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Original research article

# A novel synthetic HTB derivative, BECT inhibits lipopolysaccharide-mediated inflammatory response by suppressing the p38 MAPK/JNK and NF-κB activation pathways

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ABSTRACT

Activated microglia cells are well recognized as mediators of neuroinflammation, as they release nitric oxide and pro-inflammatory cytokines in various neuroinflammatory diseases. Thus, suppressing microglial activation may alleviate neuroinflammatory and neurodegenerative processes. In the present study, we synthesized and investigated the anti-neuroinflammatory effect of a novel HTB (2-hydroxy-4-trifuoromethylbenzoic acid) derivative in lipopolysaccharide (LPS)-stimulated microglial cells. Among the synthesized derivatives, the BECT [But-2-enedioic acid bis-(2-carboxy-5-trifluoromethyl-phenyl) ester] significantly decreased production of nitric oxide and other pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, and interleukin-6 in microglial cells. BECT also mitigated the expression of inducible nitric oxide synthase and cyclooxygenase-2 at both the mRNA and protein levels. Further mechanistic studies demonstrated that the HTB derivative inhibited phosphorylation of JNK and p38 mitogen-activated protein kinase and nuclear translocation of nuclear factor kappa-B in LPS-stimulated BV-2 microglial cells. Thus BECT, our novel synthesized compound have anti-inflammatory activity in microglial cells, and may have therapeutic potential for treating neuroinflammatory diseases.


## Introduction

Although neuroinflammation plays a critical role in brain host defense, it also contributes to the underlying neuronal loss occurring in various neurodegenerative disorders [1]. Uncontrolled activation of microglia is directly toxic to neurons, as they release various neurotoxic substances such as nitric oxide (NO), prostaglandin E2, superoxide, and proinflammatory cytokines [2–4]. Inflammatory intermediates including expression of inducible nitric oxide synthase (iNOS) and subsequent production of NO and mitogen-activated protein kinase (MAPK) signaling cascades such as c-Jun N-terminal kinase (JNK) and p38 in activated glial cells play important roles in mediating glial responses to inflammatory triggers [5,6]. Various studies have reported that suppressing the exaggerated inflammatory response initiated by activated microglial cells helps to attenuate the severity of neurodegenerative diseases [7,8]. One of the most commonly used anti-inflammatory agents are salicylates or drugs structurally resembling aspirin. One such derivative of aspirin is triflusal (2-acetoxy-4-trifluoromethylbenzoic acid). Triflusal is a fluorinated salicylate that possesses distinct pharmacologic, pharmacokinetic, and biochemical properties from those of aspirin [9,10]. Besides its antiplatelet action, triflusal reduces nuclear factor kappa-B (NF-κB), activation of microglia in excitotoxic injury and in models of Alzheimer's disease [9,11]. Following oral administration, triflusal is converted to its main deacetylated active metabolite HTB (3-hydroxy-4-trifluoromethylbenzoic acid) [12]. HTB exhibits anti-inflammatory effects by inhibiting the cyclooxygenase-2 (COX-2) protein and NF-κB [13,14]. We have synthesized HTB derivatives that may be good therapeutic agents

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in pathological conditions in which regulation of inflammatory genes constitutes a relevant step in the outcome of the neurodegenerative event.

Our previous report suggested that microglia are a good cellular model for screening potential therapeutic compounds for neuroinflammatory disorders [15]. In the present study, we report the anti-neuroinflammatory effect of a novel synthetic HTB derivative BECT [But-2-enedioic acid bis-(2-carboxy-5-trifluoromethyl-phenyl) ester] in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. BECT inhibited the production of inflammatory markers, including NO, COX-2, and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Subsequent experiments found that pretreatment with BECT abates, LPS-stimulated induction in phosphorylation of JNK and p38-MAPK and decreases the nuclear translocation of the NF-κB p65 subunit in LPS-stimulated BV-2 microglial cells. Our data indicate that inhibition JNK, p38MAPK, and NF-κB signaling might be one of the possible molecular mechanisms contributing to the anti-neuroinflammatory effect of BECT in LPS-stimulated microglial cells. These results extend our understanding of the anti-neuroinflammatory properties of BECT and suggest a possible new pharmacological agent for neuroinflammatory diseases.

## Materials and methods

### *Reagents*

LPS (*Escherichia coli* 0111:B4, Sigma, St. Louis, MO, USA), *N*-(1-naphthyl) ethylenediamine dihydrochloride, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tween-20, bovine serum albumin, dimethyl sulfoxide (Compound Diluent/(1%) Vehicle control), sodium nitrite and sulfanilamide were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), containing 4500 mg/L of D-glucose, L-arginine, 110 mg/L of sodium pyruvate, and phosphate buffered saline (PBS) as well as other cell culture reagents were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). The protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets were supplied by Roche (Indianapolis, IN, USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories Inc. (Etobicoke, Ontario, Canada). Antibodies to iNOS, JNK, phospho-JNK, p38, phospho-p38, IκB-α, p-IκB-α, and β-actin were supplied by Cell Signaling Technology (Danvers, MA, USA) and antibodies to COX-2, p65NF-κB, and nucleolin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### *Cell culture*

BV-2 cells (a mouse microglial cell line), originally developed by Dr. V. Bocchini (University of Perugia, Perugia, Italy), were used as cited in our earlier reports [15,16]. The BV-2 cell line demonstrated both phenotypic and functional properties of reactive microglia cells [17]. The cells were grown and maintained in DMEM containing 5% heat-inactivated FBS + 50 μg/mL penicillin–streptomycin and maintained in a humidified incubator at 37 °C with 5% $CO_2$.

