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Inhibition of Neuroinflammation by Synthetic Androstene Derivatives Incorporating Amino Acid Methyl Esters on Activated BV-2 Microglia

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Androstene derivatives incorporating amino acid methyl esters were prepared, and their anti-inflammatory effects were evaluated in lipopolysaccharide (LPS)-activated BV-2 microglial cells. Several compounds exhibited dose-dependent inhibition. The most active compound, methyl ((3*S*,10*R*,13*S*)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthrene-17-carbonyl)-L-phenylalaninate (**10**) significantly suppressed LPS-induced expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). Mechanistic studies revealed that compound **10** markedly inhibits phosphorylation of p38 mitogen-activated protein kinases (MAPKs) and subsequent transcription factor (NF-κB) and activator protein-1 (AP-1) activation. Furthermore, compound **10** decreased LPS-activated microglial neurotoxicity in a condition medium/HT-22 neuroblastoma co-culture model. Taken together, these results suggest **10** is a potential lead compound for the development of a novel therapeutic agent for neurodegenerative diseases.

Neuroinflammation is a common hallmark of many neurodegenerative diseases,[1] such as Alzheimer's disease[2] and Parkinson's disease.[3] The neuroinflammation is mediated mainly by activation of microglia, which are the immune cells of brain. Uncontrolled microglial activation is often found in neuronal injuries and is characterized by production of a wide range of proinflammatory cytokines and neurotoxic mediators, including nitric oxide (NO), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6).[4] The accumulated proinflammatory cytokines and neurotoxic mediators act directly on neurons to induce apoptosis.[5] Therefore, suppression of proinflammatory cytokines and free radicals that are released from activated microglial cells could be a strategy to treat neuroinflammation-related diseases.[6]

There has been much attention devoted to studying the anti-neuroinflammatory properties of synthetic compounds.[7] Our previous studies suggested that synthetic 5α-cholestan-6-one sterol analogues exert anti-inflammation properties in lipopolysaccharide (LPS)-activated BV-2 cells.[8] Some androstene sterol analogues have been shown to display significant anti-inflammatory activity,[9] and reports have also shown that the introduction of amide moieties to steroids can improve their potency.[10] Taking these results into account, we sought to obtain a series of new androstene derivatives incorporating amino acid methyl esters and to evaluate their anti-neuroinflammatory effects. In this study, new androstene derivatives were synthesized and screened for inhibitory effect on NO production in LPS-activated BV-2 cells. From these data, the C16,17 bond of the steroids, the R ($NH_2CHRCOOMe$) group of the amino acid methyl esters, and the stereochemistry of the amino acid were identified as having significant effects on the inhibitory activities of the compounds.

The synthetic routes used to access the derivatives are shown in Scheme 1. Steroidal 17-carboxylic acids (**2a**,**b**) were synthesized from pregnenolone or 16-dehydropregnenolone (**1a**,**b**) according to literature procedures.[11] Treatment of **2a** or **2b** with amino acid methyl esters in the presence of triethylamine and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) led to the desired target compounds (**3**–**20**). 17-Carboxamides **21**–**22** were obtained when the corresponding carboxylic acids were reacted with ammonia.

With the compounds in hand, we evaluated their inhibitory activity on NO production in LPS-activated BV-2 microglial cells at 20 μM, comparing with dehydroepiandrosterone (DHEA), a known androstenolone that acts as a neurosteroid.[8,12] The cytotoxic effects of the compounds were determined, using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 1), to rule out the probability that inhibition of NO production was owing to cell growth inhibition or cell death. The result showed that the 17-carboxamides exhibit decreased inhibitory activity compared with the corresponding carboxylic acids (**21** < **2a**; **22** < **2b**).

