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Pharmacological characterization of cinnamophilin, a novel dual inhibitor of thromboxane synthase and thromboxane A_2 receptor

[1]Sheu-Meei Yu, *Tian-Shung Wu & Che-Ming Teng

Department of Pharmacology, Chang Gung Medical College, Kwei-San, Tao-Yuan and *Department of Chemistry, National Cheng-Kong University, Tainan and Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan

1 The pharmacological effects of cinnamophilin, a new lignan, isolated from *Cinnamomum philippinense*, was determined *in vitro* in human platelet, rat isolated aorta and guinea-pig isolated trachea and *in vivo* in mice and guinea-pigs.

2 Cinnamophilin inhibited dose-dependently human platelet-rich plasma (PRP) aggregation induced by arachidonic acid (AA), collagen and U-46619 with IC_{50} of 5.0 ± 0.4, 5.6 ± 0.6 and $3.0 \pm 0.4\,\mu M$, respectively. The second wave of ADP- or adrenaline-induced platelet aggregation was inhibited by cinnamophilin, while the first wave was only slightly inhibited by cinnamophilin above $30\,\mu M$.

3 Cinnamophilin was found to be a thromboxane A_2 (TXA_2) receptor blocking agent in human platelet, rat aorta and guinea-pig trachea as revealed by its competitive antagonism of U-46619-induced aggregation of human-PRP, contraction of rat aortic rings and guinea-pig tracheal rings with pA_2 values of 7.3 ± 0.2, 6.3 ± 0.1 and 5.2 ± 0.2, respectively.

4 [^{3}H]-inositol monophosphate formation and the rise of intracellular Ca^{2+} caused by U-46619 in human platelet was suppressed by cinnamophilin ($10\,\mu M$).

5 Cinnamophilin induced a dose-dependent inhibition of thromboxane B_2 (TXB_2) formation, while the prostaglandin E_2 (PGE_2) formation was increased. Cinnamophilin did not affect unstimulated platelet adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels. When the platelets were challenged with AA, a dose-dependent rise in cyclic AMP was observed. Dazoxiben (a pure TX synthase inhibitor) and SQ 29548 (a pure TXA_2 receptor antagonist) did not affect cyclic AMP levels in AA-treated platelets.

6 A high concentration of cinnamophilin ($100\,\mu M$), failed to attenuate the contractile response of rat aorta to endothelin-1, angiotensin II, 5-hydroxytryptamine or noradrenaline. Contraction of tracheal rings induced by histamine, carbachol or KCl was also not inhibited by cinnamophilin ($100\,\mu M$).

7 Thirty min after intraperitoneal (i.p.) administration of cinnamophilin ($100\,\mu g\,kg^{-1}$), tail bleeding time of mice was prolonged more markedly than with indomethacin, dazoxiben or SQ 29548.

8 Intravenous administration of AA ($50\,\mu g\,kg^{-1}$) to guinea-pig induced bronchoconstriction. Cinnamophilin ($0.1\,mg\,kg^{-1}$, i.v.) was administered 1 min before AA, the bronchoconstriction response to AA was abolished.

9 It is concluded that cinnamophilin is a novel dual TX synthase inhibitor and TXA_2 receptor antagonist and that it may be a useful tool for the investigation and treatment of diseases involving TXA_2 disorders.

Keywords: Cinnamophilin; thromboxane A_2 (TXA_2) receptor antagonist; thromboxane synthase inhibitor

Introduction

The discovery of thromboxane A_2 (TXA_2) (Hamberg *et al.*, 1975) and prostacyclin (PGI_2) (Moncada *et al.*, 1976) has generated a great deal of interest in these highly potent derivatives of arachidonic acid (AA). TXA_2 is the major cyclo-oxygenase product derived from AA in blood platelets. It is an extremely potent platelet aggregator, vasoconstrictor and bronchoconstrictor (Moncada & Vane, 1987a). PGI_2 is produced by vascular endothelium and has potent biological actions opposing those of TXA_2 (Gryglewski *et al.*, 1976). Normally there is a homaeostatic balance maintained between TXA_2 and PGI_2. However, in some pathological conditions (e.g. thrombosis), the normal balance is upset allowing the detrimental properties of TXA_2 to predominate (Moncada & Vane, 1978b). Thus, drugs that inhibit TXA_2 formation or action may have a potential as antithrombotic agents.

