JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1333–1337

Received March 28, 2004
Accepted June 21, 2004
DOI 10.1211/0022357044247
ISSN 0022-3573

Structure–activity relationship studies on chalcone derivatives: potent inhibition of platelet aggregation

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Abstract

In an effort to develop potent antiplatelet agents with anti-inflammatory action, a novel series of anti-inflammatory chalcones was screened to evaluate their antiplatelet effects. Structure–activity relationships and mode of action were investigated and characterized. The antiplatelet effects of the chalcones on washed rabbit platelets and human platelet-rich plasma were evaluated. Arachidonic acid-induced platelet aggregation was potently inhibited by almost all the chalcone derivatives. Collagen-induced platelet aggregation was potently inhibited by all the chalcone derivatives at 300 μM, except for compound **4** at 100 μM. Compounds **6**, **7** and **9** significantly inhibited the aggregation of washed rabbit platelets induced by platelet-activating factor at 300 μM. Of the compounds tested in human platelet-rich plasma, compounds **2**, **8** and **9** showed significant inhibition of secondary aggregation induced by adrenaline. It is concluded that the antiplatelet effect of **2**, **8** and **9** is mainly owing to an inhibitory effect on thromboxane formation. The inhibitory effect of **6**, **7** and **9** on platelet aggregation induced by platelet-activating factor could be owing to a calcium antagonizing effect or inhibition of intracellular calcium mobilization.

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Funding: This work was partially supported by a research grand from the National Science Council of the Republic of China (NSC 90-2320-B037-042).

Introduction

Platelet aggregation is an important pathogenic factor in the development of atherosclerosis and associated thrombosis in humans (Kikimoto et al 1990). Therefore, one rational approach in the development of antithrombotic drugs is to search for inhibitors of platelet aggregation.

Nakadate et al (1985) reported that known hydroxychalcones inhibit 12-lipoxygenase and cyclooxygenase in the mouse epidermis. Some chalcone derivatives have been reported as 5-lipoxygenase and cyclooxygenase inhibitors, with anti-inflammatory or antioxidative effects (Sogawa et al 1993). A natural chalcone, broussochalcone A, was isolated from *Broussonetia papyrifera* by our laboratory: it has potent antiplatelet effects and is a potent inhibitor of cyclooxygenase (Lin et al 1997). The above findings encouraged us to continue our research into chalcone derivatives.

We recently synthesized a novel series of chalcones and demonstrated that chalcones inhibit platelet aggregation of washed rabbit platelets and human platelet-rich plasma (PRP) induced by various inducers, and cyclooxygenase activity (Lin et al 1997, 2001; Huang et al 2001). These findings suggested that chalcones may be promising antithrombotic agents. In the present study, we further describe the antiplatelet effects in washed rabbit platelets and human PRP of a variety of chalcones: structure–activity relationships and mode of action were investigated and characterized.

Materials and Methods

Chemistry

Melting points (uncorrected) were determined with a Yanaco micro-melting point apparatus (Yanaco, Kyoto, Japan). IR spectra were determined with a PerkinElmer

system 2000 FT-IR spectrophotometer (PerkinElmer, Boston, MA, USA). ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer (Varian, Palo Alto, CA, USA), and mass spectra were obtained on a JMS-HX 100 mass spectrometer (Jeol, Peabody, MA, USA). Elemental analyses were within $\pm 0.4\%$ of the theoretical values unless otherwise noted. Chromatography was performed using a flash-column technique on silica gel 60 supplied by E. Merck (Taipei, Taiwan).

Chalcones (**1**–**9**) were synthesized using Claisen-Schmidt condensation of appropriate acetophenones or hydroxyacetophenones, protected as tetrahydropyranyl ether, with appropriate aromatic aldehydes or hydroxy-aromatic aldehydes, protected as tetrahydropyranyl ether. This procedure afforded various chalcone derivatives, which have been synthesized and reported previously (Hsieh et al 2000), in a good yield (Table 1).

