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A potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom

Tur-Fu HUANG,* Jeon-Rong SHEU and Che-Ming TENG
Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan

The interaction of fibrinogen with its receptors on platelet surfaces leads to platelet aggregation. A snake-venom peptide, trigramin, has previously been demonstrated to inhibit platelet aggregation by acting as a fibrinogen-receptor antagonist. By means of gel filtration, ionic-exchange chromatography and reverse-phase h.p.l.c., a potent platelet-aggregation inhibitor, triflavin, has now been purified from the venom of *Trimeresurus flavoviridis*. The purified triflavin is a single-chain polypeptide, consisting of about 71 amino acid residues with a molecular mass of 7600 Da, and its *N*-terminal sequence is Gly-Glu-Glu-Cys-Asp. Triflavin dose-dependently inhibited human platelet aggregation stimulated by ADP, adrenaline, collagen, thrombin or prostaglandin endoperoxide analogue U46619 in preparations of platelet-rich plasma, platelet suspension and whole blood. Its IC_{50} ranged from 38 to 84 nM, depending on the aggregation inducer used and the platelet preparation. However, triflavin apparently did not affect the platelet shape change and ATP-release reactions caused by these agonists. Triflavin inhibited fibrinogen-induced aggregation of human elastase-treated platelets in a dose-dependent manner, indicating that it directly interferes with the binding of fibrinogen to its receptors on platelet membranes exposed by elastase treatment. Additionally, triflavin dose-dependently blocked ^{125}I-labelled fibrinogen binding to ADP-activated platelets. In conclusion, triflavin inhibits platelet aggregation through the blockade of fibrinogen binding to fibrinogen receptors on platelet membranes.

INTRODUCTION

Adhesion of platelets to extracellular matrices and platelet–platelet interaction are crucial events in thrombosis and haemostasis. In normal circulation, platelets cannot aggregate by themselves. When a blood vessel is damaged, platelets adhere to the disrupted surface, and the adherent platelets release some biologically active constituents and aggregate. Platelet aggregation may be initiated by a variety of physicochemical factors, such as ADP, adrenaline, thrombin, collagen, 5-hydroxytryptamine and prostaglandin endoperoxides [1–8]. The agonist binds to its specific platelet membrane receptor, resulting in the exposure of the 'cryptic' fibrinogen receptors on the platelet surface membranes via a G-protein-coupled mechanism. Currently, it is well established that the binding of fibrinogen to its receptors associated with a Ca^{2+}-dependent glycoprotein IIb–IIIa complex is the common mechanism of platelet aggregation stimulated by all these agonists [9].

Snake venoms affect platelet function in various ways: some components induce aggregation and release reactions [10–12], whereas some other components inhibit these reactions. We have previously reported that there are three main kinds of antiplatelet proteins derived from haemorrhagic snake venoms, including ADPase, α-fibrinogenase [13–15] and trigramin-like peptides, which have been reported to inhibit competitively fibrinogen binding to glycoprotein IIb–IIIa complex on platelet surfaces [16–18].

In the present study we have characterized a platelet-aggregation-inhibitory peptide, triflavin, purified from *Trimeresurus flavoviridis* snake venom, which is more potent than trigramin. It inhibited in a dose-dependent manner platelet aggregation stimulated by a variety of agonists, including ADP, collagen, adrenaline, thrombin and compound U46619; however, it apparently did not affect the initial shape change and release reactions induced by these agonists. It inhibited in a dose-dependent manner the fibrinogen-induced aggregation of elastase-treated human platelets and also ^{125}I-labelled fibrinogen binding to ADP-activated platelets, suggesting that it directly interferes with the fibrinogen binding to fibrinogen receptors associated with the glycoprotein IIb–IIIa complex. Therefore this antithrombotic peptide is a naturally occurring fibrinogen-receptor antagonist.

