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Epicatechin conjugated with fatty acid is a potent inhibitor of DNA polymerase and angiogenesis

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Abstract

Anti-cancer and anti-angiogenesis effects of green tea catechins have been demonstrated. It has been found that chemical modification of tea catechins improves their biological activities. We examined the chemical modification of epicatechin enhanced anti-cancer and anti-angiogenic effects. Epicatechin conjugated with fatty acid (acyl-catechin) strongly inhibited DNA polymerase activity, HL-60 cancer cell growth and angiogenesis. Epicatechin conjugated with palmitic acid ((2*R*,3*R*)-3′,4′,5,7-tetrahydroxyflavan-3-yl hexadecanoate, epicatechin-C16) was the strongest inhibitor in DNA polymerase α, β, λ and angiogenesis assays. Epicatechin-C16 also suppressed human endothelial cell (HUVEC) tube formation on reconstituted basement membrane, suggesting that it affected not only DNA polymerase activity but also the signal transduction pathways needed for the tube formation in HUVECs. These results suggest that acylation of epicatechin is an effective chemical modification to improve the anti-cancer activity of epicatechin.

Keywords: Angiogenesis; Aortic ring; Epicatechin; Endothelial cells; Fatty acid

Introduction

Natural polyphenols distributed in plants have diverse biological activities: antioxidant, anti-cancer, and anti-inflammation activities (Yang et al., 2001). In addition, their inhibitory effect on angiogenesis has been revealed (Cao et al., 2002). Angiogenesis is involved in tumor growth and metastasis, atherosclerosis and diabetic retinopathy (Folkman, 1995). Thus numerous efforts to develop more effective angiogenesis inhibitors have been conducted (Cao, 2004). We have found some angiogenesis inhibitors in natural products including vitamin B_6, algal polysaccharides and phenolic compounds (Matsubara et al., 2001, 2003, 2005a,b). Recently, we have reported that a polyphenolic DNA polymerase inhibitor, petasiphenol, has strong inhibitory effect on angiogenesis *in vitro* (Matsubara et al., 2004). DNA polymerases, especially DNA polymerase α, are regarded as the target of some anti-cancer drugs because DNA polymerases play central roles in DNA replication which is indispensable for the proliferation of cancer cells. Thus, it should be noted that polyphenolic DNA polymerase inhibitor has anti-angiogenic activity.

We have synthesized various derivatives of polyphenols, intending to improve biological activities of natural polyphenolic compounds. Tea catechins have been studied for their

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biological activities. The main compound of tea catechins, epigallocatechin-3-gallate (EGCG) has been the most extensively studied, and anti-angiogenic activity of EGCG has been revealed (Cao and Cao, 1999). On the other hand, other catechins including epicatechin are weaker in anti-angiogenic activity than EGCG (Lamy et al., 2002). Thus, our efforts have been conducted on synthesized potent epicatechin derivatives. We have succeeded in synthesizing 3-*O*-acylepicatechin derivatives and examined their effect on DNA polymerase activity and angiogenesis. Interestingly, 3-*O*-acylepicatechin derivatives exerted stronger inhibitory effects both on DNA polymerase activity and angiogenesis than epicatechin, suggesting the possibility of developing new anti-cancer agents. The characteristics of the acylated epicatechin as DNA polymerase and angiogenesis inhibitors are demonstrated in this report.

Materials and methods

Materials

Human recombinant vascular endothelial growth factor (VEGF) was obtained from R&D systems (MN, U.S.A). Other reagents were special grade as commercially available.

Synthesis

Optical rotation was measured with a Horiba SEPA-300 spectrometer (Horiba, Kyoto). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were taken with a JEOL LA-400 spectrometer (Tokyo, Japan). $CDCl_3$ (δ7.26 for ^{1}H and δ77.0 for ^{13}C NMR) and acetone-d_6 (δ2.00 for ^{1}H and δ30.3 and 206.0 for ^{13}C NMR) were used as the internal standards, respectively. ^{1}H and ^{13}C NMR signals were assigned by means of 1D proton decoupling technique, 2D HH-COSY, and CH-COSY. FT-IR spectra were taken with a Shimadzu FT-IR DR-8000 (Shimadzu, Kyoto). FAB mass spectra were taken with a JEOL JMS-AX500 instrument.

Synthesis of tetra-benzylated epicatechin

To a solution of (2*R*,3*R*)-3′,4′,5,7-tetrahydroxyflavan-3-ol (10 g, 0.035 mol) and K_2CO_3 (26.2 g, 0.19 mol) in *N*,*N*-dimethylformamide (500 ml) was added benzyl bromide (29.5 g, 0.17 mol) at 0 °C. After stirring for 48 h at rt, the reaction mixture was quenched with cold water and extracted with EtOAc. The organic phase was washed with water and brine and dried (Na_2SO_4). Filtration, concentration and silica gel column chromatography (hexane/EtOAc, 6/1) gave a 10.2 g of tetra-benzylated epicatechin (0.016 mol, 45%) as a white powder.