Platelet aggregation

Washed rabbit platelets were obtained from EDTA anti-coagulated PRP according to procedures described previously (Teng et al 1987). Human PRP was obtained from the supernatant after centrifugation of a 1:9 mixture of blood and sodium citrate solution (3.8%). Platelet numbers were counted with a Coulter counter (Model ZM; Beckman Coulter, Inc., Fullerton, CA, USA) and adjusted to 4.5×10^8 platelets mL^{-1}. The platelet pellets were suspended in Tyrode's solution containing (in mM): NaCl 136.8, KCl 2.8, $NaHCO_3$ 11.9, $MgCl_2$ 2.1, NaH_2PO_4 0.33, $CaCl_2$ 1.0 and glucose 11.2, with 0.35% bovine serum albumin. All glassware was siliconized. PRP or the platelet suspension was stirred at 1200 rev min^{-1} before addition of the aggregation inducer. Aggregation was measured by a turbidimetric method (O'Brien 1962). The absorbance of platelet-poor plasma or platelet-free Tyrode's solution was taken as 100% aggregation. The aggregation was measured by means of a Lumi-aggregometer (Chrono-Log Corporation, Havertown, PA, USA) connected to dual channel recorders.

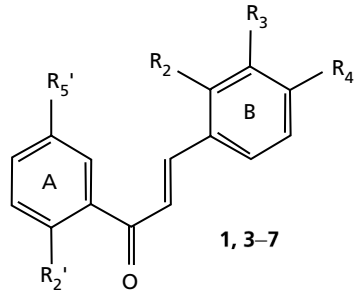
Data analysis

Data are presented as means $\pm$ s.e.m. One-way analysis of variance was used for multiple comparison, and if there was significant variation between the treatment groups and the inhibitor-treated groups, they were then compared with the control group by Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Result and Discussion

The aggregation of washed rabbit platelets induced by arachidonic acid (100 μM), collagen (10 μg mL^{-1}), thrombin (0.1 units mL^{-1}), and platelet-activating factor (2 ng mL^{-1}) was used to study the antiplatelet effects of compounds **1**–**9**. As shown in Table 2 and Figure 1, all synthetic chalcones, except for **4**, had potent antiplatelet effects on arachidonic acid-induced aggregation. The inhibitory effects of compounds **1**–**3** and **5**–**9** were concentration dependent, and the IC50 values on aggregation of washed rabbit platelets were

Table 1 Structures of chalcones **1**–**7** and dihydrochalcones **8** and **9**



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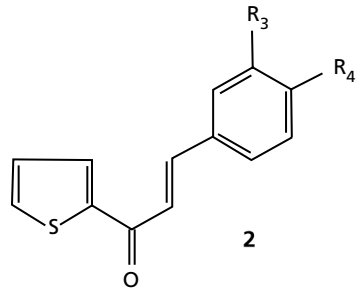

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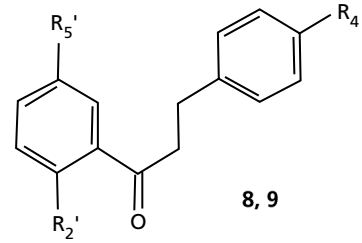

8, 9

Compound	R_2'	R_5'	R_2	R_3	R_4	Formula[a]	Anal.
1	OH	H	H	H	Cl	$C_{15}H_{11}O_2Cl$	C,H,O
2				OH	OH	$C_{13}H_{10}O_3S$	C,H,O
3	OMe	OMe	H	OH	OH	$C_{17}H_{16}O_5$	C,H
4	OH	OMe	H	OMe	OMe	$C_{18}H_{18}O_5$	C,H
5	OH	OMe	H	H	OMe	$C_{17}H_{16}O_4$	C,H
6	OH	OMe	H	H	Me	$C_{17}H_{16}O_3$	C,H
7	OH	OMe	H	H	Cl	$C_{16}H_{13}O_3Cl$	C,H,O
8	OH	OH	H	H	H	$C_{15}H_{14}O_3$	C,H
9	OH	OH	H	H	Cl	$C_{15}H_{13}O_3Cl$	C,H,O

[a]C and H analyses were within 0.4% of theoretical values.

