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Anti-inflammatory activity of the synthetic C-C biflavonoids

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Abstract

To find anti-inflammatory agents based on plant constituents, the effects of six synthetic C-C biflavonoids connecting with different positions of C-C bond between flavone monomers (**a**: 4′–4′, **b**: 4′–3′, **c**: 4′–6, **d**: 3′–6, **e**: 6–6, **f**: 4′–3) were examined on PGE_2 and nitric oxide (NO) production from lipopolysaccharide (LPS)-treated macrophages, RAW 264.7. Among the compounds tested, the biflavonoids **d**, **e**, and **f** showed a considerable inhibition of cyclooxygenase-2 (COX-2)-mediated PGE_2 production at concentrations up to 50 μM, while the derivative **c** exerted cytotoxic effects on RAW cells. Especially, the biflavonoid **e** possessed the most potent inhibitory activity of PGE_2 production with an IC50 of 3.7 μM, compared with an IC50 of 8.2–20.7 μM by ginkgetin (natural biflavonoid). Western blot and reverse transcriptase-polymerase chain reaction analyses have shown that the inhibition of PGE_2 production by these synthetic derivatives was mediated at least in part by COX-2 inhibition, but not by COX-2 down-regulation. Meanwhile, these synthetic biflavonoids did not considerably inhibit inducible nitric oxide synthase-mediated NO production at concentrations up to 50 μM. When intraperitoneally administered, the biflavonoid **e** showed a significant anti-inflammatory activity (22.2% inhibition) against rat carrageenan-induced paw oedema at 5 mg kg^{-1}. The biflavonoid **e** may be used as a synthetic lead for developing new anti-inflammatory agents.

Introduction

Various flavonoids from plant origin have been demonstrated to possess in-vivo anti-inflammatory activity (Gabor 1986). Their cellular action mechanisms of anti-inflammation include antioxidative action and inhibition of arachidonic acid metabolizing enzymes, such as phospholipase A_2 (PLA_2), cyclooxygenase (COX) and lipoxygenase (LOX) (Middleton et al 2000). In recent years, some of them, especially certain flavones and flavonols, were also revealed to regulate the transcriptional expression of pro-inflammatory enzymes/cytokines, including inducible isoform of COX (COX-2), inducible nitric oxide synthase (iNOS) and tumour necrosis factor-α, which are deeply associated with inflammatory disorders such as rheumatoid arthritis. Thus, flavonoids are suggested to have multiple mechanisms of anti-inflammatory action (Kim et al 2004).

Among the several classes of flavonoid derivatives, biflavonoids are a group of chemical entities of flavonoid dimers connected with a C-C or C-O-C bond between flavonoid monomers. Although a wealth of biflavonoids have been isolated from plants, their biological and pharmacological data are limited. Previously, certain biflavonoids were reported to inhibit phosphodiesterase (Ruckstuhl et al 1979), mast cell histamine release (Amella et al 1985), lens aldose reductase (Iwu et al 1990) and lymphocyte proliferation (Lee et al 1995). In addition, some C-C biflavonoids were synthesized and their antimicrobial activity was demonstrated (Lin et al 2001). During our study to find potential anti-inflammatory plant drugs, several biflavonoids, such as amentoflavone, ochnaflavone and ginkgetin, were for the first time found to be inhibitors of group II secretory phospholipase A_2 (PLA_2) (sPLA_2-IIA) (Chang et al 1994). Morelloflavone, a flavone–flavanone dimer, was also revealed as an sPLA_2 inhibitor (Gil et al 1997). Moreover, it was also found that certain biflavonoids, such as amentoflavone and ginkgetin, exerted inhibitory activity against COX-2-mediated PGE_2 production and iNOS-mediated NO production mainly by an inhibition of COX-2 and iNOS expression (Cheon et al 2000; Banerjee et al

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2002; Kwak et al 2002). This property is shared with that of certain types of simple flavones and flavonols as described above. Therefore, some biflavonoids from plant origin are thought to exert anti-inflammatory activity by PLA_2 inhibition or inhibition of expression of pro-inflammatory molecules (or both).