MATERIALS AND METHODS

Trimeresurus flavoviridis snake venom was purchased from Latoxan, Rosans, France, and stored at −20 °C. CM-Sephadex C-50, Sephadex G-75 and Sephadex G-50 were obtained from Pharmacia, Uppsala, Sweden. Fractogel TSK HW-50 and trifluoroacetic acid were purchased from Merck, Darmstadt, Germany. Human thrombin, AMP, ADP, collagen (bovine tendon type I), prostaglandin E_1, apyrase, BSA, elastase (type IV, pig pancreas), luciferase/luciferin mixture, acrylamide, heparin, Tris-hydrochloride, EDTA, EGTA and sucrose were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Human fibrinogen (Kabi, Stockholm, Sweden), acetonitrile (L.C. grade; Alps Chemical Co., Taipeh, Taiwan) and compound U46619 (Biomol Research Laboratories, PA, U.S.A.) were used.

Fractogel column chromatography

Fractogel TSK HW-50 was pre-equilibrated in 0.1 M-NaCl and packed in a column (3.6 cm × 66 cm) equilibrated with 0.1 M-NaCl. Crude *Trimeresurus flavoviridis* venom (1 g dissolved in the same solution) was applied to this column. Elution was carried out with 0.1 M-NaCl and the flow rate was adjusted to 25 ml/h; fractions of volume 4 ml/tube were collected. The effluent was monitored continuously at 278 nm at 5 °C with an LKB Uvicord instrument.

Rabbit platelet-aggregation assays induced by collagen (10 μg/ml) were used to monitor the antiplatelet activity during the initial stage of purification of triflavin. A 10 μl portion from every collecting tube was incubated at 37 °C with rabbit platelet

Abbreviation used: IC_{50}, inhibitor concentration causing 50% inhibition.
* To whom correspondence should be addressed.

suspension at least 2 min before the addition of collagen. The antiplatelet fraction was desalted on a Sephadex G-25 column, eluted with distilled water. However, most of the platelet-aggregation assay was performed with human platelet suspension throughout this study.

CM-Sephadex C-50 column chromatography

The antiplatelet venom fraction collected was applied to a column (3.2 cm × 65 cm) of CM-Sephadex C-50. Gradient elution with ammonium acetate was carried out in three stages: (1) 0.05 M-ammonium acetate (pH 5.0), 200 ml; (2) 0.05 M-ammonium acetate (pH 5.0) to 0.2 M-ammonium acetate (pH 6.8), 450 ml; (3) 0.6 M-ammonium acetate (pH 8.0), 650 ml. The flow rate was adjusted to 25 ml/h and the eluate (3 ml/tube) was automatically collected with an LKB collector at 4 °C.

Sephadex G-75 and Sephadex G-50 column chromatography

Sephadex G-75 and Sephadex G-50 were equilibrated in 0.01 M-ammonium bicarbonate and packed in columns (2.8 cm × 100 cm) at 4 °C. The antiplatelet fraction from the ion-exchange column was applied to this column. Elution was carried out with 0.01 M-ammonium bicarbonate. The flow rate was adjusted to 25 ml/h and the eluate (3 ml/tube) was collected. The effluent was monitored automatically at 278 nm at 4 °C with an LKB Uvicord instrument.

Reverse-phase h.p.l.c. system

Finally, the last step of purification was accomplished on a reverse-phase h.p.l.c. C_{18} column (4.0 mm × 300 mm) (Waters 501). This system consists of an injector (U6K) and a two-pump system for solvent delivery (Waters 501, h.p.l.c. pump) connected to computer software (NEC, Baseline 810).

The crude triflavin obtained after the purification by gel-filtration and ion-exchange chromatography was injected directly on to a C_{18} reverse-phase h.p.l.c. column that had been equilibrated with 0.1 % (v/v) trifluoroacetic acid in water. The column was eluted at room temperature with an acetonitrile gradient in aq. 0.1 % trifluoroacetic acid and the flow rate was adjusted to 1 ml/min. Crude triflavin (600 μl of 1 mg/ml solution) was injected on to the column and eluted by the pre-set linear acetonitrile gradient, within a 50 min period. The two-pump system utilized buffer A (0.1 % trifluoroacetic acid in distilled water) and buffer B (80 % acetonitrile plus 0.1 % trifluoroacetic acid). The elution pattern of the linear acetonitrile gradient is shown in Fig. 4. The elution was monitored at 208 nm at room temperature and the eluates were collected manually. Volatile solvent was removed by vacuum centrifugation. The dried purified protein was freezed-dried and named 'triflavin'.

