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Actions of derivatives of dehydroaltenusin, a new mammalian DNA polymerase α-specific inhibitor

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Abstract

Dehydroaltenusin was found to be an inhibitor of mammalian DNA polymerase α (pol. α) *in vitro*, but did not influence the activities of the other replicative DNA polymerases including even other vertebrate pol. α. In this study, we purified or synthesized various slightly modified derivatives of dehydroaltenusin, and using them, investigated the relationship between the chemical structure and the inhibitory effects, and the *in vitro* and *in vivo* effects of dehydroaltenusin to determine to what extent the pol. α activity inhibition influences cell proliferation. Most of the derivatives lost the enzyme species-specific inhibitory effect, suggesting that dehydroaltenusin is three-dimensionally inserted into a pocket present only in mammalian pol. α. Dehydroaltenusin inhibited the cell proliferation of the human gastric cancer cell line NUGC-3 by arresting the cells at G1/S-phase, and prevented the incorporation of thymidine into the cells, indicating that it blocks the primary step of *in vivo* DNA replication by inhibiting pol. α. This compound also induced apoptosis of the cells. Dehydroaltenusin is a mammalian pol. α-specific inhibitor useful in both of *in vivo* and *in vitro* experiments.

Keywords: DNA polymerase α; Dehydroaltenusin; Enzyme inhibitor; DNA replication; Cytotoxicity; Cell proliferation; Apoptosis

1. Introduction

We previously reported an interesting inhibitor that influenced only the activity of mammalian DNA polymerase α, termed dehydroaltenusin [1]. Dehydroaltenusin did not affect the activities of mammalian pol. δ or ε, nor pol. α from other vertebrates [1]. Since a well-known pol. α inhibitor, aphidicolin, also inhibited the activities of all eukaryotic pol. α species, and of the other replicative DNA polymerases, δ and ε [2,3], this represented the first finding of a DNA polymerase inhibitor capable not only of distinguishing among pol. α, δ and ε, but of distinguishing among eukaryotic pol. α species. Dehydroaltenusin may be a useful tool as a molecular probe to clarify the *in vivo* biological function of each of the replicative DNA polymerases if its functions *in vivo*.

At present, we are engaged in analysis of the structure and function of pol. α using dehydroaltenusin from three different viewpoints; to investigate the three-dimensional structure of the dehydroaltenusin-binding site on mammalian pol. α, to understand the precise role of each polymerase *in vivo*, and to develop a drug design strategy for cancer chemotherapy agents. Since pol. α is an essential enzyme for DNA replication and subsequently for cell division [3]. Inhibitors of mammalian pol. α are not only molecular tools useful for analyzing the polymerases, but should also be considered as a group of potentially useful cancer chemotherapy agents.

Therefore, we purified analogs of dehydroaltenusin and chemically synthesized slightly modified derivatives to examine the structural relationship between dehydroaltenusin and DNA polymerases, and to study the influence of

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Abbreviations: pol., DNA polymerase (EC 2.7.7.7); dTTP, 2′-deoxythymidine-5′-triphosphate; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide.

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dehydroaltenusin on cell proliferation using a human stomach cancer cell line, NUGC-3. Dehydroaltenusin and its derivatives represent a group of potentially useful agents to examine the precise role of each polymerase *in vivo*, and to develop a drug design strategy for cancer chemotherapy agents. They could also be useful tools as molecular probes to study the three-dimensional structure of mammalian pol. α protein.

2. Materials and methods

2.1. Materials

Nucleotides and chemically synthesized template-primers such as poly(dA) and oligo(dT)$_{12–18}$ were purchased from Pharmacia. [^{3}H]-2′-deoxythymidine-5′-triphosphate) (dTTP) (43 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. [Methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine and L-[4,5-^{3}H]-leucine were purchased from Amersham Pharmacia Biotech. All other reagents were of analytical grade and were purchased from Wako Ltd. NUGC-3, a human gastric cancer cell line (JCRB0822), was supplied by the Health Science Research Resources Bank.

