*Biochemical Pharmacology*, Vol. 46, No. 7, pp. 1165–1173, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
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# INHIBITION OF COLLAGEN-INDUCED PLATELET AGGREGATION AND ADHESION BY A PSEUDOCYANIDE DERIVATIVE OF AVICINE ISOLATED FROM *ZANTHOXYLUM INTEGRIFOLIOLUM* MERR.

FENG-NIEN KO, GEORGE HSIAO, IH-SHENG CHEN,* SHWU-JEN WU* and CHE-MING TENG†

Pharmacological Institute, College of Medicine, National Taiwan University, Tapei; and *Graduate Institute of Pharmaceutical Sciences, Kaohsiung Medical College, Kaohsiung, Taiwan

(*Received* 11 *February* 1993; *accepted* 23 *June* 1993)

**Abstract**—Avicine pseudocyanide, a derivative of avicine isolated from *Zanthoxylum integrifoliolum* Merr., inhibited collagen-induced platelet aggregation and release reaction in a concentration-dependent manner. Trimucytin is a collagen-like snake venom protein isolated from *Trimeresurus mucrosquamatus*. Avicine pseudocyanide also inhibited trimucytin (1 μg/mL)-induced platelet aggregation and release reaction concentration dependently. The $IC_{50}$ values of avicine pseudocyanide on collagen (10 μg/mL)- and trimucytin (1 μg/mL)-induced platelet aggregation were 47.3 ± 4.1 and 62.5 ± 5.6 μM, respectively. Avicine pseudocyanide at a concentration of 300 μM inhibited less than 30% of platelet aggregation induced by ADP (20 μM), AA (100 μM), U46619 (1 μM), PAF (2 ng/mL) and thrombin (0.1 U/mL). The concentration–response curve of collagen-induced platelet aggregation was shifted to the right by avicine pseudocyanide (20–100 μM) concentration dependently. The Schild plot showed that $pA_2$ and $pA_{10}$ values of avicine pseudocyanide were 4.8 and 4.3, respectively, with slope of −1.9. Avicine pseudocyanide also inhibited collagen (10 μg/mL)-induced aggregation of rabbit whole blood with an $IC_{50}$ of 145 ± 13 μM. Collagen-induced thromboxane $B_2$ formation was also inhibited by avicine pseudocyanide in a concentration-dependent manner with a maximal effect at 100 μM. However, arachidonic acid (AA)-induced thromboxane $B_2$ and prostaglandin $D_2$ formations were only partially suppressed by a high concentration of avicine pseudocyanide (300 μM). Avicine pseudocyanide (100 μM) inhibited the [$^3$H]inositol monophosphate formation and the rise of intracellular $Ca^{2+}$ concentration caused by collagen but not those caused by AA, U46619, platelet-activating factor and thrombin. In the presence of prostaglandin $E_1$, $Mg^{2+}$-dependent platelet adhesion to collagen was inhibited by avicine pseudocyanide with an $IC_{50}$ of 278 ± 16 μM. These data indicate that avicine pseudocyanide is an inhibitor of collagen-induced platelet aggregation and platelet–collagen adhesion.

Current concepts of hemostasis and thrombosis emphasize the endothelial cell as a regulatory interface between blood and tissues [1, 2]. After the integrity of the vascular endothelium is disrupted, platelets rapidly adhere to exposed subendothelial components. When fibrillar collagen, the most thrombogenic vessel wall macromolecule [3], is exposed, platelets rapidly adhere and become activated leading subsequently to aggregation and the formation of a hemostatic plug. The interaction of platelets with collagen(s) may also be the cause in a number of instances of pathological events such as thrombosis and atherosclerosis [4–6]. Thus, inhibition of platelet–collagen interaction may be a promising approach for the prevention of thrombosis.

Natural products play an important role in the history of drug development. In spite of the substantial advances that have been made in synthetic organic chemistry, natural products still remain an integral part of modern therapeutics and are widely used in many areas of the world. Medical plants have been used as traditional remedies in Oriental countries for thousands of years by a large population. In a large scale screening test, we have found many biologically active compounds isolated from plant sources. Some of them inhibited platelet aggregation. For example, magnolol and honokiol (isolated from *Magnolia officinalis*) inhibit platelet thromboxane $A_2$ formation [7]; osthole (isolated from *Angelica pubescens*) inhibits the breakdown of platelet phosphoinositides [8]; and aglafoline (isolated from *Aglaia elliptifolia*) is a specific platelet-activating factor (PAF‡) antagonist [9]. *Zanthoxylum integrifoliolum* Merr. is used in Orchid Island (Taiwan) as a folk medicine for the treatment of snake bite. In the present study, we show that avicine pseudocyanide, a derivative of avicine isolated from *Z. integrifoliolum* Merr., inhibited collagen-induced aggregation and platelet–collagen adhesion of washed rabbit platelets. Some avicine analogues are also used in this study for comparison.

† Corresponding author: Dr. C-M. Teng, Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd., 1st Section, Taipei 10018, Taiwan. Tel. 886-2-322-1742 or 866-2-356-2221; FAX 886-2-322-1742.

‡ Abbreviations: PAF, platelet-activating factor; AA, arachidonic acid; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; RIA, radioimmunoassay; PRP, platelet-rich plasma; and TCA, trichloroacetic acid.