Recently, in a continuous effort to find anti-inflammatory flavonoids, we have synthesized several biflavonoids differing in a C-C bond position, and found that they were more or less inhibitors of $sPLA_2$-IIA (Chen et al 2006). However, the regulatory potential of these derivatives against COX-2- and iNOS-mediated inflammatory responses are not known. Therefore, in this investigation, the effects of these synthetic C-C biflavonoids on COX-2-mediated prostaglandin E_2 (PGE_2) production and iNOS-mediated NO production were studied using a lipopolysaccharide (LPS)-treated macrophage cell line, RAW 264.7, and some of their cellular action mechanisms, including in-vivo effect, were examined to establish the potential for new anti-inflammatory agents.

Materials and Methods

Materials

N-(2-[Cyclohexyloxyl]-4-nitrophenyl)methane-sulfonamide (NS-398) was obtained from Biomol (Plymouth Meeting, PA) and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) was obtained from Tocris Cookson (Bristol, UK). Arachidonic acid (AA) was purchased from Cayman Chem. (Ann Arbor, MI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Duchefa Chemie (Haarlem, Netherlands). LPS (*Escherichia coli* 0127:B8) and λ-carrageenan (CGN) were purchased from Sigma Chem. (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other cell culture reagents were from Gibco BRL (Grand Island, NY). Protein assay kit was purchased from Bio-Rad (Hercules, CA). The biflavonoids used in this study (Figure 1) were synthesized according to the previously described procedure (Chen et al 2006). All compounds tested were initially dissolved in dimethyl sulfoxide (DMSO) and diluted with appropriate buffer. The final concentration of DMSO in cell culture never exceeded 0.1% (v/v).

Animals

Male Sprague-Dawley (SD) rats (4 weeks, specific pathogen-free) were obtained from Orient Co. (Korea). Rats were fed with laboratory chow (Purina Korea) and water was freely available. They were acclimatized in an animal facility (KNU) at 20–22°C, 40–60% relative humidity and 12-h light–dark cycle for at least 7 days.

RAW 264.7 cell culture and measurement of PGE_2 and NO concentration

RAW 264.7 cells obtained from American Type Culture Collection (Rockville, ML) were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin) under 5% CO_2 at 37 °C according to previously published procedures (Chi et al 2001). For a pre-treatment study, the test compounds were added to the cells in 96-well plates (2×10^5 cells/well) and the cells were incubated for 1 h. Then LPS ($1\ \mu g\,mL^{-1}$) was added and the cells were incubated further for 24 h, unless otherwise specified. Control groups received the same amount of DMSO. Cell viability was assessed by MTT assay (Mossmann 1983). For determination of NO concentration, the stable conversion product of NO, nitrite (NO_2^-), was measured from the medium using the Griess' reagent (1:1 mixture (v/v) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). Optical density was measured with a microplate reader (Spectra Max, Molecular Devices) at 550 nm. PGE_2 concentration in the medium was measured with an enzyme immunoassay (EIA) kit (Cayman Chem.) according to the manufacturer's recommended procedures. To determine the direct inhibitory activity of COX-2 by the test compounds, the cells were incubated with LPS ($1\ \mu g\,mL^{-1}$) for 24 h to fully induce COX-2 and washed thoroughly three times with serum-free DMEM. Then the test compounds were added without LPS, and the cells were incubated for another 24 h. PGE_2 concentration was measured in the medium.

Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

The expression levels of COX-2 protein and mRNA were determined by Western blot and RT-PCR analyses essentially following the previously described procedures (Chi et al 2001, 2003). The test compounds were added to RAW 264.7 cells in 6-well plates (5×10^6 cells/well) and the cells were incubated for 1 h. Then LPS ($1\ \mu g\,mL^{-1}$) was added and the cells were incubated for 20 h. The supernatant was removed and the remaining attached cells were washed twice with phosphate-buffered saline. After homogenization with a protein extraction solution (Intron Biotechnol), the homogenates were centrifuged at 10000 *g* for 10 min. Proteins were separated by a Tris-glycine gel (8%, Novex Lab.) electrophoresis and the gels were blotted to PVDF membranes. COX-2 was detected using COX-2 antibody (Cayman Chem.) followed by visualization with horseradish peroxidase-conjugated secondary antibodies and DAB reagents (Vector Lab.). For RT-PCR analysis, the cells were incubated in the presence or absence of the test compounds for 5 h and harvested. After homogenization in RLT buffer containing 1% β-mercaptoethanol for 30 s, total RNA was extracted with RNeasy mini kit (Qiagen) and the concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm. cDNAs were synthesized using RT reaction at 42°C, 50 min and 99°C, 5 min in Gene Cycler thermal cycler (Bio-Rad). Primers were synthesized on the basis of the repeated mouse cDNA sequence for COX-2 and G3PDH. The primer sequences used for PCR were as follows: COX-2 sense, 5′-ACT CAC TCA GTT TGT TGA GTC ATT C-3′, antisense, 5′-TTT GAT TAG TAC TGT AGG GTT AAT G-3′, 583 bp; G3PDH sense, 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′, antisense, 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′, 983 bp. PCR was carried out for 25–30 cycles

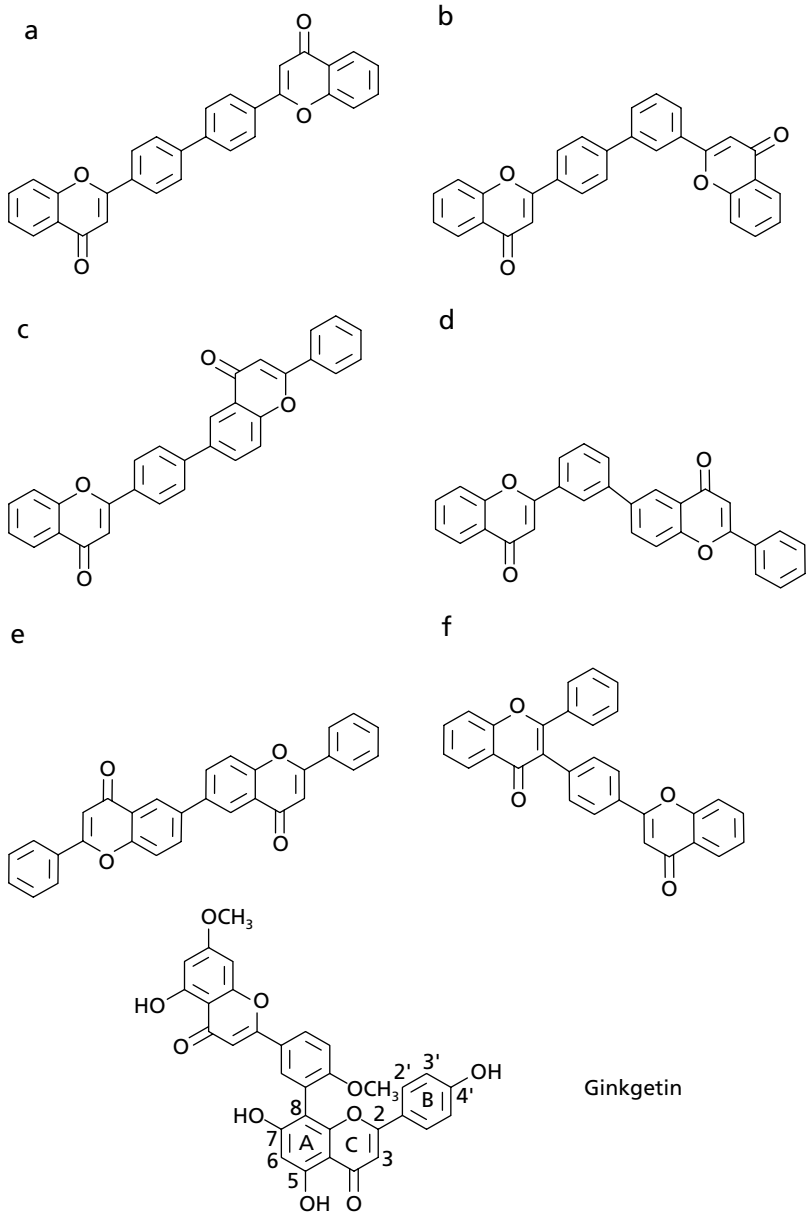

Figure 1 Chemical structures of the synthetic C-C biflavonoids (**a**–**f**) and ginkgetin.

under saturation, in 25 μL reaction mixture. After amplification, 5 μL of reaction mixture was analysed by 1.5% agarose gel electrophoresis. The bands were visualized by ethidium bromide staining for 10 min.

