

Original Research Article

Synthetic chalcone derivatives inhibit cytokine secretion via inhibition of ERK and JNK pathways in human U937 macrophage

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Abstract

Purpose: To investigate the inhibitory effects of a chalcone derivative on lipopolysaccharide (LPS)-induced interleukin (IL)-6 and IL-8 secretions and on LPS-induced mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF- κ B) activation in human U937 macrophage-like cell line.

Methods: The effects of chalcone derivative on LPS-induced secretion of IL-6 and IL-8 in endothelial cells were determined by enzyme-linked immunosorbent assay while the effects of chalcone on the activation of MAPK and NF- κ B pathway were determined by Western blotting.

Results: The results showed that 3-(5-methyl-furan-2-yl)-naphthalen-1-yl-propenone (compound 1) significantly inhibited the secretion of LPS-induced IL-6 and IL-8 in U937 macrophages. This compound also demonstrated significant suppression of c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) phosphorylation. However, the compound did not reverse the degradation of inhibitor kappa B alpha (I κ B α) and did not inhibit the phosphorylation of NF- κ B subunit and P-38 MAPK.

Conclusion: Compound 1 inhibits the secretion of cytokines via the inhibition of ERK and JNK pathways. These results suggest that chalcone derivative could act as an anti-inflammatory agent by altering cytokine secretion and inflammatory pathways.

Keywords: Inflammation, Chalcone derivative, Cytokines, MAPK, NF- κ B, Macrophage

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INTRODUCTION

Inflammation is a crucial component of the complex biological response of body tissues against detrimental stimuli [1]. However, chronic and excessive inflammation will cause several diseases such as rheumatoid arthritis and atherosclerosis. Activated macrophages serve an important role in establishing inflammation by inducing the secretion of pro-inflammatory

cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8). IL-6 is a cytokine which induces neutrophil maturation as well as cytotoxic T-lymphocyte and natural killer cell differentiation [2]. Whilst IL-8 promotes chemotaxis and directs migration of leucocytes to the target site for immune responses [3].

Chalcones (1,3-diaryl-2-propen-ones) are α , β unsaturated ketones with two aromatic rings,

which are present in edible fruits. These compounds serve their role as precursors in the biosynthesis of flavonoids and bioflavonoids. Chalcone derivatives from different moieties are synthesized in laboratories using the Claisen Schmidt condensation reaction [4]. Diverse pharmacological properties of chalcone derivatives including anti-proliferative, anti-inflammatory and immunomodulatory have been demonstrated in previous studies [5-7]. Treatment with chalcone derivative known as cardamonin was reported to be able to inhibit LPS-induced TNF- α secretion in THP-1 cells [8]. Synthetic chalcone derivatives also inhibit polymorphonuclear neutrophils' (PMNs) chemotaxis as well as chemokines secretion induced by oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) in differentiated macrophage-like cells [9,10]. However, little is known whether they can also inhibit pro-inflammatory cytokine secretion induced by bacterial endotoxin. This study aimed to investigate the effects of a synthetic chalcone derivative on LPS-stimulated cytokines secretion in human U937 macrophages.

EXPERIMENTAL

Chemicals and reagents

Synthetic chalcone derivatives 3-(5-methyl-furan-2-yl)-naphthalen-1-yl-propenone (Compound 1) (Figure 1) was obtained from the Drug and Herbal research Centre, Universiti Kebangsaan Malaysia and reported previously [23]. All cell culture reagents were purchased from Invitrogen (Burlington, ON, Canada). Lipopolysaccharides from *Escherichia coli* O26:B6 (LPS) was purchased from Sigma (St. Louis, MO, USA) and ELISA kits were purchased from eBioscience (San Diego, CA, USA). Antibodies for p-ERK1/2, p-P38 and p-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA) while antibody for I κ B α , p-p65 and β -actin were purchased from Abcam (Cambridge, MA, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

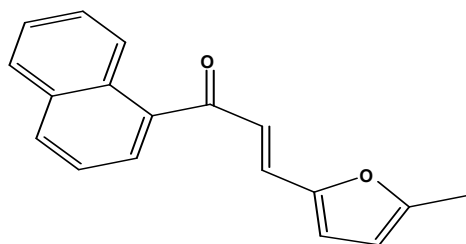


Figure 1: Chemical structures of 3-(5-methyl-furan-2-yl)-naphthalen-1-yl-propenone

Cell culture and cell differentiation

The U937 mononuclear cell line was procured from ATCC (Manassas, VA, United States). The cells were maintained in RPMI-1640 and supplemented with 10% FBS, 100 units/mL penicillin and 100 μ g/ml streptomycin. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C. They were subcultured in 75 cm² culture flasks or seeded for experiments. Differentiation of cells were done at 70% confluence. Cells were differentiated into macrophage-like cells with phorbol myristate acetate (PMA) for 24 hours and followed with overnight incubation with serum-free media [11] before treated with compounds.

