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Inhibition of Nitric Oxide Production from Lipopolysaccharide-Treated RAW 264.7 Cells by Synthetic Flavones:Structure-Activity Relationship and Action Mechanism

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Recent investigations have shown that certain flavonoids, especially flavone derivatives, inhibit nitric oxide (NO) production by inducible NO synthase (iNOS) in macrophages, which contribute their anti-inflammatory action. For the purpose of finding the optimized chemical structures of flavonoids that inhibit NO production, various A- and B-ring substituted flavones were synthesized and evaluated for their inhibitory activity using lipopolysaccharide-treated RAW 264.7 cells. It was found that the optimal chemical structures were A-ring 5,7-dihydroxyflavones having the B-ring 2',3'-dihydroxy or 3',4'-dihydroxy or 3',4'-hydroxy/methoxy (methoxy/hydroxy) groups. These structurally optimized compounds were revealed to be down-regulators of iNOS induction, but not direct iNOS inhibitors. Of these derivatives that were evaluated, 2',3',5,7-tetrahydroxyflavone and 3',4',5,7-tetrahydroxyflavone (luteolin) showed the strongest inhibition. The IC_{50} values for these compounds were 19.7 and 17.1 μM, respectively. Therefore, these compounds may have a potential as new anti-inflammatory agents.

Key words: Flavonoid, Nitric oxide, Inducible nitric oxide synthase, Anti-inflammation, Structure-activity relationship

INTRODUCTION

Of various proinflammatory molecules, nitric oxide (NO) produced by inducible NO synthase (iNOS) is speculated to participate in provoking and maintaining some inflammatory disorders such as rheumatoid arthritis. Inhibitors of iNOS inhibitors, including L-N^6-iminoethyllysine and 7-nitroindazole, were reported to show the inhibitory activity against an animal model of inflammation (Handy and Moore, 1998). Therefore, it is reasonably thought that an inhibition of iNOS activity and/or a down-regulation of the iNOS expression may possibly become one of the new therapeutic strategies for the treatment of inflammatory disorders.

In order to find new iNOS inhibitors or down-regulators of iNOS induction, various studies have been carried out and certain flavonoids have been found to show varying degrees of inhibitory activity against NO production by iNOS from macrophages or macrophage-like cell lines including RAW 264.7 cells treated with lipopolysaccharide (LPS) or LPS plus cytokines (Krol *et al.*, 1995; Soliman and Mazzio, 1998; Kim *et al.*, 1999; Liang *et al.*, 1999; Murakami *et al.*, 2000). Despite several flavonol derivatives (quercetin and kaempferol) inhibiting NO production (Kim *et al.*, 1999; Liang *et al.*, 1999; Wadsworth and Koop, 1999), the most active flavonoid compounds reported are the flavone derivatives (Sadowska-Krowicka *et al.*, 1998; Kim *et al.*, 1999; Liang *et al.*, 1999; Wakabayashi, 1999; Chi *et al.*, 2001). Examples are apigenin, luteolin, and wogonin (Table I). These compounds were found to inhibit NO production mainly through the down-regulation of iNOS expression, but not by the direct inhibition of iNOS enzyme activity. Some prenylated flavanones and biflavones also inhibited NO production by iNOS (Cheon *et al.*, 2000). All these results suggest that certain flavonoids may attenuate inflammation by modulating the expression of proinflammatory gene, iNOS, and may have a potential for being new type of anti-inflammatory agents. To date, however, no attempt has been made to find the optimized chemical structures based on the flavone molecule. Therefore, the inhibitory activity of NO production by various synthetic flavone derivatives was evaluated,

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and their action mechanisms were also studied in the present investigation.

MATERIALS AND METHODS

Chemicals

2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazinehydrochloride (AMT) was purchased from Tocris Cookson Ltd. (UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LPS (*Escherichia coli* 0127:B8) were purchased from Sigma Chem. Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were products of Gibco BRL (Grand Island, NY). The protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA). Wogonin was isolated from Scutellaria radix according to the previously described procedures (You *et al.*, 1999). All synthetic flavonoid derivatives that were used in this study were chemically synthesized and structurally identified according to the previous reports (Dao *et al.*, 2003; Dao *et al.*, 2004), and the purity was always > 95% (w/w).

