Full Research Paper

Antiplatelet Effect and Selective Binding to Cyclooxygenase (COX) by Molecular Docking Analysis of Flavonoids and Lignans

Chien-Ming Wu [1], Shu-Chun Wu [2], Wan-Jung Chung [3], Hsien-Cheng Lin [4], Kun-Tze Chen [5], Yu-Chian Chen [5], Mei-Feng Hsu [6], Jwu-Maw Yang [3], Jih-Pyang Wang [7] and Chun-Nan Lin [2,*]

1 Department of Physical Medicine and Rehabilitation, Yuan's General Hospital, Kaohsiung 802, Taiwan

2 School of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan

3 Department of Biotechnology, Chia-Nan University of Pharmacy and Science, Tainan Hsien 717, Taiwan

4 School of Technology for Medical Science, Kaohsiung Medical University, Kaohsiung 807, Taiwan

5 Department of Biological Science and Technology, China Medical University, Taichung 404, Taiwan

6 Department of Biochemistry, China Medical University, Taichung 404, Taiwan

7 Department of Education and Research, Taichung Veterans General Hospital, Taiwan 407, Taiwan

* Author to whom correspondence should be addressed; E-mail: lincna@cc.kmu.edu.tw;
Tel: (+886)7-3121101; Fax: (+886)7-5562365

Abstract: The known flavonoids ginkgetin (**1**), taiwanhomoflavone A (**2**), taiwanhomoflavone B (**3**), and taiwanhomoflavone C (**4**) and eight known lignans: justicidin B (**9**), justicidin C (**10**), justicidin D (**11**), chinensinaphthol methyl ether (**12**), procumphthalide A (**13**), procumbenoside A (**15**), and ciliatosides A (**16**) and B (**17**) were isolated from *Cephalotaxus wilsoniana* and *Justicia* species, respectively. The antiplatelet effects of the above constituents on human platelet-rich plasma (PRP) were evaluated. Of the compounds tested on human PRP, compounds **1**, **4**, **9**, and **11** showed inhibition of secondary aggregation induced by adrenaline. Compound **1** had an inhibitory effect on cyclooxygenase-1 (COX-1). Molecular docking studies revealed that **1** and the related

compounds apigenin (**5**), cycloheterophyllin (**6**), broussoflavone F (**7**), and quercetin (**8**) were docked near the gate of active site of COX-1. It indicated that the antiplatelet effect of **1**, **4**, **9**, and **11** is partially owed to suppression of COX-1 activity and reduced thromboxane formation. Flavonoids, **1**, **5**, **6**, 7, and **8** may block the gate of the active site of COX-1 and interfere the conversion of arachidonic acid to prostaglandin (PG) H_2 in the COX-1 active site.

Keywords: Flavonoids; lignans; antiplatelet; COX-1; molecular docking

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1. Introduction

In previous papers, we have reported isolation of flavonoids from *Cephalotaxus wilsoniana* (Cephalotaxaceae), lignans from *Justicia* species (Acanthaceae), and their antiplatelet, cytotoxic, and anti-inflammatory effects [1-5]. To continue screening work on antiplatelet effects of natural products, ten known lignans: diphyllin, justicidin A, justicidin B (**9**), justicidin C (**10**), justicidin D (**11**), justicidin E, chinensinaphthol methyl ether (**12**), procumphthalide A (**13**), taiwan E methyl ether (**14**), and ciliatoside A (**16**) were isolated from *J. procumbens* var. *Hayatai*.

Figure 1. Structures of **1**-**8**.

Justicidin A, **10**, **11**, **12** and **14** were shown to have antiplatelet activity on rabbit platelets against arachidonic acid (AA) induced aggregation, while justicidin A and **14** were also shown to have antiplatelet effects on platelet aggregation induced by adrenaline in human platelet-rich plasma (PRP) [5,6]. In the present paper, the antipletelet effects of flavonoids, ginkgetin (**1**), taiwanhomoflavone A (**2**), taiwanhomoflavone B (**3**), and taiwanhomoflavone C (**4**) [1] from *C. wilsoniana* and lignins, **9-13**, prcumbenoside A (**15**) [4], and ciliatosides A (**16**) and B (**17**) [3] from *Justicia* species are reported (Figures 1 and 2). Antiplatelet compounds with inhibitory effect on AA-induced platelet aggregation in washed rabbit platelets and secondary aggregation induced by adrenaline in human PRP are mainly owing to suppression of cyclooxygenase (COX) activity and reduced thromboxane formation or owing to inhibition of thromboxane synthase, leading to reduced thromboxane formation [7-9]. A more detailed understanding of COX isoform differences may aid in the design of more selective and potent inhibitors of both COX isoforms. Thus, the possible binding models of **1** and related flavonoids, apigenin (**5**), cycloheterophyllin (**6**), broussoflavonol F (**7**) and quercetin (**8**) within the COX-1 active site have been analyzed and are also reported in the present paper.