Determination of cytokines secretion

Compound 1 was dissolved in dimethylsulphoxide (DMSO) to prepare stock solution at a concentration of 50 mM. The stock solution was further diluted in serum-free media at concentrations of 12.5, 25, and 50 μ M. To determine the effects of compound 1 on LPS-induced IL-6 and IL-8 secretion, cells were pretreated with the compound for 1 h and followed by stimulation with 1 μ g/mL of LPS for the subsequent 24 h. Duration for the induction of IL-6 and IL-8 with LPS was determined based on the findings from a preliminary work. A vehicle control group received 0.1 % DMSO, a negative control group received LPS (1 μ g/mL), a positive control group received a combination of dexamethasone and LPS and treatment groups received different concentrations of chalcone derivative and LPS. Supernatants were collected and enzyme-linked immunosorbent assay (ELISA) was carried out according to the manufacturer's instructions.

Detection of protein expression by Western immunoblot

To investigate the effects of compound 1 on the activation of MAPK and NF- κ B pathway, Western blotting was used to determine the phosphorylation of ERK, JNK P-38, P65 and degradation of I κ B α . Compound 1 at concentrations 12.5, 25 and 50 μ M was added into cells for 1 h and followed by the addition of LPS for 1 h. Subsequently, cells lysate was prepared by adding lysis buffer that contain protease and phosphatase inhibitors. The concentration of protein was determined using Bradford assay. The cell lysate (20 μ g) was added in wells of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE separates proteins primarily by mass because the ionic detergent SDS denatures and

binds to proteins to make them uniformly negatively charged.

Subsequently, Western blotting technique was carried out as previously described [12]. Protein on the gel was transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% BSA and washed with TBST and followed with overnight incubation with primary antibodies such as p-ERK, p-JNK, p-P38, p-p65, anti- $\text{I}\kappa\text{B}\alpha$ (1:1000) or β -actin (1:5000). Subsequently, the membrane was washed and a secondary antibody (1:5000) was added onto the membrane for 1 h. Enhanced chemiluminescence substrate (ECL) (Perkin Elmer, USA) was added onto the membrane to visualise the bands and the relative intensity was quantified using Fusion-Capt Advance Software (Vilber Loumat, Germany).

Statistical analysis

The data were collected from three replicates of experiments and subjected to analysis using GraphPad Prism 5 software (GraphPad Software Inc, San Diego, CA, USA). The data are presented as mean \pm standard error of mean (SEM). Mean differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory effects of compound 1 on LPS-induced cytokines secretion

Concentrations of compound 1 used in this study were selected based on the concentrations that did not cause more than 10% U937 macrophage cell death, as reported in our previous work [10]. Figure 2 A shows the effect of compound 1 on LPS-induced IL-6 secretion in U937. The secretion of IL-6 by LPS-stimulated cells was significantly increased in cells treated with LPS at a concentration of 1 $\mu\text{g}/\text{mL}$. Dexamethasone (100 nM) significantly diminished IL-6 secretion induced by LPS.

Chalcone derivative (12.5 μM) significantly inhibited IL-6 secretion ($p < 0.01$) and further reduction was observed in cells treated with chalcone derivative at 25 and 50 μM ($p < 0.001$). The percentage of inhibition by the highest concentration used in this study was 36.095 %. Figure 2 B shows the effect of compound 1 on LPS-induced IL-8 secretion in U937 cells. A significant increase of IL-8 secretion was observed in cells treated with LPS. Dexamethasone significantly inhibited IL-8

secretion and chalcone derivative inhibited IL-8 secretion in a concentration dependent manner.

