Methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate is a potent cell-permeable anti-cytokine compound to inhibit inflammatory cytokines in monocyte/macrophage-like cells

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Running title

Inhibition of inflammatory cytokines by MHPAP

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ABBREVIATIONS:

MHPAP, DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IKK, IkB kinase; IL, Interleukin; LPS, lipopolysaccharide; MHPAP, Methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate; NEF, non-ester form; PBMCs, peripheral blood mononuclear cells; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

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ABSTRACT

Cytokines are signaling molecules involved in inflammation process. IL-6 is one of pivotal inflammatory cytokines associated with many human diseases. Therefore, there are on-going efforts to find a therapeutic to inhibit IL-6 and other cytokines. Methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate (MHPAP) is a phenolic amide ester, transported better than its non-ester form (NEF) in monocyte/macrophage-like cells. However, there is no information about the effects of their cell permeability on cytokines. Therefore, the effects of MHPAP and NEF on cytokines were investigated in LPS-stimulated THP-1 and human peripheral blood mononuclear cells (PBMCs). In the THP-1 cells, MHPAP significantly inhibited IL-6, IL-1beta, IL-8 and TNF-alpha ($P < 0.05$), but NEF showed no effects. MHPAP also inhibited NF-κB p65 phosphorylation in the THP-1 cells ($P < 0.05$), without significant effects on c-FOS, ATF-2 and JUN phosphorylations. Because NF-κB p65 is phosphorylated by IκB kinase (IKK), in silico analysis was performed on IKK. MHPAP was found to bind to IKK better than an IKK inhibitor ((E)-2-fluoro-4'-methoxystilbene). Furthermore, MHPAP inhibited the luminescence increased in the LPS-stimulated NF-κB-Luc2 THP-1 cells. As anticipated, MHPAP was also found to inhibit IL-6, IL-1beta, IL-8 and TNF-alpha significantly in LPS-stimulated PBMCs ($P < 0.05$). Especially, MHPAP inhibited IL-6 and IL-1beta with IC$_{50}$ of 0.85 and 0.87 μM, better than IL-8 (1.58 μM) and TNF-alpha (1.22 μM) in the cells. Altogether, the data suggest that cell-permeability may have a significant impact on MHPAP’s ability to inhibit cytokines and MHPAP may be used as a potent cell-permeable compound to inhibit inflammatory cytokines in monocyte/macrophage-like cells.
Significance Statement

Potential effects of MHPAP and NEF on inflammatory cytokines (IL-6, IL-8, IL-1beta, and TNF-alpha) were investigated in LPS-stimulated THP-1 and PBMCs. Cell transport had a great impact on cytokine inhibition in the cells. MHPAP was also found to inhibit NF-kB pathway, which was supported by in silico and NF-kB reporter (Luc)-THP-1 data. Also, in LPS-stimulated PBMCs, MHPAP significantly inhibited IL-6, IL-1beta, IL-8 and TNF-alpha, suggesting that MHPAP may be a potent cell-permeable compound to inhibit inflammatory cytokines in monocyte/macrophage-like cells.
INTRODUCTION

Inflammatory cytokines are signaling molecules secreted from various immune cells (e.g., macrophages, helper T cells and others) and deeply involved in inflammation process (Jarczak and Nierhaus, 2022; Darif et al., 2021; Karki and Kanneganti, 2021; Fajgenbaum and June, 2020). Upon bacterial/viral infections, inflammatory signaling cascades are activated, leading to a rapid release of many inflammatory cytokines (Holmes et al., 2017; Ballinger and Standiford, 2010; Falsey et al., 2013; Marom et al., 2014). Although the production of inflammatory cytokines is a necessary process for proper immune responses, the hyper-activation of cytokine signaling cascade often produces dangerous amounts of cytokines known as “cytokine storm” (Medzhitov, 2021; Karki and Kanneganti, 2021; Gu et al., 2021; Luo et al., 2021; Soy et al., 2020). Furthermore, the cytokine storm often worsens sepsis which is one of dramatic immune events followed by infections (Soy et al., 2020; Rynda-Apple et al., 2015; Angus and Wax, 2001; Chousterman et al., 2017). Although many cytokines/mediators (e.g., interleukin (IL)-1beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, TNF-alpha, MCP-1, MIP-1alpha, IFN-gamma, IFN-alpha and others) are likely up-regulated under microbial/viral infections, the blood analysis of symptomatic COVID-19 patients shows extremely high levels of several inflammatory cytokines including IL-6, TNF-alpha, IL-1beta, and IL-8 (Yalcin and Yalcin, 2021; Manik and Singh, 2022; Yalcin and Yalcin, 2021; Patel et al., 2021). Especially, a high level of IL-6 seems to be significantly associated with severe outcomes of COVID-19 infection (Kishimoto and Kang, 2022; Paces et al., 2020; Zizzo et al., 2022; McGonagle et al., 2020; Chen et al., 2020). Furthermore, IL-6 is also known to be involved in the development/progression of several chronic inflammatory diseases such as diabetes, liver diseases, rheumatoid arthritis and Crohn's disease (Akbari and Hassan-Zadeh, 2018; Schmidt-Arras and Rose-John, 2016; Shahini, 2023).
Therefore, there are on-going research efforts to find a therapeutic compound to inhibit IL-6 as well as other inflammatory cytokines (Kishimoto and Kang, 2022; Paces et al., 2020; Zizzo et al., 2022; McGonagle et al., 2020; Chen et al., 2020; Park et al., 2020).