### *NO assay*

NO production was assayed by measuring the levels of nitrite in culture medium. Accumulated levels of nitrites were measured in the cell supernatant using the Griess reaction [18]. BV-2 cells ($2.5 \times 10^4$ cells/mL) plated in 96-well plates in 200 μL culture medium were pre-treated with different BECT concentrations (12.5, 25, and 50 μM) for 60 min, and then stimulated with LPS (100 ng/mL) for 24 h. Briefly, 50 μl of culture supernatant was reacted with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine and 1 part 1% sulfanilamide in 5% $H_3PO_4$) in 96-well plates at room temperature. Nitrite concentrations were determined using standard solutions of sodium nitrite prepared in cell culture medium. Absorbance was determined at 540 nm using a microplate reader (Tecan Trading AG, Mannedorf, Switzerland). Results are representative of three independent experiments.

### *Cytotoxicity assay*

Viability of cultured cells was determined by measuring the reduction of MTT to formazan. Briefly, BV-2 cells ($2.5 \times 10^4$ cells/mL) were pre-treated with different concentrations of BECT (12.5, 25, and 50 μM) for 60 min in 96-well plates with 200 μL culture medium and then stimulated with LPS (100 ng/mL) for 24 h. Then, 0.5 mg/mL of MTT solution was added to each well. After 4 h of incubation at 37 °C, the supernatants were removed, and the formed formazan crystals were dissolved in DMSO. The absorbance at 550 nm was determined using a microplate reader. Results are representative of three independent experiments.

### *Methodology for synthesis of BECT*

To a solution of 2-hydroxy-4-(trifluoromethyl)benzoic acid (HTB) 1 (2.00 g, 9.70 mmol) in THF (150 mL) and pyridine (50 mL) at 0 °C was added fumaryl chloride (526 μL, 4.85 mmol). The reaction mixture was stirred at room temperature for 1 h and then poured into water and 1 *N* HCl solution. The solution was extracted with ethyl acetate and the organic layer was washed with water, dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude product was recrystallized with ethyl acetate/hexane (1/3) to obtain the title compound as white solid (560 mg, 23%). Mp 177 °C; IR (KBr, $cm^{-1}$) 3433, 1778, 1716; $^1$H NMR (DMSO-$d_6$) δ 7.23 (s, 2H), 7.78–7.82 (d, 2H, $J$ = 8.0 Hz), 7.87 (s, 2H), 8.15–8.20 (d, 2H, $J$ = 8.0 Hz); $^{13}$C NMR (DMSO-$d_6$) δ 121.06, 123.40, 124.28 ($J_{C\text{-}F}$ = 270.7 Hz), 127.92, 132.51, 133.46 ($J_{C\text{-}F}$ = 32.6 Hz), 133.69, 149.41, 162.47, 164.28; MS $m/z$ 491.02 ($[M+H]^+$).

### *Isolation of total RNA and reverse transcription-polymerase chain reaction*

BV-2 cells ($50 \times 10^4$ cells/mL) were cultured in 6-well plates, and total RNA was isolated by extraction with TRIzol (Invitrogen). For RT-PCR, 2.5 μg of total RNA was reverse transcribed using a First-Strand cDNA Synthesis kit (Invitrogen). Then, cDNA was amplified by PCR using specific primers as mentioned previously [15,16]. The PCR was performed using the above-prepared cDNA as a template for respective targets, as described previously [19,20]. PCR products were analyzed on 1% agarose gels. Results are representative of three independent experiments.

### *Western blot analysis*

Cells were washed twice with ice cold PBS, and total cell lysates were obtained by adding 50 or 100 μL of RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, containing fresh protease inhibitor cocktail) to the BV-2 cells ($50 \times 10^4$ cells/mL) cultured in 6-well plates. Electrophoresis and immunoblotting procedures followed those of a previous report [16]. PVDF membranes were tagged by incubating them overnight with anti-iNOS (1:1000), anti-IκB-α (1:1000), anti-p-IκB-α (1:1000), anti-p38 (1:1000), anti-phospho-p38 (1:1000), anti-JNK (1:500), anti-phospho-JNK (1:1000) anti-β-actin (1:2000), anti-COX-2 (1:1000), anti-phospho p65NF-κB (1:500), and anti-nucleolin (1:500) antibodies followed by a 1 h incubation with horseradish

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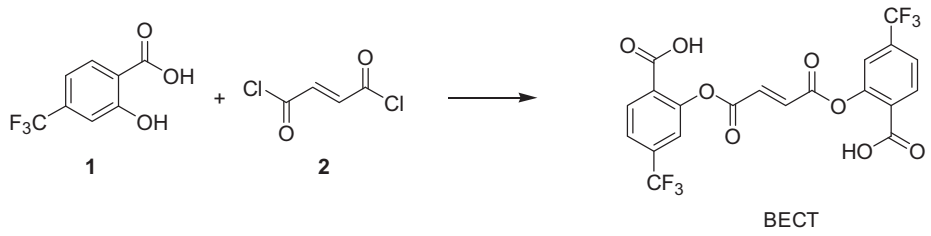



**Fig. 1.** Synthesis of novel 2-hydroxy-4-trifluoromethyl benzoic acid (HTB) derivative, BECT.

peroxidase-conjugated secondary antibody (1:1000–2000). The optical densities of the antibody specific bands were analyzed with a LAS-3000 Luminescent Image Analyzer (Fuji, Tokyo, Japan). In a parallel experiment, nuclear proteins were extracted using the method provided by the nuclear and cytoplasmic extraction kit from Thermo Scientific (Rockford, IL, USA). Results are representative of three independent experiments.