## MATERIALS AND METHODS

*Materials.* Bovine thrombin, from the Parke Davis

Co., was dissolved in 50% (v/v) glycerol for a stock solution of 100 NIH units/mL. Collagen (type I, bovine Achilles tendon), from the Sigma Chemical Co., was homogenized in 25 mM acetic acid and stored (1 mg/mL) at −70°. Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), purchased from Sigma, was dissolved in chloroform and diluted into 0.1% bovine serum albumin (BSA)–saline solution immediately prior to use. Arachidonic acid (AA), BSA, indomethacin, ethylenediaminetetraacetic acid (EDTA), luciferase-luciferin, Dowex-1 (100–200 mesh: ×8, chloride) resin, fura-2-acetoxymethyl ester, trichloroacetic acid (TCA), U46619, imidazole, prostaglandin $E_1$ ($PGE_1$) and *myo*-inositol were purchased from Sigma. *myo*-[2-$^3$H]Inositol, thromboxane $B_2$ and prostaglandin $D_2$ ($PGD_2$) radioimmunoassay (RIA) kits and $Na_2^{51}CrO_4$ (250–500 mCi/mg Cr) were purchased from Amersham, U.K.

*Platelet aggregation and ATP release reaction.* Platelet-rich plasma (PRP) was obtained from blood collected from rabbit marginal vein, anticoagulated with EDTA (100 mM, 1:14), and centrifuged for 10 min at 90 *g* and room temperature. Platelet suspension was prepared from EDTA-anticoagulated PRP according to washing procedures described previously [10]. Platelets were counted by a Coulter Counter (model ZM) and adjusted to a concentration of $3 \times 10^8$ platelets/mL. Platelet pellets were finally suspended in Tyrode's solution of the following composition (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) containing BSA (0.35%). Aggregation was measured by turbidimetry as described by O'Brien [11]. ATP released from platelets was detected by the bioluminescence method of DeLuca and McElory [12]. Both aggregation and ATP release were measured simultaneously in a Lumi-aggregometer (Chrono-Log Co., U.S.A.) connected to two dual-channel recorders. Platelet preparations were stirred at 1200 rpm. When dimethyl sulfoxide (DMSO) was used as the solvent, its final concentration was fixed at 0.5% (v/v) to eliminate the effect of the solvent.

To verify if the inhibitory effects of avicine pseudocyanide on aggregation and ATP release were reversible, platelets were incubated with avicine pseudocyanide (100 μM) for 3 min. Following that, platelets were sedimented by centrifugation and resuspended in fresh Tyrode's solution. Platelet aggregation and ATP release were then performed as described above.

*Whole blood aggregometry.* Blood anticoagulated with sodium citrate (3.8%, 1:9, v/v) was warmed up at 37° for 1 min in a Whole Blood Aggregometer (model 500, Chrono-Log Co.). After the addition of DMSO (0.5%, control) or avicine pseudocyanide (20–300 μM) and stirring at 1200 rpm for 3 min, collagen (10 μg/mL) was used to trigger the aggregation of whole blood. The aggregation was reflected by the change of the impedance between two platinum electrodes and recorded by a recorder.

*Thromboxane $B_2$ and prostaglandin $D_2$ assay.* After 6 min of platelet incubation with the inducer, EDTA (2 mM) and indomethacin (50 μM) were added to halt thromboxane and $PGD_2$ formation. After centrifugation in an Eppendorf microcentrifuge (model 5414) for 2 min, thromboxane $B_2$ and $PGD_2$ in the supernatant were assayed by RIA.

*Labeling of membrane phospholipids and measurement of the production of [$^3$H]inositol phosphate.* The method employed was modified from those of Huang and Detwiler [13] and Neylon and Summers [14]. EDTA-PRP was centrifuged at 500 *g* for 10 min. Platelet pellets were then suspended in 1 mL of $Ca^{2+}$-free and BSA-free Tyrode's solution containing *myo*-[2-$^3$H]inositol (75 μCi/mL) and EDTA (1 mM). After incubation for 2 hr at 37°, the platelets were collected by centrifugation (500 *g*, 4 min) and suspended in Tyrode's solution. Phosphoinositide breakdown was enabled by addition of aggregation inducers to platelet suspension (1 mL) in a 3.5-mL cuvette with a stirring bar driven at 1200 rpm. Incubation was continued for 6 min at 37°. An equal volume of 10% (w/v) TCA was then added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 mL of supernatant was aspirated and TCA was removed by washing with 4 × 3 vol. of diethyl ether. The aqueous phase containing inositol phosphates was adjusted to pH 7–8, diluted to 4 mL with distilled water, and applied to a Dowex-1 ion-exchange column for separation of inositol phosphates as described by Neylon and Summers [14]. All experiments were carried out in the presence of LiCl (5 mM) to inhibit inositol monophosphate phosphatase. Because concentrations of inositol bisphosphate and inositol trisphosphate were very low, we measured inositol monophosphate as an index of the total inositol phosphate formation.

*Measurement of intracellular calcium in platelets.* The method of Pollock and Rink [15] was followed. Platelets ($3 \times 10^8$ platelets/mL) were incubated with fura-2/AM (5 μM) at 37° for 45 min and then centrifuged at 500 *g*; the resultant pellet was washed with EDTA (1 mM)-containing Tyrode's solution. After centrifugation, platelets were resuspended in the Tyrode's solution containing 1 mM $Ca^{2+}$. Fluorescence (excitation 339 nm, emission 500 nm) was measured with a Hitachi Fluorescence Spectrophotometer (model F 4000). At the end of the experiment the cells were treated with 0.1% Triton X-100 followed by the addition of 10 mM ethylene glycol - bis(β - aminoethyl ether) - *N, N, N', N'* - tetraacetic acid (EGTA) to obtain the maximal and minimal fluorescence, respectively. The intracellular free calcium concentration was calculated as described for fura-2 using a $Ca^{2+}$-dye dissociation constant of 224 nM [16].