Caffedymine-type phenolic amides are phenylalanine/tyrosine-conjugated phenolic amides found in plant sources such as Coffea sp., Allium sativa, Cannabis sp., Capsicum sp. and Lycium sp. (Park, 2016; Gaikwad et al., 2019; Han et al., 2002; Ruwizhi and Aderibigbe, 2020). Several studies showed that these phenolic amide derivatives may have various biological activities including ant-inflammatory activity (Park, 2016; Gaikwad et al., 2019; Han et al., 2002; Ruwizhi and Aderibigbe, 2020; Park and Schoene, 2003; Park, 2007; Park\textsuperscript{a}, 2011; Park\textsuperscript{b}, 2011). Interestingly, our recent study even demonstrated that caffedymine-type phenolic amide esters could be transported/biotransformed into parent compounds in monocyte/macrophage-like cells, although their non-ester forms were not transported significantly in the cells (Park, 2022). However, there is no information about potential effects of their cell permeability on inflammatory cytokines in monocyte/macrophage-like cells. In fact, methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate (MHPAP, Figure 1) is one of caffedymine-type phenolic amide esters, which was reported to be transported better than its non-ester form (NEF, Figure 1) in monocyte/macrophage-like cells (Park, 2022). Therefore, in this paper, the effects of MHPAP and NEF on IL-6, TNF-alpha, IL-1beta, IL-8 were investigated in monocyte/macrophage-like cells. In this study, LPS was used as a stimulant, because LPS can bind to Toll-like receptor 4 (TLR4) to produce inflammatory cytokines including IL-6, IL-1beta, IL-8 and TNF-alpha in monocyte/macrophage-like cells, and because the activation of TLR4 is also involved in many inflammatory diseases. (Aboudounya and Heads, 2021; Coutinho-Wolino
et al., 2022). In LPS-stimulated THP-1 cells, the effects of MHPAP and NEF on the cytokines were first investigated. Then, the effects of MHPAP on MAPK and NF-κB signal pathways were investigated in the THP-1 cells, because these two signal pathways are significantly involved in the production of inflammatory cytokines in the cells (Awasthi et al., 2021; Gadina et al., 2017; Ahmed et al., 2015; Lawrence, 2009; Yeung et al., 2018). *In silico* molecular analysis was also conducted to identify a potential binding site for MHPAP in a candidate molecule in the signal pathway, because the molecular modeling has been used for years to identify potential inhibitors for targeted molecules (Park, 2017; Allen and Geldenhuys 2006; Moura et al., 2019).

Furthermore, the effect of MHPAP on NF-κB pathway was investigated in the LPS-stimulated THP-1 NF-κB-Luc2 cells. After that, the effects of MHPAP on IL-6, IL-1beta, IL-8 and TNF-alpha were also investigated in LPS-stimulated human peripheral blood mononuclear cells (PBMCs), because PBMCs are primary human blood mononuclear cells to produce these inflammatory cytokines. To my best knowledge, this is the first report about Methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate which is a potent cell-permeable compound to inhibit IL-6, IL-1beta, IL-8 and TNF-alpha in monocyte/macrophage-like cells.
MATERIALS AND METHODS