SDS/PAGE

A 20 % (w/v) polyacrylamide gel was used to judge the purity of purified triflavin [19], and to estimate molecular mass with standard proteins (Zoion Research, U.S.A.) including myoglobin fragment I (2512 Da), myoglobin fragment II (6224 Da), myoglobin fragment III (8159 Da), myoglobin fragment IV (14400 Da), myoglobin (16950 Da) and trypsin inhibitor (20400 Da).

After electrophoresis, the gel was pre-fixed with 10 % (w/v) glutaraldehyde for 30 min and stained with Coomassie Brilliant Blue for 1 h, then destained. The mobilities of the test sample and standard proteins were plotted against the molecular masses of the standard proteins on semi-log paper and the molecular mass of the purified triflavin was estimated by interpolation.

Amino acid analysis

Triflavin was hydrolysed in 6 M-HCl in a 150 °C oven in an N_2 atmosphere for 24 h. The amino acid composition was then determined with a Beckman model M121 amino acid analyser.

N-Terminal amino acid determination

This was performed on a gas-phase sequencer (model 477A; Applied Biosystems, CA, U.S.A.). Standard protocols of the manufacturer were followed with regard to both Edman degradation and separation of amino acid phenylthiohydantoin derivatives by h.p.l.c.

Preparation of human platelet-rich plasma and platelet suspension

Blood was collected from healthy human volunteers, who had not taken any medicine during the preceding 2 weeks, and mixed with 3.8 % (w/v) sodium citrate (9:1, v/v). Citrated blood was immediately centrifuged at 120 *g* for 10 min at 25 °C, and the supernatant (platelet-rich plasma) was retained. In some preliminary tests, rabbit platelet suspension was used and it was prepared essentially by the following method except that rabbit blood was withdrawn from the ear vein of New Zealand White rabbits, and anticoagulated with 3.8 % sodium citrate (9:1, v/v). Rabbit washed platelet suspension was prepared by the method of Mustard *et al.* [20] and human washed platelet suspension was prepared by the method of Mustard *et al.* [20] and Kornecki *et al.* [21]. Blood was mixed with acid/citrate/glucose (9:1, v/v). After centrifugation at 120 *g* for 10 min at room temperature, the supernatant (platelet-rich plasma) was supplemented with prostaglandin E_1 (0.5 μM) and heparin (6.4 i.u./ml), incubated for 10 min at 37 °C and centrifuged at 500 *g* for 10 min. The platelet pellet was suspended in 5 ml of Tyrode's solution, pH 7.3 [containing NaCl (11.9 mM), KCl (2.7 mM), $MgCl_2$ (2.1 mM), NaH_2PO_4 (0.4 mM), $NaHCO_3$ (11.9 mM) and glucose (11.1 mM)], then apyrase (1 unit/ml), prostaglandin E_1 (0.5 μM) and heparin (6.4 i.u./ml) were added, and the mixture was incubated for 10 min at 37 °C. After centrifugation of the suspension at 500 *g* for 10 min, the washing procedure was repeated. The washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg/ml) and adjusted to about 4.5×10^8 platelets/ml. The platelet count was monitored by a Coulter counter (model ZM). The final concentration of Ca^{2+} in the Tyrode's solution was 1 mM. Elastase-treated platelets were prepared by the method of Greenberg *et al.* and Kornecki *et al.* [22,23]. Elastase (1.25 units/10^8 platelets) was added to the platelet suspension, and the mixture was incubated for 60 min at 37 °C in the presence of apyrase (0.5 unit/ml). After centrifugation at 500 *g* for 10 min, the pellet was washed twice with Tyrode's solution, and finally suspended in Tyrode's solution containing BSA.

