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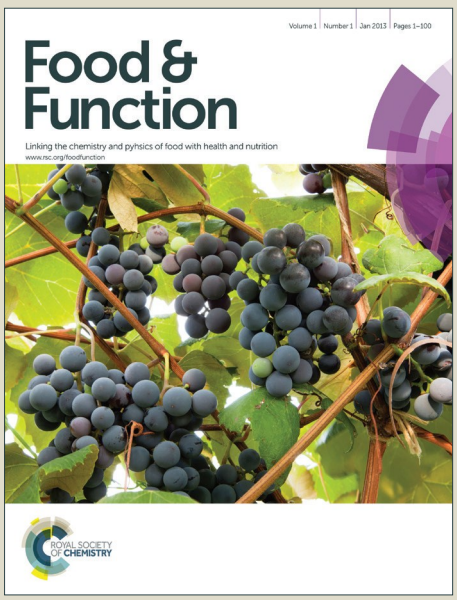


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# Inhibition of LPS-induced inflammatory mediators by 3-hydroxyanthranilic acid in macrophages through suppression of PI3K/NF-κB signaling pathways

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**Abstract**

Many tryptophan metabolites have immunomodulatory effects on various immune cells. 3-Hydroxyanthranilic Acid (3-HAA) is a tryptophan metabolite reported to have anti-inflammatory activity. The mechanism of this activity is unclear. The present study examined the immumodulatory effects and molecular mechanisms of 3-HAA on macrophages. Pretreatment of 3-HAA (0.1 - 10 μg/mL) for 2 h markedly inhibited NO and cytokine production in LPS-stimulated Raw 264.7 cells. Moreover, translocation and activation of NF-κB by LPS in the nucleus was abrogated through the prevention of IκB degradation by 3-HAA treatment. 3-HAA significantly suppressed LPS-induced PI3K/Akt/mTOR activation, whereas MAPKs were not affected by 3-HAA treatment. Furthermore, the inhibition of mTOR by 3-HAA resulted in decreased production of inflammatory mediators and NF-κB activity. Similar results were also observed in primary peritoneal macrophages. Furthermore, 3-HAA modulated macrophage polarization. Collectively, the results suggest that 3-HAA has an immunomodulatory effect that may result from inhibition of PI3K/Akt/mTOR and NF-κB activation, thereby decreasing the production of pro-inflammatory mediators.

Keywords: 3-HAA; immunomodulation; NF-κB; PI3K/Akt;mTOR

## 1. Introduction

Inflammation is a complex biological response of the body against noxious stimuli and conditions, such as infection and tissue injury.[1, 2] The inflammatory process is usually controlled to maintain a balance between initiation of inflammation and shut-down of the signal.[3] Many bacterial components and products including lipopolysaccharide (LPS) can initiate the local inflammatory responses which is mainly mediated by macrophages and mast cells.[4, 5] Activated macrophages are important in inflammatory processes and have crucial functions that include antigen presentation, phagocytosis and immunomodulation through the production of various inflammatory mediators. Additionally, persistent pro-inflammatory macrophages contribute to the development of chronic inflammatory diseases such as atherosclerosis, type 2 diabetes and hay fever.[3, 6] Thus, therapeutic intervention targeting macrophages and their products may be good strategies for preventing inflammatory diseases.

Several studies have demonstrated that macrophage activation invokes multiple downstream signaling pathways, including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB).[7-9] Activation of PI3K subsequently activates downstream signaling molecules including Akt and mammalian target of rapamycin (mTOR), which regulate a variety of biological processes like cell cycle, cell growth and protein synthesis. Recent studies have demonstrated that mTOR activated by Toll-like receptor (TLR) via PI3K/ Akt is also crucial in macrophages and monocytes for coordinating innate immunity.[10-12]

3-Hydroxyanthranilic acid (3-HAA) is a metabolite of tryptophan that is generated via the indoleamine- 2.3-dioxygenase (IDO) pathway. The importance of 3-HAA in regulating the immune system has been demonstrated.[13] 3-HAA inhibits the production of cytokines from Th1 and Th2 cells, and the expression of inducible nitrite oxide synthase (iNOS) by

68 enhancing heme oxygenase-1 (HO-1) expression in stimulated macrophages.[14, 15] How 3-
69 HAA influences the function of macrophages is unclear.

