Inhibitory effects of vitamin K_3 derivatives on DNA polymerase and inflammatory activity

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Abstract. Previously, we reported that vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone) (compound 2) inhibits the activity of human mitochondrial DNA polymerase γ (pol γ). In this study, we investigated the inhibitory effects (IEs) of vitamin K_3 and its derivatives, such as 1,4-naphthoquinone (compound 1) and 1,2-dimethyl-1,4-naphthoquinone (compound 3), on the activity of mammalian pols. Among compounds 1-3 (10 μM for each), compound 1 was the strongest inhibitor of mammalian pols α and λ, which belong to the B and X pol families, respectively, whereas compound 2 was the strongest inhibitor of human pol γ, a family A pol. However, these compounds did not affect the activity of human pol κ, a family Y pol. As we previously found a positive relationship between pol λ inhibition and anti-inflammatory action, we examined whether these vitamin K_3 derivatives are able to inhibit inflammatory responses. Among the three compounds tested, compound 1 caused the greatest reduction in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute inflammation in mouse ears. In addition, in a cell culture system using RAW264.7 mouse macrophages, compound 1 displayed the strongest suppression of tumor necrosis factor (TNF)-α production induced by lipopolysaccharide (LPS). In an *in vivo* mouse model of LPS-evoked acute inflammation, the intraperitoneal injection of compound 1 into mice suppressed TNF-α production in their peritoneal macrophages and serum. In an *in vivo* colitis mouse model induced using dextran sulfate sodium (DSS), the vitamin K_3 derivatives markedly suppressed DSS-evoked colitis. In conclusion, this study has identified several vitamin K_3 derivatives, such as compound 1, that are promising anti-inflammatory candidates.

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Abbreviations: pol, DNA polymerase (EC 2.7.7.7); TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TNF, tumor necrosis factor; NF, nuclear factor; LPS, lipopolysaccharide; TdT, terminal deoxynucleotidyl transferase; dTTP, 2'-deoxythymidine 5'-triphosphate; DMSO, dimethyl sulfoxide; IE, inhibitory effect; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; BW, body weight; BER, base excision repair; AP, apurinic/apyrimidinic; dRP, 5'-deoxyribose-5-phosphate; DSS, dextran sulfate sodium; H&E, hematoxylin and eosin

Key words: vitamin K3 derivatives, 1,4-naphthoquinone, DNA polymerase λ, enzyme inhibitor, anti-inflammatory, tumor necrosis factor-α, DSS-induced colitis

Introduction

The human genome encodes at least 15 DNA polymerases (pols) that participate in cellular DNA synthesis (1,2). Eukaryotic cells contain 3 replicative pols (α, δ and ε), 1 mitochondrial pol (γ), and at least 11 non-replicative pols [β, ζ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT), and REV1] (3,4). Pols have a highly conserved structure, which means that their catalytic subunits show little variance among species. Enzymes with conserved structures usually perform important cellular functions, the maintenance of which has evolutionary advantages. On the basis of their sequence homology, eukaryotic pols can be divided into 4 main families, termed A, B, X and Y (4). Family A includes mitochondrial pol γ, as well as pols θ and ν. Family B includes 3 replicative pols (α, δ and ε)

and pol ζ. 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. We have been studying selective inhibitors of each pol derived from natural products including food materials and nutrients for more than 15 years (5,6). We have found that vitamin K_3, but not K_1 or K_2, is a potent inhibitor of human pol γ (7-11).

Vitamin K_3 (menadione, 2-methyl-1,4-naphthoquinone) is a fat-soluble compound that contains quinone as its principle 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 (12-15). In this study, we focused on the vitamin K_3 derivatives based on 1,4-naphthoquinone that have two ketone groups at positions 1 and 4 and prepared three compounds: 1,4-naphthoquinone (compound 1), 2-methyl-1,4-naphthoquinone (i.e., vitamin K_3) (compound 2), and 1,2-dimethyl-1,4-naphthoquinone (compound 3) (Fig. 1). Compounds 1-3 consist of 1,4-naphthoquinone with no, one, and two methyl groups, respectively.

In our pol inhibitor studies, we found that pol λ-specific inhibitors, such as curcumin derivatives (16-18), display 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced anti-inflammatory activity (19-21). Although tumor promoters are classified as compounds that promote tumor formation (22), they also cause inflammation and are commonly used as artificial inducers of inflammation in order to screen for anti-inflammatory agents (23). Tumor promoter-induced inflammation can be distinguished from acute inflammation, which is exudative and is accompanied by fibroblast proliferation and granulation. The tumor promoter TPA is frequently used to search for new types of anti-inflammatory compound. TPA not only causes inflammation, but also influences mammalian cell growth (24), suggesting that the molecular basis of inflammation stems from pol reactions related to cell proliferation. However, this relationship needs to be investigated more closely.

In this study, we investigated the inhibitory effects (IEs) of the vitamin K_3 derivatives on mammalian pol activity and inflammatory responses *in vitro* and *in vivo*. In particular, we demonstrated that these compounds exert IEs against tumor necrosis factor (TNF-α) production in an animal model of lipopolysaccharide (LPS)-induced acute inflammation. In addition, the therapeutic effects of these compounds on colitis were investigated in a colitis mouse model using dextran sulfate sodium (DSS). The relationship between the pol inhibitory and anti-inflammatory effects of the vitamin K_3 derivatives is also discussed.

