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Structural Requirements of Flavonoids for Nitric Oxide Production Inhibitory Activity and Mechanism of Action

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Abstract—To clarify the structure–activity relationships of flavonoids for nitric oxide (NO) production inhibitory activity, we examined the inhibitory effects of 73 flavonoids on NO production in lipopolysaccharide-activated mouse peritoneal macrophages. Among those flavonoids, apigenin (IC_{50} = 7.7 μM), diosmetin (8.9 μM), and tetra-*O*-methylluteolin (2.4 μM), and hexa-*O*-methylmyricetin (7.4 μM) were found to show potent inhibitory activity, and the results suggested the following structural requirements of flavonoids: (1) the activities of flavones were stronger than those of corresponding flavonols; (2) the glycoside moiety reduced the activity; (3) the activities of flavones were stronger than those of corresponding flavanones; (4) the flavones and flavonols having the 4′-hydroxyl group showed stronger activities than those lacking the hydroxyl group at the B ring and having the 3′,4′-dihydroxyl group; (5) the flavonols having the 3′,4′-dihydroxyl group (catechol type) showed stronger activities than those having the 3′,4′,5′-trihydroxyl group (pyrogallol type); (6) the 5-hydroxyl group tended to enhance the activity; (7) methylation of the 3-, 5-, or 4′-hydroxyl group enhanced the activity; (8) the activities of isoflavones were weaker than those of corresponding flavones; (9) methylation of the 3-hydroxyl group reduced the cytotoxicity. In addition, potent NO production inhibitors were found to inhibit induction of inducible nitric oxide synthase (iNOS) without iNOS enzymatic inhibitory activity.

Introduction

The inorganic free radical nitric oxide (NO) has been implicated in physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation.[1] NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). In the NOS family, inducible NOS in particular is involved in pathological overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS) in various cell types including macrophages, endothelial cells, and smooth muscle cells. Nuclear factor (NF)-κB is a major transcription factor involved in iNOS, TNF-α, IL-1β, and IL-8 gene expression. NF-κB activation involves dissociation of an inhibitory subunit, IκB, which keeps NF-κB in the cytoplasm, thereby preventing activation of the target gene in the nucleus. Cellular signals lead to phosphorylation of IκB following elimination of IκB from NF-κB by proteolytic degradation. Then, the activated-NF-κB is released and translocated into the nucleus to activate transcription of its target genes.[2] Therefore, inhibition of iNOS enzyme activity or iNOS induction and inhibition of NF-κB activation may be of therapeutic benefit in various types of inflammation.[2,3]

Previously, we reported that various types of NO production inhibitors were isolated from several natural medicines, such as higher unsaturated fatty acids,[4] polyacetylenes,[5,6] coumarins,[5] flavonoids,[6–8] stilbenes,[9] lignans,[10] sesquiterpenes,[6,7,11–14] diterpenes,[8] triterpenes,[13,15] diarylheptanoids,[15–17] and alkaloids.[18] Although flavonoids, which are widely distributed in plant kingdom, have been recognized to show various biological activities including NO inhibitory activity, the structure–activity relationships of flavonoids for the biological activities including NO production inhibitory activity were not discussed satisfactorily because of insufficient numbers of examples. We previously reported the structural requirements of flavonoids for aldose reductase[19] and antigen-induced degranulation in rat basophilic leukemia (RBL-2H3) cells.[20] In the present

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study, we examined the effects of 73 flavonoids on NO production in LPS-activated mouse peritoneal macrophages and clarified several structure–activity relationships of flavonoids for NO production inhibitory activity. Furthermore, effects of the active compounds, apigenin (**8**), diosmetin (**12**), tetra-*O*-methylluteolin (**15**), and hexa-*O*-methylmyricetin (**45**), on induction of iNOS and iNOS enzymatic activity were described to clarify their mechanism of action.

