Akt Cys310-targeted inhibition by hydroxylated benzene derivatives is tightly linked to their immunosuppressive effects

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Running head: Akt-targeted immunosuppressive effect of HQ and its derivatives

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The hydroxylated benzene metabolite hydroquinone (HQ) is mainly generated from benzene, an important industrial chemical, and is also a common dietary component. Although numerous reports have addressed the tumorigenesis-inducing effects of hydroquinone, few papers have explored its molecular regulatory mechanism in immunological responses. In this study, we characterized Akt (protein kinase B)-targeted regulation by hydroxylated benzene metabolites, hydroquinone and its derivatives, in suppressing inflammatory responses using cellular, molecular, biochemical and immunopharmacological approaches. HQ down-regulated inflammatory responses such as NO production, surface levels of pattern recognition receptors (PRR), and cytokine gene expression with IC50 values that ranged from 5 to 10 μM. HQ inhibition was mediated by blocking NF-κB activation via suppression of its translocation pathway, which is composed of Akt, IKKβ, and IκBα. Of the targets in this pathway, HQ directly targeted and bound to the sulphydryl group of Cys-310 of Akt and sequentially interrupted the phosphorylation of both Thr-308 and Ser-473 by mediation of β-mercaptoethanol.
(mer-EtOH), according to the LC/MS analysis of the interaction of HQ with an Akt-derived peptide. Therefore, our data suggest that Akt and its target site Cys-310 can be considered as a prime molecular target of HQ-mediated immunosuppression and for novel anti-Akt-targeted immunosuppressive drugs.

Keywords: Hydroxylated benzene metabolite, inflammatory response, Akt Cys-310, thiolation.

INTRODUCTION

Inflammation is one of the innate immunity responses and a multiple step process that is mediated by activated inflammatory or immune cells. In particular, macrophages that differentiate from monocytes have a critical role in managing many different immunopathological events in inflammation, such as the over-production of inflammatory mediators [nitric oxide (NO) and prostaglandin E₂ (PGE₂)], which are generated by activated inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, and various chemokines and cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-12 (1,2).

The pro-inflammatory events are mainly initiated by the activation of surface receptors [pattern recognition receptors (PRRs)], such as the Toll-like receptors (TLR)-4 or TLR-2 after binding to their ligands, including lipopolysaccharide (LPS) from microorganisms (3). During the activation of PRRs, a complicated intracellular signaling machinery, including mitogen activated protein kinases (MAPKs), serine/threonine protein kinases, and non-receptor type tyrosine kinases, are up-regulated to trigger inflammatory gene expression via activation of transcription factors, such as nuclear factor (NF)-κB and activator protein (AP)-1 (4,5).

Primary [peritoneal and bone marrow (BM)-derived] macrophages and cancerous macrophage-like cells (e.g., RAW264.7 and J774 cells) induced by LPS are now regarded as a useful in vitro model for evaluating the potency of anti-inflammatory drugs and for exploring their anti-inflammatory mechanisms, due to their ability to display similar inflammatory states (6).

Hydroquinone (benzene-1,4-diol; HQ) is a benzene metabolite mainly found in cigarette smoke, coffee, industrial chemicals, and petroleum by-products and is a ubiquitous environmental pollutant. In addition, cigarette smoke-mediated allergy symptoms are caused by hydroquinone, which acts as a strong hapten (7), and previous findings that it blocked interferon (IFN)-γ production in Th1 cells (8), enhanced interleukin (IL)-4 production in CD4+ T cells (9), increased immunoglobulin E levels in antigen-primed mice (9), and blocked IL-12 production via suppression of nuclear factor (NF)-κB binding activity (7), inhibition of lymphocyte proliferation (10), and suppression of macrophage-mediated phagocytosis (11).
strongly suggest that it acts as an immunomodulator rather than as a simple toxic chemical. In particular, strong decreases in production of cytokines (IL-1β, IL-2, IL-6, IL-10, and TNF-α) and inflammatory mediators (NO and PGE₂) by HQ (12) have led to the hypothesis that this compound can be applied like a strong anti-inflammatory drug with immunosuppressive properties. Currently, the molecular inhibitory mechanisms of benzene metabolites and their molecular targets in the modulation of immune responses remain largely uncharacterized. In the present study, we investigated the molecular effect of hydroquinone on the modulation of inflammatory processes mediated by macrophages.

EXPERIMENTAL PROCEDURES

Materials - Hydroquinone, tet-butyl hydroquinone, resorcinol, curcumin, indomethacin, arachidonic acid (AA), 1,4-dithiothreitol (DTT), L-cysteine, N-acetyl-L-cysteine, α-tocopherol, phorbol-12-myristate-13-acetate (PMA), D-galactosamine, prednisolone, and lipopolysaccharide (LPS, E. coli 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). U0126 was obtained from Calbiochem. (La Jolla, CA). HQ derivatives (JS-III-49, -69, -73, -81, -87, and -89) were synthesized according to previous paper (13). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from the American Tissue Culture Center (Rockville, MD). Luciferase constructs containing NF-κB or AP-1 binding promoters were gifts from Prof. Chung, Hae Young (Pusan National University, Pusan, Korea). Crosstide (14) and Suntide (15) were synthesized from Peptron (Daejeon, Korea). All other chemicals were of Sigma grade. Phospho-or total antibodies to p85, 3-phosphoinositide-dependent kinase (PDK)1, Akt (protein kinase B) (Thr 308 and Ser 473), extracellular signal-regulated kinase (ERK), ERK kinase (MEK), p38, C-Jun N-terminal Kinase (JNK), IκBα, kinase (IKK), IκBα, p65, myelin basic protein (MBP), γ-tubulin, and β-actin were purchased from Cell Signaling (Beverly, MA) Santa Cruz Biotechnology (Santa Cruz, CA), or Upstate Biotechnology, Inc. (Lake Placid, NY). Alexa 488-conjugated secondary antibody was obtained from Invitrogen (Carlsbad, CA). Antibodies to TLR-2, TLR4, CD69, and dectin-1 were from Hycult Biotechnology (Uden, The Netherlands), BD Bioscience (San Diego, CA), and Serotec (Kidlington, Oxford, UK).

Animals - ICR and C57BL/6 male mice (6–8 weeks old, 17–21 g) were obtained from Daehan Biolink (Chungbuk, Korea) and maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied ad libitum. Studies were performed in accordance with guidelines established by
the Kangwon National University Institutional Animal Care and Use Committee.

Construction of expression vectors - GFP-fused wild type Akt construct (GFP-Akt-WT) was prepared by amplification using a typical culture method with competent *E. coli* (DH5α). pcDNA-HA, pcDNA-HA-tagged Akt, pcDNA-HA-tagged PDK1, and pcDNA-Myc-tagged PDK1 constructs were used as reported previously (16). The mutant at Cys-310 (GFP-Akt-C310A) was created by using the QuikChange site-directed mutagenesis kit (Stratagene), with the following primers: forward [F]-5'-ACC ATG AAG ACC TTT GCC GGC ACA CCT GAG TAC, and reverse [R]- GTA CTC AGG TGT GCC GGC AAA GGT CTT CAT GGT, as described by the manufacturer with pCMV5 GFP-Akt-WT as template. All constructs were confirmed by automated DNA sequencing. Sequences of the mutagenic oligonucleotides are available upon request.

Preparation of peritoneal macrophages and Bone marrow (BM)-derived macrophages - Peritoneal macrophages were obtained as reported previously (17). BM-derived macrophages were also prepared from BM-derived cells after treating GM-CSF and IL-4, as reported previously (18).

Cell culture - Peritoneal macrophage, BM-derived macrophages, RAW264.7, wild type HEK293, stable cell lines expressing the Myc-tagged PDK1 WT, derived from HEK 293 cells (19), and A21 cells were maintained in RPMI1640 or DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum in the presence or absence of G418. Cells were grown at 37°C with 5% CO₂.

Determination of NO production - RAW 264.7 cells (1 × 10⁶ cells/ml) under normal culture conditions were preincubated with each compound for 30 min and continuously activated with LPS (2 μg/ml) for 24 h. The nitrite in culture supernatants was also measured by a Griess assay, as reported previously (17).

MTT assay (colorimetric assay) for measurement of cell viability - Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as reported previously (20).