Materials and methods

Materials. Vitamin K_3 (2-methyl-1,4-naphthoquinone, compound 2) and 1,4-naphthoquinone (compound 1) were obtained from Sigma-Aldrich (St. Louis, MO). 1,2-Dimethyl-1,4-naphthoquinone (compound 3) was chemically synthesized via the radical alkylation of vitamin K_3 with acetic acid in the presence of $(NH_4)_2S_2O_8$ and $AgNO_3$, as described by Schmid *et al* (25). The structure of compound 3 was confirmed by ^{1}H NMR spectroscopy; δH (270 MHz, $CDCl_3$) 2.18 (6H, s), 7.69 (2H, dd, J = 5.9 Hz, 3.2 Hz), 8.08 (2H, dd, J = 5.9 Hz, 3.2 Hz). The structures of the three compounds are shown in Fig. 1. Each compound was purified to more than 99% purity. Chemically synthesized DNA templates such as poly(dA) were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). Nucleotide radioisotopes such as [^{3}H]-deoxythymidine 5'-triphosphate (dTTP) (43 Ci/mmol) were purchased from MP Biomedicals (Solon, OH). The oligo$(dT)_{18}$ DNA primer was customized by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). TPA and LPS were purchased from Sigma-Aldrich. DSS (MW 36,000-50,000 Da) were purchased from MP Biomedicals. For fluorescent immunostaining, the anti-mouse CD11b and the anti-mouse F4/80 antibodies were purchased from BD Biosciences (Franklin Lakes, NJ) and eBioscience (San Diego, CA), respectively. Goat anti-rat IgG antibody conjugated with Alexa Fluor 488 and phalloidin conjugated with Alexa Fluor 546 were purchased from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Mammalian pol assays. Pol α was purified from calf thymus by immunoaffinity column chromatography, as described by Tamai *et al* (26). The human pol γ catalytic gene was cloned into the pFastBac vector (Invitrogen Japan K.K., Tokyo Japan), and histidine-tagged enzyme was expressed using the Bac-to-Bac HT Baculovirus Expression System according to the manufacturer's manual (Life Technologies, MD) and purified using ProBond resin (Invitrogen Japan K.K.) (27). A truncated form of pol κ (residues 1-560) in which 6X His-tags were attached to the C-terminus was overproduced in *E. coli* and purified as described by Ohashi *et al* (28). Recombinant human His-pol λ was overexpressed and purified according to the method described by Shimazaki *et al* (29).

The reaction mixture for calf pol α was described previously (30,31). The reaction mixture for human pol γ was previously described by Umeda *et al* (27). The reaction mixtures for mammalian pols κ and λ were the same as that for calf pol α. For the pol assay, poly(dA)/oligo$(dT)_{18}$ (A/T = 2/1) and dTTP were used as DNA template-primers, and 2'-deoxynucleoside 5'-triphosphate (dNTP) was used as the substrate. The vitamin K_3 derivatives (i.e., compounds 1-3) were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. The sonicated samples (4 μl) were mixed with 16 μl of each enzyme (final amount 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 standard enzyme reaction mixture (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 15% glycerol, 10 μM poly(dA)/oligo$(dT)_{18}$ and 10 μM [^{3}H]-dTTP), and incubation was carried out at 37˚C for 60 min. The activity that occurred in the absence of an inhibitor was considered to be 100%, and the activity observed in the presence of each concentration of inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol dNTP (dTTP) into the synthetic DNA template-primers (poly(dA)/oligo$(dT)_{18}$, A/T = 2/1) for

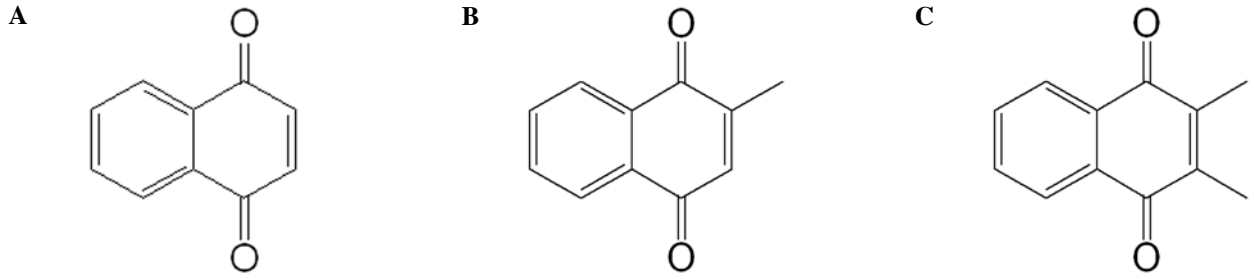

Figure 1. Structures of the vitamin K_3 derivatives. (A) 1,4-naphthoquinone (compound 1), (B) 2-methyl-1,4-naphthoquinone (vitamin K_3, compound 2), and (C) 1,2-dimethyl-1,4-naphthoquinone (compound 3).

60 min at 37˚C under normal reaction conditions for each enzyme (scintillation counts: approximately 1 pmol of incorporated radioactive nucleotides, 100 cpm) (30,31).