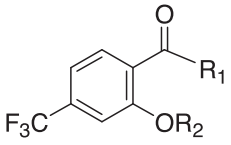
*Immunofluorescence assay*

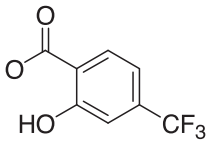
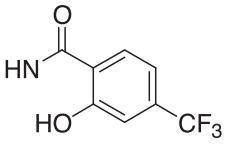
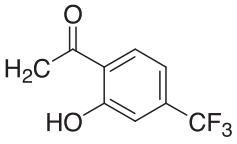
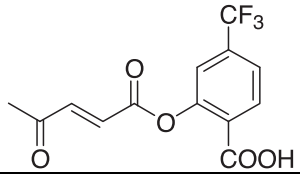
BV-2 microglia cells ($5 \times 10^4$ cells/well in 24-well plate) were cultured on sterile cover slips in 24-well plates and treated with 50 μM of BECT for 60 min followed by treatment with LPS (100 ng/mL) for 30 min to detect the intracellular location of the NF-κB p65 sub-unit. At 30 min after LPS treatment, the cells were fixed with methanol for 30 min at -20 °C and washed with PBS for 5 min. The fixed cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature, washed with PBS for 5 min, and then treated with 10% goat serum in PBS for 60 min. The permeabilized cells were treated with monoclonal mouse anti-human NF-κB p65 (1:200) (Santa Cruz Biotechnology) for 60 min at room temperature and washed in PBS for 5 min. The cells were then incubated in a 1:200 dilution of Alexa Fluor 568-labeled goat anti-mouse antibody (Invitrogen) for 60 min at room temperature and washed in PBS for 5 min. The cells were stained with 1 μM Hoechst staining solution for 30 min at 37 °C and then washed. Finally, the coverslips with cells were dried at 55 °C in an oven for 10–12 min and mounted in a 1:1 mixture of xylene and malinol.

*Statistical analyses*

Statistical analyses were conducted using GraphPad software version 5 (GraphPad, La Jolla, CA, USA). Values are presented as mean ± standard error. Significant differences between the groups

**Table 1**
HTB Derivatives inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV-2 microglia cells.



| Derivative name | $R_1$ | $R_2$ | %NO inhibition |
|---|---|---|---|
| HTB | OH | H | 7.53 |
| HTB-1 |  | H | 18.47 |
| HTB-2 |  | H | 7.51 |
| HTB-3 |  | H | 61.72 |
| HTB-4 | H |  | 30.55 |

HTB: 3-hydroxy-4-trifluoromethylbenzoic acid. Among the synthetic HTB derivatives that we screened by microglia cell-based assay, HTB-4 was identified as one among the novel synthetic compounds that strongly attenuated NO production with no significant cell toxicity observed in MTT assay in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells.

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were determined using a one-factor analysis of variance (ANOVA) followed by Tukey's *post hoc*-test. $^{*}p < 0.05$ was considered statistically significant.

## Results

### *Synthesis and purification of BECT*

2-Hydroxy-4-(trifluoromethyl) benzoic acid (HTB) was reacted with fumaryl chloride in a mixed solution of pyridine and THF at room temperature to yield BECT. The chemical structure of BECT is shown in Fig. 1. BECT was further purified by recrystallization from ethyl acetate and methanol as a white solid and was identified by NMR, IR and MS.

### *Novel HTB derivatives as inhibitors of LPS-induced microglial NO production*

Production of NO has been used as a representative measure of inflammatory activation of microglial cells [21]. Hence, the suppressive effect of the synthetic HTB derivatives (Table 1) was evaluated on NO release in LPS-stimulated microglia. BV-2 microglial cells were stimulated with LPS in the presence or absence of 10 μM of the novel synthetic derivatives for 24 h. Accumulated nitrite in the culture media was measured by the Griess reaction. Among the synthetic HTB derivatives that we screened by microglia cell-based assay, HTB-4 was identified as a novel synthetic compound that strongly attenuated NO production (Fig. 2b) with no significant toxicity seen in MTT assay (Fig. 2a) in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. We renamed HTB-4 as BECT in our further discourse.

### *Effect of BECT on NO production and cytotoxicity in LPS stimulated BV-2 microglial cells*

BV-2 microglial cells were pre-treated for 60 min with different concentrations of BECT (12.5, 25, and 50 μM), followed by LPS (100 ng/mL) treatment for 24 h. The accumulated nitrite in the culture medium estimated by the Griess reaction was used as an index of NO release. As shown in Fig. 2d; LPS treatment significantly stimulated NO production ($12.46 \pm 0.34$ μM) as compared to that in the control. The control and BECT treatments did not induce increase in NO levels. Pre-treatment with BECT (12.5, 25, and 50 μM) significantly reduced NO levels in LPS-induced BV-2 microglial cells in a dose-dependent manner to $9.32 \pm 0.48$ μM, $6.92 \pm 0.47$ μM, and $5.43 \pm 0.19$ μM, respectively. The MTT assay was performed to determine whether BECT had any cytotoxic effect on BV-2 microglia cells. Viability of control cells was used as the 100% reference value. BV-2 cells were treated with LPS (100 ng/mL) with or without different concentrations of BECT (12.5, 25, and 50 μM) for 24 h. LPS (100 ng/mL) in combination with BECT did not result in any significant cytotoxicity or changes in microglia cell viability (Fig. 2c). Hence, our results demonstrate that BECT was one of the compounds to markedly inhibit LPS-stimulated NO release with no significant cytotoxicity in BV-2 microglial cells.

