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3 **5-O-Acyl plumbagins inhibit DNA polymerase activity and suppress the**
4 **inflammatory response**

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1 Running title: Anti-inflammatory activity by C22:6-acyl plumbagin

2 *Keywords:* 5-*O*-Acyl plumbagins, C22:6-Acyl plumbagin, DNA polymerase λ , Enzyme
3 inhibitor, Anti-inflammation

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5 (Foot note)

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10 ¹ *Abbreviations used:* pol, DNA polymerase (E.C. 2.7.7.7); TNF, tumor necrosis
11 factor; LPS, lipopolysaccharide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; dsDNA,
12 double-stranded DNA; NF- κ B, nuclear factor- κ B; TdT, terminal deoxynucleotidyl
13 transferase; dTTP, 2'-deoxythymidine 5'-triphosphate; dNTP,
14 2'-deoxynucleoside-5'-triphosphate; IMP, inosine-5'-triphosphate; DMSO, dimethyl sulfoxide;
15 ELISA, enzyme-linked immunosorbent assay; IE, inhibitory effect; IC₅₀, 50% inhibitory
16 concentration; BSA, bovine serum albumin; BER, base excision repair.

1   **ABSTRACT**

2   We previously found that vitamin K₃ (menadione, 2-methyl-1,4-naphthoquinone) inhibits the
3   activity of human mitochondrial DNA polymerase (pol) γ . In this study, we focused on
4   plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), and chemically synthesized novel
5   plumbagins conjugated with C2:0 to C22:6 fatty acids (5-*O*-acyl plumbagins). These
6   chemically modified plumbagins displayed enhanced mammalian pol inhibition, with
7   plumbagin conjugated to docosahexaenoic acid (C22:6-acyl plumbagin) exhibiting the
8   strongest inhibition of pol λ among the ten 5-*O*-acyl plumbagins synthesized. C22:6-Acyl
9   plumbagin selectively inhibited the activities of mammalian pol species, but did not influence
10   the activities of other pols or DNA metabolic enzymes tested. The inhibition of pol λ , a DNA
11   repair/recombination pol, by these compounds was significantly correlated with both their
12   suppression of lipopolysaccharide (LPS) induced tumor necrosis factor- α (TNF- α) production
13   by mouse RAW264.7 macrophages and the reduction of
14   12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in the mouse ear. These
15   data indicate that 5-*O*-acyl plumbagins act as anti-inflammatory agents by inhibiting
16   mammalian pol λ . These results further suggest that C22:6-acyl plumbagin is a promising
17   anti-inflammatory candidate and that acylation could be an effective chemical modification to
18   improve the anti-inflammatory activity of vitamin K₃ derivatives, such as plumbagin.

1   **Introduction**

2   DNA-dependent   DNA   polymerase¹   (pol)   (E.C.   2.7.7.7)   catalyzes
3   deoxyribonucleotide addition to the 3'-hydroxyl terminus of primed double-stranded DNA
4   (dsDNA) molecules [1]. The human genome encodes at least 15 DNA pols which function in
5   cellular DNA synthesis [2, 3]. Eukaryotic cells contain three replicative pols (α , δ , and ϵ), one
6   mitochondrial pol (γ), and at least 11 non-replicative pols (β , ζ , η , θ , ι , κ , λ , μ , ν , terminal
7   deoxynucleotidyl transferase (TdT), and REV1) [4, 5]. Pols have a highly conserved structure,
8   with their overall catalytic subunits showing little variation among species; conserved enzyme
9   structures are usually preserved over time as they perform important cellular functions that
10   confer evolutionary advantages. Based on sequence homology, eukaryotic pols can be divided
11   into four main families, termed A, B, X and Y [4]. Family A includes mitochondrial pol γ as
12   well as pols θ and ν ; family B includes the three replicative pols α , δ , and ϵ and also pol ζ ;
13   family X comprises pols β , λ and μ , as well as TdT; and family Y includes pols η , ι and κ , in
14   addition to REV1 [5]. We have studied selective inhibitors of each pol derived from natural
15   products, including food materials and nutrients, for more than 19 years [6, 7]. We recently
16   found that vitamin K₃, but not K₁ or K₂, is a potent inhibitor of human pol γ [8, 9].

17   Vitamin K₃ (menadione, 2-methyl-1,4-naphthoquinone) is a fat-soluble compound
18   that contains quinone as its principal chemical feature. Quinones are a class of organic
19   compounds that are derived from aromatic compounds via the exchange of an even number of

1   –CH= groups for –C(=O)– groups and any necessary rearrangement of double bonds,
2   resulting in a fully conjugated cyclic dione structure. Recently, it was also reported that
3   vitamin K₃ suppresses inflammatory and immune responses [10]. In this study, we focused on
4   the related 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin, **1** of Figure 1), which has
5   the naphthoquinone skeleton in common and a hydroxyl group and a methyl group at the C-5
6   and C-2 positions respectively. Plumbagin is produced by plants of the Plumbaginaceae,
7   Droseraceae, Anastrocladaceae and Dioncophyllaceae families. The chief source of
8   plumbagin is the root of *Plumbago zeylanica* L. (also known as “Chitrak”). The root of *P.*
9   *zeylanica* L. has been used in Indian medicine for approximately 2,750 years and its
10   components possess anti-atherogenic, cardiotonic, hepatoprotective and neuroprotective
11   properties [11]. In recent studies, plumbagin has also been reported to exhibit
12   anti-inflammatory activity through inhibition of nuclear factor-κB (NF-κB) activation
13   [12-14].