Animal experiments. All animal studies were performed according to the guidelines for the 'Care and Use of Laboratory Animals' at Kobe University. The animals were anesthetized with pentobarbital before undergoing cervical dislocation. Female 8-week-old C57BL/6 mice that had been bred in-house with free access to food and water were used for all experiments. All of the mice were maintained under a 12-h light/dark cycle and housed at a room temperature of 25˚C.

Mouse TPA-induced anti-inflammatory assay. The mouse inflammatory test was performed according to Gschwendt's method (32). In brief, an acetone solution containing compounds 1, 2 or 3 (250 or 500 μg in 20 μl) or 20 μl of acetone as a vehicle control was applied to the inner part of the mouse ear. Thirty minutes after the test compound had been applied, TPA solution (0.5 μg/20 μl of acetone) was applied to the same part of the ear. Acetone, followed by TPA solution, was applied to the other ear of the same mouse as a control. After 7 h, a disk (6 mm diameter) was obtained from the ear and weighed. The IE is presented as the ratio of the increase in the weight of the ear disks: IE: {[(TPA only) - (tested compound plus TPA)]/[(TPA only) - (vehicle)] x 100}.

Cell culture of mouse macrophages. RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 4.5 g of glucose per liter plus 10% fetal calf serum, 5 mM L-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin. The cells were cultured at 37˚C in standard medium in a humidified atmosphere of 5% CO_2-95% air.

Preparation of peritoneal macrophages. Female C57BL/6 mice were intraperitoneally injected with phosphate-buffered saline (PBS), and the peritoneal cavity was washed with PBS. PBS was collected, and peritoneal macrophages were separated from PBS by centrifugation at 1,500 x g for 10 min.

Measurement of the TNF-α level in the cell culture medium. RAW264.7 cells or peritoneal macrophages were placed in a 12-well plate at $5x10^4$ cells/well and incubated for 24 h. The cells were pre-treated with compounds 1-3 for 30 min before the addition of 100 ng/ml LPS. After stimulation with LPS for 24 h, the cell culture medium was collected to measure the amount of TNF-α secreted. The concentration of TNF-α in the culture medium was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) development system (Bay Bioscience Co., Ltd., Kobe, Japan) in accordance with the manufacturer's protocol.

In vivo LPS-induced inflammatory experiment. The mice were intraperitoneally injected with 200 μl of compounds 1-3 dissolved in corn oil at 20 mg/kg body weight (BW), or 200 μl of corn oil as a vehicle control. After 30 min, the mice were intraperitoneally injected with 200 μl of 250 μg/kg BW LPS dissolved in PBS or 200 μl of PBS as a vehicle control. After 1 h, the mice were sacrificed, and blood samples were collected. The blood serum was separated by centrifugation at 15,000 x g for 10 min at 4˚C, and the TNF-α level of the serum was measured using ELISA.

In vivo DSS-induced colitis experiment. Colitis was induced by administration of sterile drinking water containing 2.5% (wt/vol) DSS. On Day 5, supplementation of the mice with DSS was discontinued, and the mice were given drinking water alone instead. On Day 8, the mice were randomly divided into 4 groups and orally injected with 20 mg/kg BW of compounds 1-3 or corn oil as a vehicle control, once daily between Days 8-11 (a total of 4 oral treatments), and then the mice were sacrificed on Day 12. Clinical assessments of the BW, stool consistency, rectal bleeding, and general appearance of the DSS-treated mice were performed daily. The colon tissue of the mice was excised, and colon length was measured.

Histological examination. The colon tissue of the mice was dissected and fixed with 4% paraformaldehyde. The paraffin-embedded tissue was sliced and stained with hematoxylin and eosin (H&E) in a blinded manner. The sections were observed using a microscope (BX51; Olympus, Tokyo, Japan), and the severity of the inflammation and epithelial damage was determined in a blinded manner.

Quantitative real-time PCR. The colon epithelial cells were derived from the mucosal layer of the colon tissue, and total-RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse-transcribed cDNA was produced using a Reverse Transcription kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions, and quantitative real-time PCR was performed with SYBR-Green PCR master mix (Applied Biosystems)

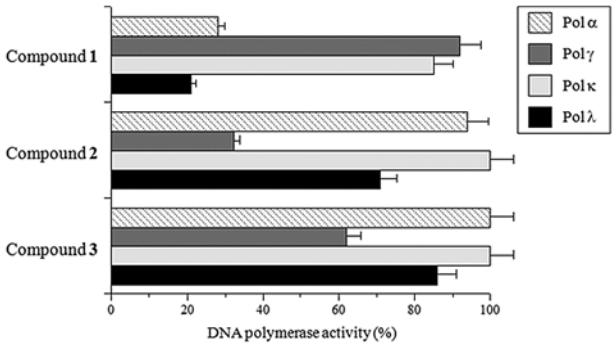

Figure 2. Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on the activity of mammalian pols. Each compound (10 μM) was incubated with calf pol α (B-family pol), human pol γ (A-family pol), human pol κ (Y-family pol), and human pol λ (X-family pol) (0.05 units each). Pol activity in the absence of the compound was taken as 100%, and the relative activity observed in the presence of the compound is shown. Data are shown as the mean ± SE (n=3).