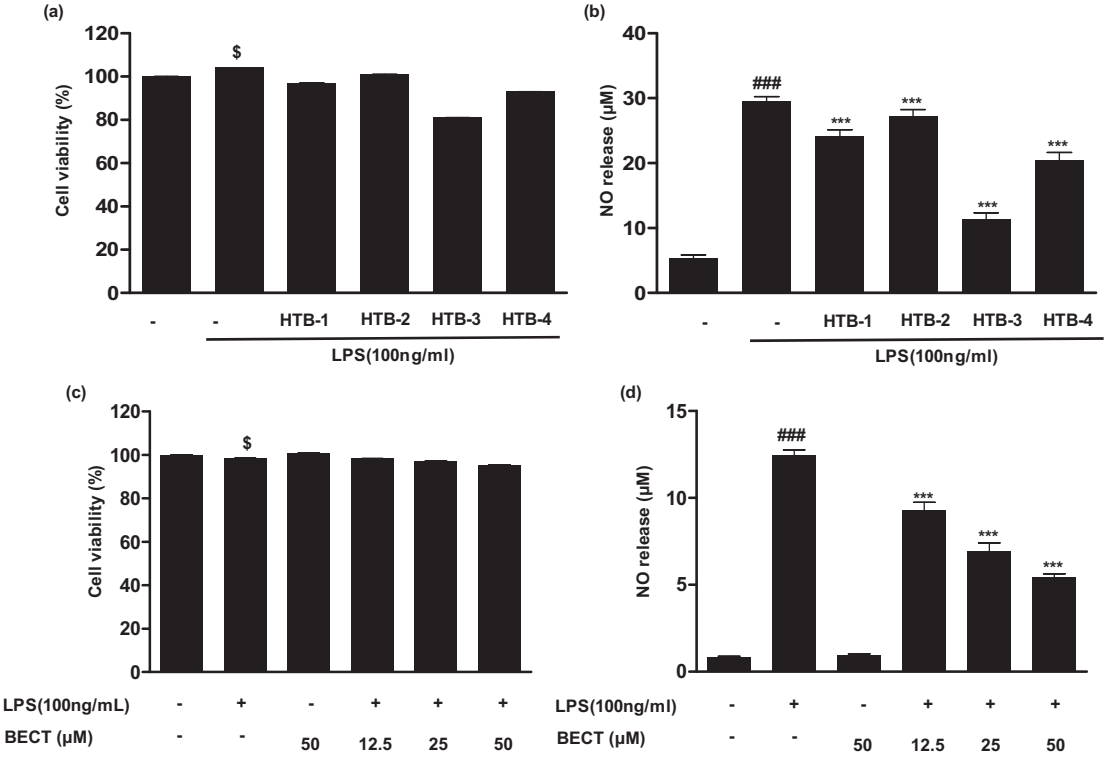


**Fig. 2.** The effect of HTB derivatives on nitric oxide (NO) production and cytotoxicity in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. BV-2 microglial cells were treated with LPS (100 ng/ml) in the absence or presence of the novel synthetic HTB derivatives (10 μM) for 24 h. (a) Cytotoxicity of each compound was assessed by MTT assays and results are expressed as a percentage of surviving cells over control cells. (c) Cells were treated with varying doses of BECT (12.5, 25, and 50 μM) and examined by MTT assay. $^{\$}p < 0.05$ compared with the control group. Significance was determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. (b) Nitrite content in the culture media was measured using the Griess reaction and the results were expressed as a percentage of released NO from LPS-stimulated BV-2 cells. Among the compounds tested, HTB-4 was one of the compounds to significantly reduce LPS-induced NO production in BV-2 cells with no significant cytotoxicity. (d) BV-2 cells were pre-treated with different concentrations of BECT (12.5, 25, and 50 μM) for 60 min before incubating with LPS (100 ng/mL) for 24 h. Data are expressed as a percentage of the control. Data are mean ± standard error (SEM) of three independent experiments. $^{\#\#\#}p < 0.001$ compared with the control group and $^{***}p < 0.001$ compared with the LPS-treated group. Significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

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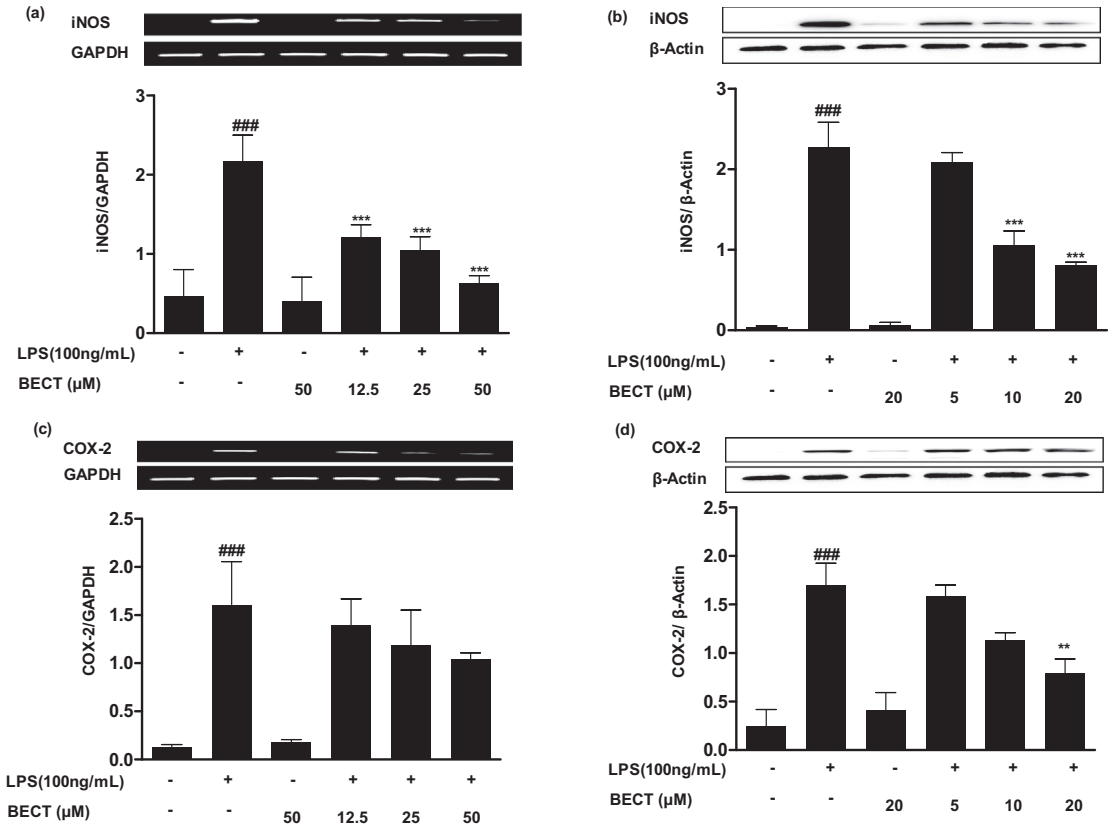