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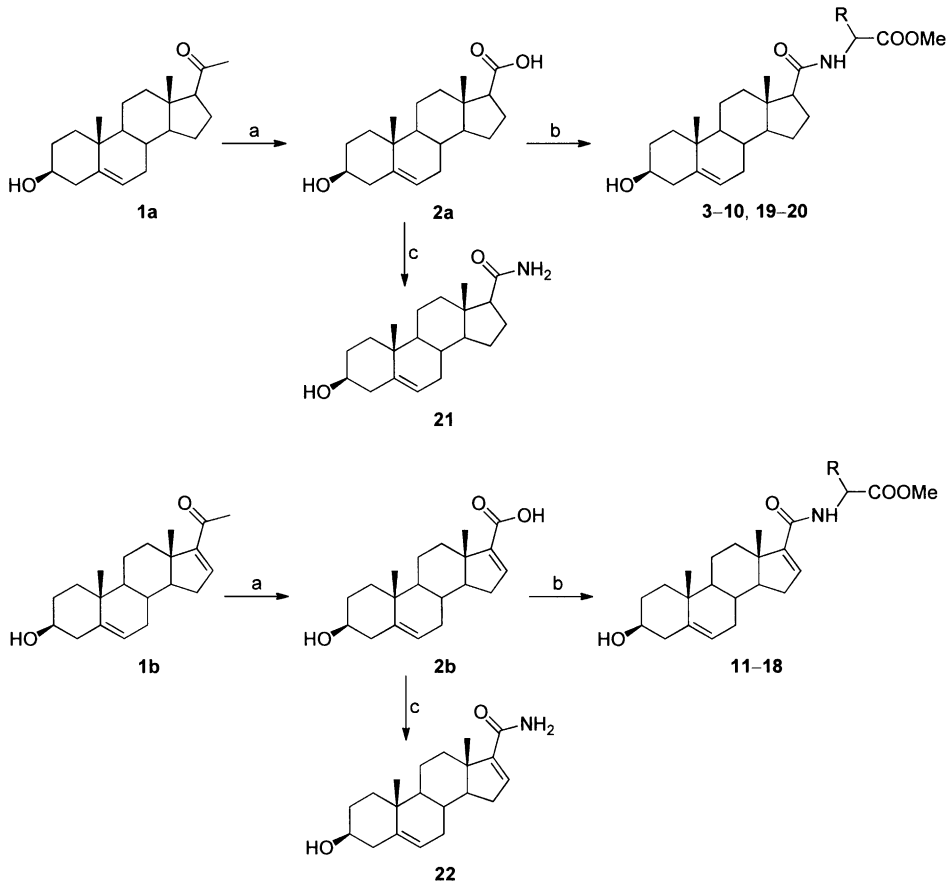

Scheme 1. *Reagents and conditions*: a) 1. NaOH, Br_2, dioxane/H_2O (7:2), −5—10 °C, 3 h; 2. 37% HCl, 92% (two steps); b) $NH_2CHRCOOMe$, HBTU, Et_3N, RT, 18 h, 68–96%; 3) NH_3/CH_3OH, HBTU, Et_3N, RT, 18 h, 91%.

The amino acid methyl ester conjugates exhibited moderate to good inhibition of NO production in LPS-activated BV-2 cells. The activity of the compounds is sensitive to the R ($NH_2CHRCOOMe$) group, and the results suggest that a larger alkyl functional group is required for potent activity (**4** > **3**; **12** > **11**). When the R group is an aromatic moiety, as in **9** and **10**, compounds exhibit good inhibitory activities. However, replacement of CH_2Ph (**10**) with *p*-$OHCH_2Ph$ (**8**) leads to a significant loss in activity. Aromatic compounds with a C-16,17 double bond exhibited decreased inhibitory activities (**9** > **16**; **10** > **17**). Further study of the impact of the stereochemistry of the amino acid was performed. The diastereomers of compounds **9**, **10** and **13** were synthesized, and the results showed that the analogues containing the natural amino acid exhibit superior activities (**13** > **18**; **9** > **19**; **10** > **20**). Among them, compounds **9**, **10** and **13** showed the most pronounced inhibition of NO production in LPS-activated BV-2 microglia without significant cytotoxicity.

The most effective compounds, **9**, **10** and **13**, were selected for evaluation in dose–response assays in LPS-activated microglia BV-2 cells. These compounds markedly decreased LPS-induced NO production in a dose-dependent manner (Figure 1 A–C). Consistent with IC_{50} determination experiments, compound **10** exhibited the most potent inhibition of NO production in LPS-activated BV-2 cells (Table 2).

The anti-inflammatory activities of compound **10** were further evaluated in other cells types and with other stimuli. LPS-activated primary microglia and LPS/IFN-γ-activated primary astrocytes were used to test further the effects of compound **10** on NO production. As shown in Figure 1 D,E, NO production in LPS-activated primary microglia and LPS/IFN-γ-activated primary astrocyte cells was decreased by compound **10**, indicating that the NO inhibitory activity of compound **10** is general. The tested concentration of compound **10** did not influence cell viability of primary microglia or primary astrocytes (data not shown).

Nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are known to be pivotal proinflammatory-associated enzymes in the brain[13] and responsible for the synthesis of NO and prostaglandin E2 (PGE-2), respectively.[14] Production of proinflammatory cytokines, such as TNF-α and IL-6, is also increased by the activated microglial cells.[15] Thus, we investigated the inhibitory effects of compound **10** on the mRNA expression of those cytokines and proinflammatory enzymes by quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis using an SYBR quantitative RT-PCR kit. As shown in Figure 2 A–D, compound **10** significantly inhibits LPS-induced iNOS, COX-2, TNF-α and IL-6 expression in BV-2 microglial cells in a dose-dependent manner. The inhibitory effects of compound **10** on iNOS, COX-2, TNF-α and IL-6 production were further assessed by Western blot analysis or enzyme-linked immunosorbent assay (ELISA). Compound **10** significantly inhibited LPS-induced iNOS, COX-2, TNF-α and IL-6 expression at the protein level (Figure 3) suggesting that compound **10** might be a potential lead for the treatment of neuroinflammation-associated neurodegenerative diseases.