70 In this study, we investigated the effect of 3-HAA on production of inflammatory mediators
71 in LPS or LPS/IFNγ-stimulated macrophages and the modulatory mechanism of 3-HAA. The
72 data demonstrate that 3-HAA blocks NO production as well as the release of cytokines by
73 inhibiting NF-κB activation by interfering with the PI3K/Akt/mTOR signaling pathway and
74 IκB degradation.

75

76

## 2. Materials and Methods

### 2.1 Reagents

Unless otherwise indicated, all chemicals including 3-HAA were purchased from Sigma Chemical Co. (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Carlsbad, CA). Metafectene PRO was purchased from Biontex (Martinsried, Germany). The reporter plasmid pGL3-NF-κB used in the luciferase assay system was obtained from Promega (Madison, WI), and pCMV-β-gal was obtained from Lonza (Walkersville, MD). IL-6 and TNF-α ELISA kits were purchased from R&D Systems (Minneapolis, MN). Antibodies against IκBα, p65, JNK, phospho-JNK (p-JNK), ERK, phospho-ERK (p-ERK), p38, phospho-p38 (p-p38), lamin A, and β-actin were purchased from Abcam Inc (Cambridge, MA). Antibodies against PI3K and Akt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-mTOR and anti-mTOR were purchased from Cell Signaling Technology (Beverly, MA).

### 2.2 Cell culture and isolation of peritoneal macrophages

The RAW 264.7 cells was purchased from ATCC (Rockville, MD) and grown in DMEM supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FBS in a humidified atmosphere containing 5% $CO_2$ at 37°C. Cells were used from the first to sixth passage.

The thioglycollate-elicited peritoneal exudates cells were obtained from C57BL/6 mice (6-8 weeks old) after they were given an intraperitoneal injection of 1 ml Brewer Thioglycollate Broth (4.05 g/100 ml) (Difco Laboratories, Detroit, ML) followed by a lavage of the

peritoneal cavity with 5 ml of medium 3–4 days later. The cells were washed twice and resuspended in RPMI-1640 containing 10% heat-inactivated FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The macrophages were isolated from the peritoneal exudate cells using the method described by Um et al.[16] The macrophages were allowed to adhere for 2–3 h at 37 °C in a 5% CO2 humidified atmosphere. All animal care procedures were conducted in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University

**2.3 Assessment of cell viability**

The cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[17] RAW 264.7 cells were seeded at a concentration of $1\times10^5$ cells/well in 96-well tissue culture plates and pretreated with various concentrations of 3-HAA (0.1, 1, 10, 50 and 100 μg/ml) for 24 h. Cell viability was measured using a quantitative colorimetric assay with MTT as an indicator of the mitochondrial activity of living cells. The extent of reduction of MTT to formazan within cells was quantified by measuring the optical density at 550 nm using a microplate reader (Molecular Device, Menlo Park, CA). The blank control only contained cell culture medium and the absorbance of untreated cultures was set at 100%. Cell viability was expressed as a percentage of the untreated control. At least three independent experiments were performed.

**2.4 Nitrite determination**

Macrophage cultures were treated with 3-HAA for 2 h. The supernatant was decanted,

followed by the addition with LPS (1 μg/ml) or LPS (1 μg/ml)+IFNγ (50U/ml) and then incubated for an additional 24 h. The amount of $NO_2^-$ accumulated in the culture supernatants was measured using a published assay system.[18] Briefly, 100 μl of the supernatant was removed from each well and placed into empty wells of a 96-well plate. After adding 100 μl Griess reagent to each well, the absorbance was measured at 550 nm using the aforementioned microplate reader. The $NO_2^-$ concentration was calculated from a $NaNO_2$ standard curve. The $NO_2^-$ levels were indicative of the amount of NO production. Griess reagent was prepared by mixing 1 part of 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part of 1% sulfanilamide in 5% concentrated $H_3PO_4$.