Materials
Phenylalanine, coumaric acid, Dimethyl sulfoxide (DMSO) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). An IKK inhibitor ((E)-2-fluoro-4’-methoxystilbene) was purchased from Cayman Chemicals. Methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate (MHPAP) was prepared as published previously (Park, 2022). For Western blots, anti-phospho-c-FOS, anti-phospho-ATF-2, anti-phospho-JUN, anti-phospho-NF-kB p65 antibodies and their respective control antibodies (c-FOS, ATF-2, JUN, NF-kB p65) were obtained from Cell Signaling Technology (Beverly, MA, USA). Also, rabbit IgG horseradish peroxidase (HRP)-linked antibody and anti-mouse IgG, HRP-linked antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Methods

Cell culture
THP-1 and human peripheral blood mononuclear cells (PBMCs) were purchased from ATCC (Manassas, VA, USA). THP-1 and human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 units/ml streptomycin. For experiments, THP-1 cells were induced to differentiate into macrophages by the incubation with phorbol 12-myristate 13-acetate (50 nmol/liter) for 48 h (Park et al., 2020). After that, differentiated THP-1 cells were cultured in RPMI 1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 units/ml streptomycin for 24 h, and treated with several concentrations of MHPAP (0-30 µM) for 10 min, followed by LPS treatment (0.2µg/ml) for 24 h. Similarly, PBMCs cells were cultured in RPMI 1640 medium containing 10% FBS, 100
units/ml penicillin, and 100 units/ml streptomycin, and treated with MHPAP (0-30 µM), then LPS (0.2µg/ml). After 24 h, the media of the cell culture were collected and used for ELISA assays.

**Assays for IL-6, IL-1beta, IL-8 and TNF-alpha proteins**

To determine the effects of MHPAP on IL-6, IL-1beta, IL-8 and TNF-alpha, the media samples were used for ELISA assays. The level of each cytokine (IL-6, IL-1beta, IL-8 and TNF-alpha) in the samples was determined using respective Human IL-6, IL-1beta, IL-8 and TNF-alpha ELISA kits from R&D systems (Minneapolis, MN, USA), according to manufacturer’s protocols.

**Phosphorylation of c-FOS, ATF-2 and JUN**

For Western blots of c-FOS, ATF-2 and JUN, blot samples were prepared using PMA-differentiated THP-1 cells treated with MHPAP (0, 10, 20, 30 µM) for 10 min, followed by LPS treatment (0.2µg/ml) for 45 min. The Western blots were generated and blotted with phospho-c-FOS, c-FOS, phospho-ATF-2, ATF-2, phospho-JUN, and JUN antibodies (Cell Signaling Technology, Danvers, MA, USA). For the blot, the amounts of protein in the samples were determined using Bio-Rad protein assay kit (Hercules, CA, USA), and c-FOS, ATF-2 and JUN antibodies (Cell Signaling Technology, Danvers, MA, USA) were used as control samples for the blot.

**Phosphorylation of NF-κB p65**

For Western blots of NF-κB p65, THP-1 cells were treated as described above, and nuclear fractions for blot samples were prepared using a nuclear extraction kit (Cayman Chemical, MA,
USA). The phosphorylation samples were generated with phospho-NF-kB p65 antibody, and the control samples were generated using NF-κB p65 antibody (Cell Signaling Technology, Danvers, MA, USA).

Molecular docking

Molecular docking was performed using an ICM-pro (MolSoft, San Diego, CA) as described previously (Park, 2017). Briefly, the pocket analysis of IKK complexes was first converted into ICM IKK complexes via conducting structure optimization, quality assessment and visualization. After that, the built-in 3D database protocol was used for a targeted screening, which was built based on ICM-pro algorithms for in silico screening. The proposed binding scores of MHPAP, NEF and (E)-2-fluoro-4′-methoxystilbene to the active pocket of each IKK complex were determined using a ranked scoring function which presents the conformational structures with most favorable binding energy (ΔE).

NF-κB reporter Assay

THP-1 NF-κB-Luc2 (ATCC TIB-202-NFκB-LUC2™) cell line was purchased from ATCC (Manassas, VA, USA), and the experiments were conducted according to the manufacturer’s protocol. Briefly, THP-1 NF-κB-Luc2 cells (~30,000 cells in 100 µl) were cultured and differentiated in 96-well plates as described above. After 72 h, the cells were treated with MHPAP (0, 10, 20, 30 µM) for 10 min, followed by LPS (0.2µg/ml) for 6 h. Then, 100 µl of Glo™ Luciferase Assay reagent (Promega, Madison, WI) was added to each well, mixed gently at room temperature for 2 min. After that, the luminescence was measured using SpectraMAX M5 (Molecular Devices, San Jose, CA).
Statistical analysis

