THE EFFECTS OF THE *CIS*- AND *TRANS*-GNETIN H ISOLATED FROM *PAEONIA SUFFRUTICOSA* AND A SYNTHETIC AURONE, (Z)-2-((5-(HYDROXYLMETHYL) FURAN-2-YL) METHYLENE) BENZOFURAN-3(2H)-ONE ON NF-κB AND MAPK PATHWAYS IN LPS-STIMULATED MACROPHAGES

By

Hyo S. Park

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Thesis Committee:
Dr. Anthony L. Farone, Chair
Dr. Elliot Altman
Dr. Norma Dunlap
Dr. Mary Farone
Dr. Anthony Newsome

I dedicate this research to my beloved grandmother, Yang-Soon Jeon, who is in heaven and Jae and Jiwoo Elina Park for their support and love.

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ABSTRACT

Inflammation is central to many disease processes such as autoimmunity and chronic inflammatory diseases, yet there are relatively few classes of compounds used as anti-inflammatory drugs. Nonsteroidal anti-inflammatory drugs (NSAIDS) and steroids have been used for many years and recently, biotherapeutic modifiers such as monoclonal antibodies and fusion proteins were developed to treat chronic inflammation, however, many patients experience serious side effects, and are thus reserved for devastating inflammatory diseases such as rheumatoid arthritis and ankylosing spondylitis. Recently, more attention has been given to herbal ingredients that have been used to treat inflammatory diseases. This is due to safety and efficacy of the herbal medicine, lower risk of side effects, lower costs, and potential use as adjunct treatments to Western medicines. In this study, *cis*- and *trans*-gnetin H and a synthetic aurone, (Z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (aurone 1) were evaluated for their mechanism of action in suppressing the intracellular proinflammatory pathways that regulate inflammatory cytokines in activated macrophages. All three compounds were found to suppress the cytokines, TNF-α, IL-1β, and IL-8, through inhibition of IKK-β which is a key regulator of the NF-κB pathway. The effects of *cis*- and *trans*-gnetin H on MAPK pathway was also investigated in THP-1 cells and showed both gnetin H compounds were able to suppress SAPK/JNK but ERK 1/2 were suppressed only by *cis*-gnetin H. Neither gnetin H were able to suppress p38 phosphorylation. Further studies using a murine cell line confirmed the effects of aurone 1 in suppressing the NF-κB pathway by concentration-dependent suppression of iNOS as

well as the production of NO which are regulated by the NF-κB pathway. The investigation of the effects of aurone 1 on MAPK mediators from human and murine macrophages suggested selective inhibition NF-κB signaling without affecting the MAPK pathway. Together, these findings suggest the gnetin H compounds and aurone 1 are anti-inflammatory compounds with therapeutic potential for the possible treatment of chronic inflammatory diseases.

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INTRODUCTION

Inflammatory diseases have been described for hundreds of years, yet understanding their pathogenesis has only evolved with an increased knowledge of the immune system. The development of pharmaceuticals for the treatments of inflammatory diseases such as rheumatoid arthritis, Crohn's disease, and psoriasis has paralleled our understanding of the pathogenic mechanisms involved. Early treatments for such diseases used natural products, such as aspirin from willow tree bark. These were followed by synthetic non-steroidal anti-inflammatory drugs (NSAIDS), and then by glucocorticoids and methotrexate, all of which have a variety of side effects ranging from gastric irritation and weight gain to bone marrow suppression (Bjarnason et al., 1993; Bongartz et al., 2006; Gabriel et al., 1991; Weinblatt, 1985). Aspirin and other NSAIDS exert their anti-inflammatory effects through various molecular mechanisms. One of the mechanisms involves the inhibition of cyclooxygenase (COX) which is responsible for formation of prostaglandin. Prostaglandins act as vasodilators and regulate smooth muscle involved in pro-inflammatory response (FitzGerald, 2003). Aspirin and NSAIDS promote 15-epi-lipoxin A4 synthesis that results in the increase of plasma nitric oxide (NO) and inhibits leukocyte-endothelium interactions, consequently exerting anti-inflammatory effects (Paul-Clark et al., 2004). Aspirin and NSAIDS exert their effect by blocking the inhibitor kappa B kinase (IKK) which is responsible for releasing sequestered pro-inflammatory transcription factors such as p65 (Yin et al., 1998). Glucocorticoids exert activity by interfering with transcription factor binding to promoter regions in inflammation response

genes, particularly cytokines (Steer et al., 2000). Methotrexate is a folate analogue that inhibits the dihydrofolate reductase required for biosynthesis of purines and pyrimidines thus suppressing proliferation of immune cells and release of adenosine, an endogenous anti-inflammatory agent (Cronstein, 2005; Cutolo et al., 2001; Gadangi et al., 1996).

A more in depth understanding of the immune mechanisms involved in the development of these diseases has led to drugs known as biological response modifiers (BRM) that target or neutralize the specific immune factors (infliximab, etanercept, and adalimumab) (Thalayasingam and Isaacs, 2011). Despite the advances in treatments for inflammatory diseases, most of the BRMs are used for the therapies of rheumatoid arthritis and inflammatory bowel disease, while effective treatments for other inflammatory diseases, such as lupus erythematosus, primary biliary cirrhosis, and scleroderma, are nonexistent (Bezalel et al., 2012; Kaplan and Gershwin, 2005).

Macrophages when activated by various types of inflammatory stimuli produce cytokines such as TNF-α through intracellular signaling pathways, such as the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Figure.1; Yamamoto and Gaynor, 2001). The NF-κB pathway, the major proinflammatory pathway, regulates inflammatory cytokine responses by activating, recruiting, and ubiquitinating various proteins and kinases (Sizemore et al., 2002). Upon activation of those various proteins and kinases, the inhibitor kappa B kinase (IKK) which is the major regulator of the NF-κB pathway, gets activated through phosphorylation which then phosphorylates NF-κB transcription factor and its antagonist, inhibitor kappa B (IκB). As it applies to

most of the kinases involved in earlier steps of signaling pathways, the NF-κB transcription factors are stored in the cytoplasm by IκB that prevents NF-κB from translocating into the nucleus and binding to its promoters (Ganchi et al., 1992). Recruitment of the necessary transcription factors results in the IKK phosphorylating IκB which then releases the transcription factors that ultimately translocate into the nucleus to promote transcription of the genes that are involved in the inflammatory response, including the production and release of biologically active cytokines such as TNF-α and interleukins (IL). The secreted cytokines induce chemotaxis, vasodilation, and cell proliferation as well as cell differentiation. The MAPK pathway regulates the proinflammatory cytokine responses in macrophages. Similar to NF-κB pathway, AP-1 transcription factor regulated by three major MAPK kinases, SAPK/JNK, ERK, and p38, modulate the transcription of proinflammatory cytokines and inducible nitric oxide synthase upon activation.

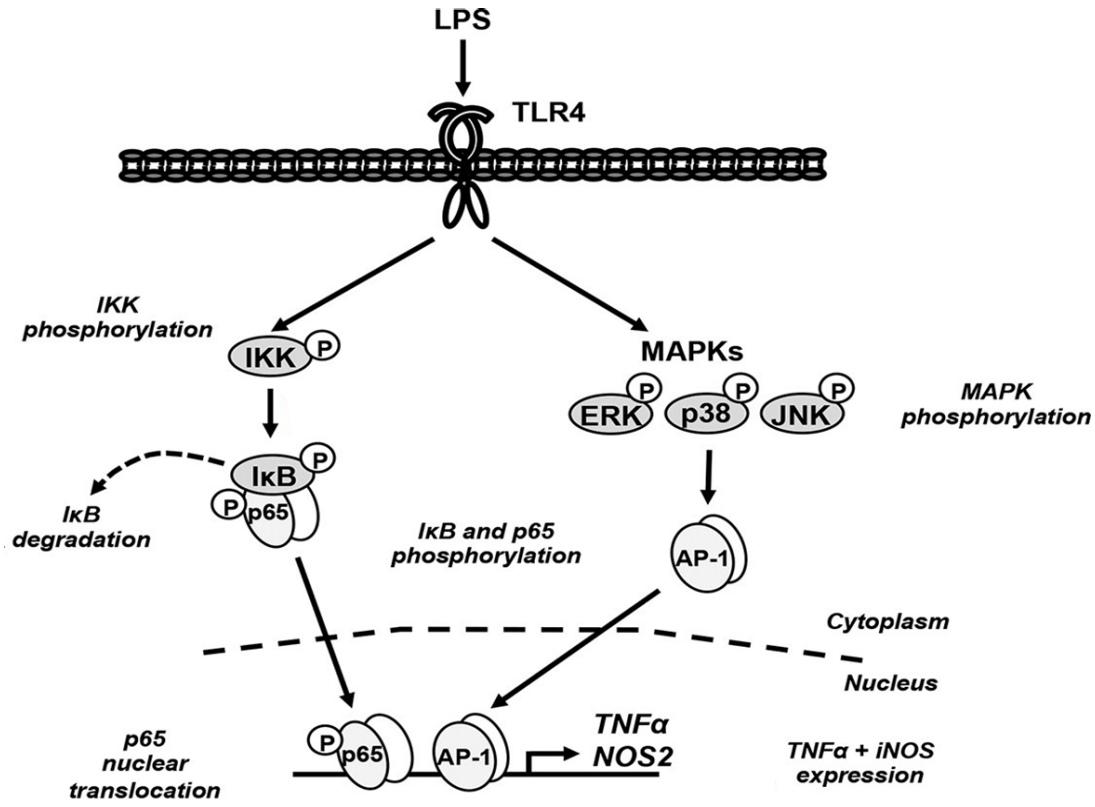

Figure 1. LPS-induced TLR4 signaling pathways.

Current treatments using recombinant proteins and monoclonal antibodies suppress inflammation by neutralizing TNF-α, the most potent cytokine secreted by cells to trigger amplification of the inflammatory response (Thalayasingam and Isaacs, 2011). Even current BRM treatments have serious side effects, including the development of resistance, malignancies, intestinal bleeding, allergic reaction, development of resistance, and are expensive for long-term treatment (Thalayasingam and Isaacs, 2011).

New and effective drugs to treat chronic inflammation are needed (O'Neill, 2006). Traditional Chinese medicine (TCM) and other ethnobotanical treatments have used plants for thousands of years to treat inflammation, although very few medicines currently used to treat inflammation are derived from botanicals. The growing attention to evaluate the efficacy and safety of the medicinal plants for medical uses has advanced various fields in bioscience and biotechnology to provide molecular mechanisms for their biological activities (Harvey et al., 2015). Also, modification and synthesis of analogs of plant derived compounds play a key role in the drug discovery field that promote development of candidates for pharmaceutical uses with supporting scientific evaluations (Dholwani et al., 2008).

In this study, the molecular mechanisms of two plant derived compounds and a synthetic aurone were evaluated for their ability to suppress proinflammatory responses in LPS-stimulated macrophages: 1) *cis*- and *trans*-gnetin H that were isolated from TCM plant, *Paeonia suffruticosa*, and 2) a synthetic aurone, (Z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (aurone 1). All three compounds showed concentration-dependent inhibition of TNF-α, IL-1β, and IL-8 in human macrophages. These effects were found to be mediated by suppression of NF-κB signaling pathway that regulates the transcription of these cytokines. The effects of the compounds were found to be through inhibition of nuclear translocation of p65 by suppressing activation of its upstream regulator, IKK-β, which is the key regulator of NF-κB activation. The activity of aurone 1 was further investigated in murine macrophages to study its effects on global NF-κB transcription, inducible nitric oxide synthase (iNOS) and nitric oxide (NO)

expression. Aurone 1 was found to suppress the global NF-κB transcription and iNOS expression resulting in reduced production of NO. The effects of the compounds on the mitogen-activated protein kinase (MAPK) mediators such as JNK, ERK, and p38 kinases were measured and showed that aurone 1 selectively inhibited NF-κB signaling without altering the MAPK pathway. Both *cis*- and *trans*-gnetin H were found to suppress SAPK/JNK, but ERK1/2 was suppressed by only *cis*-gnetin H. These results suggest the compounds exert anti-inflammatory activity in LPS-activated macrophages through inhibiting signaling pathways, NF-κB selectively by aurone 1 and broader inhibition of NF-κB and MAPK by gnetin H isomers. Together, these findings suggest the potential use of the compounds for pharmaceutical purposes as anti-inflammatory agents (Figure 2).

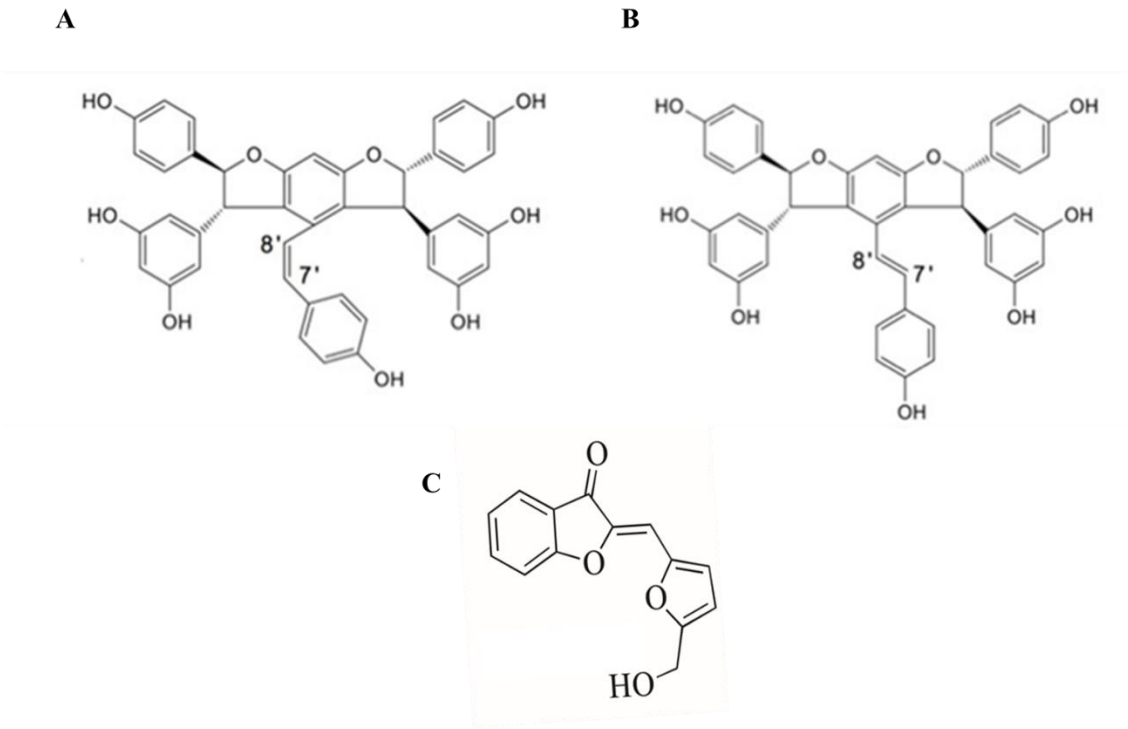

Figure 2. Structures of *cis*-gnetin H (A), *trans*-gnetin H (B), and (Z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (C).

FIRST ARTICLE

CIS- AND *TRANS*-GNETIN H FROM *PAEONIA SUFFRUTICOSA* SUPPRESS INHIBITOR KAPPA B KINASE PHOSPHORYLATION IN LPS-STIMULATED HUMAN THP-1 CELLS

Authors: Hyo S Park[a], Eric J Vick[a], Ying Gao[a], Chunnian He[b], Nadin Marwan Almosnid[a], Mary Farone[a], Anthony L Farone[a]

[a] Middle Tennessee State University, 1301 East Main Street, Murfreesboro, Tennessee 37132, USA

[b] Institute of Medicinal Plant Development, Chinese Academy of Medical Science, No. 151 Malianwa, North Road, Haidian District, Beijing 100193, PR China

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Abstract

Ethnopharmacological relevance: The inflammatory response is an important mechanism in host defense; however, overstimulation and chronic inflammation are involved in many important human diseases. Currently, tumor necrosis factor-alpha blockers such as infliximab and adalimumab along with methotrexate are used in cases of severe and chronic disease. However, there are severe side effects and limitations associated with these treatments. *Cis*- and *trans*-gnetin H are compounds isolated from the seeds of *Paeonia suffruticosa*, a medicinal plant used in traditional Chinese medicine for the

treatment of many conditions, including inflammatory diseases. In this study, we investigated possible anti-inflammatory mechanisms of *cis*- and *trans*-gnetin H against LPS-stimulated human THP-1 cells.

Material and Methods: PMA-differentiated THP-1 cells were pretreated with increasing concentrations of *cis*- and *trans*-gnetin H with or without LPS. Following treatment, cytotoxicity and the TNF-α, IL-1β, and IL-8 response were measured. We also characterized the nuclear translocation of NF-κB subunit p65 (RelA) by immunofluorescence and then investigated NF-κB activation by measuring the phosphorylation of NF-κB mediators, IKK-β, IκB α, and p65 by western blotting.

Results: We found that *cis*- and *trans*-gnetin H significantly inhibited the cytokine response in a concentration-dependent manner without affecting cell viability. *Cis*- and *trans*-gnetin H effectively inhibited nuclear translocation of p65 and phosphorylation of IKK-β, IκB α, and p65. While both compounds showed promising anti-inflammatory effects, *trans*-gnetin H was determined to be more effective in suppressing cytokine responses.

Conclusion: We demonstrated that c*is*- and *trans*-gnetin H suppress cytokine response in LPS-stimulated THP-1 cells by preventing activation of key signaling molecules, IKK-β, IκB α, and p65, involved in the NF-κB pathway and suggest the use of c*is*- and *trans*-gnetin H in potential therapies for conditions and diseases associated with chronic inflammation.

Key words: Gnetin H, *Paeonia suffruticosa*, Anti-inflammatory, Inhibitor kappa B kinase, Nuclear translocation, Traditional Chinese Medicine

1. Introduction

Control of inflammation is critical for treatment of autoimmune diseases and chronic inflammatory diseases and is achieved with steroidal and nonsteroidal anti-inflammatory drugs (NSAIDS), which comprise the major classes of anti-inflammatory compounds. NSAIDS have become one of the commonly used medications, although the negative effects of NSAIDS have raised concerns over awareness of their side effects such as gastrointestinal bleeding, hepatic and renal toxicity, and cardiovascular events (Aminoshariae et al., 2016; Bozimowski, 2015). Recently, monoclonal antibodies and fusion proteins have been developed to treat chronic inflammation (Thalayasingam and Isaacs, 2011); however, they can cause severe side effects, such as allergic reactions, malignancies, increased risk of infection, and stroke, and are limited to severe inflammatory diseases such as rheumatoid arthritis and ankylosing spondylitis (Bezalel et al., 2012; Bjarnason et al., 1993; Bongartz et al., 2006; Diamantopoulos et al., 2013). Another reason for the interest in new anti-inflammatory therapeutics is the high cost of treatment; the associated cost of inflammatory conditions well exceeds the cost associated with cancer treatment. It is increasingly recognized that inflammation contributes to many pathogenic processes, but the current classes of inflammatory drugs also have substantial side effects; this drives the search for safer and less expensive alternatives. Recently, more attention has been given to traditional Chinese medicine

(TCM) herbal ingredients that have been used to treat inflammatory diseases (Wang et al., 2013). TCM has received more attention because of its safety and efficacy, fewer side effects, lower cost, and potential use as an adjunct to Western medicine (Zhao et al., 2014). Providing a molecular basis for active ingredients found in herbal medicines further enhances their use in the treatment of serious conditions and diseases associated with chronic inflammation.

Activated macrophages produce biologically active cytokines such as tumor necrosis factor alpha (TNF-α), through the nuclear factor kappa B (NF-*κ*B) intracellular signaling pathway (Yamamoto and Gaynor, 2001a). This pathway involves numerous factors and kinases that are regulated by phosphorylation. Transcription factors that regulate the inflammatory response are sequestered in the cytoplasm by inhibitor molecules, inhibitor kappa B alpha (IκB α), which regulates NF-κB (Mercurio et al., 1997; Sizemore et al., 2002). IκB α binding to NF-κB inhibits NF-κB transactivation, translocation, and promoter binding (Ganchi et al., 1992). When the necessary factors are recruited, IκB α kinase (IKK) phosphorylates IκB α and releases NF-κB, which ultimately translocates to the nucleus and promotes the transcription and release of inflammatory genes, including the biologically active cytokines TNF-α and proinflammatory interleukins (IL). Released cytokines induce biological effects, such as chemotaxis, vasodilation, cell proliferation, and cell differentiation. TNF-α has been found to be the critical proinflammatory cytokine released by various cell types to initiate the amplification of inflammation (Aggarwal, 2003). Current therapeutics using corticosteroids, monoclonal antibodies, and

recombinant proteins suppress inflammation by preventing leukocyte activation or neutralizing TNF-α.

Cis- and *trans*-gnetin H compounds are oligostilbenes that have been isolated from the herbal plant *Paeonia suffruticosa*, an important medicinal plant that has been used in TCM for thousands of years. *Paeonia suffruticosa* has been widely used as an analgesic, anti-anaphylactic, anti-oxidative, and anti-inflammatory agent (He et al., 2010; Hu et al., 2010; Oh et al., 2003). The seeds of the plant are used in traditional medicine throughout East Asia to treat atherosclerosis, inflammation, infection, and cutaneous diseases (Choi et al., 2012; Gao et al., 2015). *Cis*- and *trans*-gnetin H compounds are isomers of resveratrol trimers; however, they differ in their olefinic moiety between C-7'/C8' (Figures 3A and 3B). Resveratrol is a stilbene produced by numerous types of plants and has anti-cancer and anti-inflammatory effects that have been studied extensively (Aggarwal et al., 2004; Manna et al., 2000). However, the anti-inflammatory mechanism of *cis*- and *trans*-gnetin H has not been fully described.

In this study, we sought to determine if the oligostilbenes *cis*-and *trans*-gnetin H could suppress inflammatory cytokines and the NF-κB pathway in PMA-differentiated human THP-1 cells stimulated with lipopolysaccharide. The THP-1 cell line has been used as an *in vitro* model to investigate roles for monocytes and macrophages in innate immunity, including their responses to various stimulants such as bacterial lipopolysaccharides (Chanput et al., 2014). THP-1 cells express MD2, CD14, and MYD88 genes as well as genes associated with inflammatory pathways, making the cell line an excellent model to

study cytokine response by LPS stimulation. Using THP-1 cells, we first examined the expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-8 and then assessed the key NF-κB inhibitory mechanism by measuring the nuclear translocation of the activated NF-κB transcription factor, p65. We further examined the expression of the activated/phosphorylated upstream kinase, IKK-β, and regulatory factor, IκB α, associated with the NF-κB pathway.

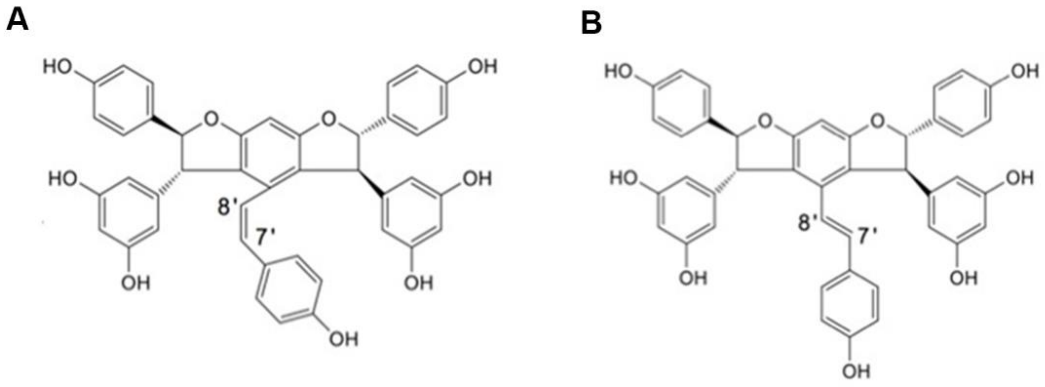


Figure 3. Chemical structures of *cis*-gnetin H (A) and *trans*-gnetin H (B).

2. Materials and Methods

2.1. Reagents

THP-1 cells (ATCC, TIB 202) were purchased from the American Type Culture Collection (Manassas, VA, USA). The γ-irradiated *Salmonella enterica* serotype Typhimurium lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (catalog number L6143, St. Louis, MO, USA). Dexamethasone, 3-(4-methylphenylsulfonyl)-2-propenenitrile **(**Bay 11-7082), dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), and RPMI 1640 culture media were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), enhanced chemiluminescence luminol (ECL) substrate, 100X penicillin/streptomycin solution, SDS-PAGE gels, and nitrocellulose membranes were obtained from Fisher Scientific (Pittsburgh, PA, USA). Alamar Blue was purchased from Life Technologies (Grand Island, NY, USA), ELISA kits and associated reagents were obtained from R&D Systems (Minneapolis, MN, USA). CellTiter-Glo® Luminescent assay was purchased from Promega (Madison, WI, USA). Bovine serum albumin (BSA) was obtained from EMD Millipore (Billerica, MA, USA). Cellomics NF-κB and BCA kits were purchased from Thermo Scientific (Waltham, MA, USA). Antibodies for the western blot analysis were purchased from Cell Signaling Technology (Denver, MA, USA).

