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In Vitro and In Vivo Anti-inflammatory Effects of a Novel 4,6-Bis ((E)-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1H)-thione

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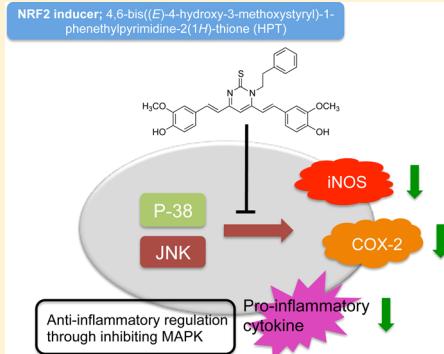
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ABSTRACT: Inflammation plays a critical defensive role in the human body. However, uncontrolled or aberrant inflammatory responses contribute to various acute and chronic diseases. The Nrf2-ARE pathway plays a pivotal role in the regulation of inflammatory markers, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). On the basis of this concept, we synthesized a novel anti-inflammatory 4,6-bis ((E)-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1H)-thione (HPT), and *in vitro* experiments using HepG2-C8 ARE-luciferase-transfected cells demonstrated the induction of Nrf2-ARE activity. In lipopolysaccharide (LPS)-induced RAW 264.7 cells, HPT treatment reduced the production of nitric oxide (NO) as well as the protein and mRNA expression levels of COX-2 and iNOS, in a dose-dependent manner. In addition, HPT suppressed the mRNA expression of inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6. In LPS-induced macrophages, HPT inhibited COX-2 and iNOS by blocking the activation of p38 and c-Jun NH₂-terminal kinase (JNK) but not extracellular signal-regulated kinase (ERK1/2). Furthermore, an *in vivo* anti-inflammatory study was performed using a TPA-induced skin inflammation mouse model, and the results showed that HPT reduced TPA-induced inflammation and attenuated the expression of COX-2 and iNOS in TPA-induced mouse skin tissue. Thus, HPT demonstrated anti-inflammatory activity both in LPS-induced RAW 264.7 cells and TPA-stimulated mouse skin and may therefore serve as a potential anti-inflammatory agent.



1. INTRODUCTION

Inflammation is a fundamental defensive reaction of the human body against bacterial and viral infections. However, uncontrolled or aberrant inflammation contributes to diverse acute and chronic human diseases.^{1,2} Prolonged inflammation produces excessive cellular reactive oxygen species (ROS), thereby aggravating the oxidation of intracellular proteins, lipids, and nucleic acids.^{3–5} This exacerbation results in either genetic changes and/or epigenetic alterations leading to the dysregulation of oncogenes and tumor-suppressor genes. Moreover, chronic inflammation and tumorigenesis are tightly coupled, and failure to halt these processes leads to carcinogenesis.^{4–7} This intriguing link between inflammation and cancer also represents a novel and promising approach for inhibiting the development and progression of malignancy.^{8,9}

The transcriptional regulation and expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in response to the bacterial endotoxin lipopolysaccharide (LPS)

are complex processes.^{10,11} Several upstream mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun NH₂-terminal kinase (JNK), are responsible for the transcriptional regulation of COX-2 and iNOS.¹² These MAPKs are activated by MAPK kinases (MAPKKs), and phosphorylated MAPKs activate several transcription factors including Elk1, ATF2, and c-Jun, which play pivotal roles in the expression of the iNOS and COX-2 genes.^{13,14} Depending on the cell type and agent, the expression of iNOS and COX-2 can be regulated by different MAPKs and upstream pathways. Six-shogaol, an anticancer compound isolated from ginger, was shown to suppress LPS-induced overexpression of iNOS and COX-2 by inhibiting ERK,¹⁵ whereas curcumin modulates COX-2 and iNOS by inhibiting p38.¹⁶ The overexpression of iNOS and COX-2 is

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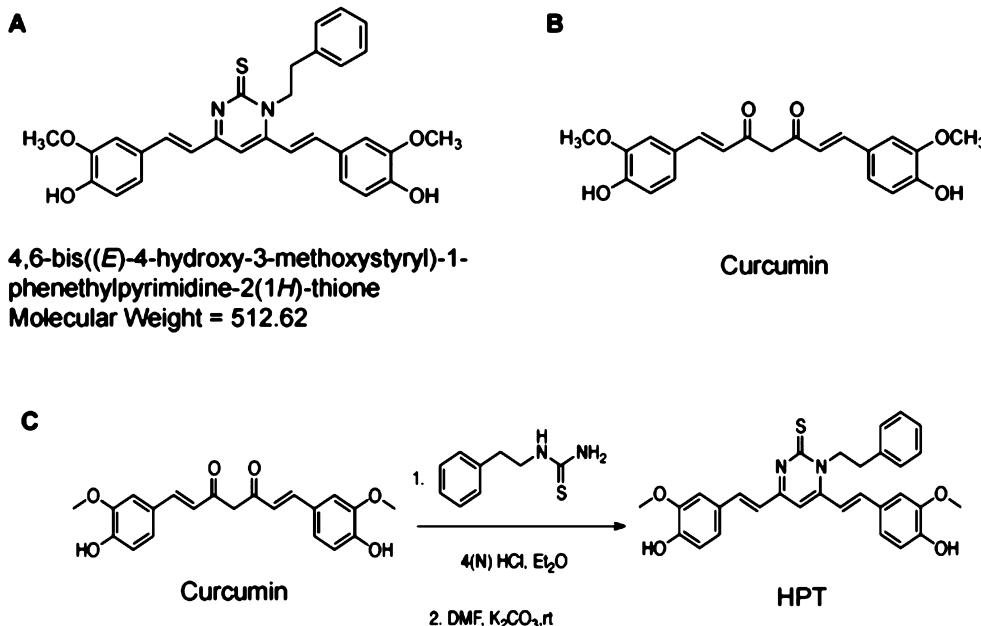


Figure 1. Structures of (A) 4,6-bis ((*E*)-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1*H*)-thione (HPT) and (B) curcumin (Cur). (C) Synthetic scheme of HPT.

frequently observed in transformed cells or tumor tissue. The topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA) to mouse skin also causes the overexpression of iNOS and COX-2 in the skin tissue.⁶ In particular, a single topical application of TPA to the ears of CD-1 mice was shown to induce time- and dose-dependent increases in edema and to raise levels of the pro-inflammatory cytokines such as interleukin (IL)-1 β and IL-6 in the skin.¹⁷