14   We previously found that longer chain unsaturated fatty acids containing
15   *cis*-configured double-bonds inhibited the activity of mammalian pols [15-17]. Therefore,
16   ten fatty acids conjugated with plumbagin (i.e., 5-*O*-acylated derivatives of plumbagin) (**3** of
17   Figure 1) were chemically synthesized from plumbagin and fatty acids (**2** of Figure 1). We
18   investigated the inhibitory effects of the ten 5-*O*-acyl plumbagins synthesized (**3a–j** of Figure
19   1) on mammalian pol activity, the production of tumor necrosis factor-α (TNF-α) induced by

- 1 lipopolysaccharide (LPS) in cultured cells and inflammation *in vivo*. The relationship between
- 2 the inhibition of members of the four families of mammalian pols and the anti-inflammatory
- 3 activity of 5-*O*-acylated derivatives of plumbagins is discussed.

1   **Materials and methods**

2   *Materials*

3   Plumbagin (**1** of Figure 1) was purchased from Sigma-Aldrich Inc. (St. Louis, MO,
4   USA). Ten 5-*O*-acyl plumbagins (**3a-j** of Figure 1), derived from the conjugation of
5   plumbagin with ten different fatty acids (**2a-j** of Figure 1), were chemically synthesized as
6   described elsewhere [18]. A chemically synthesized DNA template, poly(dA), was purchased
7   from Sigma-Aldrich Inc., and a customized oligo(dT)₁₈ DNA primer was produced by
8   Sigma-Aldrich Japan K.K. (Hokkaido, Japan). Radioactive nucleotide [³H]-labeled
9   2'-deoxythymidine-5'-triphosphate (dTTP; 43 Ci/mmol) was obtained from Moravek
10   Biochemicals Inc. (Brea, CA, USA). For Western blot analysis, anti-pol λ antibody (ab82919)
11   and horseradish peroxidase-conjugated anti-rabbit IgG antibody were obtained from Abcam
12   Inc. (Cambridge, MA, USA) and Thermo Scientific (Kanagawa, Japan), respectively. All
13   other reagents were of an analytical grade and purchased from Nacalai Tesque Inc. (Kyoto,
14   Japan).

15

16   *Enzymes*

17   Pol α was purified from calf thymus by immunoaffinity column chromatography as
18   described by Tamai et al. [19]. Recombinant rat pol β was purified from *Escherichia coli*
19   JMpβ5 as described by Date et al. [20]. The human pol γ catalytic gene was cloned into

1 pFastBac. Histidine-tagged enzyme was expressed using the BACTO-BAC HT Baculovirus
2 Expression System according to the manufacturer's instructions (Life Technologies, Frederick,
3 MD, USA) and purified using ProBound resin (Invitrogen Japan, Tokyo, Japan) [21]. Human
4 pols δ and ϵ were purified by nuclear fractionation of human peripheral blood cancer cells
5 (Molt-4) using the second subunit of pol δ and ϵ -conjugated affinity column chromatography
6 respectively [22]. A truncated form of human pol η (residues 1–511) tagged with His₆ at its
7 C-terminal was expressed in *E. coli* cells and purified as described by Kusumoto et al. [23]. A
8 recombinant mouse pol ι tagged with His₆ at its C-terminal was expressed and purified by
9 Ni–NTA column chromatography [24]. A truncated form of pol κ (residues 1–560) with 6 ×
10 His-tags attached at the C-terminus was overexpressed in *E. coli* and purified as described by
11 Ohashi et al. [25]. Recombinant human His-pol λ was overexpressed and purified according
12 to a method described by Shimazaki et al. [26]. Recombinant human His-pol μ was
13 overexpressed in *E. coli* BL21 and purified using Glutathione Sepharose 4B (GE Healthcare
14 Bio-Science Corp., Piscataway, NJ, USA) column chromatography according to the method
15 for pol λ preparation by Shimazaki et al. [26]. Pol α from a higher plant, cauliflower (*Brassica*
16 *oleracea* var. *botrytis*), was purified from the inflorescence structure according to the methods
17 outlined by Sakaguchi et al. [27]. Calf TdT, *Taq* pol, T4 pol, T7 RNA polymerase, and T4
18 polynucleotide kinase were purchased from Takara Bio Inc. (Kyoto, Japan). The Klenow
19 fragment of pol I from *E. coli* was purchased from Worthington Biochemical Corp. (Freehold,

1 NJ, USA). Bovine pancreas deoxyribonuclease I was obtained from Stratagene Cloning
2 Systems (La Jolla, CA, USA).

3

4 *Measurement of pol activity*

5   The reaction mixtures for calf pol α , rat pol β , plant pol α , and prokaryotic pols have
6 been described previously [15, 16]. Those for pol γ as well as pols δ and ϵ were previously
7 described by Umeda et al. [21] and Ogawa et al. [28] respectively. The reaction mixtures for
8 pols η , ι , and κ were the same as for pol α , and those for pols λ and μ were the same as for
9 pol β . For the pol reactions, poly(dA)/oligo(dT)₁₈ (A/T, 2/1) and dTTP were used as the DNA
10 template/primer substrate and nucleotide (dNTP; 2'-deoxynucleoside-5'-triphosphate)
11 substrate respectively. For the TdT reactions, oligo(dT)₁₈ (3'-OH) and dTTP were used as the
12 DNA primer substrate and nucleotide substrate respectively.