Flow cytometric analysis - Expression of RAW264.7 cell surface adhesion molecules under LPS treatment was determined by flow cytometric analysis as reported previously (21). Stained cells were analyzed with a FACScan (Beckton-Dickinson, San Jose, CA).

RT-PCR - For the evaluation of cytokine mRNA expression levels, total RNA from LPS-treated-RAW264.7 cells (5 × 10⁶ cells/ml) was prepared by adding TRIzol Reagent (Gibco BRL) according to the
manufacturer’s protocol. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase as reported previously (22). The primers (Bioneer, Seoul, Korea) used in this experiment are indicated as follows: TNF-α (F-5’- TTGACCTCAGCGCTGAGTTG-3’ and R- 5’-CCTGTAGCCCAACGTCGAGC-3’); IL-6 (F-5’-GTACTCCAGAAGACCAGAGG-3’ and R-5’-TGCTGGTGACAACCACGGCC-3’); and GAPDH (F-5’-CACTCACGGCAAATTCACAACGGCC-3’ and 5’-GACTCCACGACATACTCAGCAC-3’).

Luciferase Reporter Gene Activity Assay - HEK293 cells (1 × 10^6 cells/ml) were transfected with 1 μg of plasmids containing NF-κB-Luc, or AP-1-Luc as well as β-galactosidase using the calcium phosphate method in a 12-well plate according to the manufacturer’s protocol. The cells were used for experiments 48 h after transfection. Luciferase assays were performed using the Luciferase Assay System (Promega) as reported previously (23).

Preparation of cell lysates and nuclear fraction, and Immunoblotting - RAW264.7, HEK29 transfected with GFP-Akt or A21 cells (5 × 10^6 cells/ml each cell) were washed 3 times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer [20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycoltetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 g for 10 min at 4°C and stored at -20°C until needed.

Nuclear lysates were prepared with a three-step procedure (24). After treatment, cells were collected with a rubber policeman, washed with 1× PBS, and lysed in 500 μl of lysis buffer containing 50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES (pH 7.8), 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM DTT on ice for 4 min. Cell lysates were then centrifuged at 14,000 rpm for 1 min in a microcentrifuge. In the second step, the pellet (the nuclei fraction) was washed once in washing buffer, which was the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mM KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at -80°C, and then thawed on ice and centrifuged at 14,000 rpm for 5 min. Supernatant was collected as nuclear extract.

Whole cell or nuclear lysates were then analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidenedifluoride (PVDF) membrane.
Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 at room temperature. The membrane was incubated for 60 min with specific primary antibody at 4°C, washed 3 times with the same buffer, and incubated for an additional 60 min with HRP-conjugated secondary antibody. The total and phosphorylated levels of MEK, ERK, p38, JNK, IkBα, IKKβ, p85, PDK1, Akt, MBP, γ-tubulin, and β-actin were visualized using the ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

Confocal microscopy - RAW264.7 cells (1 × 10^4 cells) were plated in 12-well plates containing sterile cover slips and grown at 37°C for 24 h. The medium was then replaced with serum-free media, and the cells were allowed to grow for another 24 h before treatment. Cells were treated with HQ for 30 min followed by stimulation with LPS (2 µg/ml) for 1 h. After treatment, the cells were washed twice with PBS prewarmed to 37°C and fixed onto the cover slips by incubation in 3.7% formaldehyde for 10 min. Cells were then washed three times with PBS and permeabilized by incubation in 100% methanol for 6 min at -20°C. The cover slips were blocked in 1% BSA for 1 h at room temperature with shaking. Antibody to the NF-κB p65 subunit (1:50) was added to the 1% BSA solution and incubated for 1 h with shaking at room temperature. For nuclear staining, Hoechst solution (Sigma) was added at a final concentration of 0.5 mg/ml and incubated for 1 h in the dark. Cover slips were then washed three times each with PBS. Alexa 488-conjugated secondary antibody (1:100) in 1% BSA was then added and incubated for 1 h with shaking at room temperature. Cover slips were washed three times with PBS and mounted onto slides using Fluorescent mounting medium (DakoCytomation). The nuclear translocation of p65 was imaged by LSCM on a Zeiss LSM 510 META confocal microscope equipped with a Zeiss 37°C incubation system. Images were analysed using the Zeiss LSM Image Examiner.

Immunoprecipitation and in vitro kinase assay for PDK1 and Akt - HEK 293 cells were placed on ice and extracted with lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% (v/v) Nonidet P-40, 120 mM NaCl, 25 mM sodium fluoride, 40 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine. Lysates were centrifuged for 15 min at 12,000 × g, and Myc-PDK1 or HA-Akt protein was immunoprecipitated from 500 µg of cell-free extracts with anti-Myc 9E10 monoclonal antibody or anti-HA 12CA5 monoclonal antibody immobilized on protein G-Sepharose (Amersham Biosciences). The immune complexes were washed once with lysis buffer containing 0.5 M NaCl, followed by lysis buffer and finally with kinase assay buffer [50 mM Tris-HCl, pH 7.5, 0.1% (v/v) 2-mercaptoethanol (mer-
EtOH]. In vitro kinase assays were performed for 30 min (Akt) or 60 min (PDK1) at 30°C in a 50 µl reaction volume containing 30 µl of immunoprecipitates in kinase buffer, 100 µM Suntide (RRKDGATMKTFCGTPE) or 30 µM Crosstide (GRPRRTSSFAEG) as substrate, 10 mM MgCl₂, 1 µM protein kinase A inhibitor peptide (Alexis), and 100 µM [γ-32P]ATP (1,000–2,000 cpm/pm; Amersham Biosciences). Reactions were stopped by adding EDTA to a final concentration of 50 mM and processed as described previously (16). Protein concentrations were determined by the method of Bradford (Bio-Rad) using bovine serum albumin as a standard.

Kinase assay with purified enzymes - Kinase assay was performed by kinase profiler service from Millipore (Billerica MA). In a final reaction volume of 25 µl, human purified enzymes [Akt1, p70S6K, protein kinase A (PKA), protein kinase C (PKC)α, or serum and glucocorticoid-inducible kinase (SGK)] (1-5 mU) is incubated with the reaction buffer in the absence of mer-EtOH. The reaction is initiated by the addition of MgATP. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 5 ml of a 3% phosphoric acid solution. 10 µl of the reaction is then spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

Characterization of hydroquinone-Suntide adduct

HPLC analysis - A reverse phase HPLC system was used for the analysis of HQ as reported previously (25). The HPLC system (Waters, USA) consisted of a pump (Waters™ 600 Controller), a UV-VIS spectrophotometric detector (Waters™ 486 Tunable Absorbance Detector), an autosampler (Waters™ 717 plus Autosample), a degasser (Waters™ In-line Degasser), a reverse phase column (Luna 5µ C₁₈ analytical column (150 mm × 4.6 mm)) and an integrator (Borwin® 1.20 software). For isocratic analysis, 1% acetic acid in H₂O₂ was used as the mobile phase. The flow rate of mobile phase was 1 ml/min and the column eluate was monitored by a UV detector set at 290 nm.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry - α–cyano-4-hydroxycaffinic acid (20 mg) (Bruker Daltonics, Bremen, Germany) was dissolved in 1 ml acetone : ethanol (1 : 2, v/v), and 0.5 µl of the matrix solution was mixed with an equivalent volume of sample. Analysis was performed using an Ultraflex TOF/TOF system (Bruker Daltonics). The Ultraflex TOF/TOF system was operated in positive ion reflect mode. Each spectrum was the cumulative average of 250-450 laser shots. Mass spectra were first calibrated in the closed external mode using the peptide calibration standard II (Bruker Daltonics), sometimes using the internal statistical mode.
to achieve maximum calibration mass accuracy. Standard settings included the following: mass values, MH\(^+\) (monoisotopic); mass tolerance, varied between 75 and 100 ppm.