*Determination of platelet–collagen adhesion.* The assays previously described by Santoro [17] and Coller *et al.* [18] for adhesion of platelets to fibrillar collagen were followed with some modification: (a) Type I bovine fibrillar collagen (1 mg/mL) was diluted with distilled water to reach a final concentration of 100 μg/mL in 25 mM acetic acid. Then 0.3 mL of BSA (0.5%) or collagen solution was used to coat the wells of the polystyrene microtiter plate that had not been treated for tissue culture (Falcon 3915, Becton-Dickinson) for 2 hr at 37°. The wells were aspirated and blocked with

0.3 mL BSA (0.5%) solution at room temperature for 1 hr, and then washed three times with washing buffer of the following composition: 0.5% BSA, 0.15 M NaCl, 5 mM glucose, 0.05 M Tris–HCl, pH 7.4. (b) Washed rabbit platelets ($6 \times 10^8$ platelets/mL) were incubated with 0.5 mCi/mL $Na_2^{51}CrO_4$ at 37° for 1 hr, centrifuged at 500 *g* for 4 min, and then resuspended in $Ca^{2+}$-free Tyrode's solution; the cell number was adjusted to $10^8$ platelets/mL. Various divalent cations were added as dictated by specific experiments. (c) In the presence of $PGE_1$ (10 μM), $^{51}Cr$-labeled platelets (0.4 mL) were added to the BSA- and collagen-coated dishes and incubated at room temperature for 30 min without shaking. Non-adherent platelets were then removed by aspiration, and the plates were rinsed three times with the above washing buffer. The extent of adhesion was determined by incubating the dishes containing adherent platelets sequentially with three 0.3-mL portions of 2% sodium dodecyl sulfate (SDS) for 30 min each. The lysates were pooled, and the $^{51}Cr$ content was determined by a gamma counter (LKB).

*Isolation and preparation of avicine pseudocyanide and its analogues.* Air-dried barks of *Z. integrifoliolum* Merr. were refluxed with methanol and then concentrated under reduced pressure. Removal of a large amount of crude lupeol was made during the concentrating process. The afforded residue was acidified with 5% acetic acid for extraction of the bases. The acidic solution was made alkaline with $NH_4OH$ solution, and extracted with chloroform. The chloroform solution was shaken with 5% NaOH solution, then dried with anhydrous $K_2CO_3$ and concentrated. To the concentrated chloroform solution, 10% HCl aqueous solution was added and a mixture of quaternary benzo[*c*]phenanthridine alkaloids was precipitated. The precipitates were filtered and then reduced with $NaBH_4$ in the usual method to dihydrobases. The mixture of dihydrobases was separated by silica column chromatography using *n*-hexane as eluting solvent, and dihydroavicine (Fig. 1) was afforded. The dihydroavicine was quaternized with DDO (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) in benzene to avicine chloride (Fig. 1). Finally, avicine chloride was derived with KCN to avicine pseudocyanide (Fig. 1). Avicine pseudocyanide and its analogues were identified with authentic sample [19].

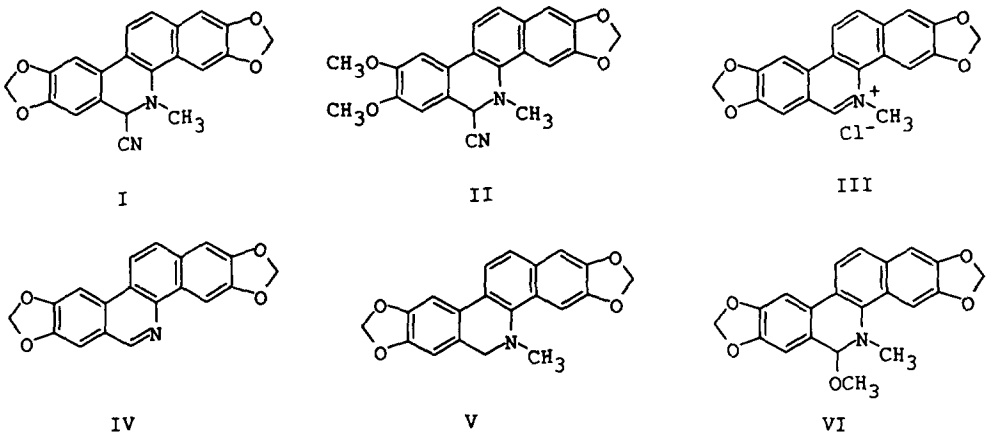
## RESULTS

*Comparison of avicine pseudocyanide and its analogues on platelet aggregation in washed rabbit platelets.* The antiplatelet activities of avicine pseudocyanide and its analogues were compared in ADP-, AA-, collagen- and PAF-induced aggregation of washed rabbit platelets. With washed rabbit platelets, ADP (20 μM), AA (100 μM), collagen (10 μg/mL) and PAF (2 ng/mL) all caused about 80–90% aggregation. Avicine chloride (50 μM) and noravicine (50 μM) did not have a significant antiplatelet effect. Dihydroavicine (300 μM) completely inhibited AA-induced platelet aggregation without having any effect on ADP-, collagen- and PAF-induced aggregation. 6-Methoxydihydroavicine (300 μM) markedly inhibited AA-, collagen- and PAF-induced platelet aggregation but did not affect ADP-induced aggregation. Avicine pseudocyanide (300 μM) slightly inhibited AA- and PAF-induced platelet aggregation. However, collagen-induced platelet aggregation was inhibited completely by avicine pseudocyanide (Table 1). Nitidine pseudocyanide (300 μM), which also possesses a cyano group at the C-6 position, only suppressed AA-, collagen- and PAF-induced platelet aggregation slightly. The properties and mechanism(s) of action of avicine pseudocyanide were then studied in the following experiments.

