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Acylated catechin derivatives: inhibitors of DNA polymerase and angiogenesis

Yoshiyuki Mizushina[1,2], Akiko Saito[3], Keiko Horikawa[4], Noriyuki Nakajima[4], Akira Tanaka[5], Hiromi Yoshida[1,2], Kiminori Matsubara[2,6]

[1]Laboratory of Food and Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan, [2]Cooperative Research Center of Life Sciences, Kobe-Gakuin University, Chuo-ku, Kobe, Hyogo 651-8586, Japan, [3]Department of Applied Chemistry, Faculty of Engineering, Osaka Electro-Communication University, Neyagawa-shi, Osaka 572-8530, Japan, [4]Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan, [5]Department of Bioresources Science, College of Technology, Toyama Prefectural University, Kosugi, Toyama 939-0398, Japan, [6]Department of Human Life Science Education, Graduate School of Education, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8524, Japan

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1. ABSTRACT

We previously found that some catechins, such as epigallocatechin-3-*O*-gallate (EGCG), inhibit the activities of eukaryotic DNA polymerases (pols). In this study, we found that catechins conjugated with fatty acid (3-*O*-acylcatechins) are stronger inhibitors of mammalian pol than epicatechins conjugated with fatty acid (3-*O*-acylepicatechins). Moreover, 3-*O*-acylcatechins are more potent inhibitors of cultured cell growth both of the human colon carcinoma cell line (HCT116 cells) and human umbilical vein endothelial cell (HUVEC) line, as well as angiogenesis by comparison with 3-*O*-acylepicatechins. Catechin conjugated with stearic acid [(2*R*,3*S*)-3',4',5,7-tetrahydroxyflavan-3-yl octadecanoate; C-C18] was the strongest inhibitor in replicative pol alpha and repair-related pol beta, as well as the cultured cell growth and angiogenesis assays in the compounds tested. C-C18 also suppressed HUVEC tube formation on reconstituted basement membrane suggesting that it affected not only pols but also signal transduction pathways in HUVECs. These data indicate that the acylated catechins target both pols and angiogenesis as anti-cancer agents. Moreover, the results suggest that acylation of catechin is an effective chemical modification to improve the anti-cancer activity of catechin.

2. INTRODUCTION

Natural polyphenols distributed in plants have diverse biological activities: anti-oxidant, anti-cancer, and anti-inflammation activities (1). Recent evidence shows that polyphenols have pharmacological effects and are useful models for the design of new drugs. Green tea catechins are a particularly attractive set of polyphenols because they have been shown to lower the risk of various cancers and suppress cancer cell growth and angiogenesis (2). Angiogenesis is involved in tumor growth and metastasis, atherosclerosis and diabetic retinopathy (3). Recently, significant resources have been directed towards developing more effective angiogenesis inhibitors (4). We have identified some angiogenesis inhibitors in natural products including vitamin B_6 (5-7), algal polysaccharides (8, 9), algal carotenoids (10, 11) and phenolic compounds (12). Recently, we have reported that some DNA polymerase (pol) inhibitors, such as a polyphenolic compound (13), glycolipids from spinach (14) as well as vitamin K_3 (15) and its derivatives (16, 17), have a strong inhibitory effect on angiogenesis *in vitro*.

Among DNA metabolic enzymes, pol catalyzes the addition of deoxyribonucleotides to the 3'-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules (18). The human genome encodes 15 pols that conduct cellular DNA synthesis (19). Eukaryotic cells reportedly contain three replicative types, namely pols alpha, delta and epsilon; mitochondrial pol gamma; and thirteen repair types, namely pols beta, delta, epsilon, zeta, eta, theta, iota, kappa, lambda, mu and nu, REV1 and terminal deoxynucleotidyl transferase (TdT) (20). DNA metabolic enzymes such as pols are not only essential for DNA replication, repair and recombination, but are also involved in cell division. Selective inhibitors of eukaryotic pols 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 (21–23).

We previously reported that certain catechins, such as epigallocatechin-3-*O*-gallate (EGCG), inhibit the activities of mammalian pols (24). Recently, our efforts have been directed towards the synthesis of potent catechin and epicatechin derivatives conjugated with fatty acids, such as 3-*O*-acylcatechins and 3-*O*-acylepicatechins, respectively. In this study, the characteristics of the acylated catechin and epicatechin derivatives as pol and angiogenesis inhibitors are discussed.

3. MATERIALS AND METHODS

3.1. Materials

(+)-Catechin (C) and (–)-epicatechin (EC) were produced by Mitsui Norin Co. Ltd. (Shizuoka, Japan). The acylated catechin and epicatechin derivatives (3-*O*-acyl-flavan-3-ols), which are conjugated fatty acids, were chemically synthesized as described previously (25, 26). The structures of all 15 synthesized compounds are shown in Figure 1. Nucleotides, such as [^{3}H]-deoxythymidine 5'-triphosphate (dTTP) (43 Ci/mmol), and chemically synthesized DNA template, such as poly(dA), were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). DNA primer, such as oligo(dT)$_{18}$, was customized by Sigma-Aldrich K. K. (Hokkaido, Japan). Human recombinant vascular endothelial growth factor (VEGF) was obtained from R&D systems (MN, USA). Other reagents were special grade as commercially available.