Mitogen-activated protein kinases (MAPKs) are well-characterized protein kinase that modulate expression of proinflmmatory cytokines and enzymes in glial cells under inflammatory conditions.[16] Conventional MAPKs comprise three main subfamilies including extracellular signal-regulated kinases (ERK1/2), p38 MAPK, and c-jun N-terminal kinase (JNK).[17] Thus, we next determined whether the anti-inflammatory properties of compound **10** occur through inhibition of a MAPK signaling pathway in LPS-activated BV-2 cells. As shown in Figure 4 A, LPS markedly induces MAPKs phosphorylation, and compound **10** suppresses the phosphorylation of p38 MAPK and JNK (Fig-

Table 1. Structures and inhibitory activity of androstene derivatives on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated microglia (20 μM).

Compd	$NH_2CHRCOOMe$	R	Inhib. [%][a]	Cell viab. [%][b]
2a	–	–	25.07 ± 2.99	108.60 ± 2.97
2b	–	–	32.23 ± 4.34	109.44 ± 3.67
3	Gly-OMe	H	22.10 ± 4.71	106.81 ± 1.59
4	L-Ala-OMe	CH_3	30.20 ± 4.72	100.78 ± 1.55
5	L-Val-OMe	$CH(CH_3)_2$	33.71 ± 4.68	92.75 ± 4.50
6	L-Pro-OMe	$(CH_2)_3$	41.08 ± 1.98	90.31 ± 1.56
7	L-Glu-$(OMe)_2$	$(CH_2)_2COOMe$	24.75 ± 3.72	94.51 ± 0.87
8	L-Tyr-OMe	*p*-$OHPhCH_2$	18.35 ± 3.99	98.73 ± 3.31
9	L-Trp-OMe	3-indole-CH_2	50.65 ± 3.56	99.82 ± 3.81
10	L-Phe-OMe	CH_2Ph	57.53 ± 2.20	102.65 ± 0.65
11	Gly-OMe	H	23.39 ± 4.31	98.26 ± 1.69
12	L-Ala-OMe	CH_3	44.01 ± 4.38	109.52 ± 2.27
13	L-Val-OMe	$CH(CH_3)_2$	44.33 ± 5.52	100.66 ± 2.97
14	L-Pro-OMe	$(CH_2)_3$	32.30 ± 3.82	93.74 ± 2.45
15	L-Glu-$(OMe)_2$	$(CH_2)_2COOMe$	26.16 ± 4.36	106.94 ± 1.06
16	L-Trp-OMe	3-indole-CH_2	42.04 ± 5.28	107.72 ± 3.3
17	L-Phe-OMe	CH_2Ph	39.04 ± 3.99	109.52 ± 2.86
18	D-Val-OMe	$CH(CH_3)_2$	33.67 ± 5.19	87.32 ± 1.25
19	D-Trp-OMe	3-indole-CH_2	34.38 ± 4.09	89.31 ± 3.03
20	D-Phe-OMe	CH_2Ph	39.82 ± 2.53	97.84 ± 3.72
21	–	–	2.76 ± 2.93	98.57 ± 4.01
22	–	–	15.33 ± 2.39	92.75 ± 5.25
DHEA	–	–	9.28 ± 1.46	101.57 ± 2.21

[a] Inhibition (%): BV-2 cells were pretreated with test compound (20 μM) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) for 24 h. The nitrite level in the cell culture medium was determined by Griess reaction. Values represent the mean ± SD of three independent experiments (performed in triplicate); [b] Cell viability was measured by using an MTT assay.

Table 2. In vitro inhibitory activity of compounds **9**, **10**, **13** on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated microglia.

Compd	IC_{50} [μM][a]
9	20.09 ± 1.30
10	17.62 ± 1.25
13	24.98 ± 1.40

[a] Values are the mean ± SD of three independent experiments (performed in triplicate).

ure 4A), whereas phosphorylation of ERK1/2 is not affected. Activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), important intracellular nuclear transcription factors that can be activated by MAPK inflammatory signal transduction pathways, play a vital part in proinflammatory enzymes and cytokines expression. Therefore, we examined the effects of compound **10** on AP-1 and NF-κB activation in LPS-activated BV-2 cells by luciferase reporter assay. Compound **10** significantly inhibits LPS-induced AP-1 and NF-κB activity in BV-2 cells (Figure 4B,C). These results suggest that the MAPK/AP-1 or MAPK/NF-κB pathway might take part in the inhibitory activity of compound **10** on iNOS, COX-2, TNF-α, and IL-6 mRNA expression in LPS-activated BV-2 cells.

