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Inhibition of prostaglandin E_2 production by 2′-hydroxychalcone derivatives and the mechanism of action

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Abstract

The effects of 14 synthetic 2′-hydroxychalcone derivatives on prostaglandin E_2 (PGE_2) production in rat peritoneal macrophages stimulated by the protein kinase C activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), were examined to clarify the structure–activity relationship. 2′,4-Dihydroxy-4′-methoxychalcone (compound **3**), 2′,4-dihydroxy-6′-methoxychalcone (compound **8**) and 2′-hydroxy-4′-methoxychalcone (compound **9**) suppressed PGE_2 production more potently than the other compounds. The IC50 (50 % Inhibitory concentration) value for compounds **3**, **8** and **9** was calculated to be 3 μM. The activity of cyclooxygenase (COX)-1 was inhibited slightly by compound **9**, but that of COX-2 was not inhibited. At concentrations that inhibited the production of PGE_2, compound **9** had no effect on the release of radioactivity from [^{3}H]arachidonic acid-labelled macrophages stimulated by TPA. Western-blot analysis revealed that the induction of COX-2 protein by TPA was inhibited by compound **9** in parallel with the inhibition of PGE_2 production. Compounds **3** and **8** had similar effects. These findings suggest that 4′-methoxyl and 6′-methoxyl groups are required for the expression of more potent inhibitory activity against PGE_2 production, and that the inhibition of PGE_2 production by these 2′-hydroxychalcone derivatives is due to the inhibition of TPA-induced COX-2 protein expression.

Introduction

Several types of prostaglandin whose synthesis is mediated by cyclooxygenase (COX), a prostaglandin endoperoxide synthase, play a significant role in inflammatory reactions (Davies et al 1984). COX has two isoforms: COX-1 is constitutively expressed in almost all types of cells (O'Neill & Ford-Hutchinson 1993; Smith et al 1994) and is probably involved in cellular housekeeping (DeWitt & Smith 1988); COX-2 is induced by several kinds of extracellular stimuli (Ali et al 1985; Ohuchi et al 1988; Nishizuka 1992; O'Sullivan et al 1992; Dubois et al 1994; Watanabe et al 1995) in inflammatory cells and tissues (Lee et al 1992; Sano et al 1992; Yamada et al 1997; Yamashita et al 1997). This suggests that the stimulation of arachidonic acid metabolism at the inflammatory site is due to the induction of COX-2 protein in the inflammatory cells by such stimuli. Recently, several kinds of selective COX-2 inhibitors have been developed (Futaki et al 1994;

Taniguchi et al 1995; Chan et al 1999; Mandell 1999). These inhibitors have fewer side effects than non-selective COX-1/COX-2 inhibitors (Masferrer et al 1994; Seibert et al 1994).

Some chalcone derivatives, such as 2′,3-dihydroxychalcone, 2′,5′-dihydroxy-4-chlorochalcone and 2′,5′-dihydroxychalcone, have been reported to be anti-inflammatory or anti-allergic agents (Hsieh et al 1998) while 3,4-dihydroxychalcone derivatives have been reported to be inhibitors of 5-lipoxygenase and COX (Sogawa et al 1993). In addition, 3′,4′,5′,3,4,5-hexamethoxychalcone inhibits the production of prostaglandin E_2 (PGE_2) and nitric oxide by inhibiting the induction of COX-2 and inducible nitric oxide synthase in lipopolysaccharide-stimulated mouse peritoneal macrophages (Herencia et al 1999). These findings suggested that some chalcones may be promising anti-inflammatory agents.

In this study, we examined the effects of 14 synthetic 2′-hydroxychalcone derivatives on PGE_2 production in rat peritoneal macrophages stimulated by the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to clarify the structure–activity relationship. Furthermore, we analysed the mechanism of action of the most potent 2′-hydroxychalcone derivatives.

Materials and Methods

2′-Hydroxychalcone derivatives

2′-Hydroxychalcone derivatives were synthesized as described previously (Lim et al 2000). Their chemical structures are shown in Table 1.

