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Platelet Anti-aggregant Activity of 2,2-Dimethylthiazolidine Hydrochloride and 2-(4-Hydroxy-3-methoxyphenyl)thiazolidine

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The characteristics of platelet anti-aggregant activity of 2,2-dimethylthiazolidine hydrochloride (I) and 2-(4-hydroxy-3-methoxyphenyl)thiazolidine (II) have been evaluated. Compounds I and II were potent inhibitors of collagen- and arachidonic acid-induced aggregation of rat and rabbit platelets (IC_{50} or IC_{100} values: 10^{-5}—10^{-4} M), while they were less effective in the cases of adenosine 5′-diphosphate 2Na, A-23187 and labile aggregation-stimulating substance. Also, both compounds inhibited platelet shape change induced by a low concentration of arachidonic acid. Compounds I (100 μM) and II (25 μM) significantly inhibited serotonin release and thromboxane B_2 formation induced by arachidonic acid in rabbit platelets. Compound I (100 μM) inhibited prostaglandin I_2 formation in rat aorta, but II (100 μM) did not. In contrast, neither compound had any effect on platelet adhesiveness. These results strongly suggest that I and II prevented platelet aggregation, shape change and serotonin release reaction *via* their inhibitory effects on arachidonic acid metabolism of platelets, and the inhibiting potency of II was higher than that of I.

Keywords——thiazolidine derivative; 2,2-dimethylthiazolidine; 2-(4-hydroxy-3-methoxyphenyl)thiazolidine; platelet aggregation; platelet shape change; serotonin release; thromboxane B_2 formation; prostaglandin I_2 formation; arachidonic acid

During an initial search for pharmacological activities of marine products, we found previously that D-cysteinolic acid prepared from sardine showed a moderate anti-aggregant activity in the collagen-induced aggregation test *in vitro* using rat platelets.[1] The preliminary studies on comparative anti-aggregant activities of D-cysteinolic acid-related compounds, including aminosulfonic and aminothiol compounds, indicated that cysteamine showed more potent activity than D-cysteinolic acid.[2] However, cysteamine was too hydrophilic and labile to utilize as an anti-platelet drug. Consequently, many condensed-ring compounds (2,3 and/or 4-substituted thiazolidines) of cysteamine and various aldehydes or ketones have been tested for platelet anti-aggregant activity. Of these compounds, 2,2-dimethylthiazolidine hydrochloride (I) and 2-(4-hydroxy-3-methoxyphenyl)thiazolidine (II) were the most potent inhibitors of collagen-induced aggregation.[2] In this paper, we should like to present the characteristics of platelet anti-aggregant activity of I and II.

Experimental

Materials——Collagen (type I from bovine achilles tendon), arachidonic acid (approx. 99%, from porcine liver) and prostaglandin (PG) I_2 were products of Sigma Chemical Co. (MO., U.S.A.). Adenosine 5′-diphosphate 2Na (ADP) was obtained from Kojin Co., Ltd. (Tokyo, Japan) and Ca-ionophore (A-23187) from Calbiochem Behring

Co. (CA., U.S.A.). 5-Hydroxy [side chain-2-^{14}C] tryptamine creatinine sulfate (^{14}C-serotonin, 57 mCi/mmol) and thromboxane B_2 (TXB_2) [^{125}I] assay system were products of Amersham (Amersham, England). All other reagents and solvents were obtained from Nakarai Chemical Ltd. (Kyoto, Japan).

Synthesis of I and II——Melting points are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-22 (90 MHz) with tetramethylsilane as an internal standard. Mass spectra (MS) were obtained with a Shimadzu QP-1000 instrument.

A suspension of cysteamine hydrochloride (1.0 g) in acetone (200 ml) was refluxed with stirring overnight. After evaporation of the solvent, the crystalline precipitates were filtered off and recrystallized to give I: 74% yield. mp 174.0—176.0 °C. MS *m/z*: 117 ($M^+ - HCl$). ^{1}H-NMR (CD_3OD): 1.80 (6H, s, C2-CH_3), 3.27—3.90 (4H, AA'BB' type, $-CH_2CH_2-$). *Anal.* Calcd for $C_5H_{12}ClNS$: C, 39.08; H, 7.89; N, 9.11. Found: C, 38.87; H, 8.03; N, 9.18.

