Abstract

We previously found that vitamin K₃ (menadione, 2-methyl-1,4-naphthoquinone) inhibits the activity of human mitochondrial DNA polymerase γ (pol γ). In this study, we focused on plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), and chemically synthesized novel plumbagins conjugated with C20 to C22:6 fatty acids (5-O-acyl plumbagins). These chemically modified plumbagins enhanced mammalian pol inhibition and their cytotoxic activity. Plumbagin conjugated with chains consisting of more than C18-unsaturated fatty acids strongly inhibited the activities of calf pol α and human pol γ. Plumbagin conjugated with oleic acid (C18:1-acyl plumbagin) showed the strongest suppression of human colon carcinoma (HCT116) cell proliferation among the ten synthesized 5-O-acyl plumbagins. The inhibitory activity on pol α, a DNA replicative pol, by these compounds showed high correlation with their cancer cell proliferation suppressive activity. C18:1-Acyl plumbagin selectively inhibited the activities of mammalian pol species, but did not influence the activities of other pols and DNA metabolic enzymes tested. This compound inhibited the proliferation of various human cancer cell lines, and was the cytotoxic inhibitor showing strongest inhibition towards HT-29 colon cancer cells (LD₅₀ = 2.9 μM) among the nine cell lines tested. In an in vivo anti-tumor assay conducted on nude mice bearing solid tumors of HT-29 cells, C18:1-acyl plumbagin was shown to be a promising tumor suppressor. These data indicate that novel 5-O-acyl plumbagins act as anti-cancer agents based on mammalian DNA replicative pol α inhibition. Moreover, the results suggest that acylation of plumbagin is an effective chemical modification to improve the anti-cancer activity of vitamin K₃ derivatives, such as plumbagin.

Citation: Kawamura M, Kuriyama I, Maruo S, Kuramochi K, Tsubaki K, et al. (2014) Anti-Tumor Effects of Novel 5-O-Acyl Plumbagins Based on the Inhibition of Mammalian DNA Replicative Polymerase Activity. PLoS ONE 9(2): e88736. doi:10.1371/journal.pone.0088736

Editor: Hitoshi Ashida, Kobe University, Japan

Received November 11, 2013; Accepted January 10, 2014; Published February 10, 2014

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Funding: This study was supported in part by the MEXT (Ministry of Education, Culture, Sports, Science and Technology, Japan)-Supported Program for the Strategic Research Foundation at Private Universities, 2012–2016. Y.M. acknowledges Grant-in-Aids for Scientific Research (C) (No. 24580205) from MEXT, and the Hyogo Science and Technology Association (Japan). I.K. acknowledges a Grant-in-Aid for Young Scientists (B) (No. 23710262) from MEXT. K.K and Y.M. acknowledge a Grant-in-Aid for A-STEP (Adaptable and Seamless Technology Transfer Program through target-driven R&D) (Exploratory Research) from JST (Japan Science and Technology Agency). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors declare that there are no conflicts of interest.

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Introduction

Cancer is a major global public health problem. Epidemiological and animal studies have indicated that chemopreventive natural products are associated with a reduced risk of cancer development [1,2]. Furthermore, selective inhibitors of DNA polymerases (pols) are considered potentially useful anti-cancer, anti-viral, anti-parasitic, and anti-pregnancy agents because some are known to suppress human cancer and normal cell proliferation, and are cytotoxic [3,4].

Pol DNA-dependent DNA polymerase, E.C. 2.7.7.7 catalyzes deoxyribonucleoside addition to the 3’-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules [5]. The human genome encodes at least 15 DNA pols, which function in cellular DNA synthesis [6,7]. Eukaryotic cells contain three replicative pols (α, δ, and ε), one mitochondrial pol (γ), and at least 11 non-replicative pols [β, ζ, η, θ, τ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT), and REV1] [8,9]. Pols have a highly conserved structure, with their overall catalytic subunits showing little variation among species; conserved enzyme structures are usually preserved over time as they perform important cellular functions that confer evolutionary advantages. Based on sequence homology, eukaryotic pols can be divided into four main families, termed A, B, X, and Y [8]. Family A includes mitochondrial pol γ as well as pols θ and τ; family B includes the three replicative pols α, δ, and ε and also pol γ; family X comprises pols β, λ, and μ as well as TdT; and family Y includes pols η, ι, and κ in addition to REV1 [9]. We have studied selective inhibitors of each pol derived from natural products including food materials and nutrients for more than 18 years [10,11]. We have found that vitamin K₃, but not K1 or K2, is a potent inhibitor of human pol γ [12,13].

Vitamin K₃ (menadione, 2-methyl-1,4-naphthoquinone, 3 of Fig. 1) is a fat-soluble compound that contains quinone as its principal chemical feature. Quinones are a class of organic compounds that are derived from aromatic compounds via the exchange of an even number of –CH= groups for –C(=O)–.
groups and any necessary rearrangement of double bonds, resulting in a fully conjugated cyclic dione structure. The toxicological properties of quinones, which act as alkylating agents, have also been examined. For example, quinones are known to interact with flavoproteins to generate reactive oxygen species (ROS) that can induce biological injury [14–17]. In this study, we focused on 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin, 1 of Fig. 1), which has the common naphthoquinone skeleton and a hydroxy group and a methyl group at the C-5 and C-2 positions, respectively. Plumbagin (1) is found in the plants of the Plumbaginaceae, Droseraceae, Ancestrocladaceae, and Dioncophyllaceae families. The chief source of plumbagin (1) is the root of Plumbago zeylanica L. (also known as “Chitrak”). Plumbagin (1) has been shown to exert anti-carcinogenic, anti-atherosclerotic, and anti-microbial effects [18–21]. The root of P. zeylanica L. has been used in Indian medicine for approximately 2,750 years and its components possess anti-atherogenic, cardiotonic, hepatoprotective, and neuroprotective properties [19]. Plumbagin (1) has potent anti-proliferative and apoptotic activities in various types of human cancers, and its mechanism of cytotoxicity is by inhibition of a PI-3 kinase for ROS generation [22].

We previously found that a vitamin K₃ (3) derivative, juglone (5-hydroxy-1,4-naphthoquinone, 4 of Fig. 1), conjugated with fatty acids such as 5-O-acyl juglones (5a–j) of Fig. 1) were stronger pol inhibitors than juglone alone (4) [23], therefore, ten 5-O-acylated derivatives of plumbagin (2a–j of Fig. 1) were chemically synthesized from plumbagin (1) and fatty acids to compare with 5-O-acyl juglones (5a–j). In this study, we first investigated the stability of 5-O-acyl plumbagins (2) and 5-O-acyl juglones (5). We also assayed the inhibitory effects of ten 5-O-acyl plumbagins (2a–j) on mammalian pol activity, cytotoxicity in human cancer cell lines and in vivo anti-tumor activity compared with plumbagin (1) and 5-O-acyl juglones (3). The relationship between the pol inhibitory and anti-tumor effects of vitamin K₃-based acylated derivatives is discussed.