**Molecular modeling study** - To understand the binding mode of action of a cysteine-HQ adduct (Mer-EtOH-HQ: MHQ), a molecular modeling study in the active site using Sybyl version 8.02 (Tripos Associates) operating under Red Hat Linux 4.0 on an IBM computer (Intel Pentium 4, 2.8GHz CPU, 1GB memory) and the X-ray crystallographic structure of an activated Akt ternary complex with GSK-peptide and AMP-PNP (26) has been carried out. The structure of the MHQ was drawn into the Sybyl package with standard bond lengths and angles and was minimized using the conjugate gradient method. The Gasteiger-Huckel charge, with a distance-dependent dielectric function, was applied for the minimization process. The 106l (PDB code) structure from the Protein Data Bank was chosen and the structure was polished following structure preparation tool in Sybyl. After this, MHQ was merged to Cys 310 for having S-S bonding and consequent complex was minimized by subset minimization tool for producing the conformationally stable complex structure.

**In vivo inflammatory models**

**Septic shock models** - C57BL/6 male mice were orally pre-treated with HQ (100 mg/kg), JS-III-49 (25 and 50 mg/kg), and indomethacin (1 mg/kg) for 4 times. Thirty min after final treatment, D-GalN (600 mg/kg) and LPS (50 \(\mu g/kg\)) were intraperitoneally co-injected, as reported previously (27). Effect of hydroquinone or its derivative on septic shock-induced mouse lethality was calculated from the number of survival mice after 7-day observations.

**Arachidonic acid-induced mouse ear edema** - ICR mice (n = 7) were orally pre-treated with JS-III-49 (25 and 50 mg/kg), and indomethacin (1 mg/kg) for 4 times. After the final treatment, AA [2% (w/v)] was applied to the ear of the mouse (25 \(\mu l/ear\)), as described previously (28). The thickness of the edema was measured with a constant-pressure thickness gauge 4 h after AA treatment. To evaluate curative efficacy, the inhibitory effect of ear edema by testing inhibitors was calculated as reported previously.

**Determination of serum TNF-\(\alpha\) level** – Ninety min after intraperitoneal injection of D-GalN (600 mg/kg) and LPS (50 \(\mu g/kg\)), blood was collected and serum samples were used to measure TNF-\(\alpha\) levels by means of an ELISA kit.

**Histological analysis of the liver** - Tissue samples taken from the liver of the mice at 8 h after challenge with LPS and GalN were fixed with 10% formalin in PBS, and then embedded in paraffin. Approximately 4 \(\mu m\) thin tissue sections were stained with
hematoxylin and eosin for histopathological examination as reported previously (29).

Statistical analysis - A Student’s t-test and one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means ± standard errors (SEM) and the results were obtained from at least three independent experiments performed in triplicate. P values of 0.05 or less were considered as statistically significant.

RESULTS

Effect of hydroquinone on LPS-induced inflammatory responses - We previously reported that HQ displays an immunosuppressive effect on various immune responses. Before starting the molecular mechanistic study on HQ-mediated effects, the effects induced by LPS in macrophages were first examined during HQ treatment. Three different types of macrophages, cancerous macrophage-like RAW264.7 cells and two primary (peritoneal and BM-derived) macrophages, were employed. As Fig. 1A shows, HQ suppressed the production of NO in a dose-dependent manner, like several kinase inhibitors, including piceatannol, PP2, and LY294002 (data not shown). Furthermore, HQ also remarkably diminished the enhanced expression of surface molecules, such as TLR4 and CD69, in response to LPS (Fig. 1B), as in the case of BAY 11-7082, a NF-κB inhibitor (data not shown). Although it has been already published that the inhibitory effect of HQ occurs at the transcriptional level, inhibitory patterns at the mRNA level and the treatment time dependency were simultaneously confirmed. In agreement with previous results (22), Fig. 1C shows that HQ was capable of reducing not only the mRNA levels of pro-inflammatory genes but also early exposure before LPS led to maximum suppression. These results therefore suggest that early events triggered by LPS exposure could be the target of HQ inhibition.

Effect of hydroquinone on LPS-induced NF-κB activation - We carefully further characterized the inhibitory effect of hydroquinone on NF-κB activation. Even though it is generally well accepted that HQ acts as a NF-κB inhibitor (7,30), whether HQ directly or indirectly blocks this transcription factor is still controversial. To address this question, NF-κB translocation signals were mainly investigated in this study. As Fig. 2A shows, this compound selectively diminished the luciferase activity mediated by NF-κB but not AP-1. Furthermore, Figs. 2B, 2C, and 2D clearly support that HQ suppressed the upstream signaling events for NF-κB (p65) translocation. Thus, phosphorylation of IKKβ and IκBα, an important process for NF-κB (p50/p65) translocation, was clearly suppressed in response to HQ exposure (Figs. 2B and 2C). In case of curcumin treatment, it also more strongly blocked IKKβ than IKKα.
(Fig. 2C). The final step of the IKK/IκBα signaling cascade, nuclear translocation of NF-κB (p65), was also diminished 1 h after LPS administration by HQ treatment in a dose-dependent manner according to immunoblotting (Fig. 2D) and confocal (Fig. 2E) analyses. Therefore, these results indicate that the molecular target of HQ could be one of upstream signaling enzymes that regulate NF-κB translocation.

Effect of hydroquinone on the upstream signaling enzymes for nuclear NF-κB translocation - The major components of the NF-κB activation pathway are now well known and consist of Akt/PDK1 and PI3K as well as protein tyrosine kinases, such as Src, Syk, and Jak2 (31). Therefore, the potential inhibitory target of HQ was first investigated by exploring the NF-κB signaling pathway. Interestingly, our data implied that Akt could be a potential target of HQ. Thus, Fig. 3A shows that inhibition of PI3K/p85 and PDK1 phosphorylation was not observed. Instead, this compound clearly blocked the phosphorylation of Akt and showed two different inhibitory patterns; phosphorylation on Thr-308 was inhibited at 2 and 5 min and Ser 473 at 5 to 15 min (Fig. 3A). On the other hand, this compound more strongly suppressed Akt phosphorylation when treated for 30 min in RAW264.7 cells (Fig. 3B, left panel). A similar effect was also obtained with peritoneal macrophages (Fig. 3B, right panel), suggesting that HQ inhibits Akt phosphorylation in both cancerous and primary cell states. Meanwhile, MAPK phosphorylation, which is required for AP-1 translocation, was not suppressed by HQ (Fig. 3C), in agreement with the AP-1-mediated luciferase activity assay (Fig. 2A).

Effect of hydroquinone on Akt kinase activity - To better characterize the Akt inhibitory mechanism, Akt kinase assay conditions were introduced. First, using specific peptide substrates, Suntide (14) and Crosstide (15), which originate from the amino acid sequences of Akt or its substrate (glycogen synthase kinase 3) (Fig. 4A), and immunoprecipitated Akt or PDK1, the direct inhibitory effect of HQ on Akt kinase activity was carefully investigated. Similarly to the aforementioned data, HQ at 100 μM strongly suppressed Akt kinase activity by up to 95% with the Crosstide substrate peptide (Fig. 4B). The Akt phosphorylation induced by insulin in 2A1 cells (HEK293 cells that stably express HA-PKB) was also remarkably diminished by HQ, implying that the inhibition seemed to be generally observable during Akt activation conditions (Fig. 4C). A particularly interesting finding was obtained in the PDK1 kinase assays with two different substrates. Namely, while phosphorylation of MBP by PDK1 was not affected by HQ, phosphorylation of Suntide was suppressed (Fig. 4D). Consistently, the activity of immunoprecipitated PDK1 was not altered when HQ was directly treated to the RAW264.7 cells (Fig. 4E), suggesting that PDK1 is not the direct target, but HQ-
mediated inhibition could be relevant to the sequence of the Akt-derived peptide Suntide (Fig. 4A).

**Effect of thiolation on hydroquinone-mediated inhibition of NO production** - It has been reported that hydroquinone and its chemical derivatives require hydroxyl groups in the *para* form for their maximum anti-inflammatory activities (32). Whether this structural feature is simply important for chemical reactions that generate or neutralize toxic radicals or specific interaction with target molecules that mediate its variable biological effects is still not fully understood.