2.2. Plant Material

Seeds of *Paeonia suffruticosa* were collected in Tongling, Anhui Province, P.R. China, and a voucher specimen has been deposited in the Seed Resource Bank of the Institution of Medicinal Plant Development, Chinese Academy of Medical Science and Peking Union Medical College (Gao et al., 2015).

2.3. Extraction and Isolation of *Cis*- and *Trans*-Gnetin H

Cis- and *trans*-gnetin H were extracted and isolated from the dried seeds of *Paeonia suffruticosa* as described previously (He et al., 2010). Briefly, the dried seeds were extracted with ethanol for 24 h at room temperature and then subfractionated using water, cyclohexane, chloroform, and ethyl acetate. *Cis*- and *trans*-gnetin H were purified from the ethyl acetate extract, further fractionated using chloroform-methanol elution followed by ODS-A C18 reversed-phase silica gel (MeOH–H_2O) and then purified by Sephadex LH-20 column chromatography. The compounds were suspended in DMSO to yield the desired concentration and stored at 4°C.

2.4. Maintenance and Differentiation of the THP-1 Cell Line

THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% complement-inactivated FBS and 1% penicillin/streptomycin solution (complete culture medium) at 37°C with 5% CO_2. The cells were adjusted to the desired concentrations for each experiment by centrifugation at 500 x g for 5 min and resuspension in complete

culture medium with 100 nM PMA. Cell concentrations were adjusted to $5x10^5$ cells/ml for most assays other than NF-κB nuclear translocation assay which used $2.5x10^5$ cells/ml. Cells were seeded onto 96-, 24-, or 12-well plates and incubated for 48 to 72 h to allow for differentiation. The cells were washed with serum-free RPMI 1640 medium before each experiment to remove undifferentiated cells.

2.5. Assessment of Cell Viability and Cytokine Response by ELISA

Differentiated THP-1 cells were pretreated with various concentrations of *cis*-gnetin H, t*rans*-gnetin H, or 1 μM dexamethasone for 1 h and then stimulated with 20 ng/ml LPS for 4 h. Dexamethasone is a synthetic glucocorticoid that suppresses cytokine responses and was used as a positive control (Abraham et al., 2006). Supernatants were collected for human cytokine ELISAs and the manufacturer's protocol was followed to assess the cytokine response. For remaining cells, relative cell viability was assessed as an internal control using Alamar Blue or CellGlow® Luminescent Assays. For Alamar Blue assays, cells were incubated with 1X Alamar Blue reagent overnight and viability was evaluated by measuring relative fluorescent units (RFU) on the *SpectraMax M2e* microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA) at Ex 560 nm and Em 590 nm. For CellTiter-Glo® luminescent assays, treated cells were lysed with the CellGlow reagent and the relative luminescence was measured using the *SpectraMax M2e* microplate reader as recommended by the manufacturer's protocol. Concentrations of DMSO (vehicle control) at 0.07, 0.13, 0.25, 0.5, and 1% were assessed for their potential effects on cytokine response as well as cell viability.

2.6. Indirect Immunofluorescence for NF-κB Nuclear Translocation

Differentiated THP-1 cells were pretreated with 50 μM *cis*-gnetin H, *trans*-gnetin H, or 10 μM Bay 11-7082 for 1 h and stimulated with 1 μg/ml LPS for 30 min. Bay 11-7082 is a compound that inhibits IKK α/β and IκB α phosphorylation and was used as a positive control (Rauert-Wunderlich et al., 2014). Treated cells were fixed, permeabilized, blocked, and stained with NF-κB (p65) primary antibody, Dylight 488 conjugated secondary antibody, and Hoechst 33342 dye, sequentially. The Hoechst and DyLight fluorophores detect changes in nuclear morphology (blue fluorescence) and NF-κB distribution (green fluorescence), respectively. Nuclear Translocation Bioapplication software on the Arrayscan VTI reader was used for image acquisition and analysis (Thermo Fisher Scientific, Waltham, MA, USA). For each well, at least 400 cells were automatically acquired and analyzed. The translocation index was calculated by measuring the average intensity difference of NF-κB between the identified cytoplasmic and nuclear regions.

2.7. Western Blot Analysis

Differentiated THP-1 cells were pretreated with various concentrations of *cis*-gnetin H, *trans*-gnetin H, or 10 μM Bay 11-7082 for 1 h and stimulated with 1 μg/ml PS for 15 min. Cells were lysed with radio-immunoprecipitation assay (RIPA) lysis buffer that contained a protease and phosphatase inhibitor cocktail. Cell lysates were then tested for protein concentration using a BCA protein assay and diluted with RIPA lysis buffer to normalize

protein concentrations. Lysates were mixed with sample loading buffer containing bromophenol blue, glycerol, sodium dodecyl sulfate (SDS), and 2-mercaptoethanol. The separated proteins were then transferred onto a nitrocellulose membrane and blocked with 5% nonfat-dried milk in 1X Tris-buffered saline (TBS) with 0.1% Tween-20 for 30 min. The blots were incubated with primary antibodies for p65, phosphorylated p-65 (Ser536), IKK β, phosphorylated IKK α/β (Ser176/180), IκB α, and phosphorylated IκB α (Ser32) at 4°C overnight or for 1 h at 22°C followed by incubation with HRP-conjugated secondary antibodies for 1 h at 22°C. The membranes were then developed by addition of ECL substrate, and images were collected by ChemiDoc XRS+ system chemiluminescence imager (Bio-Rad, Hercules, CA, USA).

2.8. Statistical Analysis

All experiments were conducted at least three times independently. Western blot band intensity analysis was performed using image lab software (Bio-Rad, Hercules, CA, USA) and statistical significance of concentration-response curves and the IC_{50} calculation were determined by GraphPad Prism version 6.0 (GraphPad, La Jolla, CA, USA). Numeric values of treated groups were compared to the control group, and results were expressed as the mean ± SEM. Statistical significance was analyzed using one-way analysis of variance followed by a Sidak test. A p value < 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of *Cis*-and *Trans*-Gnetin-H on PMA-Differentiated THP-1 Macrophages

Prior to the cytokine measurements, we assessed cell viability using Alamar Blue and CellGlow® luminescent assays to ensure cells were viable for the duration of the treatments. Pretreatment of cells with *cis*-gnetin H at 13, 25, or 50 µM followed by 20 ng/ml LPS had no significant effect on cell viability relative to the LPS alone control with both viability methods (Fig. 4). However, cells pretreated with *trans*-gnetin H at 50 µM showed less than 90% viability relative to the LPS alone control; therefore, concentrations of, 8, 15, and 30 µM *trans*-gnetin H were used for the cytokine response assay. The vehicle control, DMSO, at all of the concentrations tested showed no adverse effects on cell viability (data not shown).

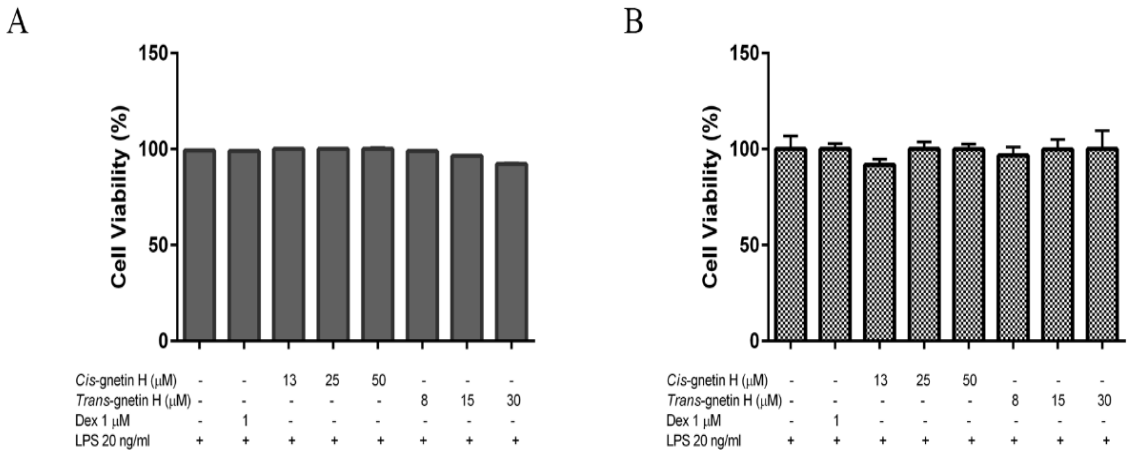
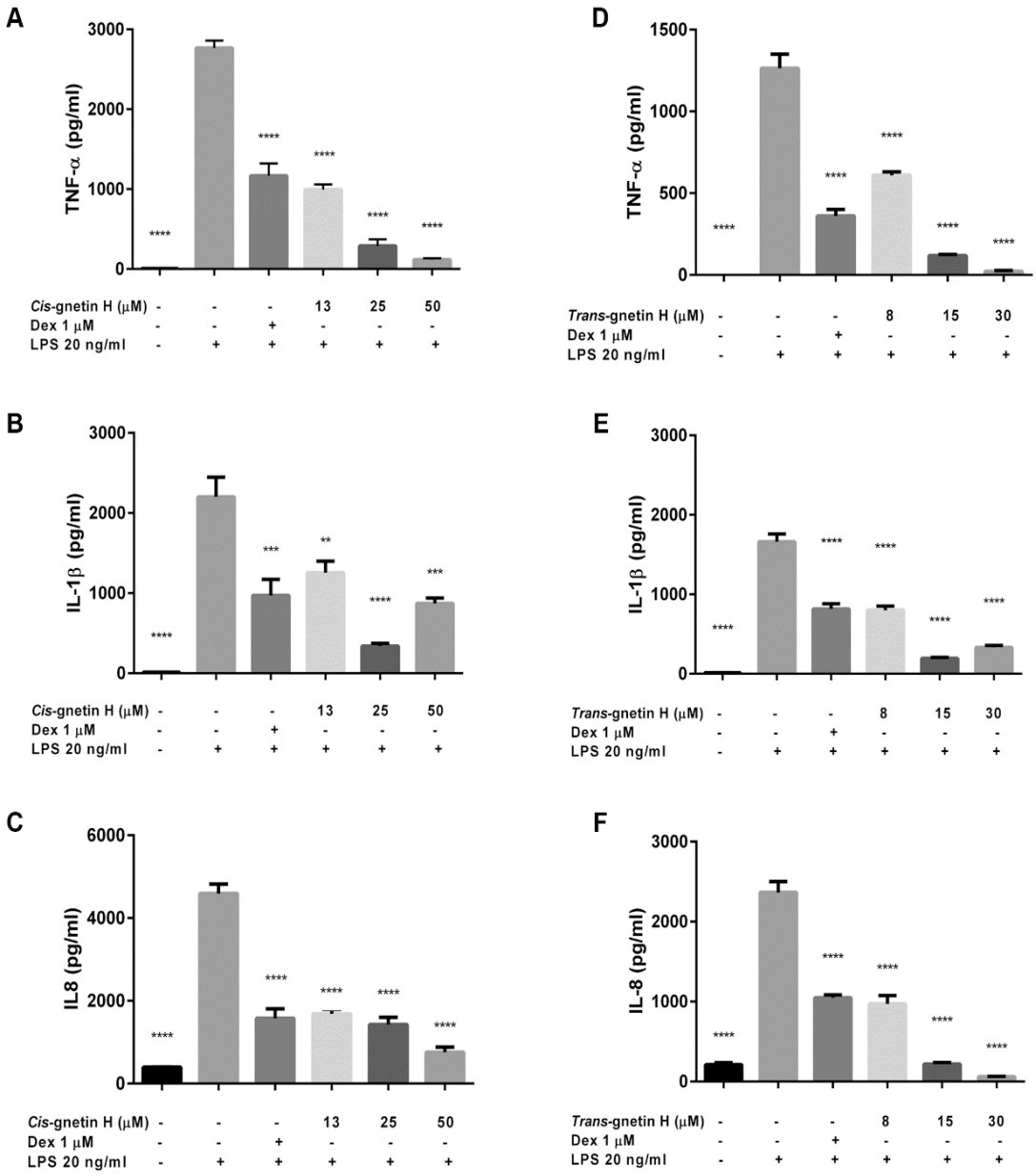


Figure 4. Cytotoxicity of *cis*- and *trans*-gnetin H on PMA-differentiated THP-1 macrophages.

PMA-differentiated THP-1 cells were treated with various concentrations of *cis*- and *trans*-gnetin H and tested for relative viability using the Alamar Blue assay (A) and luminescent assay (B). Cells were pretreated with 13, 25, 50 μM *cis*-gnetin H or 8, 15, 30 μM *trans*-gnetin H for 1 h followed by 20 ng/ml LPS for 4 h to determine relative % viability against LPS control. The results are presented as the mean ± SEM for triplicate measurements of at least three independent experiments.

3.2. The Effects of *Cis*- and *Trans*-Gnetin H on TNF-α, IL-1β, and IL-8 Response in LPS-Stimulated THP-1 Cells

We then investigated the effects of *cis*- and *trans*-gnetin H on expression of the inflammatory cytokines TNF-α, IL-1β, and IL-8 in LPS-induced THP-1 macrophages. The graphs shown in Figure 5 show that LPS at a concentration of 20 ng/ml increased the expression of TNF-α, IL-1β, and IL-8. The DMSO, at all concentrations tested, did not affect the cytokine response in LPS-treated THP-1 cells (data not shown). Dexamethasone (positive control) at a concentration of 1 μM showed 58%, 56%, and 63% inhibition of TNF-α, IL-1β, and IL-8, respectively. Cells treated with *cis*-and *trans*-gnetin H showed significant inhibition of TNF-α, IL-1β, and IL-8 ($p<0.05$). We then measured the concentration of *cis*-and *trans*-gnetin H that inhibited 50% of TNF-α (IC_{50}) and found IC_{50} values of 19 μM for *cis*-gnetin H and 6 μM for *trans*-gnetin H (Fig. 6).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Dex 1 μM - - + - - -
LPS 20 ng/ml - + + + + +

Figure 5. *Cis*- and *trans*-gnetin H inhibits TNF-α, IL-1β, and IL-8 response in LPS-stimulated THP-1 cells.

PMA-differentiated THP-1 cells were pretreated with 13, 25, and 50 μM *cis*-gnetin H or 8, 15, 30 μM *trans*-gnetin H for 1 h and then stimulated with 20 ng/ml LPS for 4 h. The secretion of TNF-α (A and B), IL-1β (C and D), and IL-8 (E and F) was determined by ELISA. The results are presented as the mean ± SEM for triplicate measurements of at least three independent experiments. **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with LPS-treated group.

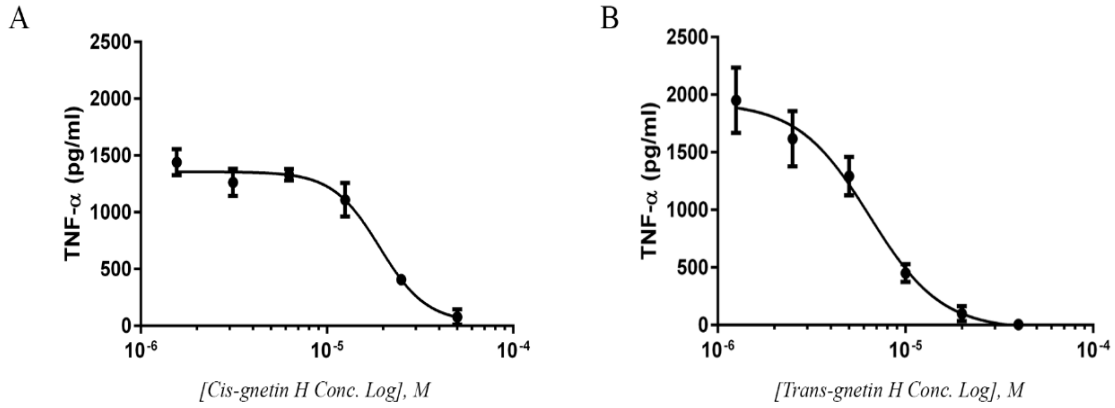


Figure 6. Concentration-response curves of *cis*- and *trans*-gnetin H on TNF-α.

PMA-differentiated THP-1 cells were pretreated with increasing concentrations of *cis*- (A) and *trans*- (B) gnetin H for 1 h and then stimulated with LPS for 4 h. Supernatants were

tested for human TNF-α by ELISA. The concentration-response curve was plotted to determine IC_{50} values with GraphPad Prism version 6.0 software. The IC_{50} value of *cis*-gnetin H was determined to be 19 μM and IC_{50} value of *trans*-gnetin H was determined to be 6 μM.

3.3. The Effects of *Cis*- and *Trans*-Gnetin H on NF-κB Transcription Factor Nuclear Translocation

We next examined the effects of *cis*- and *trans*-gnetin H on NF-κB nuclear translocation by targeting p65 (RelA), which is a significant transcription factor in the canonical NF-κB pathway. As Fig. 7A shows, p65 (green fluorescence) remained in the cytoplasm in untreated cells, whereas p65 translocated into the nucleus in LPS-challenged cells. Bay 11-7082 inhibited the p65 nuclear translocation, and both *cis*- and *trans*-gnetin H inhibited p65 nuclear translocation, as shown in Fig. 7A and B.

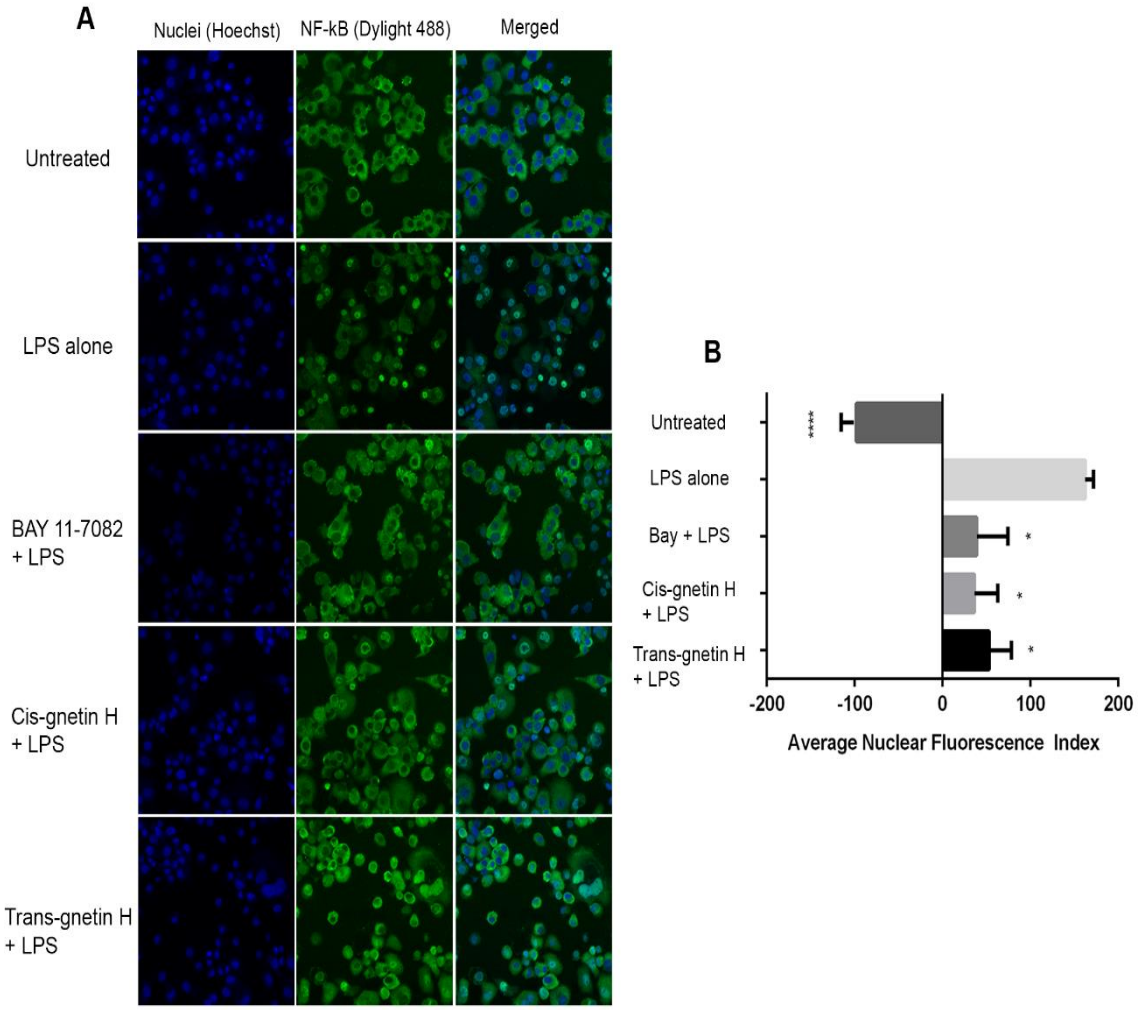


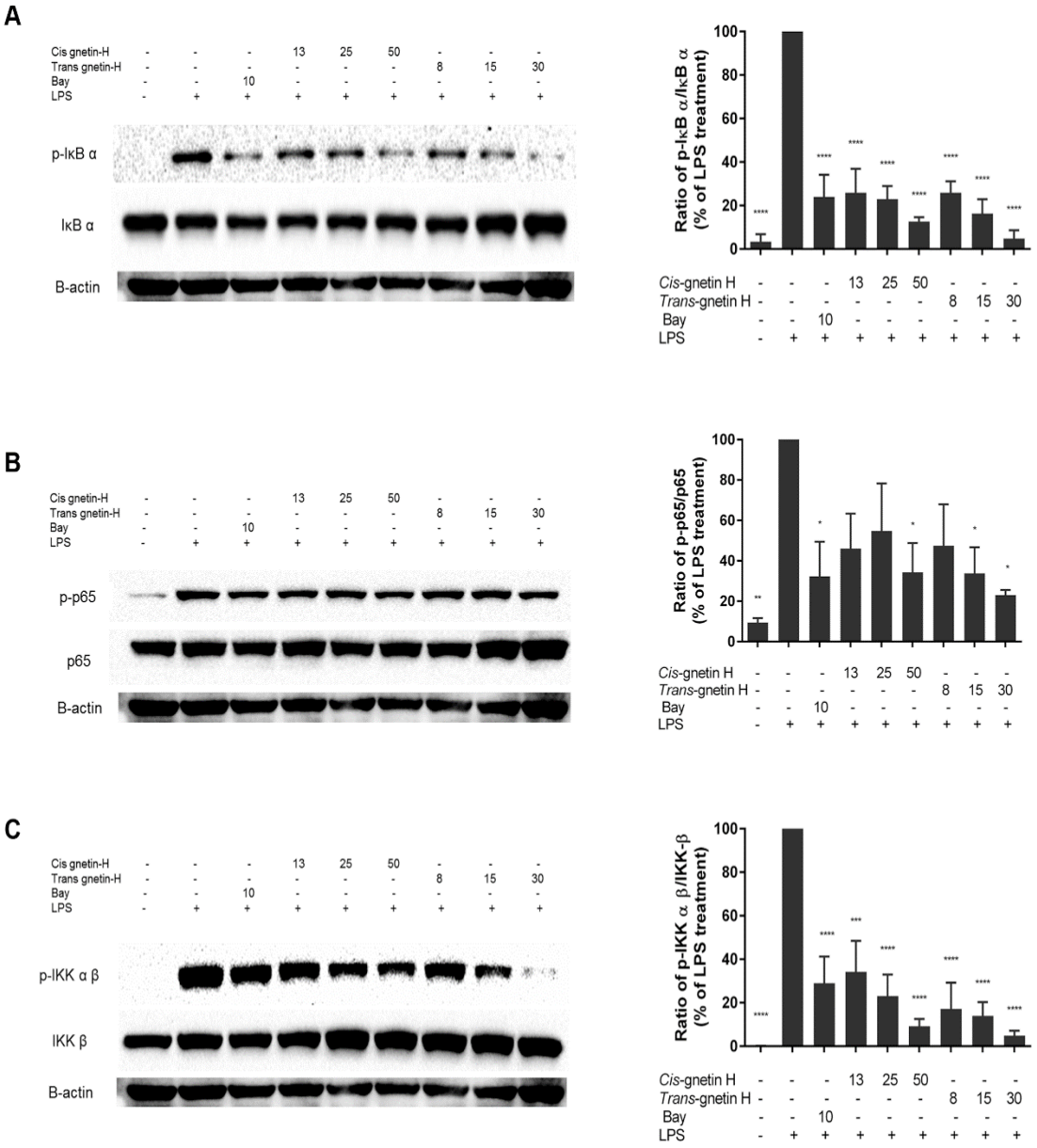
Figure 7. The effects of *cis*- and *trans*-gnetin H on the NF-κB transcription factor nuclear translocation.

PMA-differentiated cells were treated with *cis*- gnetin H at 50 μM, *trans*-gnetin H at 30 μM, or Bay 11-7082 at 10 μM for 1 h and then stimulated with 100 ng/ml LPS for 30 min. The transcription factor, p65, was stained with rabbit anti-p65 followed by Dylight 488-conjugated secondary antibody (green fluorescence) and Hoechst 33342 dye (blue

fluorescence), sequentially (A). The numeric index of nuclear fluorescence of p65 was collected using Nuclear Translocation Bioapplication software on the Arrayscan VTI reader. The results are presented as the mean ± SEM for triplicate measurements of at least three independent experiments. *$p < 0.05$, ****$p < 0.0001$ compared with LPS-treated group. (B).