Materials and Methods

Materials
A chemically synthesized DNA template, poly(dA), was purchased from Sigma-Aldrich Inc. and a customized oligo(dT)₁₈ DNA primer was produced by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). Radioactive nucleotide [³²P]-labeled 2′-deoxythymidine-5′-triphosphate (dTTP; 43 Ci/nmol) was obtained from Moravek Biochemicals Inc. (Brea, CA, USA). All other reagents were of analytical grade from Nacalai Tesque Inc. (Kyoto, Japan).

Enzymes
Pol α was purified from calf thymus by immunoaffinity column chromatography as described by Tamai et al. [24]. Recombinant rat pol β was purified from Escherichia coli JM109 as described by Date et al. [25]. The human pol γ catalytic gene was cloned into pFastBac. Histidine-tagged enzyme was expressed using the BACTO-BC AT Baculovirus Expression System according to the manufacturer’s instructions (Life Technologies, Frederick, MD, USA) and purified using ProBound resin (Invitrogen Japan, Tokyo, Japan) [26]. Human pols δ and ε were purified by nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pol δ and ε-conjugated affinity column chromatography, respectively [27]. A truncated form of human pol η (residues 1–511) tagged with His₆ at its C-terminal was expressed in E. coli cells and purified as described by Kusumoto et al. [28]. A recombinant mouse pol τ tagged with His₆ at its C-terminal was expressed and purified by Ni-NTA column chromatography [29]. A truncated form of pol κ (residues 1–560) with 6× His-tags attached at the C-terminus was overexpressed in E. coli and purified as described by Ohashi et al. [30]. Recombinant human His-pol λ was overexpressed and purified according to a method described by Shimazaki et al. [31]. Recombinant human His-pol μ was overexpressed in E. coli BL21 and purified using Glutathione Sepharose 4B (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) column chromatography according to the method for pol λ preparation by Shimazaki et al. [31]. Pol α from a higher plant, caulilower, was purified from the inflorescence structure according to the methods outlined by Sakaguchi et al. [32]. Recombinant rice (Oryza sativa L. cv. Nipponbare) pol λ tagged with His₆ at the C-terminus was expressed in E. coli and purified as described by Uchiyama et al. [33]. Calf TdT, Tαγ pol, Tα pol, Tβ RNA polymerase, and Tδ polynucleotide kinase were purchased from Takara Bio Inc. (Kyoto, Japan). The Klone fragment of pol I from E. coli was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). Bovine pancreas deoxyribonuclease I was obtained from Stratagene Cloning Systems (La Jolla, CA, USA).

Measurement of pol activity
The reaction mixtures for calf pol α, rat pol β, plant pol α, and prokaryotic pols have been described previously [34,35]; those for pol γ as well as pols δ and ε were previously described by Umeda et al. [26] and Ogawa et al. [36], respectively. Those for pols η, τ, and κ were the same as for pol α, and those for pols λ and μ were the same as for pol β. For the pol reactions, poly(dA)/oligo(dT)₁₈ (A/T, 2/1) and dTTP were used as the DNA template-primer substrate and nucleotide (dTTP, 2′-deoxyadenosine-5′-triphosphate) substrate, respectively. For the TdT reactions, oligo(dT)₁₈ (A/T, 2/1) and dNTPs were used as the DNA template-primer substrate and nucleotide (dNTP, 2′-deoxyribonucleoside-5′-triphosphate) substrate, respectively.
were next added to 16 CO2/95% air. For the cell viability assay, cells were seeded at 1.6 mg/mL NaHCO3 at 37°C in a humid atmosphere of 5% CO2 in air at 37°C, and under standard reaction conditions [34,35].

Other enzyme assays
The activities of calf thymus DNA polymerase α, T7 RNA polymerase, mouse inosine-5’-triphosphate (IMP) dehydrogenase (type II), T4 polynucleotide kinase, and bovine deoxyribonuclease I were measured in standard assays according to the manufacturer’s specifications, as described by Tamiya-Koizumi et al. [37], Nakayama and Saneyoshi [30], Mizushina et al. [39], Solhis et al. [40], and Lu and Sakaguchi [41], respectively.

Thermal transition of DNA
Thermal transition profiles of dsDNA to single-stranded DNA with or without test compound were obtained with a spectrophotometer (UV-2500; Shimadzu Corp., Kyoto, Japan) equipped with a thermostatic cell holder according to previous methods [42]. Calf thymus DNA (6 μg/mL) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% DMSO. The solution temperature was equilibrated to 75°C for 10 min, and then increased by 1°C at 2-min intervals for each measurement point. Any change in the absorbance (260 nm) of the compound itself in the spectrophotometer was automatically subtracted from that of DNA plus the compound in the spectrophotometer.

Cell culture and measurement of cancer cell viability
The following human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA): lung (A549), prostate (DU145 and PC3), colon (HCT116 and HT-29), pancreatic cancer (PANC-1). These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 1.6 mg/mL NaHCO3 at 37°C in a humid atmosphere of 5% CO2/95% air. For the cell viability assay, cells were seeded at 1 × 104 cells/well in a 96-well microplate with various concentrations of each compound. One unit of pol activity was defined as the amount of each enzyme that catalyzed the incorporation of 1 nmol dTTP into synthetic DNA template primers in 60 min, at 37°C, and incubated at 37°C for 10 min. These inhibitor-enzyme mixtures in 8 μL volumes were next added to 16 μL of enzyme standard reaction mixture and incubated at 37°C for 60 min, except for Tag pol, which was incubated at 74°C for 60 min. Activity without inhibitor was considered 100%, and the relative activity was determined for each inhibitor concentration. One unit of pol activity was defined as the amount of each enzyme that catalyzed the incorporation of 1 nmol dTTP into synthetic DNA template primers in 60 min, at 37°C, and under standard reaction conditions [34,35].