To explore the possible implication of p38 MAPK and JNK signaling pathways in anti-inflammatory activity of compound **10**, we next examined the effects of a p38 MAPK-specific inhibitor, SB203580 and a JNK-specific inhibitor, SP600125, on LPS-induced microglia activation. As shown in Figure 5, similar to

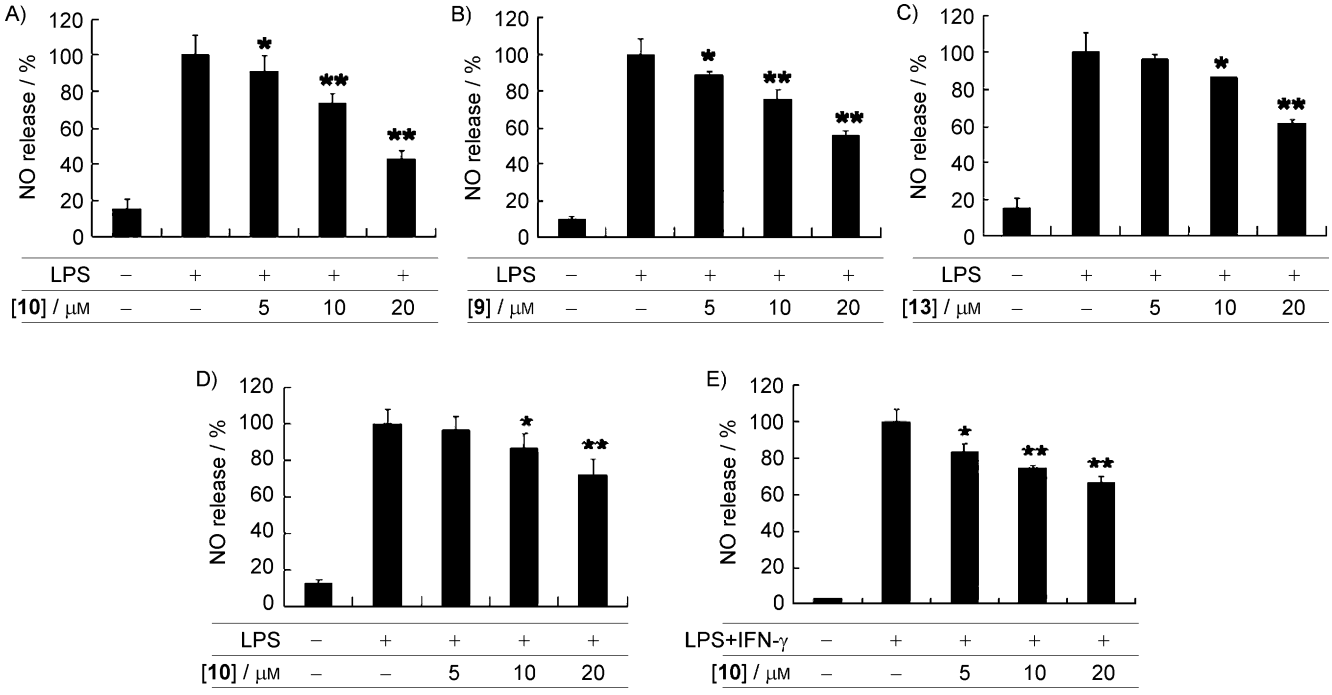

Figure 1. Inhibition of NO generation in lipopolysaccharide (LPS)-activated BV-2 cells by compounds A) **10**, B) **9**, and C) **13**, and in D) LPS-activated primary microglia and E) LPS/IFN-γ-stimulated primary astrocytes by compound **10**. Cells were pretreated with test compound (5–20 μM) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) or LPS/IFN-γ (50 U mL^{-1}) for 24 h. The nitrite level in the cell culture medium was then determined by Griess reaction. Values are the mean ± SD of three independent experiments (performed in triplicate); *$p < 0.05$ and **$p < 0.01$, compared with the LPS or LPS/IFN-γ values.