3.4. The Effects of *Cis*- and *Trans*-Gnetin H on IKK β, IκB α, and p65 in the NF-κB Pathway

We also measured LPS-induced activation of important mediators in the NF-κB pathway, IKK β, IκB α, and p65. Our results showed that in LPS-stimulated cells, IκB α phosphorylation was also significantly inhibited by the two compounds in a concentration-dependent manner (Fig. 8A). p65 phosphorylation was only significantly inhibited by *cis*-gnetin H at the highest concentration (50 μM), but both 15 and 30 μM *trans*-gnetin H showed significant inhibition of p65 phosphorylation in a concentration-dependent manner (Fig. 8B). IKK β phosphorylation was also significantly inhibited by *cis*- and *trans*-gnetin H (Fig. 8C).


A
Cis gnetin-H - - - 13 25 50 - - -
Trans gnetin-H - - - - - - 8 15 30
Bay - - 10 - - - - - -
LPS - + + + + + + + +
p-IκB α
IκB α
B-actin
Ratio of p-IκB α/IκB α (% of LPS treatment)
100
80
60
40
20
0
Cis-gnetin H - - - 13 25 50 - - -
Trans-gnetin H - - - - - - 8 15 30
Bay - - 10 - - - - - -
LPS - + + + + + + + +
B
Cis gnetin-H - - - 13 25 50 - - -
Trans gnetin-H - - - - - - 8 15 30
Bay - - 10 - - - - - -
LPS - + + + + + + + +
p-p65
p65
B-actin
Ratio of p-p65/p65 (% of LPS treatment)
100
80
60
40
20
0
Cis-gnetin H - - - 13 25 50 - - -
Trans-gnetin H - - - - - - 8 15 30
Bay - - 10 - - - - - -
LPS - + + + + + + + +
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Cis gnetin-H - - - 13 25 50 - - -
Trans gnetin-H - - - - - - 8 15 30
Bay - - 10 - - - - - -
LPS - + + + + + + + +
p-IKK α β
IKK β
B-actin
Ratio of p-IKK α β/IKK-β (% of LPS treatment)
100
80
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Cis-gnetin H - - - 13 25 50 - - -
Trans-gnetin H - - - - - - 8 15 30
Bay - - 10 - - - - - -
LPS - + + + + + + + +

Figure 8. The effects of *cis*- and *trans*-gnetin H on IKK β, IκB α, and p65 on the NF-κB pathway.

PMA-differentiated cells were pretreated with 13, 25, and 50 μM *cis*-gnetin H and 8, 15, and 30 μM *trans*-gnetin H for 1 h and stimulated with 1 μg/ml LPS for 15 min. Phosphorylated IκB α (Ser32) and total IκB α (A), phosphorylated p65 (Ser536) and total p65 (B), phosphorylated (Ser176/180) IKK-β and total IKK-α β (C) were measured by western blotting. β-actin serves as loading control. Data are represented as the mean ± SEM for at least three independent experiments. **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with LPS-treated group.

4. Discussion

Inflammation is an important component of a healthy immune response, yet it is also associated with serious human diseases. Many inflammatory diseases do not have effective treatments for control of chronic inflammation. Currently available preventive therapies for chronic inflammatory and autoimmune diseases block the cytokine response, particularly TNF-α, which is the most important cytokine involved in the inflammatory response (Bradley, 2008). Anti-TNF therapy has been clinically demonstrated as the most effective approach to control inflammation (Postal and Appenzeller, 2011). Cytokines that are released by immune cells, such as TNF-α, play a critical role in inflammatory processes by inducing chemotaxis, activation of various types of cells, and amplification

of inflammation (Thalayasingam and Isaacs, 2011; Yamamoto and Gaynor, 2001a). The present study demonstrates that *cis*- and *trans*-gnetin H can effectively suppress the expression and release of LPS-induced cytokines, TNF-α, IL-1β, and IL-8, from PMA-differentiated human THP-1 cells in a concentration-dependent manner without toxicity (Fig. 3 and 4). It is notable that the two compounds effectively suppress not only TNF-α but also interleukins. This suggests that the compounds isolated from the seeds of *Paeonia suffruticosa* exert their inhibitory effects in a broad manner through suppressing the intracellular NF-*κ*B signaling pathway, which regulates TNF-α, IL-1β, and IL-8; the regulation of these cytokines by *cis*- and *trans*-gnetin H is important because they are considered the most important proinflammatory cytokines in human disease pathogenesis (Hiscott et al., 1993; Kunsch and Rosen, 1993; Shakhov et al., 1990). It is notable that the activity levels stimulated by the two compounds were significantly different. The IC_{50} values of *cis*- and *trans*-gnetin H for inhibition of TNF-α activity show that *trans*-gnetin H is greater than three-fold more efficient than *cis*-gnetin H at inhibiting TNF-α (Fig. 5). The only difference between the structures of the two compounds is the stereochemistry of the C-7'/C-8'olefinic moiety, which is the aromatic ring structure containing a hydroxyl group. (Gao et al., 2015). It may be worth determining if the difference in biological activity is attributable to the effect of the hydroxyl group or olefin position.

NF-*κ*B pathway activation is among the most complex proinflammatory pathways, and it is an essential pathway resulting in massive production of proinflammatory cytokines (Yamamoto and Gaynor, 2001a; Yamamoto and Gaynor, 2001b). To promote transcription and production of proinflammatory cytokines, NF-κB must be translocated

into the nucleus (Fujita et al., 1992; Lee et al., 2014a). p65, also known as RelA, is one of five members of the NF-κB transcription factor family and is the most abundant and critical factor of the canonical NF-κB pathway (Sasaki et al., 2005; Schmitz and Baeuerle, 1991; Yang et al., 2003). Studies have shown that the production of the TNF-α is strongly correlated with p65 nuclear translocation (Sung et al., 2014), and our data suggest that the non-toxic levels of *cis*-and *trans*-gnetin H effectively blocked translocation of p65 to the nucleus, which is consistent with our cytokine results (Fig. 6). To investigate whether the attenuation of the p65 in the cytoplasm is upstream of the p65 phosphorylation or activation, we further explored the effects of *cis*-and *trans*-gnetin H on activation of key mediators of NF-κB pathway that lie upstream of p65 nuclear translocation.

IκB α is a NF-κB antagonist that binds to the homodimer or heterodimer of NF-κB, preventing the complex from translocating into the nucleus (Ganchi et al., 1992). To investigate the effect of p65 cytoplasm attenuation, we first determined the activation of IκB α by measuring phosphorylated IκB α, and we found that the p-IκB α expression was significantly suppressed by both *cis*-and *trans*-gnetin H (Fig. 7A). These data suggest that the attenuation of p65 in the cytoplasm is due to the antagonistic effect of IκB α. We also investigated phosphorylation of p65, which is important in nuclear transport and transactivation (Viatour et al., 2005), and observed suppressed phosphorylation (Fig. 7B). This led us to investigate the activation of IKK-β, the kinase that phosphorylates both IκB α and p65 (Sizemore et al., 2002; Yang et al., 2003). As shown by our data, IKK-β phosphorylation was significantly blocked by both *cis*-and *trans*-gnetin H (Fig. 7C), which suggests that compounds exert inhibition at the level of kinases that activate IKK

through phosphorylation or direct inhibition of IKK-β activation. The exact mechanism of LPS-induced IKK-β phosphorylation is unclear; however, IKK-β activation is required to activate the NF-κB pathway in LPS-induced macrophages (Israel, 2010; Li et al., 1999). Studies have suggested IKK-β as a potential therapeutic target for multiple diseases involving chronic inflammation such as asthma (Catley et al., 2005), bone diseases (Ruocco and Karin, 2005), and obesity (Arkan et al., 2005).

Macrophages are the initial inflammatory regulator and therefore, suppressing macrophage activation can alleviate inflammatory responses and slow the severity of disease progression caused by chronic inflammation. Our results suggest that *cis*- and *trans*-gnetin H compounds can significantly limit the cytokine response in human THP-1 macrophages through suppressing the activation of IKK-β, which is the most attractive target for suppression of the NF-κB pathway (Tak and Firestein, 2001). Recently, great attention has been given to the derivatives of resveratrol in medicinal and food chemistry as alternatives to overcome the poor bioavailability of resveratrol due to its rapid metabolism *in vivo* which compromises the biological and pharmacological benefits (Neves et al., 2012). According to recently published study on pharmacokinetics of resveratrol derivatives in humans, dimer derivatives of resveratrol which include gnetin C compounds, showed increased bioavailability by oral administration compared to the resveratrol monomer (Tani et al., 2014). Therefore, *cis*-and *trans*-gnetin H, which are trimers of resveratrol, may be worth investigating for their bioavailability through oral consumption. Experiments are in progress to better understand the mechanism and target for these compounds. In conclusion, we suggest *cis*-and *trans*-gnetin H have potential

pharmacological benefits for treating diseases and conditions that involve chronic inflammation.

5. Acknowledgments

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SECOND ARTICLE

SUPPRESSION OF LPS-INDUCED NF-ΚB ACTIVITY IN MACROPHAGES BY THE SYNTHETIC AURONE, (Z)-2-((5-(HYDROXYMETHYL) FURAN-2-YL) METHYLENE) BENZOFURAN-3(2H)-ONE

Hyo S. Park[a], David E. Nelson[a*], Zachary E. Taylor[b], James B. Hayes[a], Kirsten D. Cunningham[b], Brock A. Arivett[a], Rajarshi Ghosh[a], Larissa C. Wolf[a], Kimberley M. Taylor[b], Mary B. Farone[a], Scott T. Handy[b], Anthony L Farone[a]

[a] Middle Tennessee State University, 1301 East Main Street, Murfreesboro, Tennessee 37132, USA

[b] Middle Tennessee State University, Department of Chemistry, 1301 East Main Street, Murfreesboro, TN 37132, USA

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Abstract

Suppressing cytokine responses has frequently been shown to have promising therapeutic effects for many chronic inflammatory and autoimmune diseases. However, the severe side effects associated with the long-term use of current treatments, such as allergic reactions and increased risk of stroke, have focused attention towards the targeting of intracellular signaling mechanisms, such as NF-κB, that regulate inflammation. We synthesized a series of non-natural aurone derivatives and investigated their ability to

suppress pro-inflammatory signaling in human monocyte (THP-1) and murine macrophage-like (RAW 267.4) cell lines. One of these derivatives, (Z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (aurone 1), was found to inhibit LPS-induced secretion of the pro-inflammatory cytokines, tumor-necrosis factor α (TNFα), interleukin 1β (IL-1β), and IL-8 by THP-1 cells. To investigate the mechanism, we probed the effect of aurone 1 on LPS-induced MAPK and NF-κB signaling in both THP-1 and RAW264.7. While aurone 1 pre-treatment had no effect on the phosphorylation of ERK, JNK, or p38 MAPK, it strongly suppressed activation of IKK-β, as indicated by attenuation of Ser176/180 phosphorylation, resulting in decreased phosphorylation of p65 (ser536) as well as phosphorylation (ser32) and degradation of IκBα. Consistent with this, aurone 1 significantly reduced LPS-stimulated nuclear translocation of p65-containing NF-κB transcription factors and expression of an mCherry reporter of TNFα gene transactivation in RAW264.7 cells. Inhibition of TNFα expression at the transcription level was also demonstrated in THP-1 by qRT-PCR. In addition to its effects on cytokine expression, aurone 1 pre-treatment decreased expression of iNOS, a bona fide NF-κB target gene and marker of macrophage M1 polarization, resulting in decreased NO production in RAW264.7 cells. Together, these data indicate that aurone 1 may have the potential to function as a pharmacological agent for the treatment of chronic inflammation disorders.

Key words: Aurone, NF-κB, IKK-β, p65, iNOS, anti-inflammatory, live cell imaging, macrophage, THP-1, dual reporter RAW 264.7

1. Introduction

Inflammation is a vitally important process, which can be triggered by stress, injury, or infection, and serves to protect the body from harmful stimuli and pathogens and to facilitate the repair of damaged tissues. However, prolonged inflammation is associated with numerous chronic inflammatory and autoimmune disorders as well as cancer and neurodegenerative diseases. Studies show that suppressing the production of important pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) can effectively control inflammation (Brennan et al., 1995). Currently, monoclonal antibodies and recombinant fusion proteins that target TNFα are used to treat severe cases of chronic inflammatory and autoimmune diseases (Thalayasingam and Isaacs, 2011). However, due to the possible side effects of these therapeutics, including allergic reactions, increased risk of infections, malignancies, and stroke, attention has been refocused on targeting the specific intracellular signaling pathways that regulate cytokine production rather than the secreted cytokines themselves (Barnes and Karin, 1997; Lewis and Manning, 1999).

The nuclear factor kappa B (NF-κB) pathway is a key regulator of the cellular response to stress and pathogens, controlling the expression of genes involved in proliferation, differentiation, cell survival, cell death, and inflammation. The pathway consists of a family of five transcription factors, RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Verma et al., 1995), which share a conserved Rel homology domain (Ghosh et al., 1998). These proteins may form both homo- and heterodimers with the most prevalent forms being p65 in complex with either p50 or p52 (Schmitz and Baeuerle,

1991). The NF-κB pathway is primarily regulated by phosphorylation and ubiquitination of regulatory proteins such as inhibitor kappa B alpha (IκBα), which maintains canonical (i.e. p65-containing) transcription factors in an inactive state within the cytoplasm, and upstream kinases, the most prominent of which being the inhibitor kappa B kinase (IKK) complex that promotes the phosphorylation and proteasomal degradation of IκB proteins, allowing NF-κB transcription factors to translocate to and accumulate in the nucleus (Zandi and Karin, 1999).

Aurones are a sub-family of the flavonoids, a naturally occurring pigmented compound that provides the coloration in various yellow-flowering plants (Harborne and Williams, 2000). As a relatively minor plant component, they have only recently attracted research attention (Haudecoeur and Boumendjel, 2012). As might be expected for compounds in the flavonoid family, they display a wide range of biological activities, including anti-cancer, anti-bacterial, anti-fungal, and anti-malarial activities (Carrasco et al., 2014; Demirayak et al., 2015; Song et al., 2015; Tiwari et al., 2012). Most of the compounds studied are similar to those found in nature, meaning that the aromatic rings tend to be highly oxygenated and synthetically complex. Based upon a recent modification of the simple and efficient method reported earlier by Hawkins and Handy (2013), we have been able to synthesize a much broader range of non-natural aurone derivatives for a more comprehensive evaluation of their activities and potential for practical application.

In this study, we show that the synthetic aurone derivative (Z)-2-((5-(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one (aurone 1) exhibits anti-inflammatory

effects in both human (THP-1) and murine (RAW 264.7) macrophage-like cells, suppressing lipopolysaccharide (LPS)-induced expression of inflammatory cytokines, including TNFα at the transcriptional level. These effects were found to be mediated by inhibition of canonical NF-κB but not MAPK signaling with aurone 1 pre-treatment suppressing nuclear translocation of p65 in both human and murine cells and this was accompanied by decreased phosphorylation of p65 (ser536) and IκBα (ser 32) and decreased IκBα degradation. The effect of aurone 1 on p65 and IκBα phosphorylation was consistent with decreased IKK activity, as reported by reduced IKKβ phosphorylation (ser176/180). Finally, to test whether aurone 1 could block LPS-induced classical activation (M1) of macrophages, we measured iNOS expression and NO production and found that both were significantly reduced by aurone 1 pretreatment in RAW 264.7 cells. Together, these data show that the anti-inflammatory activity of aurone 1 is a function of its ability to inhibit canonical NF-κB signaling by directly or indirectly attenuating LPS:Toll-like receptor 4 (TLR4)-induced IKK activity without exhibiting cytotoxicity, raising the possibility that aurone 1 could be used as an anti-inflammatory drug.

2. Materials and Methods

2.1. Reagents

THP-1 (ATCC TIB 202) and RAW 264.7 (ATCC TIB 71) were purchased from American Type Culture Collection (Manassas, VA, USA). RAW 264.7 cells stably

expressing p65-EGFP fusion proteins from an endogenous p65 promoter and also incorporating a destabilized mCherry reporter of TNF-α promoter transactivation were a gift from Dr. Iain Fraser (NIH, Bethesda, MD, USA; (Sung et al., 2014)). Lipopolysaccharide (LPS; *Salmonella enterica* serotype thyphimurium), dexamethasone**,** 3-(4-methylphenylsulfonyl)-2-propenenitrile **(**Bay 11-7082), U0126 (B5556), staurosporine, dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), protease inhibitors, phenylmethanesulfonyl fluoride (PMSF), ATP, and RPMI 1640 culture media were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Corning Inc. (Corning, NY, USA). The fetal bovine serum (FBS), enhanced chemiluminescence luminol (ECL) substrate, penicillin/streptomycin, sodium pyrophosphate, SDS-PAGE gels, and nitrocellulose membranes were obtained from Fisher Scientific (Pittsburgh, PA, USA). D-luciferin was purchased from Gold-Biotechnology (St. Louis, MO, USA). Alamar blue was purchased from Life Technologies (Grand Island, NY, USA). ELISA kits as well as associated reagents were obtained from R&D Systems (Minneapolis, MN, USA). Bovine serum albumin (BSA) was obtained from EMD Millipore (Billerica, MA, USA). L-glutamine and FBS for RAW 264.7 growth medium was obtained from GE Healthcare Life Sciences (Piscataway, NJ, USA). Cellomics NF-κB and BCA kits were purchased from Thermo Scientific (Waltham, MA, USA).

2.2. Synthesis and Characterization of Aurone Derivatives

2.2.1 (Z)-2-(furan-2-yl)methylene)benzofuran-3(2H)-one 3

Synthesized according to the method reported in (Hawkins and Handy, 2013).

2.2.2 General Method for the Microwave Synthesis of Aurone Derivatives 1, 2, 4, and 5

Coumaranone (1.00 mmol) and aldehyde (1.00 mmol) were combined in a dry microwave vial. 1 mL of the deep eutectic solvent formed from a 1:2 molar ratio of choline chloride and urea was added. The reaction mixture was heated to 90°C in a CEM Discover microwave with stirring for 30 min. At this point, the reaction was cooled to room temperature and partitioned between water and methylene chloride. The organic layer was separated and concentrated to dryness *in vacuo*. Further purification was performed as noted.

2.2.3 (Z)-2-(5-hydoxymethylfuran-2-yl)methylene)benzofuran-3(2H)-one 1

The crude reaction mixture was then purified by trituration with ether to afford 48.9 mg (20%) of aurone 1 as a black solid (MP = 61-63 °C). IR (neat, thin film): 3360, 2920, 1689, 1639, 1598, 1479, 1402, 1300 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) 7.78 (d, J = 6.8 Hz, 1H), 7.64 (ddd, J = 6.8 Hz, 1H), 7.30 (d, J = 6.8 Hz, 1H), 7.22 (t, J = 6.8 Hz, 1H), 7.11 (d, J = 4.5 Hz ,1H), 6.88 (s, 1H), 6.51 (d, J = 4.5 Hz, 1H), 5.74 (d, 2H); ^{13}C NMR

($CDCl_3$, 125 MHz) 184.03, 165.66, 156.51, 147.45, 144.33, 136.44, 124.49, 123.39, 122.34, 119.35, 112.95, 110.14, 102.24, 57.78.

2.2.4 (Z)-2-(5-methylfuran-2-yl)methylene)benzofuran-3(2H)-one 2

The reaction yielded 138.6 mg (61 %) of aurone 2 as a light brown solid (MP = 62-64 °C). IR (neat, thin film): 3010, 2850, 1710, 1650, 1590, 1520, 1300, 1190, 750 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): 7.78 (d, *J* = 6.7 Hz, 1H), 7.62 (ddd, *J* = 8.5, 7, 1 Hz, 1H), 7.30 (d, *J* = 7.9 Hz, 1H), 7.20 (t, *J* = 7.4 Hz, 1H), 7.07 (d, *J* = 3.4 Hz, 1H), 6.86 (s, 1H), 6.22 (d, *J* = 3.2 Hz, 1H), 2.41 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): 183.93, 165.57, 156.54, 147.41, 144.38, 136.44, 124.50, 123.39, 122.35, 119.34, 112.96, 110.16, 102.22, 14.19.

2.2.5 (Z)-2-(2-hydroxybenzylidene)benzofuran-3(2H)-one 4

The crude solid was purified by trituration with diethyl ether to yield 120.1 mg (50 %) of aurone 4 as a yellow solid (MP = 215-216 °C). IR (neat, thin film): 3200 (br), 3100, 1710, 1650, 1600, 1450, 1380, 1120, 890, 750 cm^{-1}; ^1H NMR (Acetone D_6, 300 MHz): 8.25 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.76 (m, 2H), 7.48 (d, *J* = 9.4 Hz, 1H), 7.39 (s, 1H) 7.28 (m, 2H), 7.01 (m, *2*H). ^{13}C (Acetone-D_6, 75 MHz): 183.56, 165.72, 159.53, 146.13, 136.84, 131.81, 131.61, 124.05, 123.57, 121.82, 119.49, 118.91, 116.50, 113.08, 107.60.

2.2.6 (Z)-2-(3-hydroxybenzylidene)benzofuran-3(2H)-one 5

The crude reaction mixture was then purified by trituration with ether to afford 201.2 mg (85 %) of aurone **5** as a green-yellow solid (Decomp. = 86-89 °C). IR (neat, thin film):

3010, 1700, 1650, 1600, 1450, 1120, 890, 750 cm^{-1}; ^{1}H NMR (DMSO-D_6, 500 MHz): 7.78 (m, 2H), 7.52 (d, J = 8.7 Hz, 1H), 7.41 (s, 1H), 7.37 (d, J = 7.5 Hz, 1H), 7.28 (m, 2H), 6.83 (m, 2H). ^{13}C NMR (DMSO-D_6, 75 MHz): 184.19, 165.96, 146.69, 138.26, 133.40, 130.50, 124.87, 124.52, 123.11, 123.05, 121.45, 118.23, 118.16, 113.71, 113.16.

2.2.7 General Synthesis of Aurone Derivatives 6-9

Coumaranone (1.00 mmol) and aldehyde (1.00 mmol) were combined in a dry vial. 3.5 g of neutral alumina was then added followed by 5 ml of dichloromethane. The reaction mixture was stirred for 12 h at 25 °C. The reaction mixture was then filtered and the dichloromethane layer collected and concentrated to dryness *in vacuo*. Further purification was performed as noted.

2.2.8 (Z)-2-(4-hydroxybenzylidene)benzofuran-3(2H)-one 6

The crude solid was purified by trituration with diethyl ether to yield 81.7 mg (34 %) of aurone 6 as a yellow-orange solid (MP = 258-260 °C). IR (neat, thin film): 2980, 1700, 1560, 1300, 790 cm^{-1}; ^{1}H NMR (DMSO-D_6, 300 MHz): 7.85 (m, 2H), 7.75 (m, 2H), 7.52 (d, *J* = 8.2 Hz, 1H), 7.27 (t, *J* = 6.87 Hz, 1H), 6.91 (m, 3H); ^{13}C NMR (DMSO-D_6, 75 MHz): 183.67, 165.53, 160.46, 145.23, 137.69, 134.28, 124.62, 124.24, 123.31, 121.81, 116.76, 114.03, 113.71.

2.2.9 (Z)-2-(4-hydroxy-3-methoxybenzylidene)benzofuran-3(2H)-one 7

The resulting product was purified by trituration with ether and yielded 82.79 mg (31 %) of aurone 7 as an orange solid (Decomp = 185 °C). IR (neat, thin film): 3700, 3420, 2960, 2850, 2360, 1400 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) 7.82 (d, *J* = 7.8 Hz, 1H), 7.65 (t, *J* = 7.5 Hz, 1H), 7.49 (m, 2H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.21 (t, *J* = 7.2 Hz, 1H), 7.00 (d, *J* = 8.7 Hz, 1H), 6.87 (s, 1H), 5.97 (s, 1H), 4.00 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) 184.58, 165.76, 147.90, 146.77, 145.82, 136.62, 126.71, 124.92, 124.70, 123.44, 122.02, 115.07, 114.00, 113.42, 112.93, 56.09.

2.2.10 (Z)-2-(2-hydroxy-3-methoxybenzylidene)benzofuran-3(2H)-one 8

The crude solid was purified by trituration with diethyl ether to yield 47.5 mg (18 %) of 8 as a yellow solid (Decomp = 211 °C). IR (neat, thin film): 3300 (br), 1710, 1650, 1600, 1500, 1390, 1110, 750 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): 7.90 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.82 (d, *J* = 7.5 Hz, 1H), 7.64 (ddd, *J* = 8.4, 7.2, 0.9 Hz, 1H), 7.47 (s, 1H), 7.32 (d, *J* = 8.4 Hz, 1H), 7.21 (t, *J* = 7.5 Hz, 1H), 6.93 (m, 2H), 6.19 (s, 1H), 3.93 (s, 3H); ^{13}C NMR ($CDCl_3$, 300 MHz): 184.72, 166.06, 147.07, 146.61, 146.24, 136.72, 124.74, 123.56, 123.38, 121.97, 119.89, 118.97, 113.00, 111.99, 107.03, 56.24.

