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# Anti-inflammatory activity of JJBF11, a novel fluorinated triarylmethane derivative in LPS-stimulated macrophages

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## Abstract

Overproduction of inflammatory mediators and cytokines plays a critical role in the pathogenesis of inflammation-related diseases. In our continuing effort to develop a novel anti-inflammatory agent, bis(1,2,4-trimethoxyphenyl)(4-trifluoromethylphenyl)methane (JJBF11), a newly fluorinated triarylmethane derivative, was synthesized. In the present study, JJBF11 was investigated for its anti-inflammatory effect and the mechanism of action in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Amounts of nitric oxide (NO), as well as prostaglandin $E_2$ ($PGE_2$), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1 β) in culture media were determined by Griess reaction and ELISA, respectively. Inflammatory responsive protein levels were examined by Western blot analysis. JJBF11 significantly inhibited the production of NO generated by inducible nitric oxide synthase (iNOS) and TNF-α. The compound did not exhibit the suppressive effect on $PGE_2$ generated by cyclooxygenase-2 (COX-2) and IL-1 β. Also, JJBF11 attenuated the expression of iNOS but not COX-2 protein. Moreover, JJBF11 at 3.12-50 μM had no effect on iNOS enzyme activity. Our data suggest that JJBF11 exerts anti-inflammatory activity via inhibition of NO and TNF-α production and suppression of iNOS protein. Thus, JJBF11 has potential to be developed as a novel agent for treating inflammatory diseases.

## Introduction

Inflammation is an immune response of an organism to against various injury and pathogen. This process mediated pro-inflammatory including nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$) and cytokines such as interleukin-1β (IL-1β), and tumor necrosis factor- α (TNF-α).[1] However, the over-production of pro-inflammatory mediators are crucial for progression of inflammatory diseases.[2] NO is a crucial messenger molecule that synthesized by iNOS enzymes. That functional on microbicidal, antiviral, antiparasital and antitumoral.[3] $PGE_2$ plays an important role in regulation of the inflammatory response. They are derived from arachidonic acid (AA) and generated by cyclooxygenase (COX).[4] The inhibition of inflammatory mediators that produce by macrophage be able to retaining inflammatory diseases.[5]

Triarylmethanes (TRAMs) have been studies their numerous biological activities such as anti-oxidant, anti-tumor, anti-bacterial, anti-virus, and anti-inflammatory.[6] We have previous studies toward bis(heteroaryl) alkanes, triarylmethane analogs represent anti-

inflammatory activities.[7] Twenty analogs of bis(heteroaryl) alkane were estimated for the anti-inflammatory activities in LPS-stimulated RAW 264.7. Among them, bis[(5-methyl)2-furyl] (4-fluorophenyl) methane, which consists a fluorine atom at para-position of the benzene ring show inhibited the LPS-induced NO production comparable with aminoguanidine, a positive control. It seems like the compound which consisting a strong electron withdrawing group, are able to enhance the inhibitory effect. The incorporation of fluorine into drugs might be more potent on anti-inflammatory activity. In this study, as a part of our ongoing to develop novel anti-inflammatory agent. The JJBF11 (Figure 1) derivative of fluorinated triarylmethane was newly synthesized and evaluated for their inhibition of NO, $PGE_2$, TNF-α, and IL-1β on LPS-stimulated RAW264.7 macrophage cells. We also determined the mode of action on protein iNOS and COX-2.

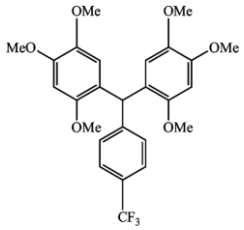


**Figure 1.** Chemical structure of bis(1,2,4-trimethoxyphenyl)(4-trifluoromethylphenyl)methane (JJBF11)

**Methodology**

*Chemical and regents*

Dulbecco' modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Penicillin/Streptomycin were purchased form Gibco/Invitrogen (NY, USA). Aminoguanidine bicarbonate, indomethacin, lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111: B4 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma (MO, USA). Antibodies for iNOS and COX-2 were purchased from BD Bioscience (CA, USA). Antibodies for GAPDH, Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were obtained from Cell Signaling Technology (MA, USA).