RAW 264.7 cell culture and measurement of NO concentration

RAW 264.7 cells obtained from American Type Culture Collection were cultured with DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO_2 at 37°C. Cells were activated with LPS according to the previously described procedures (Kim *et al.*, 1999). Briefly, cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2 h, test compounds including LPS (1 μg/mL) were added and incubated for 24 h, unless otherwise specified. Test compounds were dissolved in DMSO and were diluted with serum-free DMEM into appropriate concentrations. Cell viability was assessed with the MTT assay based on the experimental procedures of Mossmann (1983). For the determination of NO concentration, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4], and the optical density was measured at 550 nm. In order to induce iNOS and determine the inhibitory effects of iNOS activity by flavonoids, cells were pre-incubated with LPS (I μg/mL) for 24 h and completely washed three times with serum-free DMEM. Then, test compounds were added without LPS, and the cells were incubated for another 24 h. From the medium, NO concentration was measured as described above.

Western blot of iNOS

The protein level of iNOS was measured by using the Western blotting technique (Kim *et al.*, 1999). RAW cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 μg/mL) with/without the test compounds. The time course of iNOS expression was previously determined, and maximum induction was found to be 10-20 h after the LPS treatment (Chi *et al.*, 2001). Therefore, cells were harvested 20 h after the LPS/test compounds treatment. After preparing the cell homogenate, the supernatant was obtained through centrifugation at 15,000 g for 30 min. Using a Tris-glycine gel (4-15%, Novex Lab.), electrophoresis was carried out and bands were blotted to PVDF membranes. Antibody against iNOS (N32030, Transduction Lab.) was incubated and bands were visualized by treating with secondary antibody and DAB reagent (Vector Lab.).

Statistical analysis

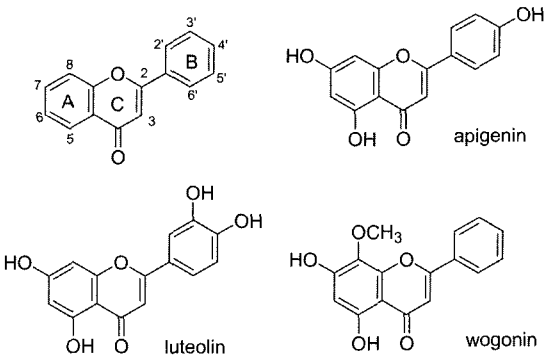
All values were represented as the arithmetic mean± SD. The unpaired Student's *t*-test was used to determine the statistical significance. All experiments were performed at least twice and they gave similar results.

RESULTS

In one typical experiment, RAW 264.7 cells (2×10^5 cells/well) produced a basal level of nitrite (a stable form of NO), 0.2±0.2 μM for 24 h of incubation (n = 3). When LPS (1 μg/mL) was treated, NO production triggered by induced iNOS increased to 33.4±1.5 μM at 24 h (n = 3). Under this condition, test compounds were added simultaneously with LPS, and inhibitory activity of NO production was evaluated.

First, in order to examine the structure-activity relationship based on the A-ring non-substituted flavone structure, various substituents were introduced to the B-ring, and their inhibitory activities of NO production were checked at 10 and 50 μM. As demonstrated in Table I, the non-substituted compound, flavone (**1-1**), did not show any inhibitory activity. Flavone derivatives having a single substitution on C-4' (**1-2**~**1-8**), even with a hydroxy or methoxy substitution, did not inhibit NO production by iNOS from LPS-treated RAW 264.7 cells up to 50 μM. No improvement of inhibitory activity was found with di-substituted flavone having chlorine at C-3',4' (**1-9**). In contrast, vicinal di-substitution at the B-ring C-2',3' or C-3',4' with the hydroxy or methoxy group enhanced the inhibitory activity of NO production (**1-10**, **1-11**, **1-13**~**1-16**). Hydroxy/methoxy (methoxy/hydroxy) substitution on B-ring C-3',4' gave a slightly higher activity than dimethoxy substitution (**1-13** and **1-14** vs. **1-16**). On the other hand, meta di-substitution at B-ring C-2',4' with the hydroxy group (**1-12**) or methoxy group (**1-17**) greatly reduced the inhibitory activity or completely abolished the inhibitory activity. Flavones having more substituents at the B-ring