General procedure for the esterification of benzylated flavan-3-ols

To a solution of (2*R*,3*R*)-3′,4′,5,7-tetra-*O*-benzylflavan-3-ol (**1**) (500 mg) in CH_2Cl_2 (10 ml) was added Et_3N (3 eq.), DMAP (5 mg) and fatty acid chlorides (lauroyl chloride, miristoyl chloride, and palmitoyl chloride, 1.5 eq.) at 0 °C. After stirring for 6–12 h at rt, the reaction mixture was quenched with water and extracted with CH_2Cl_2. The organic phase was washed with water and brine and dried (Na_2SO_4). Filtration, concentration and silica gel column chromatography (hexane/EtOAc, 6/1) gave a pure ester.

General procedure for the hydrogenation of benzyl protecting group of acyl-epicatechins

A solution of a benzylated ester (**2**–**4**) in THF/MeOH/H_2O (20/1/1) was hydrogenated over 20% $Pd(OH)_2$/C (5 mg) for 12 h at rt. Filtration and concentration afforded a colorless oil, which was purified by Cosmosil 75C-18 OPN column chromatography (MeOH–H_2O) to give a pure debenzyrated acyl-epicatechins.

*(2R,3R)-3′,4′,5,7-Tetra-O-benzylflavan-3-yl dodecanoate (**2**)*

White powder. $[\alpha]_D^{25} = -19.5$ (*c* 0.94, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) 7.47–7.28 (20H, m), 7.11 (1H, d, *J*=1.7 Hz), 6.96 (1H, dd, *J*=1.7, 8.3 Hz), 6.92 (1H, d, *J*=8.3 Hz), 6.28 (1H, d, *J*=2.2 Hz), 6.27 (1H, d, *J*=2.2 Hz), 5.45–5.41 (1H, m), 5.17 (1H, d, *J*=12.0 Hz), 5.15 (2H, s), 5.14 (1H, d, *J*=12.0 Hz), 5.02 (4H, s), 4.99 (1H, br s), 3.02 (1H, dd, *J*=4.6, 18.1 Hz), 2.95 (1H, dd, *J*=2.0, 18.1 Hz), 2.19–2.07 (2H, m), 1.45–1.37 (2H, m), 1.32–1.10 (16H, m), 0.86 (3H, t, *J*=5.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) 173.1, 158.7, 157.9, 155.5, 148.9, 148.7, 137.2, 136.9, 131.1, 128.6–127.1 (C×14), 119.7, 114.7, 113.6, 100.8, 94.6, 93.8, 77.2, 71,4, 71.3, 70.1, 69.9, 67.5, 34.2, 31.9, 29.62, 29.60, 29.4, 29.3, 29.26, 28.98, 26.0, 24.8, 22.7, 14.1; IR (neat, cm^{-1}) 3065 (m), 3034 (m), 2924 (s), 2855 (s), 2361 (w), 2338 (w), 1950 (w), 1871 (w), 1819 (w), 1782 (s), 1618 (s), 1593 (s), 1518 (s), 1454 (s), 1379 (s), 1269 (s), 1217 (s), 1184 (s), 1152 (s), 1115 (s), 1020 (s), 945 (w), 810 (m), 735 (s); FAB-MS (*m*/*z*) 856 (11), 855 ($[M+Na]^+$, 17), 834 (3.6), 833 ($[M+H]^+$, 7.5), 633 (21), 632 (28), 610 (54), 609 (100); FAB-HRMS calcd for $C_{55}H_{61}O_7$ $[M+H]^+$, 833.4417; found: 833.4448.

(2R,3R)-3′,4′,5,7-Tetrahydroxyflavan-3-dodecanoate, epicatechin-C12

Colorless amorphous solid. $[\alpha]_D^{24} = -54.0$ (*c* 0.58, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$) 6.91 (1H, br s), 6.73 (2H, br s), 5.94 (1H, d, *J*=2.4 Hz), 5.91 (1H, d, *J*=2.4 Hz), 5.33–5.32 (1H, m), 4.93 (1H, br s), 2.90 (1H, dd, *J*=4.7, 17.6 Hz), 2.78 (1H, d, *J*=17.6 Hz), 2.19–2.14 (2H, m), 1.44–1.39 (2H, m), 1.30–1.09 (16H, m), 0.88 (3H, t, *J*=6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) 175.0, 157.83, 157.79, 157.1, 145.99, 145.96, 131.3, 119.0, 115.9, 114.9, 99.1, 96.5, 95.8, 78.2, 69.9, 35.2, 33.1, 30.72, 30.70, 30.51, 30.47, 30.3, 29.9, 26.6, 26.0, 23.7, 14.4; FAB-MS (*m*/*z*) 496 (5.5), 495 ($[M+Na]^+$, 17), 494 (5.8), 474 (6.3), 473 ($[M+H]^+$, 20), 275 (13), 274 (55), 273 (100), 272 (22); FAB-HRMS calcd for $C_{27}H_{37}O_7$ $[M+H]^+$, 473.2539; found: 473.2542.