13   Plumbagin, fatty acids (**2a-j** of Figure 1) and the chemically synthesized ten
14 5-*O*-acyl plumbagins (**3a-j** of Figure 1) were dissolved in distilled dimethyl sulfoxide
15 (DMSO) to various concentrations as described in the text and sonicated for 30 s.
16 Subsequently, 4- μ L aliquots were mixed with 16 μ L of each enzyme (0.05 units) in 50 mM
17 Tris-HCl at pH 7.5, containing 1 mM dithiothreitol (DTT), 50% glycerol (by vol), and 0.1
18 mM ethylenediaminetetraacetic acid (EDTA) and held at 0 °C for 10 min. 8 μ L of
19 inhibitor-enzyme mixture was then added to 16 μ L of enzyme standard reaction mixture and

1 incubated at 37 °C for 60 min; except for *Taq* pol, which was incubated at 74 °C for 60 min.
2 Pol activity without inhibitor was considered 100%, and the relative activity was determined
3 for each inhibitor concentration tested. One unit of pol activity was defined as the amount of
4 each enzyme that catalyzed the incorporation of 1 nmol dTTP into synthetic DNA template
5 primers in 60 min at 37 °C under standard reaction conditions [15, 16].
6

7 *Other enzyme assays*

8 The activities of calf primase of pol α , T7 RNA polymerase, mouse
9 inosine-5'-triphosphate (IMP) dehydrogenase (type II), T4 polynucleotide kinase and bovine
10 deoxyribonuclease I were measured in standard assays according to the manufacturer's
11 specifications, as described by Tamiya-Koizumi et al. [29], Nakayama and Saneyoshi [30],
12 Mizushima et al. [31], Soltis et al. [32] and Lu and Sakaguchi [33] respectively.
13

14 *Measurement of cytotoxicity on mouse macrophages*

15 A mouse macrophage cell line, RAW264.7, was obtained from the American Type
16 Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in Eagle's
17 Minimum Essential Medium (MEM) supplemented with 4.5 g glucose per liter plus 10% fetal
18 calf serum, 5 mM L-glutamine, 50 units/mL penicillin and 50 units/mL streptomycin. The
19 cells were cultured at 37 °C in standard medium in a humidified atmosphere of 5% CO₂, 95%

1 air. Approximately 1×10^4 cells per well were inoculated into 96-well microplates, and then
2 5-*O*-acyl plumbagins (at final concentrations of 10 μ M) applied to each well. After incubation
3 for 24 h, the survival rate of RAW264.7 cells was determined by MTT
4 (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [34].

6 *Measurement of TNF- α in the cell culture medium of mouse macrophages*

7 RAW264.7 cells were added to a 12-well plate at 5×10^4 cells/well and incubated for
8 24 h. The cells were pretreated with 5-*O*-acyl plumbagins (at final concentrations of 1 and 5
9 μ M) for 30 min before the addition of 100 ng/mL of LPS. After stimulation with LPS for 24 h,
10 the cell culture medium was collected to measure the amount of TNF- α secreted. The
11 concentration of TNF- α in the culture medium was quantified using a commercially available
12 enzyme-linked immunosorbent assay (ELISA) (Bay Bioscience Co., Ltd, Kobe, Japan) in
13 accordance with the manufacturer's protocol.

15 *Western blotting*

16 The nuclear proteins (30-50 μ g proteins) was boiled in a quarter volume of sample
17 buffer (1 M Tris-HCl, pH 7.5, 640 mM 2-mercaptoethanol, 0.2% bromphenol blue, 4% SDS
18 and 20% glycerol) and then separated on 10% SDS polyacrylamide gels. The proteins in the
19 gels were transferred to a PVDF membrane. The membrane was blocked with 1% skimmed

1 milk in TBS-T (10 mM Tris-HCl, 100 mM NaCl and 0.5% Tween-20) and probed with
2 anti-pol λ antibody (1:1000) before being reacted with the horseradish peroxidase-conjugated
3 secondary antibody. The protein-antibody complex was detected using ChemiLumiONE
4 (Nacalai Tesque, Japan) and an Image Reader (LAS-3000 Imaging System, Fuji Photo Film).
5 The intensity of each band was analyzed using ImageJ, which was developed at the National
6 Institutes of Health.

7 8 *TPA-induced inflammatory assay in the mouse*

9 Quantifying inflammation in the mouse ear was performed according to Gschwendt's
10 method [35]. In brief, an acetone solution containing the 5-O-acyl plumbagin compounds
11 (500 μ g in 20 μ L) or 20 μ L acetone as a vehicle control was applied to the inner part of the
12 mouse ear. Thirty minutes after the test compound had been applied; TPA solution (0.5 μ g / 20
13 μ L of acetone) was applied to the same part of the ear. Acetone, followed by TPA solution,
14 was applied to the other ear of the same mouse as a control. After 7 h, a disk (6 mm diameter)
15 was obtained from the ear and weighed. The inhibitory effect (IE) is presented as the ratio of
16 the increase in weight of the ear disks:

$$17 \quad IE = [(TPA \text{ only}) - (\text{test compound} + TPA)] / [(TPA \text{ only}) - (\text{vehicle})] \times 100$$

18 19 *Pol λ knockdown using small interfering RNA (siRNA) in RAW264.7 cells*

1   The pol λ knockdown in RAW264.7 cells was performed by referring to Zucca's
2 method [36]. In brief, all of siRNA targeting pol λ was purchased from the Ambion of
3 Silencer®select siRNA. Those sequences are as follows: siRNA 80810 (si810) 5'
4 -CACUUCAACCGGUCCAUGAtt-3'   and   siRNA   80812   (si812)
5 5'-GGGCUGAACUCUUUGAGAAAtt-3'. Silencer®select Negative control #1 siRNA was
6 also used as negative control in this study. HiPerFect lipid transfection reagent (Qiagen) was
7 used for siRNA transfection to cultured RAW264.7 cells. On the day of transfection, seed 2 x
8 10⁴ cells per well of two 96-well plate in 50 μ L of DMEM medium containing serum and
9 antibiotics. One plate is for measurement of pol λ expression, another plate is for
10 measurement of TNF- α . The cells were co-transfected si810 and si812 with siRNA-lipid
11 complex (each 20 pmol siRNA+12 μ L of HiPerFect in 50 μ L of DMEM medium without
12 serum and antibiotics) in both plates. Then, cells were incubated with the transfection
13 complexes under their normal growth conditions. To evaluate pol λ expression, cells were
14 collected at 6 and 24 h post-transfection and analyzed in mRNA level by real-time PCR
15 (StepOne Real-Time PCR system, Applied Biosystems). To investigate TNF- α production
16 after siRNA transfection, cells were treated with 50 ng/mL final concentration of LPS and
17 incubated for 24 h. The cell culture medium was then collected to measure concentration of
18 secreted TNF- α in the culture medium, quantified using by ELISA.