In this study, we first attempted to identify the biological role of HQ in terms of its chemical properties using several antioxidants or thiol compounds. Fig. 5A (left panel) clearly shows that HQ inhibition of NO production could be due to electrophilic reactivity leading to thiolation. Thus, combined treatment of thiol compounds, such as L-cysteine, N-acetyl-L-cysteine, and DTT, abrogated HQ inhibition, whereas α-tocopherol at 12.5 µM, which exhibits strong anti-oxidative activity (data not shown), did not. The exact same pattern was also seen for NO production from BM-derived macrophages (Fig. 5A, right panel). Meanwhile, the abrogating effect of L-cysteine was only found when it was administered at the same time or prior to HQ treatment (Fig. 5B), suggesting that thiolation needs to precede HQ. On the other hand, the inhibitory mechanism of the *para*-type hydroquinone derivative tBHQ on NO production (Fig. 5C, left panel) seems to be the same as HQ because pre-treatment with L-cysteine prior to tBHQ also abolished its ability to suppress NO production (Fig. 5C, right panel). Therefore, these results suggest that the thiolation-inducing activity of HQ could be a major inhibitory mechanism.

**Effect of thiolation on hydroquinone-mediated inhibition of Akt kinase activity** - To obtain direct evidence linking thiolation to the regulation of Akt kinase activity, several molecular studies, including Akt kinase assays and immunoblotting analysis, were executed. As shown in Fig. 6A, L-cysteine treatment also clearly abrogated HQ-mediated inhibition of Akt phosphorylation in response to LPS at 5 and 30 min (Fig. 6A). The remarkably decreased level of Akt phosphorylation induced by insulin stimulation was also restored by co-treatment with DTT (Fig. 6B). Most of all, DTT was able to attenuate HQ-mediated inhibition of both Akt kinase activity with the Crosstide substrate (Fig. 6C) and PDK1 activity with the Suntide substrate (Fig. 6D). Therefore, our results suggest that the HQ-induced thiolation reaction could play a critical role in modulation of Akt phosphorylation and kinase activity.

**Effect of β-mercaptoethanol on formation of the hydroquinone-Suntide adduct and regulation of kinase activity** - Since HQ could be thiolated with a sulphydryl...
compound, we moved to a kinase assay system to determine why phosphorylation of the Suntide peptide but not MBP was selectively blocked by HQ in the PDK1 kinase assay (Fig. 4D). In particular, because Suntide has a cysteine residue (Fig. 4A), which is conserved in proteins with an activation loop, such as PKCa, protein kinase C-related kinase (PRK), and SGK, and important for threonine phosphorylation (14), we focused our experiments on the potential role of the sulfhydryl group of that cysteine. Interestingly, Fig. 7A indicates that HQ strongly blocked the kinase activity of Akt in a dose-dependent manner in the normal kinase buffer system with β-mercaptoethanol (mer-EtOH). However, without mer-EtOH, none of the kinases were significantly suppressed by HQ, suggesting that mer-EtOH might play a critical role in inhibition by HQ. To address this possibility, chemical conversion was examined using HPLC and MS. Surprisingly, an adduct formed with Suntide, mer-EtOH, and HQ was identified by LC-MS-MS analysis (Fig. 7B), while adduct formation between HQ and thiol compounds was not observed in PBS conditions according to the HPLC analysis (Fig. 7C). Therefore, these results suggest that HQ blocks the phosphorylation of Thr-308 through chemical modification of Cys-310 by the mer-EtOH-hydroquinone (MHQ) generated with electrophilic agents, such as mer-EtOH (Fig. 7D).

Role of Akt Cys-310 in the phosphorylation of Akt and hydroquinone-mediated inhibition of Akt kinase activity - To address the role of Cys-310 in Thr-308 phosphorylation and as a HQ binding site, a molecular biological method was employed. We first prepared a mutant Akt in which Cys-310 was substituted with alanine (Akt-C310A; Fig. 8A) and over-expressed it in HEK293 cells. The phosphorylation patterns of the mutant Akt were examined and compared to wild type (WT). As Fig. 8B displays, phosphorylation of Thr-308 was severely deficient in the mutant Akt. Similarly, Ser-473 phosphorylation was also dramatically altered, regardless of the three-fold increase in expression (Fig. 8B). More intriguingly, HQ suppression of Akt kinase activity was strongly seen in WT conditions, whereas the mutant activity was attenuated by only 50% after HQ treatment (Fig. 8C). Therefore, these results suggest that Cys-310 could play a central role in modulation of Akt phosphorylation and serve as a major binding site for HQ. To support this possibility, a docking model has been speculated using previous structural data (26). Thus, the hydroxyl group of MHQ seemed to form H-bonds with the Glu 298 and to hydrophobically interact with His-196 (Fig. 8D), which is considered as essential roles to stabilize the complex in active site. These chemical interactions presumably looked to make an environment to disturb molecular interaction between PDK1 and its substrate amino acid residue Thr-308. From this
modeling study, therefore, we propose that the MHQ bonded with Cys 310 is able to work as a Thr-308 phosphorylation blocker in Akt.

Effect of novel hydroquinone derivatives on PDK1 kinase activity, NO production, septic shock, and ear edema models - Since it has also been suggested that Cys-310 of Akt is a potentially important site for anti-cancer drug development (33,34), we further characterized the possibility that HQ could be chemically modified to be pharmacologically improved. Indeed, several derivatives (Fig. 9A) strongly blocked the phosphorylation of Suntide by the PDK1 kinase (Fig. 9B) and consequently LPS-induced NO production (Fig. 9C) with IC\textsubscript{50} values that ranged from 2 to 25 \( \mu \text{M} \) (Fig. 9D) without altering normal cell viability, except for one compound (JS-III-73) (Fig. 9E). In particular, these derivatives also worked well \textit{in vivo}. When it was orally administered, one of these compounds (JS-III-49 at 50 and 100 mg/kg) as well as HQ (100 mg/kg) strongly suppressed lethality induced by the combined treatment of D-GalN and LPS by up to 70\% (Fig. 10A). The serum level of TNF-\( \alpha \), a major cause leading to higher lethality (35), enhanced by LPS/D-GalN treatment, was also remarkably suppressed by this drug as prednisolone, a steroid drug (36), did (Fig. 10B). In agreement, liver damage induced by LPS/D-GalN treatment was revealed to be protected by JS-III-49 administration (Fig. 10C). Moreover, JS-III-49 at 50 mg/kg also significantly ameliorated ear edema induced by AA by 55\% (Fig. 10D). Although indomethacin exhibited stronger anti-edema effect than JS-III-49 (Fig. 10D), this standard drug reduced normal body weight unlike HQ derivative (data not shown). Therefore, these results suggest that HQ can be further developed chemically to generate a more powerful Akt Cys-310 targeted immunosuppressive agent for use \textit{in vivo}.

DISCUSSION
HQ is a representative of the toxic benzene type-compounds found in cigarette smoke, coffee, and numerous industrial products from petroleum companies. Although the compound has been identified as a toxic molecule, the toxicological mechanisms of HQ in various cellular and immune responses are not fully understood. Recently, we proposed that HQ could be used as an anti-inflammatory drug because it diminished the release of inflammatory mediators (21). However, most data seem to suggest that this compound rather possesses potent immunosuppressive activity. For example, HQ was reported to suppress the production or expression of various cytokines, such as TNF-\( \alpha \) (also in Fig. 1C), IFN-\( \gamma \), IL-1\( \beta \), IL-3, IL-6 (also in Fig. 1C), IL-10, IL-12 in macrophage-like RAW264.7 cells and in lymphocytes under stimulation with LPS and keyhole limpet hemocyanin (7,8,12,30). In addition to its inhibitory effects on the production of various inflammatory mediators such as NO (also in Fig. 1A) and
PGE$_2$, HQ also negatively modulated receptor-mediated phagocytosis (12), lymphocyte proliferation (8,12), up-regulation of PRR levels (Fig. 1B), and enhanced differentiation during the Th2 response (9). These results led us to explore the molecular aspects of hydroquinone inhibition and to focus on a common pathway required for cytokine production, phagocytosis, and inflammatory mediator release. The main target that is consistent with these observations is NF-κB, a common pathway in the pro-inflammatory response. Numerous papers have also suggested it is involved in HQ-mediated inhibition of a variety of cellular responses (7,21,30,37).