Table 2 Effects of chalcones and dihydrochalcones on the platelet aggregation induced by arachidonic acid, collagen, thrombin and platelet-activating factor

Compound	Platelet aggregation (%)			
	Arachidonic acid	Collagen	Thrombin	Platelet-activating factor
Control	88.6 ± 0.6	88.4 ± 1.9	91.8 ± 1.3	89.5 ± 0.9
1	3.2 ± 1.7**	22.5 ± 2.5**	83.3 ± 1.1**	48.4 ± 7.2
2	0.0 ± 0.0**	14.0 ± 2.5**	89.2 ± 1.1*	63.5 ± 4.4**
3	0.0 ± 0.0**	16.7 ± 0.8**	81.7 ± 4.3**	30.7 ± 1.7**
4	82.4 ± 2.1**	24.6 ± 3.8**	82.3 ± 5.1*	72.0 ± 5.9**
5	0.0 ± 0.0**	0.0 ± 0.0**	63.1 ± 5.2**	50.7 ± 3.0**
6	0.0 ± 0.0**	0.0 ± 0.0**	60.5 ± 10.0**	2.8 ± 2.2**
7	0.0 ± 0.0**	0.0 ± 0.0**	80.5 ± 1.3**	4.0 ± 3.2**
8	0.0 ± 0.0**	12.3 ± 3.3**	89.6 ± 0.8*	62.6 ± 3.1**
9	0.0 ± 0.0**	0.0 ± 0.0**	72.0 ± 5.2**	0.0 ± 0.0**
Aspirin	0.0 ± 0.0**	85.4 ± 3.9	91.9 ± 2.5	90.5 ± 1.2

Platelets were pre-incubated with compounds **1**–**3**, **5**–**9** (each at 300 μM), **4** (100 μM), aspirin (50 μM) or dimethylsulfoxide (0.5%, control) at 37°C for 3 min, and then arachidonic acid (100 μM), collagen (10 μg mL^{-1}), thrombin (0.1 units mL^{-1}), or platelet-activating factor (2 ng mL^{-1}) was added. Data are presented as means ± s.e.m., n = 3–5. *P < 0.05; **P < 0.01: compared with the respective control value.

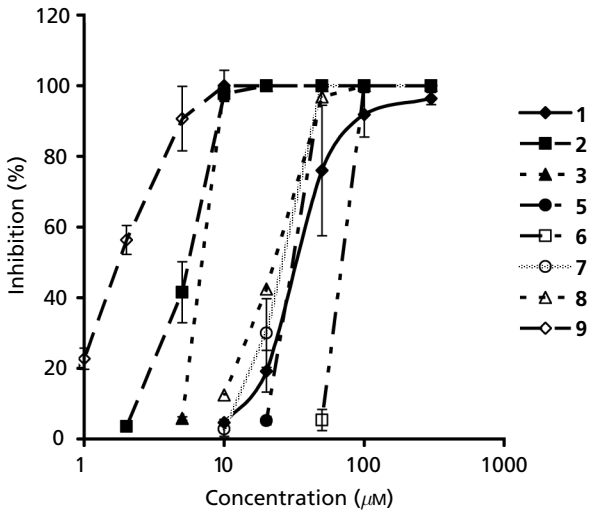

Figure 1 Effect of chalcones on platelet aggregation induced by arachidonic acid. Washed rabbit platelets were incubated with various concentrations of compounds **1**–**3** and **5**–**9**, and then arachidonic acid (100 μM) was added to trigger aggregation. Data are presented as means ± s.e.m., n = 3–5.

35.9, 5.8, 7.3, 34.2, 73.4, 28.3, 2.4 and 1.8 μM, respectively. It was clearly indicated that the A ring of the chalcone moiety substituted by a thienyl group enhanced the inhibitory effect on arachidonic acid-induced aggregation. Compound **3** had potent antiplatelet effects on arachidonic acid-induced platelet aggregation; the demethylation at C-2′ and the *O*-methylation at C-3 and C-4 (i.e. **4**) did not enhance the antiplatelet effects. The demethoxylation at C-3 of **4** (i.e. **5**), and the demethoxylation at C-3 and C-4, and a methyl or chloro group substituted at C-4 of **3** (i.e. **6** or **7**) enhanced the antiplatelet effects on arachidonic acid-induced platelet aggregation, although it was not as potent as that of compound **3**. Both of the free phenolic groups at C-3 and C-4 of chalcone derivatives may be important in the inhibition of arachidonic acid-induced platelet aggregation (Lin et al 1997). Reduction of 2′,5′-dihydroxychalcone to **8** did not enhance the inhibitory effect on arachidonic acid-induced platelet aggregation, whereas reduction of 2′,5′-dihydroxy-4-chlorochalcone to **9** did enhance the inhibitory effect on arachidonic acid-induced platelet aggregation, suggesting that the enone moiety of chalcones is not required for the inhibition of platelet aggregation induced by arachidonic acid (Lin et al 1997, 2001).