*(2R,3R)-3′,4′,5,7-Tetra-O-benzylflavan-3-yl tetradecanoate (**3**)*

White powder. $[\alpha]_D^{23}=-20.4$ (*c* 0.72, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) 7.47–7.30 (20H, m), 7.11 (1H, d, *J*=1.7 Hz), 6.96 (1H, dd, *J*=1.7, 8.3 Hz), 6.92 (1H, d, *J*=8.3 Hz), 5.46–5.41 (1H, m), 5.17 (1H, d, *J*=11.9 Hz), 5.15 (2H, s), 5.13 (1H, d, *J*=11.9 Hz), 5.12 (4H, s), 4.99 (1H, br s), 3.02 (1H, dd, *J*=4.6, 16.1 Hz), 2.94 (1H, d, *J*=16.1 Hz), 2.19–2.05 (2H, m), 1.42–1.38 (2H, m), 1.29–1.10 (20H, m), 0.88 (3H, t, *J*=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) 173.1, 158.7, 157.9, 155.4, 148.8, 148.7, 137.2 (×2), 136.9, 136.8, 131.1, 128.6, 128.5, 128.4 (×2), 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 127.2, 127.1, 119.7, 114.7, 113.6, 100.8, 94.6, 93.8, 77.2, 71.4, 71.2, 70.1, 69.9, 67.5, 34.2, 31.9, 29.7, 29.63, 29.61 (×2), 29.4, 29.3, 29.2, 29.0, 25.9, 24.8, 22.7, 14.1; IR (neat, cm^{-1}) 2924 (s), 2853 (s), 1732 (s), 1653 (s), 1558 (s), 1456 (s), 1385 (s), 1339 (m), 1151 (s), 1115 (s), 1078 (m), 1028 (m), 902 (w), 810 (w), 733 (m); FAB-MS (*m*/*z*) 885 (9.4), 884 (24), 883 ($[M+Na]^+$, 36), 862 (14), 861 ($[M+H]^+$, 29), 860 (7.9), 695 (12), 694 (44), 693 (100); FAB-HRMS calcd for $C_{57}H_{65}O_7$ $[M+H]^+$, 861.4730; found: 861.4730.

(2R,3R)-3′,4′,5,7-Tetrahydroxyflavan-3-yl tetradecanoate, epicatechin-C14

Colorless oil. $[\alpha]_D^{25}=-38.3$ (*c* 0.38, CH_3COCH_3); ^1H NMR (400 MHz, CD_3COCD_3-D_2O, 10:1) 6.94 (1H, br s), 6.76 (2H, br s), 6.02 (1H, d, *J*=2.2 Hz), 5.91 (1H, d, *J*=2.2 Hz), 5.33 (1H, br s), 4.96 (1H, br s), 2.92 (1H, dd, *J*=4.3, 17.3 Hz), 2.73 (1H, d, *J*=17.3 Hz), 2.13–2.10 (2H, m), 1.40–1.36 (2H, m), 1.25–1.08 (20H, m), 0.82 (3H, t, *J*=6.8 Hz); ^{13}C NMR (100 MHz, CD_3COCD_3-D_2O, 10:1) 173.5, 157.4, 157.3, 156.5, 145.3 (×2), 130.7, 118.6, 115.3, 114.5, 98.4, 96.2, 95.3, 77.5, 68.9, 34.5, 32.3, 30.4–29.1 (C×8), 26.2, 25.3, 23.0, 14.2; FAB-MS (*m*/*z*) 524 (15), 523 ($[M+Na]^+$, 30), 522 (6.8), 502 (6.0), 501 ($[M+H]^+$, 14), 275 (28), 274 (90), 273 (100), 272 (43); FAB-HRMS calcd for $C_{29}H_{41}O_7$ $[M+H]^+$, 501.2852; found: 501.2861.

