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Effects of Quinone Derivatives, such as 1,4-Naphthoquinone, on DNA Polymerase Inhibition and Anti-Inflammatory Action

DNA ポリメラーゼ阻害作用や抗炎症作用における 1,4-ナフトキノン等のキノン誘導体の効果について

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Key words: Quinone derivatives, 1,4-naphthoquinone, DNA polymerase λ, enzyme inhibitor, lipopolysaccharide (LPS), anti-inflammation, tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB

Effects of Quinone Derivatives, such as 1,4-Naphthoquinone, on DNA Polymerase Inhibition and Anti-Inflammatory Action

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Abstract

Previously, we reported that vitamin K_3, which consists of a quinone component, inhibits the activity of human DNA polymerase γ (pol γ). In this study, we investigated the inhibitory effects of 4 quinone derivatives (1,4-benzoquinone (BQ), 1,4-naphthoquinone (NQ), 9,10-anthraquinone (AQ) and 5,12-naphthacenequinone (NCQ)) on the activity of mammalian pols. BQ and NQ potently inhibited the activity of all the pol species: pols α, β, γ, δ, ε and λ, and NQ was a stronger pol inhibitor than BQ. Because we previously found a positive relationship between pol λ inhibition and anti-inflammatory action, we examined whether these quinone derivatives could inhibit inflammatory responses. BQ and NQ caused a marked reduction in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute inflammation in mouse ear, although AQ and NCQ did not. In a cell culture system using mouse macrophages, NQ displayed the strongest suppression in the production of tumor necrosis factor (TNF)-α induced by lipopolysaccharide (LPS) among the quinone derivatives tested. Moreover, NQ was found to inhibit the action of nuclear factor (NF)-κB. In an *in vivo* mouse model of LPS-evoked acute inflammation, intraperitoneal injection of BQ and NQ to mice led to suppression of TNF-α production in serum. These anti-inflammatory responses of NQ were more potent than those of BQ. In conclusion, this study has identified several quinone derivatives, such as NQ, that are promising anti-inflammatory candidates.

Keywords: Quinone derivatives, 1,4-naphthoquinone, DNA polymerase λ, enzyme inhibitor, lipopolysaccharide (LPS), anti-inflammation, tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB.

INTRODUCTION

The human genome encodes at least 15 DNA polymerases (pols) that conduct 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 overall catalytic subunits show little variance among species. Enzymes with conserved structures usually perform important cellular functions, the maintenance of which provides evolutionary advantages. Because not all functions of eukaryotic pols have been fully elucidated, selective inhibitors of pols are useful tools for distinguishing pols and clarifying their biological roles. We have been studying selective inhibitors of each pol from natural products including food materials and nutrients for more than 15 years [5, 6]. We have found that vitamin K3, but not K1 or K2, is a potent and specific inhibitor of human pol γ [7-10].

Vitamin K3 (menadione, 2-methyl-1,4-naphthoquinone) is a fat-soluble compound with quinone as the principle chemical feature. Quinones are a class of organic compounds that are formally derived from aromatic compounds by exchanging an even number of –CH= groups by –C(=O)– groups, with 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 been examined. For example, quinones are known to interact with flavoproteins to generate reactive oxygen species (ROS) that can induce biological injury [11-14]. In this study we focused on four major quinone derivatives that have two ketone groups at positions 1 and 4; namely 1,4-benzoquinone (BQ), 1,4-naphthoquinone (NQ), 9,10-anthraquinone (AQ) and 5,12-naphthacenequinone (NCQ). These compounds consist of a polycyclic aromatic hydrocarbon, such as benzene, naphthalene, anthracene or tetracene (Fig. 1).

In our pol inhibitor study, we have found that pol λ selective inhibitors, such as curcumin derivatives [15-17], have 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced anti-inflammatory activity [18-20]. Although tumor promoters are classified as compounds that promote tumor formation [21], they also cause inflammation and are commonly used as artificial inducers of inflammation in order to screen for anti-inflammatory agents [22]. 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 [23], suggesting that the molecular basis of the inflammation stems from the pol reactions related to cell proliferation. This relationship, however, needs to be investigated more closely.