2.2. Enzymes

DNA polymerase α was purified from calf thymus by immuno-affinity column chromatography as described previously [4]. Recombinant rat pol. β was purified from *Escherichia coli* JMpβ5 as described by Date *et al*. [5].

2.3. DNA polymerase assays

Activities of DNA polymerases were measured by the methods described previously [6,7]. For DNA polymerases, poly(dA)/oligo(dT)$_{12–18}$ and dTTP were used as the template-primer DNA and nucleotide substrate, respectively. The activity without dehydroaltenusin was considered 100%, and the remaining activities at each concentration of dehydroaltenusin were determined as percentages 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 dTTP into synthetic template-primers (i.e. poly(dA)/oligo(dT)$_{12–18}$, A/T = 2/1) in 60 min at 37° under the normal reaction conditions for each enzyme [6,7].

2.4. Investigation of cytotoxicity on cultured cells

For investigation of the *in vivo* effects of dehydroaltenusin, a human stomach cancer cell line, NUGC-3, was used. The cells were routinely cultured using Eagle's MEM (modified Eagle medium) supplemented with 10% fetal calf serum, 250 μg/mL fungizone, 300 μg/mL L-glutamine as standard medium, at 37° in a humidified atmosphere of 5% CO_2–95% air. Cytotoxicity of dehydroaltenusin was investigated as follows. High concentrations of dehydroaltenusin were dissolved in dimethylsulfoxide (DMSO) and stocked. Approximately 2×10^3 cells per well were inoculated in 96-well micro-plates, then dehydroaltenusin stock solution was diluted to various concentrations with standard medium, and applied to the each well. After incubation for 48 hr, survival rate was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [8].

2.5. Cell cycle analysis

Cellular DNA content for cell cycle analysis was determined as follows: aliquots of 3×10^5 NUGC-3 cells were inoculated into 35 cm^2 plastic culture flasks, and incubated with medium containing dehydroaltenusin diluted with 1% DMSO for 48 hr. Then, cells were collected by trypsinization and washed with cold PBS three times by centrifugation. Cells were fixed with 10 mL of PBS containing 70% (v/v) ethanol, and stored at 4°. DNA was stained with DAPI staining solution for at least 10 min at room temperature in the dark. Fluorescence intensity of 8000 stained cells was measured by flow cytometry (Patec cell counter analyzer, CCA). Cell cycle distribution was analyzed with the MULTICYCLE software program (version 2.5; Phoenix Flow Systems).

2.6. Measurement of inhibition of synthesis of DNA, RNA and protein

The effects of dehydroaltenusin on DNA, RNA and protein synthesis were examined independently. Aliquots of 1×10^4 NUGC-3 cells were inoculated into 96-well micro-plates and preincubated without dehydroaltenusin for several hours. Then, medium containing dehydroaltenusin diluted with 0.5% DMSO solution was applied to cells, and this time point was determined as 0 hr. At 0.5 hr, as probes for DNA, RNA and protein synthesis, [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine and L-[4,5-^{3}H]-leucine (final 3, 4, 4 μCi, respectively) were added. At specified time points, incubation was stopped and cell lysate was prepared to measure the incorporated radioactivity as described for the cytotoxicity assay.

2.7. Analysis of DNA fragmentation

Apoptosis was determined by assay of DNA fragmentation, by means of agarose electrophoresis. Total DNAs were extracted from 2×10^6 NUGC-3 cells following the method of Sambrook *et al*. [9] and 5 μg aliquots were separated by 1.4% (w/v) agarose gel electrophoresis in 40 mM Tris–5 mM sodium acetate–1 mM EDTA (pH 7.8) and stained with ethidium bromide. DNA bands were visualized under UV light.