9   R=H, R'=R"=OMe
14 R=OMe, R, R"= ⁻OCH₂O⁻

10 R=R'=R"=OMe, R'''=H
11 R=OMe, R, R"= ⁻OCH₂O⁻,R'''=H
13 R=H, R'=R"=R'''=OMe

12

15 R=CH₂OH, R'=OH
16 R=CH₂O-β-D-xylopyranosyl, R'=OH
17 R=CH₂O-β-D-xylopyranosyl, R'=O-β-D-
        apiofuranosyl

Figure 2. Structures of **9-17**.

2. Results and Discussion

Platelet aggregation is an important pathogenic factor in the development of atherosclerosis and associated thrombosis in human [7]. A rational approach for the discovery of antithrombotic drugs is to search for inhibitors of platelet aggregation. In previous papers, several prenylflavonoids exhibited strong inhibition of platelet aggregation induced by AA and their antiplatelet effects are partially due to an inhibitory effect on COX-1 [10,11]. To study the structure-antiplatelet activity relationships of various flavonoids and lignans, the antiplatelet effects of **1-4**, **9-13**, and **15-17** on platelet aggregation induced by adrenaline (5 µM) in human PRP were studied. Lignans, **12** and **15-16** (data not shown)

showed no antiplatelet effects in the test. While **1**, **4**, and **13** (each at 300 µM) and **9-11** (each at 100 µM) had significant antiplatelet effects on the platelet aggregation of human PRP induced by adrenaline (Table 1 and Figures 3 and 4).

Table 1. Antiplatelet effects of flavonoids, **1**-**4**, lignans, **9**-**11** and **13**, and aspirin on adrenaline-induced aggregation in human PRP.

Compound (µM)		Aggregation (%)
Control		98.7 ± 0.3
1	(300)	$49.9 \pm 3.0*$
2	(300)	97.4 ± 0.8
3	(300)	98.8 ± 1.6
4	(300)	$38.5 \pm 1.2*$
9	(100)	$50.9 \pm 5.3***$
10	(100)	$74.7 \pm 2.2**$
11	(100)	$54.9 \pm 2.0***$
13	(300)	$27.7 \pm 11.0**$
Aspirin	(50)	$29.5 \pm 1.0*$

Human PRP was pre-incubated with DMSO (0.5%, control) at 37 °C for 3 min, and then adrenaline (5 µM) was added. Data are presented as mean $\pm$ s. e. m. (n = 3).

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$: compared with the control value.

Concentration (µM)

Figure 3. Concentration-dependent inhibitory effect of **1**, **4**, and aspirin on platelet aggregation induced by adrenaline in human PRP. Human PRP was incubated with various concentrations of **1**, **4**, aspirin or DMSO (0.5%) at 37 °C for 3 min, and then adrenaline (5 µM) was added to trigger aggregation. Data are presented as means $\pm$ s. e. m (n = 3).

Figure 4. Concentration-dependent inhibitory effect of compounds **9**, **11**, **13,** and aspirin on platelet aggregation induced by adrenaline in human PRP. Human PRP was incubated with various concentrations of **9**, **11**, **13**, aspirin or DMSO (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).

The antiplatelet effects of **1, 4, 9, 11**, and **13** appeared to be concentration dependent, with IC_{50} values of 293.6 ± 18.4, 259.0 ± 22.3, 104.8 ± 25.3, 106.5 ± 39.4, and 255.1 ± 86.2 μM, respectively. Compound **12** significantly inhibited rabbit platelet aggregation induced by AA [6], but did not significantly prevent secondary aggregation induced by adrenaline on human PRP. This could be due to the higher binding capacity of plasma for this compound. The positive control, aspirin (50 μM), strongly inhibited platelet aggregation induced by adrenaline on human PRP. In adrenaline induced platelet aggregation all compounds tested in Table 1 prevented secondary aggregation (i. e. **1** and **11** in Figures 5 and 6, respectively).