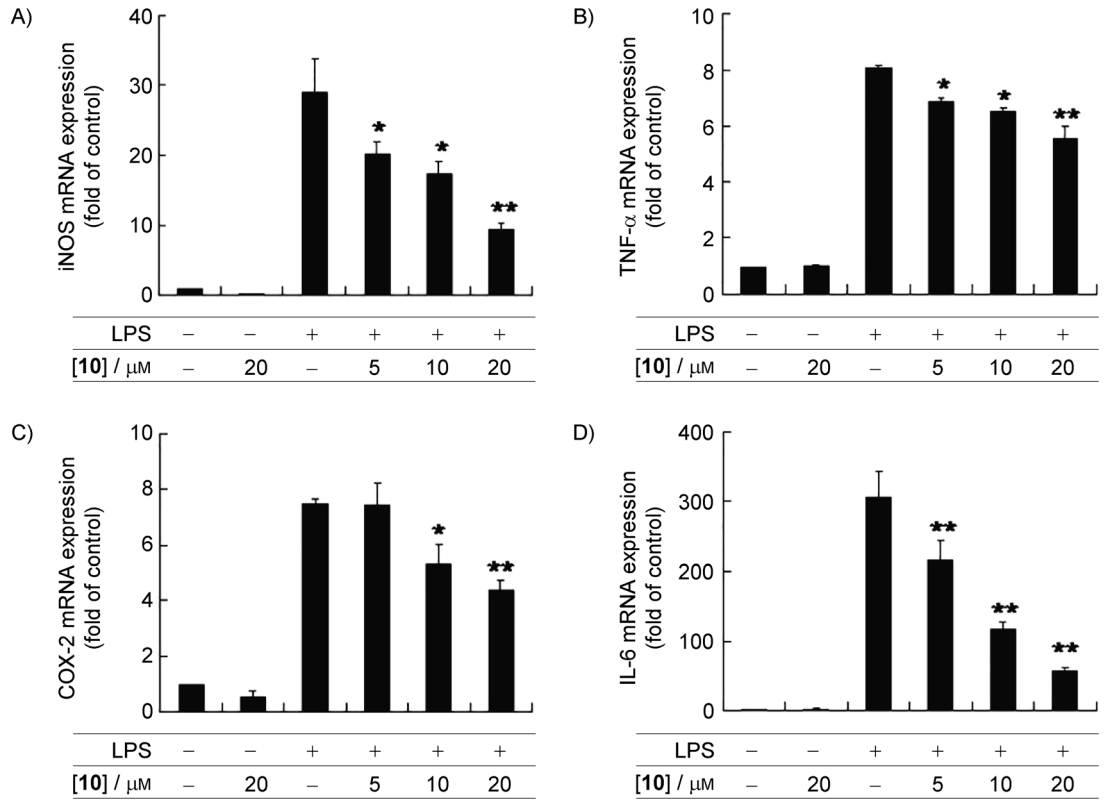

Figure 2. Inhibition of A) iNOS, B) TNF-α, C) COX-2 and D) IL-6 expression at the mRNA level in lipopolysaccharide (LPS)-activated BV-2 cells by compound **10**. BV-2 cells were pretreated with **10** (5–20 μM) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) for 6 h. The appropriate mRNA level was analyzed by SYBR green quantitative reverse transcription polymerase chain reaction (RT-PCR). Values are the mean ± SD of three independent experiments (performed in triplicate); $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the LPS value.

