Full Paper

Synthesis and Biological Evaluation of Substituted *N*-[3-(1*H*-Pyrrol-1-yl)methyl]-1,2,5,6-tetrahydropyridin-1-yl]benzamide/benzene Sulfonamides as Anti-Inflammatory Agents

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The pharmacological activities of tetrahydropyridine (THP) derivatives are dependent on the substituent ring moiety. In this study, we investigate the anti-inflammatory activities of 12 newly synthesized substituted *N*-[3-(1*H*-pyrrol-1-yl)methyl]-1,2,5,6-tetrahydrobenzamide/benzene sulfonamides (**9a–l**) in murine BV-2 microglial cells. All compounds were initially screened for attenuation of nitric oxide (NO) production in lipopolysaccharide (LPS) (1 μg/mL)-activated microglial cells. The data show that only SO_2-substituted THPs were effective at sub-lethal concentrations (IC_{50} values of 12.92 μM (**9i**), 14.64 μM (**9j**), 19.63 μM (**9k**)) relative to L-N6-(1-iminoethyl)lysine positive control ($IC_{50} = 3.1$ μM). The most potent SO_2-substituted compound (**9i**) also blocked the LPS-inducible nitric oxide synthase (iNOS) and attenuated the release of several cytokines including IL-1α, IL-10, and IL-6. These findings establish the moderate immuno-modulating effects of SO_2-substituted THP derivatives.

Keywords: Cytokines / Drug design / Microglial cells / Nitric oxide / Tetrahydropyridine

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Introduction

Central nervous system (CNS) diseases such as Alzheimer's disease (AD) [1], Parkinson's disease (PD) [2, 3], acute trauma, stroke [4, 5], amylotrophic lateral sclerosis [6], and hypoxia–ischemia [7, 8] involve chronic inflammation mediated by activated astrocytes and microglial cells [9]. While microglial cells can provide CNS protection against various pathogens, chronic activation can also contribute to sustained release of deleterious pro-inflammatory cytokines and neurotoxic substances such as nitric oxide (NO). Practical use of natural anti-inflammatory products such as omega-3 polyunsaturated fatty acids/polyphenolics or synthetic non-steroidal anti-inflammatory drugs (NSAIDs) is believed to be beneficial in attenuating neurodegenerative decline, if taken over the long term [10, 11].

Tetrahydropyridines (THPs) are known to contain inherent pharmacological properties [12], such as enzyme inhibition (monoamine oxidase) [13] or characteristic antifungal [14]/anti-inflammatory effects [15–17]. In the present study, we explore the efficacy of synthetic THP derivatives to attenuate pro-inflammatory molecules in lipopolysaccharide (LPS)-activated BV-2 murine microglial cells. The activated BV-2 model is frequently used to evaluate the efficacy and potency of immuno-modulating agents against endotoxic production of NO, interleukin-6 (IL-6) [18], tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1β), CCL_2 or NADPH oxidase [19], contributors to CNS neurodegenerative injury [19, 20]. In this study, we evaluate the anti-inflammatory activities of synthesized THP derivatives.

Results

New THP derivatives were synthesized using the methods described [21, 22]. The synthetic routes for the title compounds are outlined in Scheme 1. The O-mesitylenesulfonyl hydroxylamine (MSH) **4** was used to prepare the *N*-amino salt as an aminating agent [23]. Substituted THP derivatives **9a–l** were obtained by a series of reactions beginning with commercially available 3-[(1*H*-pyrrol-1-yl)methyl]pyridine **5**, treated with MSH **4** in dry dichloromethane. This reaction

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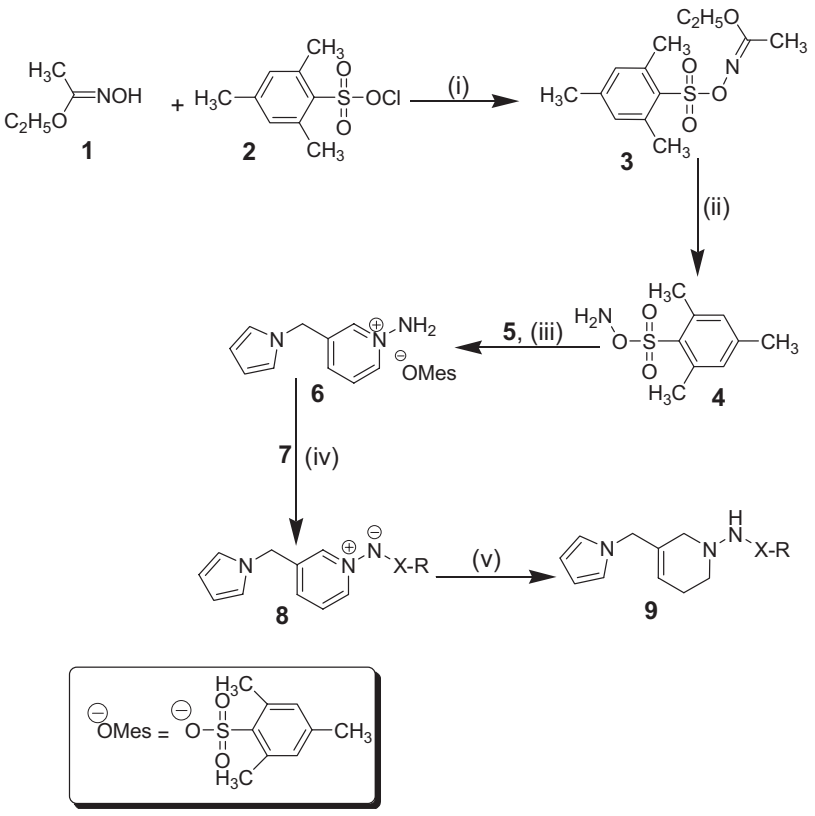


Scheme 1. Reaction conditions: (i) DMF, Et_3N, 0°C, 45 min. (ii) 70% $HClO_4$, *p*-dioxane, 0°C, 45 min. (iii) 3-[(1*H*-Pyrrol-1-yl)methyl]pyridine, CH_2Cl_2, 0°C, 5 h. (iv) 4-Substituted acyl/sulfonyl chloride, dry THF, 70°C. (v) $NaBH_4$, abs EtOH, 7 h. R = C_6H_5, 4-CH_3-C_6H_4, 4-C_2H_5-C_6H_4, 4-*n*-C_4H_9-C_6H_4, 4-OCH_3-C_6H_4, 4-F-C_6H_4, 4-Cl-C_6H_4, 4-Br-C_6H_4, 2-thiophenyl, X = CO, SO_2.

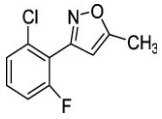
gave *N*-amino-3-[(1*H*-pyrrol-1-yl)methyl]pyridinium mesitylenesulfonate **6** in good yield. Reaction of the *N*-aminopyridinium salt with corresponding substituted acylating agents such as acylchlorides or sulfonyl chlorides, followed by treatment with a base, afforded ylides **8** as stable crystalline solids. Sodium borohydride reduction of **8a–l** in absolute ethanol furnished the title compounds **9a–l** in fair-to-good yields.