In-vivo anti-inflammatory activity

For the rat paw oedema test, 1% carrageenan dissolved in pyrogen-free saline (0.05 mL) was injected into the right hind paw of rats as previously described (Winter et al 1962). Five hours later, the swelling of the treated paw was measured using a plethysmometer (Ugo Basile, Italy). Test compounds dissolved in DMSO were administered intraperitoneally 30 min before carrageenan injection.

Statistical analysis

All values were represented as arithmetic means ± s.e. Student's unpaired *t*-test and Kruskal–Wallis test followed by Dunn's test were used to determine statistical significance.

Results

LPS-induced activation of RAW 264.7 cells is a well-characterized model for COX-2 and iNOS expression, resulting in the increased production of PGE_2 and NO. In one typical experiment, LPS treatment (1 μg mL^{-1}) for 24 h produced 52.2 ± 1.3 nM of PGE_2 and 31.1 ± 2.2 μM of NO (n = 2). The

basal level concentrations of PGE_2 and NO were 1.7 ± 0.1 nM and 0.88 ± 0.1 μM, respectively. NS-398 (COX-2 inhibitor) and AMT (iNOS inhibitor), used as reference compounds, strongly inhibited PGE_2 and NO production, respectively. When the synthetic compounds were used at 50 μM, the biflavonoids **a**, **d** and **e** showed considerable inhibition of COX-2-mediated PGE_2 production (59.3–80.1% inhibition; Table 1), while **c** and **f** exerted significant cytotoxic effects on LPS-treated RAW cells measured by MTT assay (data not shown). The concentration-dependent inhibition against PGE_2 production was examined at noncytotoxic concentrations of the biflavonoids and the IC50 values were obtained (Figure 2, Table 1). Comparing these values, the biflavonoids **d**, **e** and **f** showed a higher inhibition on PGE_2 production compared with the biflavonoids **a** and **b**. Especially, the biflavonoid **e** showed a potent inhibition, being more potent than the natural biflavonoid, ginkgetin. On the other hand, the synthetic biflavonoids did not considerably inhibit iNOS-mediated NO production at concentrations up to 50 μM, while ginkgetin strongly inhibited NO production. All these results clearly indicated that most synthetic biflavonoids in this study inhibited COX-2-catalysed PGE_2 production from LPS-treated RAW cells with different potencies depending on the chemical structures (linkage difference). Next, the possible inhibition mechanism was studied.

For elucidating the effect of the synthetic derivatives on COX-2 expression, Western blot and RT-PCR analyses were employed to measure the expression levels of COX-2 protein and mRNA, respectively. Figure 3 shows that the biflavonoids synthesized (**a**, **b**, **d**, **e**, **f**) did not inhibit COX-2 expression at non-cytotoxic concentrations, except for **c**. The biflavonoid **c** at 10 μM clearly inhibited COX-2 expression in the Western blotting experiment. However, it is not likely that this compound really down-regulates COX-2 expression since **c** (10 μM) showed cytotoxicity to the cells, checked by MTT assay. RT-PCR analysis confirmed the observation that these synthetic biflavonoids were not COX-2 down-regulators. Compound **c** only at 10 μM slightly decreased COX-2 mRNA level. Therefore, it is suggested that the synthetic biflavonoids in this study may not affect COX-2 expression level, while ginkgetin (50 μM) clearly down-regulates COX-2 expression. The other possible mechanism is a direct COX-2 inhibition. To examine this possibility, the biflavonoids at non-cytotoxic concentrations were added after full induction of COX-2 and the cells were incubated for another 24 h (post-treatment study). From this experiment, the synthetic biflavonoids were found to inhibit PGE_2 production concentration dependently (Figure 4), indicating their COX-2 inhibitory activity. Again, the biflavonoid **e** was most potent. Their IC50 values (Table 1) were roughly parallel with the inhibitory potencies found in the experiment with pre-treatment of the biflavonoids. In contrast, ginkgetin did not inhibit PGE_2 production in this experiment. On further experiment employing COX-2 inhibition screening kit, it was found that the biflavonoid **e** did inhibit COX-2 enzyme activity (data not shown). All these results strongly suggest that the some synthetic biflavonoids are COX-2 inhibitors, whereas ginkgetin is a down-regulator of COX-2 expression as previously found.