**2.5 Cytokine determination by ELISA**

Macrophages were pretreated with 3-HAA, followed by the addition of LPS (1 μg/ml) or LPS (1 μg/ml)+IFNγ (50U/ml) to the cultures for 24 h. The culture supernatants were collected and the TNF-α and IL-6 concentration in the culture supernatants was determined using Duo Set Elisa kit (R&D Systems) according to the manufacturer's instructions. Samples were assessed in triplicate using the cytokine standards provided by the manufacturer.

**2.6 Transfection and reporter assays**

RAW 264.7 cells were chosen for their high transfection efficiency. Cells ($5 \times 10^5$ cells/ml) were plated into each well of a 6-well plate. The cells were transiently co-transfected with the plasmids, pGL3-NF-κB, pCMV-β-gal and pcDNA3.1 using Metafectene PRO according to the manufacturer's protocol. Briefly, a transfection mixture containing 0.5 μg pGL3-NF-κB and 0.2 μg pCMV-β-gal was mixed with the Metafectene PRO reagent and added to the cells.

For NF-κB luciferase, the cells were transfected with 0.5 μg NF-κB luciferase reporter using Metafectene PRO. After 4 h, the cells were pretreated with 3-HAA for 2 h followed by the addition of LPS (1 μg/ml) for 4 h, and then lysed with 200 μl of lysis buffer (24 mM Tris–HCl (pH 7.8), 2 mM dithiotreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100). Aliquots (10 μl) of cell lysates were used for luciferase activity assay. The values shown represent an average of three independent transfections and each transfection was carried out in triplicate.

**2.7 Immunofluorescence assay**

The translocation of NF-κB proteins was determined by immunofluorescence microscopy. Raw 264.7 cells were grown on 22-mm diameter glass coverslips at a density of 2 x $10^6$ cells and pretreated with 3-HAA (10 μg/ml) for 2 h, and stimulated with LPS (1 μg/ml) for 4 h. Cells were washed in PBS, fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, and washed in PBS. Ice-cold methanol was added to the cells prior to incubation at −20 °C for 10 min and washing in PBS. Cells were permeabilized with 1% BSA/0.2% Triton X-100/PBS for 1 h. They were washed in PBS and incubated with antibody against NF-κB p65 overnight at 4 °C. After PBS washing, cells were incubated for 1 h with anti-rabbit IgG-fluorescein isothiocyanate (FITC) in 1% BSA/0.05% Triton X-100/PBS. Cells were washed thoroughly, and samples were mounted with glycerol/PBS (4:1) and photographed using a model BX51 fluorescent microscope (Olympus Optical Co., Ltd, Center Valley, PA).

**2.8 Western blot analysis**

Western blot analysis was performed by a modification of a technique described elsewhere.[19] After the treatment, the cells were washed twice in PBS and suspended in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin). The cells were placed on ice for 30 min. The supernatant was collected after centrifugation at 15,000 g for 20 min at 40 °C. The protein concentration was determined using a protein assay (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard. The whole lysates (20 μg) were resolved on by 7.5% SDS-polyacrylamide gel electrophoresis, transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights, IL) and probed with the appropriate antibodies. The blots were developed using an enhanced chemiluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody as a control for protein loading.

**2.9 Measurement of mRNA levels by quantitative real-time polymerase chain reaction (qRT-PCR)**

Following exposure to 3-HAA or LPS, total RNA was isolated using TRIzol (Life Technologies, Pioneer Valley, WI) according to the manufacturer's instructions. The yield and purity of the RNA were confirmed by measuring the ratio of the absorbance at 260 and 280 nm. RNA was reverse transcribed to cDNA using 0.2 μg/ml random primers, 10 mM dNTP-mix and reverse transcriptase (Promega, Madison, W1). Nested PCR was performed in a 20 μl volume comprising 3 μl cDNA, 2 μl each primer, and 2 × QuantiTect SYBR Green PCR Master Mix and the fluorescence was monitored at each cycle. The sequences of the primers corresponding to the mouse genes analyzed in this study were as follows: TNF-α