All substituted THPs (0.5–100 μM) were screened for dose-dependent inhibition of NO_2^- in LPS (1 μg/mL)-treated BV-2 cells relative to dexamethasone (steroidal anti-inflammatory) and L-NIL (selective nitric oxide synthase (iNOS) inhibitor) controls. There were no effects observed for compounds **9a–h** and **9l**, with only SO_2-substituted THPs capable of mediating anti-inflammatory effects; including **9i** X = SO_2 R = H, **9j** X = SO_2 R = CH_3, and **9k** X = SO_2 R = OCH_3 (Table 1, Fig. 1A–C). The relative potency of the most effective compound (**9i** X = SO_2 R = H) to attenuate NO_2^- was approximately fourfold weaker than L-NIL, and displayed an IC_{50} within a therapeutic range; 12.92 μM. The capability of **9i** to attenuate NO_2^- occurred independent of cell toxicity, which was observed at or above 44.6 μM (Fig. 2A and B) and corresponded to attenuating iNOS protein expression, where a complete repression was observed at 26.8 μM **9i** (Fig. 3). Compound **9i** also attenuated production/release of IL-1α, IL-6, and IL-10 determined by ELISarray profiling, but had no effect on TNFα or G-CSF (Fig. 4). Corroboration by independent enzyme-linked immunosorbent assays (ELISAs) confirmed the capability of **9i** to attenuate IL-α and IL-6 release (Fig. 5A and B), respectively. These finding establish the moderate anti-inflammatory effects of SO_2-substituted THPs.

Discussion

Microglial cells are an important part of the CNS immunological response, also playing a detrimental role in the development of neurodegenerative disorders including PD, AD, and stroke [1–7]. Likewise, the activation of microglial cells can be triggered by disease-specific pathological components such as hypoxia [24], *tau* [25], neuromelanin [26], ammonia [27], heavy metals [11], or misfolded proteins, concomitant to the extent of neurological injury [28]. Chronic microglial activation can lead to a continued release of neurotoxic substances such as pro-inflammatory cytokines and NO, the latter of which can react with superoxide to produce peroxynitrite (ONOO–), a potent neurotoxin and contributor to nitrosative stress evident not only in brain injury [29] but also in the process of aging [30]. For this reason,

Table 1. Evaluation of synthetic THPs on NO_2-inhibition in BV-2 LPS-stimulated microglial cells.

Anti-inflammatory properties of substituted tetrahydropyridines (THPs)			
Compound	**X**	**R**	**LPS-NO_2-IC_{50} (μM)**
Dexamethasone			<1.0
L-N6-(1-iminoethyl)lysine			3.16
9i	SO_2	H	12.92
9j	SO_2	CH_3	14.64
9k	SO_2	OCH_3	19.43
9a	CO	H	>100
9b	CO	CH_3	>100
9c	CO	C_2H_5	>100
9d	CO	OCH_3	>100
9e	CO	C_4H_9	>100
9f	CO	F	>100
9g	CO	Cl	>100
9h	CO	Br	>100
9l	CO		>100

IC_{50} values were calculated based on regression analysis for data obtained from mean ± SEM, $n = 4$ of a minimum of eight concentrations below 100 μM.
No anti-inflammatory effect.

novel therapeutics with a capacity to attenuate microglial activation could serve as potential neuro-protective agents. The data from this study demonstrate that novel synthetic SO_2-substituted THPs were effective in attenuating inflammatory mediators and cytokines in LPS-activated murine BV2 microglia. Future research will be required to determine safety versus efficacy or application in other inflammatory models.

In general, the preliminary evaluation of synthetic anti-inflammatory drugs at early stages requires *in vitro* screenings such as that described in this work, which aim to elucidate structure versus function with respect to attenuation of immuno-competent parameters including iNOS, TNF-α, IL-6, IL-1β, NO_2^-, and MCP-1 under various inflammatory stimuli (e.g., amyloid-beta [31, 32], oxygen–glucose deprivation [33], or LPS). [34, 35] These indicators are commonly used and represent pathological manifestation of human CNS neurodegenerative diseases [36–38]. There is a plethora of natural plant-derived [39–42] and synthetic drugs described in the literature with a capacity to downregulate microglial activation [10]. More specifically, the ability of a candidate compound to attenuate IL-6, NO, and iNOS in glial cells often equates to therapeutic efficacy in diverse *in vivo* models [43–45] and involves downregulation of nuclear factor-kappaB translocation/activation, degradation of IkappaB-alpha,

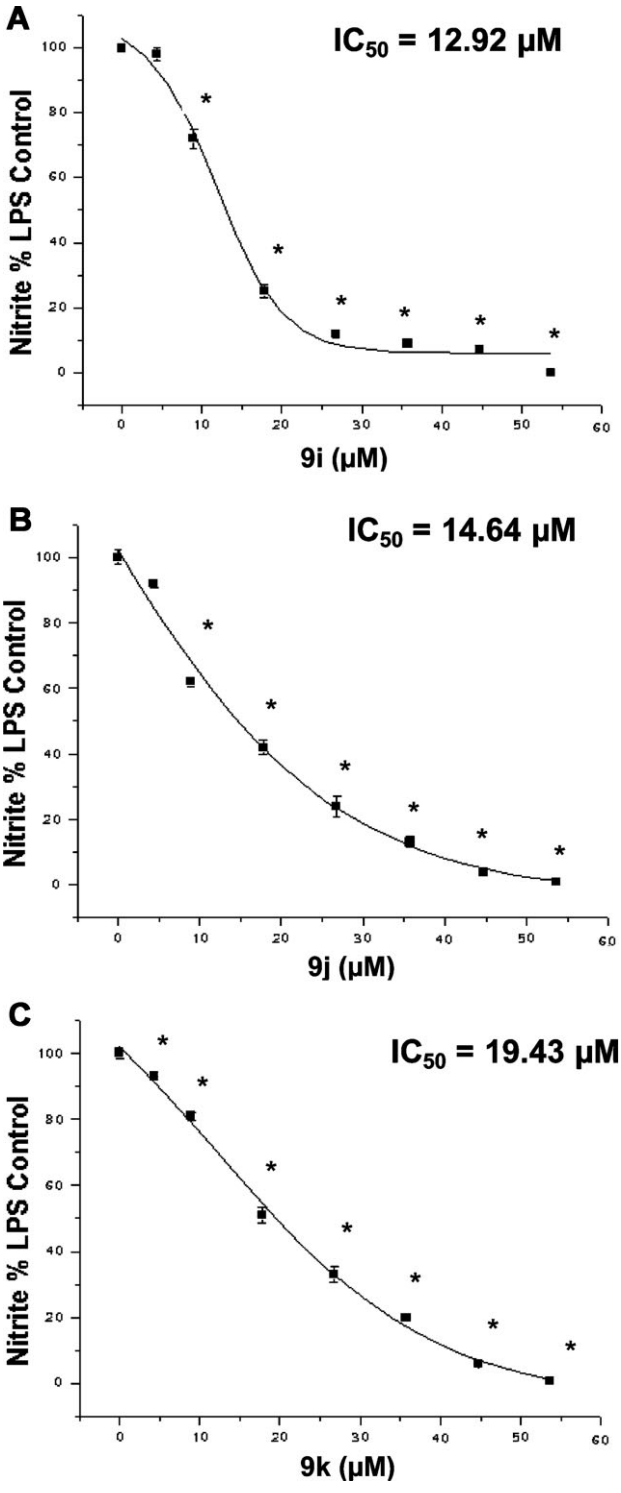


Figure 1. (A–C) The effect of SO_2-substituted tetrahydropyridines (THPs) on NO_2^- production by LPS (1 μg/mL)-activated BV-2 microglial cells at 24 h. The data represent NO_2^- as % control and are presented as the mean ± SEM, $n = 4$. Statistical differences from the control were evaluated by a one-way ANOVA, followed by a Tukey post hoc test, $^*p < 0.05$.