Table I. Inhibition of NO production from LPS-treated RAW 264.7 cells by the synthetic flavones



Compounds	2'	3'	4'	5'	% inhibition at 10 μM	% inhibition at 50 μM
1-1 (Flavone)	H	H	H	H	-[a]	-
1-2	H	H	Br	H	-	-
1-3	H	H	Cl	H	-	-
1-4	H	H	OH	H	-	-
1-5	H	H	OCH_3	H	-	-
1-6	H	H	SCH_3	H	-	-
1-7	H	H	OCF_3	H	-	-
1-8	H	H	C_6H_5	H	-	-
1-9	H	Cl	Cl	H	-	-
1-10	OH	OH	H	H	48[b]	61
1-11	H	OH	OH	H	34	68
1-12	OH	H	OH	H	-	26
1-13	H	OCH_3	OH	H	-	54
1-14	H	OH	OCH_3	H	-	43
1-15	OCH_3	OCH_3	H	H	-	23
1-16	H	OCH_3	OCH_3	H	-	40
1-17	OCH_3	H	OCH_3	H	-	-
1-18	OCH_3	OCH_3	OCH_3	H	-	33
1-19	H	OCH_3	OCH_3	OCH_3	-	32

Treatment of LPS (1 μg/mL) to RAW cells (2×10^5 cells/well) produced 37.1±0.3 μM nitrite from the basal level of 0.1±0.2 μM nitrite for a 24 h incubation period (n = 3).

$$\% \text{ Inhibition} = \left(1 - \frac{N_{L/T} - N_C}{N_L - N_C}\right) \times 100$$

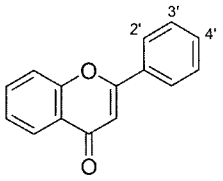
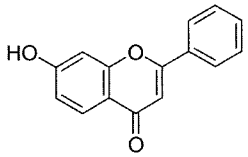
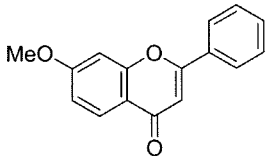
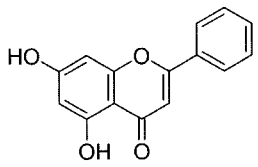
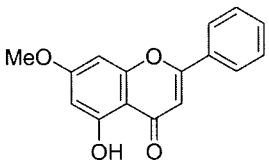
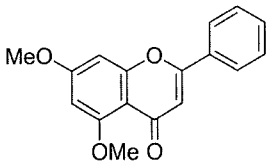
$N_{L/T}$: Nitrite concentration of RAW cells treated with LPS and test compound, N_L: nitrite concentration of RAW cells treated with LPS and vehicle, N_C: nitrite concentration of RAW cells treated with vehicle only. [a]No or less than 20% inhibition, [b]arithmetic mean of triplicate experiments.

including the C-2',3',4'-trimethoxy group (**1-18**) or C-3',4',5'-trimethoxy group (**1-19**), did not considerably increase the inhibitory activity. The substitution at the B-ring by several bulkier groups, such as the phenyl or benzyloxy group, was not favorable (data not shown). In this group of compounds, vicinal dihydroxy substitution at B-ring C-2',3' (**1-10**) or C-3',4' (**1-11**) gave the best results.