Oxidative stress is an important mediator of inflammation, and it activates the redox-sensitive pro-inflammatory signaling pathway and subsequent transcription through the direct toxic effects of ROS. Nuclear factor-erythroid-2-related factor 2 (Nrf2) is an important cytoprotective transcription factor that induces the expression of several antioxidant and phase II detoxifying enzymes.^{4,18} Nrf2 plays a central role in the regulation of inflammation because the regulation of ROS is closely related to anti-inflammatory processes.^{19,20} Our group previously showed that curcumin and isothiocyanate (ITC) induced the expression of Nrf2 and Nrf2-dependent antioxidant/detoxifying enzymes. The combinational treatment of two compounds had synergistic effects on the induction of a Nrf2-dependent cytoprotective enzyme.^{21–24} The 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) compounds are synthetic derivatives of oleanolic acid and are referred to as anti-inflammatory mediators (AIMs).^{25,26} On the basis of previous reports, our current study synthesized 4,6-bis ((*E*)-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1*H*)-thione (HPT) (Figure 1) and examined the Nrf2-ARE activation activity of HPT in HepG2-C8 ARE-luciferase-transfected cells. The anti-inflammatory potential of HPT was further evaluated in LPS-stimulated RAW 264.7 murine macrophages as well as a mouse model of TPA-induced skin inflammation.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). The anti-COX2, anti-iNOS, anti-p38, anti-ERK, anti-JNK, antiactin, and anti-Akt primary and

secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). The antiphospho-JNK, antiphospho-ERK, antiphospho-p38, and antiphospho-Akt primary antibodies were acquired from Cell Signaling Technology (Danvers, MA). Other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Synthesis of 4,6-Bis ((*E*-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1*H*)-thione (HPT). Dimethylformamide (DMF) was dried with activated 4 $^{\circ}$ A molecular sieves. NMR spectra were recorded using a Varian Mercury NMR spectrometer at 400 MHz for 1H- and 100 MHz for 13C-NMR spectra. All reported chemical shifts (ppm) are relative to the NMR solvent peak. Mass spectra were recorded on a Finnigan TSQ 700 mass spectrometer with an electron spray source. All catalytic reactions were performed under a dry argon atmosphere. Curcumin at 200 mg (0.5434 mmol) was added to 8 mL of 4 N HCl in diethyl ether. The mixture was stirred at room temperature for 30 min and then added to 117 mg (0.652 mmol) of 1-phenethylthiourea. The reaction mixture was stirred at 40 °C overnight, and the solvent was removed *in vacuo*. The crude mixture was purified using a deactivated silica gel with 75% ethyl acetate and hexane mixture (55% yield). The resulting compound was mixed with dry DMF in a flame-dried flask and purged with argon. Ten equivalents of K₂CO₃ were added to the reaction mixture and stirred in the dark at room temperature for 16 h. The DMF was evaporated *in vacuo* and acidified with 0.1 (N) HCl and methylene chloride, and it was washed with brine. The crude mixture was recrystallized from methanol, yielding 4,6-bis ((*E*-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1*H*)-thione (HPT) (33% yield; Figure 1C): 1H NMR (300 MHz, DMSO) δ d 8.12 (d, 1H), 7.78 (d, 1H) 7.61 (s, 1H) 7.15 (m) 6.83 (m, 4H) 4.91 (s, 2H) 3.20 (M).

2.3. Cell Culture and Treatment. Nrf2-mediated ARE human HepG2-C8 cells with pARE-TI-luciferase reporter gene were used, which were previously established in our laboratory.²⁷ The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. The cells were maintained in DMEM supplemented with 10% FBS (and 1% penicillin/streptomycin) at 37 °C and 5% CO₂. *E.coli* LPS (150 ng/mL) was added to induce the expression of iNOS and COX-2. The cells were treated with the test compound (0.5–8 μM) either alone or in combination with LPS for different time intervals, unless otherwise specified. Cells were treated with 0.05% DMSO as a vehicle control.

2.4. Animals. Female CD-1 (4–5 weeks old) mice were purchased from the Charles River Breeding Laboratories (Kingston, NY, USA) and used for the *in vivo* anti-inflammatory studies. The mice were maintained at a controlled room temperature (22 ± 2 °C) and humidity (60–80%) under a 12-h light/dark cycle for at least 1 week prior to use. The housing and care of the animals were performed in accordance with the guidelines established by the University's Animal Research Committee and were consistent with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.5. Cell Viability Assay. The cytotoxicity of HPT was tested in murine macrophage RAW 264.7 cells using the CellTiter 96 aqueous nonradioactive cell proliferation assay reagent (MTS) [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI). After 24 h of cell culture in 96-well plates, HPT was treated at various concentrations for another 24 h. The cells were then incubated with MTS for 1 h at 37 °C. The absorbance at 490 nm was measured using a μQuant Biomolecular Spectrophotometer (Bio-Tek Instruments, Winooski, VT).

2.6. ARE-Luciferase Activity Assay. ARE-luciferase-transfected HepG2-C8 cells were cultured in 12-well plates. The cells were treated with HPT or curcumin for 24 h, and luciferase activity was measured using a Promega luciferase kit. According to a slightly modified manufacturer's protocol, after 24 h of compound treatment, the cells were washed twice with ice-cold PBS and harvested using 1× reporter lysis buffer. The cell lysate was centrifuged at 9,660g for 5 min at 4 °C, and the supernatant was used for the luciferase activity assay. Luciferase activity was measured using a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). After normalization with protein concentration, luciferase activity was described as the fold induction of the samples as compared to vehicle control-treated cells.

2.7. Nitrite Assay. Nitrite accumulation in the culture media was used as an indicator of nitric oxide (NO) production.²⁸ Cells were cultured in 96-well culture plates for 24 h and stimulated with LPS (150 ng/mL) in the presence or absence of HPT for another 24 h. Curcumin treatment was also used as a positive control. After the isolation of supernatant fractions, equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) were added and incubated at room temperature for 10 min. Nitrite production was measured using a μQuant Biomolecular Spectrophotometer (Bio-Tek Instruments, Winooski, VT) at an absorbance 540 nm.

2.8. TPA-Induced Inflammation in Mouse Ear Skin. The induction of inflammation in mouse ear skin was performed according to the method of Huang et al.²⁹ After 1 week of acclimatization, mice were divided into the following three groups (8 mice/group): the control group, 5 μL of acetone (vehicle control); TPA group, 5 nmol TPA in 5 μL of acetone (3.08 μg/ear); and HPT group, 1.0 μmol HPT in 10 μL of acetone (513.0 μg/ear) + 5 nmol TPA in 5 μL of acetone. All experiments were conducted topically on both mouse ears. All compound treatments were performed 20 min prior to TPA treatment. Six hours after TPA treatment, all mice were sacrificed.