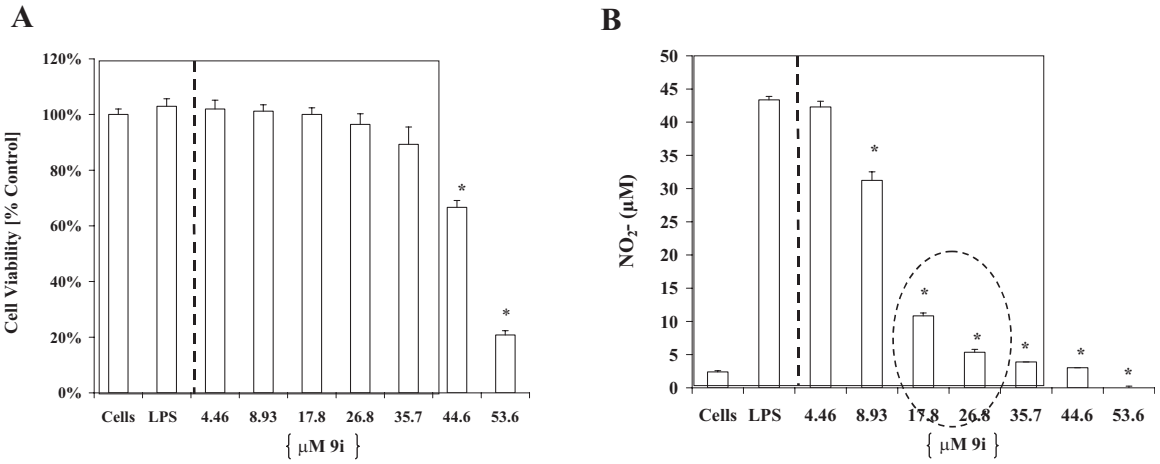


Figure 2. (A) Cell viability control for compound **9i** in LPS (1 μg/mL)-activated BV-2 microglial cells at 24 h. The data represent viability % control and are presented as the mean ± SEM, $n = 4$. Statistical differences from the LPS control were evaluated by a one-way ANOVA, followed by a Tukey post hoc test, $^{*}p < 0.05$. (B) Anti-inflammatory control for compound **9i** in LPS (1 μg/mL)-activated BV-2 microglial cells at 24 h. The data represent NO_2^- (μM) and are presented as the mean ± SEM, $n = 4$. Statistical differences from the LPS control were evaluated by a one-way ANOVA, followed by a Tukey post hoc test, $^{*}p < 0.05$.

MAPK [33, 35, 46, 47], C/EBPdelta [48], or JAK2-mediated STAT1 phosphorylation [34, 49], which indirectly controls the inflammatory process.

In this work, we report that SO_2-substituted synthetic THPs exert moderate anti-inflammatory effects, evidenced by inhibition of NO_2^-, iNOS protein expression, and various cytokines. Since the potency of these compounds is within the therapeutic range, research will be required to design THPs with greater strength, suitable, and safe for long-term use.

Experimental

Methods and materials

Hanks balanced salt solution, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) (HEPES), ethanol, dimethyl sulfoxide (DMSO), 96-well plates, general reagents and supplies were all purchased from Sigma–Aldrich Co. (St. Louis, MO) and VWR International (Radnor, PA). Imaging probes were supplied by Life Technologies (Grand Island, NY). All chemicals and solvents were purchased from Sigma–Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). The structures of the products described were confirmed by IR, ^{1}H NMR, and elemental analysis data. ^{1}H NMR spectra were recorded on Varian Gemini HX 300 MHz spectrometer. All chemical shifts expressed in parts per million (δ, ppm) are reported relative to tetramethylsilane (TMS) as internal standard for solution in $CDCl_3$ as a solvent unless otherwise specified. The IR spectra were run with KBr pellets on Perkin-Elmer FTIR 1430 spectrometer and are reported in cm^{-1}. Melting points were determined on a Mel-Temp 3.0 melting point apparatus and are uncorrected. Elemental analyses were performed with a Perkin Elmer 2400 CHN elemental analyzer, by Atlantic Microlab, Inc. (Norcross, GA). C, H, and N values are within ±0.4% of theoretical values unless otherwise noted. High-resolution mass spectra analysis was performed with Micromass AutoSpec M apparatus. The instrument was run at a mass resolution of 10,000 for the accurate mass analysis, using perfluorokerosene (PFK) as an internal calibration standard. Separations by column chromatography were performed on silica gel (200–425 mesh). All reactions and purification procedures were monitored by TLC on Whatmann AL SIL g/UV, 250 μm layer flexible plates, with visualization under UV light.

General procedure A

*Synthesis of N-amino-3-[(1H-pyrrol-1-yl)methyl]pyridinium mesitylenesulfonate (**6**)*

To an ice-cooled solution of 3-[(1*H*-pyrrol-1-yl)methyl]pyridine (**4**, 2.94 g, 18.58 mmol) in 25 mL of dry methylene chloride was added dropwise to O-mesitylenesulfonylhydroxylamine (**5**, 4.0 g, 18.58 mmol) in 10 mL of dry methylene chloride over 5 min with stirring. The resulting mixture was stirred at 0°C for 3 h, and at room temperature for 1 h; excess ether was added and the suspension was filtered. The precipitate was recrystallized from ethyl acetate–methanol (5:1 v/v) to give *N*-amino-3-[(1*H*-pyrrol-1-yl)-methyl]pyridinium mesitylenesulfonate (**6**) in 57.5% yield. ^{1}H NMR ($CDCl_3$): δ 5.20 (s, 2H, $-CH_2$), 6.26 (t, 2H, $J = 5.7$ Hz, 3′H, 4′H), 6.70 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.39–7.44 (m, 4H, 3″H, 4″H, 5″H, 5H), 7.61–7.564 (dd, 1H, $J = 6.0$, 1.8 Hz, 4H), 8.152–8.12 (m, 2H, 2″H, 6″H), 8.68 (s, 1H, 2H), 8.72 (d, 1H, $J = 6.0$ Hz, 6H).

General procedure B

*Synthesis of 1-(benzoylimino)-3-(1H-pyrrol-1-ylmethyl)-pyridinium ylide (**8a**)*

The ice-cold solution of *N*-aminopyridinium salt (**6**, 2.0 g, 5.36 mmol) in 30 mL of anhydrous tetrahydrofuran (THF) containing triethylamine was added to benzoyl chloride (**7**, 1.87 mL, 16.08 mmol) dropwise. The reaction was allowed to reflux for 12 h at 70°C. After cooling to room temperature, the reaction was quenched by adding 70 mL of saturated sodium bicarbonate ($NaHCO_3$) solution. The mixture was shaken