2.2.11 (Z)-2-(3-hydroxy-4-methoxybenzylidene)benzofuran-3(2H)-one 9

The crude solid was purified by trituration with diethyl ether to yield 45.2 mg (17 %) of 9 as a brown solid (MP = 186-187 °C). IR (neat, thin film): 3200 (br), 2920, 1700, 1650, 1600, 1290, 1120, 750 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): 7.80 (d, *J* = 4.8 Hz, 1H), 7.68 (d, *J* = 1.2 Hz, 1H), 7.65 (ddd, *J* = 8.4, 7.3, 1.4 Hz, 1H), 7.35 (m, 2H), 7.20 (t, *J* = 5.4 Hz, 1H), 6.91 (d, *J* = 4.8 Hz, 1H), 6.84 (s, 1H), 5.69 (s, 1H), 3.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): 183.75, 165.61, 150.56, 147.20, 145.60, 137.86, 125.35, 125.12, 124.72, 124.33, 121.74, 118.16, 113.93, 113.63, 112.71, 56.17.

2.3. Maintenance and Differentiation of the THP-1 and RAW 264.7 Cell Line

THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (complete culture medium) at 37°C with 5% CO_2 supplemented. Cell concentrations were adjusted to desired concentrations for each experiment by centrifugation at 500 x g for 5 min and resuspended in complete culture medium with 100 nM of PMA. Cell concentration was adjusted to $5x10^5$ cells/ml for all assays with the exception of $2.5x10^5$ cells/ml were used for NF-κB nuclear translocation assay. Cells were seeded onto 96-, 24-, or 12-well plates and incubated for 48 to 72 h to allow for differentiation. Cells were washed with serum-free RPMI 1640 medium before each experiment to remove undifferentiated cells. RAW 264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS, 200 mM L-glutamine, 1% penicillin/streptomycin, and 50 µg/ml

gentamicin (complete culture medium) at 37°C in a humidified atmosphere supplemented with 5% CO_2. Cells were seeded into tissue culture plates at 10% confluence and grown to 80% confluence within 72 h. For live cell microscopy, $1x10^5$ cells/ml were seeded into 35 mm glass-bottom (MatTek) dishes 24 h prior to imaging. For luciferase assays, $1.2x10^5$ cells/well were seeded into 24-well tissue culture-treated plates.

2.4. Alamar Blue Cell Viability Assay

Differentiated THP-1 and RAW264.7 cells were treated with a range of concentrations of aurone derivatives for 1 h and stimulated with 20 ng/ml of LPS for 4 h. Following treatment, the relative cell viability was measured by Alamar Blue assay. For Alamar Blue assays, supernatants were replaced with culture medium containing 1X Alamar Blue reagent and incubated overnight. Cell viability was assessed by measuring relative fluorescent units (RFU) on the SpectraMax M2e microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA) at Ex 560 nm and Em 590 nm. The results were expressed as a percentage relative to LPS alone control cells. The effects of the vehicle control, DMSO, on cell viability were also assessed.

2.5. Assessment of Cytokine Response by ELISA

Differentiated THP-1 cells were pretreated with a range of concentrations of aurone derivatives or 1 µM of dexamethasone for 1 h and stimulated with 20 ng/ml of LPS for 4 h. Dexamethasone is a synthetic glucocorticoid that suppresses LPS-induced pro-

inflammatory cytokine expression and was used as a control (Abraham et al., 2006). Supernatants were collected for human cytokine ELISAs and the manufacturer's protocol was followed to assess the cytokine response. Cells remaining after the supernatant collection were tested for relative viability by Alamar Blue cytotoxicity assay as previously described.

2.6. Indirect Immunofluorescence for NF-κB Nuclear Translocation

Differentiated THP-1 cells were pretreated with 50 μM of aurone 1 or 10 μM of Bay 11-7082 for 1 h and stimulated with 1 μg/ml of LPS for 30 min. Bay 11-7082 is a compound that inhibits LPS-induced activation of IKKα/β, thereby suppressing downstream IκBα phosphorylation/degradation and p65 nuclear translocation and was used as a positive control (Catalán et al., 2012). Treated cells were fixed, permeabilized, blocked, and stained with p65 (NF-κB) primary antibody, Dylight 488 conjugated secondary antibody, and Hoechst 33342 dye, sequentially. The Hoechst and DyLight fluorophores detect changes in nuclear morphology (blue fluorescence) and NF-κB distribution (green fluorescence), respectively. Nuclear Translocation Bioapplication software on the Arrayscan VTI reader was used for image acquisition and data analysis (Thermo Fisher Scientific, Waltham, MA, USA). For each well, at least 400 cells were automatically acquired and analyzed. The translocation index was calculated by measuring the average intensity difference of NF-κB between the identified cytoplasmic region and nuclear region.

2.7. Live Cell Imaging of RAW 264.7 Cells

RAW 264.7 cells were pretreated with 50 μM of aurone 1 for 1 h, stimulated with 20 ng/ml LPS, and imaged every 3 min over 5 h using a Nikon Ti-Eclipse wide-field microscope (Nikon, USA), equipped with a CoolSNAP Myo camera (Photometrics, AZ, USA), computer-controlled stage, and full environmental enclosure (InVivo Scientific, MO, USA). Cells were maintained at 37ºC with 5% CO2 in a humidified atmosphere during imaging. EGFP and mCherry fluorescence were imaged using FITC and Cy3 filters, respectively. Nikon Elements Software (Nikon, USA) was used for microscope control and image capture. Post-acquisition, images were analyzed using Fiji (Schindelin et al., 2012). Images were background subtracted and cytoplasmic:nuclear p65-EGFP and whole cell mCherry fluorescence were quantified for individual cells at each time point.

2.8. Luciferase Assay

RAW 264.7 cells were transfected with 1.5 μg endotoxin free pNF-κB-Luc (Stratagene, UK) 24 h prior to being pretreated with 50 μM of aurone 1 for 1 h, and then stimulated with 20 ng/mL LPS for 6 h. Cells were lysed in 250 μl/well of luminometry lysis buffer [25 mM Tris-phosphate, 1% $_{(w/v)}$ BSA, 0.025% $_{(w/v)}$ dithiothreitol (DTT), 1% Triton X-100, 15% $_{(v/v)}$ glycerol, 0.1 mM EDTA, 8 mM $MgCl_2$, 1x protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride] and incubated on a shaking table at 200 rpm for 15 min. A volume of 10 μl of 25 mM ATP was added to each well, and the samples were transferred in duplicates of 100 μl to an opaque-white 96-well plate. A volume of 20 μl

of 10 mM sodium pyrophosphate was added to each well prior to the addition of 100 µl of 2 mM D-luciferin. Luminescence was quantified using a SpectraMax M5 plate reader using SoftMax Pro 6.3 software (Molecular Devices, Sunnyvale, CA, USA).

2.9. Western Blot Analysis

Differentiated THP-1 or RAW 264.7 cells were pretreated with a range of concentrations of aurone 1, 10 µM of U0126, or 10 µM of Bay 11-7082 for 1 h and stimulated with 1 µg/ml of LPS for the indicated times. U0126 is a compound that inhibits MEK1/2 and was used as positive control for ERK phosphorylation inhibition (Hotokezaka et al., 2002).Cells were lysed with radio-immunoprecipitation assay (RIPA) lysis buffer that contained a protease and phosphatase inhibitor cocktail. Cell lysates were then tested for protein concentration using a BCA protein assay and diluted with RIPA lysis buffer to normalize protein concentration in all samples. Lysates were mixed with sample loading buffer containing bromophenol blue, glycerol, SDS, and 2-mercaptoethanol. The separated proteins were then transferred onto a nitrocellulose membrane and blocked with 5% BSA in 1X Tris-buffered saline with 0.1% Tween-20 for 1 h. The blots were incubated with primary antibodies at 4°C overnight followed by incubation with HRP-conjugated secondary antibodies for 2 h at 22°C. The membranes were developed by addition of ECL substrate and images were collected with a ChemiDoc XRS+ system chemiluminescence imager (Bio-Rad, Hercules, CA, USA). Western blot band intensity was quantified using Image Lab software (Bio-Rad, Hercules, CA, USA).

2.10. Antibodies

Antibodies used for Western blot analysis were as follows: Actin (A2066, Sigma; SC-1616, Santa Cruz Biotechnology, Dallas, TX, USA), and iNOS (D6B6S), IKK β (D30C6), phosphorylated IKK α/β (Ser176/180; 16A6), IκB α (L35A5), phosphorylated IκB α (Ser32; 14D4), p65 (D14E12), phosphorylated p65 (Ser536; 93H1), SAPK/JNK (9252), phosphorylated SAPK/JNK (Thr183/Tyr204; 81E11), ERK1/2 (p44/42; 137F5), phosphorylated ERK1/2 (Thr202/Tyr204; D13.14.4E), p38 (D13E1), phosphorylated p38 (Thr180/Tyr182; D3F9), anti-rabbit IgG, HRP-linked (7074), and anti-mouse IgG, HRP-linked (7076) were all purchased from Cell Signaling Technology (Denver, MA, USA).

2.11. Quantitative RT-PCR

Transcription of TNF-α (NM_000594) and beta-2-microglobin, B2M (NM_004048), in 2.5 x 10^5 PMA-differentiated THP1 cells was investigated in cells without treatment, stimulated with 20 μg/ml *Salmonella* LPS, or pretreated for 1hour with 1 μM dexamethasone or 80 μM aurone 1 followed by 4 hours of incubation with 20 ng/ml LPS. Total RNA was purified after 4 hours of LPS stimulation using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, WI, USA) per manufacturer's instructions (Jeffries et al., 2014). Total RNA concentrations and the 260/280 nm ratios of each RNA sample were assessed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). SYBR® FAST One-step qRT-PCR (KAPA Biosystems, MA, USA) was used to examine gene transcription after reaction optimization.

QuantiTect® Primer Assay TNF (QT01079561) and B2M (QT00088935) primer (Qiagen, CA, USA) optimization studies, including melt curve analyses to confirm primer specificity and efficiency, were conducted to find appropriate running conditions (Fajardy et al., 2009). Briefly, the optimized conditions were 1 ng of total RNA added to 20 μl of reaction including 150 nM of forward and reverse primers. Bio-Rad CFX Connect real-time PCR detection system (Bio-Rad, CA, USA) cycling conditions, were as follows: 42°C for 10 min, 95°C for 3 min followed by 40 cycles of 95°C for 3 s and 60°C for 20 s. Samples containing no cDNA template or no primers were used as negative template or negative reverse-transcription controls, respectively. Human XpressRef Universal Total RNA (Qiagen, CA, USA) was used as a positive control. Using optimized conditions, triplicate samples were assayed from three biological replicates (n = 9). The fold change was calculated by using $2^{-\Delta\Delta Ct}$ normalized to LPS alone (Schmittgen and Livak, 2008).

2.12. NO Assay

Nitrite concentration in culture media of cells exposed to the indicated treatments was determined as an estimate of NO production with Griess reagent using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, USA) according to the manufacturer's protocol.

2.13. Statistical Analysis

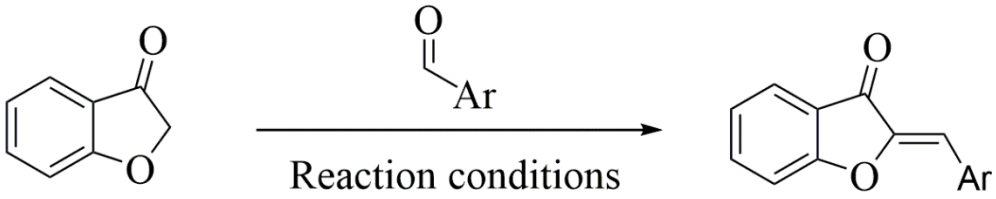
All experiments were conducted at least three times independently. Statistical significance was determined using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). Numeric values of treated groups were compared to the control group and results were expressed as mean $\pm$ SEM. Statistical significance was analyzed using one-way analysis of variance followed by the Sidak test (GraphPad Prism). A value of $p < 0.05$ was set for significance.

3. Results

3.1. Synthesis and Characterization of Aurone Derivatives

The synthetic aurones were all prepared via the standard condensation of coumaranone with the appropriate aldehyde under three different sets of conditions (Fig. 9). Four were prepared in modest yield using the conditions reported by Varma and Varma (1992) (neutral alumina in methylene chloride) (aurones 6-9). Aurone 3 was prepared using the very mild conditions reported by Hawkins and Handy (2013) using choline chloride/urea as the solvent and catalyst. Finally, four were prepared by the combination of the choline chloride/urea reaction conditions with microwave heating (aurones 1, 2, 4, and 5). This method combines very mild reaction conditions with a short reaction time, and enabled aurone 1 to be prepared in 20% yield as compared to 8% using conventional heating and a 2% yield under the Varma and Varma (1992) conditions. Another key modification of

the reaction conditions reported earlier is the observation that purification of the crude reaction product can be readily performed by simple trituration with ether instead of column chromatography. This change has generally afforded much-improved yields and certainly decreases the time required to prepare new aurone derivatives.



A = Choline Chloride/Urea, 80 C, 12 hr
B = Neutral Alumina, CH_2Cl_2
C = Choline Chloride/Urea, microwave, 90 C, 30 min

Compound	Ar	Method	Yield
1	2-(5-hydroxymethylfuryl)	C	20%
2	2-(5-methylfuryl)	C	61%
3	2-furyl	A	54%
4	2-hydroxyphenyl	C	54%
5	3-hydroxyphenyl	C	84%
6	4-hydroxyphenyl	B	34%
7	4-hydroxy-3-methoxyphenyl	B	31%
8	2-hydroxy-3-methoxyphenyl	B	18%
9	3-hydroxy-4-methoxyphenyl	B	17%

Figure 9. Synthesis of aurone derivatives.

Reaction conditions used for the synthesis of all aurone-derived compounds featuring the deep eutectic solvent, choline chloride/urea, as both the reaction medium and catalyst for the reaction as well as the use of microwave energy to greatly accelerate the reaction or the neutral alumina method (Varma and Varma, 1992).

3.2. Cytotoxicity of Aurone Derivatives in THP-1 and RAW 264.7 Cells

In order to determine the effect of aurones on inflammatory signaling by innate immune cells, we first assayed the toxicity of our aurone compounds on THP-1 cells in combination with LPS. The viability test was performed for every supernatant sample collection used for the cytokine response assay to show that suppression of cytokine response was not due to cell death. Cells pretreated with 20, 40, 80 μM of aurone 1 with LPS for 4 h had no effect on cell viability, as measured using Alamar Blue assay (Fig. 10A). However, treatment with aurone 1 at 100 μM with LPS resulted in less than 90% viability, therefore 80 μM was selected as the maximum concentration for subsequent experiments in THP-1 cells. Corresponding assays were also performed in RAW 264.7 cells with concentrations up to and including 100 μM of aurone 1with LPS exhibiting no effect on viability at 4 h post treatment (Fig. 10B). Furthermore, we also found that RAW

264.7 cells could be incubated with this higher dose of aurone 1 for at least 24 h without apparent toxicity (Fig. 10C). Table 1 shows the maximum concentrations of other aurone derivatives that were non-toxic to THP-1 cells. The vehicle control, DMSO, was also tested for toxicity and showed no effect on viability in either cell line (data not shown).

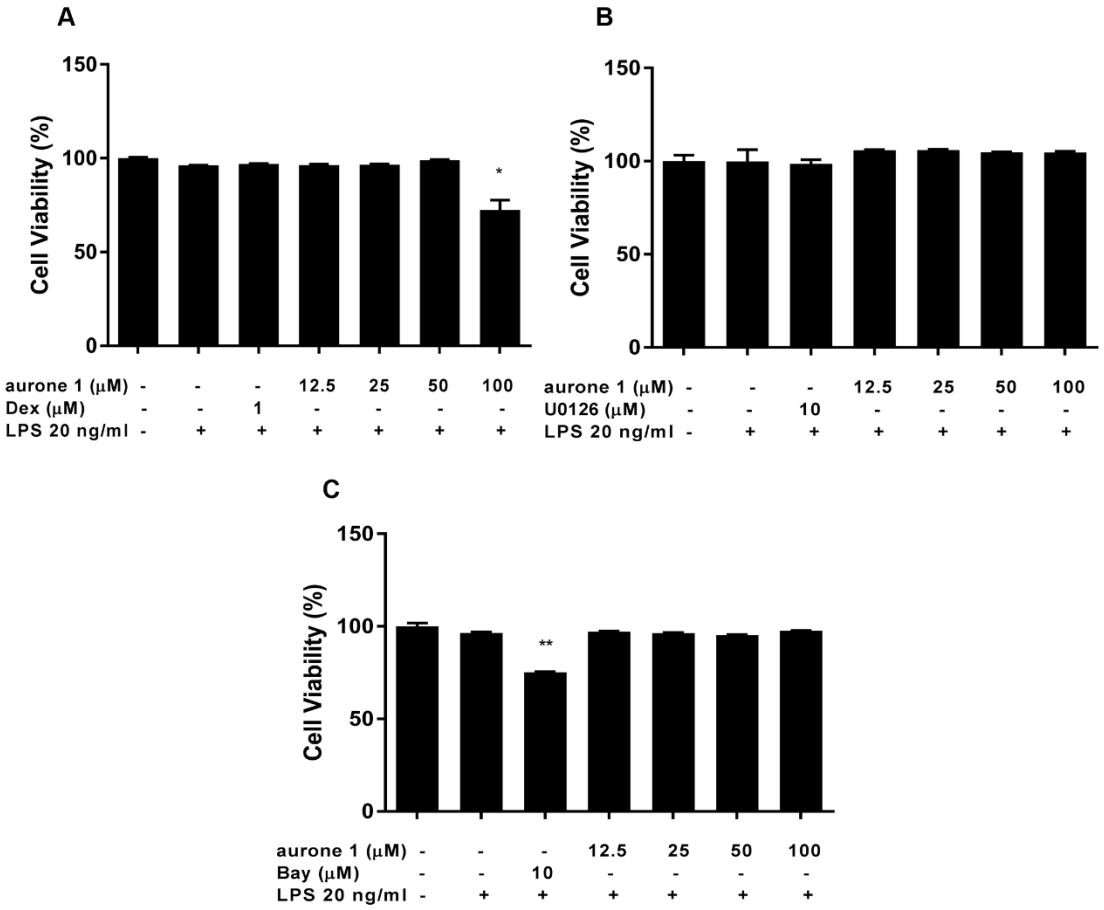


Figure 10. Cytotoxicity of aurone 1 on PMA-differentiated THP-1 and RAW 264.7 macrophages.

PMA-differentiated THP-1 or RAW 264.7 cells were treated with dexamethasone (Dex), Bay11-7082 (Bay), U0126, or the indicated concentrations of aurone 1 in combination with 20 ng/mL LPS. (A) The viability of THP-1 cells was measured 4 h post-LPS using Alamar Blue assay. The same assay was performed for RAW 264.7 cells at (B) 4 h and (C) 24 h post treatment. In all cases, the results are presented as the mean±SEM for triplicate measurements from at least 3 independent biological repeats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with no treatment control group.

3.3. The Effects of Aurone 1 on TNF-α, IL-1β, and IL-8 Response in LPS-Stimulated THP-1 Cells

We next investigated the effects of aurone derivatives on expression of the inflammatory cytokine, TNFα, by LPS-stimulated THP-1 macrophages using ELISA. LPS is recognized by the pattern recognition receptor, TLR4 with CD14 and other associating proteins on the surface of the membrane, triggering MyD88-dependent activation of a number of transcription factors that regulate the expression of TNFα and other inflammatory regulators (e.g. NF-κB and AP-1). While aurone 1 suppressed TNFα expression by 93.8% (±0.7; Fig. 11A), aurones 4, 7, and 9 at 40 μM caused only modest

inhibition (< than 15%), and all others failed to show the effect (Table 1). Therefore, only aurone 1 was selected for further study. In addition to suppressing TNFα secretion, aurone 1 also reduced expression of IL-1β, and IL-8 in THP-1 cells by 98% and 71% respectively (Fig. 11B-D), in concentration-dependent manner. The fold changes in cytokines are shown in Figure 11D and fold changes of treated groups are relative to the LPS-only group. DMSO, which was used as a vehicle for aurone 1, did not affect cytokine responses in LPS-treated THP-1 cells (data not shown).

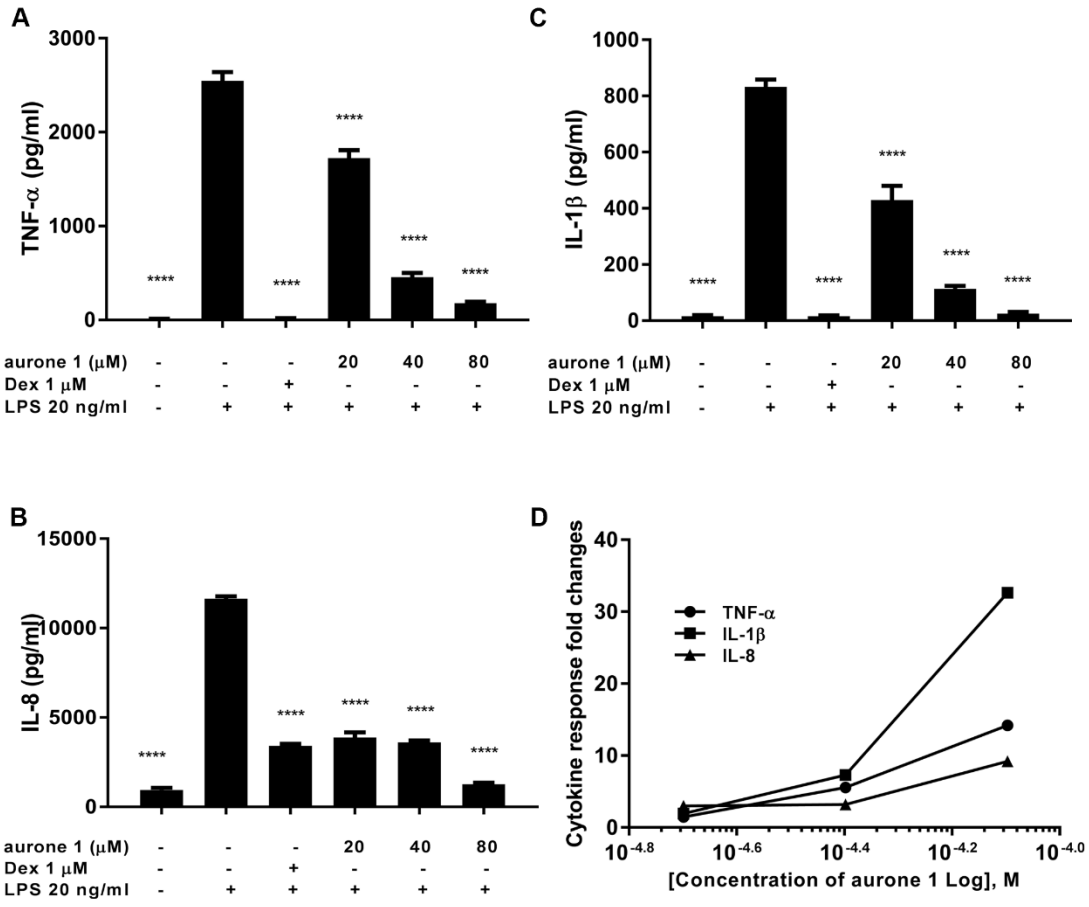


Figure 11. Aurone 1 inhibits TNFα, IL-1β, and IL-8 secretion in LPS-stimulated THP-1 cells.

PMA-differentiated THP-1 cells were pretreated with dexamethasone (Dex, a synthetic inhibitor of cytokine production) as a control or 20, 40, and 80 μM of aurone 1 for 1 h and stimulated with 20 ng/ml of LPS for 4 h. The expression of (A)TNFα, (B) IL-8, and (C) IL-1β in supernatants was determined by ELISA along with calculated fold changes of each cytokine (D) Results are presented as the mean±SEM for triplicate measurements

of at least 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared.

with LPS-treated group.

Table 1. The average percent viability and percent TNFα inhibition of the THP-1 cells treated with various concentrations of aurone derivatives with 20 ng/ml of LPS.

Data are expressed as mean ± SD of triplicates for each experiment.