A solution of the cysteamine hydrochloride (1.0 g, 8.8 mmol) and vanillin (10 mmol) in ethanol–H_2O (1 : 1, 10 ml) was stirred at room temperature overnight. After evaporation of the ethanol, the aqueous phase was washed with ether and neutralized with $NaHCO_3$. Extraction with ether and usual work-up afforded the free base of II: 64% yield. mp 182.0—183.2 °C. ^{1}H-NMR (DMSO-d_6): 2.60—4.15 (4H, m, $-CH_2CH_2-$), 3.77 (1H, s, OCH_3), 5.33 (1H, s, C2-H), 6.63—7.08 (3H, m, aromatic H). *Anal.* Calcd for $C_{10}H_{13}NO_2S$: C, 48.26; H, 5.47; N, 5.64. Found: C, 48.48; H, 5.70; N, 5.65. The hydrochloride salt: MS *m/z*: 211 ($M^+ - HCl$).

Pharmacological and Biochemical Methods——Compound I, cysteamine and acetone dissolved in saline, II and vanillin in dimethylsulfoxide (DMSO) and aspirin in methanol (MeOH) were used in *in vitro* experiments, and the final concentration of the organic solvent was less than 0.4%. In *ex vivo* experiments, I dissolved in saline and II and indomethacin suspended in 1% carboxymethylcellulose Na (CMC) were administered to animals.

Preparation of Platelet-Rich Plasma (PRP) and Washed Platelets——Rat PRP was prepared from the citrated or heparinized (for arachidonic acid-induced aggregation) blood of Wistar male rats, and the platelet count was adjusted to 10^9/ml, as previously described.[3] Rabbit PRP was prepared from the citrated blood, and the platelet count was adjusted to 4×10^8/ml. Rabbit PRP was added to 0.1 volume of Ca^{2+}, Mg^{2+}-free modified Tyrode solution–0.1% ethylenediaminetetraacetic acid (EDTA) and centrifuged at $1200 \times g$ for 10 min. The platelets were washed again and resuspended in the modified Tyrode solution without EDTA to give washed platelets (4×10^8 cells/ml).

Assay for Platelet Aggregation——Platelet aggregation studies were performed according to the turbidimetric method of Born and Cross[4] in an NKK Hema Tracer 1 aggregometer. In the collagen-, ADP-, A-23187- and arachidonic acid-induced aggregations, 25 μl of test sample was added to 225 μl of PRP 3 min prior to the induction of platelet activation, and the 50 or 100% inhibitory concentration (IC_{50} or IC_{100}, respectively) values were calculated from the concentration-response curve of the inhibition (maximum amplitude of light transmittance) of platelet aggregation by test samples.

Labile aggregation-stimulating substance (LASS)-induced aggregation was done as follows: 100 μl of rabbit washed platelets was incubated with arachidonic acid (0.4 mM) at 37 °C for 30 s. Immediately, 25 μl of the incubation mixture was transferred to 225 μl of rabbit PRP, which was preincubated with indomethacin (50 μM) and test samples at 37 °C for 2 min under stirring. The aggregation was monitored photometrically.

According to the method of Siess *et al.*,[5] the effect of test samples on platelet shape change induced by a low concentration (0.1 mM) of arachidonic acid was assayed by measuring the initial decrease in light transmittance following the stimulus addition artifact.

Assay for ^{14}C-Serotonin Release——^{14}C-Serotonin was incubated with rabbit PRP (50 nCi/ml) at room temperature for 30 min. Labelled platelets (10^8 cells/225 μl) were incubated with 25 μl of test samples and arachidonic acid (0.5 mM) at 37 °C, and after 3 min the reaction was terminated by adding 0.5 ml of cold 77 mM EDTA solution. The platelets were subsequently sedimented by centrifugation at $1200 \times g$ for 10 min. The radioactivity of ^{14}C-serotonin in the supernatant was measured in a liquid scintillation counter (Aloka LSC-1000). Release activity was expressed as a percentage of the total radioactivity incorporated into platelets.