3.2. Preparation of DNA polymerases and other DNA metabolic enzymes

Pol alpha was purified from calf thymus by immuno-affinity column chromatography, as described by Tamai *et al.* (27). Recombinant rat pol beta was purified from *Escherichia coli* JMpB5, as described by Date *et al.* (28). The human pol gamma catalytic gene 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) (29). Human pols delta and epsilon were purified by nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pols delta and epsilon-conjugated affinity column chromatography, respectively (30). Fruit fly pols alpha, delta and epsilon were purified from early embryos of *Drosophila melanogaster*, as described by Aoyagi *et al.* (31, 32). Budding yeast (*Saccharomyces cerevisiae*) pol alpha was purified by ion-exchange column chromatography as described by Hamatake *et al.* (33). Pol alpha from a higher plant, cauliflower inflorescence, was purified according to the methods outlined by Sakaguchi *et al.* (34). The Klenow fragment of pol I from *E. coli* were purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). *Taq* pol, T4 pol, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara Bio (Tokyo, Japan). Bovine pancreas DNase I were obtained from Stratagene Cloning Systems (La Jolla, CA, USA).

3.3. DNA polymerase assays

The reaction mixtures for pol alpha, pol beta, plant pols and prokaryotic pols were described previously (35–37); those for pol gamma, and pols delta and epsilon were as described by Umeda *et al.* (29) and Ogawa *et al.* (38), respectively. For pols, poly(dA)/oligo(dT)$_{18}$ (A/T = 2/1) and 2'-deoxythymidine 5'-triphosphate (dTTP) were used as the DNA template-primer and nucleotide [i.e., 2'-deoxynucleoside 5'-triphosphate (dNTP)] substrate, respectively.

The compounds were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Aliquots of 4 uL sonicated samples were mixed with 16 uL of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0˚C for 10 min. These inhibitor-enzyme mixtures (8 uL) were added to 16 uL of each of the enzyme standard reaction mixtures (final concentration of 50 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 1 mM $MgCl_2$, 15% glycerol, 10 uM poly(dA)/oligo(dT)$_{18}$ and 10 uM [^{3}H]-dTTP), 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 dNTP (i.e., dTTP) into synthetic DNA template-primers in 60 min at 37˚C under the normal reaction conditions for each enzyme (35–37).

3.4. Other DNA metabolic enzymes assays

The activities of calf primase of pol alpha, T7 RNA polymerase, T4 polynucleotide kinase and bovine DNase I were measured in standard assays according to the manufacturer's specifications, as described by Tamiya-Koizumi *et al.* (39), Nakayama *et al.* (40), Soltis *et al.* (41) and Lu *et al.* (42), respectively.

3.5. Cell culture and measurement of cell viability

The HCT116 human colon carcinoma cell line and HUVEC line were obtained from American Type Culture Collection (ATCC) (Manassas, VA). HCT116 cells were cultured in McCoy's 5A Medium supplemented with 10% foetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL). HUVECs were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 4.5 g of glucose per liter plus 10% fetal calf serum, 5 mM L-glutamine, 50 units/mL penicillin and 50 units/mL streptomycin. These cells were cultured at 37˚C in a humid atmosphere of 5% CO_2 / 95% air. For the cell growth assay, the cells were plated at 1 x 10^4 cells into each well of 96-well microplates with various concentrations of acyl-catechin compound. These compounds were dissolved in DMSO at a concentration of 10 mM as a stock solution. The stock solutions were diluted to the appropriate final concentrations with growth medium as 0.5 % DMSO just before use. The cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay (43).

3.6. *Ex vivo* angiogenesis assay

Male Wistar rats (6 week old, Clea Japan, Inc., Tokyo, Japan) were housed two to a metal cages in a room with controlled temperature (24 ± 1˚C) and a 12-h light : dark cycle (lights on, 08:00–20:00 h). They had free access to diets and deionized water. The rats were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. The *ex vivo* angiogenesis assay was performed according to slightly modified methods as described before (44, 45). Briefly, a male Wistar rat (body weight ~ 200 g) was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. A thoracic aorta was removed and washed with RPMI 1640 medium to avoid contamination with blood. It was then turned inside out, and cut into short segments about 1–1.5 mm. Collagen gel (gel matrix solution) was made with 8 volumes of porcine tendon collagen solution (3 mg/ml) (Cellmatrix Ia, Nitta Gelatin Co., Osaka, Japan), 1 volume of 10×Eagle's MEM (Gibco, New York, USA), 1 volume of reconstitution buffer (0.08 M NaOH and 200 mM HEPES). These solutions were mixed gently at 4˚C. Each aortic segment was placed in the center of a well on a 6-well culture plate and covered with 0.5 mL of gel matrix solution reconstituted as described. The solution was allowed to gel at 37˚C for 20 min. Culture medium [RPMI 1640 medium (Gibco, New York, USA) containing 1% of ITS+ (Becton Dickinson Labware, MA, USA)] with various concentration of acyl-catechin or vehicle (DMSO) was prepared as the same way described above. Then, the collagen gel was overlaid with 2 mL of culture medium. Incubation was carried out for 10 days in a fully humidified system of 5 % CO_2 in the air at 37˚C. The medium was changed on day 7 of the culture. An estimation of the length of the capillary was performed under phase-contrast microscopy by measuring the distance from the cut end of the aortic segment to the approximate mean point of capillary. Microscopic fields were photographed with a digital camera (Nikon, COOLPICKS 950). The length of the capillary was measured using Adobe Photoshop software. Each reported value represents the average of three culture samples.