2.9. Protein Lysate Preparation and Western Blotting. All cells were harvested in radioimmunoprecipitation assay (RIPA) buffer containing a protein inhibitor cocktail (Sigma, St. Louis, MO). To determine the protein concentration of cell lysates, the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) was used. An equal amount of (20 μg) total protein from each sample was resolved by 4–15% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with 5% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA), the PVDF membrane was sequentially probed with specific primary antibodies and HRP-conjugated secondary antibodies. The blots were then visualized using the SuperSignal enhanced chemiluminescence detection system and recorded using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA).

2.10. Quantitative Real-Time Polymerase Chain Reaction (qPCR). Total RNA was extracted from treated RAW 264.7

macrophages using the RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was used as the template for PCR reactions performed using the ABI7900HT system (Life technologies, Grand Island, NY). The sequences of the primers used for inflammatory-related genes are listed in Table 1.

Table 1. Murine Primers for PCR

gene	forward	reverse
GAPDH	5'-TGC TCG AGA TGT CAT GAA GG-3'	5'-TTG CGC TCA TCG TAG GCT TT-3'
IL-1β	5'-GAG TGT GGA TCC CAA GCA AT-3'	5'-CTC AGT GCA GGC TAT GAC CA-3'
IL-6	5'-AGT TGC CTT CTT GGG ACT GA-3'	5'-GCC ACT CCT TCT GTG ACT CC-3'
TNF-α	5'-ACG GCA TGG ATC TCA AAG AC-3'	5'-GGT CAC TGT CCC AGC TT-3'
iNOS	5'-GTG GTG ACA AGC ACA TTT GG-3'	5'-GGC TGG ACT TTT CAC TCT GC-3'
COX-2	5'-TCC TCC TGG AAC ATG GAC TC-3'	5'-TGA TGG TGG CTG TTT TGG TA-3'

2.11. Immunohistochemical Studies. Ear samples from each group were harvested at autopsy and fixed in 10% formalin for 24 h at room temperature. The samples were then dehydrated with a graded ethanol series, sectioned, and embedded in paraffin wax. The embedded mouse ears were serially sectioned at 4 μm. Immunohistochemistry (IHC) was performed for COX-2 and iNOS expression. Immunohistochemical analysis of the ear tissue was conducted on multiple sections per block. After antigen retrieval, anti-COX2 and anti-iNOS antibodies were used for immunostaining. The results of the IHC analysis were acquired with an Aperio Scanscope scanner (Aperio Technologies, Inc.). Five areas from the control, TPA, and TPA with HPT sections were randomly selected for analysis, and the number of positively stained cells (or the immunostaining intensity) and the total number of cells were determined by using an Image-Pro Plus Image Processing System (version 5.0; Media Cybernetics) or Aperio ScanScopeR GL system (Aperio Technologies, Vista, CA).

2.12. Statistical Analysis. Data are presented as the means ± SE for the indicated number of independently performed experiments. Statistical analyses were performed using one-way ANOVA, followed by Tukey's test. At least three independent experiments for each assay were conducted. For the determination of statistically significant correlation, tests of zero correlation were used. In both analyses, $p < 0.05$ was accepted as denoting statistical significance.

3. RESULTS

3.1. HPT Induces Transcriptional ARE-Luciferase Activation. To evaluate HPT-induced transcriptional activation of ARE, HepG2-C8-ARE luciferase cells were incubated with different concentrations (2, 4, and 8 μM) of HPT and curcumin for 24 h, and ARE induction was evaluated using a luciferase assay. The results demonstrated that HPT increased ARE induction; at a concentration of 4 μM and 8 μM, HPT stimulated ARE induction by 56% and 65%, respectively, which was statistically significant as compared to curcumin ($P < 0.01$; Figure 2).

3.2. HPT Inhibits NO Production in LPS-Induced RAW 264.7 Cells. We next evaluated the effect of HPT and curcumin on the growth of cultured murine RAW 264.7 macrophages. As illustrated in Figure 3A, HPT was a more potent inhibitor of proliferation as compared to curcumin under the same treatment conditions. To evaluate the inhibitory effect of HPT on NO production in LPS-induced RAW 264.7 cells, we measured the level of nitrite, a product of

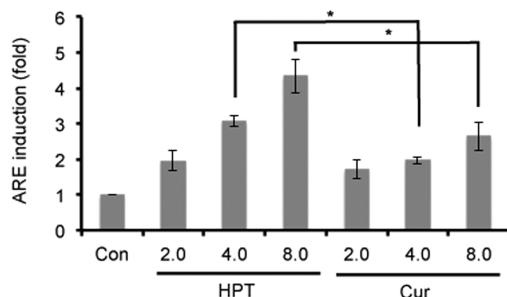


Figure 2. ARE induction activity of HPT in HepG2-C8-ARE luciferase cells. Human hepatoma HepG2-C8-ARE luciferase cells were seeded onto a 96-well plate, and the cells were treated with different concentrations (2, 4, and 8 μ M) of HPT or Cur for 24 h. The results are expressed as the mean \pm SE; *, $P < 0.01$ as compared to the curcumin-treated group.

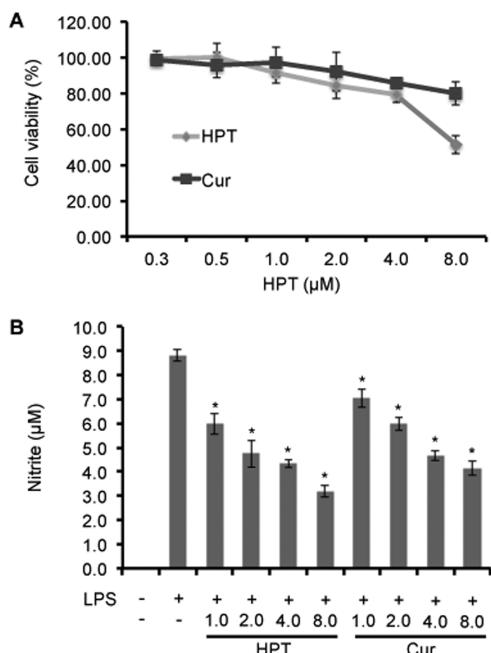


Figure 3. Inhibitory activity of HPT on cell growth and LPS-induced NO production in Raw 264.7 cells. (A) The cell growth inhibitory activity of HPT and Cur. For these experiments, RAW 264.7 cells were seeded onto a 96-well plate, and the cells were incubated with HPT or Cur at different concentrations, with DMSO as the vehicle control, for 24 h. The MTS reagent was added to each well, and the absorbance of the formazan product was read at 490 nm. (B) The inhibitory activity of HPT on LPS-induced NO production. RAW 264.7 cells were seeded onto a 96-well plate, and different concentrations of HPT or Cur were coadministered with LPS (150 ng/mL) and incubated for 24 h. Isolated supernatant fractions were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. Nitrite production was measured as the absorbance at 540 nm. Each point represents the mean \pm SE; *, $P < 0.01$ as compared to the LPS-treated group.

