λ. The chemical structures of the acetogenins (i.e., compounds 1-8), which were chemically synthesized, are shown in Fig. 1. First, the inhibitory activity of calf pol α, rat pol ß and human pol λ against 10 μM of each compound was investigated (Fig. 2A). In mammalian pols, pol α and pols ß and λ were used as representative replicative pol and repair/recombination-related pols, respectively (2,3). Compounds 5 and 6 significantly inhibited the activities of these pols, and in order of their effect, the acetogenins ranked as follows: compound 5 > compound 6 > compound 7 > compound 4 >

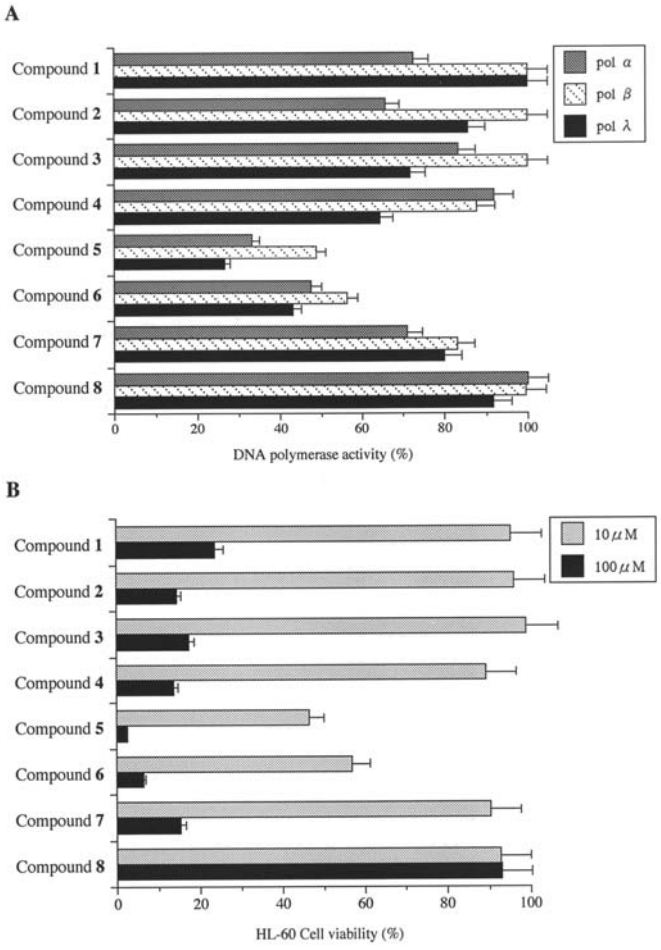

Figure 2. Effect of acetogenins on the activities of mammalian pols and human cancer cell growth. (A) Ten μM of each compound was incubated with calf pol α, rat pol ß and human pol λ (0.05 units each). Pol activities were measured as described in Materials and methods. Enzymatic activity in the absence of the compound was taken as 100%. Data are shown as the mean ± SEM of three independent experiments. (B) Each compound (10 and 100 μM) was added to the culture of a promyelocytic leukemia cell line HL-60 and incubated for 24 h. The rate of viability was determined by MTT assay (45). Cell viability of the cancer cells in the absence of the compound was taken as 100%. Data are shown as the mean ± SEM of five independent experiments.

compound 3 > compound 2 > compound 1 > compound 8. Compound 5 showed the strongest inhibition of pols α, ß and λ activities in the tested compounds, and 50% inhibition was observed at doses of 5.3, 9.6 and 2.3 μM, respectively (Table I). When activated DNA (i.e., DNA digested by bovine deoxynuclease I) was used as the DNA template-primer instead of poly(dA)/oligo(dT)$_{12\text{-}18}$ (A/T = 2/1), the mode of inhibition by these compounds did not change (data not shown).

Effects of compounds 5 on DNA polymerases and other DNA metabolic enzymes. Compound 5 also inhibited the activities of other mammalian pols tested (i.e., pols γ, δ, ε, η, ι, κ, and TdT) with IC_{50} value of 5.9-15.8 μM (Table I). The pol λ inhibitory effect of compound 5 was the strongest in mammalian pols. Furthermore, this compound inhibited animal pols from fish (cherry salmon) pol δ, and insects (fruit fly) pols α, δ and ε at almost the same concentration as the inhibition of mammalian pols. On the other hand, compound 5 had no significant influence on the activities of pols I (α-like pol) and II (ß-like pol) from plants (cauliflower) and prokaryotes such as the Klenow fragment of *E. coli* pol I, *Taq* pol and T4 pol.