19

1 *Cell viability in pol λ knockdown cells*

2   Concurrently, we examined the cytotoxicity effects of post siRNA transfection using
3 WST-1 assay (Dojindo Laboratory, Kumamoto, Japan) to formazan by the mitochondrial
4 dehydrogenase in RAW264.7 cells [37]. After 6 h or 24 h siRNA transfection, 10 μ L of
5 WST-1 reagent was added and allowed to react for 4 h at 37°C. The absorbance measured
6 against a cell free background control at a wavelength of 450 nm and a reference wavelength
7 of 630 nm by a microplate reader (Vmax-K; Molecular Devices, LLC, Sunnyvale, CA).

8

9 *Measurement of nuclear translocation of NF- κ B in pol λ knockdown cells*

10   The nuclear levels of p65 may positively is related to the activation of the NF- κ B
11 pathway. For the detection of NF- κ B/p65 translocation after pol λ knockdown by siRNA
12 transfection, the RAW 264.7 cells were seeded on 96-well plate and transfected of pol
13 λ -specific siRNA in the same way of pol λ knockdown method. After treatment with LPS for
14 24 h, the NF- κ B/p65 ActivELISA kit (Novus biological, LLC, Littleton, CO, USA) was used
15 to determine the levels of NF- κ B/p65 following the manufacturer's instructions. The
16 absorbance value at 405 nm was measured with microplate reader (Vmax-K; Molecular
17 Devices, LLC, Sunnyvale, CA, USA). The relative ratio of nuclear to cytoplasmic NF- κ B/p65
18 was calculated by the equation (absorbance value of nucleus)/(absorbance value of cytoplasm)
19 [38].

1   **Results**

2   *Effect of synthesized 5-O-acyl plumbagins on the activities of mammalian pols*

3   Initially, the inhibitory activity of plumbagin, fatty acids and chemically synthesized
4   5-O-acyl plumbagins on mammalian pols was investigated using calf pol α , and human pols γ ,
5   κ and λ . Mammalian pols, pols α , γ , κ and λ were used as the representative pols for families
6   B, A, Y and X respectively [4, 5]. Assessment of the relative activity of each pol with a set
7   concentration (10 μ M) of these compounds showed that all 5-O-acyl plumbagins were
8   stronger inhibitors of these four mammalian pols than plumbagin or fatty acids (Figure 2).
9   The plumbagins conjugated with C18 and longer chain unsaturated fatty acids (i.e., C18:1 to
10   C22:6-acyl plumbagins, **3f–j** of Figure 1) strongly inhibited the activities of pols α and γ ,
11   suggesting that the group of longer unsaturated acyl side chains might be a structural
12   characteristic of 5-O-acyl plumbagins essential for the pol inhibition. C12:0-acyl plumbagin
13   (**3d** of Figure 1) and C22:6-acyl plumbagin (**3j** of Figure 1) were the strongest inhibitors of
14   human pols κ and λ respectively among the compounds tested. When activated DNA (bovine
15   deoxyribonuclease I-treated DNA) was used as the DNA template/primer substrate instead of
16   synthesized DNA (poly(dA)/oligo(dT)₁₈ (A/T = 2/1)) and dNTP was used as the nucleotide
17   substrate instead of dTTP, the inhibitory effects of these compounds did not change (data not
18   shown).

19   In our previous studies of pol inhibitors, we have found that pol λ -selective inhibitors,

1 such as curcumin derivatives, also have anti-inflammatory activity [39, 40]. Therefore, we
2 focused on C22:6-acyl plumbagin (**3j** of Figure 1), which showed the strongest pol λ
3 inhibition of the ten 5-*O*-acyl plumbagins synthesized, in further parts of this study.

4
5 *Effects of C22:6-acyl plumbagin on the activities of various pols and other DNA metabolic*
6 *enzymes*

7 We successfully isolated eleven mammalian pol species, including pols α , β , γ , δ , ϵ , η ,
8 ι , κ , λ and μ , and purchased TdT. Pols ζ , θ , and ν and REV1 were not available at the time of
9 the study. Table 1 shows the inhibitory effect (50% inhibitory concentration; IC₅₀ value) of
10 C22:6-acyl plumbagin on the 11 mammalian pols obtained. This compound inhibited the
11 activity of all of the mammalian pols with IC₅₀ values between 5.0 and 7.1 μ M. Specifically,
12 50% inhibition of the A, B, X and Y families of pols was observed at doses of 5.0, 5.1–5.6,
13 5.8–7.1, and 6.4–6.9 μ M respectively. Therefore, the inhibitory effect of this compound on the
14 A and B families of pols was slightly stronger than that on the X and Y families of pols. For
15 comparison, the IC₅₀ values of petasiphenol, a known mammalian pol λ specific inhibitor
16 from the Japanese vegetable Fuki (*Petasites japonicus*), was 7.8 μ M [39]. Thus the pol λ
17 inhibitory activity of C22:6-acyl plumbagin was 1.4-fold more potent than that of
18 petasiphenol, highlighting the requirement for further study.