All statistical analyses were performed with Sigma Plot 11.0 (Chicago, IL). $P$ value was calculated using one-way ANOVA with Holm-Sidak method, and $P < 0.05$ was considered as statistically significant. Data points in all figures were represented as the mean ± SD of 5 samples.
RESULTS

Effects of MHPAP and NEF on inflammatory cytokines in THP-1

Potential effects of MHPAP and NEF (non-ester form) on inflammatory cytokines were investigated in LPS-stimulated THP-1 cells. The effect of MHPAP on IL-6 was first investigated in the cells. As shown in Figure 2A, MHPAP significantly inhibited IL-6 in the THP-1 cells. Because MHPAP inhibited IL-6 significantly in the cells, the effects of MHPAP on IL-1beta, IL-8 and TNF-alpha were investigated in the same THP-1 cells. As expected, MHPAP also inhibited IL-1beta, IL-8 and TNF-alpha significantly in the THP-1 cells (Figures 2B, 2C and 2D). However, NEF could not inhibit these cytokines significantly in LPS-stimulated THP-1 cells (Figures 2A-D), suggesting that cell permeability may play a significant role in the cytokine inhibition in the THP-1 cells. Based on this data, MHPAP, not NEF, is likely to a potent compound to inhibit IL-6, IL-1 beta, IL-8, and TNF-alpha in LPS-stimulated THP-1 cells.

c-FOS, ATF-2, JUN and NF-κB p65 phosphorylations in THP-1 cells

Because mitogen-activated protein kinases (MAPKs; ERK, JNK and p38) are important protein kinases in signal transduction pathways associated with cytokine expression, and because they are activated by extracellular stimuli including LPS (McGonagle et al., 2020; Chen et al., 2020; Park et al., 2020), potential effects of MHPAP on c-FOS, ATF-2, and JUN phosphorylations were investigated in LPS-stimulated THP-1 cells. Surprisingly, MHPAP did not have significant effects on c-FOS, ATF-2, and JUN phosphorylations in the THP-1 cells (The data not shown here). Nuclear factor-kappa B (NF-κB) is another key transcriptional factor involved in the expression of inflammatory cytokines including IL-6, IL-1beta, IL-8 and TNF-alpha (Ahmed et
al., 2015; Lawrence, 2009; Yeung et al., 2018). In fact, LPS can activate IκB kinase (IKK), then the activated IKK can phosphorylate NF-κB p65, leading to nuclear localization and transactivation of several downstream genes in THP-1 cells (Lawrence, 2009). Therefore, the effect of MHPAP on NF-κB pathway was investigated in LPS-stimulated THP-1 cells. As shown in Figure 3, LPS increased NF-κB p65 phosphorylation significantly in the THP-1 cells, but MHPAP was able to inhibit the phosphorylation induced by LPS in the cells. This data suggests that MHPAP may inhibit IL-6, IL-1β, IL-8 and TNF-alpha by inhibiting NF-κB signal pathway in LPS-stimulated THP-1 cells.

**Molecular Docking**

Based on a published report (Lawrence, 2009), NF-κB p65 is phosphorylated at Ser536 by IKK. Therefore, *in silico* docking experiments were performed using human IKK protein complexes as described in "Material and Methods". The docking experiments were conducted using MHPAP and NEF on three co-crystallized human IKK complexes (4KIK, 3QA8 and 3RZF). Both MHPAP and NEF were used for the molecular docking, because MHPAP is transported/biotransformed into NEF in monocyte/macrophage-like cells. In molecular modeling, most probable binding pockets, scores and ΔE values for MHPAP and NEF were calculated using ICM-pro as described previously (Park, 2017). The ΔE values of MHPAP and NEF were presented in Table 1. Similarly, the docking was also performed using a known IKK inhibitor ((E)-2-fluoro-4'-methoxystilbene) to provide the ΔE values (Table 1). As shown in Table 1, the ΔE values from MHPAP and NEF were very similar, suggesting that they may be similarly capable of binding to the selected pocket of IKK complexes. However, the ΔE value of (E)-2-fluoro-4'-methoxystilbene was not as low as those of MHPAP and NEF, suggesting that
MHPAP and NEF may be accessible to the pocket of IKK complexes better than (E)-2-fluoro-4'-methoxystilbene. Because the IKK complex (4KIK) showed the best score (Table 1), MHPAP, NEF and (E)-2-fluoro-4'-methoxystilbene was presented in the active pocket of 4KIK complex (Figure 4). In Figure 4A, all three compounds were presented together in the pocket. Also, (E)-2-fluoro-4'-methoxystilbene (Figure 4B), NEF (Figure 4C), and MHPAP (Figure 4D) were separately presented in the pocket. As shown in Figure 4B, (E)-2-fluoro-4'-methoxystilbene was configured at the left side of the pocket without a potential hydrogen bond. However, NEF was configured at the left side of the pocket like (E)-2-fluoro-4'-methoxystilbene, but with two potential hydrogen bonds (R220 and K428) (Figure 4C). In contrast, MHPAP was configured at the right side of the pocket with one potential hydrogen bond (E247) (Figure 4D). This data suggests that the hydrogen bonds may have effects on lowering binding free energy for MHPAP and NEF, regardless of the position in the pocket (Figure 4).