Even though recent articles suggested that hydroquinone was capable of preventing NF-κB from binding to its DNA promoter site (30,32), our data instead strongly implied that HQ suppressed the translocation of NF-κB. Ma et al. (2003) reported that HQ did not inhibit LPS-induced activation of IKK activity, degradation of IκBα, or translocation of activated NF-κB into the nucleus, but HQ did block the formation of NF-κB/DNA complexes (30). In contrast, we found that both phosphorylation of IκBα and IKKβ but not IKKα was completely blocked and the level of p65 translocation was dose-dependently inhibited by HQ treatment. In addition, HQ-mediated inhibition of p65 nuclear translocation was also confirmed by confocal microscopic analysis (Fig. 2E). As Fig. 2C shows, the fact that phosphorylation of IKKα was clearly seen during HQ treatment seems to explain why IKK kinase activity was not blocked by HQ during previous studies (30). A signaling cascade that links IKKβ and IκBα to the translocation of NF-κB has also been characterized by numerous findings (38,39).

The next question raised in this study is what is the target of HQ that mediates the block in NF-κB translocation? Our data strongly indicate that Akt is the only protein involved in the immunosuppressive effects of HQ. The phosphorylation patterns of Akt (Fig. 3), its kinase activity (Fig. 4), and adduct formation between HQ and Akt-derived peptide fragments (Fig. 7) are strong examples of its involvement. Akt has been recently proposed to be an important component in immune responses in a large number of studies. Akt-specific siRNA strongly blocked IκBα phosphorylation and NF-κB activation (17). Moreover, IKKβ has been identified as a substrate of Akt, according to direct binding and phosphorylation assays (39-41). The involvement of Akt in the inflammatory pathway has also been demonstrated using a variety of cell types (including dendritic cells, monocytes, and macrophages) (42,43) and by measuring several inflammatory parameters (including NO and TNF-α) (44,45). LPS treatment increased the activity of PI3K, PDK1, and Akt, as assessed by their phosphorylation levels and kinase activities (22,46). Consequently, strong inhibitors of these pathways, such as LY294002, wortmannin, and Akt inhibitors, were clearly
shown to block inflammatory events mediated by LPS (47,48). Nonetheless, Akt inhibitors are mostly developed as anti-cancer drugs because the predominant role of Akt has been more clearly demonstrated in cancer cell survival and proliferation phenomena (49,50). However, the accumulating evidence should motivate more researchers to develop promising anti-inflammatory drugs targeted to Akt.

The number of patents and papers on the Akt inhibitor development rapidly grows as more information on the functional role of Akt is attained. So far, two approaches have been generally used to develop Akt inhibitors. The first approach involves screening direct binding inhibitors targeted to the ATP binding site of Akt. Example compounds include GSK690693 (51), 6-phenylpurines (52), and conjugates of oligoarginine peptides with adenine, adenosine, adenosine-5’-carboxylic acid, and 5-isoquinolinesulfonic acid (53). Although these compounds displayed strong inhibitory effects, other side effects would be expected due to non-selectivity because most kinases share a conserved amino acid sequence in the ATP binding site. Indeed, AGC family kinases, which include cAMP-dependent PKA, cGMP-dependent kinase (PKG), and PKC have been reported to have well-conserved sequences in the ATP binding motif (54). The other way focuses on finding compounds that bind to selective allosteric sites on Akt. Indeed, most Akt inhibitor development trials have concentrated on this approach because stronger selectivity can be obtained. 2,3,5-trisubstituted pyridine derivatives (55), 2,3-diphenylquinoxaline (56), and Aminofurazans (57) are good examples of compounds with promising activity. In view of these, one of the recent interesting findings on the allosteric sites of Akt was the identification of specific amino acid residues, Cys-296 and Cys-310, in the activation (T-) loop of Akt (58). Thus, lactoquinomycin and related pyranonaphthoquinones were found to block Akt kinase activity by irreversibly binding to Cys-296 and Cys-310 in response to suppressing cancer cell proliferation (33,34). Using these compounds, it was shown that those amino acids and the adjacent residues could be another allosteric site in Akt. Similarly in our study, the benzene-type toxic molecule HQ was shown to clearly bind to Cys-310 by LC-MS-MS analyses (Fig. 7B). Furthermore, Akt-C310A, which is deficient in kinase activity as well as its phosphorylation compared to those of Akt-WT (Fig. 8B), was only partially suppressed by HQ, while the activity of WT was almost completely suppressed (Fig. 8C). Furthermore, HQ and its structural analogs have been shown to strongly block Akt phosphorylation and kinase activity (Fig. 9B) and NO production (Fig. 9C) without altering cell viability (Fig. 9E). In particular, structural modifications of HQ showed us that the activity of HQ could be improved by this strategy. Indeed, one derivative (JS-III-49) when orally administered at 50 mg/kg
strongly suppressed lethality of septic shock (Fig. 10A), enhanced levels of serum TNF-α (Fig. 10B), liver damages (Fig. 10C), and ear swelling (Fig. 10D). Therefore, these results suggest that C-310 and the adjacent sequence in Akt could be drug design targets and applicable for development of novel drugs for Akt inhibition as suggested previously (33,34). Indeed, according to a docking model postulated from the structural database of Akt (26), it is considered that HQ chemically adducted with Cys-310-Mer-EtOH can associate with Glu-298 by hydrogen bonds and with His-195 by hydrophobic interaction (Fig. 8D), to make an environment interrupting the phosphorylation of Thr-308 in Akt by a structural hindrance.

It has been speculated that the reactivity of the pyranonaphthoquinone core of lactoquinomycin and frenolicin B could cause these compounds to directly bind to the T-loop cysteine(s) and consequently interfere with the catalytic activity of Akt through decreasing phosphorylation of Thr-308 (33). Indeed, phosphorylation of Thr-308 in Suntide by PDK1 was severely reduced when Cys-310 was replaced with Ala (14), indicating the important role of Cys-310 in the initial phosphorylation stage. Interestingly, HQ was not capable of directly binding to the cysteine in Suntide, according to the HPLC analysis after incubating HQ and the peptide in PBS buffer (Fig. 7C), even though powerful thiol compounds, such as DTT, L-cysteine, and N-acetyl-L-cysteine, strikingly abrogated HQ-mediated inhibition of NO production (Fig. 5), Akt phosphorylation (Fig. 6A), and Akt kinase activity (Fig. 6C). However, mer-EtOH enabled HQ to bind to the thiol group of the cysteine in Suntide, generating a new chemical adduct molecule composed of Suntide, mer-EtOH, and HQ, according to the dose-dependent suppression of kinase activity (Fig. 7A) and LC-MS-MS analysis (Fig. 7B). Unlike the peptide analysis conditions, mer-EtOH was not used in the cellular experimental assays. In spite of this, HQ still maintained its inhibitory activity, implying that some of cellular molecules with thiol groups may work similarly to mer-EtOH to make the active, conjugated form. Indeed, several conjugates including HQ-glutathione have been reported previously (59-61), and the mitochondrial enzymes, such as NADP reductase and GSH reductase, that metabolize HQ have already been well characterized (61,62). Furthermore, it has been shown that the enzyme activity of mitochondrial dehydrogenases was enhanced by hydroquinone by up to 2.2-fold (data not shown). In particular, the fact that these molecules can act as toxins that could contribute to the toxicity of HQ seems to open the possibility that these conjugates might block Akt activity directly in a cellular system. This possibility also leads us to a new hypothesis that these toxic conjugates might be involved in mediating the various
toxicological responses of HQ, such as immunosuppression and nephrotoxicity (60).

In summary, we found that HQ-mediated down-regulation of inflammatory responses, such as NO production and cytokine gene expression from macrophage-like RAW264.7 cells and primary macrophages, was mediated by blocking NF-κB activation via suppression of the translocation pathway composed of Akt, IKKβ, and IκBα. In particular, HQ was able to bind Cys-310 and interrupt the phosphorylation of both Thr-308 and Ser-473 in Akt by thiolation of the sulfhydryl group with mer-EtOH, according to the analysis of the interaction of HQ with Suntide. Therefore, our data suggest that Akt and its target site Cys-310 can be considered a prime target of HQ and could be applied to novel anti-inflammatory drug development.

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**FOOTNOTES**

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The abbreviations used are: HQ, hydroquinone; PRR, pattern recognition receptor; NO, nitric oxide; PGE$_2$, prostaglandin E$_2$; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; AP-1, activator protein-1; IFN, interferon.