the Griess reaction. HPT concentrations of 1 μ M and 2 μ M significantly reduced LPS-stimulated NO production by 32% and 46%, respectively, whereas the same concentrations of curcumin inhibited this production of NO by 20% and 32%, respectively. Thus, HPT showed 12% and 14% stronger inhibitory activities than curcumin at the concentrations of 1 μ M and 2 μ M, respectively ($P < 0.01$; Figure 3B).

3.3. HPT Reduces iNOS and COX-2 Induction. To determine the kinetics of iNOS and COX-2 induction, LPS-induced RAW 264.7 cells were treated with 1 μ M of HPT and curcumin for periods of 6, 12, and 24 h. LPS (150 ng/mL) stimulated the expression of iNOS and COX-2 in a time-dependent manner (Figure 4A), and HPT markedly blocked

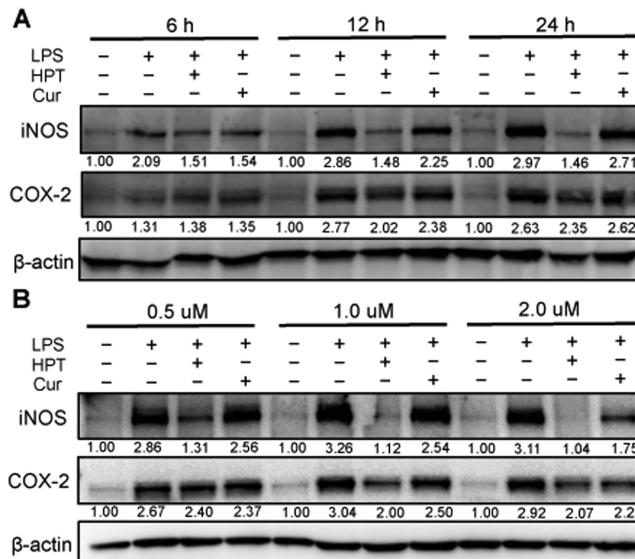


Figure 4. Effect of HPT and Cur on the expression of iNOS and COX-2 protein in LPS-induced RAW 264.7 cells. (A) iNOS and COX-2 induction by LPS was attenuated by HPT and Cur treatment in a time-dependent manner. RAW 264.7 cells were treated with LPS (150 ng/mL) alone or were cotreated with LPS and HPT or Cur (each at 1.0 μ M). The cells were harvested for protein measurements after 6, 12, or 24 h. (B) HPT and Cur suppressed the LPS-induced expression of iNOS and COX-2 in a dose-dependent manner. Different concentrations of HPT or Cur were also coadministered with LPS (150 ng/mL) for 24 h. Bands were densitometrically analyzed by using ImageJ software (<http://rsbweb.nih.gov/ij>). Each band was normalized with the density of actin. Relative intensities of protein expression were calculated in comparison with the control, which were set to 100%.

the induction of iNOS at each time point and reduced the expression of COX-2 at 24 h as compared to that of treatment with LPS only. We also treated cells with different concentrations of HPT and curcumin and found that HPT significantly inhibited the induction of iNOS and COX-2 in a dose-dependent manner (Figure 4B).

3.4. HPT Down-regulates the mRNA Levels of iNOS and COX-2 as Well as IL-1 β , IL-6, and TNF- α in LPS-Induced RAW 264.7 Cells. To evaluate the effect of HPT on the LPS-induced expression of inflammatory enzymes and proinflammatory cytokines, the mRNA levels of iNOS, COX-2, IL-1 β , IL-6, and TNF- α were analyzed by quantitative RT-PCR following treatment of RAW 264.7 cells with either LPS alone or HPT. The results showed that LPS up-regulated the mRNA expression of inflammatory genes and pro-inflammatory cytokines, such as iNOS, COX-2, IL-1 β , IL-6, and TNF- α , whereas these mRNA levels were inhibited by HPT in a dose-dependent manner (Figure 5). In particular, the levels of IL-1 β were reduced by approximately 50% after 0.5 μ M HPT treatment.