Assay for Platelet Adhesiveness——According to Salzman's method,[6] platelet adhesiveness was determined with a glass bead column technique in rats. A platelet adhesiveness measurement apparatus (Igakushoin Kikai, Tokyo) was inserted into the right carotid artery of a rat anesthetized with sodium pentobarbital. After the administration of test samples, aliquots of blood were collected through an empty polyvinyl column (0.2 × 15 cm) and then another column filled with glass beads (Superbrite 070, 0.5 g) into two plastic syringes containing 0.1 volume of 77 mM EDTA solution. Platelet counts were measured using a platelet counter (Sysmex PL-100) on the blood before and after passage through the glass bead column. The difference between the counts, expressed as a percentage of the former, was termed the adhesiveness.

Assay for TXB_2 Formation——Rabbit PRP (225 μl) and arachidonic acid (0.5 mM) were incubated with or without test samples (25 μl) at 37 °C, for 3 min, then the reaction was terminated by adding 0.1 volume of 1 mM indomethacin solution containing 77 mM EDTA. After centrifugation of the samples at $1200 \times g$ for 10 min, TXB_2, a stable breakdown product of TXA_2, in the supernatant was measured using TXB_2 radioimmunoassay kits.

Assay for PGI_2 Formation—According to the method of Harada *et al.*,[7] the formation of PGI_2 from endogeneous arachidonic acid was measured in mechanically stimulated rat aortae. Briefly, isolated thoracic aortae were gently stretched five times up to 1.5 times the original length, and then incubated in 25 mM Tris–HCl buffer (pH

7.4)–130 mM NaCl solution (1 ml) with or without test samples at 25 °C for 10 min. A 25 μl aliquot of incubation medium was transferred to 225 μl of rat PRP. After preincubation for 2 min, aggregation was induced by ADP and was monitored photometrically. The anti-aggregant activity in the medium was compared with that of authentic PGI_2.

Results

Platelet Aggregation

Platelet anti-aggregant activities of I and II in rat and rabbit PRP's are summarized in Tables I and II; the values for aspirin are included for comparison. Both compounds were potent inhibitors of platelet aggregation induced by collagen and arachidonic acid in rat and rabbit PRP's, while they were less effective in the cases of ADP and A-23187. Cysteamine was less effective than both compounds on collagen- and arachidonic acid-induced aggregation in rat PRP. Also, equimolar combinations of the raw materials (cysteamine plus acetone and cysteamine plus vanillin) showed less potency (IC_{100} values: 0.15 and 0.10 mM, respectively) than the corresponding condensed compounds (I and II) on arachidonic acid-induced aggregation in rat PRP. Therefore, anti-aggregant activity of I and II was due to their ring structures, but not the starting materials which might be produced by hydrolysis. Although there were some species differences, the rank order of inhibitory potency was I > II, aspirin for collagen, I, II > aspirin for ADP and A-23187, and II > I, aspirin for arachidonic acid.

As shown in Fig. 1, I and II (0.3 mM) had no effect on rabbit platelet aggregation induced by LASS generated from exogenous arachidonic acid in rabbit washed platelets. Aspirin (0.3 mM) also did not affect the aggregation (data not shown).

As shown in Fig. 2, arachidonic acid at a low concentration (0.1 mM) induced only platelet shape change without subsequent platelet aggregation in rabbit PRP. In this system, I (0.3 mM) and II (0.1 mM) inhibited platelet shape change. Aspirin (0.3 mM) also inhibited the

TABLE I. Platelet Anti-aggregant Activity of I and II in Rat PRP

Sample	IC_{50}[a] (mM)			IC_{100}[a] (mM)
	Collagen (10 μg/ml)[b]	ADP (4 μM)	A-23187 (20 μM)	Arachidonic acid (0.4 mM)
I	0.021	0.094	0.31	0.060
II	0.023	0.28	0.30	0.050
Cysteamine	0.070	0.17	0.98	0.20
Aspirin	0.051	>1.0	>1.0	0.080

a) Maximal sample concentrations used were 1 mM. *b*) Final concentrations of stimuli are given in parenthesis.