For evaluating the effect of A-ring substitution, the compound series of A-ring substituted flavones that have a B-ring hydroxy or methoxy group at C-2',3' or C-3',4' were obtained and examined for their inhibitory activity against NO production from LPS-treated RAW 264.7 cells. As represented in Table II, all A-ring substituted flavones having the B-ring C-2',3'- or C-3',4'-dihydroxy group showed more than 30% of inhibition at 50 μM (**2-11**, **3-11**, **4-10**, **4-11**, **5-11**, **6-11**). On the other hand, meta-dihydroxy substitution in the B-ring gave no activity or reduced activity compared to their vicinal substituents (**2-11** vs. **2-12**, **4-11** vs **4-12**). A-ring substituted flavones with hydroxy/methoxy (methoxy/hydroxy) substitution at B-ring C-3',4' showed a higher activity than flavones with dimethoxy substitution at B-ring C-3',4' (**3-13** and **3-14** vs **3-16**, **5-13** and **5-14** vs **5-16**). When the inhibitory activities of the derivatives having the B-ring C-3',4'-dihydroxy group were compared, the order of inhibition potency were approximately flavones having the A-ring C-5,7-dihydroxy group (**4-11**) > A-ring C-7-methoxy (**3-11**) = no substituent (**1-11**) > A-ring C-7-hydroxy substitution (**2-11**). The flavone derivatives having the A-ring 5-hydroxy-7-methoxy (**5-11**) and 5,7-dimethoxy group (**6-11**) showed a much reduced activity compared with flavone having no substituent at the A-ring (**1-11**). Also, flavones having the B-ring C-2',3'-dihydroxy group (**1-10**, **4-10**) showed potent inhibition as expected. When the concentration-dependency was examined using several selected derivatives, they showed the inhibitory activity of NO production in a concentration-dependent manner (Fig. 1). From this figure, the IC_{50} values were calculated as 43.2, 39.6, 50.0, 35.6, 19.7, and 17.1 μM for **1-10**, **1-11**, **2-11**, **3-11**, **4-10**, and **4-11**, respectively. From these results, the optimal chemical structures of flavones among the synthetic derivatives were determined to be A-ring C-5,7-dihydroxyflavones having the B-ring C-2',3'-dihydroxy, C-3',4'-dihydroxy (luteolin), or C-3',4'-hydroxy/methoxy (methoxy/hydroxy) group, where the formal two compounds are more potent. Within the same concentration range, AMT, a synthetic iNOS inhibitor, potently inhibited NO production. As a comparison, the IC_{50} values for AMT, chrysin (**4-1**, 5,7-dihydroxyflavone), and quercetin (3-hydroxyluteolin) were previously found to be 0.09, > 100, and 107 μM, respectively, under the same experimental conditions (Kim *et al.*, 1999).

Finally, the action mechanism of the selected flavone derivatives was evaluated. To check the inhibitory activity against the iNOS enzyme, the selected flavone derivatives were treated to pre-activated RAW cells with LPS. As shown in Fig. 1, all derivatives that were examined only

Table II. Inhibition of NO production from LPS-treated RAW 264.7 cells by the structurally optimized synthetic flavones

Compounds		2'	3'	4'	% inhibition at 10 μM	% inhibition at 50 μM
AMT					92[a]	93
	**1-10**	OH	OH	H	48[b]	61[b]
	1-11	H	OH	OH	34[b]	68[b]
	1-13	H	OCH_3	OH	-[b,c]	54[b]
	**2-11**	H	OH	OH	-	49
	2-12	OH	H	OH	-	-
	**3-4**	H	H	OH	23	42
	3-11	H	OH	OH	45	67
	3-13	H	OCH_3	OH	20	41
	3-14	H	OH	OCH_3	-	36
	3-15	OCH_3	OCH_3	H	-	-
	3-16	H	OCH_3	OCH_3	-	-
	**4-10**	OH	OH	H	59	85
	4-11 (luteolin)	H	OH	OH	69	80
	4-12	OH	H	OH	-	46
	4-13	H	OCH_3	OH	32	67
	4-14	H	OH	OCH_3	20	60
	**5-11**	H	OH	OH	-	30
	5-13	H	OCH_3	OH	-	30
	5-14	H	OH	OCH_3	-	23
	5-15	OCH_3	OCH_3	H	-	-
	5-16	H	OCH_3	OCH_3	-	-
	**6-4**	H	H	OH	-	-
	6-11	H	OH	OH	-	40
	6-13	H	OCH_3	OH	20	30
	6-14	H	OH	OCH_3	-	49

Treatment of LPS (1 μg/ml) to RAW cells (2×10^5 cells/well) produced 24.8 ± 1.4 μM nitrite from the basal level of 0.4 ± 0.2 μM nitrite for a 24 h incubation period (n = 3).

$$\% \text{ Inhibition} = \left(1 - \frac{N_{L/T} - N_C}{N_L - N_C}\right) \times 100$$

$N_{L/T}$: Nitrite concentration of RAW cells treated with LPS and test compound, N_L: nitrite concentration of RAW cells treated with LPS and vehicle, N_C: nitrite concentration of RAW cells treated with vehicle only.