Cyclo-oxygenase inhibitors (e.g. aspirin) have been criticized for their alleged capability of suppressing the synthesis not only of TXA_2 but also of PGI_2, a powerful antiaggregatory and vasodilator substance. The use of the more recently introduced thromboxane (TX) synthase inhibitors has been questioned on the basis of a possible proaggregatory activity of prostaglandin endoperoxides (PGG_2, PGH_2) accumulating after the block of TX synthase (Bertele *et al.*, 1981). TXA_2 receptor antagonists have the advantage of inhibiting the pro-aggregatory action of both TXA_2 and prostaglandin endoperoxides and also of antagonizing the effects of these agonists on smooth muscle cells (Gresele *et al.*, 1984). However, they do not suppress platelet activation mediated by TXA_2-independent agonists (high dose collagen, thrombin). Drugs with combined TX synthase inhibitory and TXA_2 receptor antagonistic properties may overcome several of the shortcomings of the pharmacological classes discussed above. Indeed, while TX synthase inhibition enhances the endogenous synthesis of antiaggregatory prostaglandins (PGI_2 and PGD_2) (Gresele *et al.*, 1987), TXA_2 receptor antagonism prevents accumulated endoperoxides from activating platelets and smooth muscle cells. The summation of these effects may theoretically lead to an increase of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in platelets and smooth muscle

[1] Author for correspondence.

cells, a phenomenon associated with strong platelet inhibition and smooth muscle relaxation.

Medicinal plants have been widely used as traditional remedies in oriental countries for hundreds of years. Recently, in a large scale screening test, we have found that cinnamophilin, a new lignan (Figure 1), isolated from *Cinnamomum philippinense*, possessed both TX synthase inhibitory and TXA_2 receptor antagonizing properties. In this study, the pharmacological effect of cinnamophilin was determined *in vitro* in human platelet, rat isolated aorta and guinea-pig isolated trachea and *in vivo* in mice and guinea-pigs.

Methods

Human platelet aggregation

Platelet-rich plasma (PRP) was obtained from blood collected from healthy individuals with sodium citrate (3.8%, 1:14) added as anticoagulant, and centrifuged for 10 min at 90 *g*. Three minutes before the addition of the aggregation inducer, PRP was preincubated with various concentrations of cinnamophilin and stirred at 1200 r.p.m. The absorbance of PRP was taken as 0% aggregation and the absorbance of platelet-poor plasma (PPP) as 100% aggregation. Adenosine 5'-triphosphate (ATP) released from platelets was detected by the bioluminescence method (DeLuca & McElory, 1978). ATP at a known concentration was used to calibrate the intensity of bioluminescence. Both the aggregation and release of ATP were simultaneously measured by a Lumi-aggregometer (Chrono-Log Co. U.S.A.) connected to a dual channel recorder. In order to eliminate the effect of the solvent on aggregation, the final concentration of dimethylsulphoxide (DMSO) was fixed at 0.5%.

Measurement of [^{3}H]-inositol monophosphate

The method was modified from those of Huang & Detwiler (1986) and Neylon & Summers (1987). Citrated human-PRP was centrifuged at 500 *g* for 10 min; the platelet pellets were then suspended in 700 µl of Ca^{2+}-free and bovine serum albumin (BSA)-free Tyrode solution (mM: NaCl 136.8, KCl 2.8, $MgCl_2$ 2.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9) containing 75 µCi ml^{-1} of [^{3}H]-*myo*-inositol. After incubation for 2 h at 37°C, the platelets were collected by centrifugation (500 *g* for 4 min) and suspended in Ca^{2+}-free Tyrode solution. Phosphoinositides breakdown was induced by adding aggregating agents to 1 ml of platelet suspension in a 3.5 ml cuvette with a stirring bar driven at 900 r.p.m., 37°C for 6 min. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 ml of supernatant was pooled and trichloroacetic acid was removed by washing with 5 × 2 vol. of diethylether. The aqueous phase containing the inositol phosphates was adjusted to pH 7–8 and diluted to 4 ml with distilled water before application to a Dowex-1 ion-exchange column for separation of the inositol phosphates as described previously by Neylon & Summers (1987). All the experiments were carried out in the presence of 5 mM LiCl to inhibit inositol-monophosphate phosphatase. Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured the inositol monophosphate as an index of the total inositol phosphates formation.

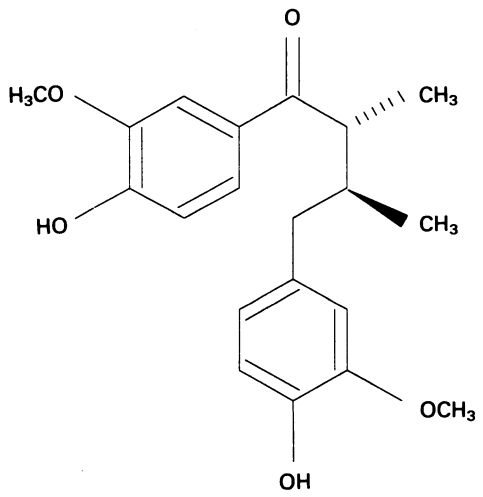

Figure 1 Chemical structure of cinnamophilin.