Table I. Anti-inflammatory activity of the vitamin K_3 derivatives (compounds 1-3) on TPA-induced edema in the mouse ear.

	Inhibitory effect, %		
Amount of compound	1	2	3
250 μg/ear	28.6±4.4	8.9±1.4	3.5±0.6
500 μg/ear	55.0±8.0	20.3±3.1	7.1±0.9

Each compound (250 or 500 μg) was applied individually to one ear of a mouse, and then after 30 min, TPA (0.5 μg) was applied to both ears. Edema was evaluated after 7 h. The inhibitory effect is expressed as the percentage of edema. Data are shown as the mean ± SE (n=6).

according to the manufacturer's protocol. The reaction conditions involved 40 cycles of two-stage PCR consisting of an initial denaturation step of 95˚C for 15 sec and annealing at 60˚C for 1 min after an initial denaturation step of 95˚C for 10 min. The primer sequences were as follows: mouse TNF-α, 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and 5'-TGGGAGTAGACAAGGTACAACCC-3'; mouse β-actin, 5'-AGAGGGAAATCGTGCGTGAC-3' and 5'-CAATAGTGATGACCTGGCCGT-3'. To allow comparisons of the mRNA expression levels, the real-time PCR data were analyzed with the ΔΔCt method and normalized to the amount of β-actin cDNA as an endogenous control.

Immunofluorescence staining. The colon tissue was frozen in O.C.T. Compound (Sakura Finetek, Tokyo, Japan) and then sectioned into 10-μm slices. The acetone-fixed sections were blocked with 10% goat serum (Vector Labs, Burlingame, CA) in PBS for 30 min at room temperature. The sections were incubated with anti-CD11b antibody (1:25) to detect monocytes including macrophages and anti-F4/80 antibody (1:25) to detect macrophages for 2 h at room temperature and then reacted with goat anti-rat IgG antibody conjugated with Alexa Fluor 488 (1:400) and Alexa Fluor 546-labeled phalloidin (1:200) for 1 h at room temperature. After being mounted with Fluorescent Mounting Medium (Dako, Denmark), the sections were observed in a blinded manner using a confocal microscope (LSM 5 Pascal; Carl Zeiss, Jena, Germany).

Statistical analysis. The data are expressed as the mean ± SD or SE of at least three independent determinations for each experiment. Statistical significance was analyzed using the Student's t-test, and a probability level of 0.05 was considered significant.

Results

Effects of the vitamin K_3 derivatives (compounds 1-3) on mammalian pol activity. Initially, we investigated the *in vitro* biochemical action of vitamin K_3 (compound 2) and its derivatives (compounds 1 and 3). The inhibition of four mammalian pols, namely calf pol α, human pol γ, human pol κ and human pol λ, induced by the administration of 10 μM of each compound was investigated. Pols α, γ, κ and λ were used as representatives of the B, A, Y and X pol families, respectively (1-3). As shown in Fig. 2, compound 1 inhibited the activity of calf pol α, whereas compounds 2 and 3 had no effect on pol α activity. Compounds 2 and 3 strongly and slightly inhibited the activity of human pol γ, respectively, whereas compound 1 had no effect on pol γ activity. None of these compounds affected the activity of human pol κ. Among the mammalian pols tested, human pol λ activity was inhibited most strongly, and the IEs of the three compounds on pol λ were ranked as follows: compound 1 > compound 2 > compound 3. The IEs of these compounds were not changed when activated DNA (i.e., bovine deoxyribonuclease I-treated DNA) and dNTP were used as the DNA template-primer and nucleotide substrate instead of synthesized DNA [poly(dA)/oligo$(dT)_{18}$ (A/T = 2/1)] and dTTP, respectively (data not shown).

Effects of the vitamin K_3 derivatives (compounds 1-3) on TPA-induced anti-inflammatory activity. In previous pol inhibitor studies, we found that pol λ inhibitors also reduce TPA-induced acute anti-inflammatory activity (6,19,20). Thus, using the mouse ear inflammatory test, we examined the anti-inflammatory activity of the vitamin K_3 derivatives. The application of TPA (0.5 μg) to the mouse ear induced edema, resulting in a 241% increase in the weight of the ear disk 7 h after its application. As shown in Table I, pretreatment with compounds 1-3 dose-dependently suppressed the inflammation, and the effects of these compounds were ranked as follows: compound 1 > compound 2 > compound 3. Therefore, the anti-inflammatory effects of these compounds displayed the same order as their IEs on mammalian pol λ (Fig. 2). These results suggest that the pol λ inhibitory activities of these compounds are positively correlated with their anti-inflammatory activities.

Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on LPS-induced inflammatory responses in cultured macrophage cells and peritoneal macrophages. Next, we investigated whether these three vitamin K_3 derivatives were able to inhibit the TNF-α production induced by LPS stimulation in cultured macrophage RAW264.7 cells and peritoneal macrophages. The inflammatory cytokine TNF-α activates

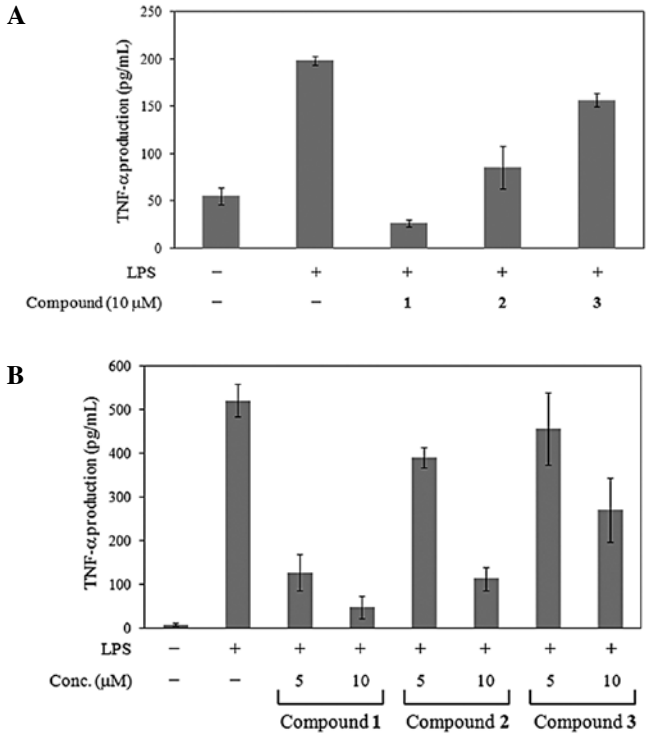

Figure 3. Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on LPS-induced TNF-α production in mouse macrophages. (A) The mouse macrophage RAW264.7 cell line was pre-treated with 10 μM of each vitamin K_3 derivative or DMSO as a vehicle control for 30 min and were then treated with 100 ng/ml LPS for 24 h. (B) Mouse peritoneal macrophages were pre-treated with 5 or 10 μM of each compound or DMSO as a vehicle control for 30 min and then with 100 ng/ml LPS for 24 h. The TNF-α concentration of the cell medium was measured by ELISA. Data are shown as the mean ± SE (n=4).

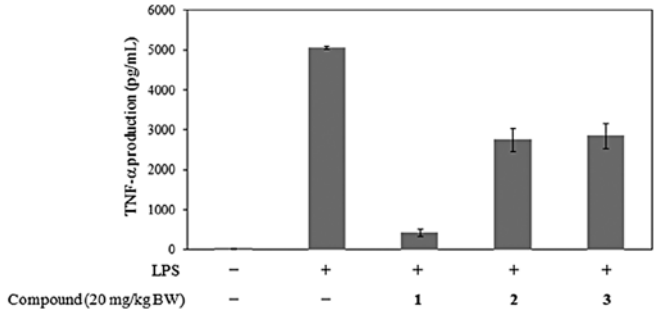

Figure 4. The inhibitory activity of the vitamin K_3 derivatives (compounds 1-3) against LPS-induced inflammation *in vivo*. Female C57BL/6 mice were intraperitoneally injected with compounds 1-3 at 20 mg/kg BW or corn oil as a vehicle control. After 30 min, the mice were intraperitoneally injected with LPS at 250 μg/kg BW or saline as a vehicle control. One hour after the LPS injection, the mice were sacrifished, and their serum TNF-α level was measured using ELISA. Treatment with corn oil and LPS was used as a positive control (TNF-α level: 5,068 pg/ml), and that with corn oil and saline was used as a negative control (TNF-α level, 26 pg/ml). Data are shown as the mean ± SE (n=3).

the major inflammation signaling pathway by binding to the TNF-α receptor (TNFR), resulting in various inflammatory diseases (33). In RAW264.7 cells, no compound showed cytotoxicity at 50 μM (data not shown). RAW264.7 cells produced 198 pg/ml of TNF-α after LPS treatment (Fig. 3A). At 10 μM, all three compounds suppressed the LPS-stimulated production of TNF-α, and the strengths of the effects of the three compounds were ranked as follows: compound 1 > compound 2 > compound 3. Fig. 3B shows the dose-dependent suppression of LPS-evoked TNF-α production in mouse peritoneal macrophages after treatment with 5 or 10 μM of each vitamin K_3 derivative. The strengths of the IEs of these compounds on the peritoneal macrophages showed the same order as in the RAW264.7 macrophage cell line; i.e., they were ranked as follows: compound 1 > compound 2 > compound 3.

Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on LPS-induced inflammation in vivo. To assess the *in vivo* anti-inflammatory effects of these three vitamin K_3 derivatives, we investigated the inhibitory activity of each compound against LPS-induced acute inflammation (Fig. 4). Treatment with 250 μg/kg BW of LPS considerably increased the serum TNF-α levels (5 ng/ml) of the mice, and the intraperitoneal injection of 20 mg/kg BW of compound 1 strongly decreased this LPS-induced TNF-α production by 94.1%. On the other hand, compounds 2 and 3 moderately suppressed the TNF-α production in mice, with compound 2 inducing a greater suppression than compound 3. Thus, the *in vivo* data obtained in the mouse study showed almost the same trend as the data obtained from the cultured mouse macrophage cells (Fig. 3). These results regarding the suppression of the inflammatory response by the vitamin K_3 derivatives displayed the same pattern as those regarding the IEs of the compounds on mammalian pol λ (Fig. 2).