Platelet-aggregation and ATP-release reactions

The turbidimetric method [24], with a Lumi-Aggregometer (Chrono-Log), was used to measure platelet aggregation. Platelet-rich plasma or platelet suspension (0.4 ml) was pre-warmed at 37 °C for 2 min in a silicone-treated glass cuvette. Triflavin or Tyrode's solution was added 1 min before addition of platelet-aggregation inducer. The reaction was allowed to proceed for at least 6 min and the extent of aggregation was expressed as the percentage of control (in the absence of triflavin). The degree of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μl of luciferase/luciferin mixture was added 1 min before the addition of agonists and ATP release was compared with that of the control.

Whole-blood aggregation was measured by the impedance method with citrated whole blood and monitored by a whole-blood aggregometer (Chrono-Log).

Effect of triflavin on ADP-induced platelet-shape change

This was studied by using the method of Holmsen *et al.* [25]. In brief, the shape change induced by ADP (20 μM) was assessed by measuring the decrease in light transmission in the presence of EDTA (5 mM).

Radiolabelling of fibrinogen

Fibrinogen was labelled with Na^{125}I (New-England Nuclear, Boston, MA, U.S.A.) with the use of Enzymobeads (Bio-Rad Laboratories), and separated from Na^{125}I by Sephadex G-25 chromatography. The specific radioactivity of ^{125}I-fibrinogen was about 80000 c.p.m./μg of protein. The 'clottability' of radiolabelled fibrinogen was about 90%. Labelled fibrinogen co-migrated with unlabelled fibrinogen on SDS/PAGE.

Effect of triflavin on ^{125}I-fibrinogen binding to ADP-activated platelets

^{125}I-fibrinogen–platelet binding assays were performed by the previously described method [16]. In brief, the incubation mixture was composed of 420 μl of platelet suspension (about 5×10^8 platelets/ml), 10 μl of ^{125}I-fibrinogen, 10 μl of ADP (final concentration 10 μM) and 10 μl of Tyrode's solution or various concentrations of triflavin. Triflavin was added to platelet suspension 2 min before the addition of ^{125}I-fibrinogen and then ADP was added 1 min later. After the addition of ADP, the incubation mixture was gently shaken and incubated for another 10 min. Then 400 μl of platelet suspension was centrifuged through 15% (w/v) sucrose at 15000 *g* in an Eppendorf centrifuge. The radioactivities of the supernatant and platelet pellets were counted separately with an LKB γ-radiation counter. The non-specific binding of ^{125}I-fibrinogen was measured in the presence of 10 mM-EDTA. The specific fibrinogen binding was obtained as the difference between the radioactivity of total binding and that of non-specific binding.

RESULTS

Purification of the platelet-aggregation inhibitor from *Trimeresurus flavoviridis* venom

By means of Fractogel TSK HW-50 column chromatography, the crude venom was separated into three fractions (Fig. 1). We tested the inhibitory activity of each fraction on platelet aggregation by using washed rabbit platelet suspension. Fraction I (tube nos. 26–54) possessed the most potent inhibitory activity towards collagen (10 μg/ml)-induced platelet aggregation. Therefore this fraction was collected, desalted, freeze-dried and further applied to a CM-Sephadex C-50 column; six subfractions were produced (Fig. 2). The third subfraction (3, tube nos. 219–280) possessed inhibitory activity towards platelet aggregation induced by collagen (10 μg/ml). Desalted freeze-dried fraction I-3 was further fractionated by Sephadex G-75 chromatography, and the third sub-subfraction (C, tube nos. 27–45) was found to be the active component (Fig. 3*a*). After gel filtration on a Sephadex G-50 column, a single symmetrical peak of inhibitory activity was obtained (tube nos. 17–43) (Fig. 3*b*). In order to obtain a highly purified component, the fraction from the Sephadex G-50 column containing inhibitory activity was further purified by C_{18} reverse-phase h.p.l.c. (Fig. 4). A single peak of inhibitory activity was eluted at approx. 25% acetonitrile, and the elution time was around 21 min (indicated by *). Other fractions, eluted at 8, 9, 19, 43 and 45 min, were inactive. The fraction eluted at 21 min was named 'triflavin' and used throughout the following experiment. The yield of this purified triflavin was about 1.5% (w/w).