Figure 5. Effect of compound **1** on the aggregation of human PRP by adrenaline. Human PRP was pre-incubated with DMSO (0.5%, control) or various concentrations of **1** for 3 min, and then adrenaline (5 μM) was added to trigger aggregation. *Adrenaline added here to trigger platelet aggregation.

Figure 6. Effect of compound **11** on the aggregation of human PRP by adrenaline. Human PRP was pre-incubated with DMSO (0.5%, control) or various concentrations of **11** for 3 min, and then adrenaline (5 μM) was added to trigger aggregation. *Adrenaline added here to trigger platelet aggregation.

Compounds **9** and **11** revealed significant antiplatelet activities on rabbit platelet aggregation induced by AA, with IC_{50} values of 8.0 ± 1.2 and 1.7 ± 0.3 μM [6], but not on adrenaline induced aggregation in human PRP with IC_{50} values of 104.8 ± 25.3 and 106 ± 39.4 μM, respectively. This could be also due to higher binding capacity of plasma for these compounds.

It is indicated that the antiplatelet effects were probably mediated through the suppression of cyclooxygenase-1 (COX-1) activity and reduced thromboxane formation or owing to the inhibition of thromboxane synthase, leading to reduced thromboxane formation [8, 9].

Compounds **1** and **4** showed antiplatelet effect on platelet aggregation of human PRP induced by adrenaline. It indicated 3', 8" linked biflavonoids such as **1** showed stronger antiplatelet effect on platelet aggregation of human PRP induced by adrenaline while a methyl group substituted at C-6 such as **2** decreased antiplatelet effect on platelet aggregation of human PRP induced by adrenaline. Previously we had reported that cilinaphthalide B, justicidin A, and taiwanin E methyl ether (**14**) were shown to have an antiplatelet effects on human PRP [5]. Among them **14** showed a strong inhibitory effect on platelet aggregation induced by adrenaline in a concentration dependent manner with an IC_{50} value of 27.6 μM [5]. From Figure 4, it is clearly indicated that 1-aryl-2,3-naphthalide lignan type compounds such **14** had a stronger antiplatelet effect than that of 4-aryl-2,3-naphthalide lignan type ones such as **11** on human PRP.

When the activity of fatty acid COX-1 from ram vesicular glands was measured in the presence of the selected compounds. Compounds **1** and **14** weakly inhibited the enzyme (Table 2).

Table 2. Inhibitory activity (O$_2$ consumption) of selective compounds, **1**, **5-8** and **14** on COX-1.

Compounds (µM)		COX-1 activity nmol/min (% inhibition)	IC$_{50}$ (µM)
Control		10.9 ± 1.1	
1	(10)	8.5 ± 1.4 (25.1 ± 6.9)	
	(30)	7.6 ± 1.7 (33.5 ± 7.5)*	> 100
	(100)	6.6 ± 1.3 (42.0 ± 6.4)*	
5 [a]			> 100
6 [b]			52.0
7 [b]			34.6
8 [a]			8.0
14	(10)	9.9 ± 1.6 (12.9 ± 8.1)	> 100
	(100)	10.2 ± 1.0 (9.9 ± 5.3)	
Indomethacin (0.3)		9.1 ± 1.1 (20.6 ± 6.2)	
	(1)	5.6 ± 1.0 (49.1 ± 6.4)**	14 ± 0.2
	(3)	3.8 ± 0.7 (61.9 ± 5.2)**	

*$P < 0.05$; **$P < 0.01$: compared with the respective control value.

[a] Data cited from Ref. 13. [b] Data cited form Ref. 10

The antiplatelet activity of **1** and **14** is partially due to suppression of COX-1 activity and reduced thromboxane formation. In crystallization experiments, it was reported that all COX-1 inhibitors interacted with the putative catalytic amino acid residue Tyr 385 and formed hydrogen bonds with Arg 120 and Tyr 355 [12].

When mapping **1** and related flavonoids **5-8** into a COX-1 model (Figures 7-11), all these compounds docked near the gate of COX-1. It could be observed that the oxygen or phenolic oxygen atom of flavonoids accept hydrogen bond from Arg 120 or Tyr 355. The scoring values given by DockScore [14] are given in Table 3. Compounds **7** and **8** indicated good qualitative agreement between experimental (Table 2) and predicted (Table 3) results. Flavonoids **1** and **5-8** may block the gate of active site of COX-1 and interfere with the conversion of arachidonic acid to prostaglandin (PG) H$_2$ in the active site of COX-1.