(forward, 5'-CCC TCA CAC TCA GAT CAT CTT CT-3'; reverse, 5'-GCT ACG ACG TGG GCT ACA G-3'); IL-1β (forward, 5'-TTG ACG GAC CCC AAA AGA TG-3'; reverse, 5'-TGG ACA GCC CAG GTC AAA G-3'); Arg-1 (forward, 5'-TCG GAG ACC GGG ACC TG-3'; reverse, 5'-GCA CCA CAC TGA CTC TTC CAT TC-3'); IL-10 (forward, 5'-GCT CTT ACT GAC TGG CAT TC-3'; reverse, 5'-CGC AGC TCT AGG AGC ATG TG-3'); and GAPDH (forward, 5'-GGT CCT CAG TGT AGC CCA AG-3'; reverse, 5'-AAT GTG TCC GTC GTG GAT CT-3').

**2.10 Statistical analyses**

All experiments were performed at least three times (unless otherwise indicated) and each result is reported as the mean ± S.E.M. For comparisons between two groups, the Student's *t* test was used. Multi-group comparisons of mean values were analyzed by a one-way ANOVA. The significant values are represented by an asterisk (* $p < 0.05$).

## 3. Results

### 3.1 Effect of 3-HAA on RAW 264.7 cell viability

To investigate if 3-HAA was cytotoxic to RAW 264.7 cells, the MTT viability assay was done using various concentrations of 3-HAA (0.1 - 100 μg/mL) or vehicle control (3 % DMSO) for 24 h. 3-HAA ranging from 0.1 to 10 μg/mL did not have effect on RAW 264.7 cells viability, while concentrations higher than 50 μg/mL were slightly, but not statistically significantly, cytotoxic to cells (Fig. 1A). In all subsequent in vitro experiments, 0.1, 1 and 10 μg/mL 3-HAA were chosen.

### 3.2 Effects of 3-HAA on LPS-induced nitrite production and release of cytokines from RAW 264.7 cells.

Since NO is recognized as a mediator of inflammatory responses, we examined the effect of 3-HAA on LPS-induced NO production. RAW 264.7 cells were not pretreated or were pretreated with 0.1, 1 and 10 μg/mL 3-HAA for 2 h before stimulation with LPS (1 μg/mL) for 24 h. As detected by ELISA, treatment with 3-HAA decreased the nitrite production in a concentration-dependent manner (Fig. 1B). Next, we then investigated the effect of 3-HAA on production of the pro-inflammatory cytokines IL-6 and TNF-α. As shown in Figs. 1C and D, 3-HAA significantly inhibited the production of both cytokines in LPS-stimulated RAW 264.7 cells. We also examined the ability of 3-HAA to polarize LPS-stimulated macrophages to M2 macrophages by real time PCR. 3-HAA inhibited the LPS-induced expressions of TNF-α and IL-1β mRNA expression (Fig. 2A), while the LPS-reduced expression of Arg-1 and IL-10 was upregulated by 3-HAA (Fig. 2B). These results suggest that 3-HAA regulates