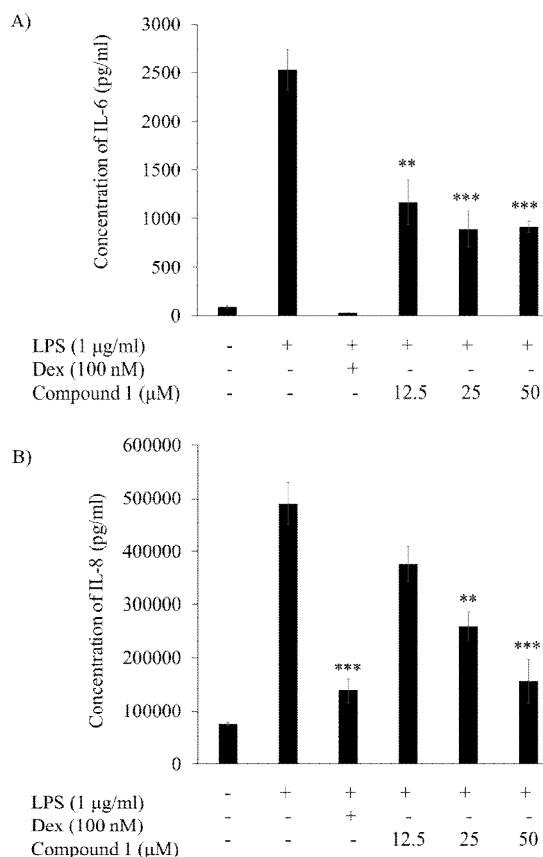


Figure 2: Effects of compound 1 on LPS-induced (A) IL-6 and (B) IL-8 secretion in U937 macrophage was determined using ELISA. Cells were pre-treated with chalcone derivative for 1 h followed by induction with LPS at 1 $\mu\text{g}/\text{mL}$ for 24 h. The data presented are mean \pm SEM ($n = 3$); ** $p < 0.01$, *** $p < 0.001$ vs negative control

Compound 1 did not suppress LPS-induced $\text{I}\kappa\text{B}\alpha$ degradation and p65 phosphorylation

Figure 3A shows that compound 1 did not inhibit the NF κ B pathway. Following treatment with LPS, degradation of $\text{I}\kappa\text{B}\alpha$ was observed. However, treatment of cells with compound 1 at concentrations ranging from 12.5 to 50 μM did not reverse the degradation of $\text{I}\kappa\text{B}\alpha$. Similarly, treatment with LPS was found to increase phosphorylation of phospho-p65. However, the chalcone derivative did not significantly suppress the phosphorylation of p65 NF κ B although there was a trend for a reduction of phosphorylation. The intensity of each band was normalised with respect to beta-actin protein expression while statistical analysis was carried out with respect to negative control.

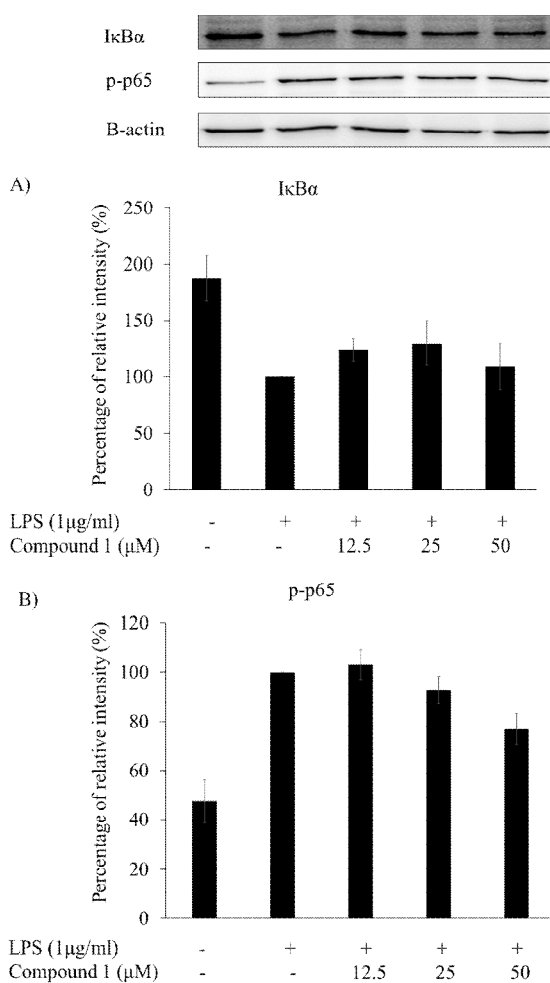


Figure 3: Effect of chalcone derivative on LPS-induced A) $\text{IkB}\alpha$ degradation and (B) p-p65 expression. Cells were pre-treated with chalcone derivatives for 1 h prior to stimulation with LPS at 1 $\mu\text{g}/\text{mL}$ for 60 min. The data represented mean \pm SEM (n = 3)