19 In contrast to its inhibition of mammalian pols, C22:6-acyl plumbagin had no effect

1 on plant pols such as cauliflower pol α , or prokaryotic pols, such as *E. coli* pol I, *Taq* pol or
2 T4 pol (Table 1). The three-dimensional structures of eukaryotic pols are likely to differ
3 greatly from those of prokaryotic pols. This compound also did not inhibit the activity of
4 other DNA metabolic enzymes, including calf primase pol α , T7 RNA polymerase, mouse
5 IMP dehydrogenase (type II), T4 polynucleotide kinase or bovine deoxyribonuclease I (Table
6 1).

7 We also performed specific assays to determine whether C22:6-acyl plumbagin
8 induced inhibition resulted from the ability of this compound to bind to DNA or to the
9 enzyme. The interaction of C22:6-acyl plumbagin with dsDNA was investigated by
10 examining the thermal transition of dsDNA by measuring its melting temperature (T_m) with
11 an excess amount of this compound (100 μ M) using a spectrophotometer equipped with a
12 thermoelectric cell holder. A thermal transition representing T_m was not observed within the
13 concentration range of the compound used in the assay, whereas a typical intercalating
14 compound used as a positive control (ethidium bromide, 15 μ M) produced a clear thermal
15 transition (data not shown). We next investigated whether an excessive amount of nucleic acid
16 (poly(rC)) or protein (bovine serum albumin (BSA)) could prevent the inhibitory effect of
17 C22:6-acyl plumbagin, to determine whether the inhibitory effect resulted from non-specific
18 adhesion of these molecules to mammalian pols or from selective binding to specific sites.
19 Poly(rC) and BSA had little or no influence on the pol inhibitory effect of this compound

(data not shown), suggesting that C22:6-acyl plumbagin selectively binds to the pol molecule.

These observations indicated that this compound does not act as a DNA intercalating agent or as a template/primer substrate.

Inhibitory effect of synthesized 5-O-acyl plumbagins on LPS-induced TNF- α production by cultured cells

Next, we investigated whether plumbagin, fatty acids (**2a–j** of Figure 1) and chemically synthesized 5-O-acyl plumbagins (**3a–j** of Figure 1) could inhibit TNF- α production by LPS stimulated cultured cells. The binding of the pro-inflammatory cytokine TNF- α to the TNF- α receptor (TNFR) can activate the NF- κ B signaling pathway, thereby initiating an inflammatory response as evident in various inflammatory diseases [41].

In cultured RAW264.7 macrophages, none of the compounds tested showed cytotoxicity at 10 μ M (Figure 3A). RAW264.7 cells produced 391 pg/mL of TNF- α after LPS treatment. The concentration of TNF- α produced in the vehicle control was set as 0% inhibition. As shown in Figure 3B, 5 μ M of plumbagin showed approximately 50% inhibition of TNF- α production, and 5 μ M of C18:1 to C22:6 unsaturated fatty acids (**2f–j** of Figure 1) only slightly suppressed TNF- α production. Conversely, 5 μ M of the ten chemically synthesized 5-O-acyl plumbagins (**3a–j** of Figure 1) were stronger inhibitors of TNF- α production than both plumbagin and fatty acids. Among these compounds, C22:6-acyl

1 plumbagin (**3j** of Figure 1) showed the strongest effect on TNF- α production, with 39.4 and
2 86.4% inhibition of TNF- α production by 1 and 5 μ M respectively.

3   Next, the effect of C22:6-acyl plumbagin (**3j**) on the expression level of pol λ protein
4 in LPS-treated RAW264.7 cells was investigated. The upper and middle panels of Figure 3C
5 shows that these macrophages underwent a more than 2.25-fold increase in the expression of
6 pol λ after LPS stimulation, but this increase was suppressed by 10 μ M of this compound. It is
7 unclear the relationship between *in vitro* pol λ inhibitory activity and the suppression of pol λ
8 protein expression by this compound in RAW264.7 cells, and we will try to elucidate it for
9 further studies. As shown in the lower graph of Figure 3C, 10 μ M C22:6-acyl plumbagin (**3j**)
10 significantly suppressed LPS-stimulated production of TNF- α . These results suggest that there
11 is the positive correlation between inflammatory induction by LPS and pol λ expression; thus,
12 not only the DNA polymerization activity but also the protein expression of pol λ is likely to
13 be important in inflammation.

15 *Effect of synthesized 5-O-acyl plumbagins on TPA-induced inflammation*

16   We examined *in vivo* the anti-inflammatory activity of plumbagin, fatty acids (**2a-j**
17 of Figure 1), and chemically synthesized 5-O-acyl plumbagins (**3a-j** of Figure 1) using the
18 mouse ear inflammatory test. Application of TPA (0.5 μ g) to the mouse ear induced edema,
19 resulting in a 241% increase in the weight of the ear disk 7 h after application. The

1 anti-inflammatory effect of the vehicle control was set at 0%. As shown in Figure 4, 500
2 $\mu\text{g}/\text{ear}$ of plumbagin and the ten fatty acids slightly suppressed the inflammation. The ten
3 chemically synthesized 5-*O*-acyl plumbagins showed stronger anti-inflammatory activity than
4 both plumbagin and the fatty acids. C22:6-Acyl plumbagin (**3j** of Figure 1) and C12:0-acyl
5 plumbagin (**3d** of Figure 1) had the first and second strongest anti-inflammatory effect on
6 TPA-induced inflammation among the compounds tested, and at 500 $\mu\text{g}/\text{ear}$ showed 61.5 and
7 51.0% inhibition of swelling respectively. In comparison 500 $\mu\text{g}/\text{ear}$ of petasiphenol (a pol λ
8 specific inhibitor) and glycyrrhetic acid (a known anti-inflammatory agent) had only a 42
9 and 40% inhibitory effect in this assay, respectively (data not shown). Thus, C22:6-acyl
10 plumbagin was 1.5-fold more potent than these compounds and may have potential as an
11 anti-inflammatory agent.