*Effects of avicine pseudocyanide on the aggregation and ATP release reaction of washed rabbit platelets.* As mentioned above, ADP (20 μM), AA (100 μM), PAF (2 ng/mL), and collagen (10 μg/mL), as well as U46619 (1 μM) and thrombin (0.1 U/mL) (Table 2), all caused 80–90% aggregation in washed rabbit platelets. Avicine pseudocyanide selectively and concentration-dependently inhibited collagen-induced platelet aggregation with an $IC_{50}$ value of 47.3 ± 4.1 μM (Table 2). A high concentration of avicine pseudocyanide (300 μM) inhibited less than 30% of platelet aggregation induced by AA, PAF, U46619 and thrombin. The aggregation caused by ADP was not affected significantly by avicine pseudocyanide (300 μM, Table 2). Indomethacin (0.1 to 1 μM) selectively inhibited AA-induced aggregation in a concentration-dependent manner with an $IC_{50}$ value of about 0.2 μM. When a concentration of 20 μM was used, indomethacin caused only a slight inhibition of collagen-induced platelet aggregation (19.3 ± 2.8% inhibition, N = 5). The concentration–response curve of collagen-induced platelet aggregation was shifted to the right by avicine pseudocyanide concentration dependently (Fig. 2A). When the data were presented as a Schild plot, $pA_2$ and $pA_{10}$ values of 4.8 (5.0 to 4.6 for the 95% confidence limit) and 4.3 (4.4 to 4.2) with a slope of −1.9 were obtained (Fig. 2B).

In addition to the inhibition of platelet aggregation, avicine pseudocyanide (20–100 μM) also inhibited collagen-induced ATP release in a selective and concentration-dependent manner (Fig. 3), without affecting AA-, PAF-, U46619- or thrombin-induced release reaction (data not shown). Inhibition of ATP release was parallel to the inhibition of platelet aggregation (Fig. 3). However, a high concentration of avicine pseudocyanide (300 μM) did inhibit AA-, PAF-, U46619- and thrombin-induced ATP release slightly (data not shown). The inhibitory effect of avicine pseudocyanide on collagen-induced platelet aggregation and ATP release was readily reversible with full restoration of platelet aggregability (data not shown).

*Effect of avicine pseudocyanide on the aggregation of whole blood.* Whole blood aggregation was measured by the impedance method instead of the turbidity method. The resistance between two electrodes was measured. After challenge for 6 min, collagen (10 μg/mL) caused aggregation of whole blood based on the increase of resistance (18.5 ± 2.2 Ω, N = 4). Avicine pseudocyanide inhibited collagen-induced aggregation of whole blood in a concentration-dependent manner with an $IC_{50}$ of 145 ± 13 μM (N = 4).



I II III

IV V VI

Fig. 1. Chemical structures of avicine pseudocyanide (I) and its analogues, nitidine pseudocyanide (II), avicine chloride (III), noravicine (IV), dihydroavicine (V) and 6-methoxydihydroavicine (VI).

Table 1. Effects of avicine pseudocyanide and its analogues on the aggregation of washed rabbit platelets induced by ADP, arachidonic acid, collagen and PAF

| | Aggregation (%) | | | |
|---|---|---|---|---|
| | ADP (20 μM) | Arachidonic acid (100 μM) | Collagen (10 μg/mL) | PAF (2 ng/mL) |
| Control | 81.7 ± 2.2 (3) | 87.3 ± 1.5 (5) | 88.2 ± 1.8 (4) | 88.1 ± 1.3 (3) |
| Avicine chloride (50 μM) | 73.3 ± 2.0* (3) | 82.8 ± 2.5 (3) | 75.3 ± 7.5 (3) | 84.9 ± 1.0 (3) |
| Avicine pseudocyanide (300 μM) | 72.8 ± 3.8 (3) | 73.5 ± 4.5† (3) | 0.0 ± 0.0‡ (3) | 66.0 ± 7.6* (3) |
| Dihydroavicine (300 μM) | 69.2 ± 8.5 (3) | 0.0 ± 0.0‡ (3) | 82.5 ± 3.7 (3) | 86.6 ± 0.6 (3) |
| 6-Methoxydihydroavicine (300 μM) | 72.9 ± 3.1 (3) | 17.5 ± 7.3‡ (3) | 8.7 ± 7.5‡ (3) | 22.6 ± 16.0† (3) |
| Noravicine (50 μM) | 74.0 ± 3.1 (3) | 83.7 ± 1.6 (3) | 91.0 ± 0.4 (3) | 90.1 ± 1.2 (3) |
| Nitidine pseudocyanide (300 μM) | 69.6 ± 5.2 (3) | 44.1 ± 16.6* (5) | 64.2 ± 6.1‡ (3) | 75.8 ± 1.9‡ (3) |

Washed rabbit platelets were preincubated with DMSO (0.5%, control) or avicine pseudocyanide or its analogues at 37° for 3 min, and then the inducer was added. Values are means ± SEM (N).

*–‡ Significantly different from the respective control: *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.