Analytical instruments for synthetic 5-O-acyl plumbagins (2a–j)
Melting points, determined on a Yanaco Micro Melting Point apparatus, are uncorrected. NMR spectra were recorded on a Bruker spectrometer (Avance 400). Chemical shifts are expressed in δ (ppm) relative to Me4Si or the residual solvent resonance, and coupling constants (J) are expressed in Hz. The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, br = broad. Infrared spectra (IR) were recorded on a HORIBA FT-720, using NaCl (neat) or KBr pellets (solid) and are reported in wavenumbers (cm⁻¹). High resolution mass spectra (HRMS) were obtained on a JEOL mass spectrometer (JMS-700 MSStation) using fast atom bombardment (FAB), or a Fourier transformation-ion cyclotron resonance-mass spectrometer, Bruker solariX (FT-ICR-MS) by using electrospray ionization (ESI) and laser desorption ionization (LDI) techniques. Analytical thin-layer chromatography (TLC) was performed on Silica Gel 60 F254 plates (Merck). Flash chromatography was carried out on SiSilFlash F60 (Silicycle).

Synthesis and characterization of 5-O-acetoxy-2-methyl-1,4-naphthoquinone (C20:0 Acyl plumbagin, 2a)
Acetic anhydride (0.5 mL) was added to a solution of plumbagin (1) (107 mg, 0.57 mmol) in pyridine (1.0 mL), and the mixture was stirred at room temperature for 4 h. After the solvent was removed, the residue was purified by silica gel chromatography (EtOAc/hexanes 1:3, v/v) to yield 2a (118 mg, 90%) as a yellow solid [44]. Mp 118–124°C; IR (KBr) 3049, 2987, 2966, 2927, 1761, 1662, 1593, 1431, 1375, 1365, 1271, 1203, 1024, 910, 785 cm⁻¹; 1H NMR (400 MHz, CDCl3) 6 (dd, J = 8.0 Hz, 0.8 Hz, 1H), 7.73 (q, J = 8.0 Hz, 1H), 7.36 (dd, J = 8.0 Hz, 0.8 Hz, 1H), 6.71 (q, J = 1.2 Hz, 1H), 2.45 (s, 3H), 2.17 (d, J = 1.2 Hz, 3H); 13C NMR (100 MHz, CDCl3) δ 184.8, 183.6, 169.4, 149.2, 146.9, 136.8, 134.4, 133.8, 129.4, 125.1, 123.4, 21.1,
General procedure for the preparation of 5-O-acyl plumbagins (2b–f) using MNBA

Et₃N (2.5–2.6 equiv.), 2-methyl-6-nitrobenzoic anhydride (MNBA) (1.5 equiv.) and N,N-dimethyl-4-aminopyridine (DMAP) (0.1 equiv.) were added to a solution of plumbagin (1) (1.0 equiv.) and carboxylic acid (1.5 equiv.) in CH₂Cl₂ at room temperature. The mixture was stirred at room temperature under a N₂ atmosphere until no further TLC changes were observed. The reaction was quenched by the addition of H₂O, and the mixture was extracted with CHCl₃. The combined extracts were washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel chromatography using EtOAc/hexanes as eluent.

Synthesis and characterization of 5-O-propanoyloxy-2-methyl-1,4-naphthoquinone (C3:0-Acyl plumbagin, 2b)

Following the general procedure, the reaction of plumbagin (1) (52 mg, 0.28 mmol) with propionic acid (30 µL, 0.41 mmol) using MNBA (143 mg, 0.41 mmol), Et₃N (96 µL, 0.69 mmol), and DMAP (3.4 mg, 0.03 mmol) for 2 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/5, v/v) to give 2b (60 mg, 89%) as a yellow solid [44]. Mp = 108–112°C; IR (KBr) 3076, 3043, 2981, 2937, 1766, 1662, 1630, 1591, 1358, 1265, 1194, 1132, 1080, 1024, 904, 881, 781 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.06 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 7.73 (t, J = 8.0 Hz, 1H), 7.36 (dd, J = 8.0 Hz, 1H), 7.23 (dd, J = 8.0 Hz, 1H)
$J = 8.0$ Hz, $1.2$ Hz, $1H$), $6.71$ (q, $J = 7.6$ Hz, $2H$), $2.16$ (d, $J = 1.2$ Hz, $3H$), $1.32$ (t, $J = 7.6$ Hz, $3H$); $13C$ NMR (100 MHz, CDCl$_3$) $\delta$ 184.8, 183.6, 172.8, 149.3, 146.8, 136.9, 134.4, 133.8, 129.4, 125.0, 123.5, 27.6, 16.0, 8.7; HRMS (FAB) calcd for C$_{14}$H$_{12}$O$_4$Na ([M+Na]$^+$) 267.0633, found 267.0639.

**Synthesis and characterization of 5-O-hexanoyloxy-2-methyl-1,4-naphthoquinone (C6:0-Acyl plumbagin, 2c)**

Following the general procedure, the reaction of plumbagin (1) (100 mg, 0.53 mmol) with caproic acid (100 $\mu$L, 0.80 mmol) using MNBA (274 mg, 0.80 mmol), Et$_3$N (185 $\mu$L, 1.33 mmol), and DMAP (6.7 mg, 0.05 mmol) for 2 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/6, v/v) to give 2c (102 mg, 68%) as a yellow solid. Mp = 45–47°C; IR (KBr) 3086, 2958, 2929, 2871, 1766, 1664, 1631, 1595, 1454, 1362, 1267, 1234, 1138, 1105, 912, 891, 791 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.99 (dd, $J = 8.0$ Hz, 1.2 Hz, 1H), 7.65 (t, $J = 8.0$ Hz, 1H), 7.28 (dd, $J = 8.0$ Hz, 1.2 Hz, 1H), 6.64 (q, $J = 1.2$ Hz, 1H), 2.66 (t, $J = 7.6$ Hz, 2H), 2.09 (d, $J = 1.2$ Hz, 3H), 1.75 (quin, $J = 7.6$ Hz, 2H), 1.41-1.28 (m, 4H), 0.87 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 184.9, 183.6, 172.2, 149.4, 146.8, 136.9, 134.4, 133.8, 129.5, 125.0, 123.6, 34.2, 31.3, 24.1, 22.4, 16.1, 14.0; HRMS (FAB) calcd for C$_{17}$H$_{18}$O$_4$Na ([M+Na]$^+$) 309.1103, found 309.1099.

