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Anti-tumor effects of dehydroaltenusin, a specific inhibitor of mammalian DNA polymerase α

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Abstract

In the screening of selective inhibitors of eukaryotic DNA polymerases (pols), dehydroaltenusin was found to be an inhibitor of pol α from a fungus (*Alternaria tennuis*). We succeeded in chemically synthesizing dehydroaltenusin, and the compound inhibited only mammalian pol α with IC_{50} value of 0.5 μM, and did not influence the activities of other replicative pols such as pols δ and ε, but also showed no effect on pol α activity from another vertebrate, fish, or from a plant species. Dehydroaltenusin also had no influence on the other pols and DNA metabolic enzymes tested. The compound also inhibited the proliferation of human cancer cells with LD_{50} values of 38.0–44.4 μM. In an *in vivo* anti-tumor assay on nude mice bearing solid tumors of HeLa cells, dehydroaltenusin was shown to be a promising suppressor of solid tumors. Histopathological examination revealed that increased tumor necrosis and decreased mitotic index were apparently detected by the compound *in vivo*. Therefore, dehydroaltenusin could be of interest as not only a mammalian pol α-specific inhibitor, but also as a candidate drug for anti-cancer treatment.

Keywords: Dehydroaltenusin; Enzyme inhibitor; DNA polymerase α; DNA replication; Cytotoxicity; Anti-tumor activity

The human genome encodes 16 DNA polymerases (pols) which control cellular DNA synthesis [1]. Eukaryotic cells reportedly contain three replicative types: pols α, δ, and ε, mitochondrial pol γ, and at least 12 repair types: pols β, δ, ε, ζ, η, θ, ι, κ, λ, μ, and σ, and REV1 [2]. Selective inhibitors of each pol are useful tools and molecular probes to distinguish pols and to clarify their biological and *in vivo* functions [3]. For example, aphidicolin is a selective inhibitor of both pol α and eukaryotic DNA replicative pols δ and ε, indicating that these pols are essential for DNA replication [4], and this inhibitor has been very useful for studying the DNA replication system [5]; however, aphidicolin cannot be an inhibitor capable of distinguishing pols α, δ, and ε. N2-butylphenyl-dGTP and 2-butylanilino-dATP

Abbreviations: DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetracetic acid; IC_{50}, 50% inhibitory concentration; LD_{50}, 50% lethal dose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; dNTP, 2′-deoxyribonucleotide 5′-triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; PBS, phosphate-buffered saline; pol, DNA-directed DNA polymerase (EC 2.7.7.7).

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are known as selective pol α inhibitors and useful for discriminating pol α from pols δ and ε [6].

We therefore established an assay to detect pol inhibitors, and screened for natural sources for more than 10 years, finding an interesting inhibitor that influenced only the activity of mammalian pol α. The agent, which was determined to be dehydroaltenusin has been reported to be isolated from the culture broth of a fungus, *Penicillium verruculosum* IAM-13756 [7,8]. We established a total chemical synthesis method for dehydroaltenusin, and succeeded in completely synthesizing the compound [9,10].

Pol α inhibitors could therefore be employed as anticancer chemotherapy agents because pol α is an essential enzyme for DNA replication and subsequently for cell division [1,2]. In this report, we report that chemically synthesized dehydroaltenusin can inhibit mammalian pol α activity, mammalian cultured cell growth, and *in vivo* solid tumor proliferation, and discuss whether the compound could help to prevent cancer, and be developed as a drug with anti-cancer activity.

Materials and methods

Materials. Dehydroaltenusin (Fig. 1) was chemically synthesized and supplied as described previously [9,10]. Nucleotides and chemically synthesized DNA template-primers such as [^{3}H]-2′-deoxythymidine 5′-triphosphate (dTTP, 43 Ci/mmol) and poly(dA), oligo(dT)$_{12–18}$ were purchased from GE Healthcare Bio-Science Corp. (Buckinghamshire, UK). A human lung cancer cell line, A-549, a human acute lymphoblastoid leukemia cell line, BALL-1, a human cervix carcinoma cell line, HeLa, and a human stomach cancer cell line, NUGC-3, were obtained from Health Science Research Bank (Osaka, Japan). The antibody for MIB-1 and its staining kit (chemMateENVISION kit) were obtained from Dako Japan (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

