Bioorganic & Medicinal Chemistry Letters 18 (2008) 4092–4094

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of azaisoflavones and their inhibitory activities of NO production in activated microglia

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ARTICLE INFO

Article history:
Received 24 January 2008
Revised 29 April 2008
Accepted 24 May 2008
Available online 13 June 2008

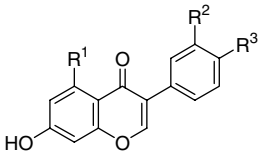
Keywords:
Azaisoflavone
Nitric oxide
iNOS

ABSTRACT

A series of azaisoflavones were synthesized and their biological activities were evaluated for nitric oxide (NO) production and inducible NO synthase (iNOS) expression in BV-2 microglia cell lines. Among these compounds, compound **8d** was the most potent with IC_{50} 7.83 μM for inhibition of NO production. Also, compound **8d** inhibited expression of iNOS in LPS-induced BV2 cells. This result suggests that compound **8d** inhibited the production of NO by suppressing the expression of iNOS.

Isoflavones are a group of naturally occurring phytochemicals that are found predominantly in legume plants (Leguminosae) (Fig. 1). Isoflavones have been reported to exhibit a variety of biological properties.[1] and may play a role in the prevention of cancers,[2–4] coronary heart diseases,[5] menopausal symptoms, and osteoporosis.[6,7]

In addition to these activities, some isoflavones have been reported to exhibit anti-inflammatory and neuroprotective functions by the inhibition of microglia activation.[8–10] Microglia, the primary immune cell in the brain, is highly involved in pathological inflammatory events in response to brain injury, infection, or inflammation.[11] Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival, it has been known that severe activation causes various autoimmune responses leading to neuronal cell death and brain injury.[12–16] Also, activation of microglial cells and consequent release of proinflammatory factors, including tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), nitric oxide (NO), and superoxide are believed to play an important role in the progression of various neurodegenerative diseases.[17–20] Among various inflammatory substances, excessive accumulation of NO has been known to be toxic to neurons. Elevated levels of NO produced by expression of inducible nitric oxide synthase (iNOS) is an important mediator of inflammation and neuronal cell death.[21] Previous studies have demonstrated that reduction of proinflammatory mediators in microglia may attenuate the severity of these disorders.[22,23] Furthermore, large amounts of NO can be converted into a highly toxic peroxynitrite ($ONOO^-$) in the presence of superoxide anion (O_2^-).



Genistein: $R^1 = OH, R^2 = H, R^3 = OH$
Biochanin: $R^1 = OH, R^2 = OCH_3, R^3 = H$
Daidzein: $R^1 = H, R^2 = H, R^3 = OH$
Daidzin: $R^1 = H, R^2 = H, R^3 = OGlc$
Orbol: $R^1 = OH, R^2 = OH, R^3 = OH$

Figure 1. Structures of naturally occurring isoflavones.

Therefore, identification of natural or synthetic compounds that inhibit inflammatory activation of microglia is a good strategy for searching new therapeutic agents for neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis. A number of studies have reported that inhibition of microglia activation by isoflavones attenuated inflammation-mediated dopaminergic neurodegeneration.[8,10]

In spite of abundant reports of isoflavones, structural requirements, and mechanisms for their neuroprotective effects have

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doi:10.1016/j.bmcl.2008.05.106

not been fully understood. Although many synthetic variations of isoflavones have been reported,[3,4,24,25] there are few reports with quinolone analogs, so-called azaisoflavones which were replaced oxygen of isoflavone skeletons with nitrogen. In our efforts to develop potent and novel anti-inflammatory neuroprotectors, we designed and synthesized azaisoflavones which was introduced extra substituents at nitrogen of the isoflavone skeleton (Fig. 2).

Synthesis of azaisoflavones is depicted in Scheme 1. Friedel–Craft's acylation of aniline or methoxyaniline with acetonitrile in the presence of boron trichloride and aluminum trichloride gave ketone **1**. Condensation of the resulting ketone **1** with benzaldehyde or *p*-anisaldehyde resulted in the key intermediate, 2′-aminochalcone **2** which was followed by *N*-acetylation to afford 2′-acetamidochalcone **3**. Rearrangement of 2′-acetamidochalcone **3** with thallium nitrate in the presence of trimethyl orthoformate gave β-ketoacetal **4** which was subsequently cyclized under acidic condition at 50 °C to yield quinolone **5**. N-alkylation of the resulting quinolone **5** with alkyl iodide in the presence of base gave alkylated quinolone **7**. Methoxyquinolones **5** and **7** were treated with HBr to obtain hydroxyquinolone **6** and **8**, respectively.