Compounds	Concentration (μM)	Mean % Viability	Mean % TNFα Inhibition
1	20	96.0±0.6	25.3±6.1
	40	96.8±0.9	51.8±13.5
	80	103.1±0.2	93.8±0.7
2	25	99.4±9.3	No Inhibition
	50	118.3±1.3	No Inhibition
	100	108.9±2.0	No Inhibition
3	13	104.3±2.2	No Inhibition
	25	102.1±0.8	No Inhibition
	50	92.2±0.9	No Inhibition
4	20	99.4±0.5	No Inhibition
	40	98.8±1.6	15.4±5.3
5	20	94.6±1.2	No Inhibition
	25	92.9±1.4	No Inhibition
	40	90.9±0.6	No Inhibition
6	20	99.8±1.5	No Inhibition
	40	98.5±0.1	No Inhibition
7	20	97.0±0.3	No Inhibition
	40	99.6±0.1	10.4±4.7
8	20	101.3±2.6	No Inhibition
	40	100.9±0.3	No Inhibition
9	20	101.8±2.1	No Inhibition
	40	100.9±1.3	14.9±7.2

3.4. Aurone 1 Inhibits TNFα Production at the Transcriptional Level

As *TNF*, *IL1B,* and *IL8* are bona fide NF-κB responsive genes (Collart et al., 1990; Hiscott et al., 1993; Kang et al., 2007; Kunsch and Rosen, 1993; Shakhov et al., 1990), we hypothesized that the reduction in LPS-induced cytokine expression in response to aurone 1 (i) occurred at the transcriptional level and (ii) was achieved through inhibition of canonical NF-κB activity. To test these hypotheses, we measured LPS-induced TNFα mRNA levels in aurone 1 pre-treated THP-1 cells by qRT-PCR and NF-κB dependent transcription in RAW 264.7 cells using a luciferase reporter assay. Here, we found that pre-treated with 80 μM aurone-1 reduced TNFα mRNA levels in differentiated THP-1 cells by 49% ($p \leq 0.0001$) (Fig. 12A). To test the effects of aurone 1 on NF-κB-dependent transcription, RAW264.7 cells were transiently transfected with the pNF-κB-Luc reporter construct, which contains the firefly luciferase gene under the control of a promoter containing 5 tandem consensus κB sites. Here, pre-treatment with aurone 1 was found to significantly inhibit LPS-induced luciferase expression in these cells (Fig. 12B). Together, these data strongly suggested that the effects of aurone 1 on cytokine expression were a consequence of NF-κB inhibition.

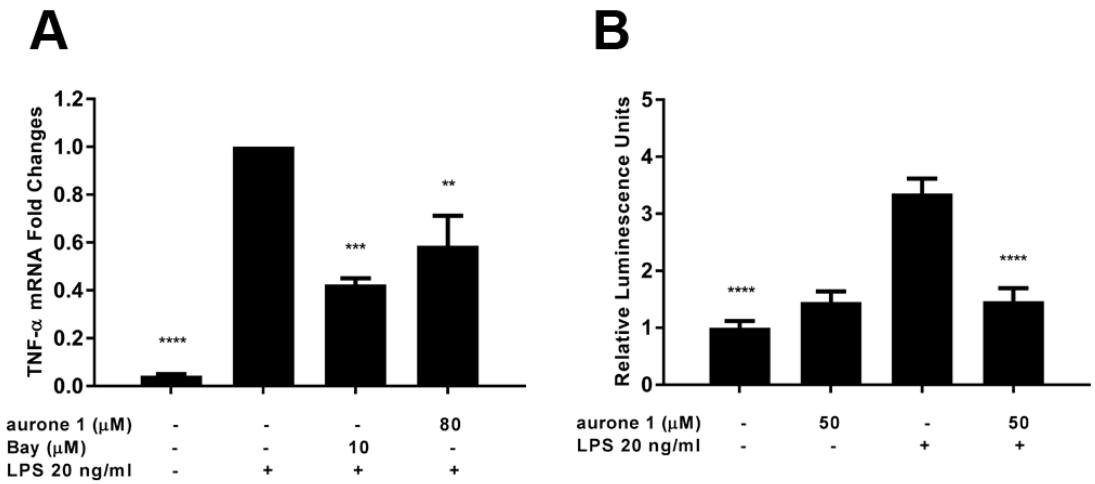


Figure 12. Aurone 1 inhibits TNFα gene transcription and transactivation of an NF-κB-dependent promoter.

(A) PMA-differentiated THP-1 cells were pretreated with 80 μM of aurone 1 or vehicle for 1 h and stimulated with 20 ng/mL of LPS for 4 h. The expression of TNFα and B2M mRNA was quantified by qRT-PCR. (B) RAW 264.7 cells containing pNF-κB-Luc were treated with 50 μM of aurone 1 or vehicle for 1 h and then stimulated with 20 ng/ml LPS for 6 h. The cells were lysed and the expression of luciferase determined by luminometry. The assay was repeated 3 times as independent experiments. Results are presented as the mean±SEM for triplicate measurements from a single representative experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with LPS-treated group.

3.5. Aurone 1 Inhibits LPS-induced Nuclear Translocation of p65 in Human and Murine Macrophages

Having shown that aurone 1 could block NF-κB dependent transcription, we tested whether this was due to the inhibition of nuclear translocation of the canonical NF-κB transcription factor, p65 (RelA). Using immunofluorescent staining of THP-1 cells challenged with LPS for 30 min, we found that pre-treatment with 50 μM aurone 1 was capable of blocking cytoplasmic-to-nuclear translocation of p65. This was comparable to the effects of the IKK inhibitor, Bay 11-7082, which blocked p65 translocation at a dose of 10 μM (Fig.13). To test whether aurone 1 had similar effects in murine macrophages and to investigate how it might alter the kinetics of the NF-κB response to LPS, we employed a previously described NF-κB dual reporter RAW 264.7 murine macrophage cell line (Sung et al., 2014). The reporter cell line, which stably expressed an EGFP fusion of p65 (p65-EGFP), also incorperates an exogenous reporter of TNFα gene transactivation based on the core murine *TNF* promoter (-1229 to -27) regulating the expression of the red fluorescent protein, mCherry. Using live cell microscopy of these dual reporter cells, we found that nuclear translocation of p65-EGFP post-LPS treatment was diminished in cells pretreated with aurone 1 and occurred slightly later (Fig. 14A-D). The reduction in nuclear p65 levels, expressed as the ratio of nuclear to cytoplasmic p65-EGFP fluorescence, was found to be statistically significant (Fig. 14E). Consistent with these data and our qRT-PCR analysis of *TNF* gene transcription in THP-1 cells (Fig. 12A), the reduction in nuclear p65 resulted in smaller fold-change in the expression of

mCherry in the murine dual reporter cells (Fig 14A, F-H). This was also found to be significant (Fig. 14I).

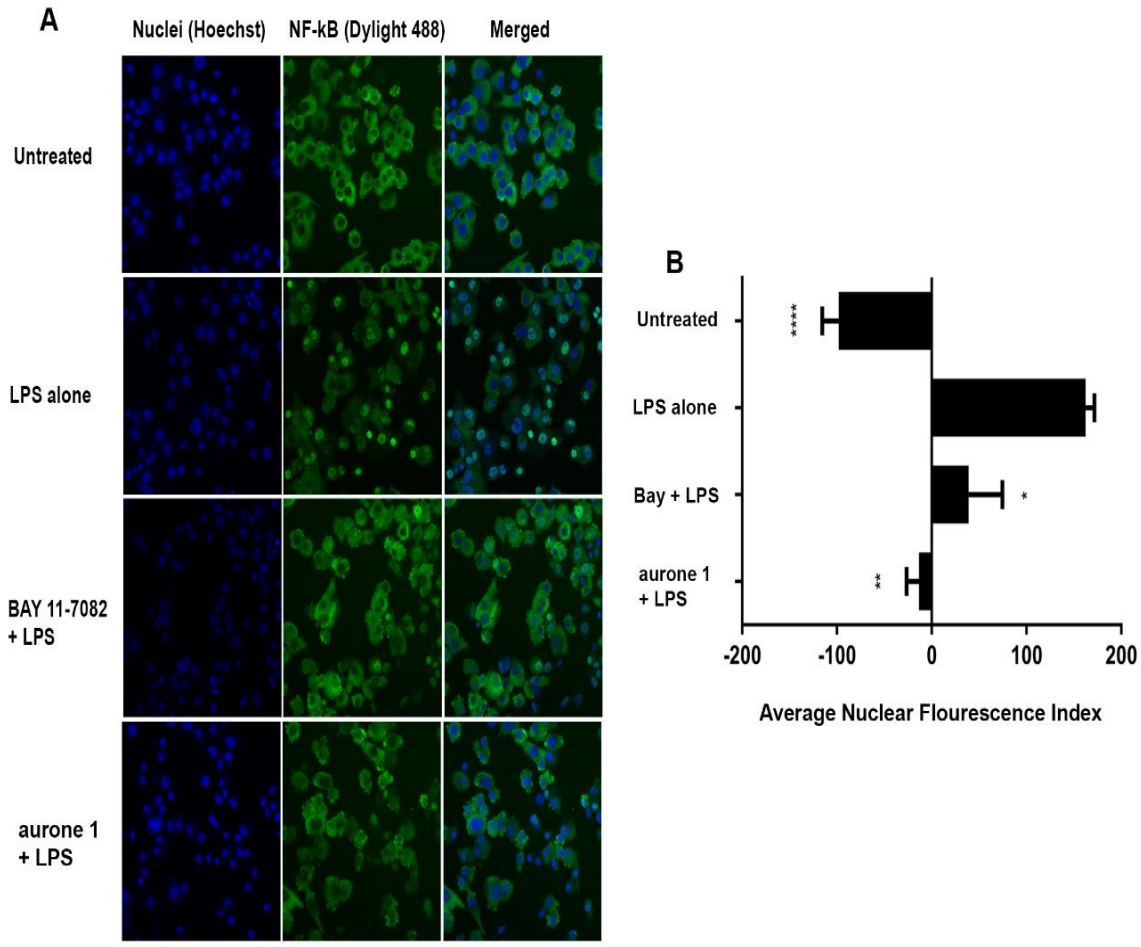


Figure 13. Aurone 1 blocks nuclear translocation of p65 in THP-1 cells.

PMA-differentiated cells were treated with 50 μM of aurone 1 or 10 μM of Bay 11-7082 for 1 h and stimulated with 100 ng/ml LPS for 30 min. (A) The transcription factor, p65, was stained with rabbit anti-p65 followed by Dylight 488-conjugated secondary antibody (green fluorescence) and Hoechst 33342 dye (blue fluorescence), sequentially. (B) The numeric index of nuclear fluorescence of p65 was collected using Nuclear Translocation Bioapplication software on the Arrayscan VTI reader. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with LPS-treated group.

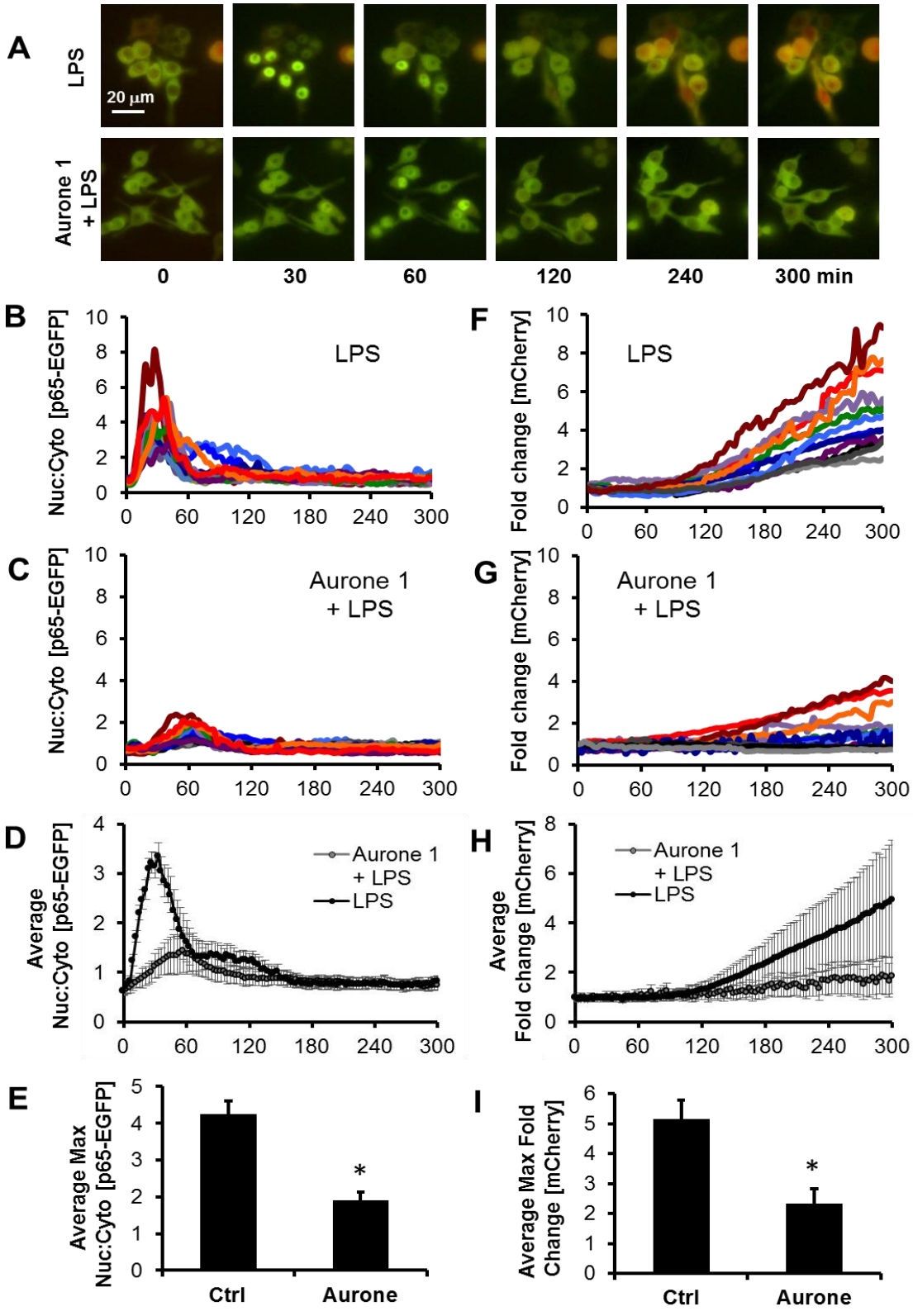
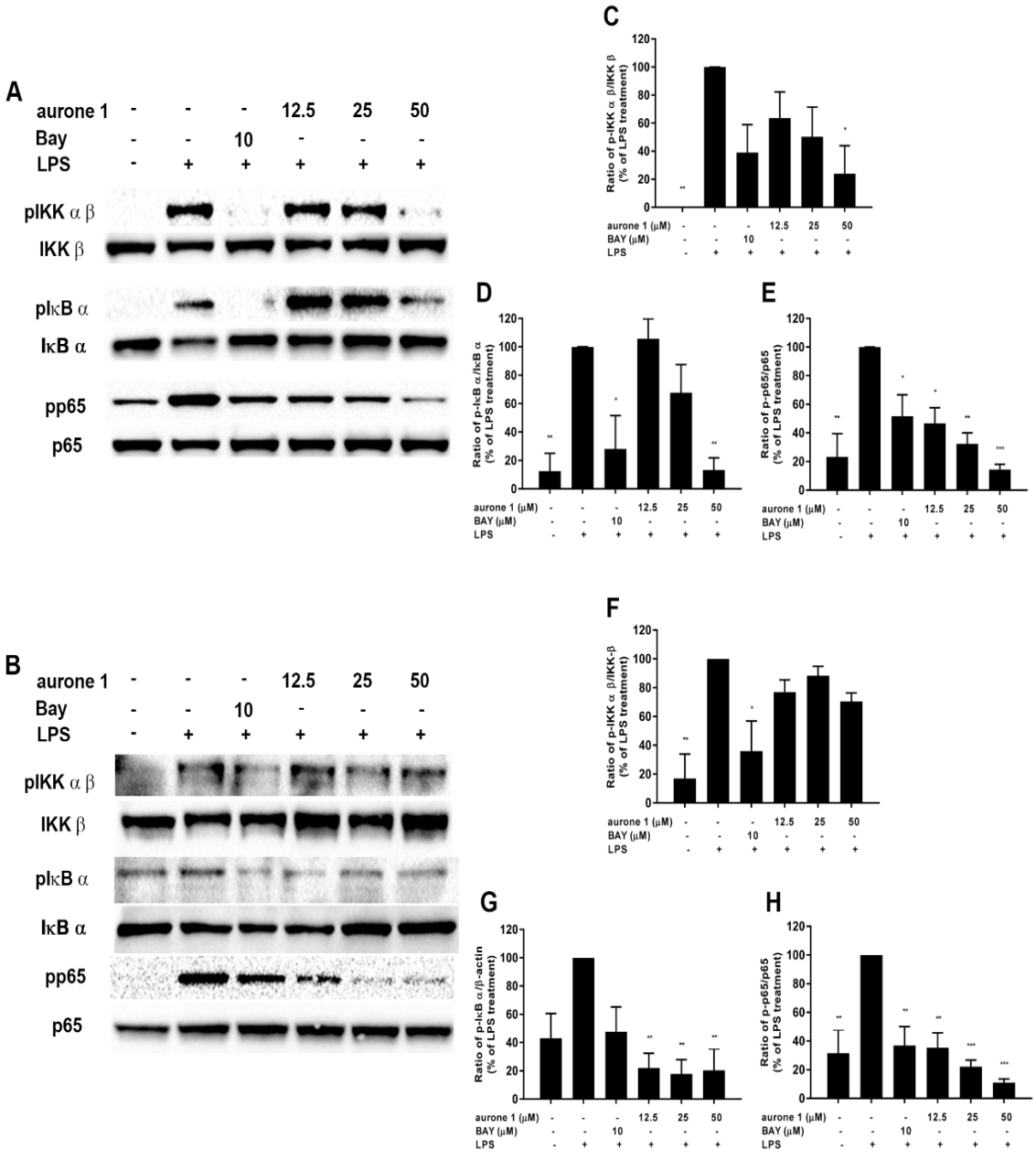

A
LPS
20 μm
Aurone 1 + LPS
0
30
60
120
240
300 min
B
Nuc:Cyto [p65-EGFP]
LPS
C
Aurone 1 + LPS
F
Fold change [mCherry]
G
D
Average Nuc:Cyto [p65-EGFP]
Aurone 1 + LPS
LPS
H
Average Fold change [mCherry]
E
Average Max Nuc:Cyto [p65-EGFP]
Ctrl
Aurone
I
Average Max Fold Change [mCherry]

Figure 14. Aurone 1 decreases LPS-induced nuclear accumulation of p65 and expression from the TNF promoter in live murine macrophages.

RAW 264.7 cells stably expressing p65-EGFP fusion protein and a destabilized mCherry reporter expressed from the TNFα promoter were treated with vehicle as a control or 50 μM of aurone 1 for 1 h and stimulated with 20 ng/ml LPS for 6 h. (A) Timecourse images of vehicle and aurone 1-treated RAW 264.7 cells. Fluorescence from p65-EGFP is represented in green and mCherry fluorescence is represented in red. Quantification of p65-EGFP nuclear:cytoplasmic (nuc:cyto) fluorescence ration for (B) 12 control cells, (C) 12 aurone-1 pre-treated cells is presented together with (D) the population averages, and (E) the average maximum amplitude of p65-EGFP nuc:cyto fluorescence. Corresponding measurements of mCherry fluorescence are also presented for (F) 12 control cells, (G) 12 aurone-1 pre-treated cells, (H) the population averages, and (I) the average maximum mCherry fluorescence. (D-E+H-I) Data is from a minimum of 44 cells per-treatment across 3 independent biological repeats. Error is presented as the SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with LPS-treated group.

3.6. Aurone 1 Inhibits IKKβ, IκBα, and p65 Phosphorylation

Since p65 nuclear translocation was attenuated by aurone 1 in both THP-1 and RAW 264.7 cells, we measured LPS-induced changes in the phosphorylation of the critical upstream regulators of p65, IKKβ and IκBα, as well as phosphorylation of p65 itself. Phosphorylation of IKKβ at ser176/180 is required for kinase activity and phosphorylation of its substrate, IκBα, at ser32/36, which stimulates its ubiquitination and proteasomal degradation (Barisic et al., 2010; Yang et al., 2003). Phosphorylation of p65 at ser536 has also been associated with increased transcriptional activity (Buss et al., 2004; Hoberg et al., 2006). Like the inhibitor of IKK activation, Bay 11-7082, aurone 1 was found to suppress the phosphorylation of all three proteins in both THP-1 (Fig. 15A and C-E) and RAW 264.7 cells (Fig. 15B and F-H). In all cases, these effects were found to be dose-dependent. However, differences were observed in the magnitude of the response between the two cell lines with aurone 1 appearing to have a greater effect on IKK phosphorylation in THP-1 cells than in RAW264.7 cells (Fig. 15C+F). Despite this, aurone 1 strongly suppressed IκBα and p65 phosphorylation – direct targets of IKKβ – even at 25 μM doses in RAW 264.7 cells (Fig. 15G+H).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Bay - - 10 - - -
LPS - + + + + +
pIKK α β
IKK β
pIκB α
IκB α
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B
aurone 1 - - - 12.5 25 50
Bay - - 10 - - -
LPS - + + + + +
pIKK α β
IKK β
pIκB α
IκB α
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Ratio of p-IKK α β/IKK β (% of LPS treatment)
aurone 1 (μM) - - - 12.5 25 50
BAY (μM) - - 10 - - -
LPS - + + + + +
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Ratio of p-IκB α/IκB α (% of LPS treatment)
aurone 1 (μM) - - - 12.5 25 50
BAY (μM) - - 10 - - -
LPS - + + + + +
E
Ratio of p-p65/p65 (% of LPS treatment)
aurone 1 (μM) - - - 12.5 25 50
BAY (μM) - - 10 - - -
LPS - + + + + +
F
Ratio of p-IKK α β/IKK-β (% of LPS treatment)
aurone 1 (μM) - - - 12.5 25 50
BAY (μM) - - 10 - - -
LPS - + + + + +
G
Ratio of p-IκB α/β-actin (% of LPS treatment)
aurone 1 (μM) - - - 12.5 25 50
BAY (μM) - - 10 - - -
LPS - + + + + +
H
Ratio of p-p65/p65 (% of LPS treatment)
aurone 1 (μM) - - - 12.5 25 50
BAY (μM) - - 10 - - -
LPS - + + + + +

Figure 15. Aurone 1 inhibits LPS-induced phosphorylation of IKK, p65, and IκBα, and decreases degradation of IκBα.

PMA-differentiated THP-1 cells and RAW264.7 cells were pretreated with 12.5, 25, and 50 μM of aurone 1 or 10 μM of Bay 11-7082 (Bay) for 1 h and stimulated with 1 μg/ml of LPS for 15 min. (A) Images of blot for phosphorylated (Ser176/180) IKK-α β and total IKK-β, phosphorylated IκBα (Ser32) and total IκBα, and phosphorylated p65 (Ser536) and total p65 in THP-1 cells were measured by Western blotting followed by densitometry (C-E). Corresponding measurements for (B) pIKK/IKK, pIκBα/actin, and p-p65/p65 in RAW 264.7 cells with densitometry results (F-H). Intensity data are represented as the mean±SEM for at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with LPS-treated group.

3.7. Aurone 1 does not Significantly Affect MAPK Phosphorylation

In addition to stimulating NF-κB activity, LPS also promotes the phosphorylation of ERK, JNK, and p38, leading to the activation of AP-1 transcription factors. As AP-1 also regulates the expression of TNFα and other pro-inflammatory cytokines at the transcriptional level (Liu et al., 2000), we hypothesized that inhibition of MAPKs may contribute to the decreased expression of TNFα seen in aurone 1 treated cells. To test this possibility, we measured the phosphorylation of MAPKs in both THP-1 and RAW264.7 cells (Fig. 16A-H). We found that aurone 1 had no statistically significant effect on the

phosphorylation of ERK (Fig. 8A+B and D+G), JNK (Fig. 16A+B and C+F), or p38 (Fig. 16A+B and E+H) across both cell lines tested, strongly indicating that aurone 1 was not affecting cytokine expression via altered NF-κB but not MAPK activity.