12

13 *Relationship between mammalian pol inhibition, suppression of TNF- α production, and*
14 *anti-inflammatory activity of 5-*O*-acyl plumbagins*

15 The relationship between the inhibition of mammalian pol families and suppression
16 of LPS-induced TNF- α production by RAW264.7 cells observed *in vitro* and the *in vivo*
17 reduction of TPA-induced inflammation was investigated by comparing the effects of
18 plumbagin and the ten synthesized 5-*O*-acyl plumbagins on these biological activities (Figure
19 5). The inhibition of pol λ , which is a DNA repair/recombination related pol of the X family,

1 by 5-*O*-acyl plumbagins showed the highest correlation with the suppression of TNF- α
2 production among those mammalian pol families tested, with a correlation coefficient of $R^2 =$
3 0.953. The inhibitory activity on pol κ , a DNA repair pol of the Y family, showed a moderate
4 correlation ($R^2 = 0.706$) with suppression of TNF- α production. In contrast, neither the effect
5 on pol α (a nuclear DNA replicative pol of the B family) nor pol γ (a mitochondrial DNA
6 replicative pol of the A family) by 5-*O*-acyl plumbagins were related to suppression of TNF- α
7 production, with a correlation coefficient between these activities of $R^2 = < 0.3$ (upper panels
8 of Figure 5A).

9   The correlation coefficients between the inhibitory activity of 5-*O*-acyl plumbagins
10 on pols α , γ , κ and λ versus the anti-inflammatory effect on TPA-treated mouse ears were R^2
11 = 0.018, 0.291, 0.790, and 0.991, respectively. Therefore, the relative effect of 5-*O*-acyl
12 plumbagins on mammalian pols was ranked as follows: pol $\lambda >$ pol $\kappa >$ pol $\gamma >$ pol α (lower
13 panels of Figure 5A). Thus, the *in vivo* data from the mouse ear study showed a high
14 correlation with the LPS-induced inflammatory response data from cultured cells (correlation
15 coefficient of $R^2 = 0.948$, Figure 5B). Furthermore, the *in vivo* anti-inflammatory effect of
16 these compounds showed the same tendency as their inhibitory effect on mammalian pol λ .

17

18 *The inflammatory response of pol λ knockdown cells*

19   Next, we investigated the effect of TNF- α production on transient knockdown of pol

1 λ in RAW264.7 cells. Pol λ expression was silenced in this cell line using two different siRNA
2 sequences from the Ambion collection of Selective Validated siRNA (si810 + si812), while a
3 scrambled siRNA served as a negative control. Cells were harvested at 6 and 24 h
4 post-transfection and the amount of pol λ expression was analyzed at mRNA level by
5 real-time PCR. As shown in Figure 6A, the expression of pol λ was 1.12 and 0.31-fold
6 down-regulated by this siRNA at 200 nM for 6 and 24 h incubation, respectively. These
7 results indicated that the 24 h incubation of post-transfection is suitable for pol λ knockdown.
8 These pol λ knockdown cells by siRNA did not influence cell proliferation and viability in
9 this condition (Figure 6B).

10 To assess the effects of pol λ down-regulation on LPS-induced TNF- α production,
11 cells were treated with this siRNA or the scrambled control and further analyzed by ELISA.
12 As shown in Figure 6C, the decrease in the production of TNF- α at 24 h post-transfection by
13 the RAW264.7 cells which were down-regulated of pol λ levels was 35.8%, suggesting that
14 pol λ expression must be closely related to TNF- α production in RAW264.7 cells, such as the
15 upper panels of Figure 5A.

16 To further investigate the relevance of pol λ knockdown effect and anti-inflammatory,
17 we measured the nuclear translocation of NF- κ B/p65 in RAW264.7 cells of post-transfection
18 of siRNA targeting pol λ . In many inflammatory responses, activation of NF- κ B is the
19 rate-limiting step of the inflammatory mechanism [42]. The translocated NF- κ B proteins work

1 as transcription factors and regulate the expression of various genes that encode
2 proinflammatory cytokines such as TNF- α . The relative nuclear to cytoplasmic ratio of
3 NF- κ B/p65 in siRNA transfected cells of 24 h was reduced by approximately 20% compared
4 with that of 6 h. These results suggested that pol λ could be potentially related to nuclear
5 translocation of NF- κ B/p65 and to cause of inflammation.

6   These results led to the speculation that the suppression of TNF- α production by
7 plumbagin and 5-*O*-acyl plumbagins might have been caused by the inhibition of DNA
8 recombination/repair related to pol λ activity. This suppression of TNF- α production may also
9 be important for the anti-inflammatory effects of these compounds *in vivo*. The molecular
10 mechanism by which 5-*O*-acyl plumbagins suppress inflammation based on pol λ inhibitory
11 activity will be addressed in future studies.