Measurement of intracellular Ca^{2+} in platelets

The method described by Rink *et al.* (1983) was used: citrated human-PRP was incubated with quin-2/AM (20 µM) for 40 min and then washed and suspended in the above Tyrode solution. Fluorescence was measured with a Hitachi Fluorescence Spectrophotometer (Ex. 339 nm, Em. 492 nm).

Measurement of platelet cyclic AMP

Platelet cyclic AMP was assayed with a commercially available RIA kit. Human-PRP was incubated with cinnamophilin or its vehicle for 3 min at 37°C with stirring, then AA (800 µM) or saline was added. After 3 min, the proteins were precipitated with 0.5 M trichloroacetic acid containing [^{3}H]-cyclic AMP for assessment of recovery. After centrifugation and chromatography the samples were assayed by RIA for cyclic AMP.

Measurement of TXB_2 and PGE_2 in human PRP

The effects of cinnamophilin on TXB_2 and PGE_2 production were determined in human PRP. Human PRP was preincubated with DMSO or various concentrations of cinnamophilin for 15 min, then AA (800 µM) was added for another 6 min incubation. The platelets were then spun down at 12,000 *g* for 5 min and the plasma was frozen until assay. Immunoreactive TXB_2 and PGE_2 were measured in unextracted, highly diluted serum samples by RIA kits.

Rat aortic contraction

Wistar rats of either sex weighing 250 to 300 g were killed by a blow to the head. The thoracic aorta was isolated and excess fat and connective tissue were removed. Vessels were cut into rings of about 5 mm in length and mounted in organ baths containing 5 ml Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.7. The tissue bath solution was maintained at 37°C and gassed with 95% O_2:5% CO_2 mixture. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. Aortae were equilibrated in the medium for 90 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph. Aortae were allowed to preincubate for 15 min with cinnamophilin before generating the cumulative concentration-response curve with each agonist for 15–30 min at 3 min intervals. Results are expressed as a percentage of the maximal control response for each agonist.

Guinea-pig tracheal contraction

Tracheae from guinea-pigs were dissected out, transferred to a dish containing Krebs solution and cut transversely between the segments of cartilage. Several of these, usually

about 5, were tied together so as to form a chain, which was then mounted in Krebs solution at 37°C, gassed with 95% O_2 plus 5% CO_2. One end of the chain was attached to a fixed pin in the bath and the other to a transducer connected to a Grass polygraph. Resting tension on each tissue was set at 1 g. Tracheae were allowed to equilibrate for at least 1 h and washed periodically. Cumulative concentration-response curves were obtained by application of various concentrations of spasmogens for 15–18 min at 3 min intervals. Tracheal rings were preincubated with cinnamophilin for 15 min, then various concentrations of spasmogens were added for 15–18 min at 3 min intervals. Results were expressed as percentage of the maximal control response for each agonist.

In vivo *studies*

Tail bleeding time in conscious mice Thirty minutes after the intraperitoneal (i.p.) administration of drugs, mice were placed in a tube holder with the tail allowed to protrude. The tail was transected at 1.5 mm from the tip and 1.5 cm of the distal portion was vertically immersed into saline at 37°C and the bleeding time was measured as described by Hornstra *et al.* (1981).

AA-induced bronchoconstriction in anaesthetized guinea-pigs

The method as previously described by Pretolani *et al.* (1987) was followed. Male Hartley guinea-pigs (350–400 g) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.). The carotid artery (for monitoring blood pressure), jugular vein (for drug administration) and trachea were cannulated. Mechanical ventilation was started (Harvard Rodent Ventilator, Model 683; 60 strokes min^{-1}) and spontaneous breathing abolished with pancuronium (2 mg kg^{-1}, i.v.). Bronchial resistance was recorded with a Bronchospasm Transducer (Ugo Basile, Model 7020) connected to a polygraph (Gould, Model 2400).

Drugs

Cinnamophilin (Figure 1) was isolated from *Cinnamomum philippinense* (unpublished data), and its purity (>99%) was confirmed by n.m.r., mass, IR and proton spectrophotometer. The following drugs were used: arachidonic acid, collagen (type I, bovine achilles tendon), ADP (tris salt), A23187, indomethacin, (−)-noradrenaline HCl, endothelin-1, angiotensin II, carbachol, quin-2/AM, histamine, luciferase-luciferin, trichloroacetic acid, bovine serum albumin (BSA), EDTA, sodium citrate and Dowex-1 (100–200 mesh: ×8, chloride) were obtained from Sigma Chemical. Co.; U-46619 (9,11-dideoxymethanoepoxy-9α,11α-$PGF_{2\alpha}$) from Biomol. cyclic AMP, *myo*-[2-^{3}H]-inositol, TXB_2 and PGE_2 RIA kits were purchased from Amersham. If drugs were dissolved in dimethylsulphoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0.1% and had no effect on the muscle contraction.