DNA polymerases. Pol α was purified from calf thymus by immunoaffinity column chromatography as described previously [11]. The amino-terminal (1–329) and the carboxyl-terminal (1280–1465) truncation mutant of the largest subunit of mouse pol α, p110, was prepared as described previously [12]. The smallest subunit of pol α, p46, was purified as described previously [13]. Pol β was purified from a recombinant plasmid expressing rat pol β [14]. The gene encoding human pol γ catalytic subunit was cloned into pFastBac. 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 Japan, Tokyo, Japan) [15]. 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 chromatography, respectively [16]. Fish pols α and δ were purified from the testis of cherry salmon (*Oncorhynchus masou*) [17]. Insect pols α, δ, and ε were purified from early embryos of fruit fly (*Drosophila melanogaster*) as described previously [18,19]. Pols I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi et al. [20]. The Klenow fragment of pol I from *Escherichia coli* was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). *Taq* pol, T4 pol, 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, USA).

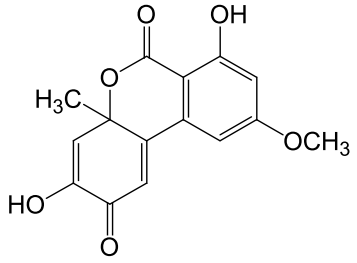

Fig. 1. Chemical structure of dehydroaltenusin.

DNA polymerase assay. The reaction mixtures for pol α, pol β, fish pols, insect pols, plant pols, and prokaryotic pols were described previously [21,22]; those for pol γ, and pols δ and ε were as described by Umeda et al. [15] and Ogawa et al. [23], respectively. For the pols, poly(dA)/oligo(dT)$_{12–18}$ (A/T = 2/1) and [^{3}H]dTTP were used as DNA template-primer and nucleotide (i.e., 2′-deoxyribonucleotide 5′-triphosphate, dNTP) substrate, respectively. Dehydroaltenusin was dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Four microliter of each sonicated sample was mixed with 16 μl of each enzyme (final 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM ethylenediaminetetracetic acid (EDTA), and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μl) were added to 16 μl of each enzyme standard reaction mixture, and incubated 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 dNTP (i.e., dTTP) into synthetic DNA template-primer in 60 min at 37 °C under the normal reaction conditions for each enzyme [21,22].

Other enzyme assays. DNA primase activity of pol α and the activities of calf terminal deoxynucleotidyltransferase, T4 polynucleotide kinase, and bovine deoxyribonuclease I were measured using standard assays according to the manufacturer's specifications as described by Tamiya-Koizumi et al. [24], Uchiyama et al. [25], Soltis et al. [26], and Lu and Sakaguchi [27], respectively.

Investigation of cytotoxicity on cultured cells. Human cancer cell lines such as A-549, BALL-1 HeLa, and NUGC-3 cells were cultured in Eagle's minimum essential medium (MEM) 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. For cell cytotoxicity, cells were plated at 3×10^5 into a 96-well microplate with various concentrations of dehydroaltenusin. Dehydroaltenusin was dissolved in DMSO at a concentration of 10 mM as a stock solution. The stock solution was diluted to the appropriate final concentrations with growth medium just before use. Cell viability was determined using MTT assay [28].

In vivo assessment of anti-tumor assay. Male BALB/c *nu/nu* mice, six weeks of age (20–22 g), were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice receiving standard laboratory chow and water ad libitum were acclimatized for one week before the experiment. For *in vivo* experiments, HeLa cells (1×10^6 cells/mouse) were subcutaneously inoculated into nude mice. At 37 days after implantation, the tumor sizes in all mice were measured at 2-day intervals. Mice bearing solid tumors that had grown to 25–35 mm^3 in volume (tumor volume = length × (width)^2 × 0.5) at 12 days after implantation were used to assess the anti-tumor effect. They were divided randomly into 2 groups ($n = 5$/group): a control group injected with 0.1 ml PBS alone, and another group injected with dehydroaltenusin dissolved in PBS at a dose of 20 mg/kg. The above administrations all took place between days 12 and 39 subsequent to implantation. All mice were injected subcutaneously 10 times at 2-day intervals with the compound and PBS alone (control). Tumor growth was measured at 2-day intervals for 27 days after implantation, and the statistics were analyzed using Student's *t* test. At the end of *in vivo* anti-tumor assay, some mice treated with dehydroaltenusin and PBS were separately examined to observe the pathohistological features of the tumors and major organs such as the lung, heart, spleen, stomach, liver, pancreas, kidney, intestine, and brain.