**FIGURE LEGENDS**

Fig. 1. The effect of HQ on LPS-induced NO release, PRR up-regulation, and cytokine mRNA expression in RAW264.7 cells or primary (peritoneal or BM-derived) macrophages treated with LPS. (A) RAW264.7 cells or primary (peritoneal or BM-derived) macrophages (1×10$^6$ cells/ml) were incubated with HQ in the presence or absence of LPS (2 µg/ml) for 24 h. Supernatants were collected and the NO concentrations in the supernatants were determined using Griess
reagent. (B) RAW264.7 cells (1×10^6 cells/ml) pretreated with HQ were incubated with LPS (2 μg/ml) for 12 h. Surface levels of TLR-2, TLR-4, dectin-1, and CD69 were analyzed by flow cytometry. (C) RAW264.7 cells (5×10^6 cells/ml) were incubated with LPS (2 μg/ml) in the presence or absence of HQ for 6 h. mRNA levels of TNF-α and IL-6 were determined by RT-PCR. Data (A and B) represent the mean ± SEM of three independent experiments performed in triplicate. The result (C) shows one representative experiment of three. * p<0.05 and ** p<0.01 compared to the control group.

Fig. 2. The effect of HQ on NF-κB activation. (A) HEK293 cells transfected with plasmid constructs containing NF-κB-Luc or AP-1-Luc (1 μg/ml each) as well as β-gal were treated with HQ in the presence or absence of PMA (0.1 μM) for 12 h. Luciferase activity was determined by a luminometer as described in the Materials and Methods. (B and C) RAW264.7 cells (5×10^6 cells/ml) were incubated with HQ, curcumin, or U0126 in the presence or absence of LPS (2 μg/ml) for 30 min. The levels of phospho-IκBα or IKK(α/β) from total lysates were analyzed by immunoblotting. (D) RAW264.7 cells (5×10^6 cells/ml) were incubated with HQ in the presence or absence of LPS (2 μg/ml) for 30 min. Then the nuclear fraction was obtained from the cells, as described in the Materials and Methods. The nuclear levels of p65 and lamin A/C were determined by immunoblotting analysis. (E) Confocal analysis of the p65 level in nuclei of LPS-activated RAW264.7 cells in the presence or absence of HQ was performed as described in the Materials and Methods. Data (A) represent the mean ± SEM of three independent experiments performed in triplicate. The results (B, C, D, and E) show one representative experiment of three. * p<0.05 and ** p<0.01 compared to the control group.

Fig. 3. The effect of HQ on NF-κB translocation signaling activation. RAW264.7 cells (A, B left panel, and C) or peritoneal macrophages (B right panel) (5×10^6 cells/ml) were incubated with HQ in the presence or absence of LPS (2 μg/ml) for 30 min. The levels of phosphorylated or total p85, PDK1, Akt, MEK, and MAPK (ERK, JNK, and p38) from total lysates were analyzed by immunoblotting. The results show one representative experiment of three.

Fig. 4. The effect of HQ on kinase activity of Akt and PDK1. (A) Amino acid sequences of Sun tide and Crosstide. (B) HQ was incubated with purified Akt prepared from the baculovirus system for 20 min. Kinase activity was measured with Crosstide (30 μM) as described in the Materials and Methods. (C) 2A1 cells (5×10^6 cells/ml) pretreated with HQ for 15 or 30 min were incubated in the presence or absence of insulin (100 μM) for 20 min. After immunoblotting, total or phospho-protein levels of Akt were identified with the total protein- or phospho-specific antibodies. (D) HQ was incubated with immunoprecipitated PDK1 prepared
from Myc-PDK1 plasmid (1 μg/ml)-transfected HEK293 cells (5×10^6 cells/ml) for 20 min. Kinase activity was measured with Suntide (100 μM) or MBP (10 ng/ml) as described in the Materials and Method. (E) HQ was incubated with immunoprecipitated PDK1 prepared from LPS-activated RAW264.7 cells (5×10^6 cells/ml) for 10 or 15 min. Kinase activity was measured with MBP (10 ng/ml) as described in the Materials and Methods. Data (B) represent the mean ± SEM of three independent experiments performed in triplicate. The results (C, D, and E) show one representative experiment done with duplicate of three. * p<0.05 and ** p<0.01 compared to the control group.

Fig. 5. The effect of thiol compounds on HQ-mediated inhibition of NO release in LPS-activated RAW264.7 cells. RAW264.7 cells (A Left) or BM-derived macrophages (A Right panel) (1×10^6 cells/ml) pretreated with various thiol compounds (L-cysteine, N-acetyl-L-cysteine, and DTT) or α-tocopherol were incubated with HQ in the presence of LPS (2 μg/ml) for 24 h. (B) The abrogative effect of L-cysteine on HQ inhibition of NO production was determined by treating with L-cysteine for various amounts of time. (C) RAW264.7 cells (1×10^6 cells/ml) pretreated with or without L-cysteine were incubated with HQ, tBHQ, or resorcinol in the presence of LPS (2 μg/ml) for 24 h. Supernatants were collected and the NO concentration in the supernatants was determined using Griess reagent. Data represent the mean ± SEM of three independent experiments performed in triplicate. * p<0.05 and ** p<0.01 compared to the control group. # p<0.05 and ## p<0.01 compared to LPS + HQ or HQ analog group with the same concentration.

Fig. 6. The effect of thiol compounds on HQ-mediated inhibition of Akt phosphorylation and kinase activity. (A) RAW264.7 cells (5×10^6 cells/ml) pretreated with L-cysteine were incubated with HQ in the presence or absence of LPS (2 μg/ml) for 5 and 30 min. The phosphorylated or total levels of Akt from total lysates were analyzed by immunoblotting. (B) 2A1 cells (5×10^6 cells/ml) pretreated with DTT were stimulated with HQ in the presence or absence of insulin (100 μM). After immunoblotting, total or phospho-protein levels of Akt were identified with the total protein- or phospho-specific antibodies. (C) Kinase activity of immunoprecipitated Akt prepared from 2A1 cells (5×10^6 cells/ml) stimulated using Griess reagent with Crosstide (30 μM) after incubation with DTT and HQ for 10 min. (D) HQ and DTT were directly incubated with immunoprecipitated PDK1 for 10 min. Kinase activity was measured with Suntide (100 μM). Data (C and D) represent the mean ± SEM of three independent experiments performed in triplicate. The results (A and B) show one representative experiment of three. * p<0.05 and ** p<0.01 compared to the control group. * p<0.05 and ** p<0.01 compared to stimulus + HQ group with the same concentration.
Fig. 7. Identification of the HQ-Suntide adduct generated in the Akt kinase assay conditions. (A) HQ was incubated with immunoprecipitated Akt or various purified enzymes (p70S6K, PKA, PKCε, and SGK) for 20 min in the presence or absence of mer-EtOH. (B) HQ and Suntide were incubated in kinase assay buffer for 30 min. The HQ-Suntide adduct was then identified by LC-MS-MS analysis. (C) HQ and L-cysteine or DTT were incubated in PBS buffer for 30 min. The HQ-L-cysteine or HQ-DTT adducts were then identified by HPLC analysis. (D) The putative chemical reaction between HQ and Suntide. Data (A) represent the mean ± SEM of three independent experiments performed in triplicate. The results (B and C) show one representative experiment done with duplicate of three. ** p<0.01 compared to the control group.

Fig. 8. The role of Cys-310 in the kinase activity of Akt and HQ inhibition. (A) Amino acid sequences of Akt-WT and the Akt-C310A mutant. (B) HEK293 cells were transfected with plasmid constructs containing GFP-Akt-WT or GFP-Akt-C310A (1 μg/ml each) for 48 h and total lysates were prepared after treatment of pervanadate (100 μM) for 15 min. After immunoprecipitation with an anti-GFP antibody, the phosphorylated or total levels of Akt and GFP were analyzed by immunoblotting. (C) HQ was directly incubated with immunoprecipitated Akt-WT or Akt-C310A prepared from HEK293 cells (5×10⁶ cells/ml) transfected with GFP-Akt or GFP-Akt-C310A (1 μg/ml) for 10 min. Kinase activity was measured with Crosstide (30 μM). (D) A putative docking model between MHQ and Akt Cys-310. The results (B and C) show one representative experiment done with duplicate of three. * p<0.05 and ** p<0.01 compared to the control group.