TABLE II. Platelet Anti-aggregant Activity of I and II in Rabbit PRP

Sample	IC_{50}[a] (mM)			
	Collagen (15 μg/ml)[b]	ADP (8 μM)	A-23187 (30 μM)	Arachidonic acid (0.5 mM)
I	0.065	0.33	>1.0	0.13
II	0.10	0.30	>1.0	0.034
Aspirin	0.10	>1.0	>1.0	0.065

a) Maximal sample concentrations used were 1 mM. *b*) Final concentrations of stimuli are given in parenthesis.

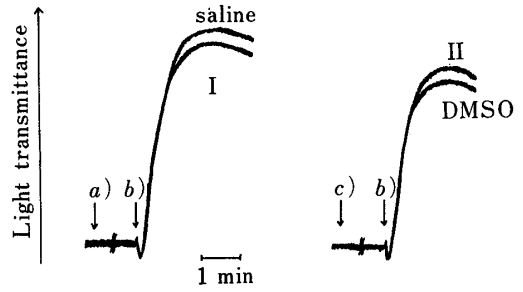

Fig. 1. Effect of I and II on LASS-Induced Aggregation of Rabbit PRP

a) PRP (225 μl)–indomethacin (50 μM)–I (0.3 mM) or saline. *b*) Washed platelet (50 μl)–arachidonic acid (0.4 mM). *c*) PRP (225 μl)–indomethacin (50 μM)–II (0.3 mM) or DMSO.

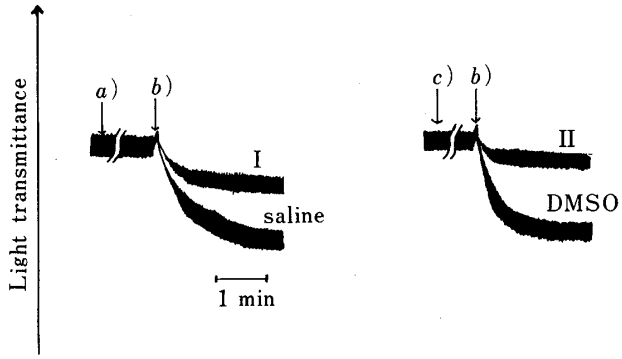

Fig. 2. Effect of I and II on Platelet Shape Change of Rabbit PRP

a) PRP (225 μl)–I (0.3 mM) or saline. *b*) Arachidonic acid (0.1 mM). *c*) PRP (225 μl)–II (0.1 mM) or DMSO.

TABLE III. Effect of I and II on Serotonin Release in Rabbit PRP

Sample	Final concentration (μM)	% ^{14}C-serotonin released by arachidonic acid (0.5 mM)[a]
Saline	—	44.6±0.7
I	100	31.3±2.3[c] (30)[b]
	250	5.6±2.3[d] (87)[b]
DMSO	—	41.7±0.8
II	25	23.5±4.1[c] (44)[b]
	50	16.5±5.3[c] (60)[b]
	100	6.3±1.3[d] (85)[b]
MeOH	—	43.1±0.8
Aspirin	100	10.0±1.2[d] (77)[b]

a) Data are mean±S.E. (n=3—4). *b*) Percent inhibition is given in parenthesis. *c*) $p<0.01$, *d*) $p<0.001$; *versus* the vehicle.

TABLE IV. Effect of I and II on Platelet Adhesiveness in Rats

Sample	Administration[a]			% adhesiveness[b]
	Dose (mg/kg)	Route	Time (min)	
Saline	—	i.v.	5	33.6±6.8
I	100	i.v.	5	34.3±3.2
1% CMC	—	i.p.	30	35.1±5.3
II	100	i.p.	30	32.9±3.9
Indomethacin	10	i.p.	30	14.0±3.2[c]

a) Sample or vehicle was administered according to the dose, route and time given in the table, before the blood collection. *b*) Data are mean±S.E. (n=4—5). *c*) $p<0.05$; *versus* the vehicle.

shape change (data not shown).