3. Results and discussion

3.1. Preparation of dehydroaltenusin and its analogs

We screened for DNA polymerase inhibitors, and found a natural compound that inhibits mammalian pol. α activity but not pol. β activity from a fungus (*Acremonium* sp.) collected from fields in the vicinity of Noda city in Chiba prefecture, Japan. Small agar plugs on PDA plates were transferred into 2 L Erlenmeyer flasks containing 1 L of culture [10]. The fungus was grown in still culture at room temperature for 21 days. The culture was filtered through cheesecloth to remove the mycelia, and fluid was extracted with CH_2Cl_2. The organic extract was concentrated *in vacuo* to give crude extract, which was separated repeatedly on silica gel column chromatography eluted with organic solvents. HPLC equipped with a photo diode array detector was occasionally used for further purification. Positive fast atom bombardment high resolution (FABHR) mass, and ^1H-, ^{13}C- and distortionless enhancement by polarization transfer (DEPT) NMR spectroscopic analyses suggested that the four compounds purified by HPLC were dehydroaltenusin (**1** in Fig. 1) and its analogs (**2**–**4** in Fig. 1). Dehydroaltenusin (**1**), altenuene (**2**), alternariol (**3**) and alternariol-9-methyl ether (**4**) previously reported as an inhibitor of myosin light chain kinase [11,12], a toxin [13], a mycotoxin [14] and a phytotoxin [14,15]. Acetyl-dehydroaltenusin (**5**) was synthesized from dehydroaltenusin as described previously [16].

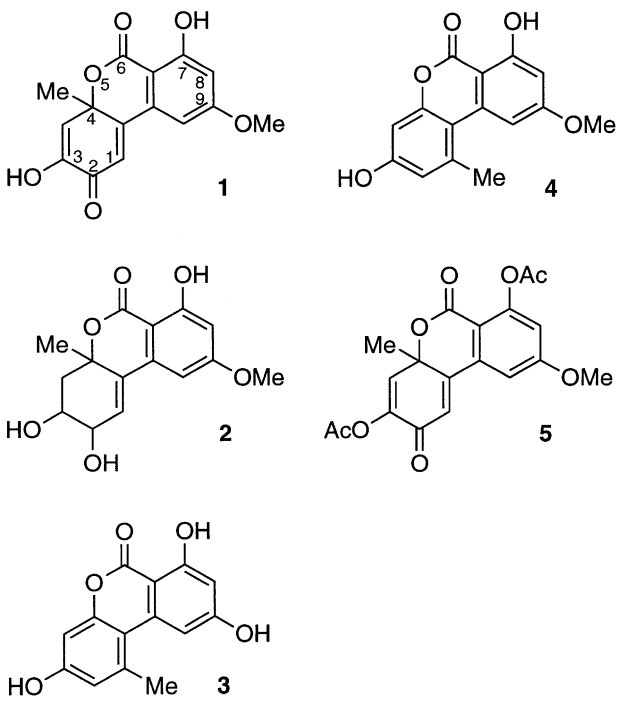

Fig. 1. Chemical structures of dehydroaltenusin and its analogs and derivative; (**1**) dehydroaltenusin, (**2**) altenuene, (**3**) alternariol, (**4**) alternariol-9-methyl ether, (**5**) acetyl-dehydroaltenusin.