In the DNA metabolic enzymes tested, compound 5 also inhibited the activities of human topos I and II with IC_{50}

Table I. IC_{50} values of compound 5 on the activities of various DNA polymerases and other DNA metabolic enzymes.

Enzyme	IC_{50} value (μM)
Mammalian DNA polymerases	
Calf DNA polymerase α	5.3±0.4
Rat DNA polymerase ß	9.6±0.8
Human DNA polymerase γ	5.9±0.4
Human DNA polymerase δ	8.4±0.7
Human DNA polymerase ε	15.8±1.4
Human DNA polymerase η	10.2±0.9
Human DNA polymerase ι	13.0±1.2
Human DNA polymerase κ	11.1±1.0
Human DNA polymerase λ	2.3±0.2
Calf terminal deoxynucleotidyl transferase	6.3±0.5
Fish DNA polymerases	
Cherry salmon DNA polymerase δ	8.8±0.7
Insect DNA polymerases	
Fruit fly DNA polymerase α	6.5±0.5
Fruit fly DNA polymerase δ	8.9±0.8
Fruit fly DNA polymerase ε	14.0±1.3
Plant DNA polymerases	
Cauliflower DNA polymerase I (α-like)	>200
Cauliflower DNA polymerase II (ß-like)	>200
Prokaryotic DNA polymerases	
E. coli DNA polymerase I (Klenow fragment)	>200
Taq DNA polymerase	>200
T4 DNA polymerase	>200
Other DNA metabolic enzymes	
Calf primase of DNA polymerase α	>200
T7 RNA polymerase	>200
Human DNA topoisomerase I	5.0±1.5
Human DNA topoisomerase II	7.5±2.0
T4 polynucleotide kinase	>200
Bovine deoxyribonuclease I	>200

Compound 5 was incubated with each enzyme (0.05 units). Enzymatic activity was measured as described in Materials and methods. Enzyme activity in the absence of the compound was taken as 100%. Data are shown as the mean ± SEM of four independent experiments.

value of 5.0 and 7.5 μM, respectively (Table I). These results suggested that the inhibitory activity of acetogenins between mammalian pols and human topos had the same tendency, although the inhibitory effect on human pol λ was approximately 3-fold stronger than that on human topo II. This compound did not inhibit the activities of the other DNA metabolic enzymes tested, including calf primase of pol α T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I.

To determine whether the inhibitor resulted in binding to DNA or the enzyme, the interaction of compound 5 with double-stranded DNA (dsDNA) was investigated based on the thermal transition of dsDNA with or without the compound. The Tm of dsDNA with an excess amount of compound 5 (100 μM) was measured using a spectrophotometer equipped with a thermoelectric cell holder. In the concentration range used, no thermal transition of Tm was observed, whereas ethidium bromide used as a positive control, a typical intercalating compound, produced a clear thermal transition. These observations indicated that compound 5 did not intercalate to DNA as a template-primer, and the compound might directly bind to the enzyme and inhibit its activity.

These results suggested that compound 5 might be a potent and selective inhibitor of animal pols and human topos. We investigated the mechanism of the inhibitory effect of acetogenins including compound 5 on human cancer cells.

Effects of acetogenins on cultured human cancer cells. Pols and topos have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Acetogenins (i.e., compounds 1-8) could therefore be useful in chemotherapy and we investigated the cytotoxic effect of eight compounds against a human promyelocytic leukemia cell line, HL-60.

As shown in Fig. 2B, 10 and 100 μM of compound 5 had the strongest growth inhibitory effect on this cancer cell line in the tested compounds, and compound 6 was the second strongest (i.e., in order of cytotoxicity: compound 5 > compound 6 > compound 7 > compound 4 > compound 3 > compound 2 > compound 1 > compound 8). The suppression of cell growth had the same tendency as the inhibition of mammalian pols among the compounds (Fig. 2A), suggesting that the cause of cancer cell influence might be the activity of pols, including replicative pols and repair/recombination pols. All compounds tested as acetogenins have a butenolide moiety or a γ-lactone moiety at the end. Compounds 1 to 7 also consist of one or two rings such as a THF ring and/or a THP ring in the center, although compound 8 has no other rings. Since compound 8 also did not influence the activities of the pols tested and HL-60 cells, the moiety of THF and/or THP rings of compounds 1 to 7 might be important for the inhibition of mammalian pols and human cancer cell growth. Furthermore, the molecular length of compounds 1 to 7 was approximately 1.9-fold longer than that of compound 8 (i.e., 33.63-37.76 Å and 19.80 Å, respectively) (data not show), 33 to 38 Å of molecular length of the compound must be essential for these inhibitory activities.