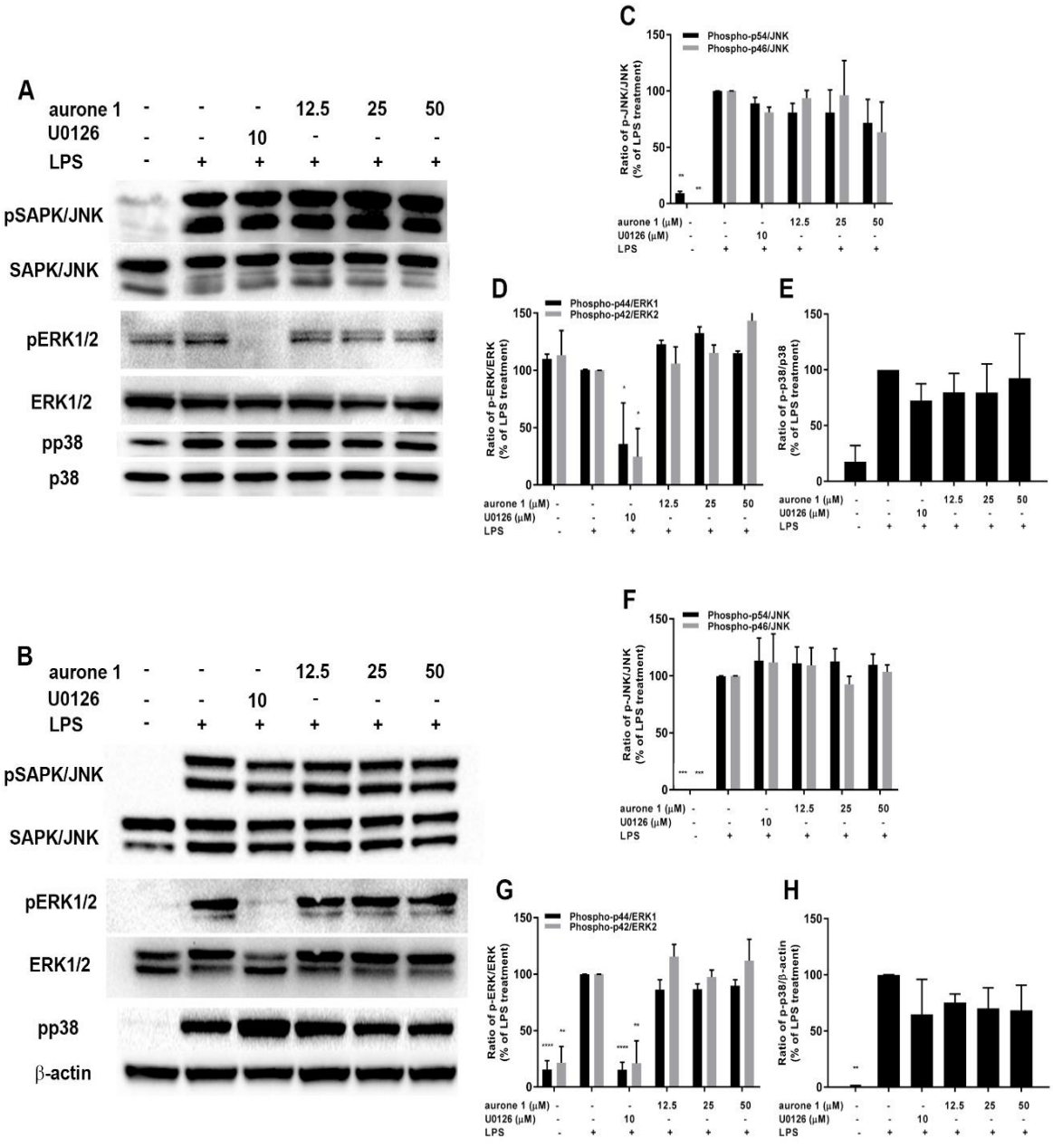

A
aurone 1 - - - 12.5 25 50
U0126 - - 10 - - -
LPS - + + + + +
pSAPK/JNK
SAPK/JNK
pERK1/2
ERK1/2
pp38
p38
B
aurone 1 - - - 12.5 25 50
U0126 - - 10 - - -
LPS - + + + + +
pSAPK/JNK
SAPK/JNK
pERK1/2
ERK1/2
pp38
β-actin
C
Phospho-p54/JNK
Phospho-p46/JNK
Ratio of p-JNK/JNK (% of LPS treatment)
D
Phospho-p44/ERK1
Phospho-p42/ERK2
Ratio of p-ERK/ERK (% of LPS treatment)
E
Ratio of p-p38/p38 (% of LPS treatment)
F
Phospho-p54/JNK
Phospho-p46/JNK
Ratio of p-JNK/JNK (% of LPS treatment)
G
Phospho-p44/ERK1
Phospho-p42/ERK2
Ratio of p-ERK/ERK (% of LPS treatment)
H
Ratio of p-p38/β-actin (% of LPS treatment)

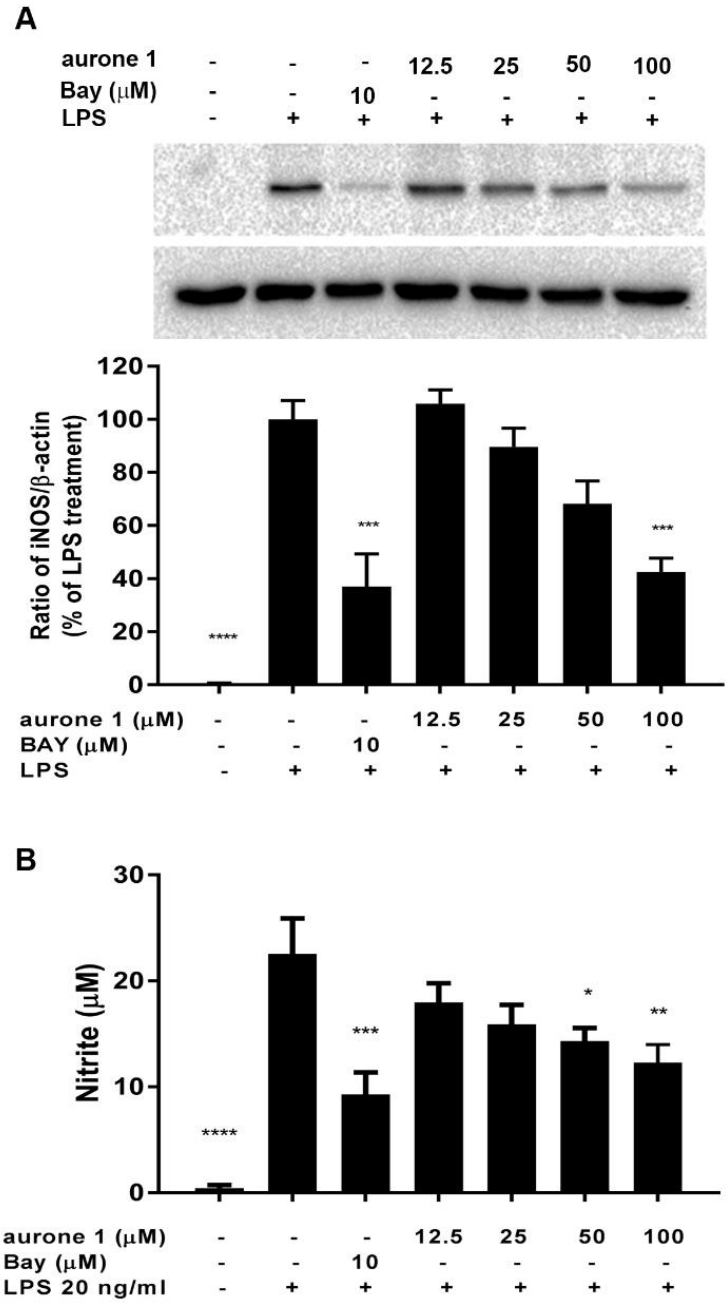
Figure 16. Aurone 1 does not significantly inhibit LPS-induced phosphorylation of MAPKs.

PMA-differentiated THP-1 cells and RAW 264.7 cells were pretreated with the indicated concentrations of aurone 1 or 10 μM of U0126 for 1 h and stimulated with 1 μg/ml of LPS for 15 min. (A) image blots of phosphorylated ERK (Thr202/Tyr204) and total ERK, phosphorylated SAPK/JNK (Thr183/Tyr185) and total SAPK/JNK, and phosphorylated p38 (Thr180/Tyr182) and total p38 were measured in THP-1 cells by Western blotting followed by densitometry (C-E). Corresponding measurements for (B) pERK/ERK, pJNK/JNK, and pp38/β-actin in RAW 264.7 cells with densitometry results (F-H) Intensity data are represented as the mean±SEM for at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with LPS-treated group.

3.8. Aurone 1 Decreases iNOS Expression and NO Production in RAW264.7 Cells

As well as pro-inflammatory cytokines, LPS is known to induce a plethora of other NF-κB-responsive genes in macrophages (Sharif et al., 2007), including *NOS2* (Xie et al., 1994), which encodes iNOS, a well-established marker of M1 macrophage polarization. To test whether this was also affected by aurone 1, we measured iNOS expression in LPS-stimulated RAW264.7 cells with and without aurone 1 pre-treatment by Western blotting. Like TNFα, we found that iNOS production was reduced by aurone 1 in a dose-

dependent manner, with 50 and 100 μM producing a 31.7 and 57.5% reduction in iNOS levels, respectively (Fig. 17A). Furthermore, production of NO, the product of iNOS, was significantly reduced in the supernatants of aurone 1 pre-treated cells (Fig. 17B). Together, these data suggest that aurone 1 can at least partially block M1 polarization of macrophages.


A
aurone 1 - - - 12.5 25 50 100
Bay (μM) - - 10 - - - -
LPS - + + + + + +
Ratio of iNOS/β-actin (% of LPS treatment)
120
100
80
60
40
20
0
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aurone 1 (μM) - - - 12.5 25 50 100
BAY (μM) - - 10 - - - -
LPS - + + + + + +
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Nitrite (μM)
30
20
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aurone 1 (μM) - - - 12.5 25 50 100
Bay (μM) - - 10 - - - -
LPS 20 ng/ml - + + + + + +

Figure 17. Aurone 1 inhibits iNOS expression and NO production in RAW 264.7 cells.

RAW 264.7 cells were pretreated with 12.5, 25, 50, and 100 μM of aurone 1 or 10 μM of Bay 11-7082 for 1 h and stimulated with 1 μg/ml of LPS for 24 h. (A) Actin and iNOS levels were measured by Western blotting followed by densitometry. (B) Nitrite concentration in cell growth medium was also analyzed 24 h post LPS by Griess assay as an indirect measurement of NO production. Western blot intensity data and nitrite concentrations are represented as the mean±SEM for at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with LPS-treated group.

4. Discussion

Inflammation is triggered by multiple factors including stress, trauma, and infection. While under normal circumstances, this process is self-limiting, resulting in an appropriate and transient inflammatory response, in the disease state inflammation can become self-perpetuating. Pro-inflammatory regulators inducing the production of further pro-inflammatory regulators in a positive-feedback loop can result in destructive chronic inflammation and is associated with the development of degenerative diseases such as cardiac disease, cancer, neurodegenerative disorders, stroke, and diabetes (Amor et al., 2010; Bastard et al., 2006; Kundu and Surh, 2008; Ridker et al., 1997). Currently

available preventive therapy for chronic inflammatory and autoimmune diseases typically targets the cytokine response with anti-TNF therapy being clinically demonstrated as the most effective approach to control inflammation (Postal and Appenzeller, 2011).

The downside of TNF blockers is that they can cause severe side effects such as allergic reactions, increased risk of infections, malignancies, and stroke, and are thus limited to severe inflammatory diseases such as rheumatoid arthritis and ankylosing spondylitis (Bezalel et al., 2012; Bjarnason et al., 1993; Bongartz et al., 2006; Diamantopoulos, 2013). Thus targeting the signaling pathways that regulate cytokine expression – including NF-κB - with small molecule-based inhibitors is seen as an attractive alternative.

The present study describes the characterization of a novel synthetic aurone, which is capable of suppressing LPS-induced expression of inflammatory cytokines and iNOS expression by blocking the activation of the canonical NF-κB pathway. The effects of this compound were also found to be highly consistent between species with the phosphorylation and nuclear translocation of p65 proteins found to be strongly suppressed in both human THP-1 and murine RAW264.7 cell lines. The utilization of live cell imaging, which has frequently been used by our and other groups to study NF-κB signaling (Ashall et al., 2009; Hayes et al., 2016; Lee et al., 2014b; Nelson et al., 2004; Sung et al., 2014), allowed us to probe both the impact of the aurone on p65 translocation and the downstream transcriptional consequences in individual cells. Here, we found that decreased nuclear accumulation of p65 resulted in a corresponding loss of expression of

the reporter gene, mCherry, which was expressed from a portion of the murine *TNF* promoter, which incorporates κB sites. Although all four target genes assayed in this study are co-regulated by MAPK, which is also induced by LPS/TLR4 signaling, we found that aurone 1 had no effect on ERK, JNK1, and p38 MAPK. We therefore can conclude that the primary mechanism by which aurone 1 suppresses the expression of these genes is via NF-κB. However, the identity of the specific molecular target of aurone 1 remains an open question and is the subject of on-going studies. Based on its contrasting effects on NF-κB and MAPK signaling, we speculate that aurone 1 may directly affect the activity of the IKK complex itself. Alternatively, aurone 1 may have a differential effect on a common upstream regulator of the two pathways. One possible candidate might be transforming growth factor β-activated kinase 1 (TAK1), a divergence point for LPS/TLR4-induced NF-κB and MAPK signaling. While the regulation of TAK1 is not yet fully understood, recent data has suggested that the ability of this protein to induce IKK and MAPK activity is somewhat separable with certain modifications, such as ubiquitination of Lys158 being required for both IKK and MAPK induction (Fan et al., 2011; Fan et al., 2010), while phosphorylation within the activation loop of TAK1 is dispensable for IKK activation (Chen et al., 2015). We also cannot rule out the possibility that aurone 1 affects other kinases that influence NF-κB activity in LPS-stimulated macrophages. Integrin-linked kinase (ILK), for example, has also been shown to phosphorylate p65 at ser536 in RAW264.7 cells with knockdown or inhibition of ILK decreasing TNFα expression in these cells (Ahmed et al., 2014).

NF-κB is a major proinflammatory regulator that is frequently targeted for anti-inflammatory drug discovery (Barnes and Karin, 1997; Karin et al., 2004; Yamamoto and Gaynor, 2001a). Although, the exact mechanism of aurone 1 suppression of NF-κB activity remains to be elucidated, we show that this novel compound can suppress the pro-inflammatory functions of both cultured human and murine macrophage cell lines without toxicity at effective doses. Therefore, we conclude that aurone 1 is an anti-inflammatory compound with therapeutic potential for the possible treatment of chronic inflammatory disorders or conditions such as endotoxic shock that involves excessive TLR4/NF-κB signaling.

5. Acknowledgements

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OVERALL CONCLUSION

Many drugs used today are derived from plants and natural products remain a major source of identification and development of novel biologically active compounds as therapeutic agents (Dias et al., 2012). This has driven herbal medicine to become more stringently tested, with increasing emphasis placed on proven product safety and efficacy (Taylor et al., 2001). Thousands of plant-based materials including synthetic compounds such as flavonoids and aurones have yielded many bioactive compounds. The advancement of synthesis technology has also eased the production of these compounds rather than isolating them from plants. This interest in bioactivity of plants or plant-derived synthetic compounds has more recently focused on anti-inflammatory compounds. Chronic inflammation has been associated with various conditions and diseases in humans such as diabetes, cancer, cardiovascular diseases, neurodegenerative diseases, autoimmune diseases, and age-related diseases (Murakami and Hirano, 2012). Current FDA approved anti-inflammatory drugs are associated with side effects that limit them for long-term uses that are frequently required to treat patients that suffer from severe chronic inflammatory and autoimmune diseases. More attention has been made to develop anti-inflammatory drugs that may relieve the complications of chronic inflammation through drugs that are selective with specific mode-of-action to inhibit proinflammatory signaling pathways such as NF-κB.

The present study describes suppression of inflammatory cytokine responses in LPS-stimulated macrophages by *cis*- and *trans*-gnetin H and a synthetic aurone, (Z)-2-((5-

(hydroxymethyl) furan-2-yl) methylene) benzofuran-3(2H)-one through inhibition of the canonical NF-κB pathway (Fig. 18). The compounds were dissolved in DMSO and the effects of DMSO on cell viability as well as TNF-α in human macrophages were tested to ensure the vehicle control had no adverse effects (Supplement Figure 1; Appendix A). The proton and caron NMR spectra data of aurones in the second article are listed in the Appendix C. The effects of the compounds are associated with indirectly blocking p65 nuclear translocation by disrupting the required activation of IKK-β, which is a key regulator of NF-κB pathway. The results from this study suggest the compounds either directly inhibit IKK-β, inhibit other kinases that are upstream of IKK-β, or both.

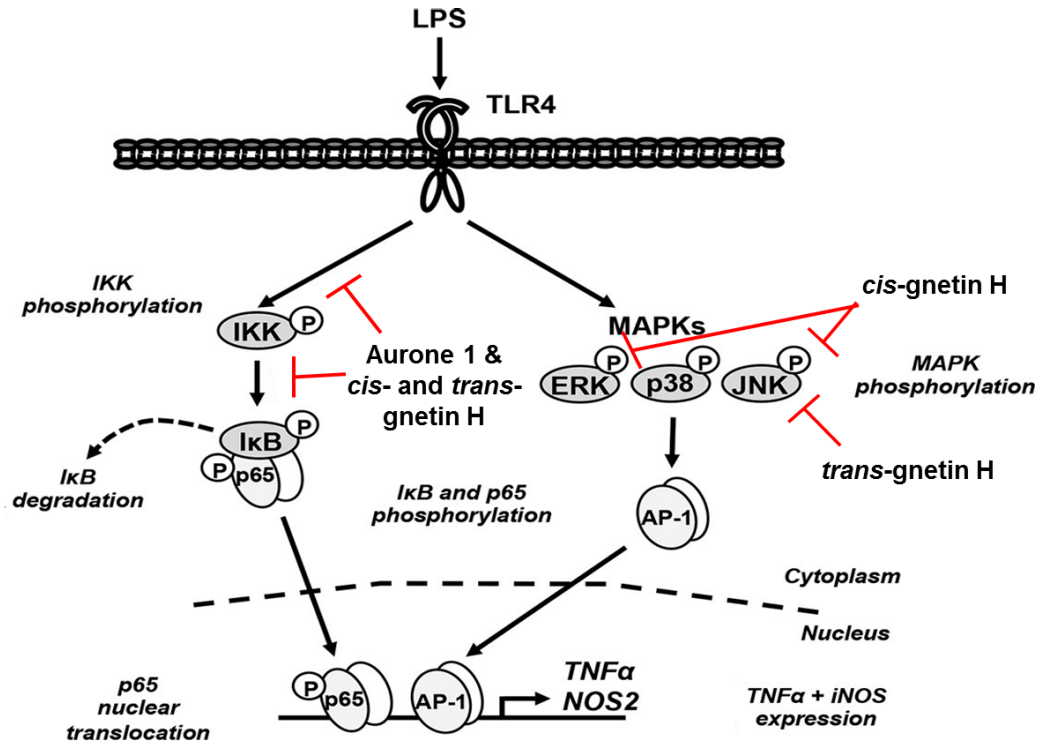

Figure 18. The effects of aurone 1 and *cis*- and *trans*-gnetin H on LPS-stimulated macrophages.

More in-depth studies are required to investigate the exact molecular mechanism of the compounds to determine their exact molecular target(s). Among the three compounds, *trans*-gnetin H was found to be the most effective compound with the greatest suppression of TNF-α and having an IC_{50} value of 6 μM. Using a live cell imaging system and the dual reporter RAW 264.7 cell line that stably expresses GFP from RelA (p65) promoter and mCherry from the TNF promoter, the effect of aurone 1 on p65

nuclear translocation was shown to be consistent with the observation seen in THP-1 cells. Interestingly, the murine cell line was not responsive to the isomers of gnetin H, therefore, the effects of *cis*- and *trans*-gnetin H were reported against the THP-1 cell line only. Aurone 1 was also found to be less toxic to RAW 264.7 cell line with LPS compared to the THP-1 cell line. Further studies using the murine cell line confirmed the effects of aurone 1 in suppressing the NF-κB pathway by concentration-dependent suppression of iNOS as well as the production of NO which are both regulated by the NF-κB pathway.

The phosphorylation of NF-κB mediators such as p65, IκB α, and IKK-β were suppressed in both THP-1 and RAW 264.7 cell lines by aurone 1 but at higher concentrations. To show significant suppression of IKK-β, 100μM of aurone 1 was required. This suggests there are some minor cell line specific suppression differences. Further, the investigation of MAPK pathway mediators such as SAPK/JNK, ERK1/2, and p38 kinases, suggested that aurone 1 selectively suppresses the NF-κB pathway without altering the MAPK pathway which is an important finding. Many small compounds have been found to show broad spectrum anti-inflammatory effects but they frequently are toxic. For example, staurosporine is a broad-spectrum kinase inhibitor and induces apoptosis. Pharmaceutical researchers are focused on decreasing the toxicity of staurosporine by developing a cell-specific delivery system, as well as constructing analogs that are less toxic (Mukthavaram et al., 2013). Also, suppression of the immune response may lead to severe infections by suppressing both pro-inflammatory signaling pathways and thus may not be an ideal strategy for treatment. The selective suppression

of the major proinflammatory pathway, NF-κB, with an intact MAPK pathway that regulates one third of the inflammatory responses would be a more attractive approach for a candidate anti-inflammatory drug. The effects of *cis*- and *trans*-gnetin H on the MAPK pathway was also investigated in THP-1 cells and showed both gnetin H compounds were able to suppress SAPK/JNK but ERK 1/2 was suppressed only by *cis*-gnetin H. Neither gnetin H was able to suppress p38 phosphorylation (Supplement Figure 2; Appendix B). This suggests that *cis*- and *trans*-gnetin H compounds have broad spectrum activities. The over-activation of ERK1/2 and JNK have been implicated in multiple pathologic conditions such as cancer, stroke, cardiac diseases, and other inflammatory diseases (Hommes et al., 2003). ERK1/2 mainly regulates cell proliferation and growth and increased activation of ERK1/2 in cancer cells has attracted attentions in drug discovery research (Kohno and Pouyssegur, 2006). JNK is activated generally through stress and regulates cell proliferation and cell death as a consequence. Increased activation of JNK is observed in cancer, stroke, neurodegenerative, and ischemic heart disease (Zhang et al., 2012). Therefore, either *cis*- or *trans*-gnetin H may be selected for inflammatory-associated diseases based on targeting a protein that reveals higher expression of activation. For example, *cis*-gentin H may be used in cancer treatments that involve tumors with increased NF-κB and MAPK pathways, whereas *trans*-gnetin H may be used in conditions that are associated with JNK overexpression (Fig. 18).

In conclusion, the present study demonstrated that gnetin H isomers and aurone 1 are anti-inflammatory compounds with therapeutic potential for the possible treatment of chronic inflammatory diseases. Better understanding of their exact molecular

mechanisms are still in question, however, future studies such as computational modeling predictions and *in vitro* multiplex kinase assays will provide more evidence in determining the mechanism of action of these compounds.

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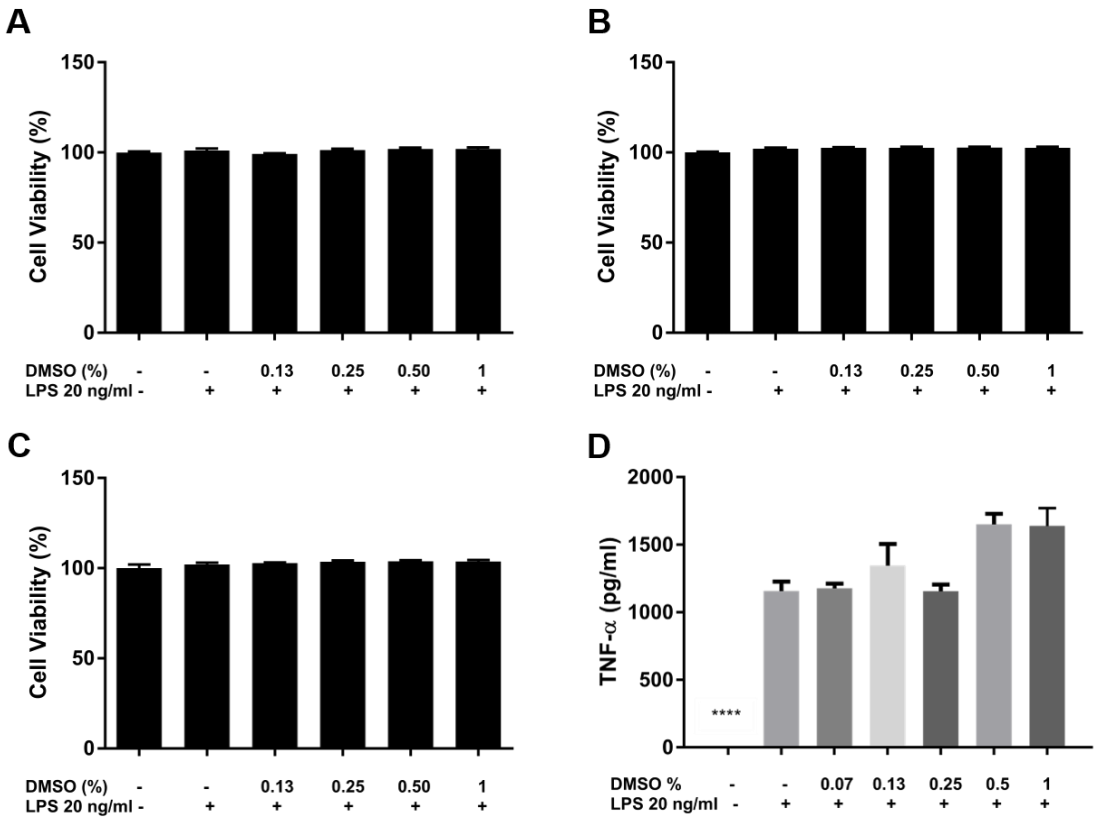
APPENDICES

APPENDIX A: VEHICLE CONTROL EFFECTS ON CELL VIABILITY AND TNF-α RESPONSE

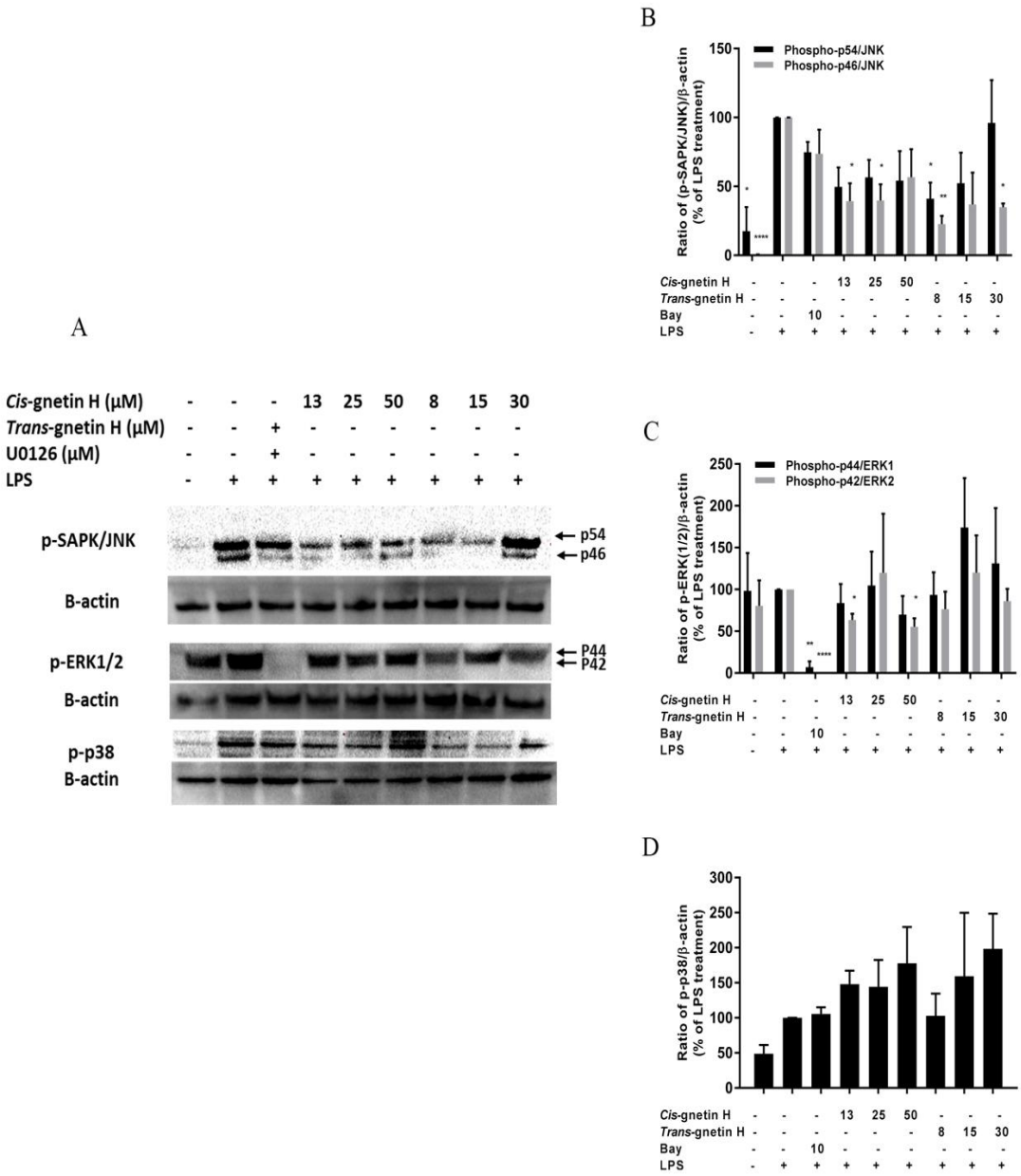


Supplement Figure 1. DMSO effect on THP-1 and RAW264.7 cell viability and THP-1 TNF-α response.