*(2R,3R)-3′,4′,5,7-Tetra-O-benzylflavan-3-yl hexadecanoate (**4**)*

White powder. $[\alpha]_D^{24}=-16.1$ (*c* 1.34, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) 7.46–7.27 (20H, m), 7.12 (1H, d, *J*=1.7 Hz), 6.95 (1H, dd, *J*=1.7, 8.3 Hz), 6.91 (1H, d, *J*=8.3 Hz), 6.29 (1H, d, *J*=2.2 Hz), 6.27 (1H, d, *J*=2.2 Hz), 5.48–5.41 (1H, m), 5.17 (1H, d, *J*=12.2 Hz), 5.14 (2H, s), 5.13 (1H, d, *J*=12.2 Hz), 5.01 (4H, s), 4.98 (1H, br s), 3.01 (1H, dd, *J*=4.4, 17.8 Hz), 2.95 (1H, dd, *J*=2.2, 17.8 Hz), 2.19–2.06 (2H, m), 1.45–1.37 (2H, m), 1.35–1.05 (24H, m), 0.88 (3H, t, *J*=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) 171.1, 158.7, 157.9, 155.4, 148.8, 148.7, 137.2, 136.84, 136.82, 131.1, 128.54 (×2), 128.48, 128.39 (×2), 127.9, 127.8, 127.74, 127.70, 127.5, 127.3, 127.2, 127.1, 119.6, 114.6, 113.5, 100.8, 94.6, 93.8, 77.1, 71.4, 71.2, 70.0, 69.8, 67.5, 34.2, 31.9, 29.64 (×2), 29.60 (×2), 29.6, 29.4, 29.3, 29.2, 28.9, 25.9, 24.8, 22.7, 21.0, 14.1; IR (neat, cm^{-1}) 3063 (w), 3034 (w), 2923 (s), 2853 (s), 1948 (w), 1809 (w), 1782 (s), 1618 (s), 1593 (s), 1520 (s), 1379 (s), 1184 (s), 1151 (s), 1028 (s), 943 (w), 810 (m), 733 (m); FAB-MS (*m*/*z*) 912 (12), 911 ($[M+Na]^+$, 19), 891 (15), 890 ($[M+H]^+$, 21), 889 (7.2), 634 (19), 633 (59), 632 (78), 631 (19), 320 (22), 319 (100); FAB-HRMS calcd for $C_{59}H_{69}O_7$ $[M+H]^+$, 889.5043; found: 889.5089.

(2R,3R)-3′,4′,5,7-Tetrahydroxyflavan-3-yl hexadecanoate, epicatechin-C16

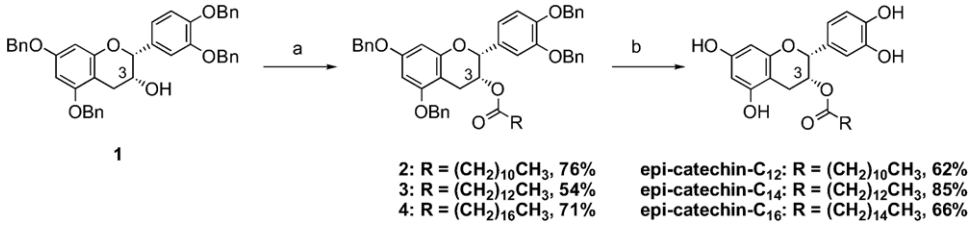
White powder. $[\alpha]_D^{25}=-42.0$ (*c* 1.22, CH_3COCH_3); ^1H NMR (400 MHz, CD_3COCD_3-D_2O, 10:1) 6.94 (1H, br s), 6.76 (2H, br s), 6.01 (1H, d, *J*=2.2 Hz), 5.91 (1H, d, *J*=2.2 Hz), 5.32–5.31 (1H, m), 4.95 (1H, br s), 2.91 (1H, dd, *J*=4.9, 17.5 Hz), 2.72 (1H, dd, *J*=2.0, 17.5 Hz), 2.16–2.10 (2H, m), 1.41–1.34 (2H, m), 1.26–1.10 (24H, m), 0.82 (3H, t, *J*=6.8 Hz); ^{13}C NMR (100 MHz, CD_3COCD_3-D_2O, 10:1) 173.4, 157.4, 157.2, 156.5, 145.3 (×2), 130.7, 118.6, 115.3, 114.5, 98.4, 96.2, 95.3, 77.5, 68.9, 34.5, 32.3, 30.4–29.2 (C×10), 26.2, 25.3, 23.0, 14.2; FAB-MS (*m*/*z*) 552 (11), 551 ($[M+Na]^+$, 29), 550 (6.1), 530 (4.4), 529 ($[M+H]^+$, 11), 275 (15), 274 (77), 273 (100), 272 (44); FAB-HRMS calcd for $C_{31}H_{45}O_7$ $[M+H]^+$, 529.3165; found: 529.3167.

