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Anti-neuroinflammatory activity of 1,5-benzodiazepine derivatives

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ABSTRACT

A series of 2,3-dihydro-1,5-benzodiazepines were synthesized and evaluated for anti-inflammatory effects in microglia cells. Among the 1,5-benzodiazepines tested, compound **3e** strongly inhibited LPS-induced nitric oxide (NO) production, with an IC_{50} value of 7.0 μM in the microglia cells. Also, compound **3e** significantly inhibited the enzymatic activity of inducible NO synthase (iNOS) without changes in iNOS protein expression or NO scavenging activity. This result suggests that compound **3e** showed anti-neuroinflammatory effects by suppressing iNOS enzyme activity.

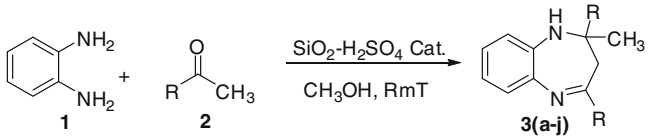
Microglia, resident macrophages, are the immune surveillance cells of the central nervous system (CNS) and play an active role in brain inflammatory, immune, and neurodegenerative processes.[1] Upon neuronal injury or inflammatory stimulation, activated microglia produce and release a variety of pro-inflammatory factors, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), that can result in neuronal cell death in neurodegenerative diseases.[2–5] Thus, microglial activation appears to play a pivotal role in the initiation and progression of neurodegenerative diseases. Therefore, modulation of activated microglia is important to the protection of neuronal cells from exposure to inflammatory substances and may have therapeutic potential in neurodegenerative diseases that are accompanied by microglial activation.

NO can be produced by three isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). Generally, eNOS and nNOS are Ca^{2+}-dependent and constitutively release small amounts of NO required for physiological function. In contrast, iNOS is Ca^{2+}-independent and is not normally expressed but can be induced by lipopolysaccharide (LPS) and various cytokines, such as interferon-γ (IFN-γ), TNF-α, and IL-1β.[6,7] NO, a neuromodulator in the CNS, participates in brain development, pain perception, memory, and neuronal plasticity.[8] However, the elevated level of NO produced by the expression of inducible iNOS is an important mediator of inflammation and neuronal cell death.[9]

Benzodiazepines are important organic molecules with a wide array of biological activities and therapeutic functions. Among the benzodiazepines, the 1,4- and the 1,5-benzodiazepines are commonly used as anxiolytic and anticonvulsive drugs. These effects are primarily mediated via the benzodiazepine receptors located in the central nervous system.[10] Also, peripheral-type benzodiazepine binding sites have been detected in microglia[11], macrophages[12], and other tissues.[13] In addition to the anxiolytic activity of the benzodiazepines, these molecules also have activity as anti-cancer[14] and anti-neuroinflammatory[15] agents.

In this study we designed and synthesized 2,3-dihydro-1,5-benzodiazepine derivatives to identify novel neuroprotective agents. Evaluation of the ten synthesized 1,5-benzodiazepine derivatives as potential anti-neuroinflammatory agents was performed using BV-2 microglia cells.

The benzodiazepine derivatives were synthesized according to the procedure shown in Scheme 1. Thus, a mixture of *o*-phenylene-



Scheme 1. Synthesis of 2,3-dihydro-1,5-benzodiazepines.

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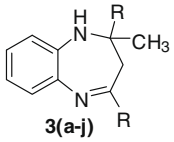
Table 1
Inhibitory activities of 1,5-benzodiazepine derivatives on NO production in LPS-activated microglia



3(a-j)

Compounds	R	Time[a] (min)	Yield[b] (%)	Inh[c] (%)	IC_{50} (μM)
3a	Methyl	30	98	28.6	14.3
3b	Ethyl	40	95	27.4	15.2
3c	Phenyl	40	92	59.8	9.4
3d	Isopropyl	60	94	26.3	16.6
3e	2-Thiophenyl	120	86	70.3	7.0
3f	3-Thiophenyl	120	82	13.2	35.2
3g	4-Chlorophenyl	50	95	28.2[d]	—
3h	3-Chlorophenyl	80	85	21.2	23.2
3i	4-Methylphenyl	50	96	34.6	14.0
3j	2-Nitrophenyl	50	95	24.9	23.2

[a] Reaction time for the preparation of **3a**–**j**.
[b] Isolated yield in the syntheses.
[c] Values obtained through three experiments and the mean inhibition of NO production relative to the LPS control at 10 μM concentration of compounds.
[d] Cytotoxic effect was observed.