Table 2. Effects of the concentration of avicine pseudocyanide on the aggregation of washed rabbit platelets induced by ADP, arachidonic acid, collagen, PAF, U46619 and thrombin

| | Aggregation (%) | | | | | |
|---|---|---|---|---|---|---|
| | ADP (20 μM) | Arachidonic acid (100 μM) | Collagen (10 μg/mL) | PAF (2 ng/mL) | U46619 (1 μM) | Thrombin (0.1 U/mL) |
| Control | 82.5 ± 3.2 (5) | 88.3 ± 1.9 (8) | 89.4 ± 0.6 (7) | 92.3 ± 1.1 (5) | 86.6 ± 1.8 (6) | 92.5 ± 0.5 (6) |
| Avicine pseudocyanide | | | | | | |
| 20 μM | | | 90.0 ± 0.6 (7) | | | |
| 50 μM | | | 31.3 ± 12.7* (7) | | | |
| 100 μM | | 81.3 ± 1.8† (8) | 3.5 ± 2.1* (7) | 86.3 ± 2.8 (5) | 81.0 ± 3.1 (6) | |
| 200 μM | | 73.7 ± 6.2† (7) | 0.0 ± 0.0* (7) | 80.5 ± 4.1† (5) | 76.0 ± 3.7† (6) | 89.2 ± 1.6 (6) |
| 300 μM | 74.6 ± 4.0 (5) | 71.9 ± 7.4† (7) | 0.0 ± 0.0* (7) | 64.1 ± 7.0* (5) | 74.4 ± 2.1* (6) | 85.0 ± 2.1‡ (6) |

Washed rabbit platelets were preincubated with DMSO (0.5%, control) or various concentrations of avicine pseudocyanide at 37° for 3 min, and then the inducer was added. Values are means ± SEM (N).

*–‡ Significantly different from the respective control: *$P < 0.001$, †$P < 0.05$, and ‡$P < 0.01$.

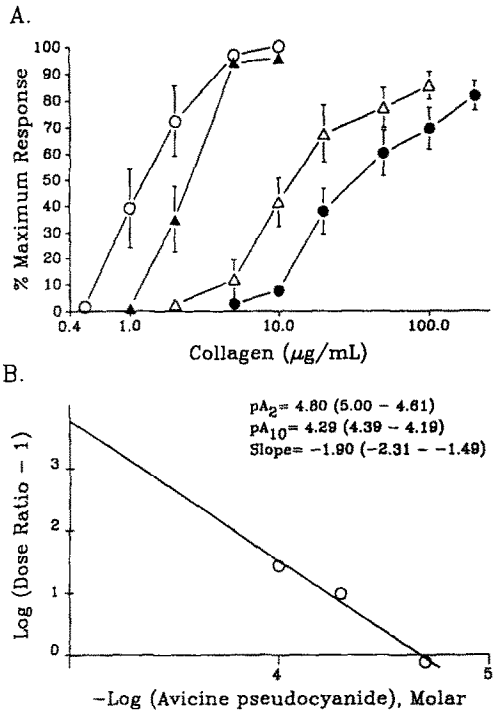



Fig. 2. (A) Concentration-dependent shifting by avicine pseudocyanide of the platelet aggregation induced by collagen. Washed rabbit platelets were incubated with DMSO (0.5%, ○—○) or various concentrations of avicine pseudocyanide (20 μM: ▲—▲; 50 μM: △—△; 100 μM: ●—●) at 37° for 3 min; then various concentrations of collagen were added to trigger the aggregation. Values are means ± SEM (N = 6). (B) Schild plot of the inhibitory effects of avicine pseudocyanide on collagen-induced platelet aggregation. The data were obtained from Fig. 2A. The equation of the regression line was $Y = -1.9 \times + 9.12$ with a correlation coefficient $r = 0.99$.

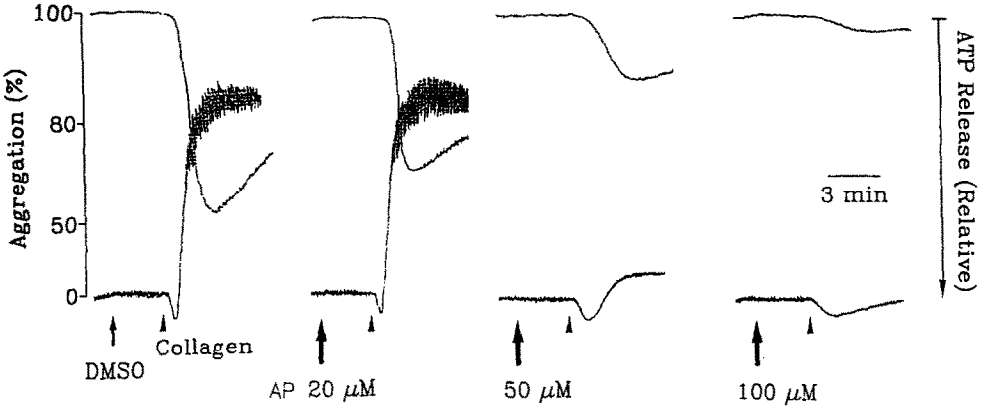



Fig. 3. Inhibitory effects of avicine pseudocyanide on platelet aggregation and ATP release induced by collagen. Washed rabbit platelets were preincubated with DMSO (0.5%) or various concentrations of avicine pseudocyanide (AP) at 37° for 3 min; then collagen (10 μg/mL) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

*Effects of avicine pseudocyanide on trimucytin-induced platelet aggregation and release reaction.* Trimucytin (1 μg/mL) caused 90.5 ± 1.1% aggregation (N = 5) of washed rabbit platelets. Avicine pseudocyanide inhibited trimucytin-induced aggregation in a concentration-dependent manner with an $IC_{50}$ of 62.5 ± 5.6 μM. In addition to the inhibition of platelet aggregation, avicine pseudocyanide (10–200 μM) also inhibited trimucytin-induced ATP release in a concentration-dependent manner (data not shown).