**NF-κB reporter Assay**

To further confirm the inhibition of NF-κB pathway by MHPAP, the THP-1 NF-κB-Luc2 cells (ATCC TIB-202-NFKB-LUC2™) were used. As shown in Figure 5, MHPAP inhibited the luminescence increased in the LPS-stimulated THP-1 NF-κB-Luc2 cells, suggesting that MHPAP can inhibit the expression of a firefly luciferase gene driven by the NF-κB response element located at the upstream of the TATA promoter in the NF-κB-Luc2. This data clearly indicates that MHPAP can inhibit IL-6, IL-1beta, IL-8 and TNF-alpha via inhibiting NF-κB signal pathway in LPS-stimulated THP-1 cells.

**Effects of MHPAP on inflammatory cytokines in PBMCs**
To validate the anti-cytokine data of THP-1 cells in human primary cells, the effects of MHPAP on inflammatory cytokines were investigated in human peripheral blood mononuclear cells (PBMCs). PBMCs were used in this study, because PBMCs are primary blood cells able to produce inflammatory cytokines. First, the potential effect of MHPAP on IL-6 was investigated in LPS-stimulated PBMCs. As shown in Figure 6A, MHPAP significantly inhibited IL-6 in the PBMCs. Likewise, MHPAP also inhibited IL-1beta, IL-8, and TNF-alpha significantly in LPS-stimulated PBMCs (Figures 6B, 6C and 6D). Because (E)-2-fluoro-4'-methoxystilbene was compared with MHPAP in the docking experiments (Table 1), the effects of (E)-2-fluoro-4'-methoxystilbene on the cytokine productions were also investigated in LPS-stimulated PBMCs. However, the compound was found to have little effects on IL-6, IL-1beta, IL-8, and TNF-alpha in the PBMCs (Data not shown here). This data suggests that MHPAP may inhibit IL-6, IL-1 beta, IL-8, and TNF-alpha better than (E)-2-fluoro-4'-methoxystilbene in LPS-stimulated PBMCs. In addition, the data suggests that MHPAP may inhibit the cytokines better in PBMCs than THP-1 cells (Figures 2 and 6).

**IC$_{50}$ of MHPAP**

The IC$_{50}$ values of MHPAP against IL-6, IL-1beta, IL-8 and TNF-alpha were determined in LPS-stimulated PBMCs. As shown in Figure 7A, MHPAP inhibited IL-6 with IC$_{50}$ of 0.85 µM. Similarly, the value of IC$_{50}$ of MHPAP on IL-1beta inhibition was also found low, approximately 0.87 µM (Figure 7B). However, the IC$_{50}$ values of IL-8 and TNF-alpha were determined to be 1.58 and 1.22 µM, respectively (Figures 7C and 7D), a bit higher than those of IL-6 and IL-1beta. Nonetheless, MHPAP was able to inhibit IL-6, IL-1beta, IL-8, and TNF-alpha significantly in LPS-stimulated PBMCs (Figures 7A-D). Altogether, the data suggests that
MHPAP may be a potent cell-permeable compound to inhibit IL-6, IL-1 beta, IL-8, and TNF-alpha in PBMCs.