3.2. Effects of dehydroaltenusin and its derivatives on the activities of mammalian DNA polymerase α and β

Fig. 2 shows the inhibition dose–response curves of dehydroaltenusin against calf pol. α and rat pol. β. The inhibitory effects of dehydroaltenusin (**1**) (Fig. 2A) and its derivatives (**2**–**5**) (Fig. 2B–E) were dose-dependent. Compounds **2** and **3** at 60 μM showed almost no inhibitory effect on either pol. α or β although the basic structure of dehydroaltenusin was three-dimensionally almost unchanged (Fig. 2B and C), indicating that the precise chemical structure is required for the inhibition. On the other hand, compounds **4** and **5** inhibited the activities of both pol. α and β (Fig. 2D and E). For pol. α, 50% inhibition by compounds **1**, **4** or **5** was observed at doses of less than 1, 11 and 8.1 μM, and almost complete inhibition was achieved at 5, 50 and 30 μM, respectively (Fig. 2A, D and E). For pol. β, concentrations of more than 60, 40 or 49 μM compounds **1**, **4** and **5** were required to achieve 50% inhibition, respectively (Fig. 2A, D and E). The inhibitory effects of compounds **4** and **5** against pol. α activity were relatively weak (Fig. 2D and E), although dehydroaltenusin was about 100-fold more effective against the activity of pol. α than that of pol. β (Fig. 2A). The effects of dehydroaltenusin on this enzyme were 10-fold stronger than that of aphidicolin, since aphidicolin, a potent inhibitor of replicative DNA polymerases, shows complete inhibition at 40 μM for mammalian pol. α [17].

Altenuene (**2**) has only a hydroxyl group at position 2 of dehydroaltenusin (Fig. 2B), and alternariol (**3**) also has a hydroxyl group at position 9 (Fig. 2C). Both agents lost the inhibitory activity. Therefore, the hydrophobic sites at these positions may be required for inhibition. The methyl ester at position 9 in dehydroaltenusin seemed to be important, because the alternariol-9-methyl ester (**4**) showed the inhibitory activities (Fig. 2C and D). Acetyl-dehydroaltenusin is acetylated at hydroxyl groups at position 3 and 7 in dehydroaltenusin. Acetylation at these positions did not affect the inhibitory effect (Fig. 2E), suggesting that for the inhibition, it is not necessary for these positions to be hydrophobic.

These results suggested that dehydroaltenusin binds three-dimensionally to a special pocket in mammalian pol. α. The structural relationship between the pol. α catalytic subunit and dehydroaltenusin should be investigated by NMR analysis and computer simulation similarly to our previous studies using fatty acids and pol. β [18–20]. Such studies are currently in process.

3.3. Cell growth inhibitory properties of dehydroaltenusin

As described in Section 1, we are engaged not only in investigating the three-dimensional structure of the