*Effects of avicine pseudocyanide on platelet*

Table 3. Effects of avicine pseudocyanide, indomethacin and imidazole on the thromboxane $B_2$ formation of washed rabbit platelets caused by arachidonic acid, collagen, PAF, U46619 and thrombin

| | Thromboxane $B_2$ (ng/mL) | | | | |
|---|---|---|---|---|---|
| | Arachidonic acid (100 μM) | Collagen (10 μg/mL) | PAF (2 ng/mL) | U46619 (1 μM) | Thrombin (0.1 U/mL) |
| Control | 432 ± 98 (4) | 289 ± 40 (6) | 16 ± 6 (5) | 0.8 ± 0.1 (6) | 166 ± 21 (5) |
| Avicine pseudocyanide | | | | | |
| 20 μM | | 225 ± 41 (6) | | | |
| 50 μM | | 8.6 ± 5.0* (5) | | | |
| 100 μM | | 2.2 ± 0.7* (6) | | | |
| 300 μM | 170 ± 34† (4) | | 0.6 ± 0.1† (5) | 0.7 ± 0.1 (6) | 2 ± 0.4* (5) |
| Indomethacin | | | | | |
| 1 μM | 3 ± 1* (4) | | | | |
| Imidazole | | | | | |
| 1 mM | 11 ± 1* (4) | | | | |

Platelets were preincubated with DMSO (0.5%, control), indomethacin (1 μM), imidazole (1 mM) or various concentrations of avicine pseudocyanide at 37° for 3 min, and then the inducer was added. Aggregation and thromboxane formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of the inducer. Values are means ± SEM (N).

*,† Significantly different from the respective control: *$P < 0.001$, and †$P < 0.05$.

*thromboxane $B_2$ formation.* The thromboxane $B_2$ level of resting platelets was 0.8 ± 0.2 ng/mL (N = 6). U46619 (1 μM) did not cause significant and PAF (2 ng/mL) caused only slight thromboxane $B_2$ formation in rabbit platelets. However, AA (100 μM), collagen (10 μg/mL) and thrombin (0.1 U/mL) caused marked thromboxane $B_2$ formation in washed rabbit platelets. Avicine pseudocyanide (20–100 μM) inhibited collagen-induced thromboxane $B_2$ formation in a concentration-dependent manner with a maximal effect at 100 μM. AA-induced thromboxane $B_2$ formation was partially suppressed by a high concentration of avicine pseudocyanide (300 μM), and was inhibited almost completely by indomethacin (1 μM) and imidazole (1 mM) (Table 3).

*Effects of avicine pseudocyanide on platelet prostaglandin $D_2$ formation.* The $PGD_2$ level of resting platelets was not detectable (<3 pg/mL). However, $PGD_2$ was formed in the presence of arachidonic acid. This $PGD_2$ formation was inhibited by indomethacin, but enhanced markedly by imidazole. Avicine pseudocyanide (300 μM) inhibited this AA-induced $PGD_2$ formation slightly but not significantly (Table 4).

*Effects of avicine pseudocyanide on the breakdown of phosphoinositides.* The breakdown of phosphoinositides has been observed in platelets activated by many agonists [20–22]. As shown in Fig. 4, collagen (10 μg/mL), PAF (2 ng/mL), U46619 (1 μM) and thrombin (0.1 U/mL) increased the [$^3$H]inositol monophosphate formation 2.3 ± 0.1-, 3.2 ± 0.7-, 1.9 ± 0.1- and 3.0 ± 0.5-fold, respectively, in the presence of indomethacin (20 μM). AA (100 μM), in the absence of indomethacin, also increased [$^3$H]inositol monophosphate formation 2.2 ± 0.6-fold. Avicine pseudocyanide (100 μM) markedly inhibited collagen-induced [$^3$H]inositol monophosphate formation without affecting the [$^3$H]inositol monophosphate formation caused by AA, PAF, U46619 and thrombin.

Table 4. Effects of avicine pseudocyanide, indomethacin and imidazole on prostaglandin $D_2$ formation induced by arachidonic acid in washed rabbit platelets

| | Prostaglandin $D_2$ (pg/mL) |
|---|---|
| Resting | <3 |
| DMSO + AA | 359 ± 130 |
| Avicine pseudocyanide + AA | 207 ± 60 |
| Indomethacin + AA | 9 ± 3* |
| Imidazole + AA | 2728 ± 655† |

Platelets were preincubated with DMSO (0.5%), indomethacin (1 μM), imidazole (1 mM) or avicine pseudocyanide (300 μM) at 37° for 3 min, and then arachidonic acid (AA, 100 μM) was added. Aggregation and prostaglandin $D_2$ formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of arachidonic acid. Values are means ± SEM (N = 4).

*,† Significantly different from the control (AA, 100 μM): *$P < 0.05$, and †$P < 0.01$.

*Effects of avicine pseudocyanide on the intracellular calcium of platelets.* In fura-2-loaded platelets, collagen, PAF, U46619 and thrombin caused an increase of intracellular free calcium in the presence of indomethacin (20 μM). AA also increased the intracellular free calcium in the absence of indomethacin. The rise was only short-lived, and intracellular free calcium declined towards the resting level within a few minutes. This was due to the aggregates interfering with the fluorescence signal and constitutes a limitation of the technique. As shown in Fig. 5, only the rise of intracellular $Ca^{2+}$ caused by collagen was markedly suppressed by avicine pseudocyanide (100 μM).