**Fig. 3.** Effect of BECT on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA and protein expression in LPS-stimulated BV-2 microglial cells. BV-2 microglial cells were pre-treated with the indicated concentrations of BECT for 60 min before incubating with LPS (100 ng/mL) for 6 h. Total RNA was extracted and further analyzed by reverse transcription polymerase chain reaction (RT-PCR). GAPDH was used as the control gene. Quantified data are shown in the lower panel. mRNA levels of (a) iNOS and (c) COX-2 were normalized to GAPDH levels and expressed as a relative change in comparison to the LPS treatment. BV-2 cells were pretreated with BECT (5, 10, and 20 μM) for 60 min and then stimulated with LPS (100 ng/ml) for 18 h. Western blot analysis was then performed to detect iNOS and COX-2 using anti-iNOS/COX-2 antibodies. The blot was stripped and reprobed with β-actin antibody to confirm equal loading. Quantification of (b) iNOS and (d) COX-2 were performed by normalization with β-actin and expressed as a relative change in comparison to the LPS treatment. Data are mean ± standard error (SEM) of three independent experiments. $^{###}p < 0.001$ compared with the control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the LPS-treated group. Significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

*Effect of BECT on LPS-stimulated iNOS and COX-2 mRNA and protein expression in LPS-stimulated BV-2 microglial cells*

Experiments involving the detection of iNOS and COX-2 mRNA and protein levels were performed by expression analysis. COX-2 is another important inflammatory enzyme that is upregulated in various neurodegenerative diseases [22]. Therefore, the effect of BECT on COX-2 expression in LPS-stimulated BV-2 microglial cells was investigated. BV-2 microglia cells were pretreated with BECT (5, 10, 12.5, 20, 25, and 50 μM) for 1 h followed by stimulation with LPS (100 ng/mL) for another 6 and 24 h. As shown in Fig. 3, pre-treatment with BECT at various concentrations significantly reduced LPS-induced iNOS mRNA (Fig. 3a) and protein expression (Fig. 3b) in a dose-dependent manner. Similarly we found that treating BV-2 cells with LPS (100 ng/mL) significantly increased COX-2 mRNA (Fig. 3c) and protein expression (Fig. 3d). Pretreatment with BECT at various concentrations inhibited LPS-induced COX-2 mRNA expression and protein level in BV-2 microglial cells in a dose-dependent manner.

*Inhibitory effect of BECT on pro-inflammatory cytokine expression in microglial cells*

Based on the earlier findings that proinflammatory cytokines released from microglia are responsible for neuronal death [23,24], we investigated the potential effects of BECT on reducing pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. BV-2 cells were pretreated with BECT (12.5, 25, and 50 μM) for 1 h in the presence or absence of LPS (100 ng/mL) for 6 h. Our data revealed that exposing BV-2 microglial cells to LPS (100 ng/mL) for 6 h resulted in a significant increase in the production of TNF-α, IL-1β, and IL-6 compared to that in the control (Fig. 4a). Pretreatment with BECT at various concentrations decreased LPS-induced mRNA expression of TNF-α, IL-1β, and IL-6 in a dose-dependent manner (Fig. 4b-d). We found a non-significant decrease in LPS-induced TNF-α and IL-1β mRNA expression levels at 12.5 and 25 μM, whereas 50 μM resulted in a significant decrease in TNF-α and IL-1β mRNA expression (Fig. 4b and c). Our results indicate that BECT suppressed production of TNF-α, IL-6, and IL-1β at the transcriptional level in response to LPS.

*Effect of BECT on LPS-induced p38, ERK, and JNK MAPK phosphorylation in BV-2 microglial cells*

MAPK signaling pathways control the release of pro-inflammatory mediators in activated microglial cells [25,26]. Therefore, we examined whether BECT influenced these signaling pathways in BV-2 microglia cells. BV-2 cells were pre-treated with various concentrations of BECT (5, 10, and 20 μM) for 60 min and then stimulated with LPS (100 ng/mL) for 30 min. We found that LPS treatment significantly increased p38MAPK and JNK phosphorylation at 30 min compared to that in the control. Pretreatment with BECT significantly inhibited LPS-stimulated upregulation of p38MAPK and JNK phosphorylation in a dose-dependent fashion