Finally, in-vivo anti-inflammatory activity was examined. Against the rat carrageenan paw oedema assay, the compound **e** showed a significant inhibition at 5 mg kg^{-1} by intraperitoneal injection (Table 2).

Discussion

This investigation has clearly shown that the synthetic C-C biflavonoids inhibited COX-2-mediated PGE_2 production from LPS-treated macrophages, at least in part, by COX-2 inhibition, but not by COX-2 down-regulation. Moreover, one of the synthetic compounds showed in-vivo anti-inflammatory activity. Although the detailed structure–activity relationship could not be obtained, it is evident that

Table 1 Inhibition of COX-2-mediated PGE_2 and iNOS-mediated NO production from LPS-treated RAW 264.7 cells by the synthetic C-C biflavonoids

Compound	PGE_2 production			NO production	
	% Inhibition at 50 μM[a]	IC50[b] (μM)	IC50[c] (μM)	% Inhibition at 50 μM	IC50[b] (μM)
NS-398	91.4 ± 6.4**[d]	<0.1	<0.1		
AMT				92.4 ± 1.5**	0.02
a (4′–4′)	63.6 ± 1.8**	>50.0	>50.0	3.3 ± 1.8	
b (4′–3′)	23.3 ± 22.7	>50.0	>50.0	—[e]	
c (4′–6)	CT[f]			CT[f]	
d (3′–6)	72.2 ± 20.6*	17.3	11.1	9.2 ± 1.5	
e (6–6)	85.8 ± 0.4**	3.7	<3.0	7.0 ± 1.1	
f (4′–3)	CT[g]	7.0	8.7	CT[g]	
Ginkgetin		8.2–20.7[h]	—[e]		10.9–25.0[h]

[a]All compounds were treated at 50 μM except the references (NS-398: 1 μM, AMT: 10 μM). [b]IC50 values obtained from the experiment of pre-treatment of the test samples, followed by 24 h incubation. [c]IC50 values obtained from the experiment of post-treatment of the test compounds after full induction of COX-2 by pre-treatment of LPS. [d]Arithmetic mean ± s.e., n = 2. *$P < 0.05$, **$P < 0.001$ (Student's *t*-test), compared with the LPS-treated control group. [e]Not active. [f]Cytotoxic to RAW cells at 10 μM by MTT assay. [g]Cytotoxic to RAW cells at 50 μM by MTT assay. [h]The range of IC50 values from five separate experiments.

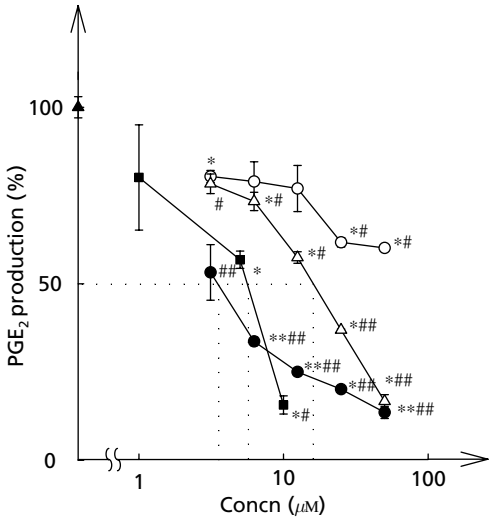

Figure 2 Inhibition of COX-2-mediated PGE_2 production from LPS-treated RAW 264.7 cells. RAW cells were pre-treated with various noncytotoxic concentrations of the test compounds for 1 h. Then LPS was added and the cells were incubated for 24 h. PGE_2 concentration was measured with ELISA. Data points and bars represented the arithmetic mean ± s.e., n = 2. $*P < 0.05$, $**P < 0.001$ (Student's *t*-test), $\#P < 0.05$, $\#\#P < 0.001$ (Kruskal–Wallis followed by Dunn's test), compared with the LPS-treated control group. **a** (○), **d** (△), **e** (●), **f** (■).