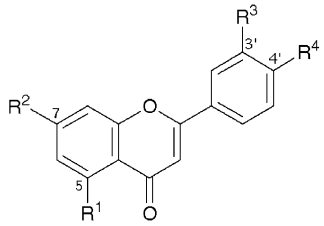
Results and Discussion

Structural requirements of flavonoids for NO production inhibitory activity

To clarify the structure–activity relationships of flavonoids for NO production, we examined the inhibitory effects of 73 flavonoids on NO production in LPS-activated mouse peritoneal macrophages. Among them, apigenin (**8**, $IC_{50} = 7.7$ μM), diosmetin (**12**, 8.9 μM), tetra-*O*-methylluteolin (**15**, 2.4 μM), and hexa-*O*-methylmyricetin (**45**, 7.4 μM) showed potent inhibitory activity (< 10 μM). The IC_{50} values of reference compounds, N^G-monomethyl-L-arginine (L-NMMA, a non-selective inhibitor of NOS), guanidinoethyldisulfide (GED, an inhibitor of iNOS), and caffeic acid phenethyl ester (CAPE, an inhibitor of NF-κB activation) were 28, 1.4, and 4.0 μM, respectively.[11] These results indicated that NO production inhibitory activities of above flavonoids were equipotent to those of the reference compounds.

Next, comparison of flavones (Table 1) with flavonols (Table 2) revealed that the activities of flavones were stronger than those of corresponding flavonols [kaempferol (**21**, 29 μM) $<$ **8**, quercetin (**23**, 36 μM) $<$ luteolin (**11**, 20 μM), tamarixetin (**25**, 25 μM) $<$ **12**, and ombuine (**27**, >30μM) $<$ pilloin (**13**, 11 μM)]. As shown in Tables 2 and 3, methylation of the 3-hydroxyl group enhanced the activity [rhamnetin (**24**, 42 μM) $<$ **26** (15 μM), **27** $<$ ayanin (**28**, 19 μM), and **41** (70 μM) $<$ **42** (43 μM)]. Whereas the cytotoxic effects of the 3-methylflavonols were weaker than those of corresponding flavonols in the MTT assay. Similarly, as shown in Tables 1–3, methylation of the 5-hydroxyl group enhanced the activity [**14** (11 μM) $<$ **15** and **30** (79 μM) $<$ penta-*O*-methylquercetin (**31**, 26 μM)], and methylation of the 4′-hydroxyl group also enhanced the activity [**11** $<$ **12**, **23** $<$ **25**, and myricetin (**39**, 99 μM) $\leqq$ mearnsetin (**40**, 84 μM)]. In addition, the flavones having the 5-hydroxyl moiety tended to show stronger activities than those lacking the 5-hydroxyl moiety [7-hydroxyflavone (**2**, 34 μM) $\leqq$ chrysin (**3**, 31 μM), 4′,7-dihydroxyflavone (**5**, 14 μM) $<$ **8**, and 3′,4′,7-trihydroxyflavone (**7**, 26 μM) $\leqq$ **11**]. Whereas, the flavones and flavonols having the 4′-hydroxyl group showed stronger activities than those lacking the hydroxyl group at the B ring [**2** $<$ **5** and **3** $<$ **8**] and having the 3′,4′-dihydroxyl moiety [**7** $<$ **5**, **11**

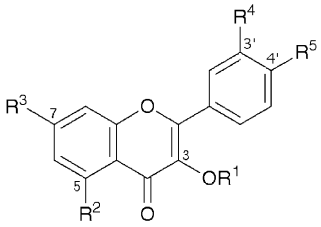
Table 1. Effects of flavones on NO production inhibitory activity in LPS-activated mouse peritoneal macrophages