In many inflammatory responses, activation of nuclear factor (NF)-κB is the rate-limiting step of the inflammatory mechanism [24]. The five members of the mammalian NF-κB family, namely p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), exist in unstimulated cells as homodimers or heterodimers bound to proteins of the IκB family [25]. The binding of NF-κB to IκB prevents NF-κB from translocating to the nucleus, thereby maintaining NF-κB in an inactive state. NF-κB proteins are characterized by the presence of a conserved 300-amino acid Rel homology domain located in the N-terminus of the protein, and this domain is responsible for dimerization with NF-κB, interaction with IκB, and binding to DNA [25]. The translocated NF-κB proteins work as transcription factors and regulate the expression of various genes that encode proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-12, which have been shown to play important roles in sustained inflammatory responses [26-28].

In this study, we investigated the inhibitory effects of BQ, NQ, AQ and NCQ on mammalian pol activity and inflammatory responses both *in vitro* and *in vivo*. We found that some of these compounds suppress NF-κB activation induced by lipopolysaccharide (LPS) in mouse macrophage cells. Moreover, we also demonstrated that the quinone derivatives exert inhibitory effects against TNF-α production in an animal model of LPS-induced acute inflammation.

MATERIALS AND METHODS

Materials

Four representative quinone derivatives, 1,4-benzoquinone (BQ), 1,4-naphthoquinone (NQ), 9,10-anthraquinone (AQ) and 5,12-naphthacenequinone (NCQ), were obtained from Sigma-Aldrich (St. Louis, MO), and these structures are shown in Fig. (**1**). Each compound was purified more than 99% pure. Chemically synthesized DNA templates such as poly(dA), and nucleotides such as [3H]-deoxythymidine 5'-triphosphate (dTTP) (43 Ci/mmol), were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). The oligo(dT)18 DNA primer was customized by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). LPS was purchased from Sigma-Aldrich. For Western blot analysis, anti-NF-κB p65 antibody, anti-β-actin antibody and horseradish peroxidaseconjugated anti-rabbit IgG antibody (i.e., secondly antibody) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Thermo Scientific (Kanagawa, Japan), respectively. 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 immuno-affinity column chromatography as described by Tamai *et al.* [29]. Recombinant rat pol β was purified from *Escherichia coli* JMpβ5 as described by Date *et al.* [30]. The human pol γ catalytic gene was cloned into pFastBac (Invitrogen Japan K.K., Tokyo Japan). Histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (Life Technologies, MD) and purified using ProBoundresin (Invitrogen Japan K.K.) [31]. 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 [32]. Recombinant human His-pol λ was overexpressed and purified according to a method described previously [33].

The reaction mixtures for calf pol α and rat pol β were described previously [34, 35]. The reaction mixtures for human pol γ, and human pols δ and ε, were previously described by Umeda *et al.* [31] and Ogawa *et al.* [36], respectively. The same reaction mixture was used for human pol λ as for rat pol β. For all pols, poly(dA)/oligo(dT)18 (A/T = 2/1) and dTTP were used as the DNA template-primer and nucleotide [2'-deoxynucleoside 5'-triphosphate (dNTP)] substrate, respectively. The quinone derivatives were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. Aliquots (4 μL) of sonicated samples 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 of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min, with the exception of *Taq* pol, which was incubated at 74 °C for 60 min. Activity in the absence of inhibitor was considered 100% activity, and the remaining activity at each inhibitor concentration 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 synthetic DNA template-primers in 60 min at 37 °C under normal reaction conditions for each enzyme [34, 35].

Animal Experiments

All animal studies were performed according to the guidelines outlined in the 'Care and Use of Laboratory Animals' of Kobe University. The animals were anesthetized with pentobarbital before undergoing cervical dislocation. Female 8-week-old C57BL/6 mice that had been bred inhouse 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 [37]. In brief, an acetone solution of the test compound (250 or 500 μg / 20 μL) or 20 μL of acetone as vehicle control was applied to the inner part of the mouse ear. Thirty minutes after the test compound was applied, a TPA solution (0.5 μg / 20 μL of acetone) was applied to the same part of the ear. To the other ear of the same mouse, methanol, followed by TPA solution, was applied as a control. After 7 h, a disk (6 mm diameter) was obtained from the ear and weighed. The inhibitory effect (IE) is presented as a ratio of the increase in weight of the ear disks: IE: {[(TPA only)-(tested compound plus TPA)] / [(TPA only)-(vehicle)] x 100}.