Compound 1 suppressed LPS-induced ERK and JNK phosphorylation but did not inhibit p-38

Figure 4 shows that compound 1 may inhibit phosphorylation of ERK and JNK but not p38. Following treatment with LPS, significant increase in expression of the three MAPKs was observed. Pretreatment with compound 1 reduced the phosphorylation of ERK back to normal level starting from 25 μM (Figure 4 A). Whilst for phosphorylation of JNK, significant reduction of phosphorylation was observed in cells pretreated with 25 μM of compound 1 but a reduction to normal level was observed in cells pretreated with 50 μM of compound 1 (Figure 4 B). Moreover, this compound did not inhibit the phosphorylation of P38 MAPK signalling pathway (Figure 4 C).

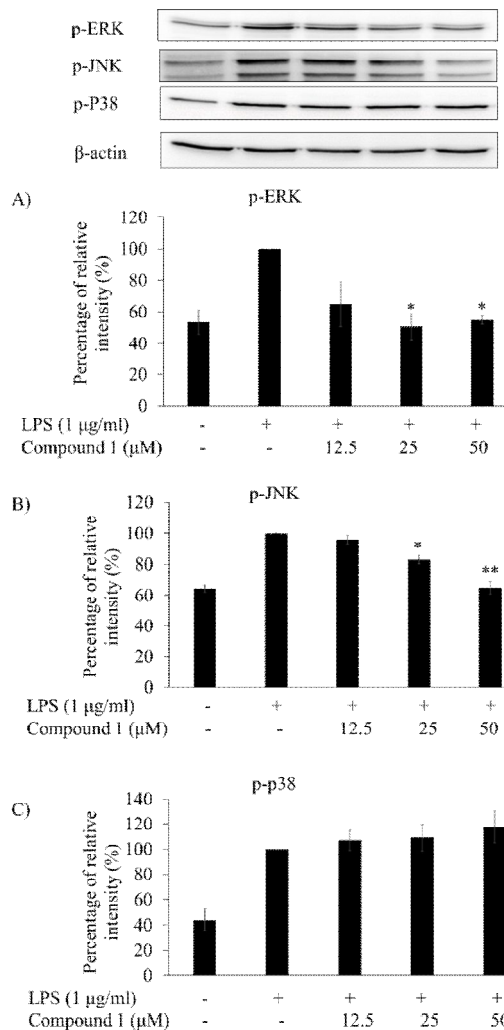


Figure 4: Effects of chalcone derivative on LPS-induced A) p-ERK, (B) p-JNK and C) p-P38 MAPK expression. Cells were pre-treated with chalcone derivatives for 1 hour prior to stimulation with LPS at 1 $\mu\text{g}/\text{mL}$ for 60 min. The data represented mean \pm SEM (n = 3)

DISCUSSION

LPS is a distinctive glycolipid comprising of a highly variable carbohydrate segment and a unique lipid A region, which can be found in the outer wall of Gram-negative bacteria [13]. A previous study reported that the presence of LPS at 50 $\mu\text{g}/\text{mL}$ or above in the plasma may increase the risk of developing atherosclerosis in humans [14]. This molecule is known to be a pathogen-associated microbial pattern (PAMP) which may activate Toll-like receptor (TLR) signalling to demonstrate cellular responses such as the secretion of interferon and pro-inflammatory cytokines [15]. A study conducted by Clahsen and Schaper revealed that the role of IL-6 as the pro-inflammatory cytokine by promoting monocytic cell migration through a number of processes such as integrin activation,

cell attachment, polymerisation of actin, chemotaxis and fibronectin-dependent migration [16]. IL-8 was also demonstrated to cause attachment of monocytic cells to human atherosclerotic plaques [17].