3.5. HPT Inhibits the Activation of p38 and JNK Signaling in LPS-Induced RAW 264.7 Cells. To elucidate

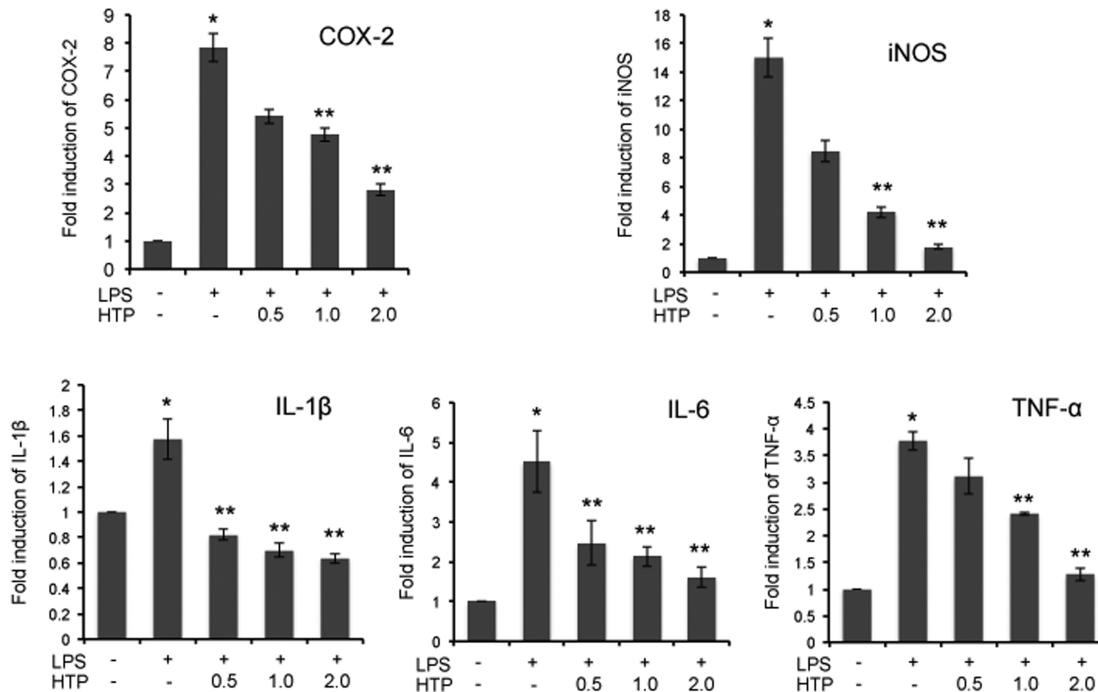


Figure 5. Inhibitory activity of HPT on LPS-induced mRNA expression for iNOS, COX-2, IL-1 β , IL-6, and TNF- α . RAW 264.7 cells were treated with LPS (150 ng/mL) either alone or were cotreated with LPS and HPT or Cur (each at 1.0 μ M). The cells were harvested for total RNA extraction at 24 h, and the mRNA levels for iNOS, COX-2, IL-1 β , IL-6, and TNF- α were measured using quantitative RT-PCR. Each point represents the mean \pm SE; *, P < 0.05 as compared to the vehicle control. **, P < 0.05 as compared to the LPS-treated group.

the mechanism responsible for the anti-inflammatory action of HPT, we examined MAPKs upstream of iNOS and COX-2. The LPS-induced activation of MAPKs, such as ERK1/2, JNK, and p38, was measured according to the levels of phosphorylated ERK1/2, JNK, and p38. The treatment with LPS significantly increased the levels of these phosphorylated proteins in RAW 264.7 cells, while the LPS treatment did not change the levels of total protein. In contrast to the LPS-treated group, cells cotreated with LPS and HPT showed significantly reduced expression of phosphorylated JNK and p38, although the expression of phosphorylated ERK1/2 was not reduced. This result indicated that HPT strongly inhibited the activation of p38 and JNK but did not block ERK1/2 activation in LPS-induced macrophages (Figure 6).

3.6. HPT Inhibits the Expression of COX-2 and iNOS in TPA-Induced Mouse Ear Tissue. TPA rapidly stimulates COX-2 induction in mouse skin,^{29,30} and our immunohistochemical analysis showed that topical application of TPA to the mouse ear significantly (6-fold) up-regulated COX-2 expression as compared to vehicle-treated control mice at 6 h after treatment (Figure 7A). However, the treatment with HPT (1.0 μ mol/ear) significantly inhibited TPA-induced COX-2 up-regulation (68%; Figure 6B). As shown in Figure 6A and C, HPT treatment also suppressed TPA-induced iNOS expression. Moreover, quantitative analysis showed that iNOS-positive cells were significantly reduced in the HPT-treated group (49%; Figure 7C).

4. DISCUSSION

Oxidative stress is an important mediator of inflammation and activates a redox-sensitive pro-inflammatory signaling pathway. Nrf2, a cytoprotective transcription factor that induces the expression of several antioxidant/detoxifying enzymes, plays a pivotal role in the regulation of inflammation.^{19,20} Many

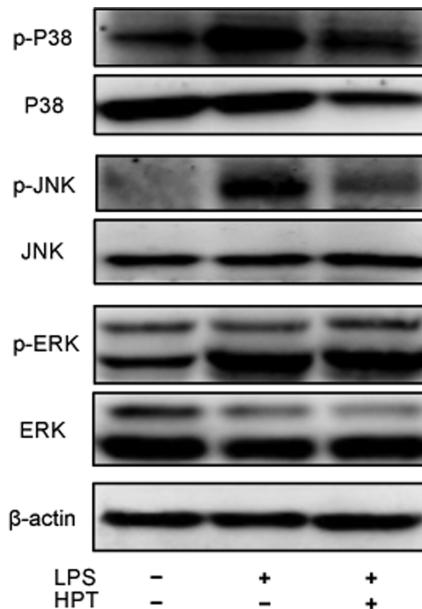


Figure 6. Evaluation of different cellular signaling pathways affected by HPT in LPS-induced RAW 264.7 cells. HPT effectively blocked the activation of p38 MAPK and JNK signaling. RAW 264.7 cells were treated with LPS (150 ng/mL) alone or were cotreated with LPS and HPT (1.0 μ M). The cells were harvested for protein measurements after 24 h.

chemopreventive compounds possess antioxidant and anti-inflammatory activities involving the Nrf2-ARE pathways.³¹ Moreover, our previous studies found that curcumin and ITC acted as strong Nrf2 inducers.^{21–24} On the basis of this finding, we synthesized HPT as a structural combination of curcumin and ITC (Figure 1). In the current study, to investigate whether