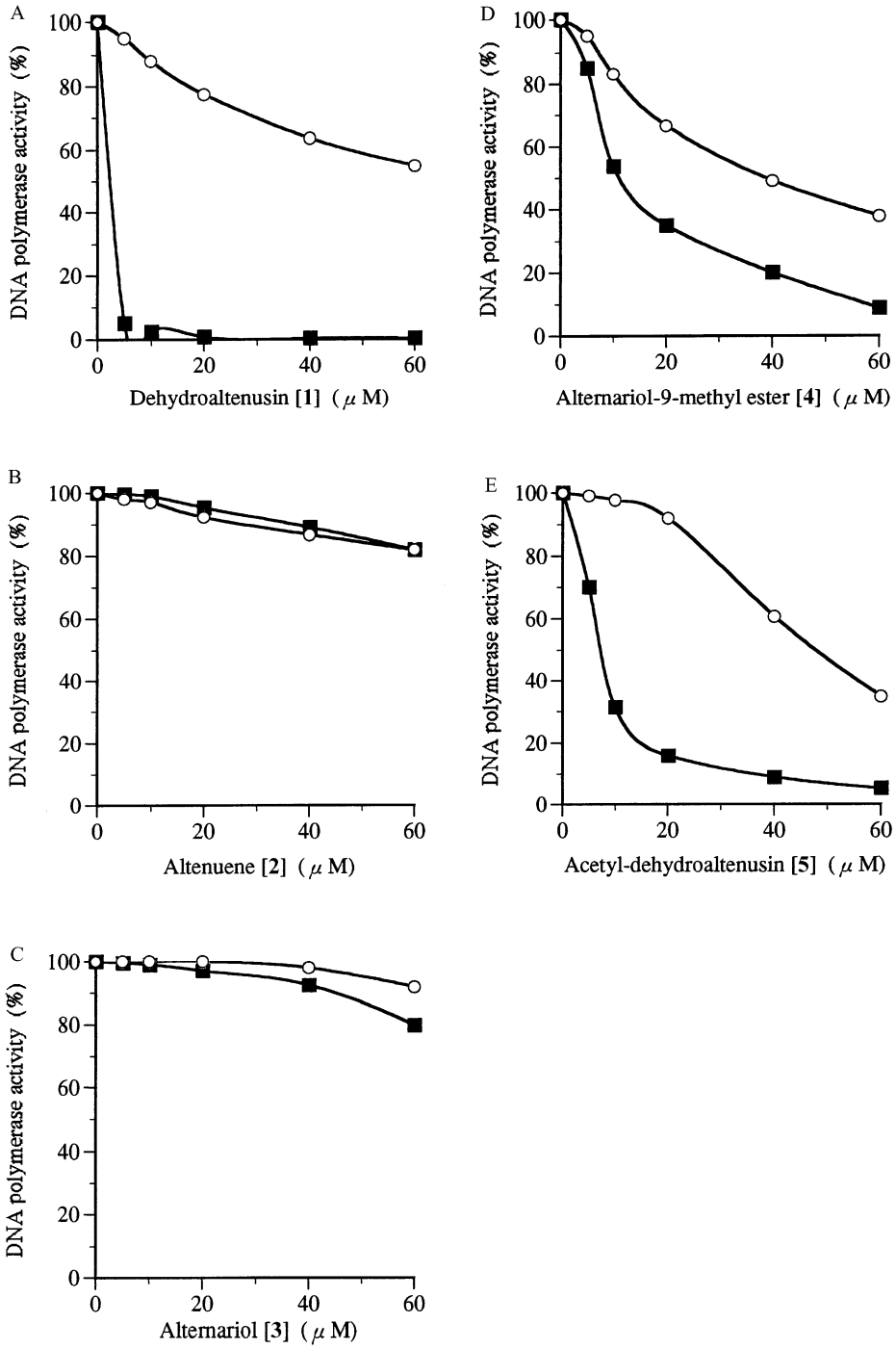

Fig. 2. Dose–response curves of dehydroaltenusin and its analogs and derivative. Effects of dehydroaltenusin (**1**), its analogs (**2**–**4**) and its derivative (**5**) on calf thymus pol. α (■) and rat recombinant pol. β (○) activities. The amount of each enzyme in the assay mixture was 0.05 unit.

dehydroaltenusin-binding site on mammalian pol. α, but also in attempts to understand the precise role of each polymerase *in vivo*, and to develop a drug design strategy for cancer chemotherapy agents. If dehydroaltenusin could act as an inhibitor *in vivo*, it would be not only a molecular tool useful for analyzing the polymerases, but may also be the lead compound of a group of potentially useful cancer chemotherapy agents. From this viewpoint, we next investigated the intracellular actions of dehydroaltenusin. Using the human gastric cancer cell line NUGC-3, we examined the cytotoxic effects of dehydroaltenusin on tumor cells (Fig. 3). As shown in Fig. 3, the growth inhibition was dose-dependent, and the LD_{50} concentration of dehydroaltenusin was 40 μM. Since the IC_{50} values of dehydroaltenusin for pol. α and pol. β were about 1 and 80 μM, respectively (Fig. 2A), the IC_{50} value for pol. α was obviously much stronger than the LD_{50} value, but the IC_{50} value for pol. β was weaker than the LD_{50} value, suggesting