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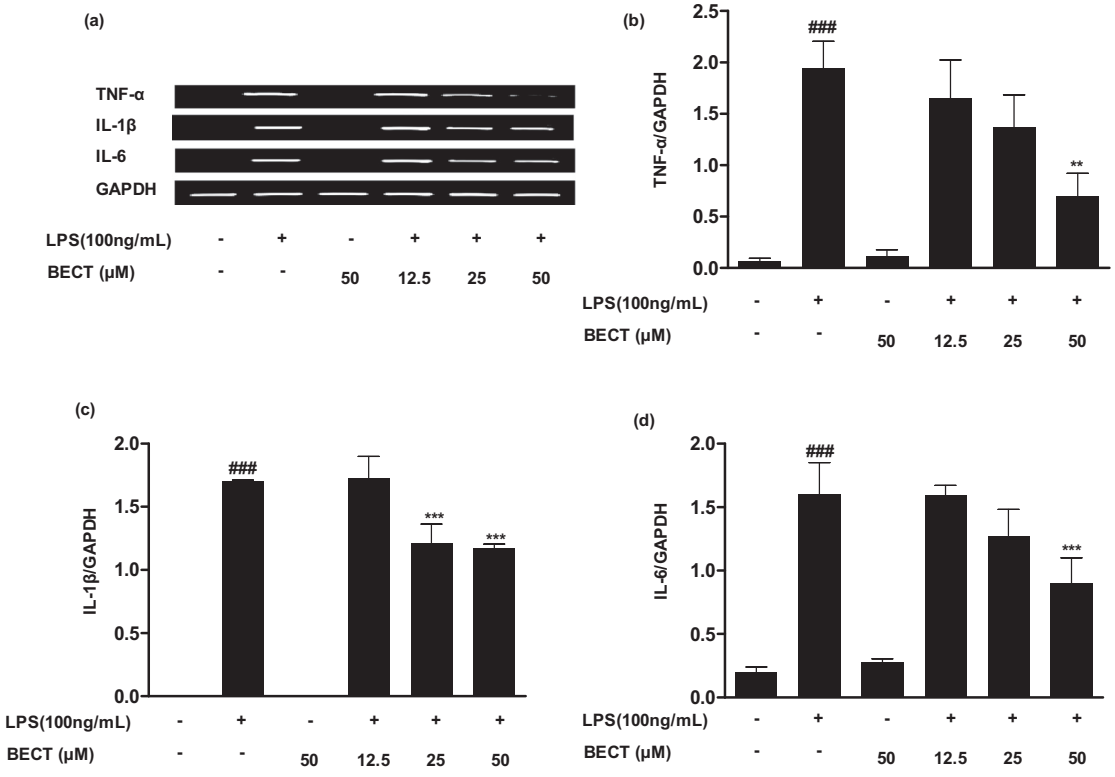


**Fig. 4.** Effects of BECT on pro-inflammatory cytokine gene expression in lipopolysaccharide (LPS)-simulated BV-2 microglial cells. BV-2 microglial cells were treated with LPS (100 ng/ml) in the absence or presence of BECT (12.5, 25, and 50 μM), and total RNA was isolated at 6 h after the treatment. (a) The levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) mRNA were determined by reverse transcription polymerase chain reaction (RT-PCR) and then subjected to densitometric quantification. Levels of (b) TNF-α, (c) IL-1β, and (d) IL-6 mRNA were normalized to GAPDH levels and expressed as relative change in comparison to the LPS treatment. Results are expressed as a ratio of TNF-α/GAPDH, IL-1β/GAPDH, and IL-6/GAPDH. $^{###}p < 0.001$ compared with the control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the LPS-treated group. Significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

(Fig. 5a and b), indicating that BECT is capable of downregulating vital signal transduction pathways involved in LPS-stimulated BV-2 microglial cells. In addition, we did not observe any BECT-induced change in the ERK signaling expression pattern in LPS-stimulated BV-2 microglial cells.

*Effect of BECT on LPS-induced IκB-α phosphorylation and NF-kB activation in microglial cells*

NF-κB plays a very important role in LPS-induced expression of iNOS, COX-2, and the production of pro-inflammatory cytokines

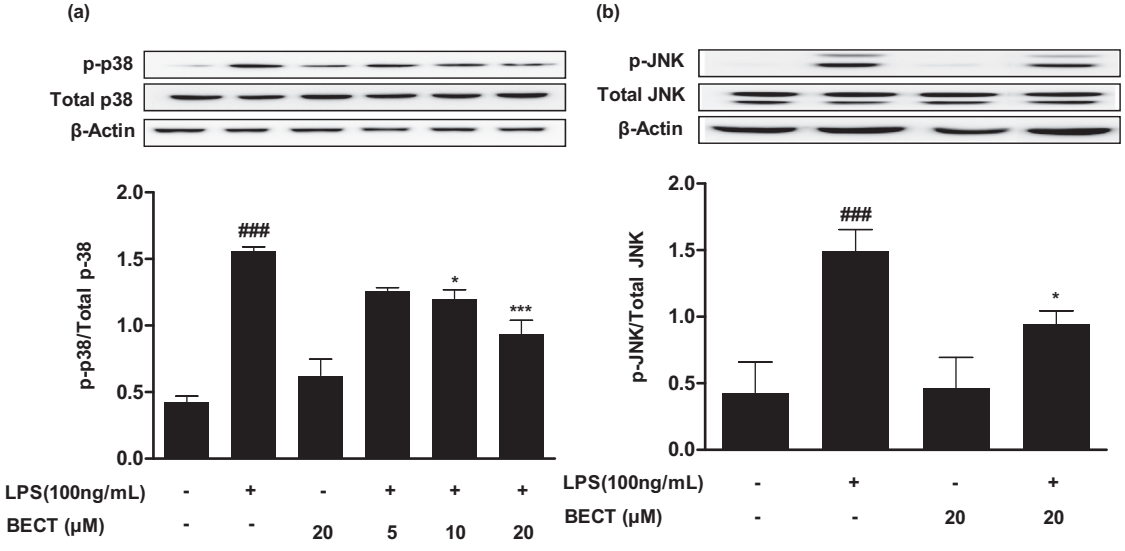


**Fig. 5.** Inhibition of lipopolysaccharide LPS-stimulated p38 and JNK phosphorylation by BECT in BV-2 microglial cells. BV-2 microglial cell lysates were obtained after a 30 min activation with LPS (100 ng/ml) in the absence or presence of BECT (5, 10, and 20 μM) and phosphorylation of (a) p38 and (b) JNK in the lysates was analyzed by Western blot. Total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Levels of phospho-mitogen activated protein kinases (MAPKs) are expressed as a relative change in comparison to the LPS treatment. Densitometric analysis of the p38 and JNK-MAPK band are shown in the lower panel. Data are mean ± standard error of three independent experiments. $^{###}p < 0.001$ compared with control group; $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with LPS-treated group. Significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