Preparation of rat peritoneal macrophages

A solution of soluble starch (Wako Pure Chemicals, Osaka, Japan) and Bacto-peptone (Difco, Detroit, MI), 5% each, was injected intraperitoneally into male Sprague-Dawley rats (400–500 g, specific pathogen-free; Charles River Japan, Kanagawa, Japan) at a dose of 5 mL per 100 g body weight. Four days later, the rats were anaesthetized with pentobarbital and peritoneal cells were harvested (Ohuchi et al 1985). The experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Japan.

Macrophage culture

The peritoneal cells were suspended in Eagle's minimal essential medium (EMEM, Nissui, Tokyo, Japan) containing 10% calf serum (Flow Laboratories, North Rydge, Australia), penicillin G potassium (18 μg mL^{-1}) and streptomycin sulfate (50 μg mL^{-1}) (Meiji Seika, Tokyo, Japan), then seeded at a density of 7.5×10^5 cells per well in 24-well plastic tissue culture dishes (Corning Glass Works, Corning, NY) in 0.5 mL of medium, and incubated at 37°C for 2 h. The dishes were then washed three times with medium to remove non-adherent cells, and the adherent cells were further incubated at 37°C for 20 h. After three washes, the adherent cells were used for subsequent experiments.

Incubation of macrophages with drugs

The adherent cells were incubated at 37°C for 8 h in 0.5 mL of medium containing 10% calf serum and various concentrations of each 2′-hydroxychalcone derivative in the presence (16.2 nM, 10 ng mL^{-1}) or absence of the protein kinase C activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA, Sigma Chemical Co., St Louis, MO) (Nishizuka 1992). TPA was dissolved in dimethyl sulfoxide (DMSO) and added to the medium. Each 2′-hydroxychalcone was also dissolved in DMSO before being added to the medium. The final concentration of DMSO was adjusted to 0.1% (v/v). The control medium contained the same amount of DMSO.

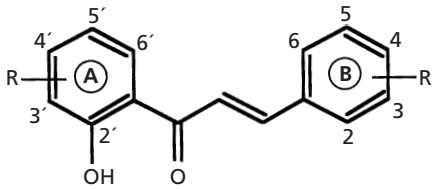
Measurement of PGE_2 concentration

Eight hours after incubation, the conditioned medium was obtained, and centrifuged at 1500 *g* and 4°C for 5 min. The concentration of PGE_2 in the supernatant fraction was then radioimmunoassayed (Ohuchi et al 1985). PGE_2 antiserum was purchased from PerSeptive Biosystems (Framingham, MA).

Determination of COX-1 and COX-2 activity in a cell-free system

The activity of COX-1 and COX-2, in a cell-free system, was determined as described by Mancini et al (1995) and Yamada et al (1997). One unit of COX-1 (isolated from sheep seminal vesicle, Cayman Chemical Co., Ann Arbor, MI) or COX-2 (isolated from sheep placenta, purity 70%, Cayman Chemical Co.) was dissolved in 210 μL of Tris-HCl (100 mM, pH 7.4) containing 10 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM reduced glutathione, 1 μM haematin and 0.5 mM phenol. The reaction mixture was pre-incubated with the drug at 37°C for 3 min, after which arachidonic acid (Sigma Chemical Co.) (0.1 μM) was added, and the mixture was

Table 1 Chemical structures of various 2′-hydroxychalcone derivatives.



Compound	A-ring modification		Compound	B-ring modification	
	R′	R		R′	R
1	H	4-OH	**9**	4′-OCH_3	H
2	4′-OH	4-OH	**10**	4′-OCH_3	3,4-di OCH_3
3	4′-OCH_3	4-OH	**11**	4′-OCH_3	4-Cl
4	5′-Cl	4-OH	**12**	4′-OCH_3	4-CH_3
5	5′-CH_3	4-OH	**13**	4′-OCH_3	4-OCH_3
6	5′-OH	4-OH	**14**	4′-OCH_3	4-$N(CH_3)_2$
7	5′-OCH_3	4-OH			
8	6′-OCH_3	4-OH			

incubated at 37°C for 3 min. To terminate the reaction, 20 μL of 1 M HCl was added to the reaction mixture. An equivalent volume of 1 M NaOH was then added to neutralize the mixture, and the amount of PGE_2 was measured by radioimmunoassay.