[a] arithmetic mean from triplicate experiments, [b]Data from Table I, [c]No or less than 20% inhibition.

weakly inhibited NO production by pre-induced iNOS over 10-50 μM, while AMT potently inhibited NO production as expected. These results indicate that the flavone derivatives are not direct iNOS inhibitors. Instead, they clearly suppressed iNOS expression as revealed by Western blotting (Fig. 2). At concentrations of 25 and 50 μM, all selected derivatives showed considerable suppression of iNOS induction. In particular, **4-10** and **4-11** potently

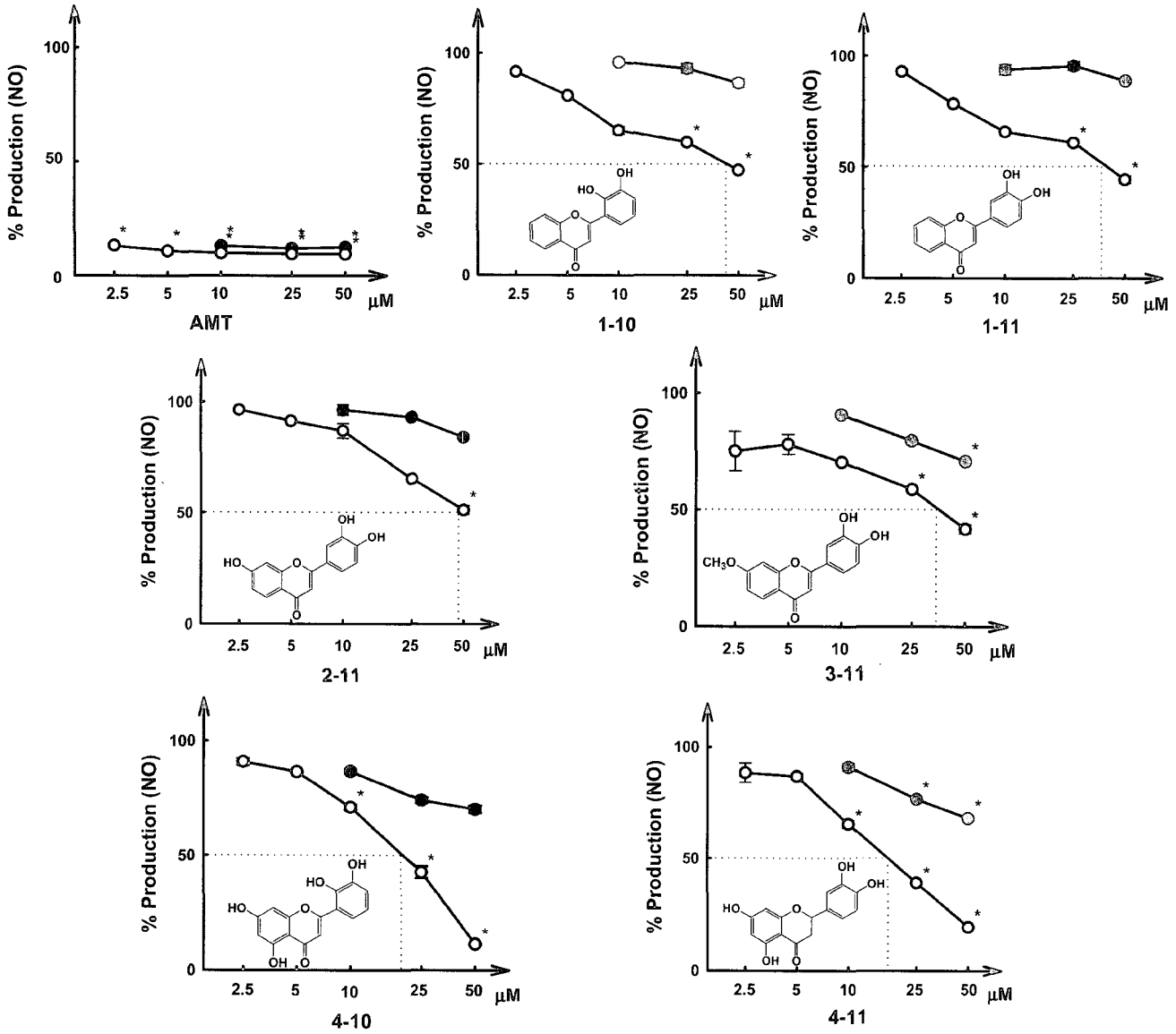


Fig. 1. Inhibition of NO production from LPS-treated RAW 264.7 cells by the selected flavones. (o) The test compounds were added to RAW cells simultaneously with LPS treatment and the cells were incubated for 24 h. The LPS-treated control group produced 34.2 ± 0.6 μM of NO compared to the basal level of 0.2 ± 0.1 μM (n = 3). (•) The test compounds were added to pre-activated cells by LPS treatment and the cells were further incubated for another 24 hrs to measure the direct inhibitory activity against iNOS. The LPS-treated control group produced 27.2 ± 0.3 μM of NO compared to the basal level of 0.1 ± 0.1 μM (n = 3). The points and bars represent the arithmetic mean ± SD from three separate experiments. *: $P < 0.001$, Significantly different from the LPS-treated control group., The dotted lines indicate IC_{50} values.

suppressed iNOS induction. At 50 μM, these compounds almost completely abrogated iNOS induction. Wogonin, which was used as a reference flavonoid also inhibited iNOS expression. In contrast, AMT increased iNOS expression. When the cytotoxicity of the selected flavonoids