Fig. 9. The effect of HQ derivatives on PDK1 kinase activity, LPS-induced NO release, and cell viability. (A) Chemical structures of HQ and the derivatives. (B) HQ and derivatives were incubated with immunoprecipitated PDK1 for 10 min. Kinase activity was measured with Suntide (100 μM). (C) RAW264.7 cells (1×10⁶ cells/ml) were incubated with HQ or derivatives in the presence or absence of LPS (2 μg/ml) for 24 h. Supernatants were collected and the NO concentrations in the supernatants were determined using Griess reagent. (D) IC₅₀ values of HQ and the derivatives were calculated from the NO inhibition profiles. (E) RAW264.7 cells (1×10⁶ cells/ml) were incubated with HQ or derivatives for 24 h. Cell viability was determined by MTT assay. * p<0.05 and ** p<0.01 compared to the control group.

Fig. 10. The effect of HQ derivative on septic shock-induced lethality and AA-induced ear edema formation. (A) Mice orally treated with HQ or JS-III-49 were intraperitoneally injected
with D-GalN (600 mg/kg) and LPS (50 μg/kg). Lethality was then observed for 7 days. (B) JS-III-49 and prednisolone were orally administered 30 min and then D-GalN (600 mg/kg)/LPS (50 μg/kg) was peritoneally injected and blood was collected after 1.5 h. Serum TNF-α levels released were assayed by ELISA. (C) The protection effect of JS-III-49 on liver damage induced by the treatment of D-GalN (600 mg/kg) and LPS (50 μg/kg) by was examined by histological analysis of liver sections as described in Materials and Methods. (D) Mice orally treated with JS-III-49 were applied with AA (2%/ear). Ear swelling was then measured with dial thickness gauge after 4 h. Data (B and D) represent the mean ± SEM of three independent experiments performed in triplicate. The results (A and C) show one representative experiment done with 7 mice of two. * p<0.05 and ** p<0.01 compared to the control group.
Fig. 1

(A) NO production (% of control) vs. Concentration (µM) for RAW264.7 cells, BM-macrophages, and Peritoneal macrophages.
Fig. 1

(B)

Surface level (MFI value)

HQ (µM)          0    0    100   100
LPS (2 µg/ml)     -    +    +    -

- no Ab
- TLR 2
- TLR 4
- dectin-1
- CD 69

**
Fig. 1

(C)

| HQ (50 μM) | LPS | Treatment time (h) |
|------------|-----|--------------------|
| -          | -   | -1                 |
| -          | +   | 0                  |
| +          | +   | +1                 |
| +          | +   | +2                 |

- TNF-α
- IL-6
- GAPDH
Fig. 2

(A)

Luciferase activity (Relative units)

| PMA (0.1 µM) | − | + | + | + | + | + |
| HQ (µM)      | − | − | 6.25 | 12.5 | 25 | 50 |

- **: Significant difference
- *: Significant difference

AP-1
NF-κB
Fig. 2

(B) 30 min

|                  | LPS (2 μg/ml) | HQ (μM) | U0126 (μM) |
|------------------|--------------|---------|------------|
|                  | -            | -       | -          |
|                  | +            | 6.25    | -          |
|                  | +            | 12.5    | -          |
|                  | +            | 25      | -          |
|                  | +            | 50      | -          |
|                  | +            | 50      | -          |

- p-IκBα
- β-actin

(C) 15 min

|                  | LPS (2 μg/ml) | HQ (μM) |
|------------------|--------------|---------|
|                  | -            | -       |
|                  | +            | 25      |
|                  | +            | 50      |

- p-IKKα/β
- p-IKKβ
- β-actin

15 min

|                  | LPS (2 μg/ml) | Curcumin (μM) |
|------------------|--------------|--------------|
|                  | -            | -            |
|                  | -            | 50           |

- p-IKKα/β
- p-IKKβ
- β-actin

5 min
Table 2: Effect of HQ on NF-κB (p65) expression.

| LPS (2 μg/ml) | 30 min |
|---------------|--------|
| -             | +      |
| HQ (μM)       | 6.25   |

Fig. 2: Western blot analysis of NF-κB (p65) and β-actin expression after treatment with LPS and HQ for 30 min.
|                     | NF-κB | Nuclei | Merged          |
|---------------------|-------|--------|-----------------|
| Normal              | ![](image) | ![image](image) | ![image](image) |
| Vehicle + LPS (2 μg/ml) | ![image](image) | ![image](image) | ![image](image) |
| HQ (50 μM) + LPS    | ![image](image) | ![image](image) | ![image](image) |
Fig. 3

(A)

|            | 2 min | 5 min | 15 min |
|------------|-------|-------|--------|
| LPS (2 μg/ml) | –     | +     | +      |
| HQ (50 μM) | –     | –     | +      |

- p-p85
- p-PDK1
- p-Akt (Thr 308)
- p-Akt (Ser 473)
- Akt
- β-actin
### Fig. 3

#### (B) Left panel

| LPS (2 μg/ml) | HQ (μM) | 30 min |
|---------------|---------|---------|
| -             | -       | +       |
| +             | -       | 3.125   |
| +             | -       | 6.25    |
| +             | -       | 12.5    |
| +             | -       | 25      |
| +             | -       | 50      |

**p-Akt (Ser 473)**

**Akt**

**β-actin**
(B) Right panel

|                | 15 min | 30 min |
|----------------|--------|--------|
| LPS (2 µg/ml)  | -      | +      | +      |
| HQ (50 µM)     | -      | -      | +      |
| p-Akt (Ser 473)| -      | +      | +      |
| Akt            | -      | -      | +      |
Fig. 3 (C)

| LPS | HQ (μM) | 6.25 | 12.5 | 25  | 50  | 100 |
|-----|---------|------|------|-----|-----|-----|
| −   | −       | +    | +    | +   | +   | +   |
| +   | +       | +    | +    | +   | +   | +   |

30 min

- **p-ERK**
- **p-MEK**
- **p-JNK**
- **p-p38**
- **β-actin**
(A) Suntide/crostide amino acid sequences

| Suntide       | RRKDGATMKTFCGTPE (14) |
|---------------|-----------------------|
| Crosstide     | GRPRTSSFAEG (15)      |
Fig. 4

(B) Akt kinase assay (Relative activity)

| Crosstide (30 μM) | HQ (μM) | Akt Kinase Assay |
|-------------------|---------|-----------------|
|                   | -       | 0.5 ± 0.2       |
|                   | +       | 8.0 ± 0.3       |
|                   | +       | 4.0 ± 0.1       |
|                   | +       | 2.0 ± 0.2       |

** indicates significant difference from control.
Fig. 4

(C)

|HQ (μM)| Insulin (100 μM)| 15 min| 30 min|
|---|---|---|---|
|−|−|+|+
|−|+|−|+
|+|+|−|+

**p-Akt (Ser 473)**

![Image of p-Akt (Ser 473) blot]

**Akt**

![Image of Akt blot]

![Bar graph showing relative intensity](chart.png)

* by guest on March 24, 2020
Fig. 4

(D)

PDK1 kinase assay (Relative activity)

| Condition          | 0 min | 10 min |
|--------------------|-------|--------|
| HQ (50 μM)         | -     | +      |
| Suntide (100 μM)   | +     | +      |
| MBP (10 ng/ml)     | -     | +      |

MBP **

10 min
Fig. 4 (E)
Fig. 5

(A) Right panel

NO production (% of control)

LPS (2 μg/ml) + + + + + + + + + +
HQ (100 μM) – + – – – – + + + + +
L-Cysteine (125 μM) – – + – – – – + – – –
N-Acetyl-L-cysteine (10 mM) – – – + – – – + – –
DTT (100 μM) – – – – + – – – + –
α-Tocopherol (12.5 μM) – – – – – + – – – +

**
#
##
Fig. 5

(A) Left panel

![Graph showing NO production (% of control) vs. LPS (2 μg/ml), HQ (μM), and DTT (100 μM). The graph includes bars representing different concentrations of LPS, HQ, and DTT, with symbols indicating significant differences.]