DNA polymerase assays

DNA polymerase α was purified from calf thymus by immuno-affinity column chromatography as described by Tamai et al. (1988). Recombinant rat DNA polymerase β was purified from *E. coli* JMpβ5 as described by Date et al. (1988). The cDNA encoding the full-length human DNA polymerase λ was constructed and purified as described previously (Mizushina et al., 2002; Shimazaki et al., 2002). The reaction mixtures for DNA polymerases were described previously (Mizushina et al., 1996, 1997). The substrates of DNA polymerases were used poly (dA)/oligo$(dT)_{12-18}$ and deoxythymidine triphosphates (dTTP) as template-primer DNA and nucleotide substrate, respectively. The synthesized compounds were dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Four microliter of sonicated samples were 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 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. The activity without the inhibitor was considered 100%, and the remaining activities at each concentration of inhibitor were determined as percentages of this value. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleotide triphosphates (i.e. dTTP) into synthetic template-primers (i.e. poly(dA)/oligo$(dT)_{12-18}$, *A*/*T*=2/1) in 60 min at 37 °C under the normal reaction conditions for each enzyme (Mizushina et al., 1996, 1997).

Cell culture and measurement of cell viability

A human promyelocytic leukemia cell line, HL-60, was obtained from the Health Science Research Bank (Osaka, Japan).



Scheme 1. Synthesis of acyl-epicatechins. Reagents: (a) RCOCl, Et_3N, DMAP, CH_2Cl_2, (b) H_2, $Pd(OH)_2/C$, THF-MeOH–H_2O (20/1/1).

The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a humid atmosphere of 5% CO_2/95% air. For the cell growth assay, HL-60 cells were plated at 3×10^5 cells into each well of 96-well microplates with various concentrations of acyl-epicatechin compound. These compounds were dissolved in dimethyl sulfoxide (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 (Mosmann, 1983).

Ex vivo angiogenesis assay

Male Wistar rats (6 week old, Clea Japan, Inc., Tokyo, Japan) were housed in 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 Okayama Prefectural University. The *ex vivo* angiogenesis assay was performed according to slightly modified methods as described before (Mori et al., 1988; Kawasaki et al., 1989). 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, U.S.A.), 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, U.S.A.) containing 1% of ITS+ (Becton Dickinson Labware, MA, U.S.A.)) with various concentration of acyl-epicatechin 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.

Endothelial cells

Human umbilical vein 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 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml recombinant human basic fibroblast growth factor (bFGF) and 10 μg/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. HUVECs at passage three to five were used in this experiment.

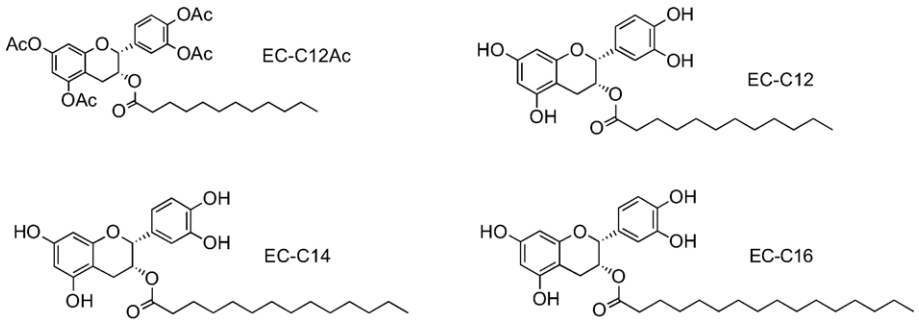


Fig. 1. Structures of acyl-epicatechins.

HUVEC tube formation assay

Tube formation assay was performed using BD Matrigel™ (Becton, Dickinson and Company, Tokyo, Japan). Briefly, solid gels were prepared according to the manufacture manual on a 96-well tissue culture plate. HUVECs (1×10^5 cells/ml) in HuMedia EG-2 medium containing acyl-epicatechin (1–100 μM) or vehicle (DMSO) were seeded 100 μl per well onto the surface of the solid gel, BD Matrigel™. 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× 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.

Statistical analysis

Values are presented as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA). Differences with $p < 0.05$ were considered significant.

Results

Synthesis of 3-O-acylepicatechin derivatives

3-*O*-Acylepicatechin derivatives (epicatechin-C12–epicatechin-C16) were synthesized as shown in Scheme 1. Benzylated (+)-epicatechin (**1**) was acylated with each fatty acid chlorides (C12, C14, and C16) to afford benzylated-3-*O*-acyl-flavan-3-ols **2**–**4** in good to excellent yields. Following hydrogenation with $Pd(OH)_2/C$ gave epicatechin-C12–epicatechin-C16 in satisfactory yields. The chemical structures are shown in Fig. 1.