Therefore, we concentrated our efforts on compound 5 in the latter part of this study. Compound 5 suppressed HL-60 cell growth in a dose-dependent manner. After 24 h of treatment, 50% cytotoxicity of this compound was observed at doses of 9.4 μM (Fig. 3). This result suggested that the LD_{50} value *in vivo* for HL-60 cells was almost the same as the IC_{50} values *in vitro* for pols and topos. We investigated in more detail which inhibition by compound 5 is effective for cancer cell proliferation, that of pols or topos.

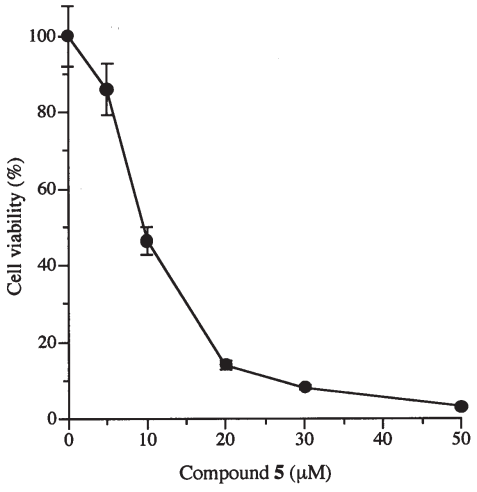

Figure 3. Effect of compound 5 on the proliferation of a human cancer cell line, HL-60. The data show the dose-response curves of growth inhibition of the human promyelocytic leukemia cell line HL-60 incubated with various concentrations of compound 5 for 24 h. Cell proliferation was determined by MTT assay (45). Data are shown as the mean ± SEM of four independent experiments.

Effects on the cell cycle progression of compound 5. Next, we analyzed whether compound 5 affected the cell cycle distribution of compound 5-treated cells (Fig. 4). The cell cycle fraction was recorded after 12 and 24 h of treatment with the LD_{50} value of the compound (i.e., 9.4 μM). Consequently, among cells treated with compound 5 for 12 h, the population of cells in the G2/M phase increased (9.6 to 15.2%), the percentage of cells in the S phase decreased from 42.1 to 34.5%, and the G1 phase was not affected (Fig. 4B). These results suggested that the actions of compound 5 blocked the G2/M phase in HL-60 cells by inhibition of topos I and II. For 24-h treatment of compound 5, the population of cells in the G1 phase significantly increased from 48.3 to 75.6%, and the S phase decreased (42.1 to 13.1%) (Fig. 4C). Dehydroaltenusin, which is a specific pol α inhibitor, inhibited the cell cycle in the G1 phase including the early-S phase (47), and classical topo inhibitors such as etoposide arrested the cell cycle at the G2/M phase (48). Compound 5, therefore, might be more effective in the inhibition of pols than topos for 24-h incubation of cells, although compound 5 inhibited the activities of mammalian pols and human topos, and the inhibitory effect for topos (IC_{50} values are 5.0-7.5 μM) was the almost same as that for pols (IC_{50} values are 2.3-15.8 μM) *in vitro* (Table I).

Effects of inhibition of synthesis of DNA, RNA and protein by compound 5 in human cancer cells. The results of cell