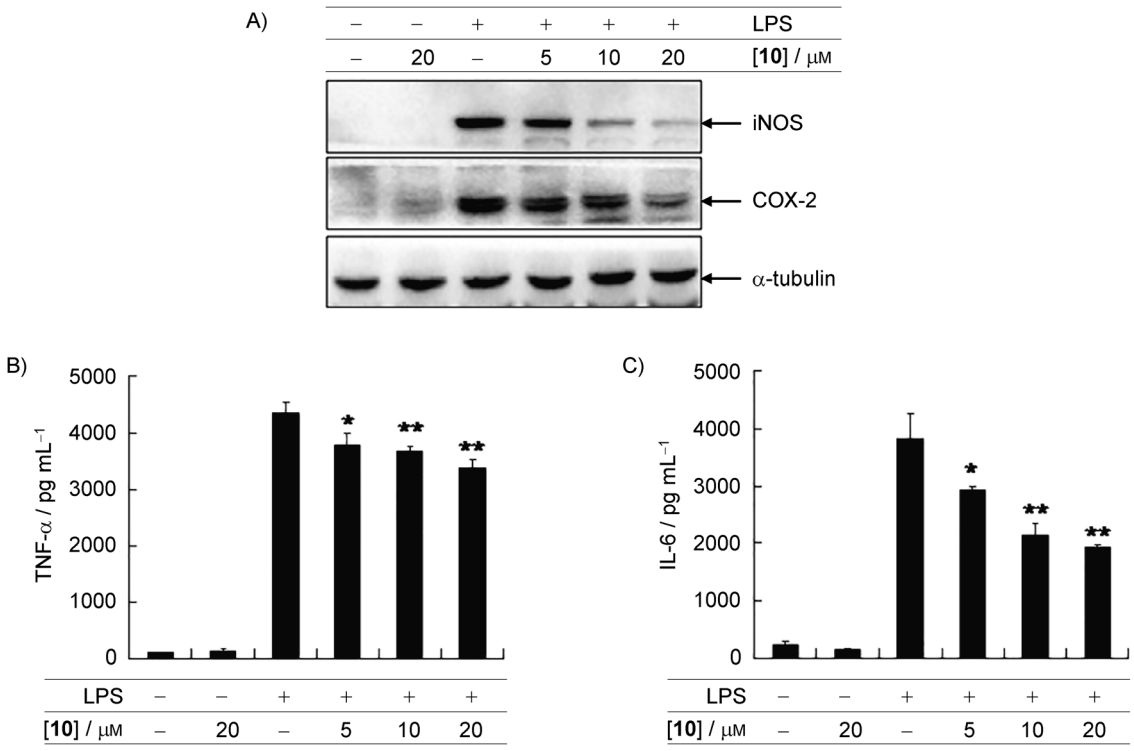

Figure 3. Inhibition of iNOS, COX-2, TNF-α and IL-6 expression at the protein level in lipopolysaccharide (LPS)-activated BV-2 cells by compound **10**. BV-2 cells were pretreated with **10** (5–20 μM) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) for 24 h. A) The iNOS and COX-2 expression levels were analyzed by Western blotting. B) TNF-α and C) IL-6 secretion levels were detected by an enzyme-linked immunosorbent assay (ELISA). Values are the mean ± SD of three independent experiments (performed in triplicate); $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the LPS value.

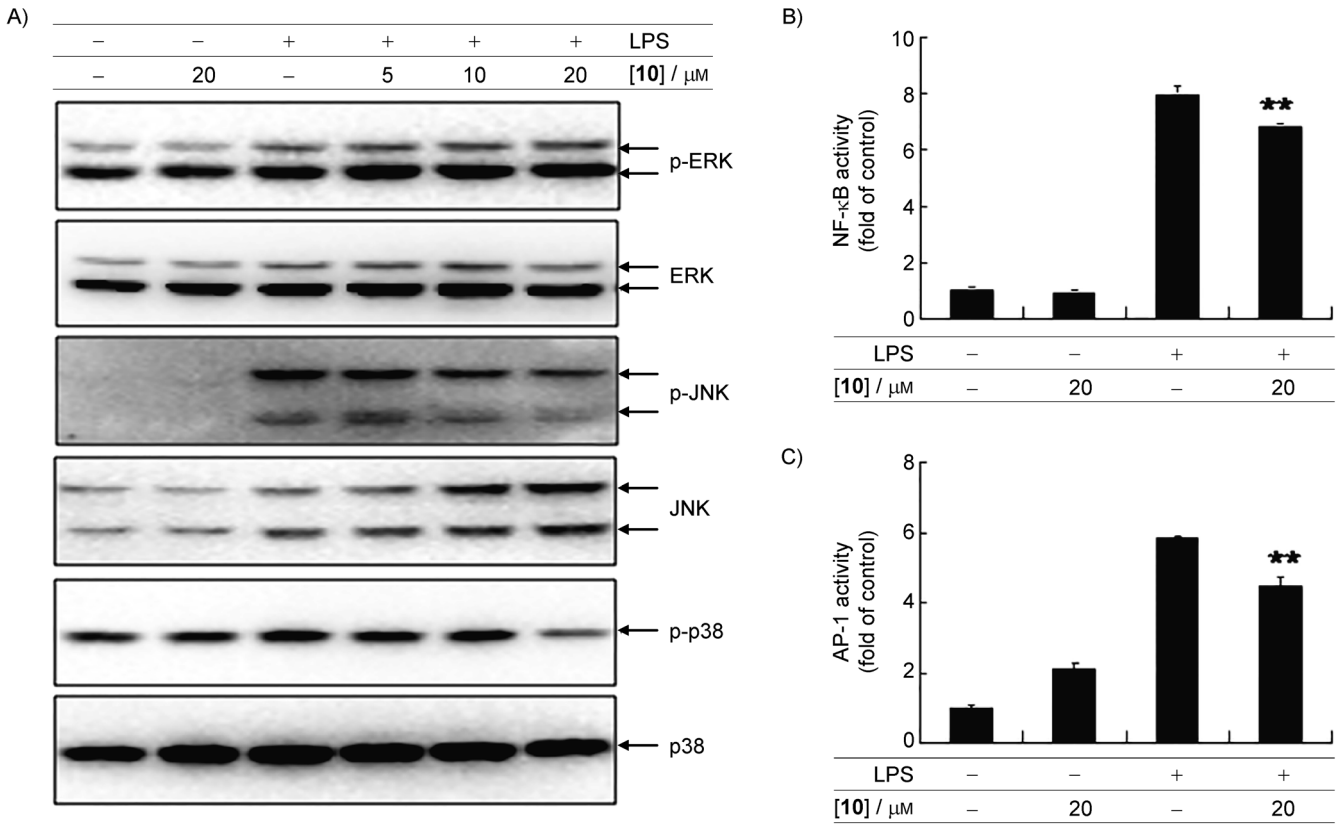

Figure 4. Inhibition of A) MAPKs, B) NF-κB and C) AP-1 activation in lipopolysaccharide (LPS)-activated BV-2 cells by compound **10**. A) BV-2 cells were pretreated with **10** (5–20 μM) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) for 20 min. The levels of phosphorylated (p) MAPKs were determined by Western blot. B,C) BV-2 cells stably expressing an NF-κB or AP-1 reporter were treated with LPS (0.1 μg mL^{-1}) following pretreatment with **10** (20 μM) for 30 min. After 16 h of LPS-stimulation, B) NF-κB or C) AP-1 activity was determined by a luciferase reporter assay. The control value was set to 1; and relative values are the mean ± SD of three independent experiments (performed in triplicate); $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the LPS value.