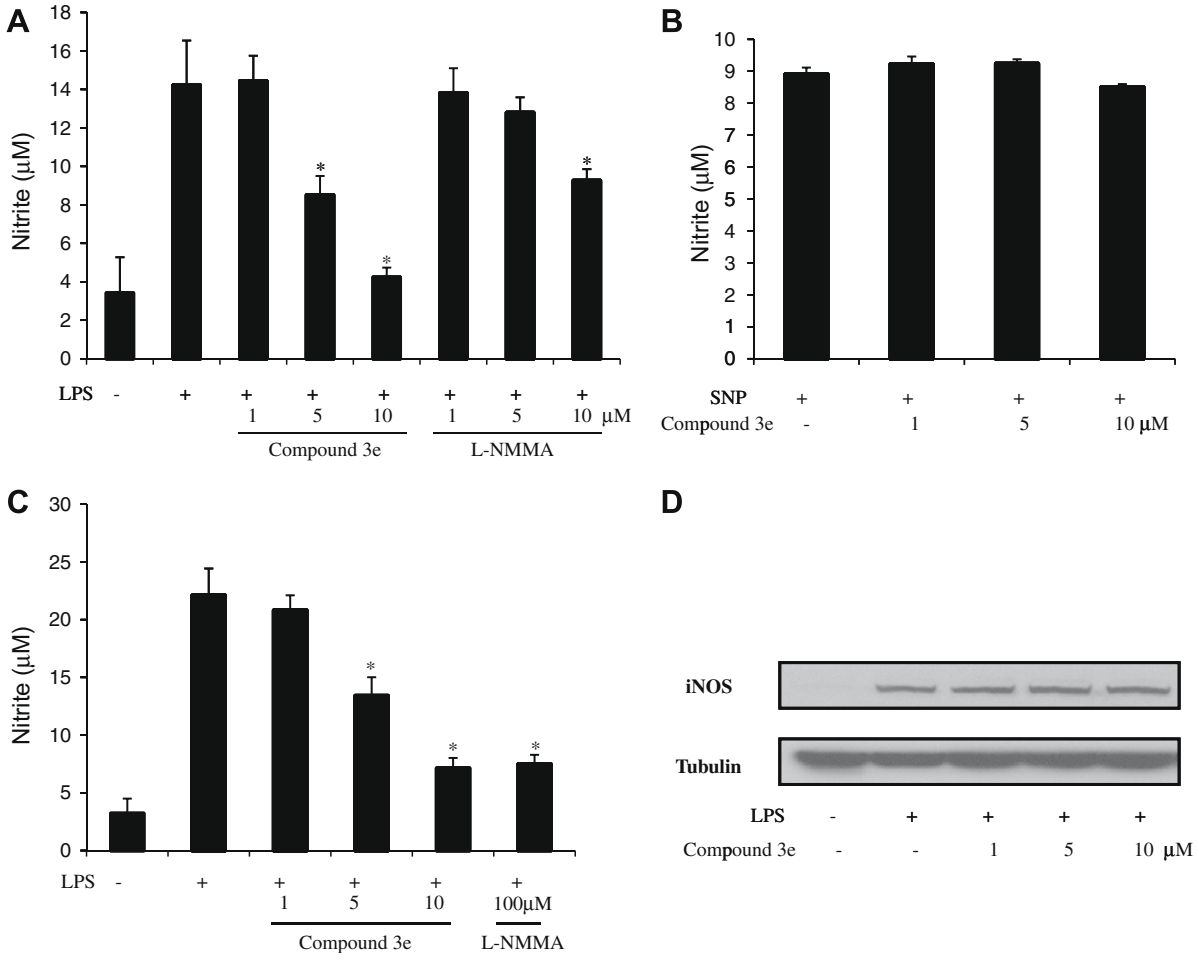


Figure 1. The inhibitory effect of compound **3e** on NO production in LPS-activated BV-2 cells. (A) The effect of compound **3e** on LPS-induced NO production in BV-2 cells. Nitrite was measured using the Griess reaction after 24 h treatment with LPS (100 ng/ml) in the presence or absence of compound **3e**. (B) The effect of compound **3e** on NO scavenging activity after a mixture of SNP and compound **3e** were incubated at 25 °C for 150 min. (C) The effect of compound **3e** on iNOS enzyme activity in BV-2 cells. (D) The effect of compound **3e** on iNOS protein expression in BV-2 cells. iNOS protein was detected using Western blot analysis after 6 h of treatment with LPS (100 ng/ml) in the presence or absence of compound **3e** (1, 5, and 10 μM). L-NMMA was used as positive control. All data were presented as the mean ± S.D. $^{*}p$ <0.05 indicates statistically significant differences from treatment with LPS alone.

diamine **1** (1 mmol), ketone **2** (2.5 mmol), and silica gel-supported sulfuric acid catalyst (0.1 mmol) in methanol was stirred at room temperature for the appropriate time[16] (Table 1). Completion of the reaction was monitored via TLC. After completion of the reaction, ethyl acetate was added to the reaction mixture, and the catalyst was recovered by filtration. The organic layer was concentrated, and the crude product was purified by silica gel column chromatography using an ethyl acetate/*n*-hexane mixture (1:9) as an eluent to afford the desired product **3**. The compounds were purified further by recrystallization with ethyl acetate and hexane to obtain the pure form with an 82–98% yield.