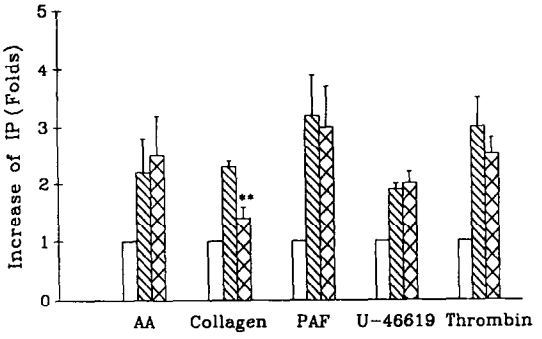



Fig. 4. Inhibitory effect of avicine pseudocyanide on the formation of inositol monophosphate in washed rabbit platelets caused by some aggregation inducers. [$^3$H]Inositol-labeled platelets were preincubated with DMSO [0.5%, for resting (□) and control (▨)] or avicine pseudocyanide (100 μM, ⊠) at 37° for 3 min; then AA (100 μM), collagen (10 μg/mL), PAF (2 ng/mL), U46619 (1 μM) or thrombin (0.1 U/mL) was added for another 6 min. Indomethacin (20 μM) was present in the medium except that challenged by AA. Increases in inositol phosphate (IP) are presented as means ± SEM (N = 3–4). Key: (**) $P < 0.01$ as compared with the respective control (without avicine pseudocyanide).

*Effects of avicine pseudocyanide on collagen–platelet adhesion.* The adhesion of $^{51}$Cr-labeled platelets to polystyrene dishes coated with fibrillar collagen was determined in medium containing 2 mM $Mg^{2+}$ and 10 μM $PGE_1$. Control $^{51}$Cr-labeled platelets in the presence of $Mg^{2+}$ and $PGE_1$ adhered to the fibrillar, type I collagen-coated microtiter walls, forming a monolayer that could be readily observed with the aid of an inverted microscope. In contrast, platelets adhered very poorly to the BSA-coated wells. The data were quantitated by solubilizing the adherent platelets in SDS and counting the released radioactivity. Approximately 20.1 ± 1.8% (N = 4) of the added platelets adhered to the collagen-coated wells. Only 0.5 ± 0.2% (N = 4) of the added platelets adhered to the BSA-coated wells. Avicine pseudocyanide inhibited this platelet–collagen adhesion in a concentration-dependent manner with an $IC_{50}$ value of about 278 ± 16 μM (Fig. 6).

## DISCUSSION

Avicine pseudocyanide selectively inhibited collagen-induced platelet aggregation and release reaction of washed rabbit platelets and whole blood in a concentration-dependent manner. The avicine pseudocyanide $IC_{50}$ values for collagen (10 μg/mL)-induced aggregation of washed platelets and whole blood were 47.3 ± 4.1 and 145 ± 13 μM, respectively.

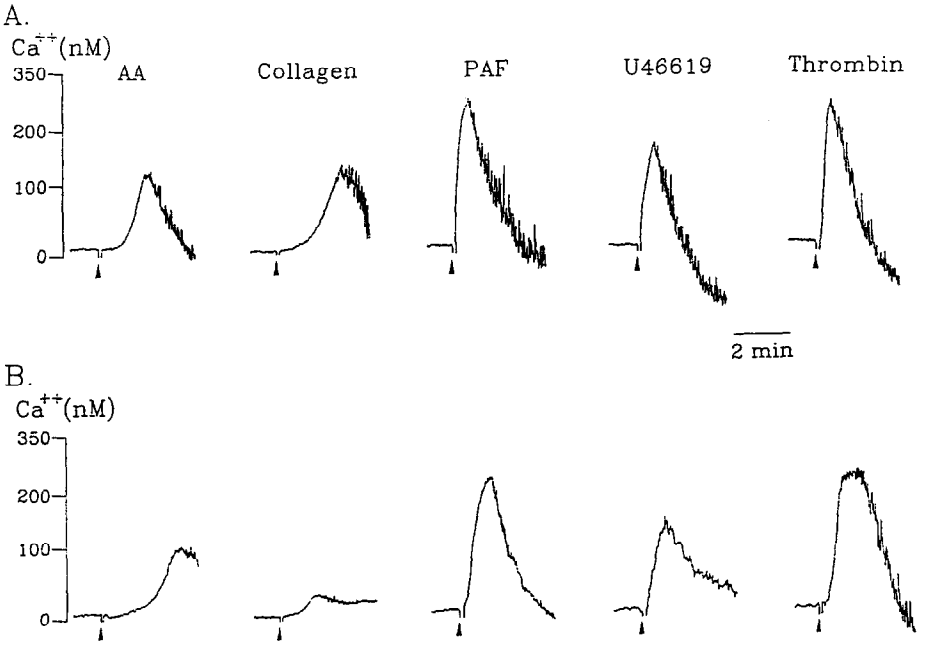



Fig. 5. Effects of avicine pseudocyanide on the increase of intracellular calcium concentration of platelets caused by some aggregation inducers. Fura-2-loaded platelets were preincubated with DMSO (0.5%, panel A) or avicine pseudocyanide (100 μM; panel B) at 37° for 3 min; then AA (100 μM), collagen (10 μg/mL), PAF (2 ng/mL), U46619 (1 μM) or thrombin (0.1 U/mL) was added (arrows). Indomethacin (20 μM) was present in the medium except that challenged by AA.