Vehicle control, DMSO, was tested for its effects on cell viability using Alamar Blue assay and TNF-α response was measure from THP-1 cells. A) THP-1 cells were pretreated with various concentrations of DMSO for 1 h and stimulated with LPS for 4 h. B) RAW 264.7 cells were pretreated with various concentrations of DMSO for 1 h and stimulated with LPS for 4 h. C) RAW 264.7 cells were pretreated with various

concentrations of DMSO for 1 h and stimulated with LPS for 24 h. D) THP-1 cells were pretreated with various concentrations of DMSO for 1 h and stimulated with LPS for 4 h. Supernatants were collected and TNF-α was measured using ELISA.

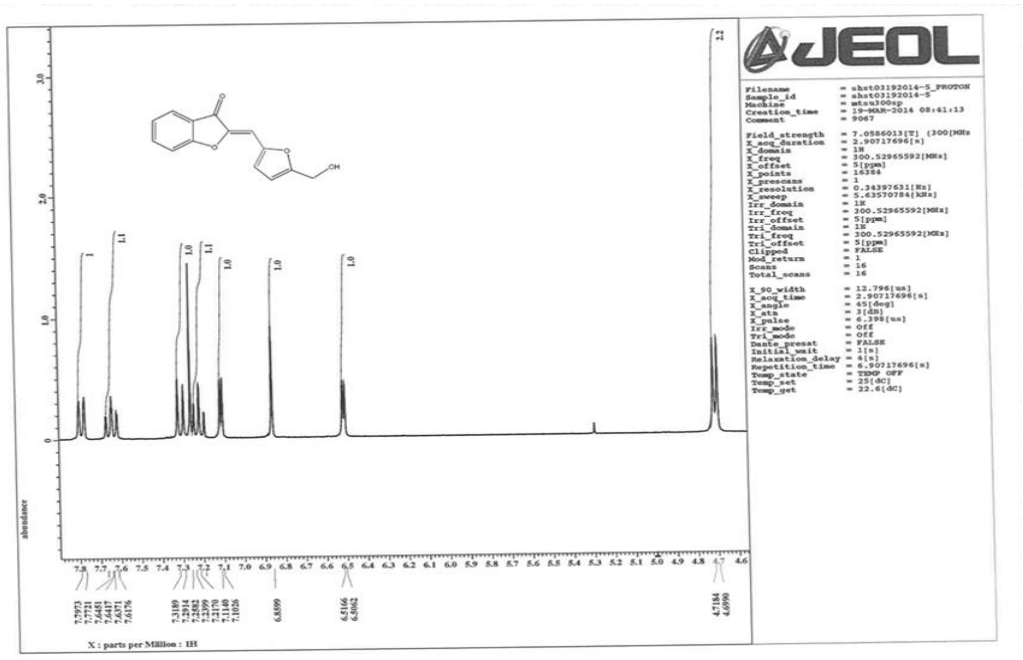
APPENDIX B: THE EFFECTS OF *CIS*- AND *TRANS*-GNETIN H ON MAPK MEDIATORS

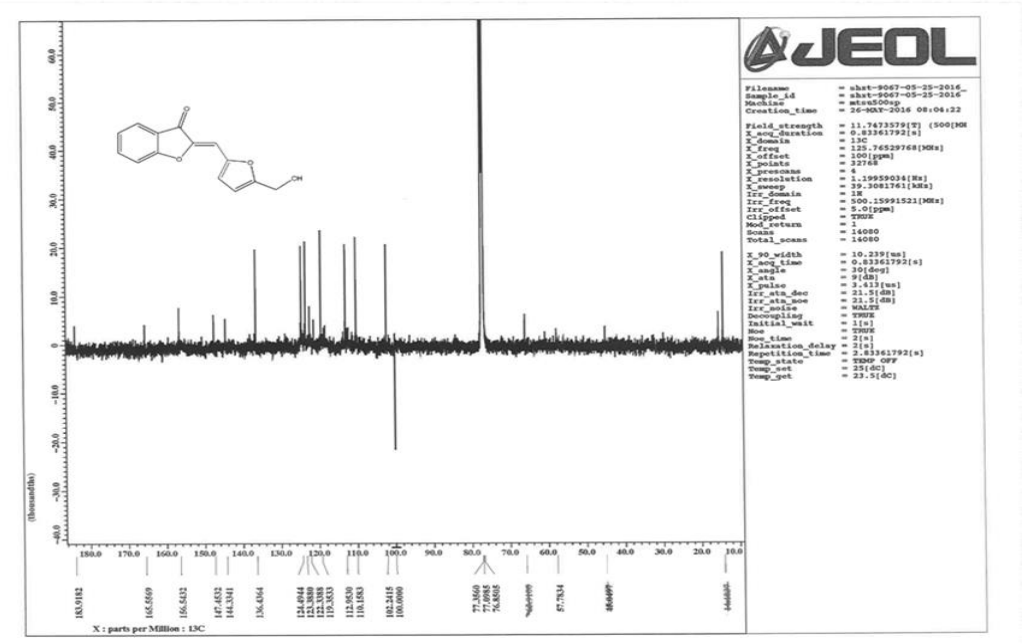


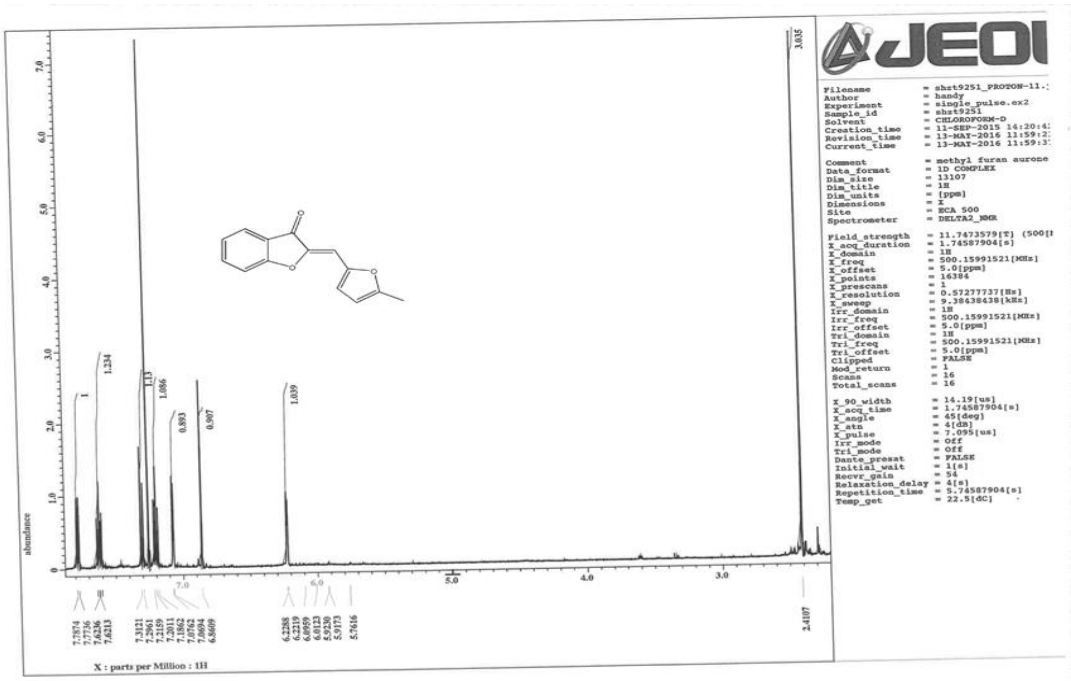
Supplement Figure 2. The effects of *cis*- and *trans*-gnetin H on JNK, ERK, and p38 phosphorylation in THP-1 cells.

PMA-differentiated cells were pretreated with 13, 25, and 50 μM *cis*-gnetin H and 8, 15, and 30 μM *trans*-gnetin H for 1 h and stimulated with 1 μg/ml LPS for 15 min. (A) Image blot of phosphorylated SAPK/JNK (Thr180/Tyr182), ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), and β-actin were measured in THP-1 cells by Western blotting followed by densitometry (B-D).