241 the function of activated macrophage by inhibiting production of NO and cytokines as well as
242 modulating macrophage polarization.
243
244 **3.3 Effects of 3-HAA on NF-kB activation and IκBα degradation in LPS-stimulated**
245 **macrophages.**
246
247 Several studies have demonstrated that NF-κB can be activated by various inflammatory
248 stimuli including LPS, which in turn induces expression of multiple genes such as
249 inflammatory cytokines, chemokines and iNOS.[20-22] Therefore, we determined whether 3-
250 HAA inhibits LPS-induced activation of NF-κB. The cells were pre-incubated with the
251 aforementioned three concentrations of 3-HAA for 2 h prior to stimulation with LPS for 4 h,
252 and transcriptional activity of NF-κB was measured. LPS treatment increased luciferase
253 activity and this increased activity was significantly attenuated by 3-HAA in a concentration-
254 dependent manner (Fig. 3A). To further clarify the inhibitory effect of 3-HAA on LPS-
255 stimulated NF-κB activation, the effect of 3-HAA on the nuclear translocation of the p65
256 proteins was examined using immunofluorescence and Western blot assays. Elevated basal
257 nuclear accumulation of p65 in LPS-stimulated cells was suppressed by exposing the cells to
258 3-HAA (Figs. 3B and 3C). Significant degradation of IκBα was observed after 30 min
259 stimulation with LPS, while treatment of cells with 3-HAA resulted in the interruption of
260 LPS-induced IκBα degradation (Fig. 3D). Collectively, these results suggest that 3-HAA
261 inhibits the activation of NF-κB, which could reduce LPS-inducible inflammatory mediator
262 production.
263
264
265 **3.4 Effects of 3-HAA on MAP kinase in LPS-stimulated RAW 264.7 cells.**

Since LPS can act via activation of MAPK signaling pathways to prompt NF-κB transcription, we investigated whether the inhibitory effect of 3-HAA on production of inflammatory mediators was regulated by inhibiting the activation of MAPK pathways.

p38, ERK and JNK phosphorylation were induced by LPS treatment, whereas the increased phosphorylation of these kinases were not attenuated in the presence of 3-HAA (Fig. 4). These data suggest that MAPK pathways are not involved in the inhibitory effect of 3-HAA.

### 3.5 Inhibitory effects of 3-HAA on the activation PI3K, Akt and mTOR by LPS

Since 3-HAA did not attenuate the phosphorylation of MAPKs, it is possible that other signaling pathways are involved in NF-κB activation. Several studies have clearly demonstrated that PI3K and Akt promote NF-κB activity and subsequent pro-inflammatory cytokine production.[23-26] We therefore examined whether 3-HAA regulated LPS-stimulated PI3K/Akt signaling. Activation of PI3K p110 and Akt was induced in cells treated with LPS for 10 or 30 min. All these activations were significantly blocked by 3-HAA in a concentration-dependent manner (Figs. 5A and 5B). We further investigated effect of 3-HAA on mTOR, one of the major targets of Akt, which influences various cellular functions including cellular growth, cell cycle control and innate immune reactions.[10] 3-HAA significantly inhibited both phosphorylation and expression of mTOR (Fig. 5C). Therefore, we next examined whether the inhibition of mTOR affects LPS-inducible inflammatory mediator production by using mTOR inhibitor rapamycin. LPS-induced elevation of NF-κB activation was abrogated in the presence of rapamycin (Fig. 6A). Additionally, LPS-induced NO and IL-6 production was significantly reduced by rapamycin, while the TNF-α level was slightly decreased (Figs. 6B, 6C and 6D). These data support our speculation that inhibitory

291 effects of 3-HAA on LPS-induced inflammatory reaction is mediated, at least partially,
292 through suppression of mTOR and PI3k/Akt pathway.
293
294 **3.6 Effects of 3-HAA in LPS/IFNγ-stimulated peritoneal macrophages.**
295
296 To further verify the effects of 3-HAA in macrophages, the inhibitory effect of 3-HAA was
297 examined in primary peritoneal macrophages. Similar results were observed in primary
298 peritoneal macrophages (Fig 7). 3-HAA treatment resulted in decrease in the production of
299 NO and cytokines in LPS/IFN γ-stimulated cells (Fig. 7A). In addition, treatment with 3-
300 HAA inhibited both NF-κB translocation and mTOR phosphorylation (Fig. 7B and 7C).
301 Overall, these results indicate that 3-HAA had similar effects in primary cells.

## 4. Discussion

Inflammation is a protective biological response to infection or harmful stimuli, which trigger the production of various inflammation mediators such as cytokines and reactive nitrogen species in leukocytes.[5] Modulation of inflammatory mediators has been considered as a promising strategy to treat and prevent chronic inflammatory diseases. The tryptophan metabolite, 3-HAA has antioxidant, immune regulatory and NO inhibitory activities.[14, 27-29] The present study elucidated molecular mechanisms by which 3-HAA inhibits LPS-induced inflammatory mediator production in macrophages.