Anti-inflammatory effects of the vitamin K_3 derivatives (compounds 1-3) on DSS-induced colitis in mice. To evaluate the therapeutic effects of the vitamin K_3 derivatives against colitis, a DSS-induced colitis mouse model was used. The DSS-treated mice were orally injected with one of the vitamin K_3 derivatives (compounds 1-3) at 20 mg/kg BW or corn oil as a vehicle control once daily (total 4 oral injections) and then were sacrificed. During the experiment, the BW of the mice was measured daily, and the relative BW was calculated as the BW of a mouse on Day 12 relative to the initial BW of the same mouse on Day 0 (Fig. 5A). As a result, it was found that compounds 1-3 significantly improved the decreased BW of the colitis mice. These compounds also reduced the DSS-induced shortening of colon length (Fig. 5B). In a histopathological examination with H&E staining (Fig. 5C), it was found that these compounds attenuated the degree of tissue injury induced by DSS.

Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on the expression of TNF-α mRNA in the colon tissue of DSS-induced colitis mice. RNA was isolated from the colon epithelia of mice treated with DSS and compounds 1-3, and the expression level of TNF-α mRNA was examined by quantitative real-time PCR (Fig. 6). In DSS-induced colitis mice, the expression level of TNF-α mRNA was elevated, and the administration of these compounds led to a reduction in TNF-α production in the colon epithelium. Among the vitamin K_3 derivatives, the attenuating effect of compound 1 was the strongest.

Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on macrophage infiltration into the colon tissue of DSS-induced