	R^1	R^2	R^3	R^4	IC_{50} (μM)
Flavone (**1**)	H	H	H	H	52
7-Hydroxyflavone (**2**)	H	OH	H	H	34
Chrysin (**3**)	OH	OH	H	H	31
Tectochrysin (**4**)	OH	OCH_3	H	H	23
4′,7-Dihydroxyflavone (**5**)	H	OH	H	OH	14
3′,4′-Dihydroxyflavone (**6**)	H	H	OH	OH	23
3′,4′,7-Trihydroxyflavone (**7**)	H	OH	OH	OH	26
Apigenin (**8**)	OH	OH	H	OH	7.7[d]
Apigenin 7-*O*-Glc (**9**)	OH	*O*-Glc	H	OH	>100 (20)[e]
Acacetin 7-*O*-Rut (**10**)	OH	*O*-Rut	H	OCH_3	>100 (13)[e]
Luteolin (**11**)	OH	OH	OH	OH	20[e]
Diosmetin (**12**)	OH	OH	OH	OCH_3	8.9[d]
Pilloin (**13**)	OH	OCH_3	OH	OCH_3	11
14	OH	OCH_3	OCH_3	OCH_3	11
15	OCH_3	OCH_3	OCH_3	OCH_3	2.4[d]
Luteolin 7-*O*-Glc (**16**)	OH	*O*-Glc	OH	OH	>100 (43)[e]
Luteolin 7-*O*-GlcA (**17**)	OH	*O*-GlcA	OH	OH	>100 (40)[e]
Diosmetin 7-*O*-Glc (**18**)	OH	*O*-Glc	OH	OCH_3	100

Glc, β-D-glucopyranosyl; GlcA, β-D-glucopyranosiduronic acid; Rut, Glc(6→1)Rha.
[a,b,c] Values in parentheses represent the inhibition (%) at [a]10 μM, [b]30 μM, or [c]100 μM. [d,e] Cytotoxic effects were observed at [d]30 μM or [e]100 μM.

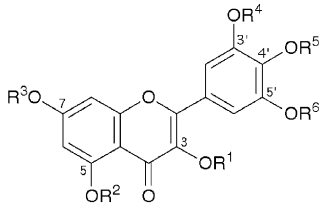
Table 2. Effects of flavonols on NO production inhibitory activity in LPS-activated mouse peritoneal macrophages—1



	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)
3-Hydroxyflavone (**19**)	H	H	H	H	H	>10 (−2)[a,d]
Izalpinin (**20**)	H	OH	OCH_3	H	H	>30 (13)[b,e]
Kaempferol (**21**)	H	OH	OH	H	OH	29
Kaempferol 3-*O*-GlcA (**22**)	GlcA	OH	OH	H	OH	>100 (4)[c]
Quercetin (**23**)	H	OH	OH	OH	OH	36
Rhamnetin (**24**)	H	OH	OCH_3	OH	OH	42
Tamarixetin (**25**)	H	OH	OH	OH	OCH_3	25
26	CH_3	OH	OCH_3	OH	OH	15
Ombuine (**27**)	H	OH	OCH_3	OH	OCH_3	>30 (21)[b,e]
Ayanin (**28**)	CH_3	OH	OCH_3	OH	OCH_3	19
29	H	OH	OCH_3	OCH_3	OCH_3	>10 (20)[a,d]
30	CH_3	OH	OCH_3	OCH_3	OCH_3	79
31	CH_3	OCH_3	OCH_3	OCH_3	OCH_3	26
Isoquercitrin (**32**)	Glc	OH	OH	OH	OH	>100 (3)[c]
Hyperin (**33**)	Gal	OH	OH	OH	OH	>100 (16)[c]
Quercetin 3,7-di-*O*-Glc (**34**)	Glc	OH	*O*-Glc	OH	OH	>100 (9)[c]
Rutin (**35**)	Rut	OH	OH	OH	OH	>100 (1)[c]
Rhamnetin 3-*O*-Rut (**36**)	Rut	OH	OCH_3	OH	OH	>100 (−3)[c]
Ombuine 3-*O*-Rut (**37**)	Rut	OH	OCH_3	OH	OCH_3	>100 (12)[c]
38	Rut	OH	OCH_3	OCH_3	OCH_3	>100 (1)[c]

Glc, β-D-glucopyranosyl; GlcA, β-D-glucopyranosiduronic acid; Gal: β-D-galactopyranosyl; Rut, Glc(6→1)Rha.
[a,b,c] Values in parentheses represent the inhibition (%) at [a]10 μM, [b]30 μM, or [c]100 μM. [d,e] Cytotoxic effects were observed at [d]30 μM or [e]100 μM.