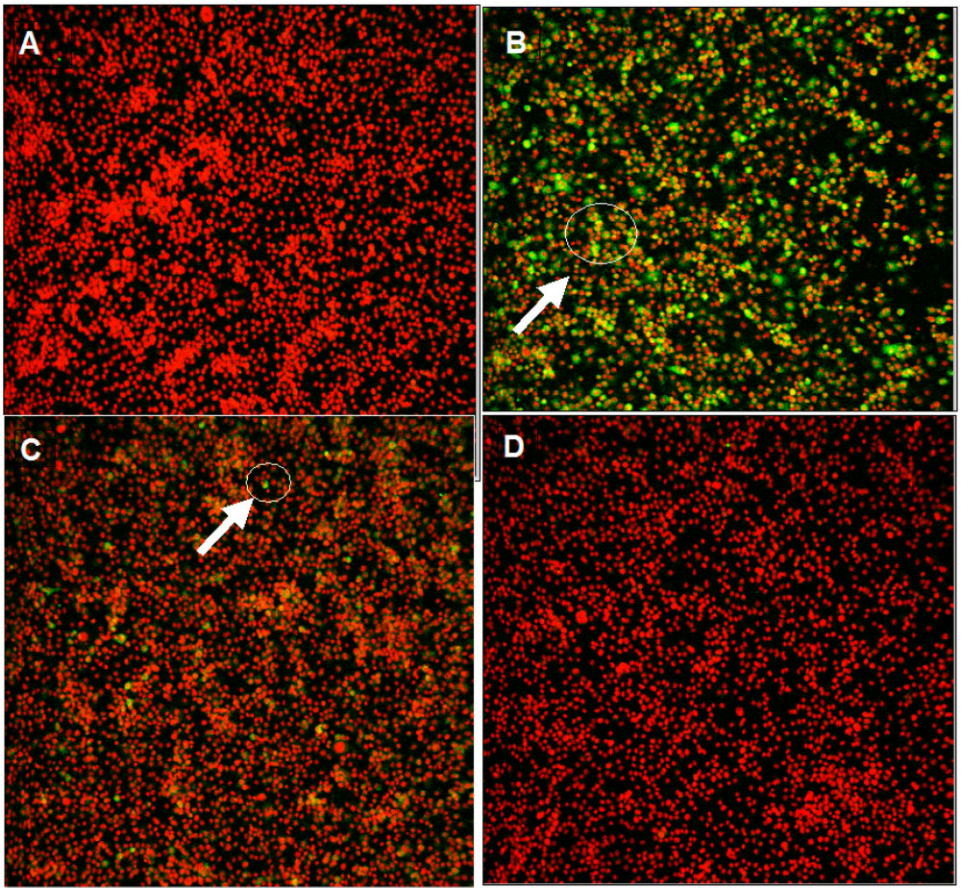


Figure 3. Complete inhibition by compound **9i** (26 μM) of iNOS protein expression in LPS (1 μg/mL)-activated BV-2 microglial cells at 24 h. Cells were fixed, permeabilized, and stained with Alexa Fluor® 488/anti-iNOS, N-terminal antibody produced in rabbit and counterstained with PI. A = untreated cells, B = LPS, C = LPS + 17.8 μM **9i**, and D = LPS + 26.8 μM **9i**.

repeatedly in a 500 mL separatory funnel and allowed to stand for a few minutes. The solution was extracted with chloroform (2 × 100 mL) and dried over anhydrous sodium sulfate, filtered, and the solvent was removed *in vacuo* giving the crude product, which was purified by column chromatography (2.5 cm × 22 cm) on silica gel (200–425 mesh) using ethyl acetate/methanol (9:1 v/v) as eluent. The resultant product **8a** was obtained as a light yellow crystalline solid in 43.9% yield, m.p. 150–151°C. The other derivatives were similarly prepared and purified. ^{1}H NMR ($CDCl_3$): δ 5.20 (s, 2H, $-CH_2$), 6.26 (t, 2H, J = 5.7 Hz, 3′H, C4′H), 6.70 (t, 2H, J = 2.1 Hz, 2′H, 5′H), 7.39–7.44 (m, 4H, 3″H, 4″H, 5″H, 5H), 7.61–7.564 (dd, 1H, J = 6.0, 1.8 Hz, 4H), 8.152–8.12 (m, 2H, 2″H, 6″H), 8.68 (s, 1H, 2H), 8.72 (d, 1H, J = 6.0 Hz, 6H). IR (KBr): (ν) 1593 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{15}N_3O \cdot 0.28H_2O$: C, 72.60; H, 5.13; N, 14.51. Found: C, 72.31; H, 5.35; N, 14.88.

*Synthesis of 1-[(4-methylbenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8b**)*

The compound **8b** was obtained following General Procedure B as off-white solid, yield 50.5%, m.p. 188–190°C; ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, $-CH_3$), 5.22 (s, 2H, $-CH_2$), 6.25 (t, 2H, J = 1.8 Hz, 3′H, 4′H), 6.69 (t, 2H, J = 1.8 Hz, 2′H, 5′H), 7.21 (d, 2H, J = 8.1 Hz, 3″H, 5″H), 7.47 (d, 1H, J = 7.8 Hz, 5H), 7.64 (t, 1H, J = 6.6 Hz, 4H), 8.04 (d, 2H, J = 7.8 Hz, 2″H, 6″H), 8.69 (s, 1H, 2H), 8.73 (d, 1H, J = 6.6 Hz, 6H). IR (KBr): (ν) 1590 (C=O) cm^{-1}. Anal. calcd. for $C_{18}H_{17}N_3O \cdot 0.05H_2O$: C, 73.76; H, 5.93; N, 14.08. Found: C, 73.98; H, 5.86; N, 14.08.

*Synthesis of 1-[(4-ethylbenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8c**)*

The compound **8c** was obtained following General Procedure B as white solid, yield 52.5%, m.p. 212–213°C; ^{1}H NMR ($CDCl_3$): δ 1.25 (t, 3H, J = 7.5 Hz, $-CH_2-CH_3$), 2.69 (q, 2H, J = 7.8, 7.5 Hz, $-CH_2-CH_3$), 5.19 (s, 2H, $-CH_2$), 6.25 (t, 2H, J = 2.1 Hz, 3′H, 4′H), 6.69 (t, 2H, J = 2.1 Hz, 2′H, 5′H), 7.22–7.25 (dd, 2H, J = 6.6, 0.6 Hz, 3″H, 5″H), 7.39–7.42 (dd, 1H, J = 5.4, 0.6 Hz, 5H), 7.54–7.59 (dd, 1H, J = 6.6, 1.5 Hz, 4H), 8.04–8.06 (dd, 2H, J = 4.2, 2.1 Hz, 2″H, 6″H), 8.68 (s, 1H, 2H), 8.72 (d, 1H, J = 5.4 Hz, 6H). IR (KBr): (ν) 1591 (C=O) cm^{-1}. Anal. calcd. for $C_{19}H_{19}N_3O \cdot 0.15H_2O$: C, 74.10; H, 5.98; N, 13.55. Found: C, 74.07; H, 6.22; N, 13.64.

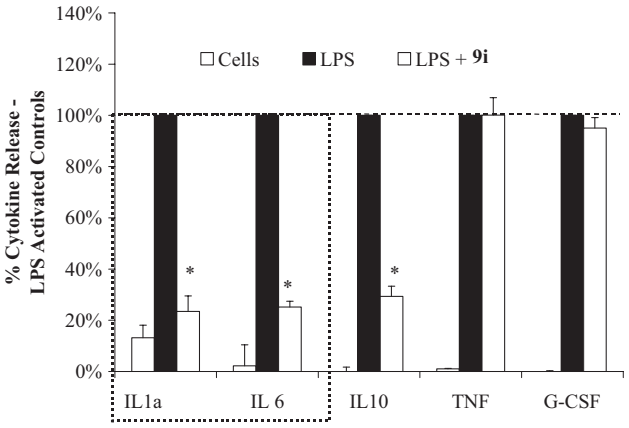


Figure 4. Effect of SO_2-substituted tetrahydropyridine **9i** (26 μM) on cytokine release in LPS (1 μg/mL)-stimulated BV-2 microglial cells at 24 h by multi-analyte ELISArray: MEM-004A – evaluated for: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17α, IFNγ, TNFα, G-CSF, and GM-CSF. The data represent cytokines released as % LPS controls for each subset, and are presented as the mean ± STD, $n = 2$. Statistical differences from the LPS control were evaluated by a Student's *t*-test, $^{*}p < 0.05$.

*Synthesis of 1-[(4-methoxybenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8d**)*

The compound **8d** was obtained following General Procedure B as light yellow solid, yield 48.5%, m.p. 175–177°C; ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 3H, $-OCH_3$), 5.23 (s, 2H, $-CH_2$), 6.26 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.70 (t, 2H, $J = 1.8$ Hz, 2′H, 5′H), 6.93 (d, 2H, $J = 8.4$ Hz, 3″H, 5″H), 7.49 (d, 1H, $J = 8.1$ Hz, 5H), 7.65 (t, 1H, $J = 6.3$ Hz, 4H), 8.13 (d, 2H, $J = 8.7$ Hz, 2″H, 6″H), 8.70 (s, 1H, 2H), 8.75 (d, 1H, $J = 6.0$ Hz, 6H). IR (KBr): (ν) 1605 (C=O) cm^{-1}. Anal. calcd. for $C_{18}H_{17}N_3O_2 \cdot 0.45H_2O$: C, 68.87; H, 5.44; N, 13.28. Found: C, 68.53; H, 5.43; N, 13.32.