- **: P < 0.01 compared to control
- *: P < 0.05 compared to control
- #: P < 0.01 compared to LPS alone
- ##: P < 0.01 compared to HQ alone

| LPS (2 μg/ml) | HQ (μM)   | DTT (100 μM) |
|---------------|-----------|--------------|
| +             | 6.25, 12.5, 25 | +, +, +      |
| +             | 6.25, 12.5, 25 | +, +, +      |
Fig. 5

(B)

| Treatment time (min) | LPS (2 µg/ml) | HQ (25 µM) | L-Cysteine (150 µM) |
|----------------------|--------------|------------|---------------------|
| 0                    | +            | -          | -                   |
| 20                   | +            | +          | +                   |
| 40                   | +            | -30        | -60                 |
| 60                   | +            | -30        | -30                 |
| 80                   | +            | +30        | -60                 |
| 100                  | +            | -60        | +30                 |
| 120                  | +            | +          | +                   |
(C) Left panel

![Graph showing NO production (%) of control against concentration (µM)]

- LPS + Hydroquinone
- LPS + Resorcinol
- LPS + tert-Butylhydroquinone

NO production (% of control) vs. Concentration (µM)
Fig. 5

(C) Right panel

![Graph showing NO production (% of control) for different compounds at varying concentrations.](image-url)
### Table

| Condition                        | 5 min | 30 min |
|----------------------------------|-------|--------|
| LPS (2 μg/ml)                    | −     | +      |
| HQ (12.5 μM)                     | −     | +      |
| L-Cysteine (800 μM)              | −     | −      |

### Graph

#### p-AKT (Ser 473)

- At 5 min, no significant changes observed.
- At 30 min, a significant increase is observed.

#### AKT

- At 5 min, no significant changes observed.
- At 30 min, a significant increase is observed.

**Significance Levels**

- *: p < 0.05
- #: p < 0.01
- **: p < 0.001
- ###: p < 0.0001
Fig. 6

(B)

|                     | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---------------------|---|---|---|---|---|---|---|
| HQ (50 μM)          | - | - | + | + |   |   |   |
| DTT (100 μM)        | - | - | - | + |   |   |   |
| Insulin (100 μM)    | - | + | + | + |   |   |   |

HQ (50 μM) and DTT (100 μM) did not have a significant effect on Akt (Ser 473) and Akt expression levels after 30 min. Insulin (100 μM) significantly increased Akt (Ser 473) and Akt expression levels.

Relative unit
Fig. 6

(D)

|            | Suntide (100 µM) | HQ (50 µM) | DTT (100 µM) |
|------------|------------------|------------|--------------|
| 0 min      | -                | -          | -            |
| 10 min     | +                | +          | +            |
| PDK1 kinase activity (Relative unit) | | | |

0.0 0.2 0.4 0.6 0.8 1.0 1.2

10 min
Fig. 7

(A)

Kinase activity (% of control)

| HQ (μM)   | 1   | 20  | 50  | 50  | 50  | 50  | 50  | 50  |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|
| Mer-EtOH (100 μM) | +   | +   | +   | -   | -   | -   | -   | -   |

- Akt1
- p70S6K
- PKA
- PKCα
- SGK

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Fig. 7

(B)
Fig. 7

Mer-EtOH

Mer-EtOH Hydroquinone (MHQ)

Suntide

p-BQ

1873.923 + 110 -2 (2H) = 1981.923

MW 1981.923

1873.923
Fig. 8

(A)

PKBα/Akt1

PH                 Kinase Regulatory
1  5            108 150                     408                480
T308                        S473

KDGATMKTFC GTPEYLAPEVL → Akt-WT
KDGATMKTFA GTPEYLAPEVL → Akt-C310A
Fig. 8

(B) IP: GFP

|                | Vector | GFP-Akt-WT | GFP-Akt-C310A |
|----------------|--------|------------|---------------|
| Akt (Thr 308)  |        |            |               |
| Akt (Ser 473)  |        |            |               |
| GFP            |        |            |               |

WB

|                | +  | - | +  | - | +  | - |

Relative unit

| GFP-Akt-WT    | 1.0 | 0.8 | 1.0 | 0.8 | 1.0 | 0.8 |
| GFP-Akt-C310A | 1.0 | 0.8 | 1.0 | 0.8 | 1.0 | 0.8 |
Fig. 8

(C)

WT
C310A

IP level:
1
2.7 : RI

Akt kinase assay
(Relative unit)

Crosstide (30 μM)
HQ (50 μM)

+ + + +
− + − +

0.0 0.2 0.4 0.6 0.8 1.0 1.2

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(D) Left panel
(D) Left panel
Fig. 9

| Entry          | R₁   | R₂   |
|----------------|------|------|
| 1 (JS-III-49)  | H    | H    |
| 2 (JS-III-81)  | CH₃  | CH₃  |
| 3 (JS-III-69)  | CH₂OH| CH₃  |
| 4 (JS-III-89)  | (CH₂)₂CH₃| CH₃ |
| 5 (JS-III-73)  | (CH₂)₃CH₃| CH₃ |
| 6 (JS-III-87)  | (CH₂)₅CH₂OH| CH₃ |

(A) Hydroquinone (HQ)

1-6

Hydroquinone (HQ)
t-butyl hydroquinone (tBHQ)

Resorcinol

Benzoquinone (BQ)

L-cysteine

DTT

Mer-EtOH
Fig. 9

(B)

![Graph showing PDK1 activity with Suntide (10 ng/ml) and Drug (50 μM) treatments.](graph.png)
**Fig. 9**

(C)

**NO production (% of control)**

- **Concentration (µM)**
  - 0
  - 1.56
  - 3.125
  - 6.25
  - 12.5
  - 25

- **Compounds**: HQ, JS-III-49, JS-III-69, JS-III-73, JS-III-81, JS-III-87, JS-III-89

The graph illustrates the dose-response relationship between concentration and NO production for various compounds.
Inhibition of NO production

IC$_{50}$ (µM)

HQ  tBHQ  BQ  49  69  73  81  87  89

Fig. 9

(D)
Fig. 9

(E)

![Graph showing cell viability (% of control) against concentration (µM) for different compounds: HQ, JS-III-49, JS-III-69, JS-III-73, JS-III-81, JS-III-87, and JS-III-89. The graph indicates a decrease in cell viability with increasing concentration for all compounds, with JS-III-89 showing the most pronounced decrease.](image-url)
Fig. 10

(A) Left panel

![Graph showing Septic shock (% Survival) over Days for different treatment groups: CMC, CMC + LPS & D-Gal, HQ (50 mg/kg) + LPS & D-Gal, HQ (100 mg/kg) + LPS & D-Gal. The graph illustrates survival rates over a 7-day period, with different symbols and line styles representing each treatment group.](http://www.jbc.org/Downloaded_from)
Fig. 10

(A) Right panel

[Diagram showing septic shock (% Survival) over days with different treatments: CMC, CMC + LPS & D-Gal, JS-III-49 (50 mg/kg) + LPS & D-Gal, JS-III-49 (100 mg/kg) + LPS & D-Gal.]
Fig. 10

(B)

Serum TNF-α levels (pg/ml)

0.5% CMC  +  +  -  -
LPS + D-gal  -  +  +  +
JS-III-49 (50 mg/kg)  -  -  +  -
Prednisolone (10 mg/kg)  -  -  -  +
Fig. 10

| Condition                  | 6 h | 6 h |
|----------------------------|-----|-----|
| LPS + D-gal                | −   | +   |
| JS-III-49 (50 mg/kg)       | −   | +   |

**X100**

![Images showing histological sections with PV and CV annotations]
Fig. 10

(D)

AA-induced ear edema (% of control)

|                      | 0.5% CMC | JS-III-49 (25 mg/kg) | JS-III-49 (50 mg/kg) | Indomethacin (10 mg/kg) |
|----------------------|----------|----------------------|----------------------|-------------------------|
|                      | +        | -                    | -                    | -                       |
| JS-III-49 (25 mg/kg) | -        | +                    | -                    | -                       |
| JS-III-49 (50 mg/kg) | -        | -                    | +                    | -                       |
| Indomethacin (10 mg/kg) | -      | -                    | -                    | +                       |
Akt Cys310-targeted inhibition by hydroxylated benzene derivatives is tightly linked to their immunosuppressive effects
Ji Yeon Lee, Yong Gyu Lee, Jaehwi Lee, Keum-Jin Yang, Ae Ra Kim, Joo Young Kim, Moo-Ho Won, Jongsun Park, Byong Chul Yoo, Sanghee Kim, Won-Jea Cho and Jae Youl Cho

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