1   **Discussion**

2   Eukaryotic cells reportedly contain 15 pol species, which belong to four families:
3   family A (pols γ , θ , and ν), family B (pols α , δ , ϵ , and ζ), family X (pols β , λ , μ , and TdT) and
4   family Y (pols η , ι , and κ , and REV1) [43, 44]. As shown in Figure 5A, plumbagin and the
5   ten 5-*O*-acyl plumbagins synthesized had pol λ (family pol X) inhibitory activity and
6   decreased LPS-induced TNF- α production and TPA-induced inflammation. However, the
7   inhibition of pols α , γ and κ (pols B, A and Y families, respectively) had less correlation with
8   these anti-inflammatory properties. Among the X family of pols, pol λ and pol β appear to
9   carry out similar functions [45]. Pol β is involved in the short-patch base excision repair
10   (BER) pathway [45-49] and plays an essential role in neural development [50]. Recently, pol
11   λ was found to possess 5'-deoxyribose-5-phosphate (dRP) lyase activity but not
12   apurinic/apyrimidinic (AP) lyase activity [51]. Pol λ is able to substitute for pol β during *in*
13   *vitro* BER, suggesting that pol λ also participates in BER. Northern blot analysis indicated
14   that transcripts of pol β are abundantly expressed in the testis, thymus, and brain in rats [52],
15   whereas pol λ is efficiently transcribed mostly in the testis [53]. Bertocci et al. reported that
16   pol λ knockout mice are not only viable and fertile but also display a normal hyper-mutation
17   pattern [54].

18   As well as causing inflammation, TPA influences cell proliferation and has tumor
19   promoting activity [55]. Therefore, anti-inflammatory agents are expected to also suppress

1 DNA replication/repair/recombination in nuclei in relation to the action of TPA. Because pol
2 λ is a repair/recombination-related pol [45], our finding that the molecular target of 5-*O*-acyl
3 plumbagins is pol λ agrees with this expected mechanism of anti-inflammatory action, and the
4 pol λ protein expression in RAW264.7 macrophages treated with LPS was correlated with
5 TNF- α production related to inflammatory induction (Figure 3C and Figure 5C). This
6 suggests that any pol λ inhibitor might also be an inhibitor of inflammation.

7 Therefore in this study we investigated the inhibitory effect of ten chemically
8 synthesized 5-*O*-acyl plumbagins on mammalian pols, which are responsible for DNA
9 replication leading to cell proliferation and DNA repair/recombination. We also explored the
10 relationship between the suppression of LPS-induced TNF- α production *in vitro* by these
11 compounds and their anti-inflammatory activity *in vivo*. Our study indicates that 5-*O*-acyl
12 plumbagins, such as C22:6-acyl plumbagin, suppress TNF- α production (Figure 3B) and may
13 be potent anti-inflammatory agents (Figure 4). To support this, we found a positive correlation
14 between the pol λ inhibitory activity and anti-inflammatory activity of these compounds
15 (Figure 5). Pol λ knockdown cells suppressed the expression of inflammatory responses such
16 as TNF- α and NF- κ B (Figure 6), suggesting that pol λ might lead to the NF- κ B activated
17 signaling pathway. The relationship between these activities, namely pol λ inhibition and
18 anti-inflammatory action, may be useful as a new and convenient *in vitro* assay to screen for
19 novel anti-inflammatory compounds.

Conclusions

In this study, we found that the chemically synthesized 5-*O*-acyl plumbagins, a conjugation of plumbagin with fatty acids, were stronger inhibitors of the mammalian pols α , γ , κ and λ representing the pol families B, A, Y, and X respectively, than plumbagin or fatty acids. Of the synthesized compounds, C22:6-acyl plumbagin showed the strongest inhibition of pol λ activity, suppression of TNF- α production in cultured cells and reduction of inflammation *in vivo*. The correlation between pol inhibition and anti-inflammatory activity for these compounds was very high for pol λ , but not for pols α , γ and κ . These results suggest that 5-*O*-acyl plumbagins, such as C22:6-acyl plumbagin, could be used as anti-inflammatory chemotherapy agents based on their inhibition of mammalian pol λ .

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- 7

(Figure legends)

Fig. 1. Structure of plumbagin (**1**), fatty acids (**2**) and 5-*O*-acyl plumbagins (**3**). “R” is the acyl chain.

Fig. 2. Inhibitory effects of plumbagin, fatty acids and chemically synthesized 5-*O*-acyl plumbagins on the activity of mammalian pols. Each compound (10 μ M) was incubated with calf pol α (B-family pol), human pol γ (A-family pol), human pol κ (Y-family pol) or human pol λ (X-family pol) (0.05 units each). Inhibition of pol activity by the vehicle control was taken as 0%. Data are shown as the mean $\pm$ SD (n = 3).

Fig. 3. Inhibitory effects of plumbagin, fatty acids and chemically synthesized 5-*O*-acyl plumbagins on LPS-induced TNF- α production by the mouse macrophage cell line RAW264.7. (A) RAW264.7 cells were added 10 μ M of each compound and incubated for 24 h, and the rate of cultured cell growth inhibition was determined by MTT assay [34]. Cell growth inhibition of the cells in the absence of the compound was taken as 100%. Data are shown as the mean $\pm$ SD (n = 5). (B) RAW264.7 cells were pretreated with either 1 or 5 μ M of each compound or a vehicle control (base TNF- α concentration, 72 pg/mL) for 30 min and then treated with 100 ng/mL LPS for 24 h (LPS induced TNF- α concentration, 391 pg/mL). The TNF- α concentration in the cell culture medium was measured by ELISA. Inhibition of

1 LPS-induced TNF- α production by the vehicle control was taken as 0%. Data are shown as
2 the mean $\pm$ SD (n = 4). (C) RAW264.7 cells were pre-treated with 10 μ M C22:6-acyl
3 plumbagin (**3j**) for 30 min, and then incubated with 100 ng/mL of LPS for 24 h. The cultured
4 cells were collected, and the expression level of pol λ (upper panel) and β -actin (middle
5 panel) were evaluated by Western blot analysis. The intensity of each band was analyzed, and
6 the values relative to non-treatment with LPS are represented at the lower edge of the image.
7 The TNF- α concentration in the cultured media were measured by ELISA (lower panel). Data
8 are shown as the mean $\pm$ SD (n = 4).