compound **10**, both SB203580 and SP600125 inhibited LPS-induced NO production and activation of NF-κB and AP-1. Furthermore, both compound **10** and SB203580 or SP600125 synergistically suppressed NO production in LPS-activated BV-2 microglial cells (Figure 5 A,B). Thus, these results further confirm that these two signaling pathway are actively implicated in the anti-inflammatory action of compound **10**.

Overactivated microglia can induce neuron death and promote the cascade reaction of neuron degeneration via release of proinflammatory cytokines and neurotoxic mediators, thus suppression microglia activation could have neuroprotective effects.[18] To examine whether compound **10** has neuroprotective properties, microglia-conditioned medium and neuron co-culture in vitro were used.[19] The cell toxicity of HT-22 hippocampal cells was evaluated with conditioned media (CM) from LPS-activated BV-2 cells. HT-22 cell viability was significantly decreased after treated with CMs harvested from LPS-activated BV-2 cells (Figure 6 A). However, pretreatment of compound **10** in LPS-activated BV-2 cells significantly weakened the cell toxicity of HT-22 cells (Figure 6 B). To investigate whether compound **10** could protect against oxidative stress-induced HT-22 cell death, we examined the cell viability of HT-22 cells after hydrogen peroxide treatment for 24 h with or without compound **10**. The results revealed that compound **10** does not affect hydrogen peroxide-induced HT-22 cell death. These results suggest that compound **10** could have a neuroprotective effect via inhibiting microglial neurotoxicity.

In conclusion, a series of androstene derivatives incorporating amino acid methyl esters was synthesized. Their inhibitory activities were evaluated on NO production in LPS-activated BV-2 cells. Amino acid methyl ester conjugates were found to have moderate to good inhibitory effects on NO production in LPS-activated BV-2 cells with no or weak cell toxicity at 20 μM, and the C16,17 bond of the steroids, the R ($NH_2CHRCOOMe$) group of amino acid methyl esters, and the stereochemical configuration of the amino acid had important effects on the inhibitory activities of these compounds. The most potent compound (**10**) also significantly suppressed LPS-induced expression of proinflammatory cytokines and enzymes, such as iNOS, COX-2, TNF-α and IL-6. In addition, blockade of MAPK/AP-1 or NF-κB likely mediated the anti-inflamamtory effects of compound **10** in microglial cells. Furthermore, compound **10** inhibited the neurotoxicity in a microglial CM/HT-22 neuroblastoma co-culture system.

These results demonstrate that compound **10** possesses potent anti-neuroinflammatory and neuroprotective properties, and thus it might be a potential lead for the development of a novel therapeutic agent for neurodegenerative diseases. The molecular mechanisms on neuroprotection and anti-inflammatory effects in vivo require further investigation.