**Synthesis and characterization of 5-O-dodecanoyloxy-2-methyl-1,4-naphthoquinone (C12:0-Acyl plumbagin, 2d)**

Following the general procedure, the reaction of plumbagin (1) (53 mg, 0.28 mmol) with lauric acid (85 mg, 0.43 mmol) using MNBA (147 mg, 0.43 mmol), Et$_3$N (100 $\mu$L, 0.72 mmol), and DMAP (3.5 mg, 0.03 mmol) for 19 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/9, v/v) to give 2d (90 mg, 86%) as a yellow crystal; Mp 41–42°C; IR (KBr) 2922, 2850, 1755, 1654, 1632, 1593, 1464, 1358, 1271, 1230, 1188, 1144, 939, 918, 893, 787 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.06 (dd, $J = 8.0$ Hz, 1.2 Hz, 1H), 7.73 (t, $J = 8.0$ Hz, 1H), 7.35 (dd, $J = 8.0$ Hz, 1.2 Hz, 1H), 6.71 (q, $J = 1.2$ Hz, 1H), 2.73 (t, $J = 7.6$ Hz, 2H), 2.16 (d, $J = 1.2$ Hz, 3H), 1.81 (quin, $J = 7.6$ Hz, 2H), 1.38-1.27 (brm, 16H), 0.88 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 184.9, 183.6, 172.2, 149.4, 146.8, 136.9, 134.4, 133.8, 129.5, 125.0, 123.6, 34.2, 31.9, 29.6 (2C), 29.5, 29.3, 29.2, 24.4, 22.7, 16.1, 14.1; HRMS (ESI-LDI) calcd for C$_{23}$H$_{30}$O$_4$Na ([M+Na]$^+$) 393.2036, found 393.2032.

**Synthesis and characterization of 5-O-octadecanoyloxy-2-methyl-1,4-naphthoquinone (C18:0-Acyl plumbagin, 2e)**

Following the general procedure, the reaction of plumbagin (1) (102 mg, 0.54 mmol) with stearic acid (231 mg, 0.81 mmol) using MNBA (280 mg, 0.81 mmol), Et$_3$N (189 $\mu$L, 1.36 mmol), and DMAP (6.6 mg, 0.05 mmol) for 19 h gave the crude product.

Figure 3. Stability of C18:1-acyl plumbagin (2f) and C18:1-acyl juglone (5f) under basic conditions. C18:1-acyl plumbagin (2f) (A) and C18:1-acyl juglone (5f) (B) were treated with 1 equivalent of Triton B in 1,4-dioxane and MeOH. The mixtures were monitored by UV-vis spectroscopy over time. Conditions: $1.1 \times 10^{-3}$ M, 25°C, light path length = 1 mm.

doi:10.1371/journal.pone.0088736.g003
The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/10, v/v) to give 2e (139 mg, 56%) as a yellow solid. Mp = 67–70 °C; IR (KBr) 2922, 2850, 1759, 1660, 1593, 1541, 1514, 1471, 1273, 1142, 1105, 893, 789 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.99 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 7.65 (t, J = 8.0 Hz, 1H), 7.27 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 6.63 (q, J = 1.2 Hz, 1H), 2.66 (t, J = 7.6 Hz, 2H), 2.09 (d, J = 1.2 Hz, 3H), 1.74 (quin, J = 7.6 Hz, 2H), 1.31–1.19 (brm, 28H), 0.81 (t, J = 7.6 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 184.9, 183.6, 172.2, 149.4, 146.8, 136.9, 134.4, 133.8, 129.5, 125.4, 123.6, 34.2, 31.9, 29.7 (5C), 29.6 (2C), 29.6, 29.5, 29.3, 24.4, 22.7, 16.0, 14.1; HRMS (FAB) calcd for C₂₉H₄₂O₄Na ([M+Na]⁺) 477.2981, found 477.2988.

Synthesis and characterization of 5-O-oleoyl-2-methyl-1,4-naphthoquinone (C₁₈:₁-Acyl plumbagin, 2f)

Following the general procedure, the reaction of plumbagin (1) (100 mg, 0.53 mmol) with oleic acid (0.25 mL, 0.79 mmol) using MNBA (275 mg, 0.80 mmol), Et₃N (106 µL, 1.33 mmol), and DMAP (6.5 mg, 0.05 mmol) for 24 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/20, v/v) to give 2f (166 mg, 69%) as yellow oil. IR (neat) 3005, 2925, 2834, 1770, 1644, 1595, 1456, 1271, 1190, 1107, 895, 783 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.06 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 7.72 (t, J = 8.0 Hz, 1H), 7.34 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 6.70 (q, J = 1.2 Hz, 1H), 5.40–5.35 (m, 2H), 2.73 (t, J = 7.6 Hz, 2H), 2.16 (d, J = 1.2 Hz, 3H), 1.97 (brm, 4H), 1.81 (quin, J = 7.6 Hz, 2H), 1.47–1.27 (brm, 20H), 0.88 (t, J = 7.6 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 184.9, 183.6, 172.2, 149.4, 146.8, 136.9, 134.4, 133.8, 129.5, 125.4, 123.6, 34.2, 31.9, 29.7 (5C), 29.6 (2C), 29.6, 29.5, 29.3, 29.2, 29.1, 27.2, 27.2, 24.4, 22.7, 16.1, 14.1; HRMS (FAB) calcd for C₂₉H₄₀O₄Na ([M+Na]⁺) 475.2824, found 475.2821.

General procedure for the preparation of acyl plumbagins (2g–j) via acyl chlorides

Oxalyl chloride (3.0–3.6 equiv.) was added to a solution of carboxylic acid (1 equiv.) in CH₂Cl₂ at 0 °C. The mixture was stirred at room temperature for 3–4.5 h. The solvent was removed to yield crude acyl chloride. A solution of the acyl chloride (2.2–5.8 equiv.), plumbagin (1) (1 equiv.) and DMAP (0.1 equiv.) in pyridine was stirred at room temperature. The mixture was stirred at room temperature under a N₂ atmosphere until no further TLC changes were observed. The reaction was quenched by the addition of H₂O, and the mixture was extracted with CHCl₃. The extracts were washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel chromatography using hexanes/ethyl acetate as eluent.