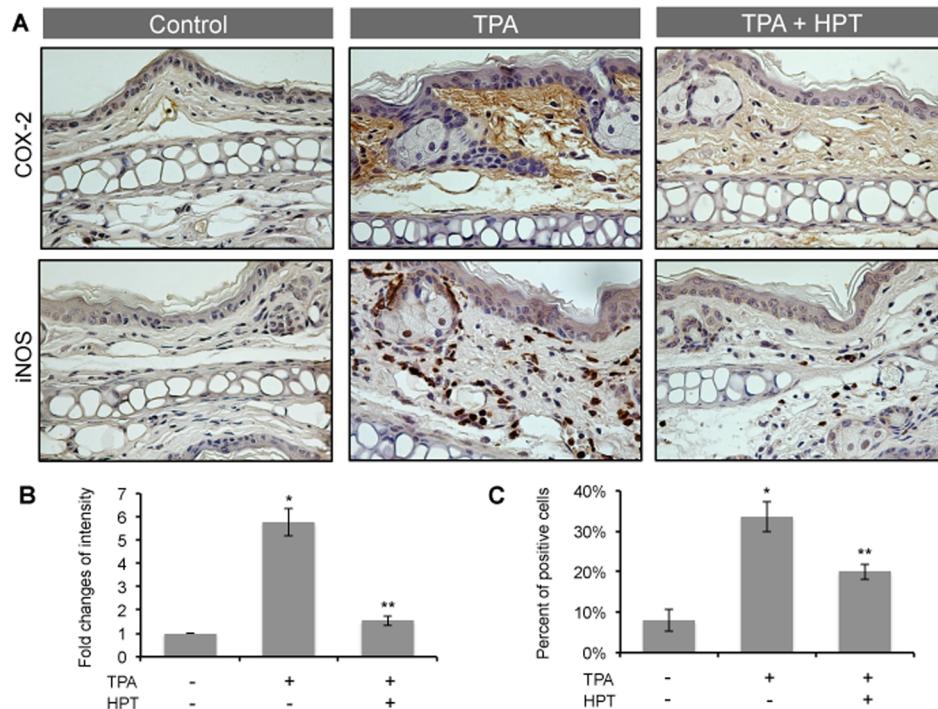


Figure 7. Topical application of HPT reduces TPA-induced up-regulation of COX-2 and iNOS. (A) Representative images of IHC performed on mice ears. All compound treatments were performed 20 min prior to TPA treatment. Six hours after TPA treatment, all mice were sacrificed. Original magnification, 400 \times . (B) Quantification of COX-2 expression, with the fold-change in the staining intensity over the control, shown as the mean \pm SE ($n = 8$ /group). (C) Quantification of iNOS expression, with the percentage of positively stained cells among the total cells, shown as the mean \pm SE ($n = 8$ /group); *, $P < 0.05$ as compared to vehicle-treated animals. **, $P < 0.05$ as compared to vehicle/TPA-treated animals.

Nrf2-ARE induction would generate anti-inflammatory effects, we examined Nrf2-ARE induction following HPT treatment in ARE-luciferase-transfected HepG2-C8 cells, the anti-inflammatory effects of HPT in LPS-induced RAW 264.7 cells, and an *in vivo* TPA-induced mouse model of skin inflammation.

In accordance with previously published results,^{21–24} our data show that HPT, which represents the structural combination of curcumin and ITC, significantly induced ARE-luciferase activity in ARE-luciferase-transfected HepG2 C8 cells (Figure 2). Because Nrf2 plays a critical role in the regulation of COX-2 and iNOS expression,²⁰ we hypothesized that HPT would demonstrate anti-inflammatory activity.

The gaseous signaling molecule NO is commonly involved in cell interactions at a low concentration. However, excessive NO generated by iNOS modulates inflammation and plays a key role in the regulation of immune responses. Thus, regulating the production of NO constitutes an important event during inflammatory processes.^{32,33} HPT treatment of LPS-induced RAW 264.7 cells reduced the production of nitrite in a dose-dependent manner, which indicated a potential anti-inflammatory effect of HPT. Moreover, treatment with HPT decreased the expression of COX-2 and iNOS at both the mRNA and protein levels in a dose-dependent manner, which demonstrated a significant inhibitory effect of HPT in LPS-induced RAW 264.7 cells. Pro-inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, play a pivotal role in mediating the inflammatory response to pathologic stimuli.^{34,35} TNF- α was shown to up-regulate COX-2 in β -amyloid-injected mice,³⁶ and IL-1 β induces both COX-2 and iNOS through p38 MAPK activation³⁷ and stimulates NO formation in the absence of LPS.³⁸ Moreover, the overproduction of IL-1 β was shown to promote cell/tissue damage during an inflammatory response.³⁹

The up-regulation of iNOS and COX-2 is mediated by multiple pathways, which vary according to the cell type and the predominant cytokines.⁴⁰ The results of the current study found that HPT was more effective than curcumin at inhibiting iNOS and COX-2 in LPS-induced RAW 264.7 cells (Figures 3B and 4). In addition, HPT demonstrated a stronger ability to inhibit the expression of inflammatory genes, such as *iNOS* and *COX-2*, and pro-inflammatory cytokine genes, such as *IL-1 β* , *IL-6*, and *TNF- α* (Figure 5).

MAPKs play an important role in controlling a wide range of physiological processes.⁴¹ ERK functions in the control of cell division, proliferation, differentiation, and survival, which indicates that inhibitors of this enzyme may be useful as anticancer agents.^{41,42} JNK is activated in response to various environmental stresses, such as UV radiation and inflammatory cytokines, and is a critical regulator of transcription.^{41,43} p38 is also activated by inflammatory cytokines, growth factors, and UV light and contributes to diseases such as asthma and autoimmunity.^{41,43} In particular, the activation of ERK and p38 MAP kinase occurs in response to inflammatory stimuli such as LPS, IL-1 β , and TNF- α , and the induction of key inflammatory enzymes, such as COX-2 and iNOS, is attributed to ERK and p38 activation.^{16,44} Thus, the inhibition of p38 and JNK MAP kinases plays a pivotal role in regulating the transcription of COX-2, iNOS, and pro-inflammatory cytokines during inflammatory processes. According to these previous observations, the inhibition activity of HPT on the phosphorylation of p38 and JNK MAP kinases in LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Figure 6) indicates that p38 and JNK MAP kinases are involved in the anti-inflammatory actions of HPT. Interestingly, HPT did not change the expression level of *IκB α* in LPS-induced RAW 264.7 cells (data not shown),

which revealed that the anti-inflammatory effect of HPT did not seem to be regulated by the NF- κ B pathway. There has been increasing evidence recently showing that Nrf2 regulates the anti-inflammatory reactions through MAPK pathway.^{45,46} The Nrf2-regulated anti-inflammatory effect of HPT correlated with previous findings. Thus, the Nrf2-regulated MAPK pathway should be a potential anti-inflammatory mechanism of HPT.

To investigate the anti-inflammatory effects of HPT *in vivo*, we assessed the inhibitory potential of HPT against TPA-induced COX-2 and iNOS expression in mouse skin. Topical application of TPA to the skin induces an inflammatory response by attracting inflammatory cells, increasing the levels of pro-inflammatory mediators, and inducing hyperplasia.⁴⁷ TPA-induced skin inflammation is also involved in the enhanced expression of iNOS and COX-2.^{6,48,49} We found that topical application of HPT to the mouse ear strongly inhibited TPA-induced overexpression of COX-2 and iNOS and that these *in vivo* data on the inhibitory effect of HPT on COX-2 and iNOS were in agreement with the *in vitro* data. Thus, these observations along with the results from the LPS-induced macrophage experiments confirm the anti-inflammatory effects of HPT.