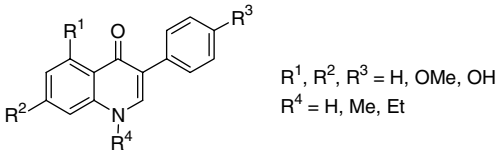
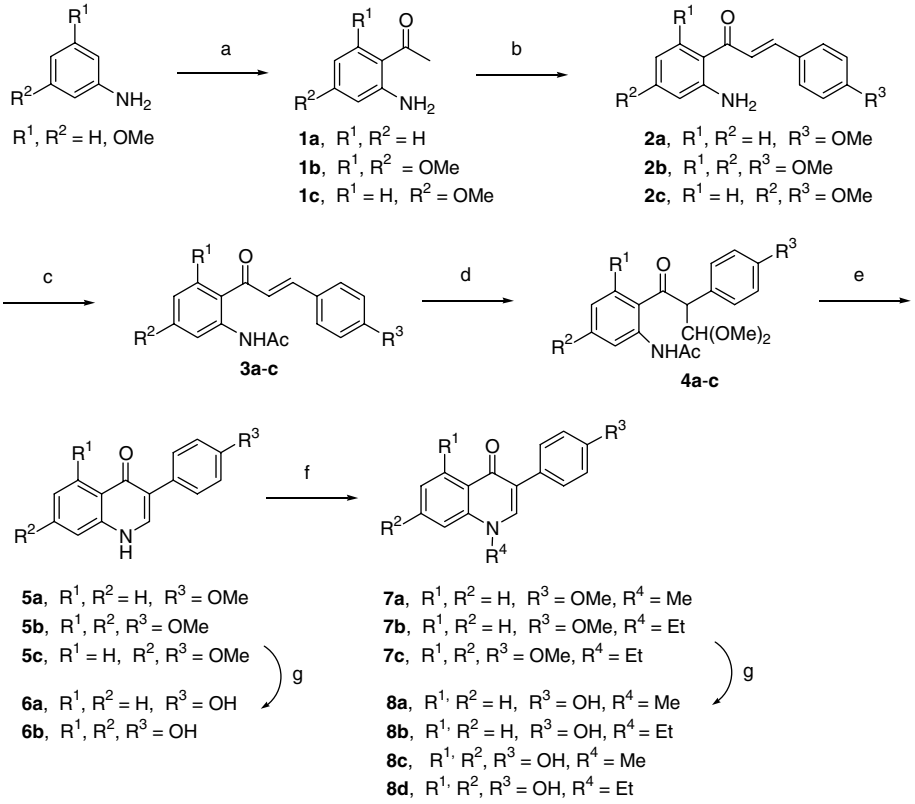


Figure 2. Targeted structure of azaisoflavones.

The inhibitory activities of NO production by azaisoflavones were evaluated in microglia cell culture system.[26] A murine microglia cell line, BV-2 cells, was stimulated with 100 ng/mL of LPS in the presence or absence of samples for 24 h. Nitrite in the culture media, a soluble oxidation product of NO, was determined by the Griess reaction. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

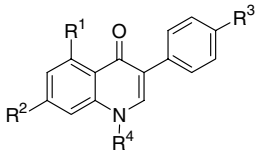
The inhibitory activities of the prepared compounds on NO production are given in Table 1. Inhibitory activities of the effective compounds were ranged in 14.9–58.1% at 10 μM concentrations. The cell viability assay revealed that compounds **5a** and **5c** possess cytotoxicity while the rest of the compounds did not show any significant cytotoxicity at 10 μM concentration. The cytotoxicity of the compound **5a** was removed by either converting the methoxy group to hydroxy (**6a**) or introducing alkyl substituent at N1 position of the isoflavone (**7a** and **7b**). Among the prepared compounds, compound **8d**, which has trihydroxy and *N*-ethyl substituent, was the most potent. Compound **8d** inhibited the NO production in a dose-dependent manner with IC_{50} value of 7.83 μM in LPS-activated BV-2 cells. Inhibitory activity of compound **8d** was higher than that of L-N^G-monomethylarginine (L-NMMA), a classical iNOS inhibitor (Fig. 3).

It has been found that the activity of hydroxy-substituted azaisoflavones were superior to methoxy-substituted ones as compared compounds **6a, b**, and **8a, b, d** with **5a, b** and **7a, b, d**, respectively. The effects of substituents at N1 position of azaisoflavone were investigated. Introduction of *N*-alkyl substituents on compound **5a** eliminated cytotoxicity and brought out the activity in both cases of methyl and ethyl substitutions. However, the *N*-alkyl substitution of **6a** and **6b**, which already have



Scheme 1. Synthesis of 5,7-dihydroxy-3-(4-hydroxyphenyl)-1-alkyl-1*H*-quinolin-4-one. Reagents and conditions: (a) CH_3CN, BCl_3, $AlCl_3$, dichloroethane, 80 °C, 20 h; (b) benzaldehyde or *p*-anisaldehyde, NaOH, EtOH, rt, 10 h; (c) Ac_2O, pyridine, rt, 3 h; (d) thallium(III) nitrate, $HC(CCH_3)_3$, rt, 4 h; (e) 5% HCl, EtOH, 50 °C, 2 h; (f) R_4I, K_2CO_3, DMF, rt, 3 h; (g) HBr, AcOH, 80 °C, 3 days.