**DISCUSSION**

Bacterial/viral infectious diseases are a leading cause for human suffering and death (Holmes et al., 2017; Ballinger and Standiford, 2010; Falsey et al., 2013; Marom et al., 2014). Under the infections, inflammatory cytokines are significantly up-regulated in patients, often leading to an auto-amplifying cytokine surge called “cytokine storm” (Medzhitov, 2021; Karki and Kanneganti, 2021; Gu et al., 2021; Luo et al., 2021, Chousterman et al., 2017, Falsey et al., 2013; Marom et al., 2014; Chousterman et al., 2017). Recently, coronavirus disease-2019 (COVID-19) has caused a devastating health crisis worldwide (Yalcin and Yalcin, 2021; Manik and Singh, 2022; Yalcin and Yalcin, 2021; Patel et al., 2021). During COVID-19 infection, several cytokines were reported to be overexpressed in the infected people (Yalcin and Yalcin, 2021; Patel et al., 2021). Among the cytokines, particularly, a high level of IL-6 was reported to be highly associated with grave outcomes of COVID-19 infection. Furthermore, the dysfunction of IL-6 was reported to be associated with the progress of several human inflammatory diseases (e.g., diabetes, liver diseases, rheumatoid arthritis, cardiovascular disease and inflammatory bowel disease) (Akbari and Hassan-Zadeh, 2018; Schmidt-Arras and Rose-John, 2016; Ridker and Rane, 2021; Shahini, 2023), suggesting that the inhibition of IL-6 may be beneficial in mitigating a broad range of human diseases (Yalcin and Yalcin, 2021; Patel et al., 2021; Kishimoto and Kang, 2022; Paces et al., 2020; Zizzo et al., 2022; McGonagle et al., 2020). Therefore, there are on-going research efforts to find potent therapeutic agents to inhibit
inflammatory cytokines, especially IL-6 (Park, 2017; Allen and Geldenhuys 2006; Moura et al., 2019; Chakraborty et al., 2020; Szollosi et al., 2018).

For years, in our laboratory, natural/synthetic compounds have been investigated to discover a new cytokine inhibitor. Interestingly, our recent study showed that caffedymine-type phenolic amide esters (CTPAEs) could be transported better than their non-ester forms in monocytic/macrophage-like cells (Park, 2022). In fact, the class of CTPAEs is composed of several compounds (Park, 2022), and MHPAP is one of CTPAEs, which is reported to be transported/biotransformed better than its non-ester form (NEF) as well as other CTPAEs in monocyte/macrophage-like cells (Park, 2022). However, there is no information about potential effects of MHPAP and NEF on inflammatory cytokines. Therefore, in this study, their effects on inflammatory cytokines were investigated in LPS-stimulated THP-1 cells, because LPS can binds to Toll-like receptor 4 (TLR4) to activate MAPK and NF-kB pathways and because the activation of the pathways can lead to the production of inflammatory cytokines (e.g., IL-6, IL-1beta, IL-8 and TNF-alpha) in monocyte/macrophage-like cells (Aboudounya and Heads, 2021; Coutinho-Wolino et al., 2022). As shown in Figure 2, MHPAP significantly inhibited IL-6, IL-1beta, IL-8 and TNF-alpha ($P < 0.02$) in LPS-stimulated THP-1 cells. However, NEF was found to have little effects on the inhibition of IL-6, IL-1beta, IL-8 and TNF-alpha in the cells (Figure 2). This data clearly suggests that cell permeability may have a significant impact on cytokine inhibition in LPS-stimulated THP-1 cells.