3.7. Endothelial cells

HUVECs were purchased from Kurabo Industries (Osaka, Japan). Cells were grown in the medium, HuMedia EG 2 (Kurabo Industries, Osaka, Japan), which was modified MCDB 131 medium containing 2% fetal bovine serum (FBS), 10 ng/ml recombinant human epidermal growth factor (EGF), 1 ug/mL hydrocortisone, 50 ug/mL gentamicin, 50 ng/mL amphotericin B, 5 ng/mL recombinant human basic fibroblast growth factor (bFGF) and 10 ug/mL heparin, at 37˚C in a humidified 5% CO_2. Subcultures were obtained by treating the HUVEC cultures with 0.025% trypsin-0.01% EDTA solution. HUVEC at passage three to five were used in this experiment.

3.8. HUVEC tube formation assay

Tube formation assay was performed using an *In vitro* Angiogenesis Assay Kit (Chemicon International, Inc., Temecula, CA, U.S.A.). Briefly, solid gels were prepared according to the manufacture manual on a 96-well tissue culture plate. HUVECs (1 X 10^5 cells/mL) in HuMedia EG-2 medium containing acyl-catechin (1–100 uM) or vehicle (DMSO) were seeded 100 uL per well onto the surface of the solid gel, ECMatrixTM. The cells were incubated for 12 h at 37˚C in a CO_2 incubator. Tube formation was observed under an inverted light microscope at 40 X magnification. Microscopic fields were photographed with a digital camera (Nikon, COOLPICKS 950). The total length of tube structures in each photograph was measured using Adobe Photoshop software. Each reported value represents the average of three samples.

4. RESULTS

4.1. *In vitro* DNA polymerase inhibition

4.1.1. Effect of the derivatives of 3-*O*-acylcatechin and 3-*O*-acylepicatechin on the activities of mammalian pol activity

The 15 compounds of the acylated catechin and epicatechin derivatives (3-*O*-acyl-flavan-3-ols) are prepared (Figure 1). (+)-Catechin (C) and its acylated derivatives are compound 1 and compounds 2–9, respectively, and (–)-epicatechin (EC) and its acylated

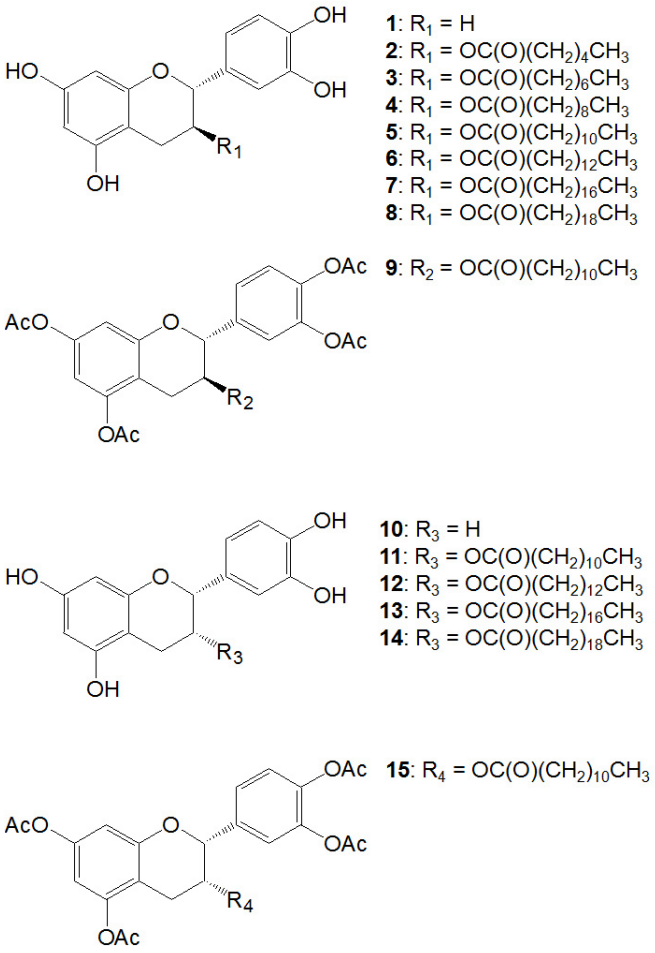

Figure 1. Chemical structures of the derivatives of acylated catechin (compound 1–9) and epicatechin (compound 10–15). Compound 1, (+)-catechin (C); compound 2, C-C6; compound 3, C-C8; compound 4, C-C10; compound 5, C-C12; compound 6, C-C14; compound 7, C-C16; compound 8, C-C18; compound 9, acetylated compound 5 (C-C12Ac); Compound 10, (–)-epicatechin (EC); compound 11, EC-C12; compound 12, EC-C14; compound 13, EC-C16; compound 14, EC-C18; and compound 15, acetylated compound 11 (EC-C12Ac).

derivatives are compound 10 and compounds 11–15, respectively. The purification grade of each compound was >98%. The inhibitory activity of calf pol alpha and rat pol beta against each compound was then investigated. For mammalian pols, pols alpha and beta were used as representative replicative pol and repair-related pols, respectively (19, 20).