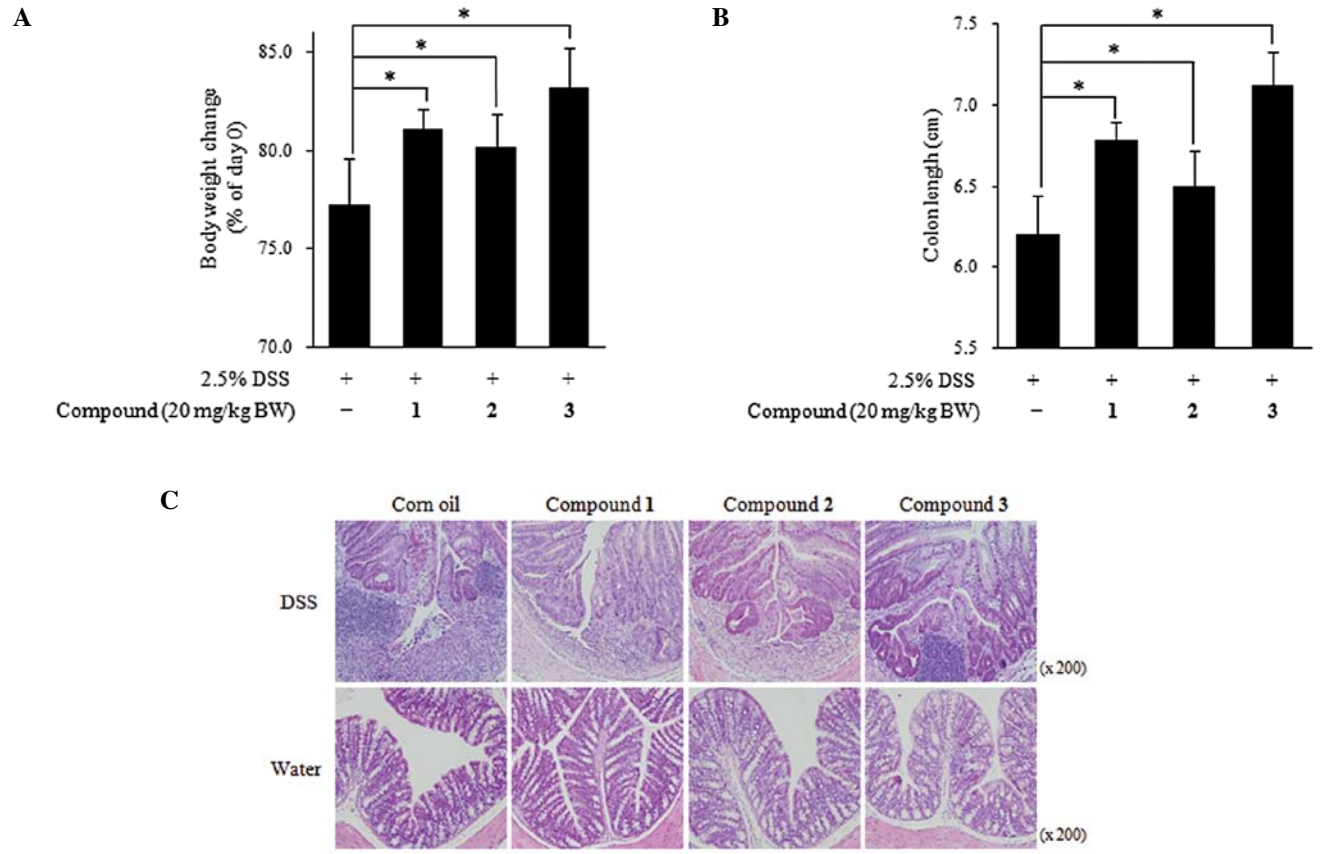

Figure 5. Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on DSS-induced colitis in mice. Colitis was induced in mice by the administration of DSS. C57BL/6J mice were administered 2.5% DSS in their drinking water for 5 days (Day 0-5) and were subsequently given drinking water alone. The mice were orally injected with compounds 1-3 at 20 mg/kg BW or corn oil as a vehicle control once daily between Days 8-11 (a total of 4 oral injections), and then the mice were sacrificed on Day 12. (A) The BW change of the mice challenged with DSS and the vitamin K_3 derivatives. During the experiment, the BW of the mice was measured daily, and the relative BW of the mice on Day 12 compared to the initial BW of the mice on Day 0 was calculated. The initial BW of the DSS-treated mice was 20.5±0.50 g. Values are represented as means ± SD (n=10). The asterisks indicate a significant difference according to the Student's t-test ($P<0.05$). (B) The colon length of mice challenged with DSS and the vitamin K_3 derivatives. The length of inflamed colon tissue was measured on Day 12. Data are shown as the mean ± SD (n=10). The asterisks indicate a significant difference according to the Student's t-test ($P<0.05$). (C) Histopathological evaluation of the colon tissue of mice challenged with DSS and the vitamin K_3 derivatives. H&E staining was performed using colon sections obtained from mice treated with DSS and compounds 1-3 (upper) or water alone and these compounds (lower). Magnification, x200.

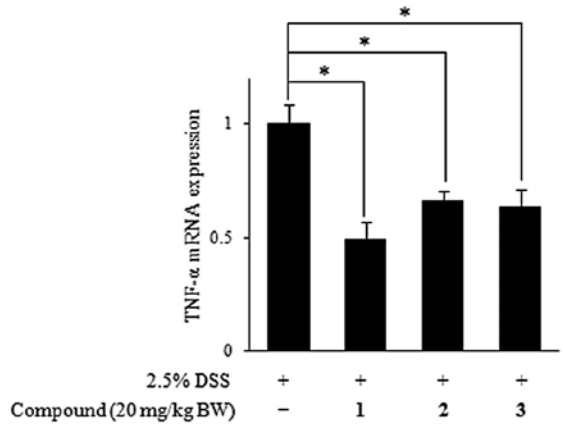

Figure 6. Inhibitory effects of the vitamin K_3 derivatives (compounds 1-3) on the expression of TNF-α mRNA in the colon tissue of DSS-induced colitis mice. RNA was isolated from the colon epithelia of mice challenged with DSS and compounds 1-3, and the expression of TNF-α mRNA was examined by quantitative real-time PCR, as described in the Materials and methods section. The mRNA expression level was normalized to that of β-actin as an internal standard. The data are shown as relative values compared with those of the mice injected with DSS alone and are shown as the mean ± SE (n=4). The asterisks indicate a significant difference according to the Student's t-test ($P<0.05$).

colitis mice. The frozen colon sections obtained from the mice were stained with anti-CD11b antibody to detect monocytes including macrophages (Fig. 7A) and anti-F4/80 antibody to detect macrophages (Fig. 7B). As a result, it was found that compounds 1-3 significantly attenuated macrophage infiltration into the large intestinal submucosa of the mice; therefore, the vitamin K_3 derivatives may be useful as therapeutic anti-inflammatory drugs.

Discussion

We have shown here that 1,4-naphthoquinone (compound 1) is the strongest inhibitor of mammalian pols, especially pols α and λ (Fig. 2), and that this compound is also the strongest inhibitor of the inflammatory response among the three vitamin K_3 derivatives examined in this study (compounds 1-3) (Table I and Figs. 3-7). Compounds 1-3 have none, one, and two methyl groups in addition to their 1,4-naphthoquinone backbone, respectively; therefore, the number of methyl side groups was found to be very important for the inhibitory activities of vitamin K_3 derivatives. As reported previously, the phenolic compound curcumin, which is a known anti-inflammatory