Table 3. Effects of flavonols on NO production inhibitory activity in LPS-activated mouse peritoneal macrophages—2



	R^1	R^2	R^3	R^4	R^5	R^6	IC_{50} (μM)
Myricetin (**39**)	H	H	H	H	H	H	99
Mearnsetin (**40**)	H	H	H	H	CH_3	H	84
41	H	H	CH_3	H	H	H	70
42	CH_3	H	CH_3	H	H	H	43
43	H	H	CH_3	H	CH_3	H	24[e]
44	H	H	CH_3	CH_3	CH_3	H	>10 (2)[a,d]
45	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	7.4
Myricitrin (**46**)	Rha	H	H	H	H	H	>100 (15)[c]
47	Rha	H	CH_3	H	CH_3	H	>100 (10)[c]
48	Rha	H	H	CH_3	CH_3	H	>100 (9)[c]
49	Rha	H	CH_3	CH_3	CH_3	H	56
50	Rha	H	CH_3	CH_3	CH_3	CH_3	62

Rha, α-L-rhamnopyranosyl.
[a,b,c] Values in parentheses represent the inhibition (%) at [a]10 μM, [b]30 μM, or [c]100 μM.
[d,e] Cytotoxic effects were observed at [d]30 μM or [e]100 μM.

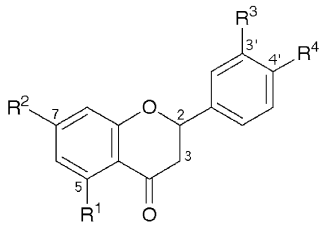
<8, and **23** ≦ **21**], and the flavonols having the 3′,4′-dihydroxyl group showed stronger activities than those having the 3′,4′,5′-trihydroxyl group [**40** <**25**, **39** <**23**, **41** <**24**, and **42** <**26**].

Furthermore, comparison of flavones with flavanones (Table 4) revealed that the 2–3 double bond moiety enhanced the activity [flavanone (**51**, >100 μM) <flavone (**1**, 52 μM), liquiritigenin (**52**, 85 μM) <**5**, and eriodictyol (**56**, >100 μM) <**11**]. As shown in Table 5, isoflavones were weaker than those of corresponding flavones [daidzein (**59**, 33 μM) <**5** and genistein (**61**, 26 μM) <**8**]. As shown in Tables 1–6, glycosidic compounds (**9**, **10**, **16**, **17**, **22**, **32**–**38**, **46**–**48**, **54**, **55**, **57**, **58**, **60**, **62**, **64**, **65**, and **67**–**69**) and flavan-3-ols (**71** and **72**) lacked the activity (>100 μM), expect for diosmetin 7-*O*-β-D-glucopyranoside (**18**, 100 μM), **49** (56 μM), **50** (62 μM), and (−)-epigallocatechin (**73**, 65 μM).

Recently, Kim et al. examined the naturally occured flavonoids for NO production inhibitory activity in LPS-activated RAW 264.7 cells and discussed following structural requirements: (a) the strongly active flavonoids possessed the 2–3 double bond and 5,7-dihydroxyl group; (b) the 8-methoxyl group and 4′- or 3′,4′-vicinal substitutions favorably affected inhibitory activity; (c) the 2′,4′-(*meta*)-hydroxyl substitution abolished the inhibitory activity; (d) the 3-hydroxyl moiety reduced the activity; (e) flavonoid glycosides were not active regardless of the types of aglycons.[21]

In the present study, the results of large number of flavonoids using mouse peritoneal macrophages were in agreement with the previous report by Kim et al., except for the 2′,4′-dihydroxyl substituted flavonoids, and the structural requirements of flavonoids for NO production