In the presence of 25 uM of catechin (compound 1) or epicatechin (compound 10) there was no detectable effect on the activities of pols alpha and beta, whereas the same concentration of acylated derivatives caused significant inhibition of enzyme activity (Figure 2). The inhibitory effect of the derivatives conjugated with longer chain fatty acids was stronger than that of the corresponding shorter acylated derivatives. Compound 8 [(2*R*,3*S*)-3',4',5,7-tetrahydroxyflavan-3-yl octadecanoate; C-C18], which is conjugated with C18-fatty acid (stearic acid), was the most potent inhibitor tested. Interestingly, compound 5 (C-C12), which is the C12-fatty acid acylated catechin derivative, inhibited the activities of pols alpha and beta, whereas compound 9 (C-C12Ac), which is the acetylated compound 5, had no such inhibitory effect. These results suggested that both the acyl-chain group and hydroxyl group of catechin are essential for the inhibitory activity. For the 3-*O*-acylcatechins derivatives (compounds 1–9), the effect of the nine compounds can be ranked as follows: compound 8 (C-C18) > compound 7 (C-C16) > compound 6 (C-C14) > compound 5 (C-C12) > compound 4 (C-C10) > compound 3 (C-C8) > compound 2 (C-C6) > compound 1 (C) = compound 9 (C-C12Ac). When the acyl-chain of the catechin derivative was equivalent to that of the epicatechin derivatives, 3-*O*-acylcatechins (compounds 2–8) were always found to be stronger inhibitors of pols than 3-*O*-acylepicatechins (compounds 11–14). Therefore, the stereochemistry of the 3-*O*-position in the catechin may be important for the inhibition. The inhibitory effect of these compounds on pol alpha showed almost the same tendency as that observed for pol beta. When activated DNA (i.e., DNA digested by bovine deoxyribonuclease I) and dNTP were used as the DNA template-primer and nucleotide substrate, respectively, instead of

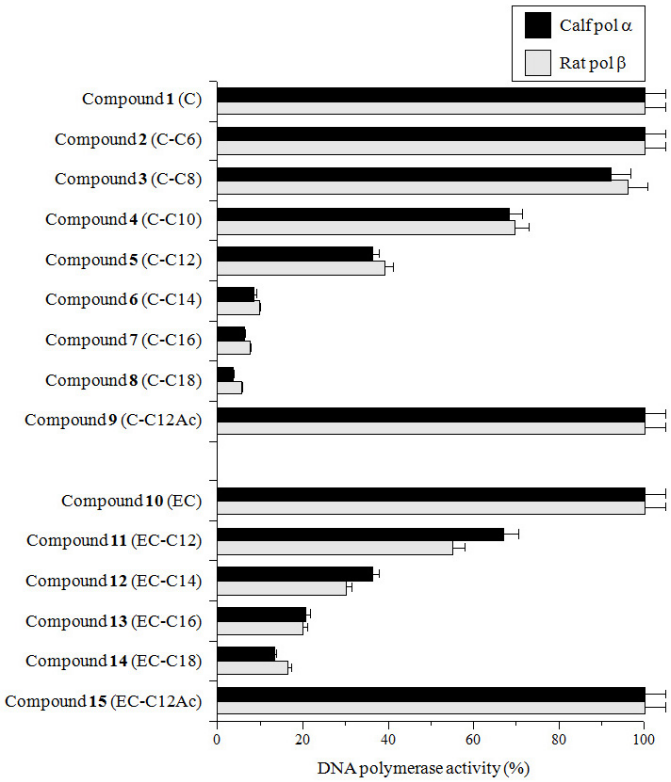

Figure 2. Effect of acylated catechin and epicatechin derivatives (compounds 1–15) on the activity of mammalian pols. Each compound at 25 uM was incubated with calf pol alpha and rat pol beta (0.05 units each). Pol activity in the absence of the compounds was taken as 100% (35–37). Data are the mean ± S.E. of three independent experiments.

poly(dA)/oligo(dT)$_{18}$ (A/T = 2/1) and dTTP, the mode of inhibition by these compounds did not change (data not shown).

4.1.2. Effect of compound 8 (C-C18) on various pols and other DNA metabolic enzymes

Of all the compounds (compounds 1–15) investigated in this study compound 8 (C-C18) was the strongest inhibitor of mammalian pols alpha and beta (Figure 2). Therefore, we decided to focus our studies on compound 8. The IC_{50} values for the inhibition of DNA replicative pols, such as pols alpha, delta and epsilon, mitochondrial pol gamma, and DNA repair-related pol beta for compound 8 ranged between 9.4–14.1 uM (Table 1). The pol alpha inhibitory effect of this compound was the strongest among these five major classes of mammalian pols tested. Compound 8 also inhibited the activities of pols alpha, delta and epsilon from insect (*Drosophila melanogaster*) and pol alpha from protistan (*Saccharomyces cerevisiae*) as well as mammalian pols. By contrast, this compound did not inhibit the activity of pol alpha from plant (cauliflower), prokaryotic pols, such as *Escherichia coli* pol I (Klenow fragment), *Taq* pol and T4 pol, and other DNA-metabolic enzymes such as calf primase of pol alpha, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I (DNase I). As a result, compound 8 was shown to be a potent and selective inhibitor of mammalian pols, especially DNA replicative pol alpha.

To determine whether the inhibition resulted from binding to DNA or binding to the enzyme, the interaction of compound 8 with dsDNA was investigated by measuring the thermal transition of dsDNA with or without the compound. The melting temperature (Tm) of dsDNA mixed with an excess amount of compound 8 (200 uM) was measured using a spectrophotometer equipped with a thermoelectric cell holder. In the temperature range (75–98˚C) used, no thermal transition (Tm) was observed. By contrast, 15 uM of ethidium bromide (EtBr), a typical intercalating compound that was used as a positive control, produced a clear thermal transition. These observations indicate that compound 8 binds directly to the enzyme to inhibit its activity, rather than intercalating with the DNA as a template-primer.