*Synthesis of 1-[(4-butylbenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8e**)*

The compound **8e** was obtained following General Procedure B as off-white color solid, yield 57.3%, m.p. 197–198°C; ^{1}H NMR ($CDCl_3$): δ 0.92 (t, 3H, $J = 7.2$ Hz, $-CH_2-CH_2-CH_2-CH_3$), 1.36 (q, 2H, $J = 7.8$ Hz, $-CH_2-CH_2-CH_2-CH_3$), 1.62 (q, 2H, $J = 5.1$ Hz, $-CH_2-CH_2-CH_2-CH_3$), 2.65 (t, 2H, $J = 7.8$, Hz, $-CH_2-CH_2-CH_2-CH_3$), 5.19 (s, 2H, $-CH_2$), 6.25 (t, 2H, $J = 2.1$ Hz, 3′H, 4′H), 6.69 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.21 (d, 2H, $J = 8.1$ Hz, 3″H, 5″H), 7.41 (d, 1H, $J = 7.8$ Hz, 5H) 7.54–7.59 (dd, 1H, $J = 6.6$, 1.5 Hz, 4H), 8.02–8.05 (dd, 2H, $J = 4.8$, 1.8 Hz, 2″H, 6″H), 8.68 (s, 1H, 2H), 8.71 (d, 1H, $J = 6.3$ Hz, 6H). IR (KBr): (ν) 1592 (C=O) cm^{-1}. Anal. calcd. for $C_{21}H_{23}N_3O$: C, 75.65; H, 6.95; N, 12.60. Found: C, 75.56; H, 6.90; N, 12.40.

*Synthesis of 1-[(4-fluorobenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8f**)*

The compound **8f** was obtained following General Procedure B as light yellow color solid, yield 46.8%, m.p. 197–198°C; δ 5.21 (s, 2H, $-CH_2$), 6.26 (s, 2H, 3′H, 4′H), 6.70 (s, 2H, 2′H, 5′H), 7.07 (t, 2H, $J = 8.4$ Hz, 3″H, 5″H), 7.45 (d, 1H, $J = 8.1$ Hz, 5H), 7.61 (t, 1H, $J = 7.8$ Hz, 4H), 8.14 (t, 2H, $J = 8.1$ Hz, 2″H, 6″H), 8.66 (s, 1H, 2H), 8.71 (d, 1H, $J = 5.7$ Hz, 6H). IR (KBr): (ν) 1604 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{14}FN_3O$: C, 69.14; H, 4.78; N, 14.23. Found: C, 68.95; H, 4.48; N, 13.88.

*Synthesis of 1-[(4-chlorobenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8g**)*

The compound **8g** was obtained following General Procedure B as light yellow color solid, yield 51.5%, m.p. 197–198°C; δ 5.21 (s, 2H, $-CH_2$), 6.26 (t, 2H, $J = 3.0$ Hz, 3′H, 4′H), 6.70 (s, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.37 (d, 2H, $J = 8.7$ Hz, 3″H, 5″H), 7.45 (d, 1H, $J = 7.5$ Hz, 5H), 7.61 (t, 1H, $J = 6.3$ Hz, 4H), 8.07 (d, 2H, $J = 7.8$ Hz, 2″H, 6″H), 8.66 (s, 1H, 2H), 8.71 (d, 1H, $J = 6.0$ Hz, 6H). IR (KBr): (ν) 1603 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{14}ClN_3O$: C, 65.49; H, 4.53; N, 13.48. Found: C, 65.12; H, 4.25; N, 13.19.

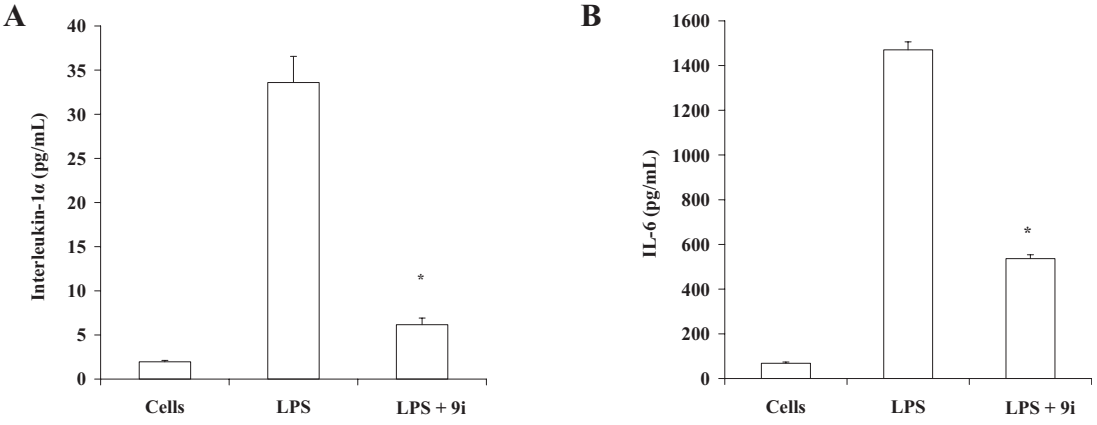


Figure 5. (A) Effect of SO_2-substituted tetrahydropyridine compound **9i** (26 μM) on interleukin-1α release in LPS (1 μg/mL)-stimulated BV-2 microglial cells at 24 h. The data represent interleukin-1a released as (pg/mL) and presented as the mean ± SEM, $n = 6$. Statistical differences from the LPS control were evaluated by a Student's *t*-test, $^{*}p < 0.001$. (B) Effect of SO_2-substituted tetrahydropyridine compound **9i** (26 μM) on interleukin-6 release in LPS (1 μg/mL)-stimulated BV-2 microglial cells at 24 h. The data represent interleukin-6 released as (pg/mL) and presented as the mean ± SEM, $n = 6$. Statistical differences from the LPS control were evaluated by a Student's *t*-test, $^{*}p < 0.001$.

*Synthesis of 1-[(4-bromobenzoyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8h**)*

The compound **8h** was obtained following General Procedure B as brown color solid, yield 56.9%, m.p. 197–198°C; δ 5.23 (s, 2H, $-CH_2$), 6.27 (s, 2H, 3′H, 4′H), 6.70 (s, 2H, 2′H, 5H), 7.47 (d, 2H, $J = 8.7$ Hz, 3″H, C5″-H), 7.54 (d, 1H, $J = 7.8$ Hz, 5H), 7.64 (t, 1H, $J = 8.7$ Hz, 4H), 8.02 (d, 2H, $J = 8.1$ Hz, 2″H, 6″H), 8.67 (s, 1H, 2H), 8.74 (d, 1H, $J = 6.6$ Hz, 6H). IR (KBr): (ν) 1601 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{14}BrN_3O$: C, 57.32; H, 3.96; N, 11.80. Found: C, 56.95; H, 3.91; N, 11.62.

*Synthesis of 1-[(phenylsulfonyl)imino]-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8i**)*

The compound **8i** was obtained following General Procedure B as light yellow crystalline solid in 43.9% yield, m.p. 140–142°C; ^{1}H NMR ($CDCl_3$): δ 5.20 (s, 2H, $-CH_2$), 6.26 (t, 2H, $J = 5.7$ Hz, 3′H, 4′H), 6.70 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.34–7.44 (m, 4H, 3″H, 4″H, 5″H, 5H), 7.49–7.50 (dd, 1H, $J = 1.8$, 1.2 Hz, 4H), 7.66–7.69 (m, 2H, 2″H, 6″H), 8.16 (s, 1H, 2H), 8.34 (d, 1H, $J = 3.0$ Hz, 6H). IR (KBr): (ν) 1332, 1205 (SO_2) cm^{-1}. Anal. calcd. for $C_{16}H_{15}N_3O_2S$: C, 61.32; H, 4.82; N, 13.41. Found: C, 61.21; H, 4.74; N, 13.16.