Measurement of radioactivity released from [^{3}H]arachidonic acid-labelled macrophages

Peritoneal cells (7.5×10^5 cells), collected as described in preparation of rat peritoneal macrophages, were incubated at 37°C for 2 h in 0.5 mL of medium containing 10% calf serum per well in 24-well plastic tissue culture dishes (Corning Glass Works). The dishes were then washed three times to remove non-adherent cells, and further incubated at 37°C for 18 h in 0.5 mL of medium containing 10% calf serum. The cells were again washed three times and incubated at 37°C for 20 h in 0.5 mL of medium containing 10% calf serum and 3.7 kBq of [^{3}H]arachidonic acid (2.26 TBq mmol^{-1}, Du Pont New England Nuclear, Boston, MA). The adherent cells were washed three times with medium to remove free [^{3}H]-arachidonic acid, and incubated at 37°C for the periods indicated in 0.5 mL of medium containing 10% calf serum and various concentrations of each 2′-hydroxychalcone in the presence or absence of TPA (16.2 nM, 10 ng mL^{-1}). The conditioned medium was withdrawn at 1, 2 and 4 h, then centrifuged at 1500 *g* and 4°C for 5 min, and the radioactivity in the supernatant fraction was determined (Ohuchi et al 1988).

Western-blot analysis of COX-1 and COX-2

Peritoneal cells (3×10^6 cells), collected as described in preparation of rat peritoneal macrophages, were incubated at 37°C for 2 h in 2 mL of medium containing 10% calf serum in 6-well plastic tissue culture dishes (Corning Glass Works). The dishes were then washed three times to remove non-adherent cells, and the adherent cells were further incubated at 37°C for 20 h. After three washes, the cells were incubated at 37°C for 6 h in 2 mL of medium containing 10% calf serum in the presence or absence of TPA (16.2 nM, 10 ng mL^{-1}) and various concentrations of each 2′-hydroxychalcone. The cells were then washed three times with phosphate-buffered saline (PBS, pH 7.4), dipped in 150 μL of ice-cold lysis buffer (HEPES, 20 mM; Triton-X 100, 1%; glycerol, 10%; sodium fluoride, 1 M; *p*-nitrophenylene phosphate, 2.5 mM; phenylmethylsulfonylfluoride, 10 μg mL^{-1}; Na_3VO_4, 1 mM; leupeptin, 5 μg mL^{-1}; EDTA, 1 mM; pH 7.4) for 15 min, and disrupted by a Handy Sonic Disrupter (UR-20P, TOMY, Tokyo, Japan). The lysis buffer containing the disrupted cells was centrifuged at 13000 *g* and 4°C for 20 min. The supernatant fraction obtained was boiled for 5 min in 3 × sample buffer (Tris, 50 mM; sodium dodecyl sulfate, 4% (v/v); glycerol, 10% (v/v); 2-mercaptoethanol, 4% (v/v); and bromophenol blue, 0.05 mg mL^{-1}; pH 7.4) at a ratio of 2:1 (v/v), loaded on an acrylamide gel (8%) and subjected to electrophoresis (150 min at 125 V). Western blotting for COX-1 and COX-2 was carried out as described previously (Kim et al 1999). The level of

COX-1 and COX-2 protein was quantified by scanning densitometry, and the individual band density value for each point was expressed as the relative density signal.

Statistical analysis

All the values are expressed as the means ± s.d. Comparisons between TPA-control and other groups were performed using Dunnett's test and the unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

Effects of A-ring-modified 2′,4-dihydroxychalcone derivatives on TPA-induced PGE_2 production in rat peritoneal macrophages

When rat peritoneal macrophages were incubated at 37°C for 8 h in medium containing TPA (16.2 nM), PGE_2 production was greatly increased (Table 2). In the presence of 10 μM of each 2′,4-dihydroxychalcone derivative (A-ring-modified compounds **1**–**8**, Table 1), PGE_2 production stimulated by TPA was significantly inhibited (Table 2). At 3 μM, compounds **1**, **3**, **6** and **8** significantly inhibited TPA-stimulated PGE_2 production (Table 2). Among the eight compounds, 2′,4-dihydroxy-4′-methoxychalcone (**3**) and 2′,4-dihydroxy-6′-methoxychalcone (**8**) had the most potent inhibitory effect (Table 2).