Effects of acyl-epicatechin compounds on DNA polymerase activities

The effect of synthesized acyl-epicatechin compounds on mammalian DNA polymerase α, β and λ was investigated. DNA polymerase α and β, λ are replicative and repair-related DNA polymerases in nuclei, respectively (Bebenek and Kunkel, 2004). As shown in Table 1, epiatechin-C12 to epicatechin-C16 inhibited the activity of calf DNA polymerase α (pol α), rat DNA polymerase β (pol β) and human DNA polymerase λ (pol λ). Epicatechin-C16 was the strongest inhibitor of these enzymes, with 50% inhibition doses of 2.2–15.0 μM. The inhibitory effect of the compound on DNA polymerase λ was 6.7-fold stronger than that on other polymerases. On the other hand, both epicatechin and acetylated epicatechin-C12Ac were not effective for these inhibitory activities. This result suggested that the acyl chain group and hydroxyl group is essential for the inhibitory effect.

Table 1
IC_{50} values of acyl-epicatechins on DNA polymerase α, β and λ activities

Sample	IC_{50} values (μM)		
	pol α	pol β	pol λ
Epicatechin	>200	>200	>200
Epicatechin-C12	30.1	28.0	12.5
Epicatechin-C14	19.1	16.6	5.6
Epicatechin-C16	15.0	14.8	2.2
Epicatechin-C12Ac	>200	>200	>200

Table 2
Ki values of epicatechin-C16 for mammalian DNA polymerases

DNA polymerase	poly(dA)/oligo$(dT)_{12-18}$		dTTP	
	Ki (μM)	Inhibitory mode	Ki (μM)	Inhibitory mode
α	5.7	Competitive	8.4	Non-competitive
β	7.8	Competitive	9.8	Non-competitive
λ	1.1	Competitive	1.4	Non-competitive

The inhibitory mode and Ki values of epicatechin-C16 for DNA polymerases were determined with respect to each of poly(dA)/oligo$(dT)_{12-18}$ (DNA template-primer) and dTTP (nucleotide substrate) from Dixon plots.

Next, to elucidate the mechanism of inhibition of epicatechin-C16 on mammalian DNA polymerases, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentrations was studied. In kinetic analysis, poly (dA)/oligo$(dT)_{12-18}$ and dTTP were used as the template-primer DNA and dNTP substrate, respectively. Double reciprocal plots of the results showed that the epicatechin-C16-induced inhibition of DNA polymerase activities was competitive and non-competitive with the DNA template and the dNTP substrate, respectively (Table 2), suggesting that the compound directly binds to the DNA template binding site of the enzyme. The inhibition constant (Ki) values of epicatechin-C16, obtained from Dixon plots, were found to be 1.1–7.8 μM and 1.4–9.8 μM for the DNA template and substrate dTTP, respectively (Table 2). Because the Ki values for the DNA template-primer were smaller than those for the dNTP substrate, the affinity of epicatechin-C16 is greater for the enzyme–DNA template-primer binary complex than for the enzyme–nucleotide substrate complex.

Effects of acyl-epicatechin compounds on cancer cell growth

Epicatechin-C12 to epicatechin-C16 (10 μM each) also inhibited human promyelocytic leukemia (HL-60) cell growth for 2 days (48 h), and epicatechin-C16 was the strongest of the acyl-epicatechin compounds tested (Fig. 2). The IC_{50} values of epicatechin-C12, -C14 and -C16 were 10.8, 5.9, and 3.5 μM, respectively. The measurement of IC_{50} values was 0.1–200 μM. The human cell growth inhibitory effect of acyl-epicatechin

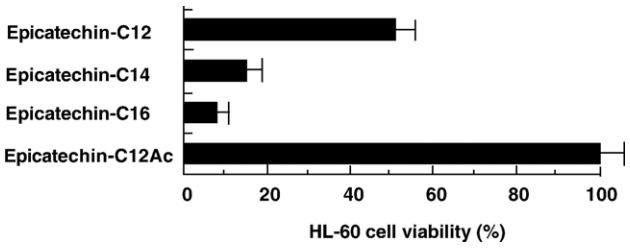


Fig. 2. Effect of acyl-epicatechins (10 μM each) on the proliferation of HL-60 cells. After incubation for 2 days (48 h) with compound, cell proliferation was determined by MTT assay. Data are shown as means of four independent experiments.