Data analysis

In each experiment, agonist dose-response curves in the presence of cinnamophilin were related to the control dose-response curves, of which the maximum response was taken as 100%. In most experiments, three or four concentrations of cinnamophilin were tested and the slopes of the resulting Schild plots were used to assess competitive antagonism. The pA_2 values were calculated for each concentration of cinnamophilin according to: $pA_2 = -\log([\text{antagonist}]/[\text{dose ratio} - 1])$ (Mackay, 1978).

The experimental results are expressed as the mean ± s.e. mean and accompanied by the number of observations. Statistical significance was assessed by Student's t test and P values less than 0.05 were considered significant.

Results

Effects of cinnamophilin on platelet aggregation and ATP release

Cinnamophilin inhibited dose-dependently human platelet-rich plasma (PRP) aggregation induced by arachidonic acid (AA) (800 μM), collagen (10 μM) and the prostaglandin endoperoxide analogue, U-46619 (1 μM) with IC_{50} of 5.0 ± 0.4 μM, 5.6 ± 0.6 μM and 3.0 ± 0.4 μM, respectively (Table 1). In human PRP, ADP (5 μM) and adrenaline (10 μM) caused biphasic aggregation. The second wave of ADP- or adrenaline-induced platelet aggregation was suppressed by cinnamophilin (IC_{50} = 6.0 ± 0.6 and 7.6 ± 0.9 μM, respectively), while the first wave was only slightly reduced by cinnamophilin above 30 μM. The release of ATP from platelets challenged by AA, collagen, U-46619, ADP or adrenaline was also inhibited by cinnamophilin; this inhibition was paralleled by its inhibitory effect on aggregation (Table 1). The aggregation and ATP release induced by the calcium ionophore, A23187, was slightly affected by cinnamophilin concentrations up to 30 μM (Table 1).

Cinnamophilin (0.3–10 μM) produced a parallel shift to the right of the concentration-response curve of U-46619; there was no depression of the maximum response to U-46619 even with the highest dose of cinnamophilin (10 μM) tested (Figure 2a). When the data were represented as a Schild plot, pA_2 and pA_{10} values of 7.3 (7.1–7.5 for 95% confidence limits) and 6.3 (6.1–6.5) with a slope of 0.95 were obtained (Figure 2b). At various times after the platelet aggregation trigged by U-46619 (1 μM), deaggregation of platelets was caused by cinnamophilin (3 μM) but not by indomethacin (20 μM). The earlier the addition of cinnamophilin, the more rapid was the reversal of platelet aggregation (Figure 3).

Effects of cinnamophilin on platelet phosphoinositides breakdown and intracellular Ca^{2+}

To see if signal transduction after TXA_2 receptor activation was blocked by cinnamophilin, human PRP were labelled with [^{3}H]-*myo*-inositol and quin-2/AM. Phosphoinositides breakdown was observed in platelets activated by many agonists. We found that thrombin (0.1 u ml^{-1}), collagen (10 μM), PAF (20 ng ml^{-1}) and U-46619 (1 μM) increased inositol monophosphate formation 280 ± 30%, 150 ± 20%, 180 ± 30% and 160 ± 20%, respectively (after subtraction of basal levels). Only the formation of inositol monophosphate by U-46619 was almost completely inhibited by cinnamophilin (10 μM) (Figure 4). In quin-2/AM-loaded platelets, the net increases of intracellular Ca^{2+} caused by thrombin, collagen,

Table 1 Effects of cinnamophilin on platelet aggregation and ATP release in human platelet-rich plasma (PRP)

Inducer	*Aggregation* IC_{50} (μM)	*ATP release* IC_{50} (μM)
Arachidonic acid (800 μM)	5.0 ± 0.4	4.3 ± 0.2
Collagen (10 μM)	5.6 ± 0.6	5.0 ± 0.5
U-46619 (1 μM)	3.0 ± 0.4	2.5 ± 0.2
A23187 (5 μM)	>30	>30
ADP (5 μM)		
first wave	>30	–
second wave	6.0 ± 0.6	5.0 ± 0.4
Adrenaline (10 μM)		
first wave	>30	–
second wave	7.6 ± 0.9	7.1 ± 0.3

Human PRP was incubated with cinnamophilin for 3 min, then arachidonic acid (800 μM), collagen (10 μM), U-46619 (1 μM), A23187 (5 μM), ADP (5 μM) or adrenaline (10 μM) was added to trigger the aggregation and ATP release.