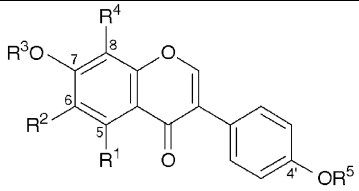
Table 4. Effects of flavanones on NO production inhibitory activity in LPS-activated mouse peritoneal macrophages



	R^1	R^2	R^3	R^4	IC_{50} (μM)
Flavanone (**51**)	H	H	H	H	> 100 (48)[c]
Liquiritigenin (**52**)	H	OH	H	OH	85
53	H	OCH_3	H	OH	38
Liquiritin (**54**)	H	OH	H	*O*-Glc	> 100 (21)[c]
55	H	OCH_3	H	*O*-Glc	> 100 (32)[c]
Eriodictyol (**56**)	OH	OH	OH	OH	> 100 (38)[c]
(2*S*)-Eriodictyol 7-*O*-GlcA (**57**)	OH	O-GlcA	OH	OH	> 100 (26)[c]
(2*R*)-Eriodictyol 7-*O*-GlcA (**58**)	OH	O-GlcA	OH	OH	> 100 (19)[c]

Glc, β-D-glucopyranosyl; GlcA, β-D-glucopyranosiduronic acid.
[a,b,c] Values in parentheses represent the inhibition (%) at [a]10 μM, [b]30 μM, or [c]100 μM.

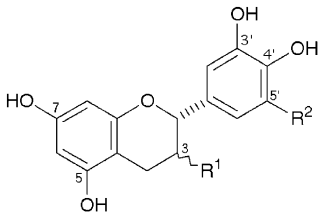
Table 5. Effects of isoflavones on NO production inhibitory activity in LPS-activated mouse peritoneal macrophages



	R^1	R^2	R^3	R^4	R^5	IC_{50} (μM)
Daidzein (**59**)	H	H	H	H	H	33
Daidzin (**60**)	H	H	Glc	H	H	> 100 (28)[c]
Genistein (**61**)	OH	H	H	H	H	26
Genistin (**62**)	OH	H	Glc	H	H	> 100 (26)[c]
Tectorigenin (**63**)	OH	OCH_3	H	H	H	31
Tectoridin (**64**)	OH	OCH_3	Glc	H	H	> 100 (9)[c]
Tectorigenin 7-*O*-Glc-Xyl (**65**)	OH	OCH_3	Glc(6→1)Xyl	H	H	> 100 (1)[c]
Glycitein (**66**)	H	OCH_3	H	H	H	> 100 (45)[c]
Glycitin (**67**)	H	OCH_3	Glc	H	H	> 100 (24)[c]
Glycitein 7-*O*-Glc-Xyl (**68**)	H	OCH_3	Glc(6→1)Xyl	H	H	> 100 (12)[c]
Puerarin (**69**)	H	H	H	Glc	H	> 100 (12)[c]
Biochanin A (**70**)	OH	H	H	H	CH_3	30

Glc, β-D-glucopyranosyl; Xyl, β-D-xylopyranosyl.
[a,b,c] Values in parentheses represent the inhibition (%) at [a]10 μM, [b]30 μM, or [c]100 μM.

Table 6. Effects of flavan-3-ols on NO production inhibitory activity in LPS-activated mouse peritoneal macrophages



	R^1	R^2	IC_{50} (μM)
(+)-Catechin (**71**)	β-OH	H	> 100 (−2)[c]
(−)-Epicatechin (**72**)	α-OH	H	> 100 (33)[c]
(−)-Epigallocatechin (**73**)	α-OH	OH	65

[a,b,c] Values in parentheses represent the inhibition (%) at [a]10 μM, [b]30 μM, or [c]100 μM.