Table 2 Effects of A-ring-modified 2′,4-dihydroxychalcone derivatives on TPA-induced PGE_2 production in rat peritoneal macrophages.

Treatment	PGE_2 (ng mL^{-1})	
None	1.34 ± 0.37***	
TPA	8.75 ± 0.29	
	3 μM	10 μM
TPA + compound **1**	6.13 ± 0.72**	4.61 ± 0.90***
TPA + compound **2**	8.00 ± 1.82	5.12 ± 0.53***
TPA + compound **3**	3.79 ± 1.23***	1.55 ± 0.67***
TPA + compound **4**	7.36 ± 2.13**	6.06 ± 1.14*
TPA + compound **5**	9.34 ± 1.72	6.63 ± 1.36*
TPA + compound **6**	6.00 ± 0.29***	3.78 ± 0.84***
TPA + compound **7**	8.47 ± 0.29	6.44 ± 0.63**
TPA + compound **8**	4.30 ± 0.94***	1.50 ± 0.32***

Rat peritoneal macrophages (7.5×10^5 cells) were incubated at 37°C for 8 h in 0.5 mL of medium in the presence of TPA (16.2 nM) and the indicated concentrations of each compound. Values are the means ± s.d. from four samples; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs TPA control.

Table 3 Effects of B-ring-modified 2′-hydroxy-4′-methoxychalcone derivatives on TPA-induced PGE_2 production in rat peritoneal macrophages.

Treatment	PGE_2 (ng mL^{-1})	
None	1.40 ± 0.35***	
TPA	9.17 ± 1.73	
	3 μM	10 μM
TPA + compound **3**	4.00 ± 0.49***	1.42 ± 0.27***
TPA + compound **9**	3.78 ± 1.98***	1.78 ± 0.32***
TPA + compound **10**	8.26 ± 0.24	5.57 ± 0.34*
TPA + compound **11**	8.42 ± 1.98	5.86 ± 1.92*
TPA + compound **12**	7.82 ± 2.52	4.39 ± 0.85**
TPA + compound **13**	6.32 ± 1.37	4.98 ± 2.10*
TPA + compound **14**	7.29 ± 1.49	4.51 ± 0.50***

Rat peritoneal macrophages (7.5×10^5 cells) were incubated at 37°C for 8 h in 0.5 mL of medium in the presence of TPA (16.2 nM) and the indicated concentrations of each compound. Values are the means ± s.d. from four samples; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs TPA control. For comparison, effects of 2′,4-dihydroxy-4′-methoxychalcone (compound **3**) are shown.

Effects of B-ring-modified 2′-hydroxy-4′-methoxychalcone derivatives on TPA-induced PGE_2 production in rat peritoneal macrophages

The effects of six compounds (compounds **9**–**14**, B-ring-modified 2′-hydroxy-4′-methoxychalcone derivatives, Table 1) on TPA (16.2 nM)-induced PGE_2 production were examined. Except for 2′-hydroxy-4′-methoxychalcone (compound **9**), none of the compounds at 3 μM inhibited TPA-induced PGE_2 production at 8 h (Table 3). At 10 μM, these compounds significantly inhibited PGE_2 production. Among the six compounds, **9** was the most potent inhibitor, with almost the same level of activity as 2′,4-dihydroxy-4′-methoxychalcone (Table 3).

Effects of 2′-hydroxy-4′-methoxychalcone (compound 9) on the enzyme activity of isolated COX-1 and COX-2

The direct effects of 2′-hydroxy-4′-methoxychalcone (compound **9**) on isolated COX-1 and COX-2 were examined. The COX-1/COX-2 inhibitor indometacin

Table 4 Effects of 2′-hydroxy-4′-methoxychalcone (compound **9**) on the activity of isolated COX-1 and COX-2.