PAF and U-46619 were 380 ± 20 nM, 225 ± 15 nM, 338 ± 30 nM and 316 ± 10 nM, respectively. Only the increase of intracellular Ca^{2+} by U-46619 was almost completely suppressed by cinnamophilin (10 μM) (net increase about 20 ± 15 nM).

Effects of cinnamophilin on arachidonic acid metabolism

In human PRP, cinnamophilin induced a dose-dependent inhibition of TXB_2 producton; PGE_2 formation, instead, was dose-dependently increased by the cinnamophilin (Figure 5).

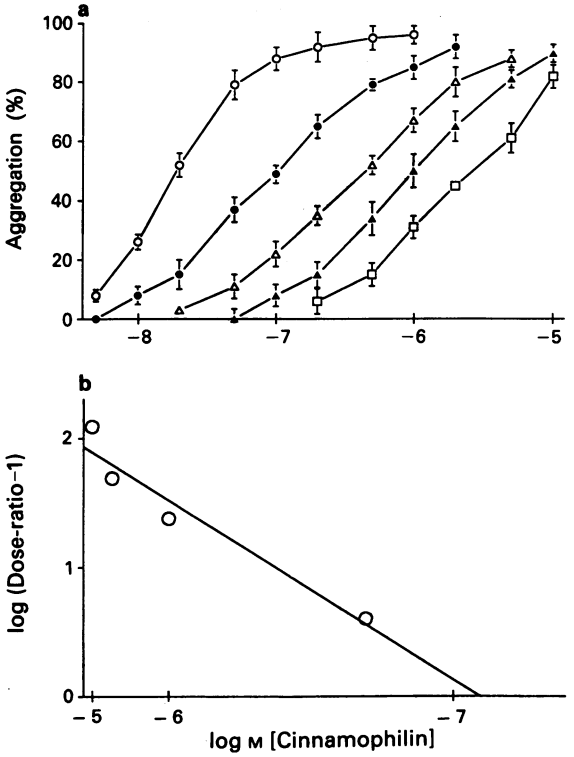

Figure 2 (a) Concentration-dependent shift induced by cinnamophilin on platelet aggregation induced by U-46619. Human platelet-rich plasma were incubated with dimethylsulphoxide (0.5%, ○) or various concentrations of cinnamophilin (0.3 μM, ●; 1 μM, △; 3 μM, ▲; 10 μM, □) for 3 min, then various concentrations of U-46619 were added to trigger the aggregation. Each point represents the mean ± s.e.mean ($n = 6$). (b) Schild plot of (a). The equation of the regression line was $y = -0.95 \times + 6.9$ with $r = 0.99$.

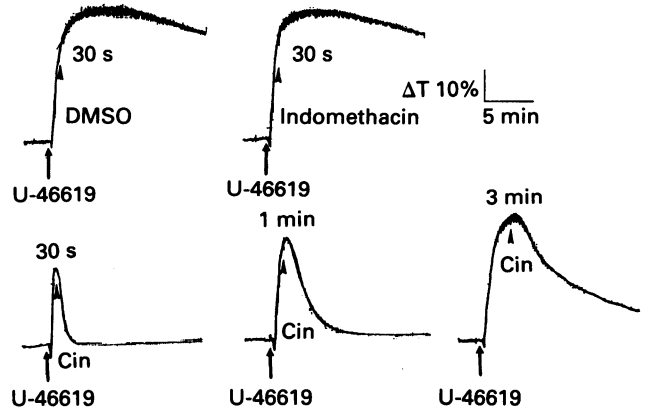

Figure 3 Reversal effect of cinnamophilin on the aggregation of human platelet-rich plasma induced by U-46619. Platelet aggregation was induced by U-46619 (1 μM) for 30 s, 1 min or 3 min, then dimethylsulphoxide (DMSO, 0.5%), indomethacin (20 μM) or cinnamophilin (Cin, 3 μM) was added.

In sheep seminal vesicle microsomes, cinnamophilin also induced a dose-dependent increase of PGE_2 formation (data not shown). Thus cinnamophilin was an inhibitor of platelet TX synthase, but had no effect on cyclo-oxygenase activity.

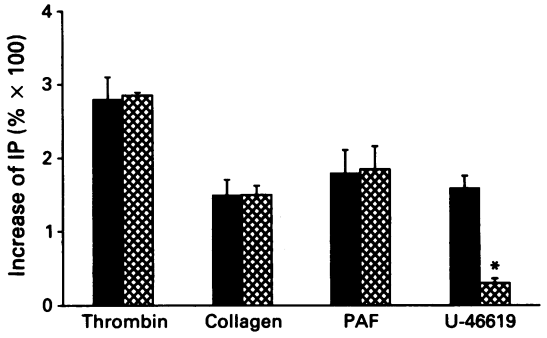

Figure 4 Effect of cinnamophilin on the formation of inositol monophosphate caused by thrombin, collagen, PAF and U-46619 in human platelet-rich plasma (PRP). [^{3}H]-*myo*-inositol-labelled platelets were incubated with DMSO (solid columns) or cinnamophilin (10 μM, cross-hatched columns) for 3 min, then thrombin (0.1 u ml^{-1}), collagen (10 μM), PAF (20 ng ml^{-1}) or U-46619 (1 μM) was added for another 6 min. Percentage increases of inositol monophosphate (IP) (after subtraction of basal levels) are expressed as the mean ± s.e.mean ($n = 6$). *$P < 0.001$ denotes significantly different from stimulated platelets which were not cinnamophilin-treated.