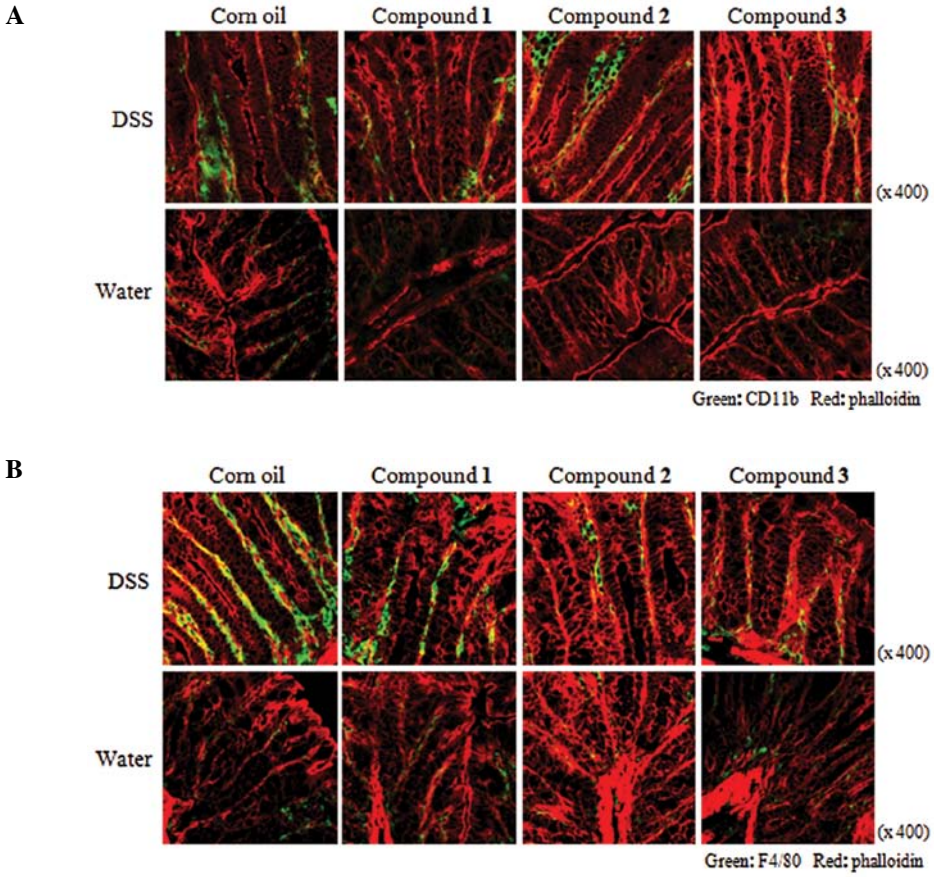

Figure 7. Immunohistochemical staining in mice challenged with DSS and the vitamin K_3 derivatives (compounds 1-3). Frozen colon tissue obtained from mice treated with DSS and compounds 1-3 (upper) or water alone and compounds 1-3 (lower) was stained with (A) anti-CD11b antibody and (B) anti-F4/80 antibody, as described in the Materials and methods section. The frozen tissue was also stained with Alexa Fluor 546-labeled phalloidin to observe the form of the colon tissue. The green and red signals represent CD11b and F4/80 or phalloidin, respectively. Magnification, x400.

agent, is a pol λ-specific inhibitor (7,19,20). Intriguingly, pol λ is also the principle molecular target of the vitamin K_3 derivatives based on 1,4-naphthoquinone.

Eukaryotic cells reportedly contain 15 pol species, which belong to four families; namely, family A (pols γ, θ and ν), family B (pols α, δ, ε and ζ), family X (pols β, λ and μ, and TdT), and family Y (pols η, ι and κ, and REV1) (3,4). Among the X pol family, pol λ has an unclear biochemical function, although it seems to work in a similar way to pol β (34). Pol β is involved in the short-patch base excision repair (BER) pathway (35-38) and also plays an essential role in neural development (39). Recently, pol λ was found to possess 5'-deoxyribose-5-phosphate (dRP) lyase activity, but not apurinic/apyrimidinic (AP) lyase activity (40). Pol λ is able to substitute for pol β during *in vitro* BER, suggesting that pol λ also participates in BER. Northern blot analysis indicated that pol β transcripts are abundantly expressed in the testes, thymus, and brain in rats (41), whereas pol λ is mostly transcribed in the testes (42). Bertocci *et al* (43) reported that mice in which pol λ expression had been knocked out were not only viable and fertile, but also displayed a normal hypermutation pattern.

In addition to causing inflammation, TPA influences cell proliferation and has physiological effects on cells because it possesses tumor promoter activity (24). Therefore, anti-inflammatory agents are expected to suppress DNA replication/repair/recombination in nuclei. As pol λ is a repair/recombination-related pol (34), our finding that the molecular target of the vitamin K_3 derivatives, such as compound 1, is pol λ is in good agreement with this predicted mechanism of anti-inflammatory agents. As a result, pol λ inhibitors may also inhibit inflammation.

We have investigated the IEs of the vitamin K_3 derivatives on mammalian pols, which are responsible for DNA replication leading to cell proliferation and DNA repair/recombination, as well as the relationship between the degree to which they suppress LPS-evoked TNF-α production and their anti-inflammatory activity. In addition, the degree to which cytokine production was attenuated by the three vitamin K_3 derivatives in a mouse colitis model exhibited almost the same pattern as their suppression of LPS-induced inflammatory reactions. The molecular mechanism linking the LPS-induced inflammatory response and anti-inflammatory activity in the TPA-induced ear edema model is unknown. As a result, we found a positive correlation between the pol inhibitory activity and anti-inflammatory activity of the vitamin K_3 derivatives. The relationship between these activities may provide a new and convenient *in vitro* assay for screening for novel anti-inflammatory compounds.

Recently, we found that vitamin K_3, but not K_1 or K_2, suppressed inflammation in *in vitro* cell culture experiments and *in vivo* animal experiments (10). Since vitamin K_3 (2-methyl-1,4-naphthoquinone) has a 1,4-naphthoquinone backbone, this

structure must be important for the above mentioned activities. Although the vitamin K group of vitamins share a methylated 1,4-naphthoquinone ring structure, the aliphatic side chains attached to the 3-positions of vitamins K_1 and K_2 may reduce their pol inhibitory and anti-inflammatory activities (10). Since 1,4-naphthoquinone (i.e., compound 1) was found to be a stronger inhibitor of pol and inflammation than vitamin K_3 (i.e., compound 2), compound 1 could be the strongest inhibitor of pol and inflammation among the vitamin K_3 derivatives.

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