Treatment	% of control	
	COX-1	COX-2
None	100	100
NS-398 (0.01 μM)	93.3 ± 5.2	95.5 ± 14.2
NS-398 (0.1 μM)	86.6 ± 13.5	57.7 ± 13.2***
NS-398 (1 μM)	82.2 ± 14.2	29.3 ± 6.6***
Indometacin (0.01 μM)	96.1 ± 23.3	93.3 ± 17.7
Indometacin (0.1 μM)	44.4 ± 22.8***	41.1 ± 5.6***
Indometacin (1 μM)	6.2 ± 4.4***	12.2 ± 9.6***
Compound **9** (3 μM)	84.4 ± 17.6	103.3 ± 13.2
Compound **9** (10 μM)	68.8 ± 8.1***	108.8 ± 16.9
Compound **9** (30 μM)	49.5 ± 12.4	102.6 ± 6.7

One unit of COX 1 (isolated from sheep seminal vesicle) and one unit of COX 2 (isolated from sheep seminal placenta) dissolved in 210 μL of 100 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 1 mM reduced glutathione, 1 μM haematin and 0.5 mM phenol were incubated for 3 min at 37°C in the presence of the indicated concentration of 2′-hydroxy-4′-methoxychalcone (compound **9**), indometacin or NS-398. Arachidonic acid (0.1 μM) was then added, and incubation continued at 37°C for another 3 min. PGE_2 concentrations in the reaction mixture were then radioimmunoassayed, and mean concentrations of control group was set to 100%. Values are the means ± s.d. from four samples; ***$P < 0.001$ vs corresponding control.

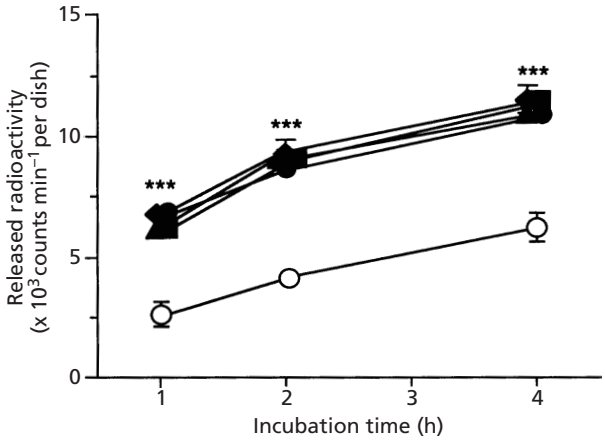


Figure 1 Effects of 2′-hydroxy-4′-methoxychalcone (compound **9**) on the release of radioactivity from [^{3}H]arachidonic acid-labelled macrophages. Rat peritoneal macrophages (7.5×10^5 cells) were incubated at 37°C for 20 h in medium containing 10% calf serum and 3.7 kBq of [^{3}H]arachidonic acid. After three washes, the cells were incubated at 37°C for the periods indicated in 0.5 mL of medium in the presence of TPA (16.2 nM) and various concentrations of compound **9** (■, 0 μM; ●, 3 μM; ▲, 10 μM and ◆, 30 μM). Radioactivity released into the medium was determined. Released radioactivity from non-stimulated macrophages is shown by open circles. Values are the means from four samples with s.d. shown by vertical bars; ***$P < 0.001$ vs non-stimulated control.

inhibited the activity of COX-1 and COX-2 (Table 4) in a concentration-dependent manner over the range 0.1–1 μM. NS-398, a specific inhibitor of COX-2, inhibited COX-2 activity in a concentration-dependent manner at 0.1–1 μM (Table 4), but showed no inhibitory effect on COX 1 at such concentrations (Table 4). Compound **9** partially inhibited COX-1 activity but did not inhibit COX-2 activity, at 10 and 30 μM (Table 4). Compounds **3** and **8** had almost the same effect as compound **9** (data not shown).