Cell Culture of Mouse Macrophage

A mouse macrophage cell line, RAW264.7, was obtained from 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 units/mL penicillin and 50 units/mL streptomycin. The cells were cultured at 37 °C in standard medium in a humidified atmosphere of 5% CO2-95% air.

Preparation of Peritoneal Macrophages

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

Measurement of TNF-α Level in the Cell Culture Medium

The RAW264·7 cells or the peritoneal macrophages were placed in a 12-well plate at 5 x 104 cells/well and incubated for 24 h. The cells were pretreated with the quinone derivatives 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.

Cell Treatment and Preparation of Nuclear Proteins

RAW264.7 cells on a 6-well plate at 5 × 105 cells/well were incubated with 10 μM of the quinone derivatives or DMSO (1 μL/mL) as a vehicle control for 30 min followed by treatment with 100 ng/mL LPS for 30 min. After treatment, cells were harvested with lysis buffer consisting of 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2 and 0.5 mM DTT containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL leupeptin and 5 μg/mL aprotinin) and phosphatase inhibitors (10 mM NaF and 1 mM Na3VO4) and stood on ice for 15 min with occasional mixing. The mixture was centrifuged at 1,000 x *g* for 10 min at 4 °C. The precipitate was suspended in extraction buffer consisting of 20 mM Hepes, pH 7.6, 20% (v/v) glycerol, 500 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1.0 mM DTT and 0.1% (v/v) Nonidet P-40 containing the same protease and phosphatase inhibitors. The suspension was then gently mixed on a rotary device for 1 h at 4 °C before being centrifuged at 15,000 x *g* for 20 min at 4 °C. The resulting supernatant was used as a nuclear extract. The protein concentration was measured using a bicinchoninic acid (BCA) assay kit, in accordance with the manufacturer’s protocol.

Western Blotting

The nuclear proteins (30-50 μg protein) were boiled in a quarter-volume of sample buffer (1 M Tris-HCl, pH 7.5, 640 mM 2-mercaptoethanol, 0.2% bromphenol blue, 4% SDS and 20% glycerol) and then separated on 10% SDS polyacrylamide gels. Each gel was then electroblotted onto a PVDF membrane. The membrane was blocked with 1% skimmed milk in Tris-buffered saline TBS-T (10 mM Tris-HCl, 100 mM NaCl and 0.5% Tween-20) and probed with anti-NF-κB p65 antibody (1:500) and anti-β-actin antibody (1:5,000) before being reacted with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 and 1:20,000, respectively). The protein-antibody complex was visualized with ChemiLumiONE (Nacalai Tesque, Kyoto, Japan) and detected using an Image Reader (LAS-3000 Imaging System, Fuji Photo Film, Tokyo, Japan). The intensity of each band was analyzed using ImageJ, which was developed at the National Institute of Health.

***In Vivo* LPS-Induced Inflammatory Experiment**

Mice were intraperitoneally injected with quinone derivatives 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 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 in the serum was measured using ELISA.

Statistical Analysis

All data are expressed as the means ± SE of at least three independent determinations for each experiment. Statistical significance was analyzed using Student's t-test, and a level of probability of 0.05 was used as the criterion of significance.

RESULTS

Effect of the Quinone Derivatives on Mammalian Pol Activity

Initially we investigated the *in vitro* biochemical action of four commercially purified quinone derivatives (BQ, NQ, AQ, and NCQ). The inhibitory activity of six mammalian pols, calf pol α, rat pol β, human pols γ, δ, ε and λ, by each compound was investigated. Pol α, pols β and λ, pol γ and pols δ and ε were used as representative replicative pol, repair/recombination-related pols, mitochondrial pols and replication/repair pols, respectively [1-3]. As shown in Table **1**, BQ and NQ inhibited the activity of all of the mammalian pols, whereas AQ and NCQ had no effect on pol activity. Of the mammalian pols investigated in this study, pol α was most strongly inhibited by BQ and NQ. The inhibitory effect of BQ and NQ on pols can be ranked as follows: pol α > pol λ > pol ε > pol δ > pol γ > pol β. NQ displayed a stronger inhibitory effect against these pols than BQ. For example, 50% inhibition of BQ and NQ against calf pol α activity was observed at a dose of 8.30 and 6.65 μM, respectively. We also tested the effect of using activated DNA (i.e., bovine deoxyribonuclease I treated DNA) with dNTP as the DNA template-primer and nucleotide substrate instead of synthesized DNA [poly(dA)/oligo(dT)18 (A/T = 2/1)] and dTTP, respectively. However, the inhibitory effects of these compounds were unchanged (data not shown).