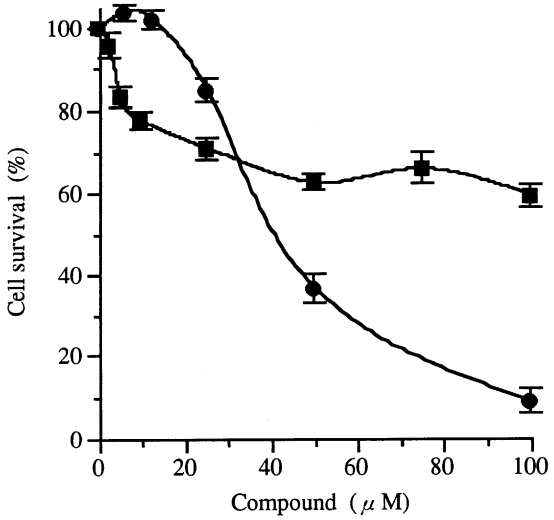

Fig. 3. Effects of dehydroaltenusin on proliferation of NUGC-3 cancer cells. Dose–response curves of growth inhibition of the human stomach cancer cell line NUGC-3 by dehydroaltenusin (●) or aphidicolin (■). The assays were carried out under the conditions described in Section 2 with the compounds at the indicated concentrations. Survival rate was determined by MTT assay [8]. Data are shown as means ± SEM for three independent experiments.

that the growth inhibition was due mostly to prevention of pol. α activity *in vivo*. Aphidicolin which is a pol. α, δ and ε inhibitor [2,3] also suppressed the cell growth, but the LD_{50} value was more than 100 μM (Fig. 3). The inhibitory effect of cell growth by dehydroaltenusin was stronger than that by aphidicolin. NUGC-3 cells is a adherent cell line. BALL-1 cells (human acute lymphoblastoid leukemia cell line) as a non-adherent cell line were also investigated and obtained the same cell growth inhibitory results as the NUGC-3 cells (data not shown). If the growth inhibition by dehydroaltenusin was based on preventing the pol. α activity *in vivo*, RNA priming might not be affected because it does not inhibit the primase activity in pol. α [1].

To confirm that dehydroaltenusin inhibits the cell proliferation, we analyzed whether cell cycle-dependent arrest by dehydroaltenusin occurred by flow cytometry. As shown in Fig. 4, the NUGC-3 cells were arrested in G1/S-phase by incubation with this agent at 50 μM for 48 hr. Dehydroaltenusin might block the early step of S-phase. The results were more directly confirmed by the incorporation experiment. Fig. 5A–C show the incorporation of [^{3}H]-labeled thymidine, [^{3}H]-uridine and [^{3}H]-leucine into NUGC-3 cells, respectively. Dehydroaltenusin inhibited only the incorporation of [^{3}H]-thymidine into the cells. The [^{3}H]-thymidine incorporation was decreased by 45% of control in the presence of 30 μM dehydroaltenusin (Fig. 5A). Neither [^{3}H]-uridine nor [^{3}H]-leucine incorporation were affected by dehydroaltenusin (Fig. 5B and C). These observations indicated that dehydroaltenusin must inhibit cell growth by blocking the primary step of DNA replication, i.e. by inhibiting pol. α activity *in vivo*.