inhibitory activity were suggested as follows: (1) the activities of flavones were stronger than those of corresponding flavonols; (2) the glycoside moiety reduced the activity; (3) the activities of flavones were stronger than those of corresponding flavanones; (4) the flavones and flavonols having the 4′-hydroxyl group showed stronger activities than those lacking the hydroxyl group at the B ring and having the 3′,4′-dihydroxyl group; (5) the flavonols having the 3′,4′-dihydroxyl group (catechol type) showed stronger activities than those having the 3′,4′,5′-trihydroxyl group (pyrogallol type); (6) the 5-hydroxyl group tended to enhance the activity; (7) methylation of the 3, 5, or 4′-hydroxyl group enhanced the activity; (8) the activities of isoflavones were weaker than those of corresponding flavones; (9) methylation of the 3-hydroxyl group reduced the cytotoxicity.

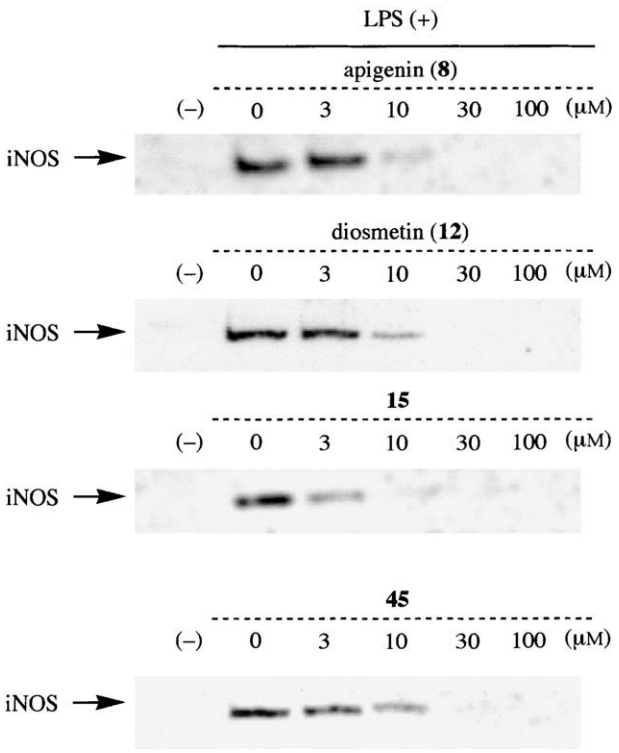


Figure 1. Effects of apigenin (**8**), disometin (**12**), **15**, and **45** on iNOS induction in LPS-activated mouse macrophages.

Effects of 8, 12, 15, and 45 on iNOS induction and iNOS enzyme activity

The effects of four potent active flavonoids (**8**, **12**, **15**, and **45**) on iNOS induction and iNOS enzyme activity were examined. iNOS protein was detected at 130 kDa after 12-h incubation with LPS by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-Western blotting analysis. Inhibitory effects of wogonin and **11** using RAW 264.7 cells and **8** using J774A.1 cells on iNOS induction were reported previously.[21,22] In agreement with the previous studies, **8** suppressed iNOS induction in LPS-activated macrophages. In addition, other active flavonoids (**12**, **15**, and **45**) also suppressed the iNOS induction in a concentration-dependent manner (Fig. 1). However, these compounds did not inhibit iNOS enzyme activity (L-NMMA, $IC_{50} = 31$ μM).

These findings suggested that the active flavonoids (**8**, **12**, **15**, and **45**) inhibited the upstream signaling pathway of NF-κB activation followed by iNOS expression, thereby preventing NO production.

In conclusion, inhibitory activities of 73 flavonoids against NO production in LPS-activated macrophages were examined and some structural requirements (vide ante) were clarified. Furthermore, the active flavonoids [apigenin (**8**), diosmetin (**12**), tetra-*O*-methylluteolin (**15**), and hexa-*O*-methylmyricetin (**45**)] inhibited iNOS induction without iNOS enzyme activity. On the basis of above-mentioned evidence, those active flavonoids (**8**, **12**, **15**, and **45**) may be useful for the treatment of various inflammatory diseases.