In fact, the activation of MAPK and NF-kB signal transduction pathways are closely associated with cytokine productions in the cells (McGonagle et al., 2020; Chen et al., 2020; Park et al.,
2020; Ahmed et al., 2015; Lawrence, 2009; Yeung et al., 2018). Therefore, the effects of MHPAP on signal transcriptional factors (e.g., c-FOS, ATF-2, c-JUN, NF-κB p65) were investigated in LPS-stimulated THP-1 cells. Surprisingly, MHPAP did not inhibit the phosphorylations of MAPK-related transcriptional factors (c-FOS, ATF-2, and JUN) significantly in LPS-stimulated THP-1 cells. However, MHPAP significantly inhibited NF-κB p65 phosphorylation (Figure 3), suggesting that MHPAP may inhibit inflammatory cytokines by inhibiting NF-κB pathway. In recent years, the knowledge of molecular/cellular roles of NF-κB pathway has expanded considerably, and the contribution of NF-κB pathway in inflammation have been keenly investigated as an attractive target for new therapies. In silico modeling is a molecular tool widely used to find potential inhibitors, because it can identify a potential compound for targeted protein molecules with velocity and rationality (Park, 2017; Allen and Geldenhuys 2006; Moura et al., 2019). Therefore, in this study, in silico modeling was performed using a docking software program (ICM-pro) as described in "Material and Methods". The docking was first performed using MHPAP and NEF, then the docking of a known IKK inhibitor ((E)-2-Fluoro-4'-Methoxystilbene) was also performed. The resulting lowest energy (ΔE) values of MHPAP, NEF and (E)-2-Fluoro-4'-Methoxystilbene in the most favorable pocket were calculated and presented in Table 1. As shown in Table 1, the scores from MHPAP and NEF were very similar, suggesting that they may have a similar binding affinity to the pocket in IKK complexes. However, the score of (E)-2-fluoro-4'-methoxystilbene was not as low as those of MHPAP and NEF. Additionally, one hydrogen bond (E247) and two hydrogen bonds (R220 and K428) were found for MHPAP and NEF, respectively. However, no hydrogen bond was found for (E)-2-fluoro-4'-methoxystilbene, suggesting that MHPAP and NEF may be preferably accessible to the pocket better than (E)-2-Fluoro-4'-Methoxystilbene (Figure 4). The docking
study also suggests that NEF may be as good as MHPAP in inhibiting IKK, if transported inside cells. In fact, this data may explain why MHPAP can be better than NEF, related to the cytokine inhibition in the cells. Furthermore, this data suggests that NF-κB pathway inhibition may be an underlying mechanism for the inhibition of IL-6, IL-1beta, IL-8 and TNF-alpha in LPS-stimulated THP-1 cells. Therefore, this supposition was further investigated using the LPS-stimulated NF-κB reporter (Luc)-THP-1 cells. As shown in Figure 5, the inhibition of NF-kB pathway was confirmed by the NF-κB reporter (Luc)-THP-1 data that MHPAP inhibited luminescence increased in the LPS-stimulated NF-κB reporter (Luc)-THP-1 cells. In fact, all these data confirm that MHPAP can inhibit NF-κB pathway, thereby inhibiting the productions of IL-6, IL-1beta, IL-8 and TNF-alpha in LPS-stimulated THP-1 cells.

Thus far, the data indicates that MHPAP may be a potent cell-permeable compound to inhibit IL-6, IL-1beta, IL-8 and TNF-alpha in LPS-stimulated THP-1 cells. However, there may be some limitations in the THP-1 data, because THP-1 is a transformed cell line, which may show inconsistent/atypical characteristics, compared to normal cells. Therefore, potential effects of MHPAP on IL-6, IL-1beta, IL-8 and TNF-alpha were also investigated in PBMCs, because PBMCs are primary human cells to produce inflammatory cytokines. As shown in Figure 7, MHPAP were found to significantly inhibit IL-6, IL-1beta, IL-8 and TNF-alpha with IC₅₀ of 0.85, 0.87, 1.6, and 1.2 µM, respectively in LPS-stimulated PBMCs. This data clearly indicates that MHPAP may be a potent cell-permeable compound to inhibit IL-6, TNF-alpha, IL-1beta, and IL-8 in monocyte/macrophage-like cells (e.g., PBMCs). In fact, this data may have a good utility for the compounds whose efficacy is limited by poor transport in the cells. For instance, compounds with poor cell-permeability may turn into more effective therapeutics by a simple
esterification process, if suitable for this process. Altogether, this study suggests that MHPAP is a potent cell-permeable compound which may be used as a candidate therapeutic to inhibit IL-6, TNF-alpha, IL-1beta, and IL-8 in monocyte/macrophage-like cells.
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Author contributions

Conducted experiments: Jae Park.
Contributed new reagents or analytic tools: Jae Park.
Performed data analysis: Jae Park.
Wrote or contributed to the writing of the manuscript: Jae Park.
References


Footnotes

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This article contains no datasets generated or analyzed during the current study.
Figure legends

Figure 1) **The chemical structures.** A) Non-ester form (NEF; *N*-coumaroylphenylalanine) and B) Ester form (MHPAP; (Methyl 2-[3-(4-hydroxyphenyl)prop-2-enoylamino]-3-phenylpropanoate).

Figure 2) **Effects of MHPAP and NEF on IL-6, IL-1beta, IL-8 and TNF-alpha in THP-1.** The productions of IL-6, IL-1beta, IL-8, and TNF-alpha were determined at the concentrations of 0, 10 and 20 µM MHPAP or NEF (*N*-coumaroylphenylalanine) in LPS-stimulated PMA-differentiated THP-1 cells. Data points are shown as the means ± SD (n=5). The *P* value was calculated using one-way ANOVA with the Holm-Sidak method. The asterisks (*) denote significant differences (*P* < 0.05) between the LPS (0.2 µg/mL) and MHPAP (10 and 20 µM).