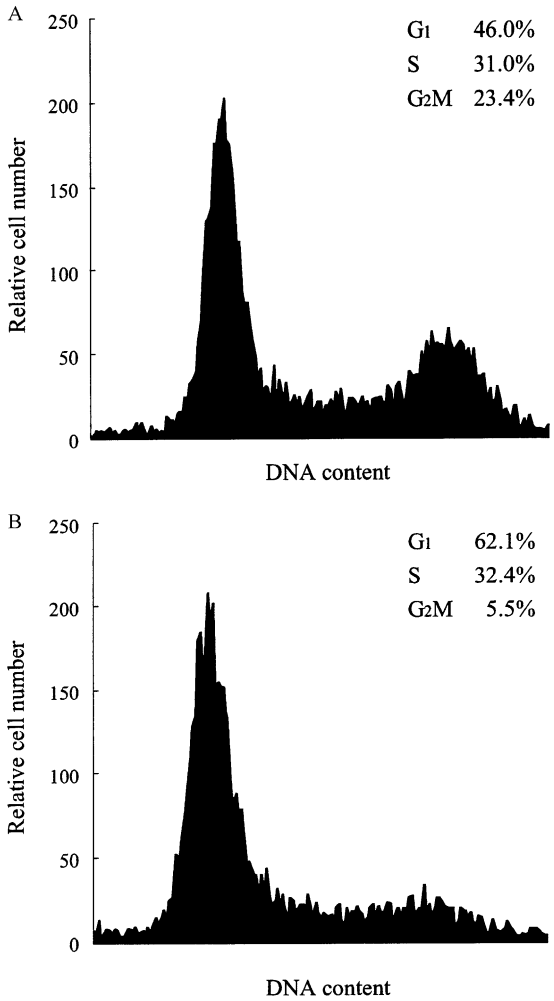

Fig. 4. Flow cytometric analysis of cell cycle perturbation by dehydroaltenusin. NUGC-3 cells were incubated without (control, A) or with 50 μM dehydroaltenusin (B) for 48 hr. DNA was stained with DAPI solution. Fluorescence intensity of the 8000 stained cells was measured by flow cytometry. All experiments were performed three times.

3.4. Effect of dehydroaltenusin on apoptotic cell death

To examine whether the decrease of cell numbers by dehydroaltenusin (Fig. 3) was due to apoptosis, DNA fragmentation was analyzed by electrophoresis. DNA ladder formation was dose-dependently observed in NUGC-3 cells treated with 0–100 μM dehydroaltenusin for 48 hr (Fig. 6). The ladders appeared with 75 μM dehydroaltenusin as shown in Fig. 6. Therefore, both the inhibition of *in vivo* DNA synthesis and the apoptotic effect were performed in the cells at the concentration of more than 75 μM (Figs. 5 and 6). Such ladders were not evident for the initial 12 hr but were apparent at 24 hr and thereafter (data not shown). This indicates that the inhibition of pol. α activity by dehydroaltenusin has a strong apoptotic effect on a human cancer cells. The effect of dehydroaltenunsin must occur in the combination of the growth arrest and the cell death.