Experimental

Preparations of flavonoids

All test flavonoids were prepared with some chemical modifications, as described previously.[19,20]

Reagents

Lipopolysaccharide (LPS, from *Salmonella enteritidis*) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was from Dojindo; caffeic acid phenethyl ester (CAPE) and guanidinoethyldisulfide (GED) were from Calbiochem; RPMI 1640 was from Gibco; protease inhibitor cocktail was from Boehringer Mannheim; anti-mouse iNOS antibody (monoclonal) was from Transduction Laboratories; anti-mouse IgG antibody conjugated to horseradish peroxidase and the enhanced chemiluminescense (ECL™) kit were from Amersham; thioglycolate (TGC) medium was from Nissui Seiyaku; iNOS (from mouse macrophages) was from Cayman; other chemicals were from Wako. Nitrocellulose membranes (0.25 μm) were purchased from Bio Rad; 96-well microplates and culture dishes (6 cm) were from Nunc; and centrifugal filter units (Ultrafree™-MC) were from Milipore.

Screening test for NO production

Screening test for NO production was performed as described previously.[4–17] Briefly, peritoneal exudate cells (5×10^5 cells/well) collected from the peritoneal cavities of male ddY mice and were suspended in 200 μL of RPMI 1640 supplemented with 5% fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent.

Cytotoxicity was determined by MTT colorimetric assay. Briefly, after 20 h incubation with test compounds, MTT (10 μL, 5 mg/mL in PBS) solution was added to the wells. After a further 4 h in culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). When O.D. of sample-treated group reduced below 80% of O.D. in the vehicle-treated group, the test compound was considered to exhibit cytotoxic effect. L-NMMA, GED, and CAPE were used as reference compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated by the following formula and IC_{50} was determined graphically ($N = 4$).

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

A−C : NO_2^- concentration (μM)

[A : LPS (+), sample (−); B : LPS (+), sample (+); C : LPS (−), sample (−)]

Detection of iNOS

Detection of iNOS were performed as described previously with slight modifications.[8–11,15] TGC-Induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h. After washing, the culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/mL LPS and test compound for 12 h. Cells were collected in lysis buffer [150 mM NaCl, 10 mM Tris, protease inhibitor cocktail (1 tab/50 mL), 0.1% Triton X-100, 2 mM EGTA, pH 7.4] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer. For SDS-PAGE, aliquots of 40 μg of protein from each sample were subjected to electrophoresis in 7.5% polyacrylamide gels. Following electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membrane. The membrane were incubated with 5% nonfat dried milk in Tris-buffered saline (T-TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in T-TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL™ and X-ray film (Hyperfilm-ECL™, Amersham).

iNOS enzyme activity

NOS activity was measured by monitoring the conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline, as described previously with a slight modification.[9a,10,14] Briefly, test sample solution (5 μL) and 40 μL of substrate and coenzyme solution [100 μM L-arginine (containing 50 nCi L-[^{14}C]-arginine), 1 mM NADPH, 3 μM tetrahydrobiopterin (BH_4), 1 μM flavin adenine dinucleotide (FAD), 1 μM flavin mononucleotide (FMN) in 25 mM Tris–HCl buffer (pH 7.4)] were pre-incubated at 37 °C for 5 min. iNOS (20 mU/5 μL) was then added to the reaction mixture. After incubation at 37 °C for 20 min, the reaction was terminated by addition of 400 μL of cold buffer containing 5 mM EDTA and 50 mM HEPES (pH 5.5). The substrate was adsorbed on AG 50W X-8 ion-exchange resin (Na^+ form, 60–70 mg) packed in centrifugal filter units. The L-citrulline, which is ionically neutral at pH 5.5, flowed through the column completely, and was mixed with a scintillation cocktail and radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman). Test compound was dissolved in DMSO and diluted with Tris–HCl buffer (pH 7.4) (final DMSO concentration was 2%).

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