*Cell viability test by MTT assay*

RAW 264.7 macrophage cells were maintained in 24-well plate ($1\times10^5$ cells/well). After an overnight incubation, cells were treated with the test compound for 24 hours before the MTT assay as described by Srisook et al.[8]

*Determination of nitrite, $PGE_2$ production and cytokines*

RAW264.7 cells were seeded into 24-well plate ($1\times10^5$ cells/well) for overnight and treated with compound in the absence or presence of LPS for 24 hours. Culture media was determined the nitrite concentration as an indicator for NO using Griess reaction as previously described by Srisook et al.[8] The supernatant of culture media was collected for determination of PGE2, TNF-α and IL-1β by using $PGE_2$ competitive enzyme immunoassay kit (Arbor assay, MI, USA.), mouse TNF-α and IL-1β/IL-1F2 quantikine® ELISA (R&D systems®, MN, USA.) according to manufacturer's instructions, respectively.

*Indirect determination of iNOS activity*

This experiment was modified from Tsao, Lee Huang Kuo, & Wang.[9] RAW264.7 cells were seeded in 24-well plate ($1\times10^5$ cells/well). After overnight growth, the cells were triggered with LPS for 24 hours. Therefore, the cells were washed twice with HBSS and treated with the test compound for 6 hours. Finally, supernatants were collected and measured the nitrite concentration by Griess reaction.

*Protein preparation and Western blot analysis*

RAW264.7 cells were harvested with cold PBS and lysed in RIPA lysis buffer. Cells lysate were centrifuge at 12,000 g for 10 minutes at 4 ºC. The protein was separated by SDS-PAGE. For Western blot analysis, iNOS and COX-2 were determined by the method of Bualpool et al.[10]

*Statistical analysis*

All results were expresses as mean ± S.D. of two independent experiments. Data were analyzed statistical significance by one-way analysis of variance (ANOVA) and Tukey's test for multiple comparison. Values of $p<0.05$ were considered to be significant.

**Results and Discussion**

*Effect of JJBF11 on cell viability and NO production*

As shown in Figure 2A, JJBF11 at 50 µM showed the %cell viability of 66.3 ± 8.9, that without significant cytotoxicity. However, the cytotoxic at 12.5-25 µM was more than 50%. Treatment with JJBF11 significantly suppressed LPS-induced NO production with $IC_{50}$ values of 7.06 ± 0.62 µM (Figure 2B). Nevertheless, JJBF11 did not decrease the $PGE_2$ production by treatment at concentration of 12.5 and 50 µM (Figure 2C).