Of the compounds tested in the collagen-induced aggregation, all of them showed potent inhibitory effect at 300 μM (Table 1), except for **4** at 100 μM. Compounds **1** and **7** showed significant inhibition of platelet aggregation induced by collagen in a concentration-dependent manner, with IC50 values of 81.2 and 34.6 μM, respectively. Compounds **6**, **7** and **9** showed significant inhibition of platelet aggregation induced by platelet-activating factor at 300 μM (Tables 1 and 2; Figures 2 and 3); compound **9** did not show inhibitory effects at concentrations lower than 300 μM (data not shown). The effect appeared to be concentration-dependent and the IC50 values of **6** and **7** were 120.7 and 68.5 μM, respectively. The above results indicated that chlorination of 2′-hydroxychalcone at C-4 or *O*-methylation of 2′,5′-dihydroxy-4-chlorochalcone at C-5′ did not enhance the inhibitory effect on platelet aggregation induced by collagen (Lin et al 1997, 2001).The OMe at C-4 of **5** replaced by a Me group (i.e. **7**) markedly enhanced the inhibitory effect on platelet aggregation induced by platelet-activating factor.

The antiplatelet effects of **2**, **8** and **9** on platelet aggregation induced by adrenaline (5 μM) in human PRP were also studied. As shown in Table 3 and Figure 4, all three

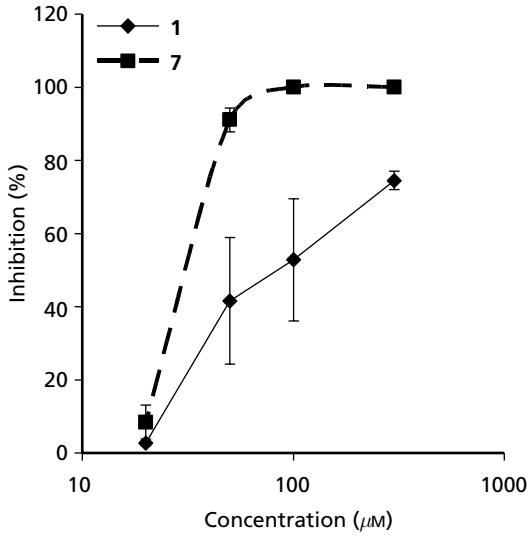

Figure 2 Effect of chalcones on platelet aggregation induced by collagen. Washed rabbit platelets were incubated with various concentrations of compounds **1** and **7**, and then collagen ($10\,\mu g\,mL^{-1}$) was added to trigger aggregation. Data are presented as means ± s.e.m., n = 3–5.

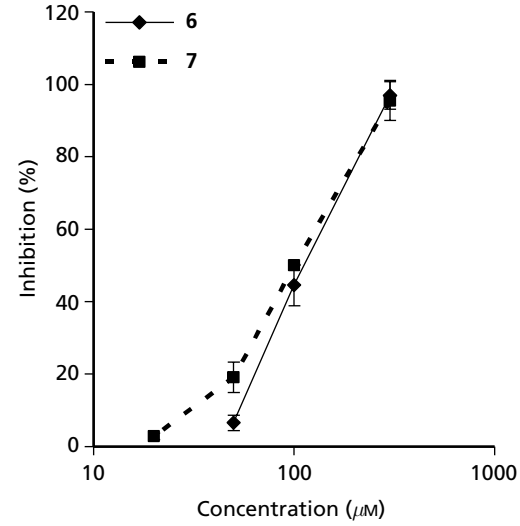

Figure 3 Effect of chalcones on platelet aggregation induced by platelet-activating factor. Washed rabbit platelets were incubated with various concentrations of compounds **6** and **7**, and then platelet-activating factor ($2\,ng\,mL^{-1}$) was added to trigger aggregation. Data are presented as means ± s.e.m., n = 3–5.

compounds had significant antiplatelet effects on adrenaline-induced platelet aggregation. This effect appeared to be concentration-dependent. In adrenaline-induced platelet aggregation, all three compounds prevented secondary aggregation (i.e. **9** in Figure 5). Aspirin was used in the present study as a positive control. Aspirin (50 μM) strongly inhibited platelet aggregation induced by epinephrine in human PRP (Table 3).