Activated macrophages induce the production of pro-inflammatory cytokines.[30] NO is a versatile molecule that acts on a variety of cellular functions including immune defenses, inflammation and neurotransmission.[31] It also has an important role in maintaining normal physiological conditions under low concentrations. On the other hand, overproduction of NO can be toxic and pro-inflammatory.[32, 33] IL-6 and TNF-α have main roles in the acute phase of inflammation by stimulating immune cells.[34-36] In the present study, we examined the effect of 3-HAA on the production of both NO and cytokines in LPS-stimulated Raw 264.7 cells and LPS/IFNγ-stimulated peritoneal macrophages. The data demonstrate that 3-HAA inhibited LPS-induced NO production as well as release of cytokines. Since macrophage polarization has been known to be involved in the inflammatory response caused by microbial products like LPS,[37] the modulation of macrophage polarization might be important for the treatment of inflammatory diseases. Therefore, we examined the effect of 3-HAA on macrophage polarization. The result showed that 3-HAA treatment regulated not only the LPS-increased expression of M1 markers but also the LPS-reduced expression of M2 markers in LPS-treated macrophages. Collectively, these results suggest that 3-HAA has an inhibitory effect on the production of pro-inflammatory mediators and a modulatory effect on

327 macrophage polarization in activated macrophages.

328 The effect of both NO and pro-inflammatory cytokine in immune regulation is exerted
329 though multiple mechanisms. Stimulation of macrophage by LPS activates MAPKs that lead
330 to the production of inflammatory mediators.[38-40] Presently, phosphorylations of MAPKs
331 were clearly detected in LPS-stimulated cells. However, the increased phosphorylation of
332 MAPKs was not inhibited by 3-HAA treatment. Thus, these data suggest that inhibition of
333 LPS induced inflammatory mediators by 3-HAA is not mediated by MAPK signaling
334 pathways.

335 PI3K/Akt signaling pathway has also been implicated in the production of NO and cytokine
336 in LPS-stimulated macrophages.[7, 41] Therefore, we investigated the effect of 3-HAA on the
337 PI3K/Akt signaling pathway in LPS-stimulated RAW 264.7 cells. 3-HAA significantly
338 abolished phosphorylation of both PI3K and Akt, suggesting that the inhibitory effect of 3-
339 HAA on production of inflammatory mediators is associated with activation of the PI3K/Akt
340 signaling pathway in LPS-induced macrophages.

341 Activation of the serine/threonine kinase Akt can up-regulate NF-κB activity in various cell
342 types.[23] NF-κB is essential in inflammation by prompting transcription of pro-inflammatory
343 genes.[42] Furthermore, it has been suggested that inducible NF-κB activation requires the
344 nuclear translocation of p65 through the phosphorylation and degradation of IκBα.[22, 43] In the
345 current study, the activation of NF-κB was concentration-dependently blocked by 3-HAA
346 through the inhibition of IκBα degradation and subsequent p65 nuclear translocation in LPS-
347 stimulated Raw 264.7 cells. Moreover, our data showed that 3-HAA blocked the LPS/ IFNγ-
348 translocated NF-κB into the nuclues in peritoneal macrophages. Thus, the present data
349 suggest that this inhibitory mechanism is associated with the suppressive effect of 3-HAA on
350 production of inflammatory mediators.