Synthesis and characterization of 5-O-linoleoyloxy-2-methyl-1,4-naphthoquinone (C₁₈:₂-Acyl plumbagin, 2g)

Following the general procedure, linoleoyl chloride was prepared by treatment of linoleic acid (110 mg, 0.39 mmol) with oxalyl chloride (100 µL, 1.16 mmol) for 4 h. The reaction of...
plumbagin (1) (34 mg, 0.18 mmol) with the crude linoleoyl chloride using DMAP (2.2 mg, 0.02 mmol) in pyridine for 2 h gave the crude product. The crude product was purified by silica gel column chromatography (toluene/hexanes = 1/10, v/v) to give 2g (31.9 mg, 39%) as yellow oil. IR (neat) 3008, 2927, 2854, 1768, 1664, 1595, 1462, 1358, 1271, 1190, 1009, 1026, 982, 895, 783 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.06 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 7.73 (t, J = 8.0 Hz, 1H), 7.35 (dd, J = 8.0 Hz, 1.2 Hz, 1H), 6.71 (q, J = 1.2 Hz, 1H), 5.42-5.33 (m, 4H), 2.78 (t, J = 7.6 Hz, 2H), 2.73 (t, J = 7.6 Hz, 2H), 2.16 (d, J = 1.2 Hz, 3H), 2.08-2.03 (m, 4H), 1.81 (quin, J = 7.6 Hz, 2H), 1.36-1.26 (m, 14H), 0.94-0.86 (m, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 184.9, 183.6, 172.2, 149.4, 146.8, 136.9, 134.4, 133.8, 132.0, 130.1, 129.5, 128.0, 127.9, 125.0, 123.5, 34.2, 32.5, 31.5, 29.6, 29.5, 29.3, 29.2, 29.1, 27.2, 25.6, 24.4, 22.6, 16.1, 14.1; HRMS (FAB) calcd for C₂₉H₃₈O₄Na ([M+Na]+) 473.2668, found 473.2666.

Synthesis and characterization of 5-O-linolenoyloxy-2-methyl-1,4-naphthoquinone (C18:3-Acyl plumbagin, 2h)
Following the general procedure, ω-linolenic chloride was prepared by treatment of ω-linolenic acid (200 mg, 0.72 mmol) with oxalyl chloride (200 µL, 2.33 mmol) for 4.5 h. The reaction of plumbagin (1) (25 mg, 0.13 mmol) with the crude ω-linolenoyl chloride using DMAP (1.6 mg, 0.01 mmol) in pyridine for 18 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/20, v/v) to give 2h (32 mg, 54%) as yellow oil; IR (neat) 3010, 2927, 2856, 1768, 1664, 1595, 1462, 1358, 1271, 1107, 974, 912, 783 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.05 (dd, J = 7.6 Hz, 1.2 Hz, 1H), 7.71 (t, J = 7.6 Hz, 1H), 7.34 (dd, J = 7.6 Hz, 1.2 Hz, 1H), 6.71 (q, J = 1.2 Hz, 1H), 5.41-5.33 (m, 6H), 2.83-2.71 (m, 6H), 2.16 (d, J = 1.2 Hz, 3H), 2.12-2.00 (m, 4H), 1.81 (quin, 2H), 1.48-1.30 (brm, 8H), 0.98 (t, J = 7.6 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 184.9, 183.6, 172.2, 149.4, 146.8, 136.9, 134.4, 133.8, 132.0,

Figure 5. Effect of plumbagin (1) and 5-O-acyl plumbagins (2a–j) on the proliferation of HCT116 human colon carcinoma cells. Each compound (10 and 100 µM) was added to cultured HCT116 cells. The cells were incubated for 48 h, and the rate of proliferation inhibition was determined by MTT assay. Cell proliferation inhibition of the cancer cells in the absence of the compound (control) was taken as 100%. Data are shown as the mean ± SD (n = 5). ** P<0.01 and * P<0.05 vs. controls.
doi:10.1371/journal.pone.0088736.g005
Following the general procedure, eicosapentaenoyl chloride was prepared by treatment of eicosapentaenoic acid (194 mg, 0.64 mmol) with oxalyl chloride (200 μL, 2.33 mmol) for 3 h. The reaction of plumbagin (1) (20 mg, 0.10 mmol) with the crude eicosapentaenoyl chloride using DMAP (1.3 mg, 0.01 mmol) in pyridine for 4 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hexanes = 1/20, v/v) to give 4i (40 mg, 80%) as yellow oil. IR (neat) 3012, 2962, 1768, 1664, 1595, 1446, 1358, 1271, 1190, 1126, 897, 783, 717 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.06 (dd, J = 7.6 Hz, 1.2 Hz, 1H), 7.72 (t, J = 7.6 Hz, 1H), 7.34 (dd, J = 7.6 Hz, 1.2 Hz, 1H), 6.70 (q, J = 7.6 Hz, 2H), 5.50-5.35 (m, 10H), 2.90-2.72 (m, 10H), 2.27 (q, J = 7.6 Hz, 2H), 2.16 (d, J = 1.2 Hz, 3H), 2.08 (quin, J = 7.6 Hz, 2H), 1.90 (quin, J = 7.6 Hz, 2H), 0.97 (t, J = 7.6 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 184.8, 183.6, 171.9, 149.3, 146.8, 136.9, 134.4, 133.8, 132.0, 129.4, 129.0, 128.9, 128.5, 128.3, 128.2, 128.1, 128.1, 127.8, 127.0, 125.0, 123.5, 33.6, 26.6, 25.6, 25.6, 25.5, 24.3, 20.5, 16.1, 14.3; HRMS (ESI-LDI) calcd for C₃₁H₃₆O₄Na ([M+Na]⁺) 495.2506, found 495.2505.

Synthesis and characterization of 5-O-docosahexaenoyloxy-2-methyl-1,4-naphthoquinone (C22:6-Acyl plumbagin, 2j)

Following the general procedure, docosahexaenoyl chloride was prepared by treatment of docosahexaenoic acid (210 mg, 0.64 mmol) with oxalyl chloride (200 μL, 2.33 mmol) for 3 h. The reaction of plumbagin (1) (20 mg, 0.11 mmol) with the crude docosahexaenoyl chloride using DMAP (1.3 mg, 0.01 mmol) in pyridine for 3 h gave the crude product. The crude product was purified by silica gel column chromatography (EtOAc/hex-
### Table 1. IC₅₀ of C18:1-acyl plumbagin (2f) on the activities of mammalian pols, other species pols, and various DNA metabolic enzymes.

| Enzyme                          | IC₅₀ values (µM) |
|---------------------------------|------------------|
| **Mammalian pols**              |                  |
| [A family of pols]              |                  |
| Human pol γ                     | 4.5±0.40 **      |
| [B family of pols]              |                  |
| Calf pol α                      | 4.8±0.38 **      |
| Human pol δ                     | 5.3±0.41 **      |
| Human pol ε                     | 4.9±0.40 **      |
| [X family of pols]              |                  |
| Rat pol β                       | 10.3±0.46 **     |
| Human pol λ                     | 9.2±0.43 **      |
| Human pol μ                     | 9.5±0.48 **      |
| Calf TdT                        | 10.6±0.50 **     |
| [Y family of pols]              |                  |
| Human pol η                     | 7.9±0.42 **      |
| Mouse pol ε                     | 8.2±0.43 **      |
| Human pol κ                     | 7.6±0.41 **      |
| **Plant pol**                   |                  |
| Cauliflower pol α               | >100             |
| Rice pol λ                      | >100             |
| **Prokaryotic pols**            |                  |
| E. coli pol I                   | >100             |
| Taq pol                         | >100             |
| T4 pol                          | >100             |
| **Other DNA metabolic enzymes** |                  |
| Calf primase of pol α           | >100             |
| T7 RNA polymerase               | >100             |
| Mouse IMP dehydrogenase (type II)| >100             |
| T4 polynucleotide kinase        | >100             |
| Bovine deoxyribonuclease I      | >100             |