4.1.3. Mode of inhibition of mammalian pols alpha and beta by compound 8 (C-C18)

Next, to elucidate the mechanism by which compound 8 (C-C18) inhibits mammalian pols alpha and beta, the extent of inhibition as a function of the DNA template-primer or dNTP substrate concentrations was studied. In kinetic analysis, poly (dA)/oligo(dT)$_{18}$ and dTTP were used as the synthetic DNA template-primer and dNTP substrate, respectively. Double reciprocal plots of the obtained data show that compound 8-induces inhibition of the activities of both pol alpha and pol beta in a competitive and non-competitive fashion with the DNA template-primer and the dNTP substrate, respectively (Table 2).

Table 1. IC_{50} values of compound 8 (C-C18) against the activity of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} value (uM)
Mammalian DNA polymerases	
Calf DNA polymerase alpha	9.4 ± 0.5
Rat DNA polymerase beta	14.1 ± 0.8
Human DNA polymerase gamma	13.5 ± 0.7
Human DNA polymerase delta	10.6 ± 0.5
Human DNA polymerase epsilon	10.9 ± 0.6
Insect DNA polymerases	
Fruit fly DNA polymerase alpha	11.7 ± 0.6
Fruit fly DNA polymerase delta	13.8 ± 0.7
Fruit fly DNA polymerase epsilon	14.0 ± 1.0
Protistan DNA polymerase	
Budding yeast DNA polymerase alpha	13.2 ± 0.7
Plant DNA polymerase	
Cauliflower DNA polymerase alpha	> 200
Prokaryotic DNA polymerases	
E. coli DNA polymerase I	> 200
Taq DNA polymerase	> 200
T4 DNA polymerase	> 200
Other DNA metabolic enzymes	
Calf primase of DNA polymerase alpha	> 200
T7 RNA polymerase	> 200
T4 polynucleotide kinase	> 200
Bovine deoxyribonuclease I	> 200

Compound 8 was pre-incubated with each enzyme. Enzyme activity in the absence of the compounds was taken as 100%. The data shown are the means ± S.E. of three independent experiments.

Table 2. *Ki* values of compound 8 (C-C18) for mammalian DNA polymerases alpha and beta

Substrate	poly(dA)/oligo(dT_{18})		dTTP	
	Ki (uM)	Inhibitory mode	*Ki* (uM)	Inhibitory mode
Calf pol alpha	3.6	Competitive	5.3	Non-competitive
Rat pol beta	7.4	Competitive	9.3	Non-competitive

The inhibitory mode and inhibition constant (*Ki*) values of compound 8 for pols were determined with respect to each of poly(dA)/oligo(dT_{18}) (DNA template-primer) and dTTP (nucleotide substrate) from Lineweaver-Burk plots and Dixon plots, respectively.

These results suggest that the compound binds directly to the DNA template binding site of the enzyme. The inhibition constant (*Ki*) values of compound 8, obtained from Dixon plots, were found to be 3.6–7.4 uM and 5.3–9.3 uM for the DNA template-primer and dTTP, substrate, respectively. Because the *Ki* values for the DNA template-primer were smaller than those for the dNTP substrate, the affinity of compound 8 must be greater for the enzyme–DNA template-primer binary complex than for the enzyme–nucleotide substrate complex.

4.2. Suppression of cultured cell growth

Pols have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Therefore, these synthesized catechin derivatives (compounds 1–15) are promising chemotherapeutic candidates. To clarify the cytological effects of these compounds, their influence on survival of two cultured cell lines, such as human colon carcinoma (HCT116) cells and human umbilical vein endothelial cells (HUVECs), was tested by MTT assay (43). Anti-cancer chemotherapy agents inhibit the growth of human cancer cells such as HCT116 cells, and many inhibitors of angiogenesis suppress the proliferation of endothelial cells such as HUVECs. As shown in Figure 3, neither 25 uM of compound 1 (C), compound 10 (EC) and their acetylated derivatives (compounds 9 and 15) influenced the growth of HCT116 cells. However, 25 uM of the derivatives of 3-*O*-acylcatechins (compounds 2–8) and 3-*O*-acylepicatechins (compounds 11–14) suppressed cell growth. Compound 8 (C-C18) had the strongest growth inhibitory effect on the HCT116 cancer cell line among the compounds tested, and the LD_{50} value was 12.5 uM. This compound also dose-dependently suppressed the growth of other human cancer cell lines such as A549 (human lung cancer), BALL-1 (human leukemia), HL-60 (human promyelocytic carcinoma), Molt-4 (human leukemia) and NUGC-3 (human gastric cancer) with a range of LD_{50} values of between 10–30 uM (data not shown). Intriguingly, the LD_{50} value of compound 8 is approximately the same as the IC_{50} value for mammalian pol activity, suggesting that the growth inhibition is primarily a function of the effect of compound 8 on the replicative pols, such as pol alpha. The growth suppression of these compounds on HUVECs showed the same tendency as that on HCT116 cells, but the inhibitory effect on HUVECs was stronger. A significant correlation was found between the cultured cell proliferation and the inhibition of mammalian pols such as pol alpha (see Figure 2 and Figure 3). The acylated catechin and epicatechin derivatives may be able to penetrate the cells more efficiently to reach the nucleus and interact with pol alpha. The resultant inhibition of pol alpha activity by the compound may lead to the observed cell growth suppression. The effect of the various compounds on angiogenesis will be addressed in the next part of this study.