*Synthesis of 1-[[(4-methylphenyl)sulfonyl]imino)-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8j**)*

The compound **8j** was obtained following General Procedure B as off-white solid in 63.7% yield, m.p. 168–169°C; ^{1}H NMR ($CDCl_3$): δ 2.38 (S, 3H, $-CH_3$), 5.11 (s, 2H, $-CH_2$), 6.22 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.56 (t, 2H, $J = 1.8$ Hz, 2′H, 5′H), 7.15–7.18 (dd, 2H, $J = 8.1$, 0.6Hz, 3″H, 5″H), 7.46–7.48 (dd, 2H, $J = 5.2$, 1.2 Hz, 2″H, 6″H), 7.57–7.60 (dd, 2H, $J = 4.8$, 1.5 Hz, 5H, 4H), 8.16 (s, 1H, 2H), 8.34 (d, 1H, $J = 2.7$ Hz, 6H). IR (KBr): (ν) 1286, 1134 (SO_2) cm^{-1}. Anal. calcd. for $C_{17}H_{17}N_3O_2S$: C, 62.36; H, 5.23; N, 12.83. Found: C, 62.12; H, 5.13; N, 12.66.

*Synthesis of 1-[[(4-methoxyphenyl)sulfonyl]imino)-3-(1H-pyrrol-1-ylmethyl)pyridinium ylide (**8k**)*

The compound **8k** was obtained following General Procedure B as off-white solid in 60.0% yield, m.p. 153–154°C; ^{1}H NMR ($CDCl_3$): δ 3.81 (s, 3H, $-OCH_3$), 5.15 (s, 2H, $-CH_2$), 6.22 (t, 2H, $J = 2.4$ Hz, 3′H, 4′H), 6.56 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.83–6.86 (dd, 2H, $J = 4.5$, 2.1 Hz, 3″H, 5″H), 7.46–7.48 (dd, 2H, $J = 3.9$, 0.6 Hz, 2″H, 6″H), 7.62–7.65 (dd, 2H, $J = 4.5$, 2.1 Hz, 5H, 4H), 8.18 (s, 1H, 2H), 8.35 (d, 1H, $J = 3.0$ Hz, 6H). IR (KBr): (ν) 1280, 1137 (SO_2) cm^{-1}. Anal. calcd. for $C_{17}H_{17}N_3O_3S \cdot 0.05H_2O$: C, 59.34; H, 4.86; N, 11.97. Found: C, 59.30; H, 4.98; N, 12.20.

*Synthesis of 1-(([3-(2-chloro-6-fluorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl)imino)-3-(1H-pyrrol-1-ylmethyl) pyridinium ylide (**8l**)*

The compound **8l** was obtained following General Procedure B as off white color solid, yield 46.3%, m.p. 149–150°C; ^{1}H NMR ($CDCl_3$): δ 2.81 (s, 3H, $-CH_3$), 5.15 (s, 2H, $-CH_2$), 6.26 (t, 2H, $J = 2.1$ Hz, 3′H, 4′H), 6.66 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.03–7.09 (ddd, 1H, $J = 8.1$, 1.2, 1.5, 0.9 Hz, 3″H), 7.23–7.35 (m, 3H, 5H,4′′H, 5″H), 7.56 (t, 1H, $J = 6.6$ Hz, 4H), 8.37 (s, 1H, 2H), 8.47 (d, 1H, $J = 6.0$ Hz, 6H). IR (KBr): (ν) 1608 (C=O) cm^{-1}. Anal. calcd. for $C_{21}H_{16}ClFN_4O_2$: C, 61.39; H, 3.93; N, 13.64. Found: C, 61.08; H, 3.94; N, 13.28.

General procedure C

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]benzamide (**9a**)*

A solution of 1-(benzoylimino)-3-(1*H*-pyrrol-1-ylmethyl)pyridinium ylide (**8a** 0.80 g, 2.55 mmol) in dichloromethane/ethanol (1:1 v/v, 40 mL) was added dropwise to a stirred suspension of sodium borohydride (0.38 g, 10.21 mmol) in 20 mL of absolute ethanol over a period or 30 min. The resulting solution was stirred for 7 h at 0°C. The excess sodium borohydride was treated with 50 mL of distilled water and the reaction mixture was allowed to warm up to room temperature. It was then extracted with dichloromethane (200 mL) and dried over anhydrous sodium sulfate. The dichloromethane filtrate was evaporated *in vacuo* and the product chromatographed on a column of silica gel using ethyl acetate/hexane (3:2 v/v) as an eluent. The solid obtained was further crystallized from dichlormethane/hexane (3:2 v/v) and furnished **9a** as a white solid, 48.9% yield, m.p. 149–150°C; ^{1}H NMR ($CDCl_3$): δ 2.42 (brs, 2H, 5H), 3.28 (t, 2H, $J = 5.1$ Hz, 6H), 3.51 (s, 2H, 2H), 4.41 (s, 2H, $-CH_2$), 5.68 (m, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.61 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.93 (brs, –NH, D_2O exchangeable), 7.38–7.52 (m, 3H, 3″H, 4″H, 5″H), 7.73 (d, 2H, $J = 7.2$ Hz, 2″H, 6″H). IR (KBr): (ν) 3213 (NH), 1638 (C=O) cm^{-1}; HRMS (EI) m/z [M^+] calcd. for $C_{17}H_{19}N_3O$, 281.1528; found 281.1525. Anal. calcd. for $C_{17}H_{19}N_3O \cdot 0.05H_2O$: C, 72.13; H, 6.51; N, 14.85. Found: C, 72.34; H, 6.79; N, 14.89.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-methylbenzamide (**9b**)*

The compound **9b** was obtained following General Procedure C as white color solid, yield 54.5%, m.p. 145–147°C; ^{1}H NMR ($CDCl_3$): δ 2.38 (s, 3H, $-CH_3$), 2.35 (brs, 2H, 5H), 3.08 (t, 2H, $J = 5.1$ Hz, 6H), 3.38 (s, 2H, 2H), 4.39 (s, 2H, $-CH_2$), 5.64 (m, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.62 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.93 (brs, –NH, D_2O exchangeable), 7.23 (d, 2H, $J = 8.1$ Hz, 3″H, 5″H), 7.61 (d, 2H, $J = 8.1$ Hz, 2″H, 6″H). IR (KBr): (ν) 3202 (NH), 1634 (C=O) cm^{-1}. Anal. calcd. for $C_{18}H_{21}N_3O$: C, 73.19; H, 7.17; N, 14.23. Found: C, 73.12; H, 7.40; N, 14.09.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-ethylbenzamide (**9c**)*

The compound **9c** was obtained following General Procedure C as off-white color solid, yield 61.8%, m.p. 143–145°C; ^{1}H NMR ($CDCl_3$): δ 1.23 (t, 3H, $J = 7.8$ Hz, $-CH_2-\mathit{CH_3}$), 2.35 (brs, 2H, 5H), 2.68 (q, 2H, $J = 7.5$ Hz, $-\mathit{CH_2}-CH_3$), 3.08 (t, 2H, $J = 5.1$ Hz, 6H), 3.38 (s, 2H, 2H), 4.39 (s, 2H, $-CH_2$), 5.64 (m, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.62 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.93 (brs, –NH, D_2O exchangeable), 7.23 (d, 2H, $J = 8.1$ Hz, 3″H, 5″H), 7.61 (d, 2H, $J = 8.1$ Hz, 2″H, 6″H). IR (KBr): (ν) 3183 (NH), 1625 (C=O) cm^{-1}. Anal. calcd. for $C_{19}H_{23}N_3O$: C, 73.76; H, 7.49; N, 13.58. Found: C, 73.61; H, 7.52; N, 13.36.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-methoxybenzamide (**9d**)*