Table 1
Inhibitory activities of azaisoflavones on the NO production in LPS-activated microglia



Compound	R^1	R^2	R^3	R^4	Inh.[a] (%)	IC_{50} (μM)
5a	H	H	OMe	H	na[b]	—
5b	OMe	OMe	OMe	H	23.2	20.5
5c	H	OMe	OMe	H	na	—
6a	H	H	OH	H	29.5	15.6
6b	OH	OH	OH	H	45.0	10.9
7a	H	H	OMe	Me	31.9	18.8
7b	H	H	OMe	Et	14.9	25.8
7c	OMe	OMe	OMe	Et	27.3	17.6
8a	H	H	OH	Me	47.4	9.91
8b	H	H	OH	Et	27.0	18.5
8c	OH	OH	OH	Me	39.3	13.3
8d	OH	OH	OH	Et	58.1	7.83

[a] Values mean the inhibition of NO production relative to the LPS control at 10 μM concentration of compounds ($n = 3$).
[b] Not active but only toxic.

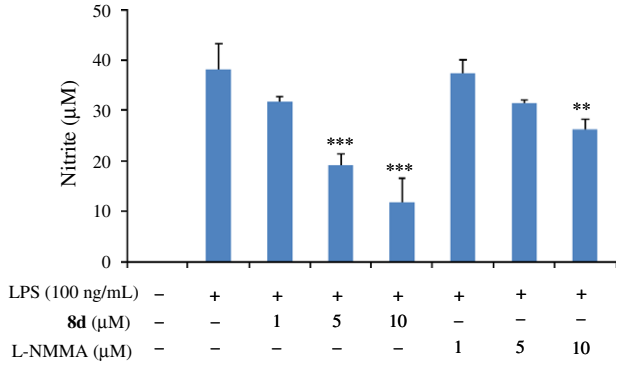


Figure 3. Inhibitory effect of **8d** and L-NMMA on NO production in LPS-activated BV-2 cells. Nitrite was measured by Griess method after 24 h treatment with **8d** and L-NMMA as positive control (1, 5, and 10 μ M) during LPS-activation. Data were presented as means ± SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant differences from treatment with LPS alone.

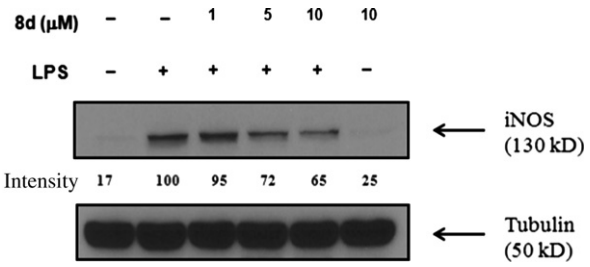


Figure 4. Effect of compound **8d** on the expression of iNOS protein in LPS-stimulated BV-2 cells. BV-2 cells were treated for 6 h with **8d** (1–10 μM) during LPS (100 ng/mL) activation. Equal amounts of protein loading was confirmed by blotting with anti-tubulin. The results are representatives of three independent experiments.

hydroxy substituents on the azaisoflavone ring, did not show any meaningful effect on the activity and even decreased the activity.

To further understand the mechanism of the action of **8d**, we investigated the effect of **8d** on the expression of iNOS protein in LPS-activated BV-2 cells by Western blot analysis. As shown in Figure 4, compound **8d** strongly inhibited expression of iNOS in dose-dependent manners. These results suggested that the inhibition of LPS-induced NO production by compound **8d** occurred mainly through inhibition of iNOS expression.

In summary, we synthesized azaisoflavone derivatives and evaluated their inhibitory activities of NO production in LPS-activated microglia. Among the prepared compounds, *N*-ethyltrihydroxy azaisoflavone **8d** was the most potent. Compound **8d** inhibited NO production through the suppression of iNOS expression. Further study of the structural modification, underlying mechanism and biological activity in vivo are underway. Our result implies the prepared azaisoflavones can act as good leads for the development of useful therapeutic agents to modulate inflammatory damage caused by activated microglia.

Acknowledgments

This work was supported by Grant (R01-2006-000-10515-0) from the Basic Research Program of the Korea Science & Engineering Foundation and Seoul R&BD Program (10541).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.106.

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