Table 3. Docking results on COX-1 enzyme for **1** and **5-8**.

Compound	DockScore	Van der Waals force (kcal/mol)	Interaction with following amino acids near the gate of active site of COX-1
1	59.13	-32.91	Arg120
5	50.65	-16.67	Arg120[a], Tyr355
6	53.93	-33.85	Arg120
7	63.17	-34.14	Arg120, Tyr355
8	60.24	-2.01	Arg120

[a] Compound **5** forms two hydrogen bonds with Arg120.

Figure 7. Compound **1** docked near the gate of active site of COX-1. The hydrogen bonding interaction is shown as green broken line.

Figure 8. Compound **5** docked near the gate of active site of COX-1. The hydrogen bonding interaction is shown as green broken line.

Figure 9. Compound **6** docked near the gate of active site of COX-1. The hydrogen bonding interaction is shown as green broken line.

Figure 10. Compound **7** docked near the gate of active site of COX-1. The hydrogen bonding interaction is shown as green broken line

Figure 11. Compound **8** docked near the gate of active site of COX-1. The hydrogen bonding interaction is shown as green broken line.

Conclusions

In the present study, we had further found biflavonoid, **1**, tetraflavonoid, **4**, and lignans, **9** and **11** showed inhibition of secondary aggregation induced by adrenaline in human PRP. It indicates that the antiplatelet effects of these compounds are partially due to an inhibitory effect on thromboxane formation [7-9]. Additionally, compounds **1**, **5**, **6**, **7**, and **8** showed inhibitory effect on COX-1 activity (Table 3) [10,13]. It indicated a mode of action including the interference in arachidonic acid binding channel might be possible.

3. Experimental Section

General

Optical rotation: *JASCO* model *DIP-370* digital polarimeter. UV Spectra: *JASCO* UV-vis spectrophotometer. IR Spectra: *Perkin-Elmer 2000 FT-IR* spectrophotometer. ^{1}H and ^{13}C-NMR Spectra: *Varian Unity-400* spectrophotometers, at 400 (^{1}H) and 100 (^{13}C) MHz, with Me$_4$Si as internal standard; δ in ppm, *J* in Hz. Mass Spectra: *JMX-HX100MS* spectrometer.

Extraction and Isolation

Compounds **1**-**4**, **15**, and **17** have been previously identified and reported [1,3,4]. Whole plants of *Justicia procumbens* var. *Hayatai* (6.5 kg) were chipped and extracted with MeOH at room temperature. The MeOH extract was applied to a Si gel column and eluted with CHCl$_3$ to yield justicidin E (5 mg). Elution with *n*-hexane-EtOAc (8:1) yielded diphyllin (13 mg) and **11** (11 mg).

Elution with *n*-hexane-EtOAc (4:1) yielded justicidin A (105 mg), **10** (15 mg), **14** (35 mg), **12** (10 mg). Elution with *n*-hexane-EtOAc (3:1) yielded **9** (12 mg). Elution with *n*-hexane-EtOAc (2:1) yielded **16** (15 mg). Elution with EtOAc-acetone (5:1) yielded **13** (15 mg). The isolated compounds were identified by spectroscopic methods and comparison with the spectroscopic data reported in literature [3,5,6,15,16].

Platelet aggregation

Platelet aggregation in human PRP assays were performed by the method described previously [7].

COX-1 activity

The reaction mixture (0.1 M Tris-HCl, PH 8.0, 5 mM tryptophan, 8 mM hematin, test drugs, and 10 μg/mL of ram seminal vesicles COX was incubated for 3 min at 30 °C. The reaction was initiated by adding 100 μM AA. The velocity of oxygen consumption in the reaction mixture was monitored continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (Model 5300) [17].

Molecular docking study

Docking experiments were performed using DS modeling 1.2 (Accelrys Inc.). The coordinates for the x-ray crystal structure of the enzyme COX-1 was obtained from the RCSB Protein Data Bank and hydrogens were added. The ligand molecules were constructed using DS Modeling 1.1 and energy-minimized. The docking experiment on COX-1 was carried out by superimposing the energy-minimized ligand on FLP in the PDB file 1CQE after which FLP was detected. The chemistry at HARvard Molecular Mechanics force field (CHARMm) was employed for all docking purposes. The optical binding orientation of the ligand-enzyme site obtained after docking was further minimized for 1000 iterations using the conjugate gradient method until a convergence of 0.001 Kcal/mol-Å. [18]

Data analysis

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

Acknowledgements

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