Compounds were incubated with each enzyme (0.05 units). Enzyme activity in the absence of an inhibitor (control) was taken as 100%; data, mean ± SD (n = 3). **P<0.01 vs. controls.

doi:10.1371/journal.pone.0088736.t001

Stability of C18:1-acyl plumbagin (2f) and C18:1-acyl juglone (5f) under basic conditions

A 2.2×10⁻² M solution of Triton B in MeOH was added to a 2.2×10⁻² M solution of each sample in 1,4-dioxane. Then, the mixture was diluted with 1,4-dioxane to a concentration of 1.1×10⁻³ M. Then, the stability of each compound was monitored by UV-vis spectroscopy. UV-vis spectra were measured at 25°C on a UV-vis spectrophotometer (JASCO V-650, Tokyo, Japan). For comparison, UV-vis spectra of 1.1×10⁻³ M solution of C18:1-acyl plumbagin (2f) and C18:1-acyl juglone (5f) in 1,4-dioxane and MeOH (19/1, v/v) were measured.

Statistical analysis

All data are expressed as the mean value ± the standard deviation (SD) of at least three independent determinations for each experiment. Statistical significance between each experimental group was analyzed using Student’s t-test, and a probability level of 0.01 and 0.05 was used as the criterion of significance.

Results and Discussion

Synthesis of 5-O-acyl plumbagins (2a–j)

Synthesis of 5-O-acyl plumbagins (2a–j) is summarized in Fig. 2. C2:0-Acyl plumbagin (2a) was prepared by treatment of plumbagin (1) with acetic anhydride in pyridine in 90% yield (Fig. 2A). 5-O-Acyl plumbagins 2b–f were prepared by condensation of plumbagin (1) with the corresponding carboxylic acids, using MNBA with triethylamine and DMAP in CH₂Cl₂ (Fig. 2B). 5-O-Acyl plumbagins 2g–j were prepared by acylation of plumbagin (1) with acyl chlorides in the presence of DMAP in pyridine (Fig. 2C).

Stability of C18:1-acyl plumbagin (2f) and C18:1-acyl juglone (5f) under basic conditions

We have found that C18:1-acyl plumbagin (2f) is more stable than C18:1-acyl juglone (5f) under basic conditions. C18:1-acyl plumbagin (2f) and C18:1-acyl juglone (5f) were treated with Triton B (benzyltrimethylammonium hydroxide) in 1,4-dioxane and MeOH. As shown in Fig. 3, UV spectra of the mixture were recorded at different reaction times. Almost no changes in UV spectra of C18:1-acyl plumbagin (2f) were observed before and after treatment of Triton B (Fig. 3A). UV spectra of the mixture of C18:1-acyl plumbagin (2f) and Triton B rarely changed during the reaction. In contrast, the changes in UV absorption of C18:1-acyl juglone (5f) were clearly observed (Fig. 3B). UV absorption at 675 nm and 400 nm was observed soon after the addition of Triton B. The absorption at 675 nm suggests the formation of an extended aromatic compound by the reaction of C18:1-acyl juglone with Triton B in 1,4-dioxane and MeOH. The absorption at 675 nm increased with reaction time. We observed weak and broad absorption in the wavelength range 300-700 nm. These results suggest that C18:1-acyl juglone (5f) readily decomposes under basic conditions.

Effect of synthesized 5-O-acyl plumbagins (2a–j) on the activities of mammalian pols

Initially, the inhibitory activity of each 5-O-acyl plumbagin (2a–j) toward mammalian pols was investigated using calf pol α, and human pol γ, k, and λ. In mammalian pols, pol α, γ, k, and λ were used as the representative pols for families B, A, Y, and X, respectively [9,9]. Assessment of the relative activity of each pol at
a set concentration (10 μM) of plumbagin (1) and the ten chemically synthesized compounds showed that some 5-O-acyl plumbagins were stronger inhibitors of these four mammalian pols than plumbagin (1) (Fig. 4). The plumbagins conjugated with C18 and longer chain unsaturated fatty acids (i.e., C18:1 to C22:6-acyl plumbagins 2f–j) strongly inhibited the activities of pols α and γ, suggesting that the group of unsaturated longer acyl side chains might be an important structural characteristic of 5-O-acyl plumbagin for pol inhibition. C22:6-acyl plumbagin (2j) was the strongest inhibitor of human pols κ and λ, and C18:0-acyl plumbagin (2e) did not inhibit pol α activity. When activated DNA (bovine deoxyribonuclease I-treated DNA) was used as the DNA template-primer substrate instead of synthesized DNA [poly(dA)/oligo(dT)18 (A/T = 2/1)] and dNTP was used as the nucleotide substrate instead of dTTP, the inhibitory effects of these compounds did not differ (data not shown).

Effect of synthesized 5-O-acyl plumbagins (2a–j) on cultured human cancer cells

Pols have emerged as important cellular targets for chemical intervention in the development of anti-cancer agents [2]. Therefore, the synthesized 5-O-acyl plumbagins (2a–j) could be useful in chemotherapy. Hence, we investigated the cytotoxic effect of these compounds against HCT116 human colon carcinoma cells. As shown in Fig. 5, 100 μM plumbagin (1) approximately 50% suppressed cell proliferation, but 10 μM of this compound did not. Of the ten synthesized compounds, the nine 5-O-acyl plumbagins except for C18:0-acyl plumbagin (2e) were stronger cell proliferation inhibitors than plumbagin (1). C18:1-Acyl plumbagin (2f) and C18:2-acyl plumbagin (2g) had the first and second strongest proliferation inhibitory effect on HCT116 cells among the compounds tested, and at 10 μM compound these cells showed less than 40 and 60% of the cell proliferation rate, respectively.