Table 3 Antiplatelet effects of compounds **2**, **8** and **9**, and aspirin on adrenaline-induced aggregation in human platelet-rich plasma

Compound	Aggregation (%)
Control	96.6 ± 1.6
2	42.6 ± 8.0*
8	48.8 ± 7.3*
9	36.5 ± 4.0**
Aspirin	29.5 ± 1.0**

Human platelet-rich plasma was pre-incubated with dimethylsulfoxide (0.5%, control), **2**, **8**, **9** (each at 50 μM) or aspirin (50 μM) at 37°C for 3 min, and then adrenaline (5 μM) was added. Data are presented as means ± s.e.m., n = 3. *$P < 0.05$; **$P < 0.01$: compared with the control.

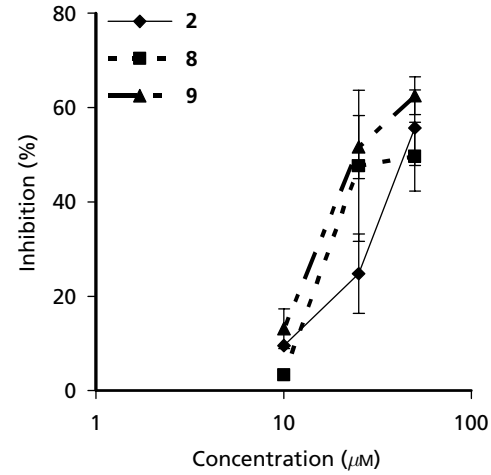

Figure 4 Concentration-dependent inhibitory effect of compounds **2**, **8** and **9** on platelet aggregation induced by adrenaline in human platelet-rich plasma. Human platelet-rich plasma was incubated with various concentrations of compounds **2**, **8**, **9** or dimethylsulfoxide (0.5%) at 37°C for 3 min, and then adrenaline (5 μM) was added to trigger aggregation. Data are presented as means ± s.e.m., n = 3–4.

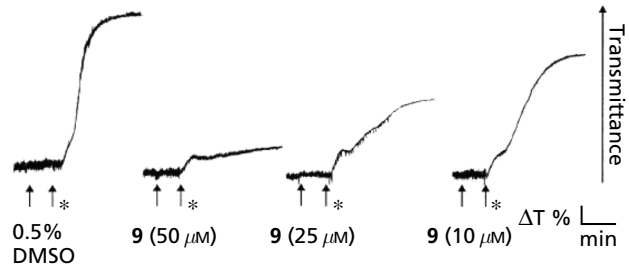

Figure 5 Effect of compound **9** on the aggregation of human platelet-rich plasma induced by adrenaline. Human platelet-rich plasma was pre-incubated with dimethylsulfoxide (0.5%, control) or various concentrations of **9** for 3 min, and then adrenaline (5 μM) was added to trigger aggregation. *Adrenaline added here to trigger platelet aggregation.

Conclusions

In the present study, compounds **2**, **8** and **9** are potent inhibitors of arachidonic acid-induced platelet aggregation in washed rabbit platelets and secondary aggregation induced by adrenaline in human PRP. The antiplatelet effects of **2**, **8** and **9** are probably mainly mediated through the suppression of cyclooxygenase activity and reduced thromboxane formation or owing to the inhibition of thromboxane synthase, leading to reduced thromboxane formation (Mitchell & Sharp 1964; Mustard et al 1975; Weiss 1983). Compounds **6**, **7** and **9** (each at 300 μM) inhibited platelet aggregation induced by platelet-activating factor, which did not cause thromboxane formation (Teng et al 1987). This inhibition could be owing to the calcium antagonizing effect or inhibition of intracellular calcium mobilization. Further experiments are needed to elucidate their mechanism of action. Compounds **8** and **9** have been shown to have inhibitory effects on nitric oxide production and inducible nitric oxide synthase protein expression (Hsieh et al 2000; Ko et al 2003), and **9** also inhibited cyclooxygenase-2 activity in RAW 264.7 cells (Huang et al 2001), suggesting that **9** could be developed as a potential antithrombotic and antioxidant agent to reduce the risk of cardiovascular disease.

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