The compound **9d** was obtained following General Procedure C as off-white color solid, yield 57.6%, m.p. 151–152°C; ^{1}H NMR ($CDCl_3$): δ 3.81 (s, 3H, $-OCH_3$), 2.36 (brs, 2H, 5H), 3.06 (t, 2H, $J = 5.1$ Hz, 6H), 3.34 (s, 2H, 2H), 4.38 (s, 2H, $-CH_2$), 5.62 (m, 1H, 4H, olefinic), 6.15 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.63 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.91 (brs, –NH, D_2O exchangeable), 7.24 (d, 2H, $J = 8.1$ Hz,

3″H, 5″H), 7.60 (d, 2H, $J = 8.1$ Hz, 2″H, 6″H). IR (KBr): (ν) 3193 (NH), 1615 (C=O) cm^{-1}. Anal. calcd. for $C_{18}H_{21}N_3O_2$: C, 69.43; H, 6.80; N, 13.49. Found: C, 69.28; H, 7.01; N, 13.31.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-butylbenzamide (**9e**)*

The compound **9e** was obtained following General Procedure C as white color solid, yield 56.3%, m.p. 148–149°C; ^{1}H NMR ($CDCl_3$): δ 0.92 (t, 3H, $J = 7.2$ Hz, $-CH_2-CH_2-CH_2-CH3$), 1.36 (q, 2H, $J = 7.5$ Hz, $-CH_2-CH_2-CH_2-CH_3$), 1.60 (q, 2H, $J = 5.1$ Hz, $-CH_2-CH_2-CH_2-CH_3$), 2.35 (brs, 2H, 5H), 2.63 (t, 2H, $J = 8.1$ Hz, $-CH_2-CH_2-CH_2-CH_3$), 3.08 (t, 2H, $J = 5.4$ Hz, 6H), 3.32 (s, 2H, 2H), 4.39 (s, 2H, $-CH_2$), 5.64 (m, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.62 (t, 2H, $J = 2.1$ Hz, 2′H, 5H), 6.92 (brs, –NH, D_2O exchangeable), 7.23 (d, 2H, $J = 8.4$ Hz, 3″H, 5″H), 7.60 (d, 2H, $J = 8.1$ Hz, 2″H, 6″H). IR (KBr): (ν) 3192 (NH), 1635 (C=O) cm^{-1}. Anal. calcd. for $C_{21}H_{27}N_3O$: C, 74.74; H, 8.06; N, 12.45. Found: C, 74.70; H, 8.07; N, 12.26.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-fluorobenzamide (**9f**)*

The compound **9f** was obtained following General Procedure C as off-white color solid, yield 48.6%, m.p. 152–153°C; ^{1}H NMR ($CDCl_3$): δ 2.36 (brs, 2H, 5H), 3.08 (t, 2H, $J = 5.1$ Hz, 6H), 3.31 (s, 2H, 2H), 4.39 (s, 2H, $-CH_2$), 5.67 (m, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.61 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.91 (brs, –NH, D_2O exchangeable), 7.14 (t, 2H, $J = 8.4$ Hz, 3″H, 5″H), 7.87 (dd, 2H, $J = 2.1$, 8.1 Hz, 2″H, 6″H). IR (KBr): (ν) 3190 (NH), 1636 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{18}FN_3O$: C, 68.21; H, 6.06; N, 14.04. Found: C, 67.96; H, 6.01; N, 13.91.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-chlorobenzamide (**9g**)*

The compound **9g** was obtained following General Procedure C as off-white color crystalline solid, yield 52.8%, m.p. 147–149°C; ^{1}H NMR ($CDCl_3$): δ 2.54 (brs, 2H, 5H), 3.59 (t, 2H, $J = 5.1$ Hz, 6H), 3.83 (s, 2H, 2H), 4.43 (s, 2H, $-CH_2$), 5.71 (s, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 2.1$ Hz, 3′H, 4′H), 6.60 (t, 2H, $J = 1.8$ Hz, 2′H, 5′H), 6.95 (brs, –NH, D_2O exchangeable), 7.36 (t, 2H, $J = 6.9$ Hz, 3″H, 5″H), 7.75 (d, 2H, $J = 8.4$ Hz, 2″H, 6″H). IR (KBr): (ν) 3187 (NH), 1634 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{18}ClN_3O$: C, 64.66; H, 5.75; N, 13.31. Found: C, 64.51; H, 5.69; N, 13.21.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-bromobenzamide (**9h**)*

The compound **9h** was obtained following General Procedure C as light yellow color solid, yield 60.8%, m.p. 154–155°C; ^{1}H NMR ($CDCl_3$): δ 2.70 (brs, 2H, 5H), 4.01 (t, 2H, $J = 5.1$ Hz, 6H), 4.23 (s, 2H, 2H), 4.49 (s, 2H, $-CH_2$), 5.76 (m, 1H, 4H, olefinic), 6.15 (t, 2H, $J = 1.8$ Hz, 3′H, 4′H), 6.62 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.91 (brs, –NH, D_2O exchangeable), 7.54 (d, 2H, $J = 8.7$ Hz, 3″H, 5″H), 7.75 (d, 2H, $J = 8.4$ Hz, 2″H, 6″H). IR (KBr): (ν) 3185 (NH), 1635 (C=O) cm^{-1}. Anal. calcd. for $C_{17}H_{18}BrN_3O$: C, 56.68; H, 5.04; N, 11.66. Found: C, 56.50; H, 4.95; N, 11.53.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]benzenesulfonamide (**9i**)*

The compound **9i** was obtained following General Procedure C as light yellow color solid, yield 54.2%, m.p. 125–127°C; ^{1}H NMR ($CDCl_3$): δ 2.13–2.14 (m, 2H, 5H), 2.72 (t, 2H, $J = 6.0$ Hz, 6H), 2.91 (s, 2H, 2H), 4.24 (s, 2H, $-CH_2$), 5.45 (brs, –NH, D_2O exchangeable), 5.57 (m, 1H, 4H, olefinic), 6.12 (t, 2H, $J = 2.1$ Hz, 3′H, 4′H), 6.50 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.44–7.49 (m, 3H, 3″H, 4″H,5″H), 7.84 (d, 2H, $J = 8.1$ Hz, 2″H, 6″H). IR (KBr): (ν) 3069 (NH), and 1332, 1164 (SO_2) cm^{-1}. Anal. calcd. for $C_{16}H_{19}N_3O_2S$: C, 60.54; H, 6.03; N, 13.24. Found: C, 60.32; H, 5.89; N, 12.98.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-methylbenzenesulfonamide (**9j**)*

The compound **9j** was obtained following General Procedure C as white color flakes, yield 58.5%, m.p. 122–124°C; ^{1}H NMR ($CDCl_3$): δ 2.14–2.15 (m, 2H, 5H), 2.73 (t, 2H, $J = 6.0$ Hz, 6H), 2.91 (s, 2H, 2H), 3.87 (s, 3H, $-OCH_3$), 4.25 (s, 2H, $-CH_2$), 5.40 (brs, –NH, D_2O exchangeable), 5.57 (m, 1H, 4H, olefinic), 6.12 (t, 2H, $J = 2.4$ Hz, 3′H, 4′H), 6.50 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 6.93 (d, 2H, $J = 9.0$ Hz, 3″H, 5″H), 7.79 (d, 2H, $J = 9.0$ Hz, 2″H, 6″H). IR (KBr): (ν) 3065 (NH), and 1333, 1165 (SO_2) cm^{-1}. Anal. calcd. for $C_{17}H_{21}N_3O_2S$: C, 61.61; H, 6.39; N, 12.68. Found: C, 61.48; H, 6.31; N, 12.57.