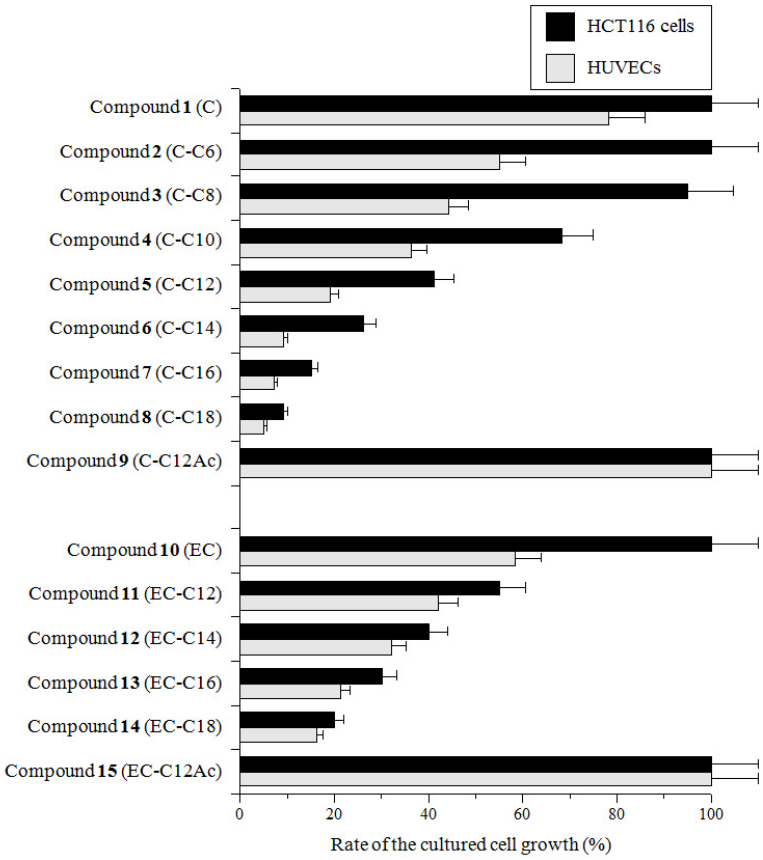

Figure 3. Effect of acylated catechin and epicatechin derivatives (compounds 1–15) on the proliferation of human colon carcinoma (HCT116) cell and human umbilical vein endothelial cell (HUVEC) growth. Each compound at 25 uM was added to a culture of HCT116 cells or HUVECs. HCT116 cells and HUVECs were incubated for 24 and 72 h, respectively, and the rate of cell growth inhibition was determined by MTT assay for the cells (43). Rate of the cultured cell growth in the absence of the compound was taken as 100%. Data are the mean ± S.E. of five independent experiments.

4.3. Angiogenesis inhibition

4.3.1. Effect of the derivatives of 3-*O*-acylcatechin and 3-*O*-acylepicatechin on *ex vivo* angiogenesis

It has been shown that tea catechins have anti-angiogenic activity (46). Among the tea catechins, EGCG is the most effective anti-angiogenic agent. The *ex vivo* anti-angiogenic effect of compounds 1–15 was investigated in a rat aortic ring model (Figure 4). This angiogenesis method is widely used to evaluate anti-angiogenic agents (47–49). In this model, fibroblastic fusiform cells migrate from the ends of the aortic rings over a period of 2 to 3 days and spread in the collagen gel and then microvessels appear from the ends of aortic rings after 5 to 6 days (Figure 5A). Interestingly, the inhibitory effects of all the acylated derivatives of catechins (compounds 2–8) and epicatechins (compounds 12 and 13) were stronger than catechin (compound 1) and epicatechin (compound 10), respectively (Figure 4). Moreover, the inhibitory action increased with the length of the acyl moiety. 3-*O*-Acylcatechin derivatives were stronger suppressors of the *ex vivo* angiogenesis than 3-*O*-acylepicatechin derivatives. The anti-angiogenic activity by these compounds was correlated with pol inhibition (Figure 2) and suppression of the cultured cell proliferation (Figure 3). Compound 6 (C-C14) and compound 7 (C-C16) completely inhibited microvessel outgrowth, although fibroblastic fusiform cells migrated from the ends of the aortic rings (data not shown). By contrast, compound 8 (C-C18) completely suppressed fibroblastic fusiform cell migration and microvessel outgrowth as shown in Figure 5A. Therefore, compound 8 was the most effective inhibitor of angiogenesis. Moreover, compound 8 inhibited angiogenesis in a dose-dependent manner and was statistically significant at greater than 50 uM (Figure 5B).

4.3.2. Effect of compound 8 (C-C18) on HUVEC tube formation

The effect of compound 8 (C-C18) was examined in an *in vitro* angiogenesis model using HUVECs. HUVECs inoculated on a reconstituted basement membrane (Matrigel™) migrated, then attached to each other, and finally formed tube structures (Figure 6A). Compound 8 suppressed HUVEC tube formation in a dose-dependent manner (Figure 6A and B). Vascular endothelial growth factor (VEGF) strongly stimulated HUVEC migration, and the effect of compound 8 on HUVEC migration on gelatin coated Boyden chambers was examined. Compound 8 did not affect HUVEC migration

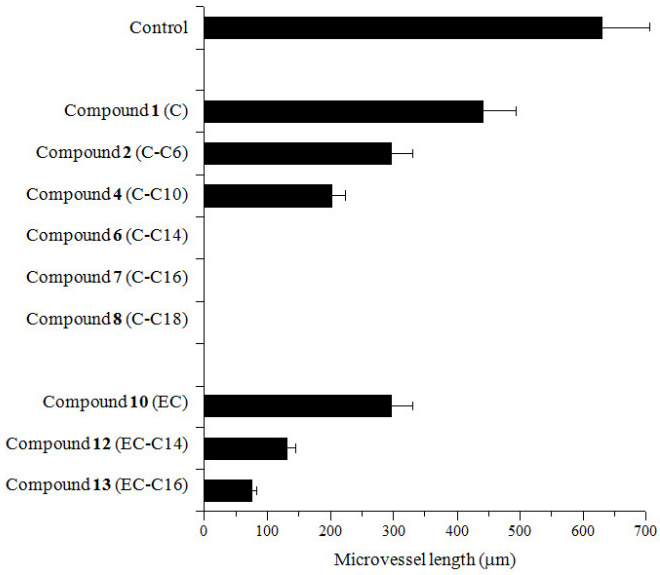

Figure 4, Effect of acylated catechin and epicatechin derivatives (compounds 1–15) (100 uM each) on ex vivo angiogenesis using a rat aortic ring. Microvessel length was measured on day 7 of the culture (47–49). Data are the mean ± S.D. of three independent experiments.