Effects of 2′-hydroxy-4′-methoxychalcone (compound 9) on the release of radioactivity from [^{3}H]arachidonic acid-labelled rat peritoneal macrophages

Incubation of [^{3}H]arachidonic acid-labelled macrophages in medium containing TPA (16.2 nM) significantly increased the release of radioactivity into the medium at 1–4 h (Figure 1). But the TPA-induced release of radioactivity from [^{3}H]arachidonic acid-labelled macrophages was not affected by compound **9** at concentrations of 3–30 μM (Figure 1). Compounds **3** and **8** had almost the same effect as compound **9** (data not shown).

Effects of 2′-hydroxy-4′-methoxychalcone (compound 9) on the protein levels of COX 1 and COX 2 in rat peritoneal macrophages

Western-blot analysis revealed that the COX-1 level in macrophages was unchanged by treatment with TPA (16.2 nM) when determined at 6 h (Figure 2). Treatment with compound **9** at 30 μM in the presence of TPA did not affect protein levels of COX 1 at 6 h (Figure 2) either. In contrast, the COX-2 level at 6 h was increased by treatment with TPA (16.2 nM) (Figure 2). In the presence of compound **9**, the induction of COX-2 protein at 6 h caused by TPA was inhibited in a concentration-dependent manner at 3–30 μM (Figure 2), in accordance with the potency with which PGE_2 production was inhibited (Table 3).

Compounds **3** and **8** had almost the same effect as compound **9** (data not shown).

Discussion

Recently, we reported that the isoflavones tectorigenin and tectoridin isolated from the rhizomes of *Belamcanda chinensis*, a Korean medicinal plant, inhibit PGE_2 production in TPA- or thapsigargin-stimulated rat per-

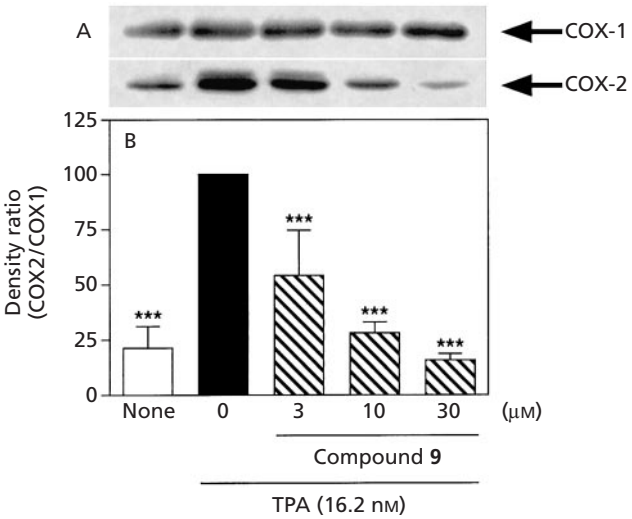


Figure 2 Effect of 2′-hydroxy-4′-methoxychalcone (compound **9**) on COX-2 protein induction in TPA-stimulated rat peritoneal macrophages. Rat peritoneal macrophages (3×10^6 cells) were incubated at 37°C for 6 h in 2 mL of medium containing TPA (16.2 nM) and the indicated concentration of compound **9**. The protein level of COX-1 and COX-2 was determined by Western-blot analysis (A). The density ratios of COX-2 protein to COX-1 protein were calculated, and the mean value of the density ratio in the TPA-treated control group was set to 100 (B). Values are the means from five samples with s.d. shown by vertical bars; $***P < 0.001$ vs corresponding TPA control.