Effect of the Quinone Derivatives on TPA-Induced Anti-Inflammatory Activity

In a pol inhibitor study, we previously found that there is a relationship between pol λ inhibitors and TPA-induced acute anti-inflammatory activity [6, 18, 19]. Thus, using the

mouse ear inflammatory test, we examined the anti-inflammatory activity of the quinone derivatives. 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 application. As shown in Table **2**, pretreatment with 500 μg/ear of BQ or NQ suppressed inflammation by 43.3 and 55.0%, respectively. By contrast, AQ and NCQ had almost no effect on the level of inflammation. Therefore, the anti-inflammatory effect correlated with the inhibitory effect on mammalian pols including pol λ, which was strongly inhibited by both BQ and NQ (Table **1**). These results suggest that inhibition of pol λ inhibitory activity has a positive correlation with the observed anti-inflammatory activity.

Inhibitory Effect of Quinone Derivatives on LPS-Induced Inflammatory Responses in Cultured Macrophage Cells and Peritoneal Macrophages

Next, we investigated whether these 4 quinone derivatives can inhibit both the reduction of TNF-α production in peritoneal macrophages and nuclear translocation of NF-κB p65 induced by LPS stimulation in cultured macrophage RAW264.7 cells. The inflammatory cytokine TNF-α activates the NF-κB signaling pathway by binding to the TNF-α receptor (TNFR) and thereby initiates an inflammatory response, resulting in various inflammatory diseases [38]. In RAW264.7 cells, cytotoxicity of each BQ, NQ, AQ and NCQ at 10 μM was not observed, because the LD50 values were 66, 62, >200, >200 μM, respectively. As shown in Fig. (**2A**), the cells produced 198 pg/mL of TNF-α after LPS treatment. BQ and NQ at 10 μM significantly suppressed this LPS-stimulated production of TNF-α, although AQ and NCQ displayed almost no effect on TNF-α production. The inhibitory effect of NQ was stronger than that of BQ. NQ dose-dependently suppressed the LPS-stimulated production of TNF-α, and at 10 μM, the significant suppressive effect of the compound on TNF-α production was confirmed, with a reduction of 84.1% (Fig. **2B**). Fig. (**3**) shows the suppression of LPS-evoked TNF-α production in peritoneal macrophages derived from mice by 10 μM of the quinone derivatives. The inhibitory effect in the peritoneal macrophages showed the same tendency as that in the macrophage cell line RAW264.7. The strength of the inhibitory effect of the 4 quinones can be ranked as follows; NQ > BQ >> NCQ > AQ.

NF-κB is known to be the rate-controlling factor for inflammatory responses. We therefore, examined the inhibitory effects of the quinone derivatives on the LPS-induced nuclear translocation of NF-κB in RAW264.7 cells (Fig. **4**). By Western blot analysis, it was revealed that the amount of NF-κB nuclear translocation in RAW264.7 cells was 2.29-fold higher after LPS treatment, and that 10 μM of NQ was sufficient to completely inhibit the LPS-stimulated nuclear translocation of NF-κB. These results demonstrate that NQ can suppress the nuclear translocation of NF-κB by inhibiting the production of TNF-α. BQ did not inhibit the NF-κB nuclear translocation, although this compound suppressed LPS-evoked inflammatory responses. The effects of these compounds on the molecular mechanism of anti-inflammation will be addressed in future studies.