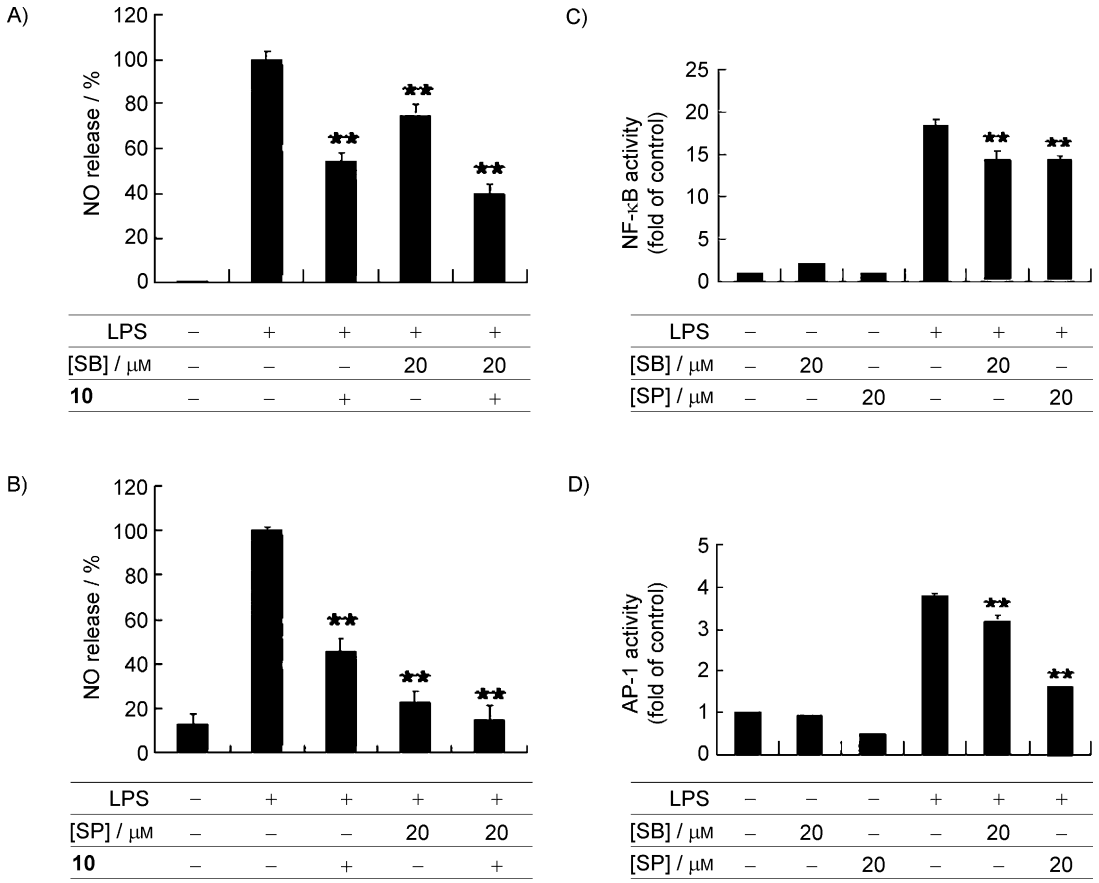

Figure 5. Effects of p38 MAPK-specific inhibitor SB203580 (SB) and JNK-specific inhibitor SP600125 (SP) in lipopolysaccharide (LPS)-induced microglial activation. BV-2 cells were pretreated with **10** (20 μM) and SB203580 (A) or SP600125 (B) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) for 24 h. The nitrite level in the cell culture medium was determined. BV-2 cells stably expressing NF-κB (C) or AP-1 (D) reporter were treated with LPS (0.1 μg mL^{-1}) following pretreatment with SB or SP for 30 min. After 16 h of LPS-stimulation, the NF-κB or AP-1 activity was determined by a luciferase reporter assay. In panels C,D, control values were set to 1, and relative values are the mean ± SD of three independent experiments (performed in triplicate). In panels A,B, values are the mean ± SD of three independent experiments (performed in triplicate); * $p < 0.05$ and ** $p < 0.01$ compared with the LPS value.

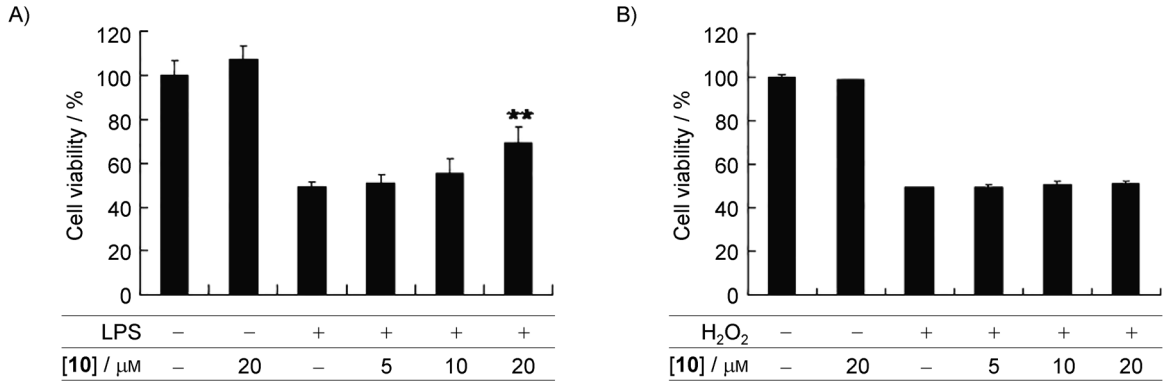

Figure 6. Neuroprotective effects of compound **10** on microglial neurotoxicity. A) BV-2 cells were pretreated with **10** (5–20 μM) for 30 min and then stimulated with or without LPS (0.1 μg mL^{-1}) for 24 h. The conditioned mediums (CMs) of BV-2 cells treated with: nothing (control), **10** alone, LPS alone, and LPS/**10** were collected, and the CMs were added to HT-22 cells. After 36 h, HT-22 cell viability was determined. B) HT-22 cells were pretreated with **10** (5–20 μM) for 30 min and then stimulated with or without H_2O_2 (600 μM) for 24 h. HT-22 cell viability was determined. Values are the mean ± SD of three independent experiments (performed in triplicate); * $p < 0.05$ and ** $p < 0.01$ compared with the LPS or H_2O_2 value.

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Keywords: androstene derivatives · microglia · neuroinflammation · neuroprotection

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