was checked in LPS-treated RAW cells using the MTT assay, all derivatives except **4-11** (luteolin) showed less than a 20% reduction of viability over 2.5-50 μM (data not shown). Thus, it is reasonably thought that the inhibitory activity of NO production by the tested flavones is not mainly due to the cytotoxic effect on RAW cells. Luteolin, however, showed a 33% reduction in the MTT assay at 50 μM, indicating that the inhibitory effect of NO production at this concentration might be at least partly associated with the cytotoxicity of this compound.

DISCUSSION

The purpose of this study was to find out the optimal chemical structures of flavones that inhibit NO production by iNOS and to evaluate their action mechanism. For this purpose, RAW 264.7 cells, a mouse macrophage-like cell

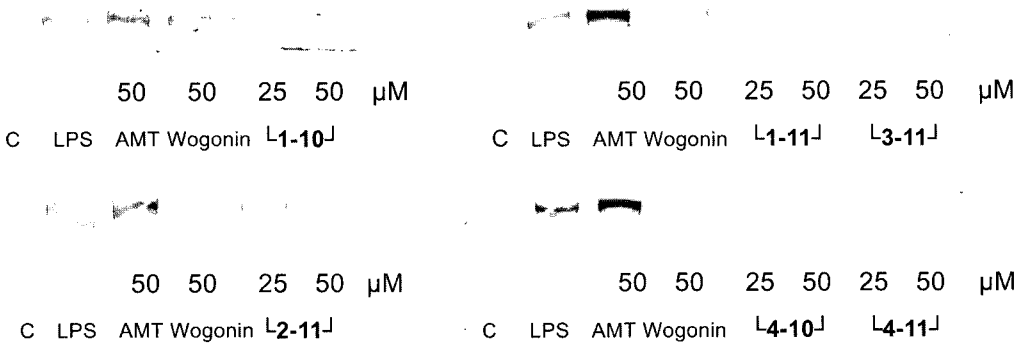


Fig. 2. Suppression of iNOS induction by the synthetic flavones (Western blot). Each flavone was incubated with LPS (1 μg/mL) in RAW cells for 20 h. Wogonin was used as a reference flavone that down-regulates iNOS expression. C: Control without LPS, Note: All synthetic flavones selected showed suppression of iNOS expression at 25 and 50 μM.

line, were used because it has been well established that a large amount of NO is produced by iNOS induced by LPS treatment. From the results of this study, the A-ring C-5,7-dihydroxy B-ring C-2',3'-dihydroxy or C-3',4'-dihydroxy or C-3',4'-hydroxy/methoxy (methoxy/hydroxy) flavones are optimal chemical structures showing potent inhibitory activity of NO production by iNOS. It was also found that the inhibition of NO production by these flavones is mediated mainly by iNOS down-regulation, but not by iNOS inhibition.