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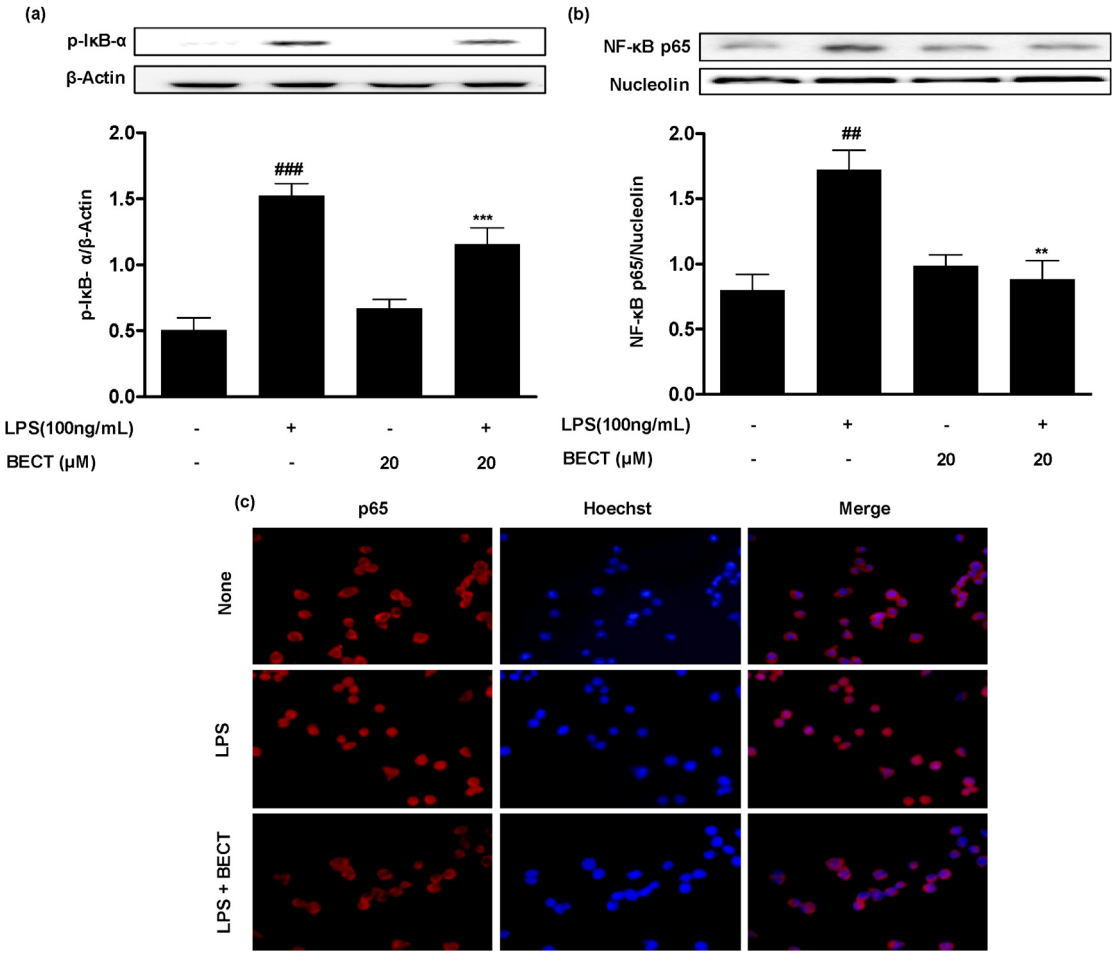


**Fig. 6.** Effect of BECT on inhibiting p-IκB-α and p65 nuclear factor NF-κB activation in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. Cells were treated with the respective dose (20 μM) of BECT, 60 min before LPS (100 ng/mL) treatment. Total nuclear and whole cell proteins were electrophoresed followed by immunoblotting using (a) anti-p-IκB-α and (b) NF-κB p65. Densitometric analysis of p-IκB-α and NF-κB p65 is shown in the lower panel. Results are expressed as a ratio of p-IκB-α/β-actin and NF-κB p65/nucleolin. BV-2 microglial cells were seeded at a density of $5 \times 10^4$ cells/well on a 24-well plate. (c) Cells were treated with BECT (20 μM) for 60 min and then stimulated with LPS (100 ng/mL) for 30 min. Sub-cellular location of the p65NF-κB subunit was determined by immunofluorescence assay. Data are mean ± standard error of three independent experiments. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the LPS-treated group. Significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

[27]. Activation of NF-κB includes IκB-α phosphorylation and subsequent nuclear translocation of the NF-κB p65 subunit into the nucleus. Based on these reports, BV-2 microglial cells were treated with 20 μM of BECT for 1 h with or without LPS (100 ng/mL) for 30 min to examine whether BECT regulates the NF-κB pathway, and the levels of the p-IκB-α and NF-κB p65 subunits were investigated. As shown in Fig. 6a, exposing BV-2 cells to LPS significantly induced IκB-α phosphorylation. Treatment of LPS-stimulated BV-2 microglial cells with BECT significantly reduced IκB-α phosphorylation. As shown in Fig. 6b, stimulating BV-2 cells with LPS (100 ng/mL) for 30 min significantly increased nuclear translocation of the NF-κB p65 subunit. Pretreatment of BV-2 cells with BECT (20 μM) significantly inhibited p65NF-κB nuclear translocation in response to LPS; nucleolin was used as the internal control (Fig. 6b). Additionally, BECT treatment did not induce any significant increase in p65NF-κB nuclear translocation. NF-κB levels were also confirmed by immunofluorescence assay, and we confirmed translocation of p65 NF-κB into the nucleus within 30 min of LPS stimulation, which was inhibited by treatment with 20 μM BECT (Fig. 6c).