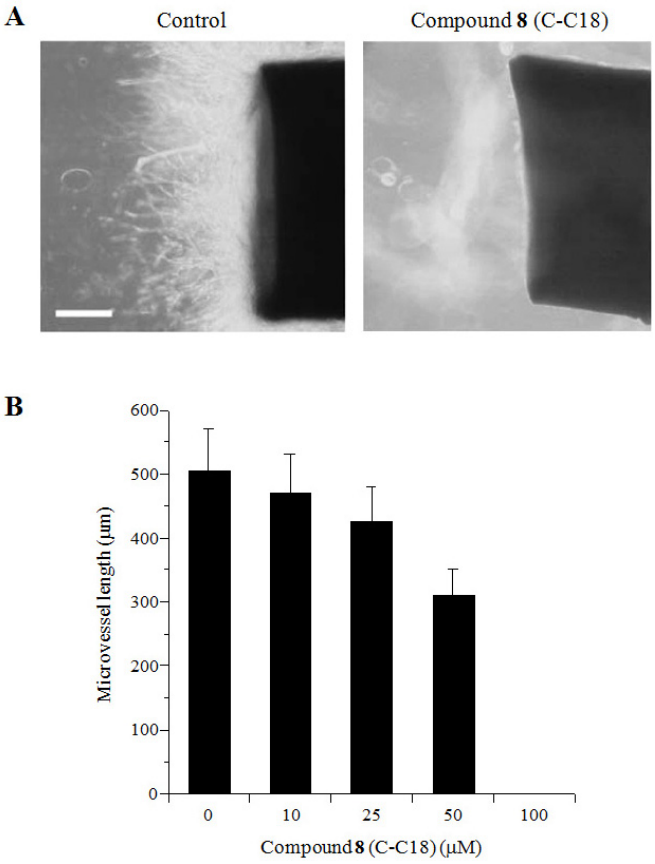

Figure 5. Suppressive effect of compound 8 (C-C18) in the ex vivo angiogenesis model. (A) The inhibitory effect of compound 8 (100 uM). Bar equals 500 um. (B) Dose-response results of compound 8. Microvessel length was measured on day 7 of the culture (32-34). Data are the mean ± S.D. of three independent experiments.

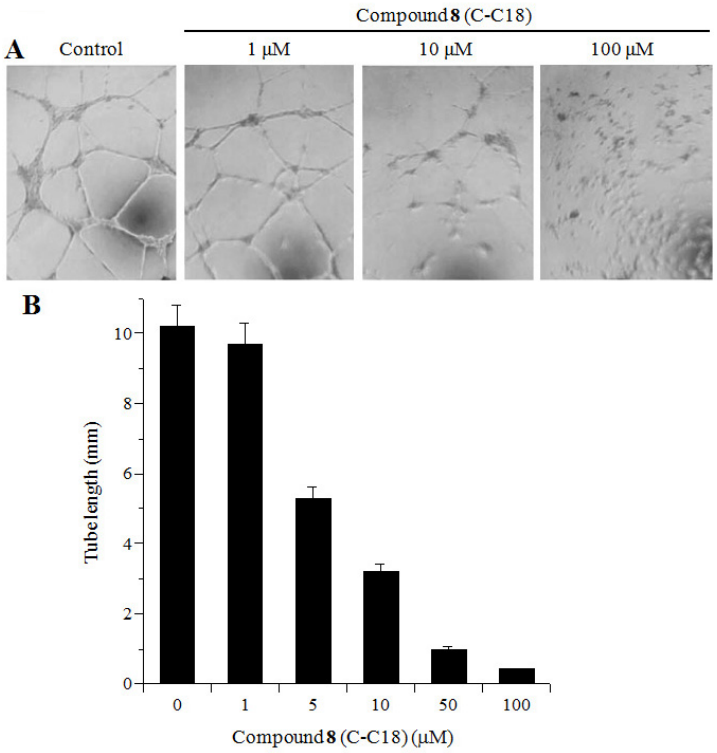

Figure 6. Dose-response effect of compound 8 (C-C18) on HUVEC tube formation on reconstituted basement membrane. (A) Photographs. (B) Capillary length was measured. Cells were plated on reconstituted gel and observed 12 h later. Data are the mean ± S.D. of three independent experiments.

induced by VEGF at the examined concentration of 1 to 100 uM (data not shown). Taken together, the above results show that compound 8 exerts its anti-angiogenic activity through inhibiting pol activity and endothelial cell tube formation.

5. DISCUSSION

Many organisms are known to contain as many as 15 types of pol (19), which catalyze DNA replication, repair and recombination (19, 20). Pols, especially pol alpha, are regarded as the target of some anti-cancer drugs because they play a central role in DNA replication, which is indispensable for the proliferation of cancer cells. Thus, pol inhibitors are promising candidates as anti-cancer chemotherapy agents. Based on this idea, we have isolated many new pol inhibitors over the past 15 years from natural sources, particular from food materials (22, 50). Moreover, we have chemically synthesized the pol inhibitors and related compounds.