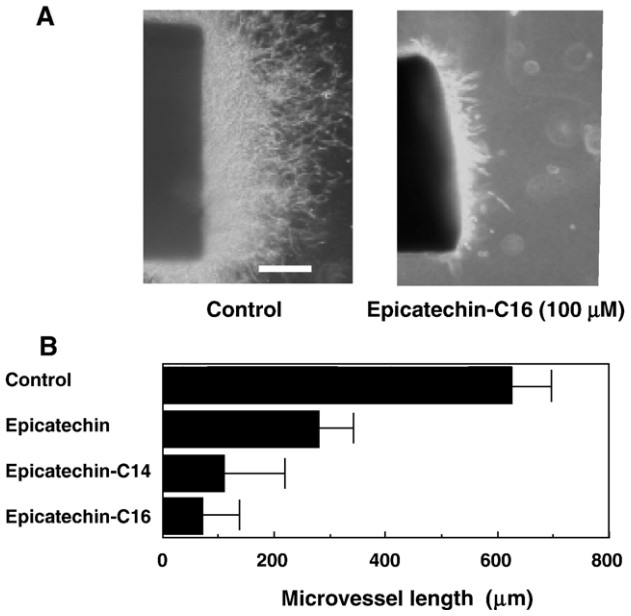


Fig. 3. Effect of acyl-epicatechins (100 μM each) on *ex vivo* angiogenesis using a rat aortic ring. Representative result of the inhibitory effect of epicatechin-C16 (100 μM) (A). Bar equals 500 μm. Microvessel length was measured on day 7 of culture. Values are means ± SD (n = 3) (B).

compounds was the same tendency as the inhibitory activity of mammalian DNA polymerases.

Effects of acyl-epicatechin compounds on ex vivo angiogenesis

It has been shown that tea catechins have anti-angiogenic activity (Cao and Cao, 1999). Epigallocatechin-3-gallate (EGCG) is the most effective anti-angiogenic catechin among them. We also examined the effect of acyl-epicatechins on angiogenesis in a rat aortic ring model. This angiogenesis method is widely used to evaluate anti-angiogenic agents (Nicosia and Ottinetti, 1990; Kruger et al., 2000; Zogakis et al., 2002). In this model, fibroblastic fusiform cells migrated from the ends of the aortic rings after 2 to 3 days, and they spread in the collagen gel. Microvessels appeared from the ends of aortic rings after 5 to 6 days and elongated (Fig. 3A). Since the amounts of synthesized acyl-epicatechins were limited, we could examine epicatechin-C14 and -C16, which yielded enough amounts for angiogenesis assays. Inhibitory effects of acyl-epicatechins were stronger than epicatechin (Fig. 3B). Epicatechin-C16 was the most effective inhibitor to angiogenesis within the compounds so far examined. The inhibitory effect of epicatechin-C16 was in a dose-dependent manner and statistically significant at higher than 50 μM (Fig. 4).

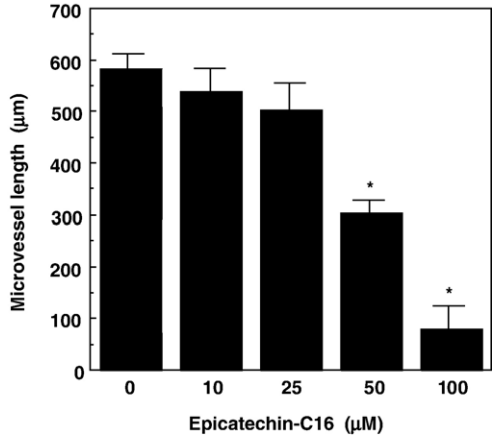


Fig. 4. Suppressive effect of epicatechin-C16 in *ex vivo* angiogenesis model. Microvessel length was measured on day 7 of culture. Values are means ± SD (n = 3). Mean with an asterisk (*; p < 0.01) is significantly different from control.

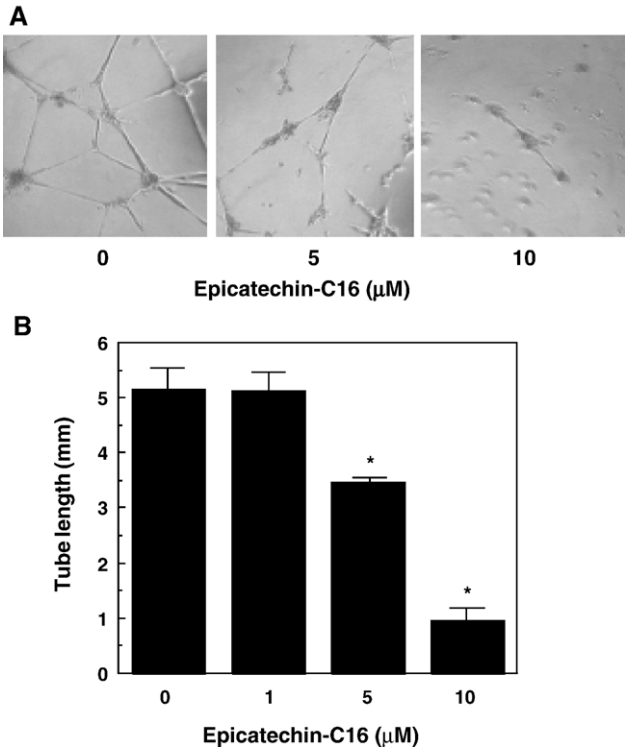


Fig. 5. Effect of epicatechin-C16 on HUVEC tube formation on reconstituted basement membrane. Cells were plated on reconstituted gel and observed 12 h later (A). Capillary length was measured, and values are means ± SD (n = 3). Means with an asterisk (*; p < 0.01) are significantly different from control (B).