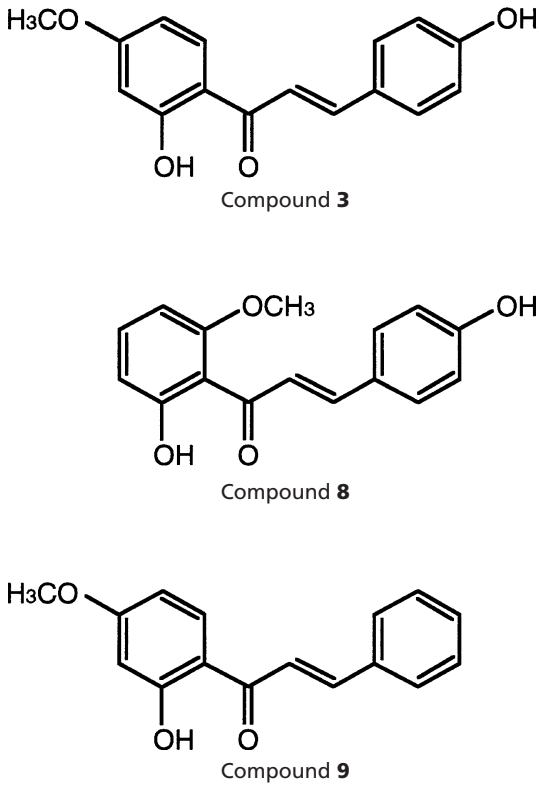


Figure 3 Chemical structures of compounds **3** (2′,4-dihydroxy-4′-methoxychalcone), **8** (2′,4-dihydroxy-6′-methoxychalcone) and **9** (2′-hydroxy-4′-methoxychalcone).

itoneal macrophages by inhibiting the induction of COX-2 protein (Kim et al 1999). We suggested that the anti-inflammatory activity of *Belamcanda chinensis* rhizome extract is due to the inhibition of prostanoid production dependent upon COX 2 in the inflammatory cells. Because COX-2 protein is induced by several kinds of stimuli in inflammatory cells (Lee et al 1992; Sano et al 1992; Yamada et al 1997; Yamashita et al 1997), inhibitors of COX-2 protein induction might be candidates for the new-type non-steroidal anti-inflammatory drugs. During the course of the study on inhibitors of COX-2 protein induction in rat peritoneal macrophages, we found that certain types of chalcone derivatives inhibit PGE_2 production by inhibiting the induction of COX-2 protein. This study was undertaken to clarify the structure–activity relationship of 14 2′-hydroxychalcone derivatives.

Among the 2′-hydroxychalcone derivatives, compounds **3**, **8** and **9** (Figure 3) had the most potent inhibitory effect. Substitution of the 4′-methoxyl group of compound **3** with a 4′-hydroxyl group (compound **2**) decreased the inhibitory activity. Substitution with a 5′-methoxyl group (compound **7**) also decreased the inhibitory activity, while replacing the 4′-methoxyl group of compound **3** with a 6′-methoxyl group (compound **8**) did not change the inhibitory effect. Therefore, a methoxyl group at 4′ or 6′ is essential for the expression of the inhibitory activity of 2′-hydroxychalcone derivatives. Among the 2′-hydroxychalcones whose 5′ position was substituted with a chloro group (compound **4**), methyl group (**5**), hydroxyl group (**6**) or methoxyl group (**7**), 2′,5′-dihydroxychalcone (**6**) had the strongest inhibitory activity, but the activity did not exceed that of compounds **3** or **8**. Addition of a chloro group (compound **11**), methyl group (**12**), methoxyl group (**13**), or *N*-dimethyl group (**14**) at position 4 of the B ring decreased the inhibitory potency of compound **9** whose B ring had no substitution.

These findings indicate that for 2′-hydroxychalcones, the addition of a 4′-methoxyl group or 6′-methoxyl group is important to the potent inhibitory activity against TPA-induced PGE_2 production. As for the B ring of 2′-hydroxy-4′-methoxychalcones, substitution with another group at position 4 decreased the inhibitory activity of compound **3**. The inhibitory activity of compounds **10** and **13** was much less than that of compounds

3 and **9**, indicating that the addition of a methyl group at position 3 and 4 of the B ring lowers the inhibitory activity. Dehydration at position 4 of compound **3** did not affect the inhibitory activity, indicating that the hydroxyl group at position 4 of the B ring is not essential for the expression of the inhibitory activity.