In conclusion, the results of the present study demonstrate that the novel, synthetic compound HPT acts as an anti-inflammatory agent. HPT treatment inhibited the expression of the inflammatory gene and the production of pro-inflammatory cytokine in LPS-stimulated RAW 264.7 cells, and this inhibition activity of HPT could be attributed to the inhibition of p38 and JNK MAPK phosphorylation. Additionally, HPT reduced TPA-induced mouse skin inflammation. HPT treatment attenuated the overexpression of COX-2 and iNOS in TPA-induced mouse ears. On the basis of these anti-inflammatory effects of HPT, we are currently performing bioavailability studies as well as examining the potential for HPT to serve as a potent anticancer reagent.

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ABBREVIATIONS

AIM, anti-inflammation mediator; ARE, antioxidant response element; BCA, bicinchoninic acid; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; HPT, 4,6-bis ((E)-4-hydroxy-3-methoxystyryl)-1-phenethylpyrimidine-2(1H)-thione; IHC, immunohistochemistry; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; ITC, isothiocyanate; JNK, c-Jun NH₂-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MPAKK, MAPK kinase; Nrf2, nuclear factor-erythroid-2-related factor 2; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; ROS, oxidative stress; TPA, 12-O-tetradecanoylphorbol-13-acetate

REFERENCES

- (1) Aggarwal, B. B., Shishodia, S., Sandur, S. K., Pandey, M. K., and Sethi, G. (2006) Inflammation and cancer: how hot is the link? *Biochem. Pharmacol.* 72, 1605–1621.
- (2) Lu, H., Ouyang, W., and Huang, C. (2006) Inflammation, a key event in cancer development. *Mol. Cancer. Res.* 4, 221–233.
- (3) Kong, A. N., Mandlikar, S., Yu, R., Lei, W., and Fasanmade, A. (1999) Pharmacodynamics and toxicodynamics of drug action: signaling in cell survival and cell death. *Pharm. Res.* 16, 790–798.
- (4) Lee, J. H., Khor, T. O., Shu, L., Su, Z. Y., Fuentes, F., and Kong, A. N. (2013) Dietary phytochemicals and cancer prevention: Nrf2 signaling, epigenetics, and cell death mechanisms in blocking cancer initiation and progression. *Pharmacol. Ther.* 137, 153–171.
- (5) Schetter, A. J., Heegaard, N. H., and Harris, C. C. (2010) Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* 31, 37–49.
- (6) Chun, K. S., Cha, H. H., Shin, J. W., Na, H. K., Park, K. K., Chung, W. Y., and Surh, Y. J. (2004) Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF- κ B. *Carcinogenesis* 25, 445–454.
- (7) Hussain, S. P., and Harris, C. C. (2007) Inflammation and cancer: an ancient link with novel potentials. *Int. J. Cancer* 121, 2373–2380.
- (8) Bartsch, H., and Nair, J. (2006) Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbecks Arch. Surg.* 391, 499–510.
- (9) Hussain, S. P., Hofseth, L. J., and Harris, C. C. (2003) Radical causes of cancer. *Nat. Rev. Cancer* 3, 276–285.
- (10) Chun, K. S., and Surh, Y. J. (2004) Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem. Pharmacol.* 68, 1089–1100.
- (11) Kleinert, H., Pautz, A., Linker, K., and Schwarz, P. M. (2004) Regulation of the expression of inducible nitric oxide synthase. *Eur. J. Pharmacol.* 500, 255–266.
- (12) Johnson, G. L., and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298, 1911–1912.
- (13) Kumar, S., Boehm, J., and Lee, J. C. (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discovery* 2, 717–726.
- (14) Bebien, M., Salinas, S., Becamel, C., Richard, V., Linares, L., and Hipskind, R. A. (2003) Immediate-early gene induction by the stresses anisomycin and arsenite in human osteosarcoma cells involves MAPK cascade signaling to Elk-1, CREB and SRF. *Oncogene* 22, 1836–1847.
- (15) Pan, M.-H., Hsieh, M.-C., Hsu, P.-C., Ho, S.-Y., Lai, C.-S., Wu, H., Sang, S., and Ho, C.-T. (2008) 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *Mol. Nutr. Food Res.* 52, 1467–1477.
- (16) Camacho-Barquero, L., Villegas, I., Sanchez-Calvo, J. M., Talero, E., Sanchez-Fidalgo, S., Motilva, V., and Alarcon de la Lastra, C. (2007) Curcumin, a *Curcuma longa* constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis. *Int. Immunopharmacol.* 7, 333–342.
- (17) Huang, M. T., Liu, Y., Ramji, D., Lo, C. Y., Ghai, G., Dushenkov, S., and Ho, C. T. (2006) Inhibitory effects of black tea theaflavin derivatives on 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and arachidonic acid metabolism in mouse ears. *Mol. Nutr. Food Res.* 50, 115–122.
- (18) Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010) Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biol. Med.* 49, 1603–1616.
- (19) Keum, Y. S., Jeong, W. S., and Kong, A. N. (2004) Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. *Mutat. Res.* 555, 191–202.
- (20) Cheung, K. L., Lee, J. H., Khor, T. O., Wu, T.-Y., Li, G. X., Chan, J., Yang, C. S., and Kong, A.-N. T. (2012) Nrf2 knockout enhances intestinal tumorigenesis in *Apcmin/+* mice due to attenuation of anti-oxidative stress pathway while potentiates inflammation. *Mol. Carcinog.* DOI: 10.1002/mc.21950.