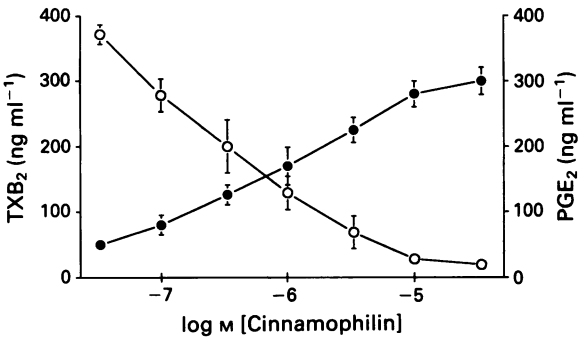

Figure 5 Effects of cinnamophilin on the thromboxane B_2 (○) and prostaglandin E_2 (●) synthesis induced by arachidonic acid (AA) in human PRP. Cinnamophilin was preincubated with platelets for 15 min, then AA (800 μM) was added. Each point represents the mean ± s.e.mean ($n = 4$).

Table 2 Effects of cinnamophilin, dazoxiben and SQ 29548 on platelet cyclic AMP levels

	Unstimulated	*AA-stimulated*
	(pmol ml^{-1})	
Control	28 ± 4	21 ± 4
Cinnamophilin (μM)		
1	–	22 ± 3
3	–	29 ± 3*
10	29 ± 5	35 ± 2**
Dazoxiben (μM)		
30	29 ± 4	22 ± 3
SQ 29548 (μM)		
30	28 ± 5	21 ± 3

Human platelet-rich plasma (PRP) were incubated with drugs for 3 min at 37°C with stirring, then saline or AA (800 μM) was added. Values are expressed as mean ± s.e.mean ($n = 5$–7). *$P < 0.05$, **$P < 0.01$ as compared with the respective control.

Effects of cinnamophilin on platelet cyclic AMP

In control experiments, 28 ± 4 pmol ml^{-1} of cyclic AMP in human PRP was measured. Cinnamophilin did not affect platelet cyclic AMP levels up to 10 μM (Table 2). When the platelets were preincubated with cinnamophilin and challenged with AA, a dose-dependent rise in cyclic AMP was observed (Table 2). Dazoxiben (a specific TX synthase inhibitor) and SQ 29548 (a specific TXA_2 receptor antagonist) did not affect cyclic AMP levels in AA-untreated or AA-treated platelets.

Effects of cinnamophilin on U-46619-induced rat aorta and guinea-pig contractions

Cumulative addition of U-46619 (0.005–10 μM) caused a stepwise increase of contractions of rat aorta and guinea-pig trachea. Cinnamophilin inhibited these responses in a concentration-dependent manner and shifted the dose-response curve to the right in a parallel fashion. The pA_2 values for cinnamophilin toward U-46619-induced responses in rat aorta and guinea-pig trachea were 6.3 ± 0.1 and 5.2 ± 0.2, respectively. (Figure 6a,b).