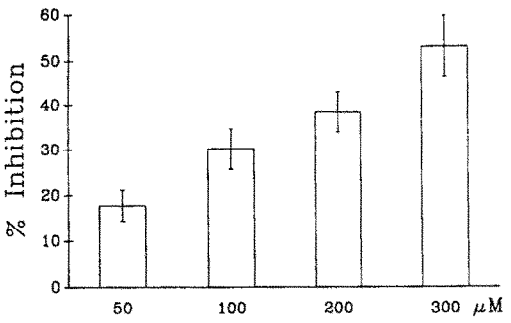



Fig. 6. Inhibitory activity of avicine pseudocyanide on the adhesion of platelets to collagen. The adhesion of $^{51}Cr$-labeled platelets to fibrillar collagen substrates was determined over a 30-min period in medium containing 2 mM $Mg^{2+}$ and 10 μM $PGE_1$ in the presence of DMSO (0.5% control) or various concentrations of avicine pseudocyanide. About 20.1 ± 1.8% of the added platelets adhered to the collagen-coated wells in the control condition. Values are means ± SEM (N = 4).

Trimucytin is a collagen-like platelet aggregation inducer isolated from *Trimeresurus mucrosquamatus* snake venom [23]. Both trimucytin- and collagen-induced platelet aggregation and release reaction were inhibited by the mouse anti-human platelet GPIa monoclonal antibody, MAB 1988, concentration dependently. Avicine pseudocyanide also inhibited trimucytin (1 μg/mL)-induced aggregation and release reaction in a concentration-dependent manner. The $IC_{50}$ of avicine pseudocyanide on trimucytin-induced platelet aggregation was 62.5 ± 5.6 μM. A high concentration of avicine pseudocyanide (300 μM) inhibited less than 30% of platelet aggregation caused by ADP, AA, PAF, U46619 and thrombin. This indicates that avicine pseudocyanide is a selective collagen inhibitor in washed rabbit platelets. The concentration–response curve of collagen-induced platelet aggregation was shifted to the right by avicine pseudocyanide. Maximal response of collagen-induced aggregation could be achieved at sufficient collagen concentration. In addition, the inhibitory effects of avicine pseudocyanide on collagen-induced platelet aggregation and release reaction were easily washed out. Thus, avicine pseudocyanide may be a reversible collagen inhibitor.

Thromboxane $A_2$ is an important mediator of the release reaction and aggregation in platelets [24]. Formation of thromboxane $B_2$, a stable metabolite of thromboxane $A_2$, induced by collagen was inhibited completely by 100 μM avicine pseudocyanide. Avicine pseudocyanide at a concentration of 300 μM inhibited only partially AA-induced thromboxane $B_2$ formation. The AA-induced $PGD_2$ formation was also inhibited by avicine pseudocyanide (300 μM) partially but not significantly. Thus, avicine pseudocyanide inhibition of collagen-induced thromboxane $B_2$ formation is on an earlier step before cyclooxygenase. Phosphoinositide breakdown is an important pathway in signal transduction of agonist-induced platelet activation. Indeed, the primary biological signal induced by many aggregation inducers is clearly the increase of phosphatidylinositol breakdown [25, 26]. This process generates two active products, diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C, leading to protein phosphorylation and release reaction. Inositol trisphosphate triggers calcium mobilization from intracellular compartments [27]. The rise of intracellular $Ca^{2+}$ is very important for both the release reaction and the activation of phospholipase $A_2$, which is a rate-limiting enzyme for the generation of arachidonic acid. Avicine pseudocyanide (100 μM) selectively inhibited collagen-induced inositol phosphate formation and the rise of intracellular $Ca^{2+}$ concentration without affecting those caused by AA, PAF, U46619 and thrombin. Thus, it may suppress the collagen-induced platelet aggregation, release reaction, thromboxane formation and rise of intracellular $Ca^{2+}$ concentration by the inhibition of collagen-induced phosphoinositide breakdown. The possibility that a high concentration of avicine pseudocyanide (300 μM) may suppress cyclooxygenase activity cannot be ruled out. This also explains why thrombin-induced thromboxane $B_2$ formation was inhibited by a high concentration of avicine pseudocyanide (300 μM).

Platelet adhesion to fibrillar collagen and monomeric collagen are divalent cation dependent and supported by $Mg^{2+}$ [17]. The platelet–collagen adhesion in the presence of $Mg^{2+}$ and $PGE_1$ was also inhibited by avicine pseudocyanide with a much higher concentration ($IC_{50}$: 278 ± 16 μM) than inhibition on collagen-induced aggregation ($IC_{50}$: 47.3 ± 4.1 μM). It has been documented that not only the native, triple helical structure of collagen is required for activation of platelet aggregation, but also that polymerization of monomeric collagen molecules into collagen fibers is required for platelet activation and aggregation [28–30]. However, both fibrillar and monomeric collagen effectively support platelet adhesion [17]. It has been proposed that collagen-induced platelet aggregation and collagen–platelet adhesion probably result from multiple interactions of components in the platelet membrane with multiple sites available on the collagen fibril, and various receptors for collagen have also been proposed [31, 32]. Furthermore, platelet adhesion to collagen may involve sites in the collagen molecule distinct from those more directly associated with aggregation [33]. Thus, the differential $IC_{50}$ of avicine pseudocyanide on collagen-induced aggregation and collagen–platelet adhesion may be due to distinct sites in the collagen molecule, and/or different receptors in the platelet membrane are involved in aggregation and adhesion.

Avicine pseudocyanide selectively inhibited collagen-induced platelet responses and platelet-collagen adhesion in washed rabbit platelets. Further investigation of its *in vivo* anti-thrombogenic activity is warranted.

*Acknowledgement*—This work was supported by a research grant of the National Science Council of the Republic of China (NSC82-0420-B002-055-M13).

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