Table 2. LD50 values of C18:1-acyl plumbagin (2f) on the proliferation of human cancer cells.

| Human cancer cell line     | LD50 values (μM) |
|----------------------------|------------------|
| A549 (lung cancer)         | 15.4±1.7 **      |
| DU145 (prostate cancer)    | 8.8±0.9 **       |
| HCT116 (colon cancer)      | 6.5±0.6 **       |
| HeLa (cervical cancer)     | 21.0±2.5 **      |
| HepG2 (hepatocellular liver cancer) | 7.4±0.8 ** |
| HT-29 cells (colon cancer) | 2.9±0.4 **       |
| MCF-7 (breast cancer)      | 7.2±0.7 **       |
| PANC-1 (pancreatic cancer) | 16.1±1.8 **      |
| PC3 (prostate cancer)      | 10.0±1.1 **      |

The nine human cancer cell lines were incubated with C18:1-acyl plumbagin (2f) for 48 h. Cell viability was determined by MTT assay, and this viability in the absence of an inhibitor (control) was taken as 100%; data, mean ± SD (n=5). **P<0.01 vs. controls.

doi:10.1371/journal.pone.0088736.t002

The possible relationship between the observed inhibition of four mammalian pol families and HCT116 human colon cancer cell proliferation inhibitory activity was confirmed by comparing the effects of plumbagin (1) and the ten synthesized 5-O-acyl
plumbagins (2a–j) on these biological activities (Fig. 6). The effect of 10 μM of these compounds on the relative activity of pol α, which is a DNA replicative pol of the B family, showed the highest correlation with the effect of 100 μM of these compounds on the cancer cell proliferation among those mammalian pol families tested, with a correlation coefficient of 0.844. The relative activity of pol γ, a mitochondrial DNA replicative pol of the A family, showed a moderate correlation ($R^2 = 0.545$) with the cancer cell proliferation rate. Conversely, neither the activities of pols κ and λ, which are DNA repair-related pols of the Y and X families,

Figure 8. In vivo anti-tumor effects of C18:1-acyl plumbagin (2f). Nude mice bearing HT-29 solid tumors were administered with PBS (control), vitamin K₃ (3), juglone (4), C18:1-acyl juglone (5f), and C18:1-acyl plumbagin (2f) at a dose of 5 mg/kg. (A) Inhibitory effect on tumor volume in nude mice. (B) Body weight changes of nude mice; data, means ± SE (n = 6). * $P<0.05$ vs. controls.

doi:10.1371/journal.pone.0088736.g008
respectively, were related to cytotoxicity, with a correlation coefficient between these activities and cytotoxicity of <0.25. These results led to the speculation that the inhibition of the activities of DNA replicative pols, such as pols α and γ; in particular, inhibition of both the A and B families of pols by compounds 1 and 2a–j might cause the suppression of human cancer cell proliferation.

In particular, C18:1-acyl plumbagin (2f) showed the strongest inhibition of pol γ of the ten synthesized 5-O-acyl plumbagins (2a–j) tested. This compound also exhibited the strongest effects on cancer cell proliferation suppression (Fig. 5). Therefore, C18:1-acyl plumbagin (2f) was used in the latter part of this study.

Effects of C18:1-acyl plumbagin (2f) on the activities of various pols and other DNA metabolic enzymes

As described briefly in the Introduction, we succeeded in obtaining eleven mammalian pol species, including pols α, β, γ, δ, ε, η, τ, κ, λ, and μ, and TelT; however, pols ζ, θ, and v, and REV1 were not yet available at the time of the study. Currently, eukaryotes are thought to express at least 15 species of pols [6,7], and we are still in an era when most pols are very difficult to obtain. This compound inhibited the activity of all of the mammalian pols with IC_{50} values of 4.5–10.6 μM, and 50% inhibition of the A, B, X, and Y families of pols was observed at doses of 4.5, 4.8–5.3, 9.2–10.6, and 7.6–8.2 μM, respectively; therefore, the inhibitory effect of this compound on mammalian pols that could be obtained. This compound inhibited the activity of all of the mammalian pols with IC_{50} values of 4.5–10.6 μM, and 50% inhibition of the A, B, X, and Y families of pols was observed at doses of 4.5, 4.8–5.3, 9.2–10.6, and 7.6–8.2 μM, respectively; therefore, the inhibitory effect of this compound on the A and B families of pols was 1.5–2-fold stronger than that on the X and Y families of pols. Because the IC_{50} values of aphidicolin, a known eukaryotic DNA replicative pol α, δ, and ε inhibitor, were 20, 13, and 16 μM, respectively [45], the pol inhibitory activity of C18:1-acyl plumbagin (2f) was >2-fold more potent than that of aphidicolin.

In contrast, C18:1-acyl plumbagin (2f) had no effect on plant pols such as cauliflower pol α or rice pol λ, or prokaryotic pols, such as E. coli pol I, Taq pol, or T4 pol (Table 1). The three-dimensional structures of eukaryotic pols are likely to differ greatly from those of prokaryotic pols. This compound did not inhibit the activity of other DNA metabolic enzymes, such as calf primase pol α, 7 RNA polymerase, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase, or bovine deoxyribonuclease I. These results suggest that 5-O-acyl plumbagins (2) may be selective inhibitors of mammalian pols; in particular, plumbagin (1) conjugated with unsaturated fatty acids, such as C18:1-acyl plumbagin (2f), potently inhibited the activities of the A and B families of pols.

Influence of C18:1-acyl plumbagin (2f) on the hyperchromicity of dsDNA

Specific assays were performed to determine whether C18:1-acyl plumbagin (2f)-induced inhibition resulted from the ability of the compound to bind to DNA or the enzyme. The interaction of C18:1-acyl plumbagin (2f) with dsDNA was investigated by studying its thermal transition. For this, the melting temperature (T_m) of dsDNA in the presence of an excess of C18:1-acyl plumbagin (2f) (100 μM) was observed using a spectrophotometer equipped with a thermostatic cell holder. As shown in Fig. 7, a thermal transition (i.e., T_m) from 75 to 90°C was not observed within the concentration range used in the assay, whereas a typical intercalating compound, such as ethidium bromide (EtBr, 15 μM), was used as a positive control, an obvious thermal transition was observed.

The question of whether the inhibitory effect of C18:1-acyl plumbagin (2f) resulted from nonspecific adhesion to mammalian pols or from its binding to these enzymes was investigated by determining if an excessive amount of nucleic acid [poly(rC)] or protein (BSA; bovine serum albumin) prevented the inhibitory effect of C18:1-acyl plumbagin (2f). Poly(rC) and BSA had little or no influence on pol inhibition by C18:1-acyl plumbagin (2f) (data not shown), suggesting that this compound selectively bound to the pol molecule. These observations indicated that C18:1-acyl plumbagin (2f) did not act as a DNA intercalating agent or as a template-primer substrate.