Figure 3) **Effects of MHPAP on NF-κB p65 phosphorylation.** PMA-differentiated THP-1 cells were treated with MHPAP (0, 10, 20, 30 µM), followed by the treatment of LPS (0.2 µg/mL) for 45 min, and the nuclear extract samples for blots were prepared as described in “Materials and Methods”. P-NF-κB p65 is a phosphorylated NF-κB p65. The asterisks (*) denote significant differences in the phosphorylation levels of P-NF-κB p65 (*P* < 0.05) between the LPS (0.2 µg/mL) and MHPAP (10, 20, 30 µM).

Figure 4) **In silico docking.** The docking of MHPAP, NEF (*N*-coumaroylphenylalanine) and (E)-2-fluoro-4′-methoxystilbene to IκB kinase (4KIK) was presented in an active pocket. (A) All three compounds were presented together in the pocket. (B) (E)-2-fluoro-4′-methoxystilbene was presented in the pocket. (C) NEF was presented in the pocket. (D) MHPAP was presented in the pocket. 4KIK is an IKK (IκB kinase) crystal structure from Protein Data Bank (PDB).

Figure 5) **Effects of MHPAP on luciferase activity in NF-κB reporter (Luc)-THP-1 cells.** NF-κB reporter (Luc)-THP-1 cells were treated with several concentrations of MHPAP (0, 10, 20, 30 µM), followed by the treatment of LPS (0.2 µg/mL) for 5 h. Then, luciferase activity was measured as described in “Materials and Methods”. The asterisks (*) denote significant differences in the luminescence (*P* < 0.05) between the LPS (0.2 µg/mL) and MHPAP (10, 20, 30 µM).

Figure 6) **Effects of MHPAP on IL-6, IL-1beta, IL-8 and TNF-alpha in PBMCs.** The productions of IL-6, IL-1beta, IL-8, and TNF-alpha were determined at the concentrations of 0, 1, 5 µM MHPAP in LPS-stimulated PBMCs. Data points are shown as the means ± SD (n=5). The *P* value was calculated using one-way ANOVA with the Holm-Sidak method. The asterisks (*) denote significant differences (*P* < 0.05) between the LPS (0.2 µg/mL) and MHPAP (1 and 5 µM).

Figure 7) **IC50 of MHPAP in PBMCs.** IC50 curves for IL-6 (A), IL-1beta (B), IL-8 (C), and TNF-alpha (D) were determined as described in “Materials and Methods”. The cytokine productions were determined at the concentrations of MHPAP (0, 0.1, 0.2, 0.5, 1, 5, 10 and 20 µM) in LPS-stimulated PBMCs cells. Data points are shown as the means ± SD (n=5).
Table 1. ΔE values of MHPAP, non-ester form (NEF) and a IκB kinase (IKK) inhibitor. Binding energy values (ΔE) were calculated using a docking program ICM-pro as described in "Material and Methods". Average data represents the means ± S.D. (n=5). (E)-2-fluoro-4'-methoxystilbene was used as an IKK inhibitor. 4KIK, 3QA8 and 3RZF are IKK crystal structures from Protein Data Bank (PDB).

<table>
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<tr>
<th>PDB_ID</th>
<th>MHPAP ΔE (Kcal/mol)</th>
<th>NEF ΔE (Kcal/mol)</th>
<th>IKK inhibitor ΔE (Kcal/mol)</th>
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<tr>
<td>4KIK</td>
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<td>-9.5±1.6</td>
</tr>
</tbody>
</table>
A) Non-ester form (NEF)

B) Ester form (MHPAP)
Figure 3

- P-NF-kB p65
- NF-kB p65

The graph shows the relative blot signal (%) for NF-kB p65 and p-NF-kB p65 under different conditions of LPS (0, 10, 20, 30 microM) and MHPAP (0, 0, 10, 20, 30 microM). The bars indicate the mean signal with error bars representing the standard deviation. Asterisks (*) denote statistically significant differences compared to the control (0 microM).
Fig 5

![Graph showing relative luminescence as a function of MHPAP concentration and LPS presence.](image-url)
Figure 6

(A) IL-6 (pg/ml) levels

(B) IL-1β (pg/ml) levels

(C) IL-8 (pg/ml) levels

(D) TNF-α (pg/ml) levels

- 0 1 2 3 4 MHPAP (microM)
- 0 1 2 3 4 LPS

* indicates significant difference from control.