Inhibitory Effect of the Quinone Derivatives on LPS-Induced Inflammation *In Vivo*

To assess the anti-inflammatory effects of these 4 quinone derivatives *in vivo*, we investigated the inhibitory activity of each compound against LPS-induced acute inflammation (Fig. **5**). Treatment with 250 μg/kg BW of LPS significantly increased the serum TNF-α level (5 ng/mL) and intraperitoneal injection of 20 mg/kg BW of BQ and NQ strongly decreased this LPS-induced TNF-α production to 77.5% and 92.1%, respectively. Thus, the *in vivo* data obtained in the mouse study gave the same trend as the data obtained from cultured mouse macrophage cells (Fig. **2** and Fig. **3**).

DISCUSSION

We have shown here that NQ and BQ potently inhibit the activity of mammalian pols, especially pols α and λ (Table **1**), and that these two compounds are promising novel therapeutic agents for chronic inflammation (Table **2** and Figs. **2** to **5**). As reported previously, a phenolic compound, curcumin, which is a known anti-inflammatory agent, is a pol λ-specific inhibitor [6, 18, 19]. Intriguingly, the principle molecular target of the quinone derivatives is also pol λ.

Eukaryotic cells reportedly contain 15 pol species consisting of 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 family of pols, the biochemical function of pol λ is unclear, although pol λ appears to work in a similar way to pol β [39]. Pol β is involved in the short-patch base excision repair (BER) pathway [40-43], as well as playing an essential role in neural development [44]. Recently, pol λ was found to possess 5'-deoxyribose-5-phosphate (dRP) lyase activity, but no apurinic/apyrimidinic (AP) lyase activity [45]. 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 transcripts of pol β are abundantly expressed in the testis, thymus and brain in rats [46], whereas pol λ is efficiently transcribed mostly in the testis [47]. Bertocci *et al.* reported that mice in which pol λ is knocked down are not only viable and fertile, but also display a normal hyper-mutation pattern [48].

As well as causing inflammation, TPA influences cell proliferation and has physiological effects on cells because it is a tumor promoter activity [23]. Therefore, anti-inflammatory agents are expected to suppress both mammalian cell proliferation and DNA replication/repair/recombination in nuclei in relation to the action of TPA. Because pol λ is a repair/recombination-related pol [39], our finding that the molecular target of quinone derivatives, such as BQ and NQ, is pol λ is in good agreement with this expected mechanism of anti-inflammatory agents. As a result, a pol λ inhibitor could also be an inhibitor of chronic inflammation.

We have investigated the mode of action of the quinone derivatives inhibition of pols, which are responsible for DNA replication leading to cell proliferation and DNA repair/recombination, as well as the relationship between the degree of the suppression of LPS-evoked TNF-α production and anti-inflammatory activity. As a result, we found a positive relationship between the pol inhibitory and anti-inflammatory activities. The correlation between these activities may be useful as a new and convenient *in vitro* assay to screen for novel anti-inflammatory compounds.

Recently, we found that vitamin K3, but not K1 or K2, suppressed the inflammatory effects on *in vitro* cultured cell experiments and *in vivo* animal experiments [9]. Since vitamin K3 (2-methyl-1,4-naphthoquinone) has a NQ backbone, this structure must be important for the activities. Although vitamin K group of vitamins share a methylated NQ ring structure, the aliphatic side chain attached at the 3-position of vitamins K1 and K2 may reduce the pol inhibitory and anti-inflammatory activities. Since NQ was stronger inhibitor of pol and inflammation than vitamin K3 [9], NQ could be the strongest inhibitor in the quinone derivatives.

CONCLUSION

We have shown that the quinone derivatives BQ and NQ inhibited the activity of mammalian pols, and suppress LPS-evoked inflammatory responses both *in vitro* and *in vivo*.

Furthermore, the effects of NQ are stronger than those of BQ. Our study is the first to demonstrate that NQ reduces NF-κB activation and TNF-α production. BQ has no effect on NF-κB nuclear translocation, although this compound shows anti-inflammatory action. BQ and NQ also suppressed the mouse ear inflammation stimulated by TPA. The molecular mechanism linking the LPS-induced inflammatory response and anti-inflammatory activity in the model of TPA-induced ear edema is unknown. Because activated NF-κB has been observed in a model of TPA-induced ear edema [49], the anti-inflammatory effects of NQ may be, at least in part, dependent on the inhibition of NF-κB activation. Our study indicates that NQ is useful as an NF-κB inhibitor and may be a potent chemopreventive agent against inflammation.