The present study found that compound 1 significantly suppressed LPS-induced IL-6 and IL-8 secretion in human U937 macrophages. This result is consistent with previous data that reported the inhibitory effects of natural chalcone and chalcone derivatives on proinflammatory cytokines secretion. Several natural chalcones have been demonstrated to inhibit proinflammatory cytokines secretion. For instance, naringenin caused significant inhibition on LPS induced IL-1 β , IL-6, TNF- α and IL-8 secretion stimulated by LPS in macrophage cells [18]. Moreover, butein, a natural chalcone, was found to attenuate the secretion of IL-6, IL-8 and TNF- α induced by calcium ionophore A23187 (PMACI) in human mast cells [19]. A chalcone derivative, trans-1,3-diphenyl-2,3-epoxypropane-1-one (DPEP) has shown that it is able to inhibit LPS-induced IL-6, IL-1 β and TNF- α secretion in murine RAW264.7 cells in a concentration-dependent manner [20]. In the present study, compound 1 exhibited significant inhibition on LPS-induced IL-6 and IL-8 secretion, possibly due to the presence of naphthalene and methyl furan groups in the compound. Consistent with our findings, a synthetic hydroxypropenone bearing methyl furan group demonstrated that it is able to alleviate the secretion of IL-1 β , TNF- α and IL-6 as well as prostaglandin induced by LPS in murine RAW264.7 cells [11]. Moreover, it has been demonstrated previously that compound 1 can also inhibit proinflammatory cytokines secretion induced by oxidised phospholipids [10]. Oxidised phospholipids such as OxPAPC did not produce cytokines particularly MCP-1 and IL-8 through common inflammatory pathways that include NF- κ B and MAPK, suggesting that this compound may affect cytokine secretion by targeting various types of signalling pathways.

NF κ B comprises of a family of transcription factors which are essential to control inflammation, immune responses, and several cellular processes (survival, differentiation, and proliferation). In resting cells, inactive NF κ B is held as dimers in the cytoplasm and are associated with a regulatory element known as inhibitory kappa B (I κ B) proteins. Upon stimulation, activated I κ B kinase complex will cause phosphorylation, ubiquitination, and subsequent I κ B proteins degradation to release NF κ B. This is followed by translocation of NF κ B to the nucleus and initiation of transcription for

target genes [21]. In the present study, we showed that the induction with LPS at 1 μ g/mL caused significant phosphorylation of NF κ B p65 protein and degradation of I κ B α protein. Treatment with compound 1 inhibited neither the phosphorylation of NF κ B p65 proteins nor the degradation of I κ B α proteins. In contrast, Ban and colleagues reported the inhibitory potential of 2'-hydroxy chalcone derivatives which may inhibit I κ B α degradation and attenuate the activation of transcription factors, NF κ B and AP-1 [22]. 2',4'-dihydroxy-6'-isopentyloxychalcone (JSH) was demonstrated to bind competitively on MD-2, a subunit of the receptor complex for LPS, thus preventing the activation of NF κ B pathways [23].

MAPKs are serine/threonine protein kinases which function as key regulators of diverse cellular processes such as gene induction, cell survival, proliferation, differentiation, and inflammation. Conventional MAPKs consist of the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), P38 isoforms (α , β , γ , and δ) and ERK5. Activation of MAPK signaling pathways involves three sequentially activated kinases: a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK), which are triggered by a phosphorylation cascade upon exposure to stimuli [24]. DPEP was reported to inhibit phosphorylation of p38, ERK and JNK MAPKs induced by LPS in RAW264.7 cells [32]. A chalcone isolated from the heartwood of *Pterocarpus marsupium*, α -dihydroxychalcone-glycoside (α -DHC), markedly suppressed LPS-induced phosphorylation of the three protein kinases in RAW264.7 cells [25]. In the present study pre-treatment of the cells with compound 1 did not inhibit phosphorylation of the P38 MAPK pathways. However, it can cause remarkable inhibition of JNK and ERK MAPKs phosphorylation. This suggests that compound 1 might exert its inhibitory effect on LPS-mediated cytokines secretion by suppressing ERK and JNK MAPKs pathways but not cytosolic NF κ B signalling pathways.

CONCLUSION

The present findings reveal that compound 1 exhibits potential anti-inflammatory property by significantly reducing LPS-induced pro-inflammatory cytokines secretion in U937 macrophages. The underlying mechanism of this inhibitory potential is most likely caused by suppressing the phosphorylation of ERK and JNK MAPK signaling pathways. These results suggested that chalcone derivative could act as the antiinflammatory agents by altering cytokine secretion and inflammatory pathways.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The experimental design of the study was conducted by Norsyahida Mohd Fauzi, Malina Jasamai Norazrina Azmi, Endang Kumolosasi and Nor Syafinaz Yaakob. The synthesis of chalcone derivatives was synthesised by Malina Jasamai and the bioassays were carried out by Lee Jian Sian.

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