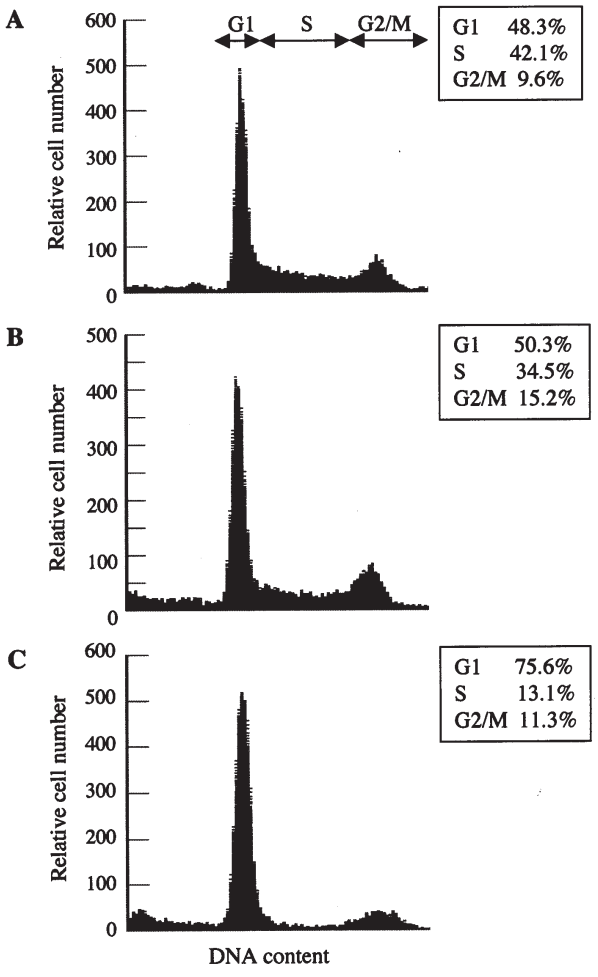

Figure 4. The effect of compound 5 on the cell cycle. HL-60 cells were incubated with 9.4 μM compound 5 for 0 h (A), 12 h (B) and 24 h (C). Cell cycle distribution was calculated as the percentage of cells in G1, S and G2/M phases. All experiments were performed three times.

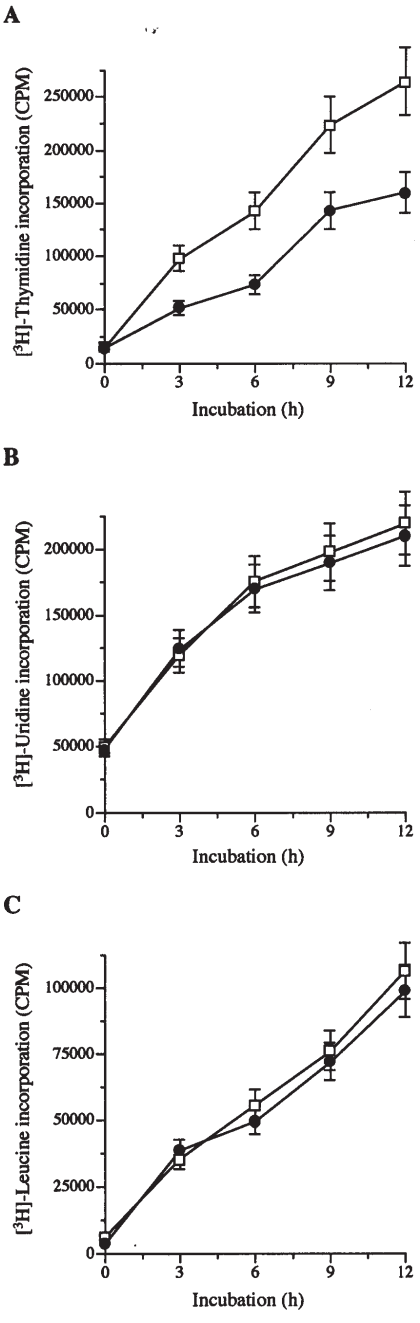

Figure 5. Measurement of DNA, RNA and protein synthesis in HL-60 cells incubated with compound 5. HL-60 cells ($1x10^4$) were incubated without (open square) or with 9.4 μM compound 5 (control, closed circle) from 0 h, and radiolabeled thymidine, uridine or leucine was added at -0.5 h. (A-C) Show the incorporation of radiolabeled thymidine, uridine and leucine, respectively. Each point represents the average of triplicate experiments and bars indicate SD.

cycle analysis were more directly confirmed by the incorporation experiment. Fig. 5A-C shows the incorporation of [^{3}H]-labeled thymidine, [^{3}H]-uridine and [^{3}H]-leucine into HL-60 cells, respectively. Compound 5 inhibited only the incorporation of [^{3}H]-thymidine time-dependently into the cells. The [^{3}H]-thymidine incorporation was decreased by 41.8% of the control level in the presence of the LD_{50} value of compound 5 (i.e., 9.4 μM) for 12 h (Fig. 5A). Neither [^{3}H]-uridine nor [^{3}H]-leucine incorporation was affected by the compound (Fig. 5B and C). These observations indicated that compound 5 must inhibit cell growth by blocking the primary step in the replication of DNA. The effect of the compound on HL-60 cells in Figs. 4 and 5 would suggest that compound 5 is distributed in the nucleus, and the mechanism of its action might be the inhibition of replicative pols (i.e., pols α, δ and ε) rather than topos; therefore, cell cycle regulation was induced by the inhibition of pol activity by compound 5.