Some structural-activity relationships of various flavonoid derivatives isolated from plant extracts were previously reported (Kim *et al.*, 1999). While flavanones and flavonoid glycosides were not active, several flavones/isoflavones/flavonols showed considerable inhibition of NO production from LPS-treated RAW 264.7 cells, which strongly suggests an importance of the C-2',3'-double bond. It was also found that the B-ring meta-substitution was not favorable. These structural-activity relationships were confirmed again by the present investigation and other studies (Raso *et al.*, 2001). Luteolin is found to be one of the flavonoids having optimal chemical structure. In addition, a substitution with the hydroxy group at C-3 such as in the flavonol structure was revealed to decrease the inhibitory activity (luteolin vs quercetin). A similar result was previously observed when apigenin was more active than kaempferol (3-hydroxyapigenin) (Liang *et al.*, 1999), and flavones were more active than the corresponding flavonols (Kim *et al.*, 1999; Raso *et al.*, 2001). Whereas substitution with the A-ring C-5,7-dihydroxy group is important, flavones with some more substituents at the A-ring also possess inhibitory activity of NO production. For instance, wogonin (5,7-dihydroxy-8-methoxyflavone) and oroxylin A (5,7-dihydroxy6-methoxyflavone) strongly inhibit NO production with IC_{50} values of 17 μM and 5-10 μg/mL, respectively (Kim *et al.*, 1999; Chen *et al.*, 2000), while 5,6,7-trihydroxyflavone (baicalein) showed less inhibitory activity (Wakabayashi, 1999). Even in these compounds, the A-ring C-5,7-dihydroxy group is certainly the essential structure. Therefore, it may be concluded that the A-ring C-5,7 B-ring C-2',3'- or C-3',4'-tetrahydroxy flavones are within the optimized structures, if not the best. These compounds may be used *in vitro* and *in vivo* as representative flavonoids having a down-regulating activity of iNOS. A-ring C-5,7-dihydroxyflavones that have additional C-6- and/or C-8-substituents should be synthesized and their inhibitory activities need to be further investigated.

Up to now, most flavonoids showing an inhibitory activity of NO production by iNOS were found to suppress iNOS expression as a major cellular mechanism, but not to inhibit iNOS activity (Kim *et al.*, 1999; Liang *et al.*, 1999; Wadsworth and Koop, 1999; Cheon *et al*, 2000; Chen *et al.*, 2000; Chi *et al.*, 2001). These previous findings were confirmed by the present study demonstrating that the structurally optimized flavones also suppressed iNOS induction. Thus, it is strongly suggested that the down-regulating capacity of iNOS expression may be the common property of flavonoids that inhibit NO production. The suppressive mechanisms of iNOS induction by flavonoids have been extensively studied by several investigators. Although the detailed mechanism(s) is not fully understood, flavonoids are found to inhibit the activation of transcription factor(s), such as nuclear factor-κB or activator protein-1 depending on their chemical structures (Liang *et al.*, 1999; Chen *et al.*, 2000; Cho *et al.*, 2003). Each flavonoid inhibits transcription factor activation at least partly through the inhibition of several different protein kinases and/or mitogen activated protein (MAP) kinase pathways (Ferriola *et al.*, 1989; Liang *et al.*, 1999; Wadswoth and Koop, 2001; Cho *et al.*, 2003). However, the exact point(s) that is affected needs to be further investigated using the structurally optimized flavonoids obtained from the present study in order to derive the common action mechanism of flavonoids. Another study to find *in vivo* activity is now under investigation.

In conclusion, the A-ring C-5,7-dihydroxy B-ring C-2',3'-dihydroxy or C-3',4'-dihydroxy or C-3',4'-hydroxy/methoxy (methoxy/hydroxy) flavones are optimal chemical structures showing a potent inhibitory activity of NO production by iNOS. These synthetic flavone derivatives inhibit NO production through iNOS down-regulation, but not through iNOS inhibition. Thus, these compounds may possibly be useful as anti-inflammatory agents.

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