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ABBREVIATIONS

AP = Apurinic/apyrimidinic
AQ = 9,10-Anthraquinone
BER = Base excision repair
BQ = 1,4-Benzoquinone
BW = Body weight
DMSO = Dimethyl sulfoxide
dNTP = 2'-Deoxynucleoside 5'-triphosphate
dRP = 5'-Deoxyribose-5-phosphate
dTTP = 2'-Deoxythymidine 5'-triphosphate
ELISA = Enzyme-linked immunosorbent assay
IE = Inhibitory effect
IL = Interleukin
LPS = Lipopolysaccharide
NCQ = 5,12-Naphthacenequinone
NF = Nuclear factor
NQ = 1,4-Naphthoquinone
PBS = Phosphate buffered saline
pol = DNA polymerase (E.C. 2.7.7.7)
TdT = Terminal deoxynucleotidyl transferase

TNF = Tumor necrosis factor
TPA = 12-*O*-Tetradecanoylphorbol-13-acetate

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Figure Legends

Fig. (1). Structure of quinones of heterocyclic aromatic compounds. (A) 1,4-Benzoquinone (BQ), (B) 1,4-Naphthoquinone (NQ), (C) 9,10-Anthraquinone (AQ) and (D) 5,12-Naphthacenequinone (NCQ).

Fig. (2). Inhibitory effects of the quinone derivatives on LPS-induced production of TNF-α in mouse macrophage cell line RAW264.7. (**A**) RAW264.7 cells were pretreated with 10 μM of the quinone derivatives as a vehicle control for 30 min and then treated with 100 ng/mL LPS for 24 h. TNF-α concentration in the cell medium was measured by ELISA. (**B**) TNF-α dose-response curves of NQ (0 to 100 μM) in RAW264.7 cells. Data are shown as the mean ± SE (n=4). *Significant difference according to Student's t-test ($p<0.05$).

Fig. (3). Inhibitory effects of the quinone derivatives on LPS-evoked production of TNF-α in peritoneal macrophages from mice. Peritoneal macrophages were pretreated with 10 μM of each compound as a vehicle control for 30 min and then with 100 ng/mL LPS for 24 h. TNF-α concentration in the cell medium was measured by ELISA. Data are shown as the mean ± SE (n=4). *Significant difference according to Student's t-test ($p<0.05$).

Fig. (4). Inhibitory effects of the quinone derivatives on nuclear translocation of NF-κB in RAW264.7 cells. The cells were incubated with 10 μM of each compound or DMSO (none), as a vehicle control for 30 min, and then with 100 ng/mL LPS for 30 min. The nuclear proteins were prepared from the cells and subjected to Western blot analysis for evaluation of the nuclear translocation of NF-κB p65. The intensity of each band was analyzed, and the values relative to treatment without LPS (negative control) are represented at the lower edge of the image.

Fig. (5). The inhibitory activity of the quinone derivatives against LPS-induced inflammation *in vivo*. Female C57BL/6 mice were intraperitoneally injected with each compound 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 sacrificed, and the TNF-α level in serum was measured using ELISA. Treatment with corn oil and LPS was a positive control (TNF-α level, 5,068 pg/mL), and that with corn oil and saline was a negative control (TNF-α level, 26 pg/mL). Data are shown as the mean ± SE (n=3). *Significant difference according to Student's t-test ($p<0.05$).

Table 1. IC_{50} Values of the Quinone Derivatives against the Activity of Mammalian DNA Polymerases

Mammalian pol	IC_{50} values (μM)			
	BQ	NQ	AQ	NCQ
Calf pol α	8.30 ± 0.42	6.65 ± 0.35	>200	>200
Rat pol β	189 ± 9.5	128 ± 6.4	>200	>200
Human pol γ	105 ± 5.4	73 ± 3.7	>200	>200
Human pol δ	94.8 ± 4.6	67.2 ± 3.5	>200	>200
Human pol ε	92.1 ± 4.6	61.4 ± 3.2	>200	>200
Human pol λ	86.4 ± 4.2	55.7 ± 2.8	>200	>200

Each compound was incubated with the six representative enzymes. Enzymatic activity was measured as described in *Materials and Methods*. Enzyme activity in the absence of compound was taken as 100%. Data are shown as the mean ± SE (n=3).