APPENDIX C: PROTON AND CARBON NMR SPECTRA DATA FOR SYNTHETIC AURONES



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Irr_freq = 500.15991521[MHz]
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Tri_mode = Off
Dante_presat = FALSE
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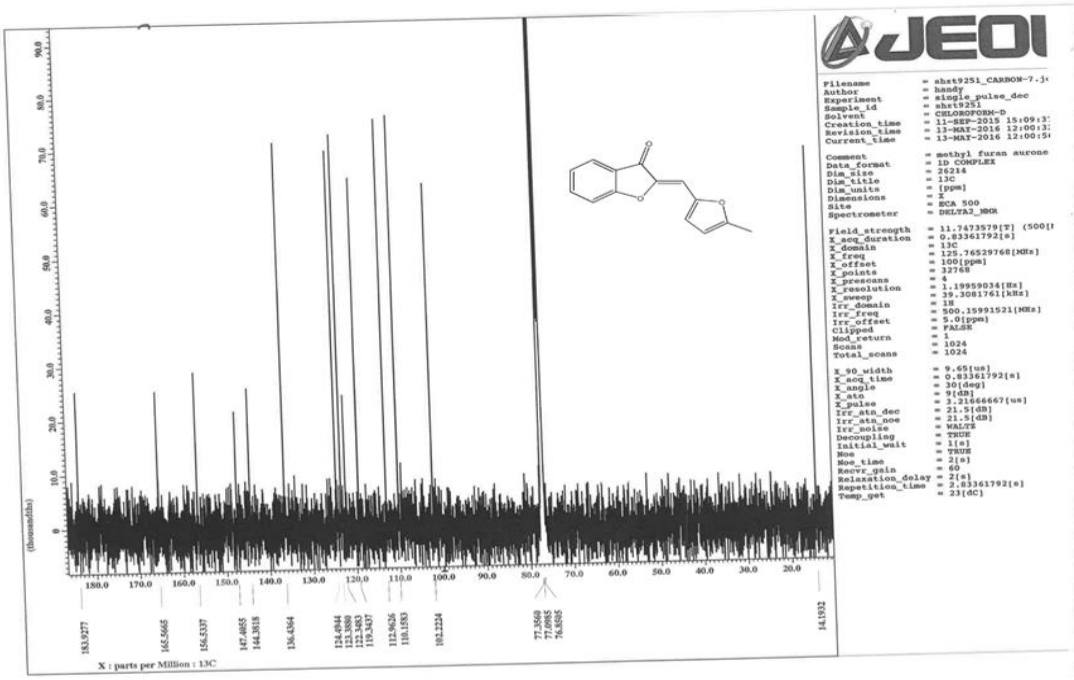
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X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
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X_acq_time = 0.83361792[s]
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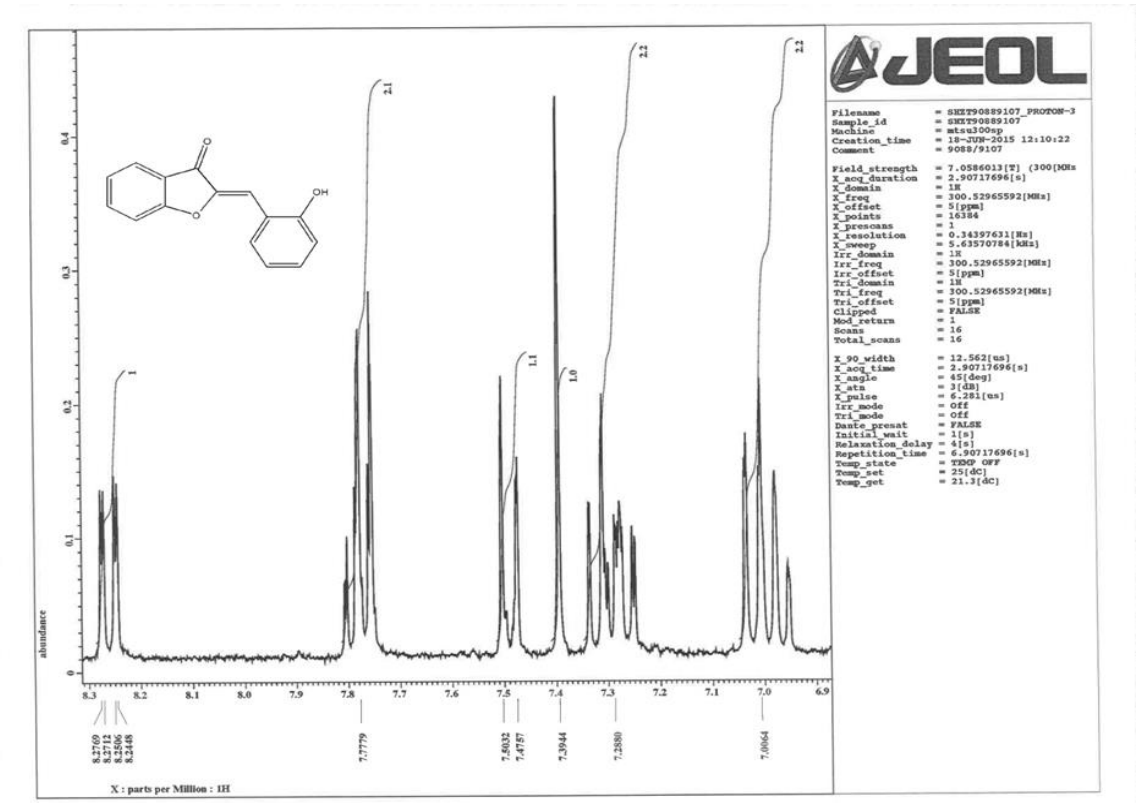
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Irr_freq = 300.52965592[MHz]
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Dante_presat = FALSE
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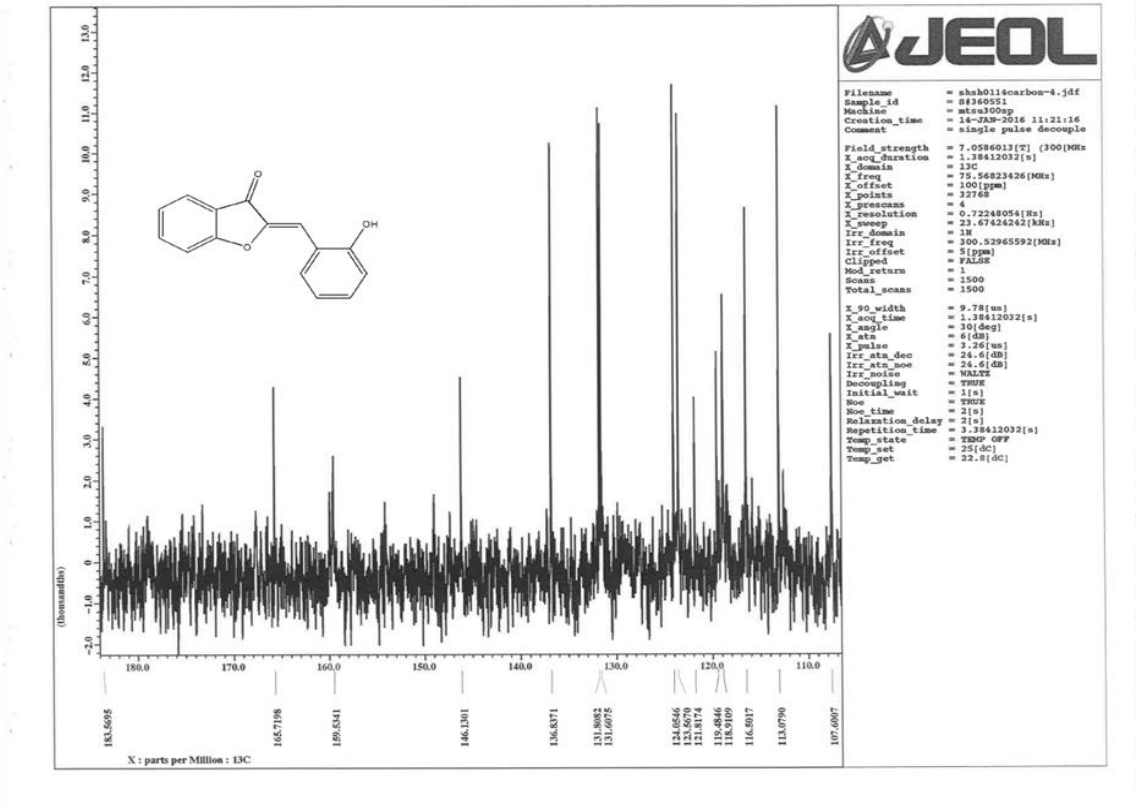
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X_sweep = 23.67424242[kHz]
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Noe_time = 2[s]
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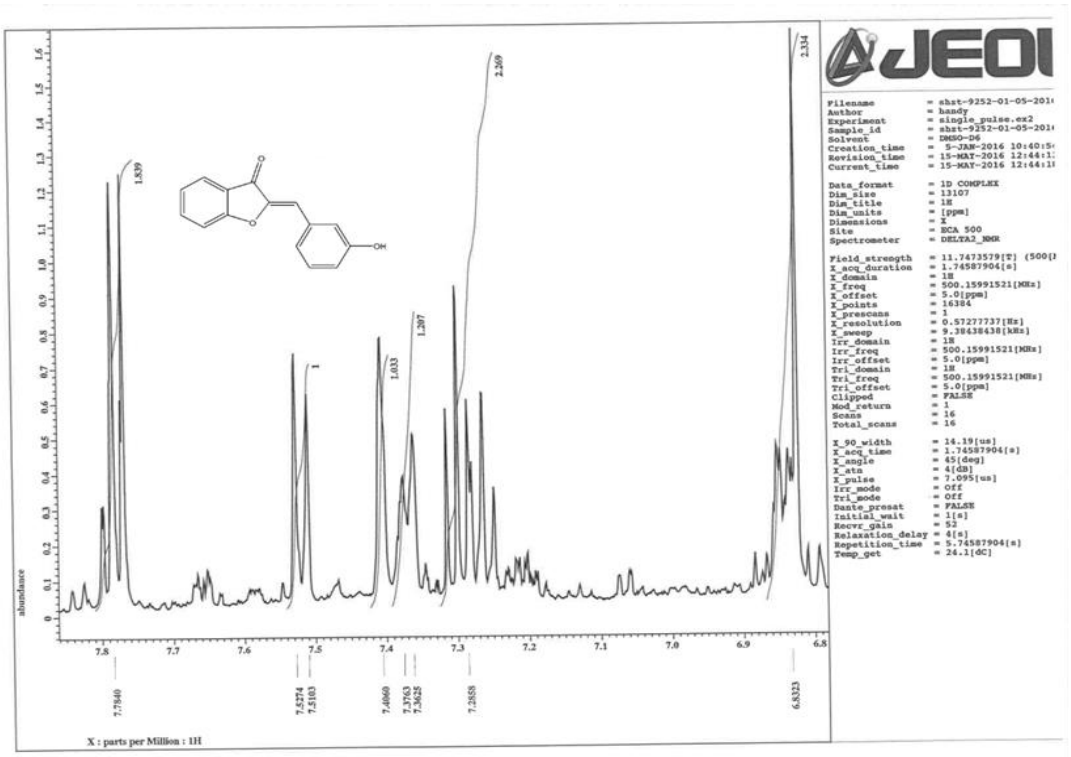
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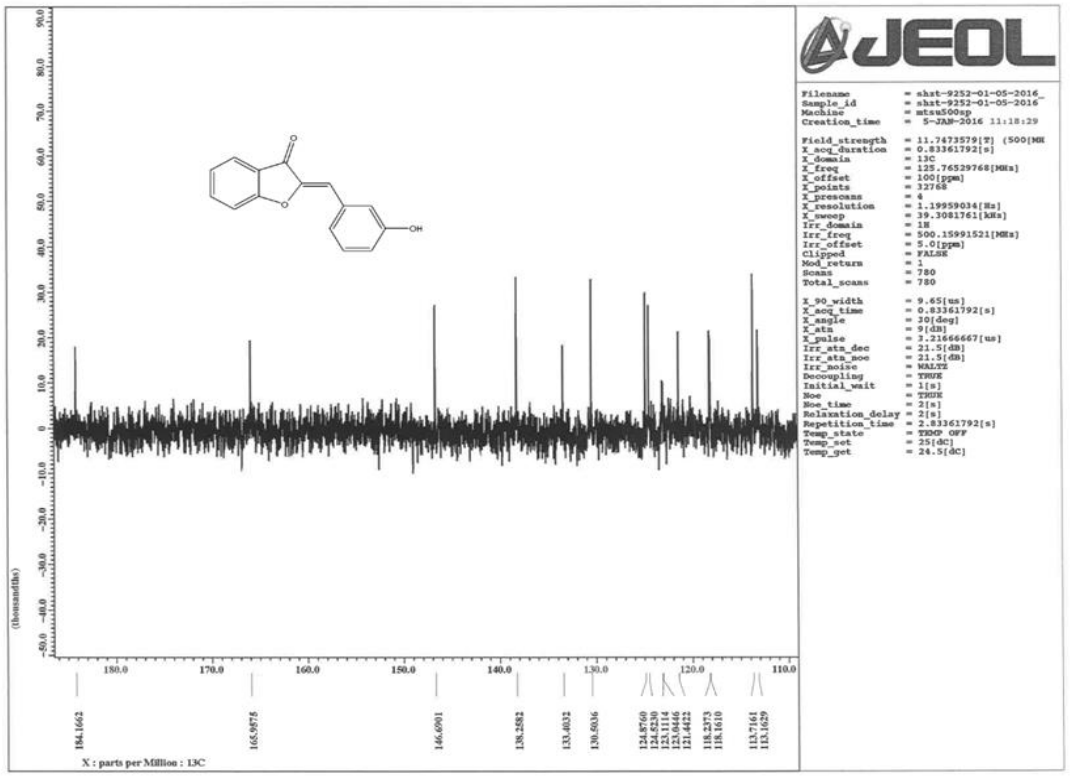
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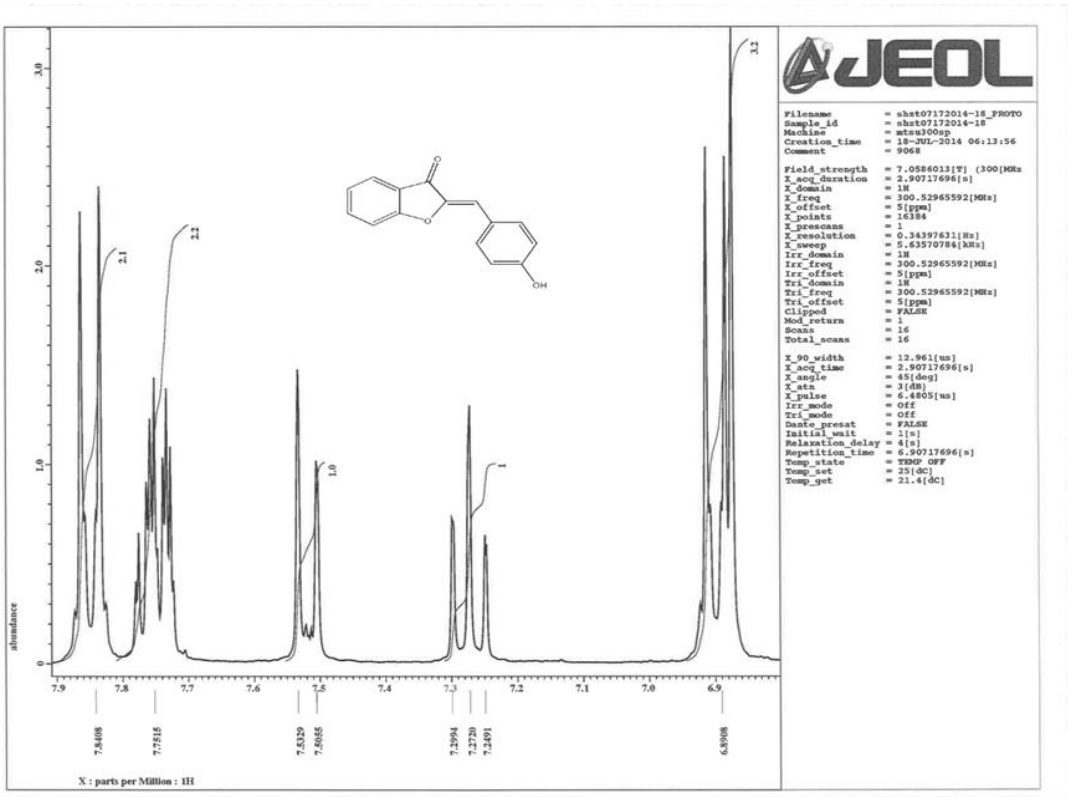
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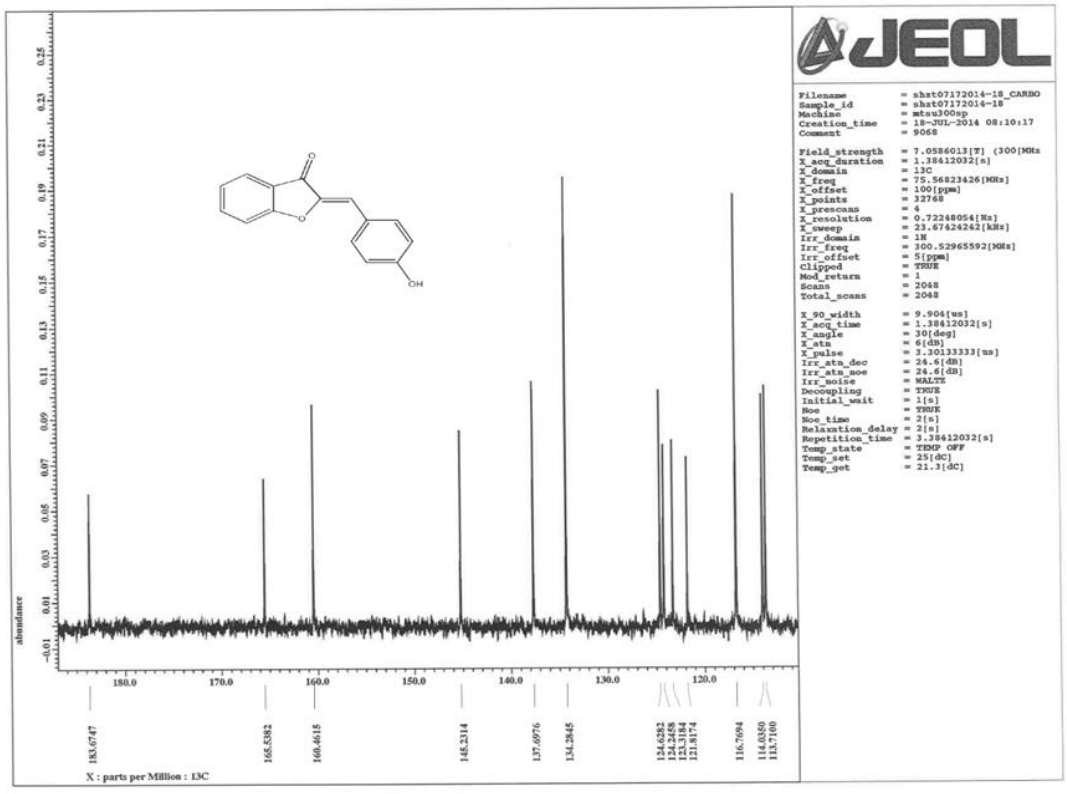
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Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
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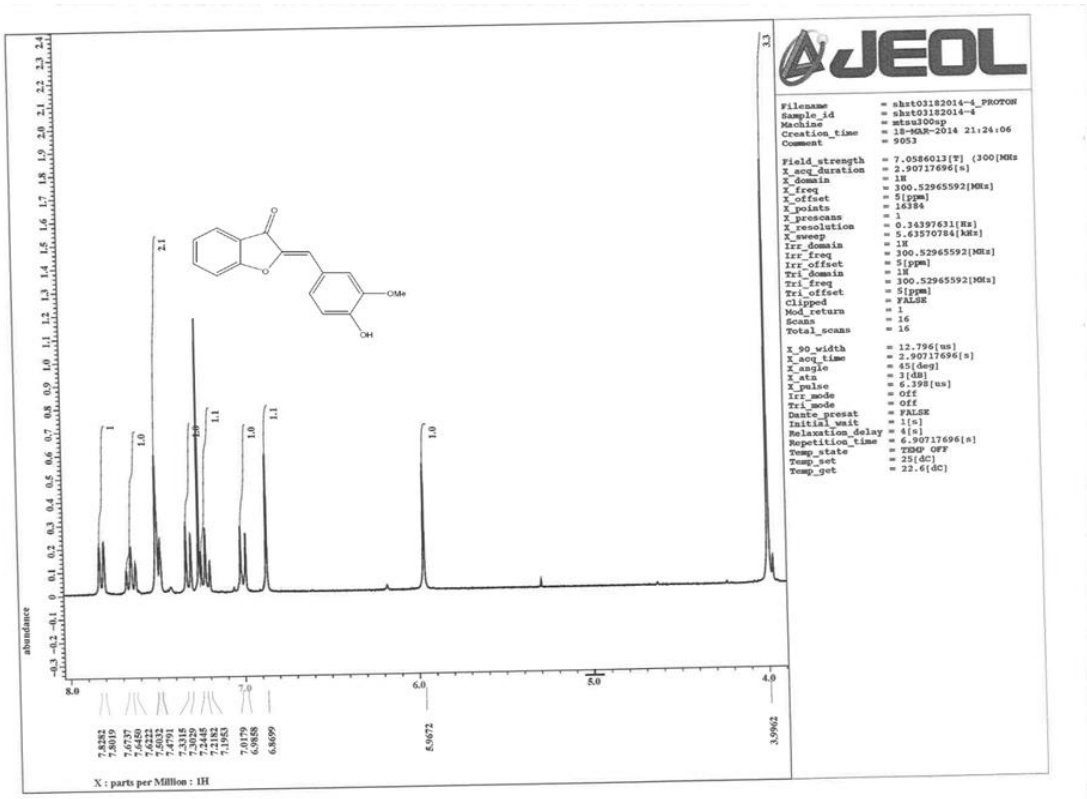
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Dante_presat = FALSE
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OMe
OH
abundance
7.8252
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7.6737
7.6480
7.6222
7.6032
7.4791
7.3315
7.3029
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7.1953
7.0179
6.9858
6.8699
5.0672
3.5962
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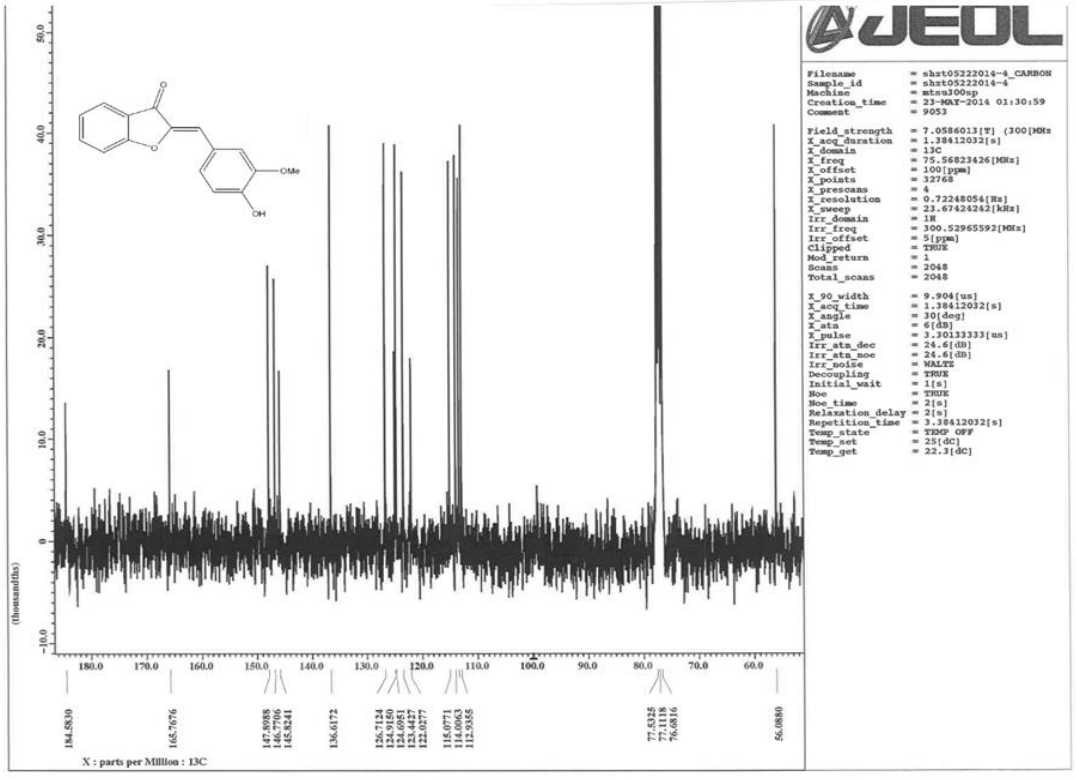
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Irr_freq = 300.52965592[MHz]
Irr_offset = 5[ppm]
Clipped = TRUE
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X_acq_time = 1.38412032[s]
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Irr_atn_noe = 24.6[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Relaxation_delay = 2[s]
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Temp_set = 25[dC]
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OMe
OH
(thousandths)
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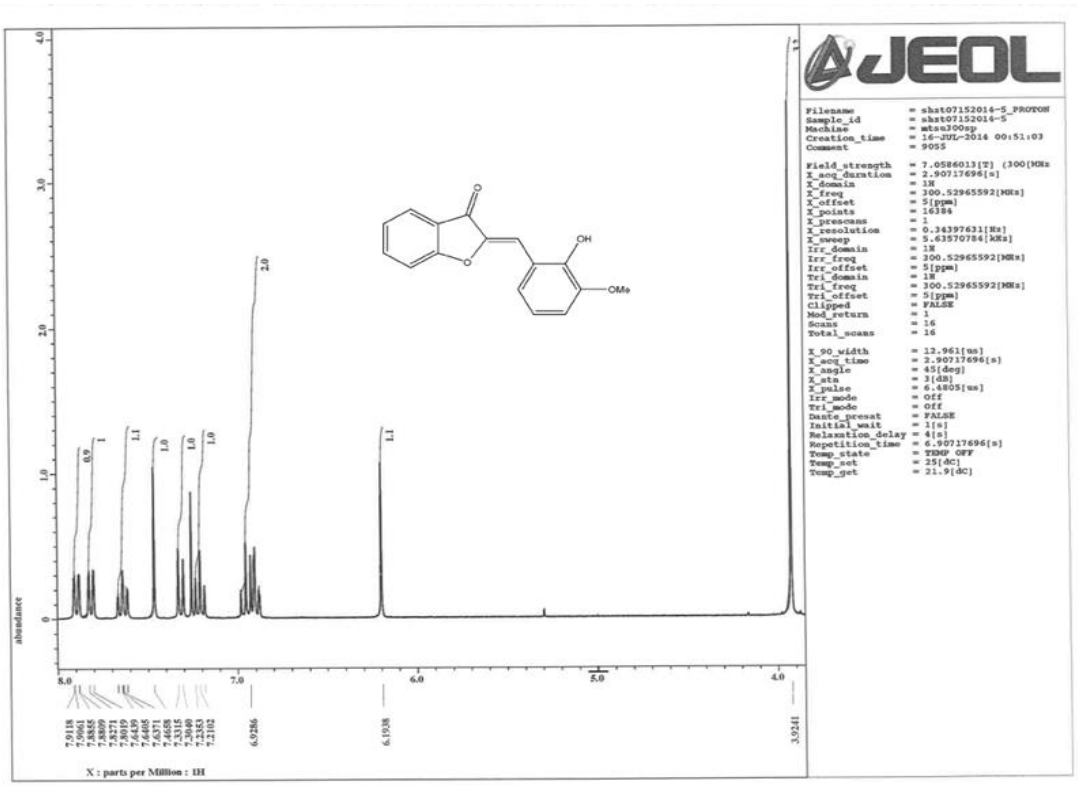
146.7706
145.8241
136.6172
126.7124
124.6080
124.0051
123.4427
122.0277
116.0771
114.6963
112.9385
77.5325
77.1118
76.6816
56.0880
X : parts per Million : 13C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Filename = shst07152014-S_PROTON
Sample_id = shst07152014-S
Machine = mtsa300sp
Creation_time = 16-JUL-2014 00:51:03
Comment = 9055
Field_strength = 7.0586013[T] (300[MHz
X_acq_duration = 2.90717696[s]
X_domain = 1H
X_freq = 300.52965592[MHz]
X_offset = 5[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.34397631[Hz]
X_sweep = 5.63570784[kHz]
Irr_domain = 1H
Irr_freq = 300.52965592[MHz]
Irr_offset = 5[ppm]
Tri_domain = 1H
Tri_freq = 300.52965592[MHz]
Tri_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 12.961[us]
X_acq_time = 2.90717696[s]
X_angle = 45[deg]
X_atn = 3[dB]
X_pulse = 6.4805[us]
Irr_mode = Off
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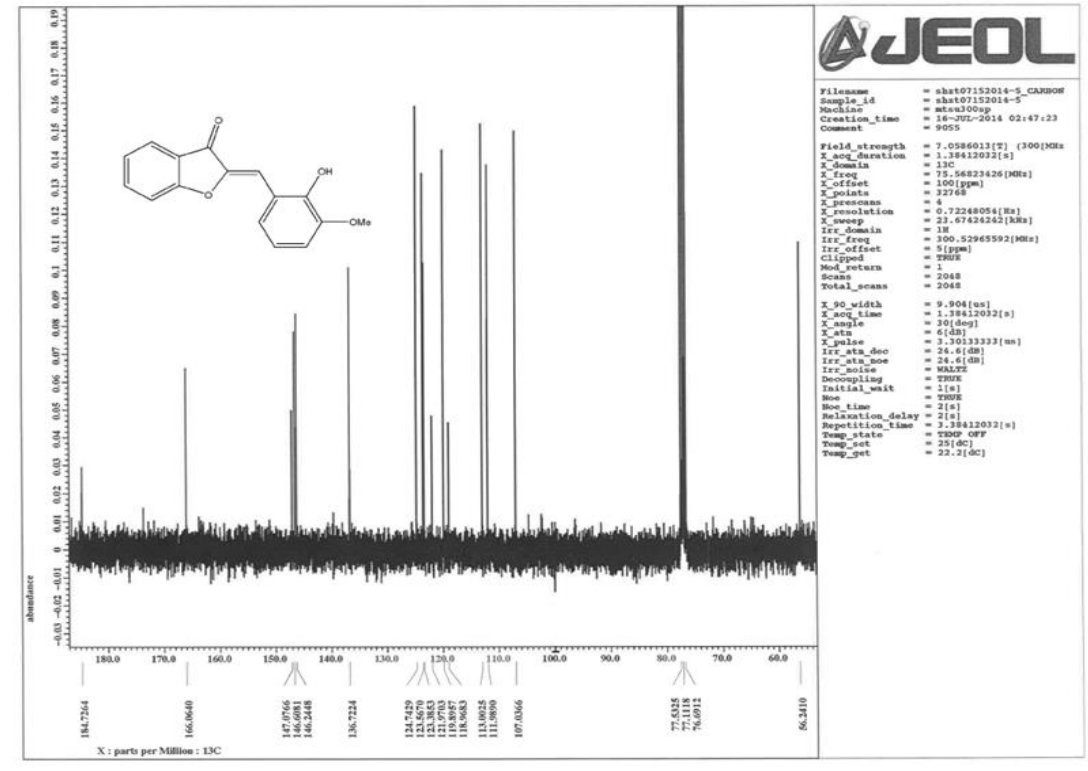
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 6.90717696[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.9[dC]
OH
OMe
abundance
X : parts per Million : 1H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Filename = shst07152014-S_CARBON
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Machine = mtsa300sp
Creation_time = 16-JUL-2014 02:47:23
Comment = 9055
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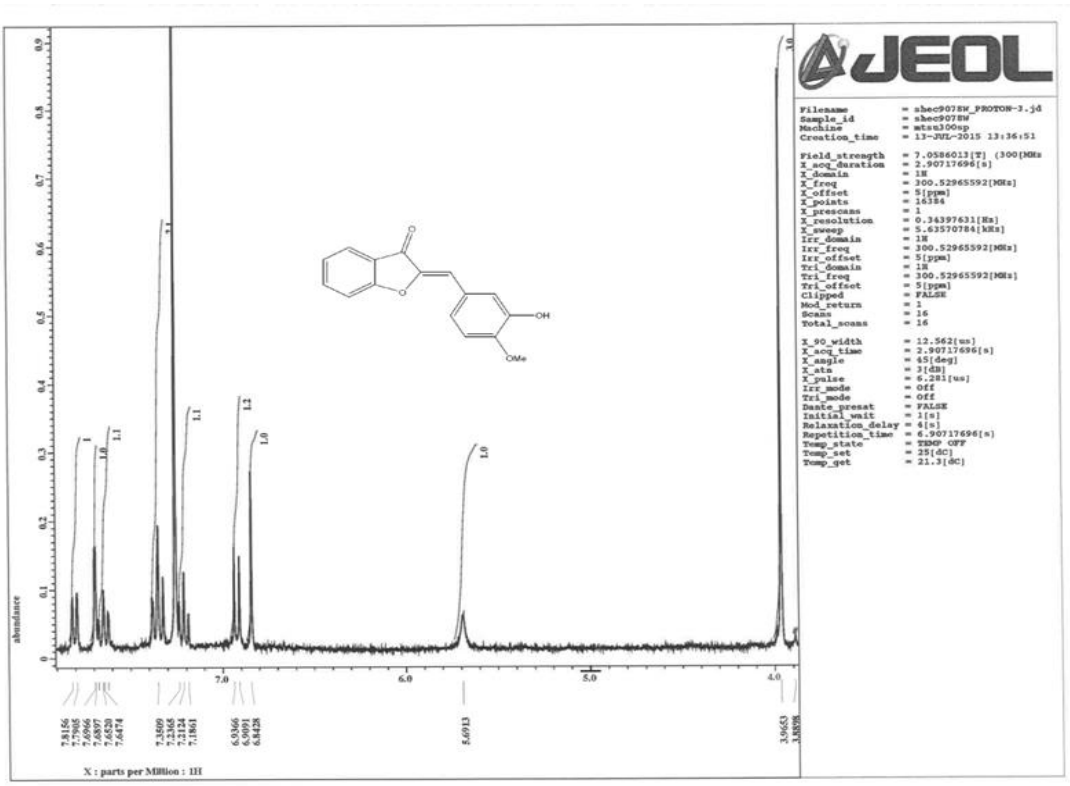
X_acq_duration = 1.38412032[s]
X_domain = 13C
X_freq = 75.56823426[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.72248054[Hz]
X_sweep = 23.67424242[kHz]
Irr_domain = 1H
Irr_freq = 300.52965592[MHz]
Irr_offset = 5[ppm]
Clipped = TRUE
Mod_return = 1
Scans = 2048
Total_scans = 2048
X_90_width = 9.904[us]
X_acq_time = 1.38412032[s]
X_angle = 30[deg]
X_atn = 6[dB]
X_pulse = 3.30133333[us]
Irr_atm_dec = 26.6[dB]
Irr_atm_noe = 24.6[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Relaxation_delay = 2[s]
Repetition_time = 3.38412032[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 22.2[dC]
OH
OMe
abundance
X : parts per Million : 13C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Sample_id = shec907SW
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Creation_time = 13-JUL-2015 13:36:51
Field_strength = 7.0586013[T] (300[MHz
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X_domain = 1H
X_freq = 300.52965592[MHz]
X_offset = 5[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.34397631[Hz]
X_sweep = 5.63570786[kHz]
Irr_domain = 1H
Irr_freq = 300.52965592[MHz]
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Tri_domain = 1H
Tri_freq = 300.52965592[MHz]
Tri_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 12.562[us]
X_acq_time = 2.90717696[s]
X_angle = 45[deg]
X_atn = 3[dB]
X_pulse = 6.281[us]
Irr_mode = Off
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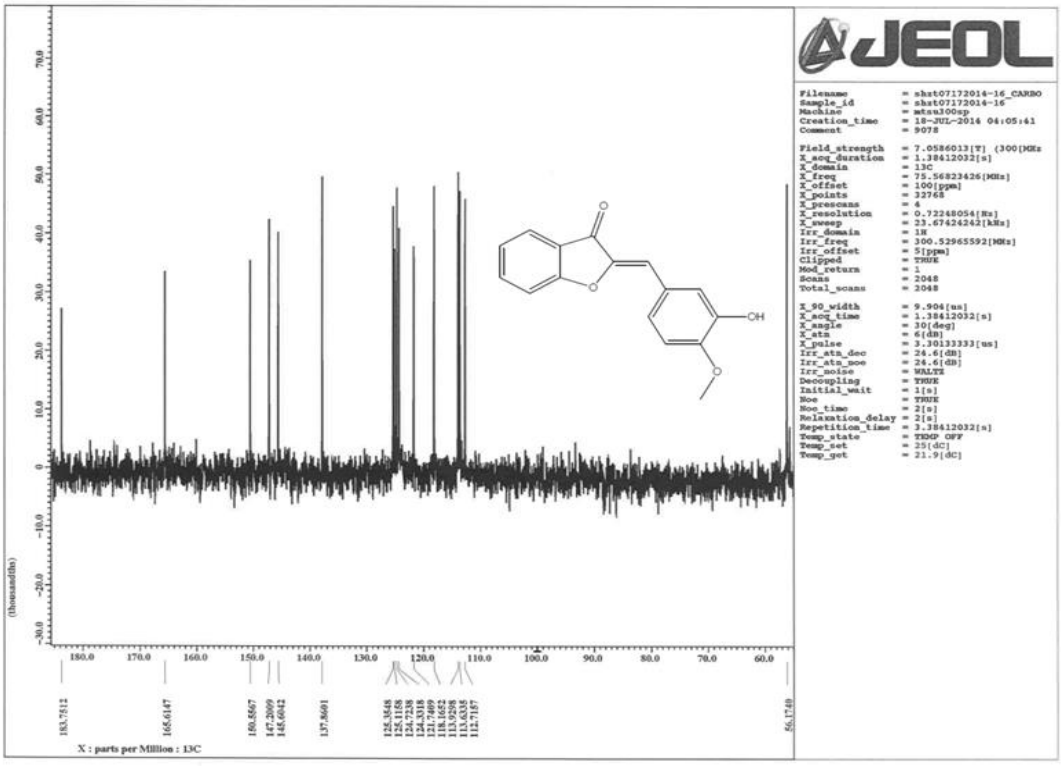
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 6[s]
Repetition_time = 6.90717696[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.3[dC]
OH
OMe
absolute
X : parts per Million : 1H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Filename = shst07172014-16_CARBO
Sample_id = shst07172014-16
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Field_strength = 7.0586013[T] (300[MHz
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X_domain = 13C
X_freq = 75.56823426[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.72248054[Hz]
X_sweep = 23.67424242[kHz]
Irr_domain = 1H
Irr_freq = 300.52965592[MHz]
Irr_offset = 5[ppm]
Clipped = TRUE
Mod_return = 1
Scans = 2048
Total_scans = 2048
X_90_width = 9.906[us]
X_acq_time = 1.38412032[s]
X_angle = 30[deg]
X_atn = 6[dB]
X_pulse = 3.30133333[us]
Irr_atn_dec = 24.6[dB]
Irr_atn_noe = 24.6[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Relaxation_delay = 2[s]
Repetition_time = 3.38412032[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.9[dC]
OH
(thousandths)
X : parts per Million : 13C

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Expected completion date	May 2017
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