351 One of the main targets of Akt is mTOR which regulates multiple cellular functions and

innate immunity.[10-12] Hence, we further determined the involvement of mTOR in inhibitory mechanism of 3-HAA. The present data showed that 3-HAA treatment resulted in a decrease in LPS or LPS/ IFNγ-induced mTOR activation. In addition, inhibition of mTOR attenuated LPS-induced NF-κB activation as well as LPS-induced production of NO and IL-6, but had little effect on LPS-induced TNF-α activation. These results are consistent with previous studies that LPS-induced TNF-α expression is not responsive to rapamycin.[44, 45] It is also interesting to note that 3-HAA had not a synergistic effect with rapamycin in inducing NF-κB activity and NO production, whereas both agents exhibited a strong inhibitory effect. The interrelationship of rapamycin and 3-HAA is probably very complex. However, it is plausible that an interdependence exists between activation or blocking NF-κB activity and NO production. Additionally, it has been suggested that mTOR is involved in mediating PI3K/Akt-associated activation.[11] Based on these findings, inhibitory effect of 3-HAA on the production of inflammatory mediators is probably associated with PI3K/Akt/mTOR pathway in activated macrophages.

Our results are in conflict with reports that mTOR inhibition by rapamycin increases the release of pro-inflammatory cytokines and NF-κB activity,[46, 47] and that rapamycin treatment can inhibit production of pro-inflammatory mediators and activation of NF-κB.[10, 45, 48-50] These discrepancies might be explained by differences in cell types and stimulation conditions. In addition, we cannot rule out the possibility that the effect of 3-HAA on LPS-induced inflammatory response is mediated through other factors such as HO-1 which is related to regulate NO production in LPS-stimulated macrophages.[14] Nonetheless, the simplest explanation of our data is that the PI3K/Akt/mTOR and NF-κB pathways participate in the mechanisms of 3-HAA effects on NO and cytokine production on macrophages.

In summary, 3-HAA inhibited the production of inflammatory mediators in LPS-induced Raw 264.7 cells. These inhibitory effects resulted from the repression of PI3K/Akt/mTOR

activation and downstream NF-κB activation, which implicates 3-HAA as a novel chemotherapeutic agent for chronic inflammatory diseases. Further studies are needed to confirm this speculation *in vivo*.

**Conflict of interest**

The authors have no conflate of interests to declare.

383 **Legends**

384

385 **Fig. 1** Effects of 3-HAA on RAW 264.7 cells. (A) Effect of 3-HAA on RAW 264.7 cell
386 viability. Cells were treated with indicated concentrations of 3-HAA or vehicle control (3 %
387 DMSO) for 24 h and viability was measured using the MTT assay. The results are expressed
388 as percentage of viable cells compared to untreated cells. (B-D) NO production and release of
389 cytokines in LPS-treated cells. The cells were incubated with a medium in the absence or
390 presence of 3-HAA (0, 0.1, 1, 10) for 2 h followed by the stimulation with LPS (1 μg/mL)
391 treatment for 24 h. Concentrations of nitrite and cytokines in medium were determined as
392 described in Materials and Methods. * Significantly different from LPS-induced cells not
393 treated with 3-HAA.

394

395 **Fig. 2** Effect of 3-HAA on the expression of polarization markers in LPS-stimulated
396 macrophages. RAW 264.7 cells were incubated with a medium containing 3-HAA (10 μg/mL)
397 for 2 h followed by the stimulation with LPS (1 μg/mL) for 20 h. The levels of TNF-α, IL-1β,
398 Arg-1 and IL-10 mRNA were determined by qRT-PCR. GAPDH served as the internal
399 control. One of the three separate experiments is shown. The mRNA levels of these
400 polarization markers are in arbitrary units, and data are normalized to the respective amount
401 of GAPDH mRNA. # Significantly different from untreated control. * Significantly different
402 from LPS-stimulated cells not treated with 3-HAA ($p<0.05$).