Table 2. Anti-Inflammatory Activity of the Quinone Derivatives in the Mouse Ear Inflammation Test

Amount of Compound	Inhibitory Effect (%)			
	BQ	NQ	AQ	NCQ
250 μg/ear	22.8 ± 3.6	28.6 ± 4.4	2.2 ± 0.6	1.5 ± 0.4
500 μg/ear	43.3 ± 6.5	55.0 ± 8.0	3.5 ± 0.9	2.5 ± 0.7

A sample of test compound (250 or 500 μg) was applied to one mouse ear for 30 min and then TPA (0.5 mg) was applied to both ears of the mouse. The level of edema was evaluated after 7 h. The inhibitory effect is expressed as the percentage ratio of edema. Data are shown as the mean ± SE (n=6).

Fig. (1)

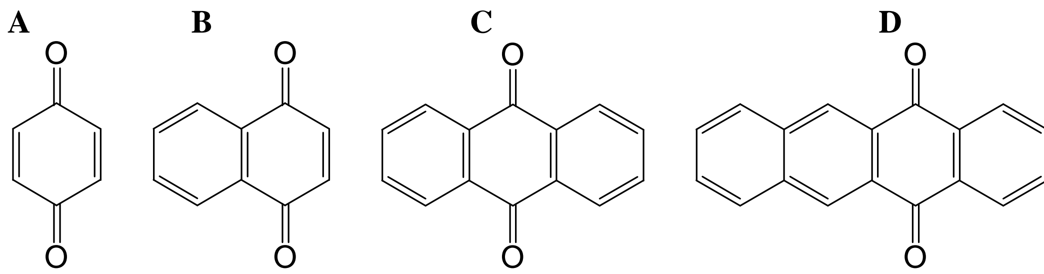
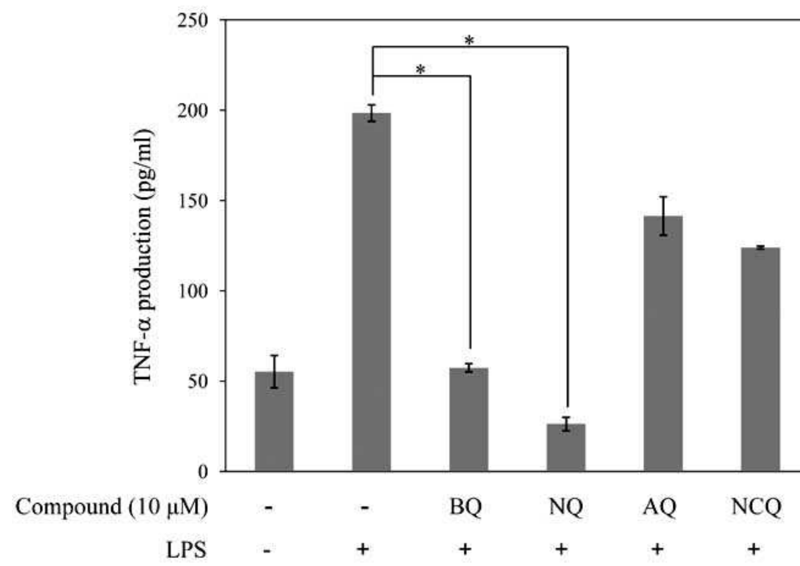


Fig. (2)