the effects of the synthetic biflavonoids on PGE_2 production are quite different depending on the positions of C-C linkages. The biflavonoids **d**, **e** and **f** showed a higher activity than the derivatives **a** and **b**, compound **e** being most potent. At present, we do not know what kinds of structural requirements provide a higher activity. However, it is worth mentioning that the less active derivatives **a** and **b** have a C-C bond between the B-rings of two flavonoid monomers (4′–3′, 4′–4′). It was also found that the synthetic biflavonoids (**c**, **f**) showed significant cytotoxicity on RAW cells under the conditions tested. These cytotoxic biflavonoids have a common structure of 4′-linkage to the next flavone A- or C-ring.

Some natural biflavonoids were previously demonstrated to inhibit the expression of pro-inflammatory molecules. For example, ginkgetin, bilobetin and ochnaflavone were found to down-regulate iNOS expression from LPS-treated RAW 264.7 cells (Cheon et al 2000). Amentoflavone was proved to inhibit COX-2 expression and this inhibition was mediated by an inhibition of NF-κB activation (Banerjee et al 2002). Unlike these natural biflavonoids, the synthetic C-C biflavonoids in this study were not down-regulators of COX-2 expression. Instead, some of them were proved to be COX-2 inhibitors. These results were not expected since most natural biflavonoids tested previously did not have the capacity to inhibit COX-2 directly. In this respect, the synthetic derivatives in this study are unique to the biflavonoid family. To the

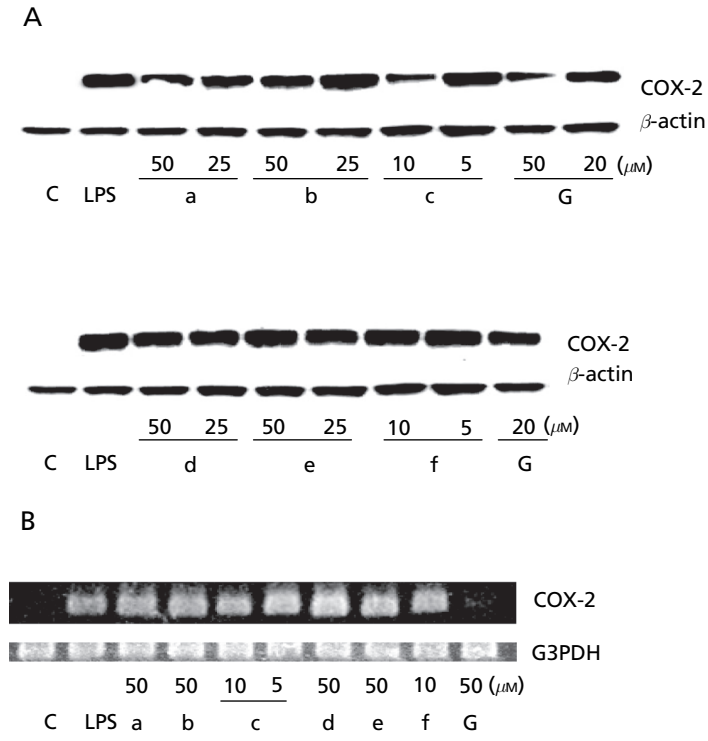

Figure 3 Effect on COX-2 expression from LPS-treated RAW 264.7 cells. A. Western blot: RAW 264.7 cells were incubated in the presence or absence of LPS and the test compounds for 20 h. B. RT-PCR: RAW 264.7 cells were incubated in the presence or absence of LPS and the test compounds for 5 h. Control (C), LPS-treated control (LPS), ginkgetin (G).