To clarify the mechanism by which compounds **3**, **8** and **9** inhibit the TPA-induced production of PGE_2, the direct effects of these compounds on COX-1 and COX-2 were examined. Under conditions in which indometacin inhibits the activity of both COX-1 and COX-2, and NS-398 inhibits the activity of COX-2 but not COX-1, these compounds, at concentrations of 10 and 30 μM, slightly inhibited COX-1 activity but did not inhibit COX-2 activity (Table 4). Because TPA-induced production of PGE_2 at 8 h in rat peritoneal macrophages depends on the TPA-induced expression of COX-2 protein (Yamada et al 1997), direct partial inhibition by these compounds does not contribute to the inhibition of the TPA-induced PGE_2 production at 8 h. Therefore, the inhibition of the TPA-induced PGE_2 production by these compounds is not due to the direct inhibition of COX-1 and COX-2.

Second, we examined the effect of these compounds on the TPA-induced release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages. Because the release of radioactivity was not suppressed by these compounds (Figure 1), it was suggested that inhibition of the TPA-induced production of PGE_2 is not due to the inhibition of phospholipase A_2. Finally, we examined the effects of these compounds on the protein levels of COX-1 and COX-2. It was demonstrated that these compounds inhibit the TPA-induced induction of COX-2 protein, but have no effect on COX-1 protein levels (Figure 2). From these observations, we suggest that the inhibition of TPA-induced PGE_2 production by these compounds is due to the inhibition of COX-2 protein induction. The molecular mechanism of action of these compounds in the suppression of the TPA-induced COX-2 protein induction remains to be clarified.

Recently, it has been reported that 3′,4′,5′,3,4,5-hexamethoxychalcone inhibits the production of PGE_2 and nitric oxide by inhibiting the induction of COX-2 and inducible nitric oxide synthase, respectively, in lipopolysaccharide-stimulated mouse peritoneal macrophages (Herencia et al 1999). Together with these findings, our results suggest that certain types of chalcone derivatives are potentially new non-steroidal anti-inflammatory drugs. Inhibition of the induction of COX-2 protein might be a novel therapeutic approach for inflammatory diseases. Specific inhibitors of COX-2 protein induction might act as specific inhibitors of COX-2, such as NS-398 (Futaki et al 1994), nimesulide (Taniguchi et al 1995), rofecoxib (Chan et al 1999) and celebrex (Mandell 1999), having fewer side effects on the gastrointestinal tract than the non-selective, COX-1-directed anti-inflammatory drugs, including aspirin and indometacin. Therefore, the suppression of COX-2 activity in the inflammatory cells is one of the targets in the development of non-steroidal anti-inflammatory drugs. However, COX inhibition results in increased COX-2 protein expression in the murine macrophage cell line J774 (Pang & Hoult 1996) and transient over-production of PGE_2 in rat hepatocytes and peritoneal macrophages (Callejas et al 1999). Furthermore, some inhibitors of COX-1 and COX-2 are less effective at more inflamed sites, because the supply of arachidonic acid determines effectiveness (Hamilton et al 1999).

It remains to be elucidated whether compounds **3**, **8** and **9** show anti-inflammatory activity in-vivo.

Conclusions

Among the 14 synthetic 2′-hydroxychalcone derivatives, 2′,4-dihydroxy-4′-methoxychalcone (compound **3**), 2′,4-dihydroxy-6′-methoxychalcone (**8**) and 2′-hydroxy-4′-methoxychalcone (**9**) were the most potent inhibitors of TPA-induced prostaglandin E_2 production in rats. Investigation of the structure–activity relationship revealed that the addition of a 4′-methoxyl or 6′-methoxyl group is important to increase the inhibitory activity of 2′-hydroxychalcone. Compounds **3**, **8** and **9** inhibited TPA-induced COX-2 protein induction, but showed no effect on TPA-induced release of radioactivity from [^{3}H]arachidonic acid-labelled macrophages. These compounds did not inhibit COX-2 activity but slightly inhibited COX-1. Because TPA-induced PGE_2 production is mainly dependent on TPA-induced COX-2, the inhibition of the TPA-induced PGE_2 production by these compounds is due to the inhibition of COX-2 protein induction by TPA.

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