A



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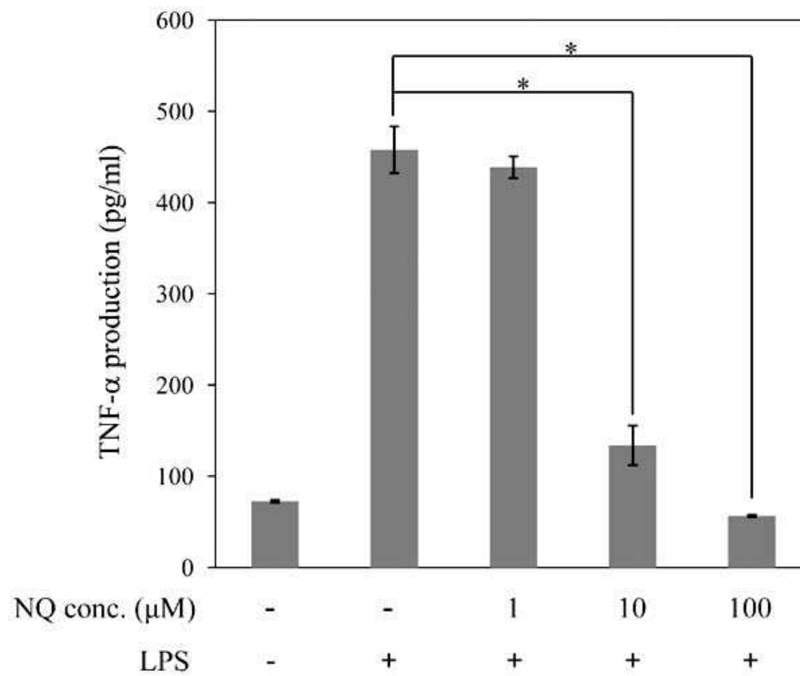

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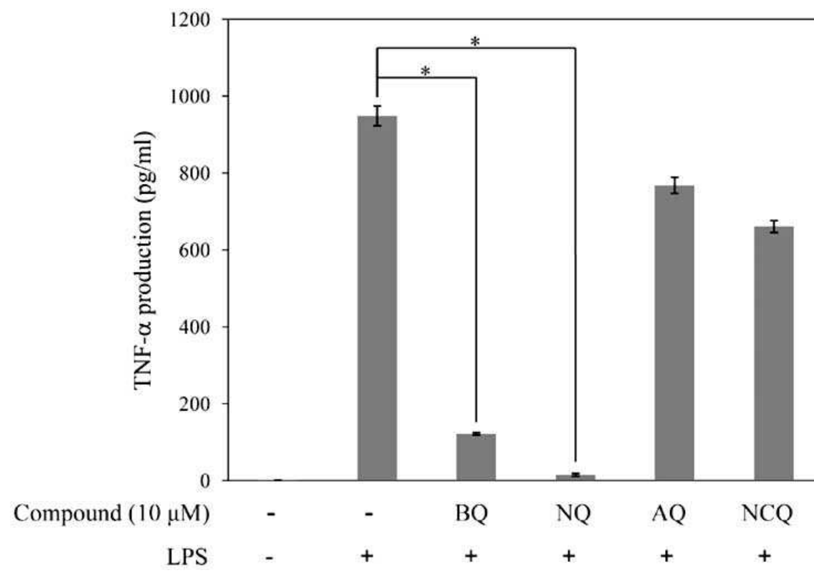

Fig. (4)

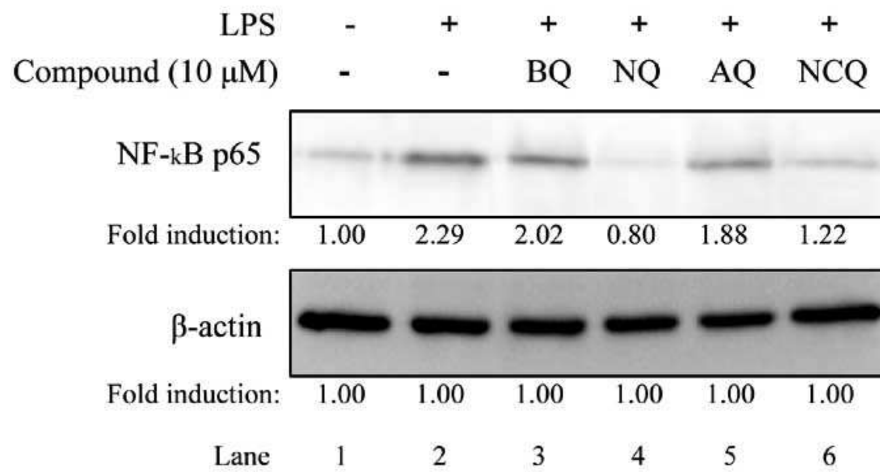

Fig. (5)