A series of acylated derivatives of catechin (compounds 1–9) and epicatechin (compounds 10–15), which are polyphenols found in green tea, were used in this study (Figure 1). These compounds were then investigated to determine the relationship between their chemical structure and respective anti-cancer activities. These results indicate that catechin derivatives (compounds 2–8) exert stronger inhibitory effects on pol activity, cultured cell proliferation and angiogenesis compared to epicatechin derivatives (compounds 11–14) (Figures 2–4). Compound 8 (C-C18) was the strongest inhibitor among the synthesized acylated catechin derivatives. The *in vitro* inhibition of cancer cell proliferation and induction of anti-angiogenesis by compound 8 may be caused by the inhibition of pols, especially replicative pol alpha. We are trying to investigate whether the specificity of compound 8-mediated inhibition of mammalian pol activity is another major concern for further studies.

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels. The process of angiogenesis is involved in various pathological states including solid tumor growth, diabetic retinopathy, and age-related macular degeneration (3). Inhibition of angiogenesis is an important target for cancer treatment because metastasis as well as solid tumor growth is facilitated through angiogenesis (4). Interestingly, administration of drugs exhibiting anti-angiogenic activity has been shown to reduce the risk of brain diseases in older patients (51). Given that angiogenesis-related diseases pose serious medical problems worldwide, there has been much research devoted to developing effective angiogenesis inhibitors.

Various biological activities of natural polyphenols have been reported. It has been shown that catechins in green tea lower the risk of cancer and

cardiovascular diseases (2). EGCG is the most abundant compound present in green tea and has been the most extensively studied in terms of its biological action. EGCG has strong anti-cancer and anti-angiogenic effects (52). In addition, it has been shown that esterification of epigallocatechin increases anti-virus activity (53). Moreover, epicatechin and its metabolite protect human fibroblast cells from oxidative-stress-induced cell death (54). These observations suggest that chemical modification of green tea catechins could improve the biological activity of epigallocatechin.

It has been demonstrated that EGCG strongly inhibits endothelial cell tube formation, but catechin (50 uM) exerts no effect (55). The different effects can be ascribed to their respective action on the phosphorylation of the VEGF receptor. EGCG inhibits tyrosine phosphorylation of the VEGF receptor, whereas catechin does not (55). HUVECs migrate, attach to each other, and form tube-like structures on reconstituted basement membrane. This morphological change is an important process in forming new blood vessels. Although compound 8 strongly inhibits HUVEC tube formation (Figures 5 and 6), it does not affect HUVEC migration stimulated by VEGF (data not shown). This result suggests that compound 8 does not inhibit VEGF receptor phosphorylation. Therefore, compound 8 appears to target the interaction of endothelial cells or signal transduction pathways involved in endothelial cell tube formation. Although the exact mechanism by which compound 8 inhibits pol activity is unknown, the present study suggests that the stereochemistry of the fatty acid moiety is an important factor that affects the formation of the DNA template-primer complex or configuration of the catalytic domain (24, 56). The acylated derivatives of catechin and epicatechin exert a growth suppressive effect on the cultured cells, such as HCT116 cancer cells and HUVECs, by inhibiting pol activity, which are critical enzymes for cell proliferation. In addition, compound 8 strongly inhibits HUVEC tube formation indicating that this compound can target other molecules involved in neovascularization.

We synthesized several derivatives of catechin and epicatechin, such as 3-*O*-acylcatechin and 3-*O*-acylepicatechin, respectively (3-*O*-acyl-flavan-3-ols) (25, 26). 3-*O*-Acylcatechin derivatives have been synthesized previously (53, 57) in order to improve their physical properties, such as the solubility of hydrophilic catechins in lipidic matrices. Among the hydroxy groups of catechin derivatives, modification at the 3'-position of the C-ring is most suitable for this purpose. In addition, the phenolic hydroxy groups, especially on the B-ring, are important for the anti-oxidant activity of catechin derivatives. Indeed, it is reported that the inhibitory activity of the activation of the Epstein-Barr virus early antigen (EBV-EA) is increased by esterification of epigallocatechin with fatty acid (53). These findings led us to surmise whether esterification of catechin with fatty acid might enhance anti-cancer activity. Thus, acylated catechin derivatives with a long alkyl chain, such as compound 8, are promising candidates for developing a novel series of anti-angiogenic agents.

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Abbreviations: EGCG: Epigallocatechin-3-*O*-gallate, DMSO: dimethyl sulfoxide, dNTP: 2'-deoxynucleoside 5'-triphosphate, dsDNA: double-stranded DNA, dTTP: 2'-deoxythymidine 5'-triphosphate, HUVEC: human umbilical vein endothelial cell, pol: DNA polymerase, VEGF: vascular endothelial growth factor

Key Words: Acylated catechin derivative, Angiogenesis, Anti-cancer, Catechin, Cultured cell growth, DNA polymerase, E.C.2.7.7.7, Enzyme inhibitor, HUVEC

Send correspondence to: Yoshiyuki Mizushina, Laboratory of Food and Nutritional Sciences, Department of Nutritional Science, Kobe-Gakuin University, 518 Arise, Ikawadani-cho, Nishi-ku, Kobe, Hyogo 651-2180, Japan, Tel: 81-78-974-1551 (ext. 3232), Fax: 81-78-974-5689, E-mail: mizushin@nutr.kobegakuin.ac.jp

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