The inhibitory activities of NO production by the 2,3-dihydro-1,5-benzodiazepine derivatives were evaluated using LPS-activated microglia cells. A murine microglia cell line, BV-2, was stimulated with 100 ng/ml LPS for 24 h in the presence or absence of the samples. The presence of nitrite, a soluble oxidation product of NO, in the culture media was determined by Griess reaction. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

As shown in the Table 1, the inhibitory activities of the 1,5-benzodiazepines varied according to the structure of R. The benzodiazepine containing a phenyl ring at the R position showed good inhibitory activity (**3c**), as compared with alkyl groups. However, the activity declined as substituents were attached to the phenyl ring (**3g–j**), indicating that the BV-2 cell is very sensitive towards both steric and electronic differences in position R.

The highest NO-inhibitory effect on BV-2 cells among the compounds tested in this study was obtained with the benzodiazepine containing 2-thiophene ring (**3e**). Interestingly, the activity sharply changed depending upon the attachment position of the thiophene ring. The compound with the 3-thiophenyl group (**3f**) gave poor inhibitory activity. Since the steric difference between 2-thiophenyl and 3-thiophenyl group is marginal and can be ignored in this case, the activity difference might be due to the electronic effects.

INOS expression is induced in activated microglia and mediates NO synthesis. Thus, we investigated the effect of **3e** on the activity[17] and expression[18] of iNOS protein in LPS-induced BV-2 cells. Also, we evaluated the NO scavenging activity[19] of compound **3e**. As shown in Figure 1, compound **3e** did not have a significant inhibitory effect on the expression of iNOS and NO scavenging activity but did significantly inhibit iNOS enzyme activity. Therefore, the inhibitory effect of compound **3e** on NO production may be partly due to inhibition of iNOS enzyme activity.

In summary, we have reported a new class of 1,5-benzodiazepines that demonstrate anti-neuroinflammatory activity in microglia cells. Compound **3e** has shown the highest NO-inhibitory effect on BV-2 cells. Compound **3e** showed inhibitory effects on NO production through the suppression of iNOS enzyme activity. These results suggest that compound **3e** may be of beneficial therapeutic potential for neuroinflammatory diseases through the inhibition of microglial activation and is a suitable target for further structure activity relationship studies. Further studies are required to elucidate the precise mechanisms underlying the anti-inflammatory activity of compound **3e**.

Acknowledgments

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16. *Catalyst preparation*: To a slurry of silica gel (10 g) in dry diethyl ether (50 ml), commercially available sulfuric acid (3 ml) was added with shaking for 5 min. The solvent was evaporated under reduced pressure, resulting in free-flowing silica gel-supported sulfuric acid (SiO_2–H_2SO_4), which was then dried at 110 °C for 3 h.
17. For the assay the iNOS enzyme activity in intact cells, BV-2 cells were plated in 100 mm tissue culture dishes (4×10^6 cells) and incubated with LPS (100 ng/ml) for 12 h. Then, the cells were washed twice with PBS, and cells were harvested and plated in a 96-well plate (2×10^6 cells/well), and incubated in the presence or absence of different concentrations of compound **3e** for a further 12 h. The amount of NO in the supernatant was detected by Griess reaction.
18. Western blot was performed to analyze iNOS expression. BV-2 cells were seeded in a 6-well plate and exposed to LPS (100 ng/ml) in the presence or absence of compound **3e** for 6 h. The protein sample (40 μg for each) from the BV-2 cell extract was separated by 8% SDS–PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with primary antibodies (rabbit anti-iNOS; Transduction Laboratories, San Diego, CA, USA) and secondary antibodies (goat anti-rabbit IgG; Amersham Pharmacia Biotech). The blots were developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech).
19. Sodium nitroprusside (SNP) was dissolved in phosphate buffered saline (PBS) at 100 mM. This SNP solution (50 μl) was mixed with 950 μl PBS containing various concentrations of compound **3e** (1, 5 and 10 μM). These mixtures were incubated at 25 °C for 2.5 h, and nitrite formed through the combination of oxygen and NO released from the SNP, as measured by the Griess reaction.