Effects of compound 5 on apoptosis in human cancer cells. To examine whether the decrease in cell numbers caused by compound 5 was due to apoptosis, DNA fragmentation was analyzed by electrophoresis. A DNA ladder formation was dose-dependently observed in HL-60 cells treated with the LD_{50} value of the compound (i.e., 9.4 μM), and ladders were apparent at 6 h (Fig. 6). In cell cycle analysis, compound 5-treated cells were in the sub-G1 phase (Fig. 4B and C). These results suggested that apoptotic effects were evident in the cells, and the effect of the compound must involve a combination of growth arrest and cell death. Compound 5 therefore should be considered the lead compound of potentially useful cancer chemotherapy agents.

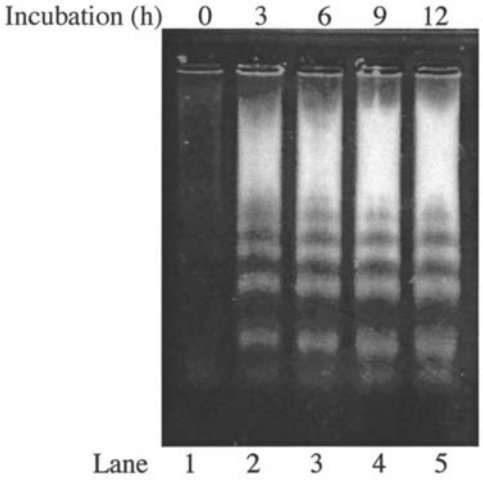

Figure 6. Detection of intracellular DNA ladder formation in HL-60 cells treated with 9.4 μM compound 5 for 0 h (lane 1), 3 h (lane 2), 6 h (lane 3), 9 h (lane 4) and 12 h (lane 5). Following cell lysis, total DNA was extracted and analyzed by agarose gel electrophoresis.

Discussion

As described in this report, we found that chemically synthesized acetogenins inhibited the activities of mammalian pols and human cancer cell growth. In particular, compound 5 showed the strongest inhibition in the tested acetogenins, and it was revealed that the inhibition of pol activity by compound 5, which is an inhibitor of mammalian pols and topos, influenced not only cell proliferation but also the cell cycle.

Most acetogenins exhibit potent and selective *in vitro* anti-tumor activities. For example, mucocin (compound 1) is reported to show remarkable inhibitory activities against A-549 (lung cancer) and PACA-2 (pancreatic cancer) solid tumor lines (49). As the mode of action, blockage of the mitochondrial NADH-ubiquinone oxidoreductase in complex I, which is a membrane-bound and essential enzyme for ATP production, is discussed (49). Furthermore, these natural products were also shown to inhibit a ubiquinone-linked NADH oxidase found in the plasma membrane of specific tumor cell lines, including some which show multidrug resistance (50). Very little research, however, has been pursued on the inhibition of acetogenins against pol and topo activities until now, probably due to the small quantities of natural products. Since the total synthesis of bioactive acetogenins such as compound 5 could be possible (16-24), these compounds should be provided and studied in pharmaceutical research throughout the world.

Compound 5 used in this study directly inhibited animal pols and human topos activities (Table I), but it did not bind to DNA (data not shown). These observations suggested that there may be some structural similarity between both enzymes at the compound 5-binding site, although the characteristics of both pols and topos, including their modes of action, amino acid sequences and three-dimensional structures, are markedly different. We previously reported that several inhibitors, long chain fatty acids (37,38) and triterpenoids (51,52), of mammalian pol ß could also inhibit topo II activity, and both enzymes have structural homology at the DNA-binding site (53-55), and the DNA-binding site of long chain fatty acids on the pol was the same domain (i.e., the N-terminal 8-kDa domain of pol ß) as that of triterpenoids (53,56). Therefore, compound 5 was expected to have similar characteristics. Topos and pols have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents; therefore, information concerning the structural characteristics of these inhibitors could provide valuable insight for the design of new anti-cancer agents.

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