^{14}C-Serotonin Release

As shown in Table III, I (100 and 250 μM) and II (25, 50 and 100 μM) significantly inhibited the release of ^{14}C-serotonin from rabbit platelets induced by arachidonic acid, as did aspirin (100 μM). Their inhibitory potencies were approximately similar to those in arachi-

Table V. Effect of I and II on TXB_2 Formation in Rabbit PRP

Sample	Final concentration (μM)	TXB_2 formation induced by arachidonic acid (0.5 mM) (ng/10^8 platelets)[a]
Saline	—	55.5±2.7
I	100	38.8±3.6[c] (30)[b]
	250	22.3±3.9[d] (60)[b]
DMSO	—	58.8±2.1
II	25	46.4±1.9[c] (21)[b]
	50	29.3±2.7[d] (50)[b]
	100	12.4±0.9[d] (79)[b]
MeOH	—	53.9±1.5
Aspirin	100	9.3±1.1[d] (83)[b]

a) Data are mean±S.E. (n=4—5). *b*) Percent inhibition is given in parenthesis. *c*) $p<0.01$, *d*) $p<0.001$; *versus* the vehicle.

Table VI. Effect of I and II on PGI_2 Formation in Isolated Rat Aorta

Sample	Final concentration[a] (μM)	PGI_2 formation[b] (pmol)
Saline	—	84±8
I	100	48±8[d] (42)[c]
DMSO	—	80±5
II	100	80±5 (0)[c]
MeOH	—	82±7
Aspirin	100	8±4[e] (90)[c]

a) On incubation with arterial strips. *b*) Data are mean±S.E. (n=4). *c*) Percent inhibition is given in parenthesis. *d*) $p<0.05$, *e*) $p<0.001$; *versus* the vehicle.

donic acid-induced aggregation in rabbit PRP.

Platelet Adhesiveness

As shown in Table IV, I (100 mg/kg, i.v.) and II (100 mg/kg, i.p.) had no effect on platelet adhesiveness in rats, differing from indomethacin (10 mg/kg, i.p.).

TXB_2 Formation

As shown in Table V, I (100 and 250 μM) and II (25, 50 and 100 μM) significantly inhibited TXB_2 formation during arachidonic acid-induced aggregation in rabbit PRP, like aspirin (100 μM). Their inhibitory potencies approximately accorded with those in the platelet aggregation and release reaction tests induced by arachidonic acid in rabbit PRP.

PGI_2 Formation

The effect of I and II on PGI_2 formation in isolated rat aortae is shown in Table VI, in comparison with that of aspirin. The final concentrations of test samples were 100 μM which could completely inhibit arachidonic acid-induced aggregation in rat PRP. In this system, I significantly inhibited PGI_2 formation by 42%, but II had no effect. On the other hand, aspirin strongly inhibited PGI_2 formation by 90%.

Discussion

During a series of processes of platelet activation, I and II inhibited platelet aggregation,

shape change and serotonin release reaction but not platelet adhesion. In the platelet aggregation studies, both compounds were more effective in the cases of collagen and arachidonic acid than ADP, A-23187 and LASS (PG endoperoxides and TXA_2). It has been proposed that collagen activates platelets *via* receptor binding, phosphatidylinositol (PI) response, Ca^{2+} mobilization, phospholipase (PL) C activation and promotion of arachidonic acid metabolism, followed by the release reaction, and arachidonic acid *via* its direct participation in arachidonic acid metabolism coupled with PI response.[8] Therefore, it seemed that both compounds, especially II, which had a greater effect on arachidonic acid-induced aggregation of platelets, inhibited arachidonic acid metabolism rather than the other processes such as PI response, PLC activation and Ca^{2+} mobilization, which are involved in the actions of collagen,[8] ADP[9] and/or A-23187.[10] On platelet activation, arachidonic acid is physiologically converted to TXA_2 which is a strong stimulator of platelet aggregation and release reaction, *via* PG endoperoxides by various enzymatic reactions including fatty acid cyclooxygenase, PG hydroperoxidase and TX synthetase systems.[11] Both compounds inhibited the formation of TXB_2, a stable metabolite of TXA_2, at the concentrations which inhibited the platelet aggregation and release reaction. Furthermore, neither compound affected the direct aggregating action of TXA_2, as shown in the LASS-induced aggregation test. These results strongly suggest that the compounds inhibit the formation of TXA_2.