- (21) Xu, C., Yuan, X., Pan, Z., Shen, G., Kim, J.-H., Yu, S., Khor, T. O., Li, W., Ma, J., and Kong, A.-N. T. (2006) Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. *Mol. Cancer Ther.* 5, 1918–1926.
- (22) Xu, C., Huang, M. T., Shen, G., Yuan, X., Lin, W., Khor, T. O., Conney, A. H., and Kong, A. N. (2006) Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. *Cancer Res.* 66, 8293–8296.
- (23) Shen, G., Xu, C., Hu, R., Jain, M. R., Gopalkrishnan, A., Nair, S., Huang, M. T., Chan, J. Y., and Kong, A. N. (2006) Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin. *Mol. Cancer Ther.* 5, 39–51.
- (24) Cheung, K., Khor, T., and Kong, A.-N. (2009) Synergistic effect of combination of phenethyl isothiocyanate and sulforaphane or curcumin and sulforaphane in the inhibition of inflammation. *Pharm. Res.* 26, 224–231.
- (25) Suh, N., Wang, Y., Honda, T., Gribble, G. W., Dmitrovsky, E., Hickey, W. F., Mau, R. A., Place, A. E., Porter, D. M., Spinella, M. J., Williams, C. R., Wu, G., Dannenberg, A. J., Flanders, K. C., Letterio, J. J., Mangelsdorf, D. J., Nathan, C. F., Nguyen, L., Porter, W. W., Ren, R. F., Roberts, A. B., Roche, N. S., Subbaramaiah, K., and Sporn, M. B. (1999) A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res.* 59, 336–341.
- (26) Konopleva, M., Tsao, T., Ruvolo, P., Stiouf, I., Estrov, Z., Leysath, C. E., Zhao, S., Harris, D., Chang, S., Jackson, C. E., Munsell, M., Suh, N., Gribble, G., Honda, T., May, W. S., Sporn, M. B., and Andreeff, M. (2002) Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood* 99, 326–335.
- (27) Yu, R., Mandlikar, S., Lei, W., Fahl, W. E., Tan, T. H., and Kong, A. N. (2000) p38 mitogen-activated protein kinase negatively regulates the induction of phase II drug-metabolizing enzymes that detoxify carcinogens. *J. Biol. Chem.* 275, 2322–2327.
- (28) Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite, and [¹⁵N]nitrate in biological fluids. *Anal. Biochem.* 126, 131–138.
- (29) Huang, M.-T., Ho, C.-T., Wang, Z. Y., Ferraro, T., Lou, Y.-R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J. D., and Conney, A. H. (1994) Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res.* 54, 701–708.
- (30) Surh, Y.-J., Lee, J.-Y., Choi, K.-J., and Ko, S.-R. (2002) Effects of selected ginsenosides on phorbol ester-induced expression of cyclooxygenase-2 and activation of NF- κ B and ERK1/2 in mouse skin. *Ann. N.Y. Acad. Sci.* 973, 396–401.
- (31) Wu, T. Y., Khor, T. O., Saw, C. L., Loh, S. C., Chen, A. I., Lim, S. S., Park, J. H., Cai, L., and Kong, A. N. (2011) Anti-inflammatory/anti-oxidative stress activities and differential regulation of Nrf2-mediated genes by non-polar fractions of tea chrysanthemum zawadskii and licorice glycyrrhiza uralensis. *AAPS J.* 13, 1–13.
- (32) Nathan, C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* 6, 3051–3064.
- (33) Guzik, T. J., Korbut, R., and Adamek-Guzik, T. (2003) Nitric oxide and superoxide in inflammation and immune regulation. *J. Physiol. Pharmacol.* 54, 469–487.
- (34) Jin, C. Y., Lee, J. D., Park, C., Choi, Y. H., and Kim, G. Y. (2007) Curcumin attenuates the release of pro-inflammatory cytokines in lipopolysaccharide-stimulated BV2 microglia. *Acta Pharmacol. Sin.* 28, 1645–1651.
- (35) Chi, H., Barry, S. P., Roth, R. J., Wu, J. J., Jones, E. A., Bennett, A. M., and Flavell, R. A. (2006) Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2274–2279.
- (36) Medeiros, R., Figueiredo, C. P., Pandolfo, P., Duarte, F. S., Prediger, R. D. S., Passos, G. F., and Calixto, J. B. (2010) The role of TNF- α signaling pathway on COX-2 upregulation and cognitive decline induced by β -amyloid peptide. *Behav. Brain Res.* 209, 165–173.
- (37) Guan, Z., Baier, L. D., and Morrison, A. R. (1997) p38 mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E2 biosynthesis stimulated by interleukin-1 β . *J. Biol. Chem.* 272, 8083–8089.
- (38) Kitade, H., Sakitani, K., Inoue, K., Masu, Y., Kawada, N., Hiramatsu, Y., Kamiyama, Y., Okumura, T., and Ito, S. (1996) Interleukin 1 beta markedly stimulates nitric oxide formation in the absence of other cytokines or lipopolysaccharide in primary cultured rat hepatocytes but not in Kupffer cells. *Hepatology* 23, 797–802.
- (39) Dinarello, C. A. (1999) Cytokines as endogenous pyrogens. *J. Infect. Dis.* 179, S294–S304.
- (40) Surh, Y.-J., Chun, K.-S., Cha, H.-H., Han, S. S., Keum, Y.-S., Park, K.-K., and Lee, S. S. (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF- κ B activation. *Mutat. Res.-Fund. Mol. M.* 480–481, 243–268.
- (41) Johnson, G. L., and Lapidat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298, 1911–1912.
- (42) Roux, P. P., and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68, 320–344.
- (43) Wagner, E. F., and Nebreda, A. R. (2009) Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat. Rev. Cancer* 9, 537–549.
- (44) Dean, J. L., Brook, M., Clark, A. R., and Saklatvala, J. (1999) p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J. Biol. Chem.* 274, 264–269.
- (45) Al-Huseini, L. M. A., Aw Yeang, H. X., Sethu, S., Alhumeed, N., Hamdam, J. M., Tingle, Y., Djouhri, L., Kitteringham, N., Park, B. K., Goldring, C. E., and Sathish, J. G. (2013) Nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (Nrf2) modulates dendritic cell immune function through regulation of p38 MAPK-cAMP-responsive element binding protein/activating transcription factor 1 signaling. *J. Biol. Chem.* 288, 22281–22288.
- (46) Bak, M.-J., Jun, M., and Jeong, W.-S. (2012) Procyanidins from wild grape (*Vitis amurensis*) seeds regulate ARE-mediated enzyme expression via Nrf2 coupled with p38 and PI3K/Akt pathway in HepG2 cells. *Int. J. Mol. Sci.* 13, 801–818.
- (47) Lewis, J. G., and Adams, D. O. (1987) Early inflammatory changes in the skin of SENCAR and C57BL/6 mice following exposure to 12-O-tetradecanoylphorbol-13-acetate. *Carcinogenesis* 8, 889–898.
- (48) Chun, K.-S., Kim, S.-H., Song, Y.-S., and Surh, Y.-J. (2004) Celecoxib inhibits phorbol ester-induced expression of COX-2 and activation of AP-1 and p38 MAP kinase in mouse skin. *Carcinogenesis* 25, 713–722.
- (49) Murakawa, M., Yamaoka, K., Tanaka, Y., and Fukuda, Y. (2006) Involvement of tumor necrosis factor (TNF)- α in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin edema in mice. *Biochem. Pharmacol.* 71, 1331–1336.