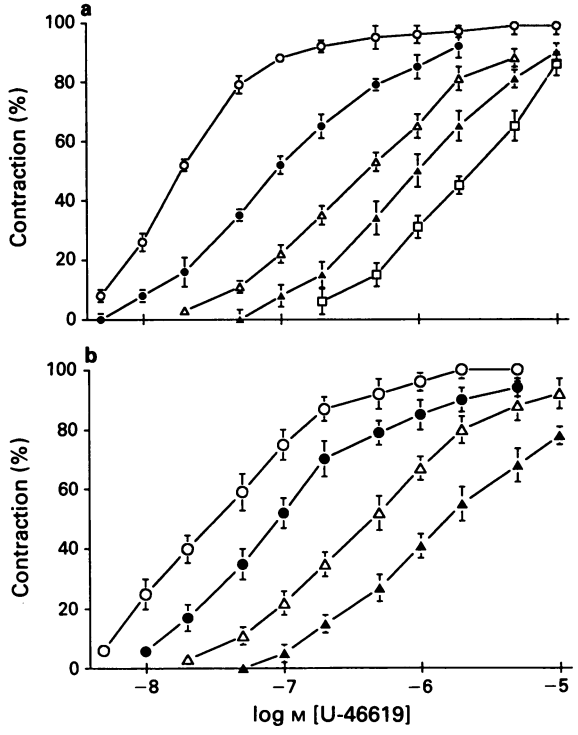

Figure 6 Antagonism of U-46619-induced contractions by cinnamophilin in rat aorta and guinea-pig trachea. Rat aortic rings were preincubated with 0.1% dimethylsulphoxide (DMSO, ○) or cinnamophilin (3 μM, ●; 10 μM, △; 30 μM, ▲; 100 μM, □) at 37°C for 15 min (a). Tracheal rings were preincubated with 0.1% DMSO (○) or cinnamophilin (10 μM, ●; 30 μM, △; 100 μM, ▲) for 15 min (b). Cumulative concentrations of U-46619 were used in (a) and (b). Each point represent the mean ± s.e.mean ($n = 6$).

Table 3 Effect of cinnamophilin on mice tail bleeding time

	Tail bleeding time (s)
Control	60.3 ± 5.4
Cinnamophilin 100 μg kg^{-1}	160.6 ± 17.5***
Indomethacin 100 μg kg^{-1}	110.9 ± 19.9*
Dazoxiben 100 μg kg^{-1}	96.8 ± 10.8**
SQ 29548 100 μg kg^{-1}	99.6 ± 11.9**

Drugs were administered i.p. 30 min before test. Values are expressed as mean ± s.e.mean ($n = 8$–12). *$P<0.05$, **$P<0.01$, ***$P<0.001$ denote values significantly different from the control values (DMSO-treated).

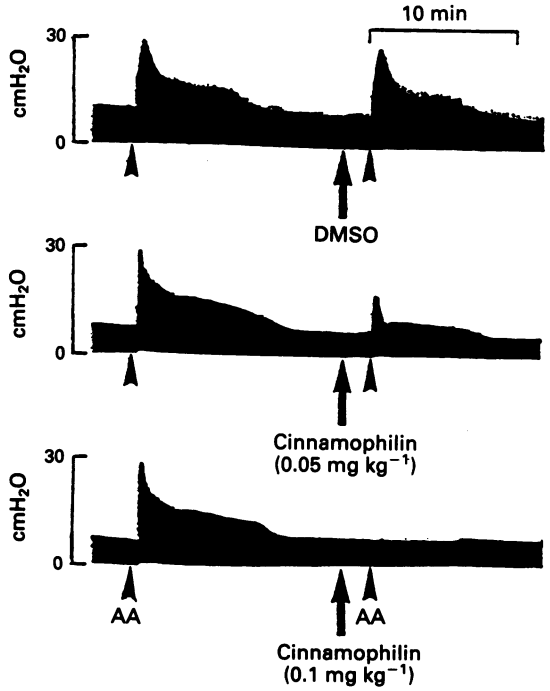

Figure 7 Inhibition by cinnamophilin of the bronchoconstriction induced by arachidonic acid (AA) in guinea-pigs. Cinnamophilin (0.05, 0.1 mg kg^{-1}, i.v.) was administered 1 min before AA (50 μg kg^{-1}, i.v.). Data from one of the three experiments are presented.

To determine the specificity of cinnamophilin on other receptors of rat aorta, endothelin-1 (3 nM), angiotensin II (300 nM), 5-hydroxytryptamine (5-HT) (10 μM) or noradrenaline (3 μM) was used. Cinnamophilin, at a concentration (100 μM) sufficient to inhibit the U46619-induced contraction of the aorta, failed to attenuate the contractile response of rat aorta to endothelin-1, angiotensin II, 5-HT or noradrenaline. Tracheal ring contraction induced by histamine, carbachol or KCl was also not antagonized by cinnamophilin (100 μM) (data not shown).

Effects of cinnamophilin on tail bleeding time of mice

Thirty minutes after i.p. administration of cinnamophilin, tail bleeding time of mice was prolonged more markedly than after indomethacin, dazoxiben and SQ 29548 (Table 3).

Effects of cinnamophilin on AA-induced bronchoconstriction in guinea-pig

Intravenous administration of AA (50 μg kg^{-1}) to guinea-pig induced a reversible bronchoconstriction. When cinnamophilin (0.1 mg kg^{-1}) was administered (i.v.) 1 min before AA, the bronchoconstrictor response to AA was blocked (Figure 7). In contrast, the bronchoconstriction induced by PAF (50 ng kg^{-1}) was not affected by cinnamophilin (1 mg kg^{-1}).