9
10 **Fig. 4.** Anti-inflammatory activity of plumbagin, fatty acids and chemically synthesized
11 5-*O*-acyl plumbagins on TPA-induced edema in the mouse ear. Each compound (500 μ g) was
12 applied individually to one ear of a mouse, and after 30 min TPA (0.5 μ g) was applied to both
13 ears. Edema was evaluated after 7 h. The inhibitory effect is expressed as the decrease in the
14 percentage of edema. The degree of inflammation in the vehicle control was taken as 0%
15 inhibition. Data are represented as the means $\pm$ SD (n = 6).

16
17 **Fig. 5.** The relationship between mammalian pol inhibition by 5-*O*-acyl plumbagins and the
18 suppression of TNF- α production and reduction of inflammation. (A) Inhibitory activities of
19 10 μ M 5-*O*-acyl plumbagins on calf pol α (B-family pol), human pol γ (A-family pol), human

1 pol κ (Y-family pol) and human pol λ (X-family pol) versus inhibitory effect of 5 μ M
2 5-*O*-acyl plumbagins on LPS-induced TNF- α production in a cell-culture system (upper
3 panels, white symbols). The inhibitory activities of 5-*O*-acyl plumbagins on pols versus the
4 anti-inflammatory effect of 500 μ g 5-*O*-acyl plumbagins on TPA-induced edema in the mouse
5 ear (lower panels, black symbols). (B) Inhibitory effect of 5 μ M 5-*O*-acyl plumbagins on
6 LPS-induced TNF- α production in cultured cells versus the anti-inflammatory effect of 500
7 μ g 5-*O*-acyl plumbagins on TPA-induced inflammation of the mouse ear. The values of
8 correlation (R^2) are shown in each figure.

9

10 **Fig. 6.** Pol λ knockdown induces the suppression of TNF- α and NF- κ B productions. NT, not
11 transfected; NC, negative control (Silencer®select Negative control #1 siRNA); si810 + si812,
12 pol λ -specific siRNAs. (A) Real-time PCR measuring the mRNA levels for the pol λ gene in
13 RAW264.7 cells after 6 and 24 h from transfection with 200 nM siRNA oligonucleotides. The
14 ratio of mRNA expression of pol λ in NT versus NC or si810 + si812 is indicated on this
15 panel. Data are represented as the means $\pm$ SD ($n = 3$). (B) Effects of Pol λ knockdown on cell
16 viability in RAW264.7 cells. Cell viability was measured after 6 and 24 h siRNA transfection
17 by WST-1 assay [37]. The cell viability of NT was taken as 100%. Data are represented as the
18 means $\pm$ SD ($n = 5$). (C) Inhibitory effects of pol λ knockdown on LPS-induced TNF- α
19 production in RAW264.7 cells after 6 and 24 h siRNA transfection. The TNF- α concentration

1 in the cell culture medium was measured by ELISA. LPS-induced TNF- α production by NT
2 was taken as 100%. Data are represented as the means $\pm$ SD (n = 5). (D) The relative ratio of
3 nuclear to cytoplasmic NF- κ B/p65 in the cells transfected with siRNA targeting pol λ . The
4 relative ratio of nuclear to cytoplasmic NF- κ B/p65 was measured after 6 and 24 h siRNA
5 transfection by the NF- κ B/p65 ActivELISA kit [38]. Data are represented as the means $\pm$ SD
6 (n = 3).

Table 1. IC₅₀ of C22:6-acyl plumbagin (**3j**) on the activities of mammalian, plant and prokaryotic pols and various DNA metabolic enzymes.

Enzyme	IC ₅₀ values (μM)
– Mammalian pols –	
[A-Family of pol]	
Human pol γ	5.0 ± 0.28
[B-Family of pols]	
Calf pol α	5.1 ± 0.30
Human pol δ	5.6 ± 0.31
Human pol ε	5.3 ± 0.30
[X-Family of pols]	
Rat pol β	6.0 ± 0.35
Human pol λ	5.8 ± 0.34
Human pol μ	6.2 ± 0.36
Calf TdT	7.1 ± 0.40
[Y-Family of pols]	
Human pol η	6.9 ± 0.39
Mouse pol ι	6.5 ± 0.34
Human pol κ	6.4 ± 0.32
– Plant pol –	
Cauliflower pol α	>100
– Prokaryotic pols –	
<i>E. coli</i> pol I	>100
<i>Taq</i> 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); data are mean ± SD (n=3).

- 2a:** Acetic acid (C2:0)
2b: Propionic acid (C3:0)
2c: Capronic acid (C6:0)
2d: Lauric acid (C12:0)
2e: Stearic acid (C18:1)
2f: Oleic acid
2g: Linoleic acid (C18:2)
2h: α -Linolenic acid (C18:3)
2i: Eicosapentaenoic acid (C20:5)
2j: Docosahexaenoic acid (C22:6)

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6 **Fig. 1.** Onodera et al.

Fig. 2. Onodera et al.

1 **A**

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5 **Fig. 3A.** Onodera et al.

1 **B**

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5 **Fig. 3B.** Onodera et al.

1 **C**

2

3

4

5 **Fig. 3C.** Onodera et al.

Fig. 4. Onodera et al.

1 **A**

2

3 **B**

4

5

6

7 **Fig. 5. Onodera et al.**

1 **A**

B

2

3 **C**

D

4

5

6

7 **Fig. 6.** Onodera et al.

Graphical Abstract

5-*O*-Acyl plumbagins inhibit DNA polymerase activity and suppress the inflammatory response

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