PGI_2, which is generated from arachidonic acid by fatty acid cyclooxygenase and PGI_2 synthetase in endothelial cells of blood vessels, strongly inhibits platelet aggregation.[12] Therefore, the inhibition of PGI_2 formation would be disadvantageous to the anti-platelet effect of drugs. It is well known that aspirin, a cyclooxygenase inhibitor, inhibits PGI_2 formation in vessels. In contrast, II had no effect on PGI_2 formation in rat aortae at the concentration which could completely inhibit the arachidonic acid-induced aggregation. It was reported that certain phenol compounds such as 2-aminomethyl-4-*tert*-butyl-6-iodophenol (MK. 447) and guaiacol act as essential factors in the fatty acid cyclooxygenase and PG hydroperoxidase systems.[13] These phenol compounds, which are known to be hydrogen donors for peroxidase, stimulate PG hydroperoxidase activity, which is responsible for the conversion of PGG_2 to PGH_2 in a seminal vesicle preparation, while they have less effect on fatty acid cyclooxygenase.[13,14] These findings may explain the differences between the effects on arachidonic acid-induced aggregation and PGI_2 formation in the cases of II (having a 3-hydroxy-4-methoxyphenyl substituent) and I (having no phenyl group). Furthermore, it is more likely that II inhibits TXA_2 synthetase, which is responsible for the conversion of PGH_2 to TXA_2, since this compound inhibited the formation of TXB_2 from arachidonic acid but not the formation of PGI_2. However, it is necessary to consider the differences in the tissue specificities of arachidonic acid and PG converting enzymes.[11] In any case, it is clear that I and II inhibit arachidonic acid metabolism in the platelet.

Platelet shape change is the first measurable physiological platelet response preceding other responses such as platelet aggregation and release reaction.[15] Low concentrations of arachidonic acid induce platelet shape change in parallel to formation of phosphatidic acid (PA), which reflects stimulation of PLC, without platelet aggregation or serotonin release.[5] Moreover, endoperoxides and TXA_2 are responsible for arachidonic acid-induced stimulation of PLC, since formation of PA is blocked by aspirin, indomethacin (inhibitors of cyclooxygenase) and *N*-methylimidazole (an inhibitor of TX synthetase).[5] Therefore, we consider that I and II inhibited platelet shape change by inhibiting the conversion of arachidonic acid.

The factors affecting platelet retention (adhesion plus aggregation) are red blood cells, platelets and some plasma proteins. In particular, ADP released from red cells is believed to initiate platelet retention on glass beads and this presumably occurs when blood and beads are mixed together.[16] Therefore, the result that I and II had no effect on platelet adhesion may be partly due to their relatively weak potencies to inhibit ADP-induced aggregation. Although

indomethacin is known to be less effective on ADP-induced aggregation, it inhibited platelet adhesion. It was reported that products of platelet or vascular arachidonic acid metabolism do not play a role in the process of platelet adhesion.[17] It is possible that indomethacin reduced platelet adhesion by stabilizing the red cell membranes.[18]

Although II may be only a moderately strong inhibitor of platelet aggregation, compared with known inhibitors of arachidonic acid metabolism,[19] it is noteworthy that this compound has the ability to inhibit arachidonic acid metabolism in platelets without stimulating prostacyclin formation in blood vessels, differing from aspirin.

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