Discussion

The present study has demonstrated that cinnamophilin has both TX synthase inhibitory and TXA_2 receptor antagonizing activities. Thromboxane-dependent pathways of platelet acti-

vation (AA, collagen, second wave of ADP and adrenaline) were inhibited, whereas the independent ones (first wave of ADP, adrenaline and A23187) were not inhibited by cinnamophilin. TX synthase inhibition was determined as a drop in the formation of TXB_2 in AA-stimulated PRP. Furthermore, this inhibition was accompanied by an increase in the formation of PGE_2. Cinnamophilin did not affect basal cyclic AMP levels, therefore excluding a direct action of cinnamophilin on adenylate cyclase or cyclic AMP-dependent phosphodiesterase. This whole series of effects is a typical feature of TX synthase inhibition (Gresele *et al.*, 1984; 1988). Cinnamophilin also behaves as a TXA_2 receptor antagonist. The profiles of action of cinnamophilin upon U-46619-induced contraction of rat aortic rings, guinea-pig tracheal rings and aggregation of human platelets (with pA_2 value of 6.3 ± 0.1, 5.2 ± 0.2 and 7.3 ± 0.2, respectively), indicate that cinnamophilin is a competitive TXA_2 receptor antagonist. This conclusion is supported by the lack of depression of the maximum responses to U-46619 and slopes of the Schild regression not significantly different from unity. Cinnamophilin does not exert any antagonism on the endothelin-1-, angiotensin II- or noradrenaline-induced contraction of the rat aorta and histamine-, carbachol- or KCl-induced contraction of the guinea-pig trachea. This indicated that cinnamophilin is a selective antagonist of the TXA_2 receptor. AA has been shown to cause TXA_2-mediated bronchoconstriction in the anaesthetized guinea-pig (Vargaftig & Doa Hai, 1972; Greenberg *et al.*, 1984). Cinnamophilin significantly inhibited AA-induced increases in airways resistance. *In vivo*, cinnamophilin produced a more marked prolongation of the tail bleeding time in mice than indomethacin, dazoxiben and SQ 29548. These results indicate that cinnamophilin is an effective inhibitor of TX synthase and TXA_2 receptor not only *in vitro*, but also *in vivo*.

TXA_2 plays a pivotal role in platelet activation and is involved in the development of thrombosis. TX synthase inhibitors suppress TXA_2 formation and increase the synthesis of the antiaggregatory prostaglandins (PGI_2 and PGD_2); however, accumulated PGH_2 may interact with platelet and vessel wall TXA_2 receptors, thus reducing the antithrombotic effects of this class of drug. TXA_2 receptor antagonists block the activity of both TXA_2 and PGH_2 on platelets and the vessel wall. Recently, ridogrel and picotamide have been developed (Gresele *et al.*, 1991). Both drugs reduce formation of TXA_2 and increase levels of PGI_2 and PGD_2, and competitively inhibit the binding and action of TXA_2. However, ridogrel shows a dissociation between the TX synthase inhibitory activity ($IC_{50} = 15$ nM) and the receptor antagonistic activity ($IC_{50} = 2$ μM) (De Clerck *et al.*, 1989; Hoet *et al.*, 1990). Indeed, in an ideal agent the two pharmacological activities should be balanced to allow both a suppression of TX synthase and antagonism of TXA_2 receptors of >95% at equivalent plasma concentrations. The potency ratio of cinnamophilin as a TX synthase inhibitor and as a receptor antagonist is approximately 1; thus cinnamophilin is likely to exert simultaneously both effects at active concentrations. Picotamide is relatively weak ($IC_{50} = 100$ μM) (Gresele *et al.*, 1989); in addition, this drug may possess other pharmacological actions beyond its activities on TXA_2 (Gresele *et al.*, 1990).

Dazoxiben (a pure TX synthase inhibitor) and SQ 29548 (a pure TXA_2 receptor antagonist) did not increase intraplatelet cyclic AMP in AA-stimulated platelets. However, cinnamophilin possessing the combination of a TX synthase inhibitory activity with a TXA_2 receptor antagonistic action may lead to an increase in cyclic AMP levels in activated platelets. This enhancement is probably caused by the simultaneous stimulation of platelet adenylate cyclase by PGE_2 produced in increased amounts after TX synthase inhibition (Gresele *et al.*, 1984; 1987), and by the neutralization of the adenylate cyclase inhibitory effects of accumulated prostaglandin endoperoxides (Avdonin *et al.*, 1985).

A potentiation of the platelet-suppressing activity of a TX synthase inhibitor by a TXA_2 receptor antagonist *in vitro* has been described (FitzGerald *et al.*, 1985). Thus, cinnamophilin acting at more than one level of the AA-metabolic pathway appears to be a promising approach to antithrombotic therapy.

All of these data indicate that cinnamophilin is a novel dual TX synthase inhibitor and TXA_2 receptor antagonist and that it may be a useful tool for the investigation and treatment of diseases involving TXA_2 disorders.

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