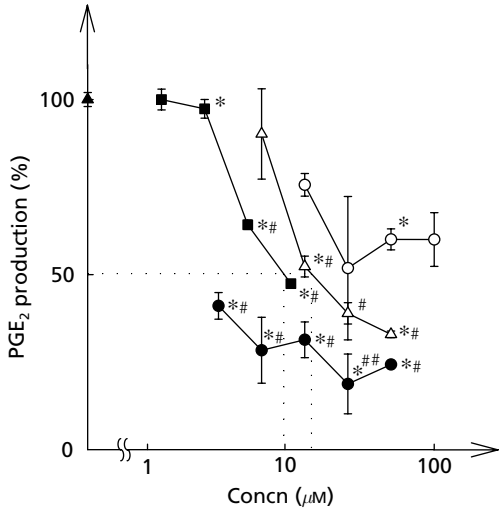

Figure 4 Inhibition of pre-induced COX-2-mediated PGE_2 production from LPS-treated RAW 264.7 cells. RAW cells were treated with LPS for 24 h. After washing, the test compounds were added without LPS and incubated further for 24 h. PGE_2 concentration was measured with ELISA. Data points and bars represented the arithmetic mean ± s.e., n = 2. $*P < 0.05$, $**P < 0.001$ (Student's *t*-test), $\#P < 0.05$, $\#\#P < 0.001$ (Kruskal–Wallis followed by Dunn's test), compared with the LPS-treated control group. **a** (○), **d** (△), **e** (●), **f** (■).

Table 2 Inhibition of rat carrageenan paw oedema

Compound	Dose (mg kg^{-1})[a]	Oedema increased (mL)	% Inhibition
Vehicle	—	0.04 ± 0.01	—
Carrageenan	—	0.83 ± 0.05	—
Prednisolone	10.0	0.41 ± 0.02**##	53.3
	5.0	0.64 ± 0.02**#	24.0
e	5.0[b]	0.65 ± 0.02*	22.1
	2.5	0.74 ± 0.06	11.7

[a]All compounds were intraperitoneally administered 30 min before carrageenan injection. [b]Due to the poor solubility, this dose was the highest concentration to be obtained. $*P < 0.05$, $**P < 0.001$ (Student's *t*-test), $\#P < 0.05$, $\#\#P < 0.001$ (Kruskal–Wallis followed by Dunn's test), compared with the carrageenan-treated control group (n = 5).

author's best knowledge, there has been only one report demonstrating COX-2 inhibitory biflavonoid. Tetrahydroamentoflavone, isolated from the seeds of *Semecarpus anacardium*, was found to be a COX-2 inhibitor (Selvam & Jachak 2004). Some of the flavonoid derivatives in our investigation are the first synthetic biflavonoids having COX-2 inhibitory action. The structural requirement for COX-2 inhibition in these biflavonoid molecules is not clear, but it is suspected that the lack of substituent(s) on the biflavonoid backbone structure may be related. This speculation needs to be further elucidated with more synthetic biflavonoids having structural diversities.

Along with several natural biflavonoids (Chang et al 1994; Gil et al 1997), the synthetic biflavonoids **a**–**f** were previously proved to possess $sPLA_2$-IIA inhibitory activity (Chen et al 2006). Since $sPLA_2$ is deeply associated with several inflammatory disorders (Murakami & Kudo 2004), an inhibition of $sPLA_2$-IIA is important for exerting anti-inflammatory activity. Therefore, the synthetic biflavonoids in this study may have dual mechanisms of anti-inflammation, and COX-2 and PLA_2 inhibition. The PLA_2 inhibitory capacity of the synthetic biflavonoids might also contribute to the inhibitory activity of PGE_2 production from RAW cells described above.

Conclusion

In this study, six synthetic C-C biflavonoids having different connecting linkages were evaluated for anti-inflammatory activity in-vitro and in-vivo. Some of them inhibited COX-2-mediated PGE_2 production from LPS-treated RAW 264.7 cells at least in part by COX-2 inhibition, but not by COX-2 down-regulating capacity. They did not considerably inhibit iNOS-mediated NO production at concentrations up to 50 μM. A most potent PGE_2 inhibitory biflavonoid (**e**), having a C-C bond between 6–6 positions of each flavone molecule, also showed anti-inflammatory activity against rat carrageenan paw oedema assay. The biflavonoid **e** may be used as a synthetic lead for developing new anti-inflammatory agents.

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