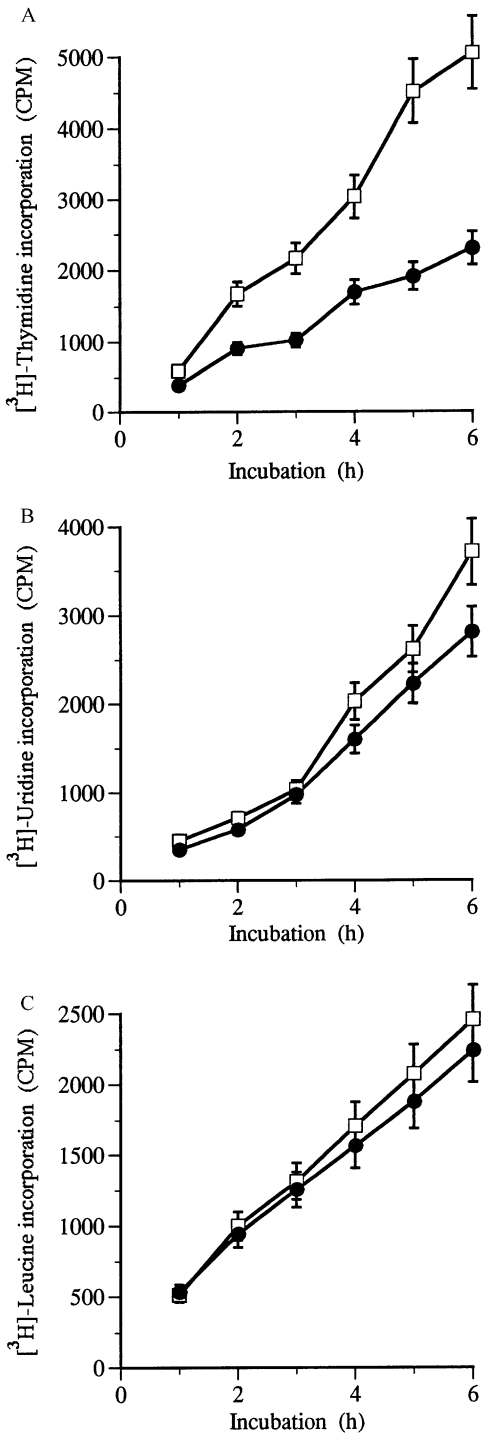

Fig. 5. Measurement of DNA, RNA and protein synthesis in NUGC-3 cells incubated with dehydroaltenusin. 1×10^4 NUGC-3 cells were incubated without (control, □) or with 30 μM dehydroaltenusin (●) from 0 hr, and radiolabeled thymidine, uridine or leucine was added at 0.5 hr. These three metabolites were measured simultaneously. (A–C) The incorporation of thymidine, uridine and leucine, respectively. Each point represents the average of triplicate experiments and bars indicate SD.

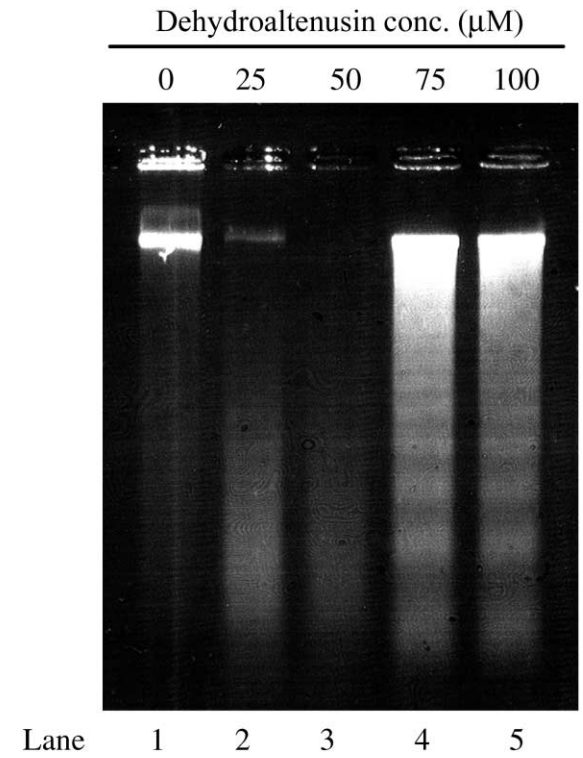

Fig. 6. DNA fragmentation of dehydroaltenusin-treated cells on agarose gel electrophoresis. Lanes 1–5, NUGC-3 cells (2×10^6) were incubated for 48 hr with 0, 25, 50, 75 and 100 μM dehydroaltenusin, respectively. Total DNA were then extracted and analyzed by 1.4% agarose gel electrophoresis.

The results of dehydroaltenusin in Figs. 4 and 5 would suggest that the mechanism of dehydroaltenusin action is the inhibition of pol. α. However, aphidicolin reversibly inhibits various replicative DNA polymerases without killing them [21]. Therefore, an additional mechanism using a yet unknown activity of dehydroaltenusin causes its apoptotic functions. Especially since dehydroaltenusin also inhibits myosin light chain kinase [12].

As described in Section 1, we have been engaged in analyzing the structure and function of pol. α using dehydroaltenusin from three different viewpoints. This agent could be a useful inhibitor not only to investigate the three-dimensional structure of the dehydroaltenusin binding site on mammalian pol. α, but also to analyze the precise role of pol. α *in vivo*. The results of the present study indicated that dehydroaltenusin is useful as a mammalian pol. α-specific inhibitor both *in vivo* and *in vitro*. In addition, we also investigated whether the agent could be a useful key drug to develop a design strategy for cancer chemotherapy agents. As shown in this paper, the agent should also be considered as the lead compound of a group of potentially useful cancer chemotherapy agents.

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