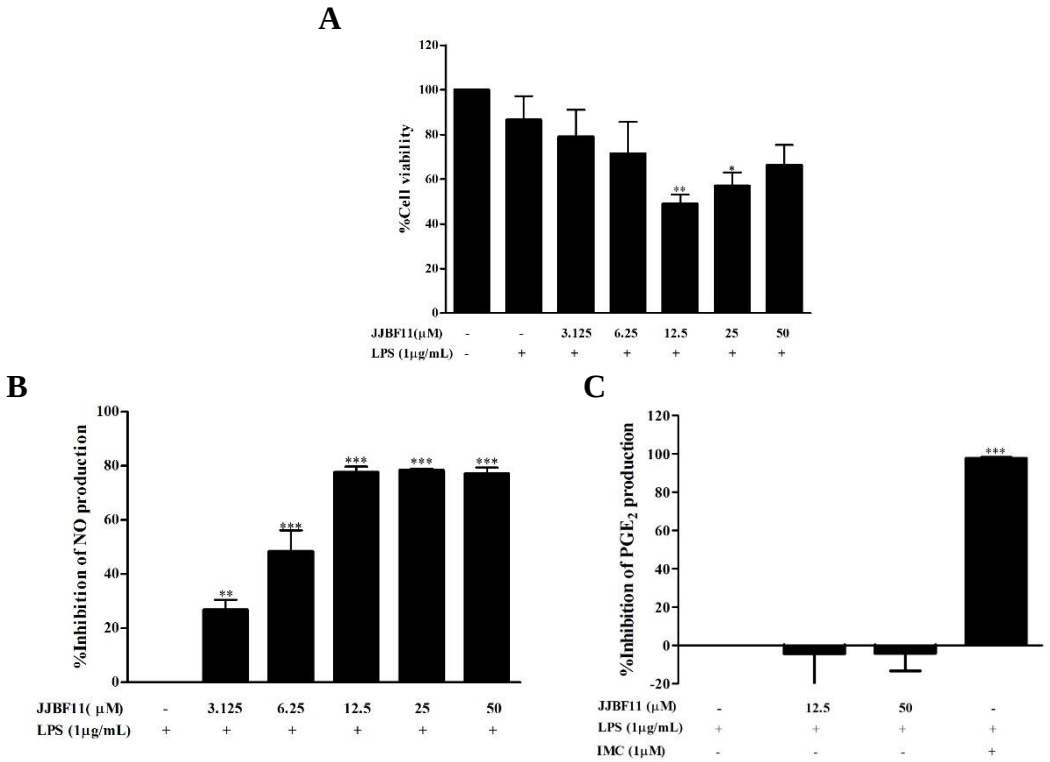


**Figure 2.** Effect of JJBF11 on LPS-activated NO, $PGE_2$ production and cell viability. (A) Cell viability were presented as percentage of unstimulated cells. The percentage inhibition of NO (B) and $PGE_2$ (C) production were determined in comparison to LPS-stimulated cells.

*Effect of JJBF11 on LPS-activated iNOS and COX-2 expression*

The iNOS and COX-2 enzyme are involved in the activation-induced synthesis of NO and $PGE_2$. To determine the mechanisms underlying the inhibitory effect of JJBF11 on NO production, we examined that JJBF11 affected on iNOS activity and its expression. As shown in Table 1, treatment with JJBF11 did not show an inhibitory effect on iNOS activity at all concentration, while aminoguanidine inhibited iNOS activity with %inhibition of iNOS

activity value 39.2 ± 1.7 at a concentration of 50 µM. Moreover, JJBF11 markedly block LPS-induced iNOS protein expression at concentration as 3.125-12.5 µM were significantly suppressive ($P$<0.001) (Figure 3A). However, JJBF11 did not suppress COX-2 expression at all concentrations tested (Figure 3B). Consequently, JJBF11 treatment did not inhibit $PGE_2$, that correlate with COX-2 protein. Besides, the similar observation was reported that allyl isothiocyanate (AITC) suppress LPS-activated iNOS, but did not suppress LPS-activated COX-2 expression.[11] The expression of iNOS and COX-2 were described whether, both genes are regulated by different of transcription factor.[3,12] Taken together, these result suggest that JJBF11 probably suppressed the regulatory signaling pathway with control only iNOS expression but not COX-2 gene. Thus, these results shown that the inhibitory action of JJBF11 on LPS-stimulated NO production mainly causes from the regulatory effect at the protein level.

**Table 1** The effect of JJBF11 on iNOS activity[a]

| Concentration (µM) | %Inhibition of iNOS activity[b] | |
|---|---|---|
| | Aminoguanidine | JJBF11 |
| 3.125 | - | -6.3±1.8 |
| 6.25 | - | -11.5±7.5 |
| 12.5 | - | -11.4±6.9 |
| 25 | - | 0.4±0.6 |
| 50 | 39.2±1.7 | -5.9±3.9 |

[a]All data show mean ± SD of at least two independent experiment with triplicate samples.
[b]The percentage inhibition of NO production of treatment was established in comparison to LPS-activated cells.