Collectively, these results suggested that C18:1-acyl plumbagin (2f) might be a potent and specific inhibitor of mammalian pols. Subsequently, we investigated whether pol inhibition by C18:1-acyl plumbagin (2f) resulted in reduced human cancer cell proliferation.

Effect of C18:1-acyl plumbagin (2f) on cultured human cancer cell lines

C18:1-acyl plumbagin (2f) treatment for 48 h suppressed the proliferation of various human cancer cells in a dose-dependent manner. As shown in Table 2, C18:1-acyl plumbagin (2f) prevented the proliferation of nine human cancer cell lines, such as A549, DU145, HCT116, HeLa, HepG2, HT-29, MCF-7, Panc-1, and PC3 cells, with LD_{50} values of 2.9–21.0 μM. These results suggested that this compound could have suppressive activity against the different type of cancer cell lines. In particular, this compound showed the strongest cell proliferation suppression in the colon cancer cell lines, HCT116 and HT-29, with LD_{50} values of 6.5 and 2.9 μM, respectively. These dose-response curves by MTT detection were the same as that obtained by trypan blue staining (data not shown), suggesting that C18:1-acyl plumbagin (2f) might cause a direct toxic effect on the cells. Because these LD_{50} were similar to the IC_{50} for pols (Table 1), this inhibition must be mostly led by the function of pols, such as DNA replicative pol α. C18:1-acyl plumbagin (2f) more strongly suppressed the proliferation of these human cancer cell lines than aphidicolin, which is an inhibitor of eukaryotic DNA replicative pols (data not shown).

Effect of C18:1-acyl plumbagin (2f) on in vivo anti-tumor activity

Because the cell proliferation inhibitory effect of C18:1-acyl plumbagin (2f) was the strongest on HT-29 cells among the nine human cancer cell lines tested (Table 2), this cell line was used in vivo anti-tumor assays. In this assay, we investigated whether C18:1-acyl plumbagin (2f) was more stable and/or had stronger bioactivity than C18:1-acyl juglone (5f) in vivo; thus, C18:1-acyl plumbagin (2f) was compared with vitamin K_{3} (3), juglone (4), and C18:1-acyl juglone (5f).

HT-29 cells were subcutaneously injected into 40 nude mice. At 12 days after the implantation, these nude mice were sorted five groups (one control group and four treatment groups) and each mouse group contained 6 mice bearing solid tumor volume of 96–102 mm³. The sorted nude mice were injected with each test compound dissolved in PBS (5 mg/kg) at 1-day intervals until 40 days. As shown in Fig. 8A, these compounds suppressed tumor growth as compared with the control (PBS) group, and the decreased rates of tumor volume at 40 days following injection with vitamin K_{3} (3), juglone (4), C18:1-acyl juglone (5f), and C18:1-acyl plumbagin (2f) were 7.0, 9.2, 10.3, and 30.5%, respectively.
respectively. C18:1-acyl plumbagin (2f) showed more than a 3-fold stronger anti-tumor effect than the other compounds tested, suggesting that C18:1-acyl plumbagin (2f) must be stable in vivo, but C18:1-acyl juglone (5f) did not. A significant correlation was found between 5-O-acyl plumbagins (2) and the inhibition of mammalian pols, especially DNA replicative pol α (Fig. 1A). C18:1-Acylic plumbagin (2f) may be able to penetrate cancer cells of tumor in mouse and reach the nucleus, inhibiting the activities of pols and then the inhibition of pol activity by this compound may lead to cell proliferative suppression and prevent tumor growth.

None of the nude mice showed any significant loss of body weight throughout the experimental period (Fig. 3B). It was also noted that the main visceral organs, such as the liver, lung, kidney, spleen, heart, stomach, small intestine, large intestine, pancreas, and testis of all the groups showed no toxic or degenerative histological appearance (data not shown); therefore, C18:1-acyl plumbagin (2f) is of interest as a candidate material for anti-cancer treatment.

Conclusions

We previously found that vitamin K<sub>3</sub> (2-methyl-1,4-naphthoquinone), juglone (5-hydroxy-1,4-naphthoquinone) and vitamin K<sub>3</sub> conjugated with fatty acids inhibited the activity of mammalian pols. In this study, 5-O-acyl plumbagins (2), which are plumbagins (5-hydroxy-2-methyl-1,4-naphthoquinone) conjugated with fatty acid, were produced to establish an efficient chemical synthesis method. In the synthesized ten 5-O-acyl plumbagins (2a–j), 2c–j are novel compounds. These synthesized compounds were stronger inhibitors of the mammalian pols α, γ, κ, and λ, representing the pol families B, A, Y, and X, respectively, than plumbagin (1). Of the synthesized compounds, C18:1-acyl plumbagin (2f) showed the strongest suppression of human cancer cell proliferation. The human cancer cytotoxicity of this compound was realized through the inhibition of pols, which are essential for DNA replication as well as cell division. Because C18:1-acyl plumbagin (2f) potently inhibited the activities of replicative pols, such as pols α and γ and suppressed human cancer cell proliferation, they might show in vivo anti-tumor activity without any side effects. The in vivo anti-tumor effect of C18:1-acyl plumbagin (2f) was stronger than that of C18:1-acyl juglone (5f) because C18:1-acyl plumbagin (2f) is more stable than C18:1-acyl juglone (5f) under basic conditions. These results suggested that 5-O-acyl plumbagins (2), such as C18:1-acyl plumbagin (2f), could be used as anti-cancer chemotherapy agents based on their mammalian pol inhibition.

Acknowledgments

We are grateful for the following donations: calf pol β by Dr. M. Takeamura of Tokyo University of Science (Tokyo, Japan); rat pol β, and human pols δ and ε by Dr. K. Sakaguchi of Tokyo University of Science (Chiba, Japan); human pol γ by Dr. M. Suzuki of Nagoya University School of Medicine (Nagoya, Japan); mouse pol η and human pol 1 by Dr. F. Hanaoka of Gakushuin University (Tokyo, Japan) and Dr. C. Masutani of Nagoya University (Nagoya, Japan); human pol κ by Dr. H. Ohmori of Kyoto University (Kyoto, Japan); and human pols λ and μ by Dr. O. Koivai of Tokyo University of Science (Chiba, Japan). This study was carried out on mass spectrometers at the Joint Usage/Research Center (JURC) at Institute for Chemical Research, Kyoto University.

Author Contributions

Conceived and designed the experiments: KK YM. Performed the experiments: MK IK SM KK. Analyzed the data: KT HY YM. Wrote the paper: KK YM.

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