Effect of epicatechin-C16 on HUVEC tube formation

The effect of epicatechin-C16 was examined in *in vitro* angiogenesis model using HUVECs. HUVECs inoculated on reconstituted basement membrane (Matrigel™) migrated, then attached each other, and finally formed tube structures (Fig. 5A). Epicatechin-C16 suppressed HUVEC tube formation in a dose-dependent manner (Fig. 5A, B).

Taken together, epicatechin-C16 exerted the anti-angiogenic activity through inhibiting DNA polymerase and endothelial cell tube formation.

Discussion

Various biological activities of natural polyphenols have been reported. It has been suggested that catechins in green tea

could lower the risk of cancer and cardiovascular diseases. Epigallocatechin-3-gallate (EGCG) is the most abundant compound present in green tea and has been the most extensively studied. EGCG has strong anti-cancer and anti-angiogenic effects (Cao et al., 2002). In addition, it has been shown that esterification of epigallocatechin increases anti-virus activity (Uesato et al., 2000) and that epicatechin and its metabolite have a protective effect on human fibroblast cells from oxidative-stress-induced cell death (Spencer et al., 2001). These observations suggest that chemical modification of green tea catechins could improve the biological activities. In this study, we have examined the effects of acyl-epicatechins as anti-cancer agents.

We have synthesized acyl-epicatechins (epicatechin-C12 to C16) and found that acylation of epicatechins enhances their inhibitory effects on DNA polymerase activity, cancer cell growth and angiogenesis. These observations suggest that acyl-epicatechins might be useful as potent anti-cancer agents. Among acyl-epicatechins, epicatechin-C16, the longest acyl-chain epicatechin, exerted the strongest inhibitory effects on all assays showing that the length of acyl-chain is critical for their biological activities. The exact mechanism of how acyl-epicatechins inhibit DNA polymerase in an acyl-chain length dependent manner is not clear at this moment. However, the present study suggests that the stereochemistry of the fatty acid moiety is also important in showing the inhibitory effect on DNA polymerase, by affecting the template-primer binding or the configuration of catalytic domain (Murakami-Murofushi et al., 1995; Mizushina et al., 2005). Acyl-epicatechins exert a suppressive effect on HL-60 cancer cell growth by inhibiting DNA polymerases, which are critical enzymes for cell proliferation. The inhibitory effects of acyl-catechins on DNA polymerase and angiogenesis were correlated with the length of acyl-chain, suggesting that their anti-angiogenic effects would be exerted by suppressing endothelial cell proliferation. In addition, epicatechin C-16 strongly inhibits HUVEC tube formation suggesting that epicatechin C-16 could target other molecules involved in neovascularization.

EGCG, a tea catechin having anti-angiogenic activity, also inhibits HUVEC tube formation. As a mechanism, it has been demonstrated that EGCG inhibits tyrosine phosphorylation of vascular endothelial growth factor (VEGF) receptor. VEGF is a highly pro-angiogenic protein. Epicatechin (50 μM) does not inhibit phosphorylation of VEGF receptor induced by VEGF, however, epicatechin-3-gallate inhibits the phosphorylation in the same manner as EGCG (Lamy et al., 2002). These observations suggest that acyl-epicatechin could attenuate the phosphorylation of VEGF receptor. To clarify the exact mechanism of the anti-angiogenic effect of acyl-epicatechin, further study will be conducted.

From our results that acyl-epicatechins not only inhibit cancer cell growth directly but also inhibit angiogenesis, they are likely to be potent anti-cancer agents. Recently, anti-angiogenic effects of natural polyphenols have considerable attention, because they are small molecules and act as angiogenesis inhibitors by oral administration (Cao and Cao, 1999; Bråkenhielm et al., 2001). Their characteristics would have advantages for long period anti-angiogenic therapy to arrest tumors during their dormant stage. Natural polyphenol-derivatives with enhanced their anti-angiogenic activity, as shown in this study, would be useful for anti-angiogenic therapy. To reveal the advantage of acyl-epicatechins, we should address the issues of the derivatives: specificity for endothelial cells and stability and metabolic fate *in vivo* in the future.

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