## Discussion

One of the most popular prototype synthetic anti-inflammatory drugs worldwide is aspirin. Nevertheless, the mechanism behind the therapeutic effects of aspirin and salicylate derivatives are far from being established and remain open for discussion. Initial experiments using aspirin, triflusal and HTB showed that both 4-trifluoromethyl derivatives were more potent than aspirin; thus, indicating that the presence of the acetyl moiety in 4-trifuoromethylbenzoic acid is not a key requirement for inhibiting NF-κB activation [13]. With respect to the above structure to activity profile we added new substitutions to HTB and synthesized novel HTB derivatives that have inhibitory activities against microglial activation and might also attenuate the progression of neuroinflammatory disease. HTB is the main deacetylated metabolite of triflusal with structural analogy to aspirin [13]. The purpose of this study was to address the effect of a novel HTB derivative on inhibiting LPS-induced inflammatory response in BV-2 microglial cells. As NO is a vital proinflammatory mediator and plays an important role in neuroinflammatory diseases, the HTB derivatives were screened for their ability to inhibit NO release in response to LPS using a BV-2 microglia cell-based assay [28]. Among the battery of HTB derivatives screened, few synthetic compounds reduced NO release in LPS-stimulated BV-2 microglial cells. Among these derivatives, BECT was one of the compounds exhibiting significant inhibitory effect on NO release with least cytotoxicity in LPS-stimulated BV-2 microglial cells. The results from our experiment matched a similar finding in which HTB decreased

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the release of NO in response to LPS and IgG/ovalbumin [13]. Further studies on BECT were performed based on its strong inhibitory effect against NO production in microglial cells. Neuroinflammation is a process that results primarily from an abnormally high or chronic activation of microglia. This overactive state results in increased levels of inflammatory and oxidative stress molecules, which can lead to neuronal damage or death. Therefore, inhibiting excess microglial activation might be a therapeutic approach to prevent the progression of neurodegenerative diseases [29].

Activated microglia produce several potentially neurotoxic mediators such as iNOS and COX-2 [30,31]. We found that BECT dose-dependently reduced iNOS and COX-2 mRNA and protein expression levels in LPS-stimulated BV-2 microglial cells. Our results were in agreement with earlier reports showing decrease in COX-2 release by HTB [14,32]. Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are crucial mediators during neuroinflammation [33]. Our mRNA expression profile analysis results indicated a dose dependent decrease in mRNA expression in TNF-α, IL-1β, and IL-6. NF-κB is a key transcription factor implicated in the regulation of proinflammatory cytokines [34] with iNOS [35] and COX-2 [36]. LPS increases nuclear translocation of the NF-κB p65 subunit through IκB-α/β degradation and promotes transcription of target genes [37]. In parallel to these reports, HTB is also reported to suppress the activation of IκB-β, but has no effect on IκB-α [32]. Hence, we further studied the effects of BECT on LPS-induced IκB-α phosphorylation and nuclear translocation of NF-κB by immunoblot and immunofluorescence staining. In our experiments, we observed that BECT decreased IκB-α phosphorylation and nuclear translocation of NF-κB p65 significantly as confirmed by protein levels and the immunofluorescence assay. Our data agree with earlier experiments demonstrating that HTB exhibits its effect by inhibiting NF-κB [32]. It can be concluded from our data that the inhibitory effect of HTB on NF-κB activation and COX-2 expression paralleled each other. Although concentration of BECT required for significant inhibition of cytokines is much higher than the concentration required for inhibition of NFkB, iNOS, or COX-2, our data suggest that BECT suppresses LPS-induced NF-κB activity to regulate iNOS, COX-2, and TNF-α expression.

MAPKs play critical roles integrating and processing cellular responses to a number of diverse extracellular signals that lead to inflammatory responses [26]. JNK and p38 MAPK are the main cause for the release of inflammatory mediators including COX-2 [38] and cytokines [39]. Therefore, we further evaluated the effect of BECT on upstream JNK and p38MAPK signaling pathways. HTB has been reported to have no significant effect on the activity of JNK and p38 MAPK [32]. But, we found that BECT significantly attenuated JNK and p38MAPK phosphorylation in LPS-stimulated BV-2 microglia cells. This discrepancy in activity of HTB and BECT can be addressed on the fact that, BECT is structurally a bit different from HTB in terms that it has two trifluoromethylbenzoic acid moieties. So with different pharmacophores, BECT is expected to adopt different biological profile as compared to HTB. In our study we found BECT to inhibit phosphorylation of JNK and p38 which have already been reported to incite inflammatory response. Our finding that BECT inhibits phosphorylation of IkB-α, JNK and p38 is an added asset since HTB lacks it. Our results suggest that inhibition of JNK, p38MAPK, and NF-κB signaling might be one of the possible molecular mechanisms contributing to the anti-neuroinflammatory effect of BECT in LPS-stimulated microglial cells.

In conclusion, our results demonstrate that BECT, a novel synthetic HTB derivative, elicited potent anti-inflammatory effects on LPS-stimulated microglial cells. HTB has already been reported to have good penetration through the blood brain barrier [40] and the same feature might be followed by BECT. In comparison to the parent compound HTB, BECT was found to have better profile against NO inhibition and also exhibited inhibition of JNK, p38MAPK, and NF-κB signaling as mechanistic pathways involved in modulation of inflammatory mediators. Thus, our results suggest that BECT is a novel HTB derivative which has good anti-neuroinflammatory activity and hence should be further assessed by additional mechanistic and preclinical studies for its use as an anti-neuroinflammatory agent.

## Authors' contributions

Seong-Mook Kang, Ju-Young Park, Byung-Wook Kim, Park Jeong In were carried out the execution of experiments. Preparation of manuscript with figures and referencing was done by Sandeep Vasant More. Planning and interpretation of experimental data were done by Sung-Hwa Yoon and Dong-Kug Choi.

## Conflict of interest

Authors have no conflict of interest.

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