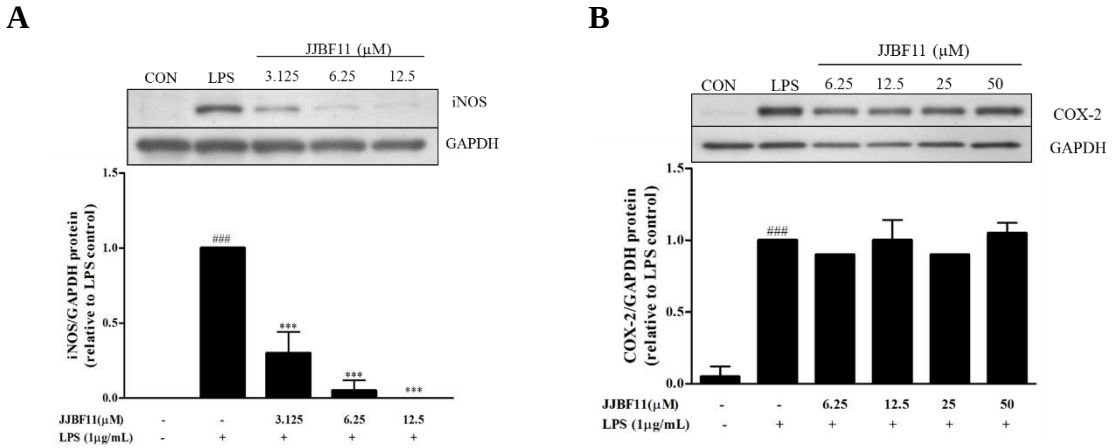


**Figure 3.** Effect of JJBF11 on iNOS (A) and COX-2 (B) protein expression were determined by Western blot analysis.

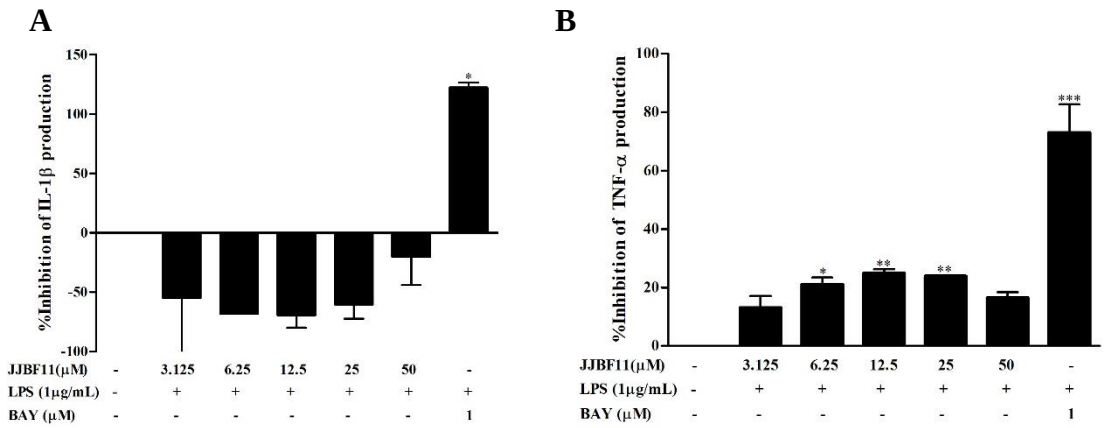


**Figure 4.** Effect of JJBF11 on IL-1β(A) and TNF-α (B) production were determined by ELISA

*Effect of JJBF11 on LPS-induced cytokines production in RAW264.7 macrophages*

To examine whether JJBF11 have their ability to decrease LPS-induced IL-1β and TNF-α production was determined by ELISA. The levels of cytokines in culture media from cells were measured after treatment with LPS alone or combination with JJBF11 for 24 hours. As shown in Figure 4, LPS clearly enlarged the production of both IL-1β and TNF-α. JJBF11 inhibited the LPS-induced TNF-α production at a dosage of 6.25-25 μM were significantly repressive (Figure 4A), while IL-1β was not affected by treatment (Figure 4B). Nevertheless, BAY11-7082, an inhibitor of NF-κB activation, suppressed both IL-1β and TNF-α. NF-κB plays a key role regulator for the transcription of TNF-α and IL-1β.[13] These results demonstrate that JJBF11 inhibit only TNF-α but not IL-1β may be involved in another regulation steps.

## Conclusion

In summary, JJBF11, a newly synthesized fluorinated triarylmethane derivative, was estimated its anti-inflammatory activities against LPS-induced NO, $PGE_2$, TNF-α and IL-1β. We founded that JJBF11 suppresses NO production in LPS-stimulated RAW 264.7 macrophages with $IC_{50}$ values of 7.06 ± 0.62 μM via repressed iNOS protein expression. In addition, treatment of cells with JJBF11 also decreased TNF-α production. Moreover, the fluorinated triarylmethane derivative, JJBF11, might be a lead compound for developing the anti-inflammatory agent.

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