403

404 **Fig. 3** Effect of 3-HAA on NF-κB activation and IkB degradation in LPS-stimulated
405 macrophages. (A) RAW 264.7 cells were transfected with a pGL3-NF-κB-Luc reporter
406 plasmid and pCMV-β-gal, pretreated with 3-HAA for 2 h and treated with LPS (1 μg/mL) for
407 4 h. Luciferase activity in the cells was measured. (B) Cells were incubated with indicated

concentrations of 3-HAA for 2 h and stimulated for 4 h with LPS. The cells were fixed and incubated with anti-NF-κB antibody followed by FITC conjugated α-rabbit secondary antibody. Hoechst nuclear staining was also performed. The images were visualized by confocal immunofluorescence microscopy. Bars denote = 10 μm (C) RAW 264.7 cells were untreated or pre-incubated with various concentrations of 3-HAA for 2 h prior to exposure to LPS for 4 h. Cytoplasmic and nuclear levels of NF-κB p65 were determined by Western blotting. α-Tubulin and Lamin A were used as loading control for cytosolic and nuclear protein fractions, respectively. (D) RAW 264.7 cells were pre-incubated with or without 3-HAA (10 μg/mL) for 2 h and then treated with LPS (1 μg/mL) for indicated times. IκBα degradation was analyzed by Western blotting with anti-IκBα antibody. β-actin protein level was considered as an internal control. The results illustrated are from a single experiment and a representative of three separate experiments.

**Fig. 4** Influence of 3-HAA on phosphorylation of MAPKs in LPS-stimulated macrophages. 3-HAA was added to cells for 2 h before LPS (1 μg/mL) stimulation. Whole cell lysates were extracted 15 min after the stimulation. The levels of phosphorylated p38, JNK and ERK were analyzed by Western blotting as described in Materials and Methods. The relative intensities are expressed as the ratio of phospho-MAPK to total MAPK.

**Fig. 5** 3-HAA represses LPS-induced phosphorylation of PI3-p110, Akt and mTOR. Cells were pre-incubated with 3-HAA for 2 h and then protein samples were extracted at 10 min (for PI3-p110), 30 min (for Akt) or 1 h (for mTOR) after LPS (1 μg/mL) stimulation. The whole cell lysates were analyzed by Western blot. The levels of unphosphorylated Akt and β-actin protein were considered as internal controls. The intensity of the bands was quantitated by densitometry. * Significantly different from LPS-induced cells not treated with 3-HAA (p

433 < 0.05).

434

435 **Fig. 6** Effect of mTOR inhibition on the suppressive effects of 3-HAA against LPS-induced
436 inflammation. (A) RAW 264.7 cells were transfected with a pGL3-NFκB-Luc reporter
437 plasmid and pCMV-β-gal plasmid, and then pretreated with rapamycin for 30 min. The cells
438 were treated with 3-HAA for 2 h followed by LPS for 4 h. Luciferase activity in the cells was
439 measured. (B-D) Cells were pre-incubated with rapamycin for 30 min prior to incubation
440 with medium in the absence or presence of 3-HAA (10 μg/mL) for 2 h, followed by
441 stimulation with LPS (1 μg/mL) for 24 h. Concentrations of nitrite and cytokines in the media
442 were determined as described in Materials and Methods. * Significantly different from LPS-
443 induced cells not treated with 3-HAA.

444

445 **Fig. 7** Inhibitory effects of 3-HAA in LPS/IFN-γ-stimulated peritoneal macrophages. (A)
446 Peritoneal macrophages were treated with 3-HAA (10 μg/mL) for 2 h and then stimulated
447 with LPS (1 μg/mL)/IFN-γ (50U/ml) for 24 h. Concentrations of nitrite and cytokines in
448 medium were measured as described in Materials and Methods. (B) Cells were pre-incubated
449 with 3-HAA for 2 h and then protein samples were extracted at 4 h after LPS (1 μg/mL)/IFN-
450 γ (50U/ml) stimulation. Cytoplasmic and nuclear levels of NF-κB p65 were detected by
451 Western blotting to analyze the translocation of NF-κB. α-Tubulin and Lamin A were used as
452 loading controls for cytosolic and nuclear protein fractions, respectively. (C) Cells were pre-
453 treated with the indicated concentrations of 3-HAA for 2 h before stimulation with LPS (1
454 μg/mL)/IFN-γ (50U/ml) for 1 h. The whole cell lysates were analyzed by Western blotting.
455 The levels of β-actin protein were used as internal controls. * Significantly different from
456 LPS-induced cells not treated with 3-HAA.

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458 **Notes and References**

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