*Synthesis of N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-4-methoxybenzenesulfonamide (**9k**)*

The compound **9k** was obtained following General Procedure C as off-white color solid, yield 65.5%, m.p. 132–134°C; ^{1}H NMR ($CDCl_3$): δ 2.13–2.15 (m, 2H, 5H), 2.42 (s, 3H, $-CH_3$), 2.72 (t, 2H, $J = 6.0$ Hz, 6H), 2.92 (s, 2H, 2H), 4.25 (s, 2H, $-CH_2$), 5.37 (brs, –NH, D_2O exchangeable), 5.56 (m, 1H, 4H, olefinic), 6.12 (t, 2H, $J = 2.1$ Hz, 3′H, 4′H), 6.50 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.24 (d, 2H, $J = 9.0$ Hz, 3″H, 5″H), 7.74 (d, 2H, $J = 8.7$ Hz, 2″H, 6″H). IR (KBr): (ν) 3078 (NH), and 1331, 1163 (SO_2) cm^{-1}. Anal. calcd. for $C_{17}H_{21}N_3O_3S$: C, 58.77; H, 6.09; N, 12.09. Found: C, 58.57; H, 5.98; N, 11.95.

*Synthesis of 3-(2-chloro-6-fluorophenyl)-5-methyl-N-[3-((1H-pyrrol-1-yl)methyl)-1,2,5,6-tetrahydropyridin-1-yl]-1,2-oxazole-4-carboxamide (**9l**)*

The compound **9l** was obtained following General Procedure C as off-white color solid, yield 56.2%, m.p. 188–189°C; ^{1}H NMR ($CDCl_3$): δ 2.10 (brs, 2H, 5H), 2.77 (s, 3H, $-CH_3$), 2.80 (t, 2H, $J = 5.7$ Hz, 6H), 3.02 (s, 2H, 2H), 4.29 (s, 2H, $-CH_2$), 5.46 (m, 1H, 4H, olefinic), 6.14 (t, 2H, $J = 1.8$ Hz, 3′H, 4H), 6.17 (brs, –NH, D_2O exchangeable), 6.55 (t, 2H, $J = 2.1$ Hz, 2′H, 5′H), 7.17 (t, 1H, $J = 8.4$ Hz, 3″H), 7.35 (d, 1H, $J = 7.5$ Hz, 5″H), 7.46 (t, 1H, $J = 5.7$ Hz, 4″H). IR (KBr): (ν) 3185 (NH), 1634 (C=O) cm^{-1}; HRMS (EI) m/z [M^+] calcd. for $C_{21}H_{20}ClFN_4O_2$ 414.1259; found 414.1252. Anal. calcd. for $C_{21}H_{20}ClFN_4O_2 \cdot 0.35H_2O$: C, 59.90; H, 4.70; N, 13.02. Found: C, 59.89; H, 4.79; N, 13.02.

Biological studies

Cell culture

BV-2 microglial cells were kindly provided by Blasi et al. [50] and cultured in high glucose (4500 mg/mL) DMEM containing phenol red, 5% FBS, 4 mM L-glutamine, and penicillin/streptomycin (100 U/0.1 mg/mL). Culture conditions were maintained (37°C in 5% CO_2/atmosphere) and every 2–5 days, the media was replaced and cells sub-cultured. For experiments, plating media consisted of DMEM (minus phenol red),

5% FBS, penicillin/streptomycin (100 U/0.1 mg/mL) and 3 mM L-glutamine. All experimental compounds were dissolved in DMSO (20 mg/mL) and dilutions were prepared in sterile HBSS + 5 mM HEPES, adjusted to a pH of 7.4. Activation of BV-2 cells was established using 1 μg/mL of LPS from *Escherichia coli* O111:B4 for 24 h in experimental media, maintaining cell density at 0.5×10^6 cells/mL.

Cell viability

Cell viability was quantified using resazurin (alamar blue) indicator dye [51]. A working solution of resazurin was prepared in sterile phosphate buffered saline (PBS)–phenol red (0.5 mg/mL) and added (15% v/v) to each sample. Samples were returned to the incubator for 6–8 h, and reduction of the dye by viable cells (to resorufin, a fluorescent compound) was quantitatively analyzed using a microplate fluorometer, model 7620, version 5.02 (Cambridge Technologies, Inc., Watertown, MA) with settings at 550/580 (excitation/emission) wavelengths. The data were expressed as percent of live untreated controls.

NO_2^- determination

Quantification of nitrite (NO_2^-) was determined by a colorimetric method using the Griess reagent. The Griess reagent was prepared by mixing an equal volume of 1.0% sulfanilamide in 0.5 N HCl and 0.1% *N*-(1-naphthyl)-ethylenediamine in deionized water. The Griess reagent was added directly to the cell supernatant suspension and incubated under reduced light at room temperature for 10 min. A standard curve for NO_2^- was generated from dilutions of sodium nitrite ($NaNO_2$) (1–100 μM) prepared in plating medium. Controls and blanks were run simultaneously and subtracted from the final value to eliminate interference. Samples were analyzed at 550 nm on a UV microplate spectrophotometer (model 7600, version 5.02, Cambridge Technologies, Inc.).

iNOS protein expression

After 24 h treatment, cells were fixed in 4% paraformaldehyde/permeabilized in 0.1% Triton X-100 in PBS and incubated with anti-iNOS, N-terminal antibody produced in rabbit (Sigma–Aldrich) for 2 h at 37°C. Samples were washed in PBS and subsequently incubated with anti-rabbit Alexa Fluor® 488 conjugate for 2 h at 37°C. Samples were counterstained with propidium iodide and photographically observed using a Nikon TE 900 inverted microscope and a Nikon PCM 2000 confocal microscope. Data acquisition was done using C-imaging systems confocal PCI-Simple software (Compix, Inc., Cranberry Township, PA, USA).

Inflammatory cytokines

Multi-analyte ELISArray

Murine inflammatory cytokines were quantified using a multi-analyte sandwich-based ELISArray MEM-004A (SABiosciences, Qiagen, Inc., Valencia, CA), which simultaneously quantifies 12 pro-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17α, IFNγ, TNFα, G-CSF, and GM-CSF. Very briefly, cell supernatants were removed after 24 h and stored at −20°C. Supernatant from each sample was thawed and incubated with capture antibodies for 2 h at RT along with a mixed antigen standard cocktail. After washing, samples were then re-incubated with biotinylated detection antibodies for 1 h at RT. After a 2nd washing process, samples were incubated with avidin–horseradish peroxidase conjugate for 30 min, re-washed, and incubated with a development reagent. After addition of the stop solution, OD was evaluated on a spectrophotometer at 450 nm with a wavelength correction at 570 nm. Data was evaluated as % cytokines production and released of the LPS control.

IL-1α and IL-6

OmniKine™ Murine IL-6 (Catalog # OK-0187) and Murine IL-1α (Catalog # OK-0181) quantitative "Sandwich" ELISA (Assay Biotechnology Company, Inc., Sunnyvale, CA) were used for detection and quantification of IL-6 and IL-1α produced/released within the range of 62–4000 pg/mL. Experiments were performed according to the manufacturer's guidelines (Assay Biotech) and quantified using a spectrophotometer at 450 nm (with correction wavelength set to 570 nm).

Data analysis

Statistical analysis was performed using Graph Pad Prism (version 3.0; Graph Pad Software, Inc., San Diego, CA, USA) with significance of difference between the groups assessed using a one-way ANOVA, followed by Tukey post hoc means comparison test, or Student's *t*-test. IC_{50}s were determined by regression analysis using Origin Software (OriginLab, Northampton, MA).

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