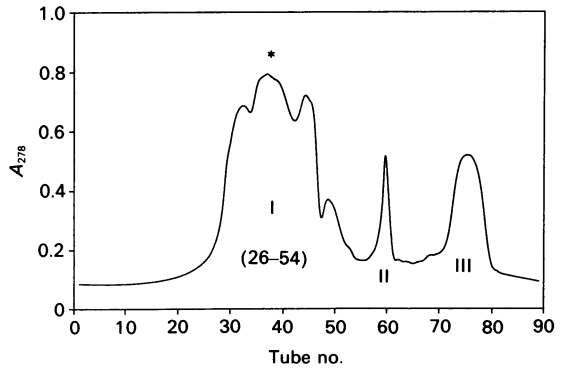

Fig. 1. Fractogel TSK HW-50 column chromatography of *Trimeresurus flavoviridis* venom

The crude venom (1 g) was applied to a column (3.6 cm × 66 cm) with a bed volume of 400 ml. NaCl (0.1 M) was used as eluent. The flow rate was adjusted to 25 ml/h and fractions (4 ml/tube) were collected. The eluate was monitored continuously at 278 nm at 5 °C with an LKB Uvicord instrument. Fraction I (*, tube nos. 26–54), which possessed antiplatelet activity, was collected.

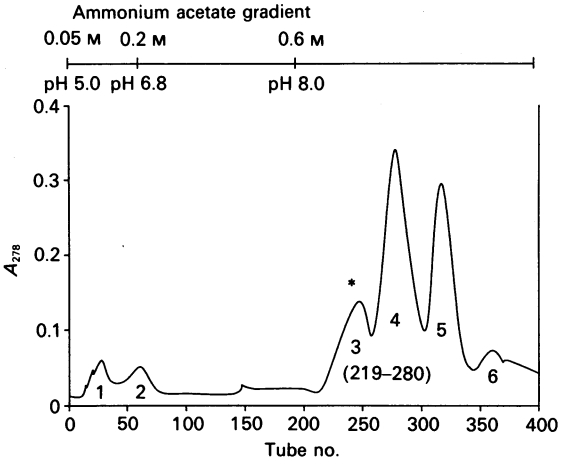

Fig. 2. CM-Sephadex C-50 column chromatography of fraction I

Fraction I obtained from the Fractogel TSK HW-50 column (Fig. 1) was applied to a column (3.2 cm × 65 cm) packed with CM-Sephadex C-50. Ammonium acetate solution was used as eluent in the gradient indicated. The flow rate was adjusted to 25 ml/h and fractions (3 ml/tube) were collected. The antiplatelet fraction (3*, tube nos. 219–280) was collected for further purification.

Determination of amino acid composition and molecular mass of triflavin

The purified platelet-aggregation inhibitor (triflavin) was shown to be a single peptide chain by SDS/PAGE since its mobility was the same in the presence and in the absence of 2% (v/v) 2-mercaptoethanol. The molecular mass was estimated by SDS/PAGE to be around 7600 Da as compared with standard markers (results not shown). It was composed of about 71 amino acid residues per molecule in which the contents of arginine, aspartic acid, glycine, alanine and half-cystine were high, as shown in Table 1. So far, we have not completed its sequence analysis; however, the partial *N*-terminal sequence was shown to be Gly-Glu-Glu-Cys-Asp-Cys-Gly-Ser-Pro-Ser-Asn-Pro-Cys-Cys-Asp-Ala-Ala-Thr...

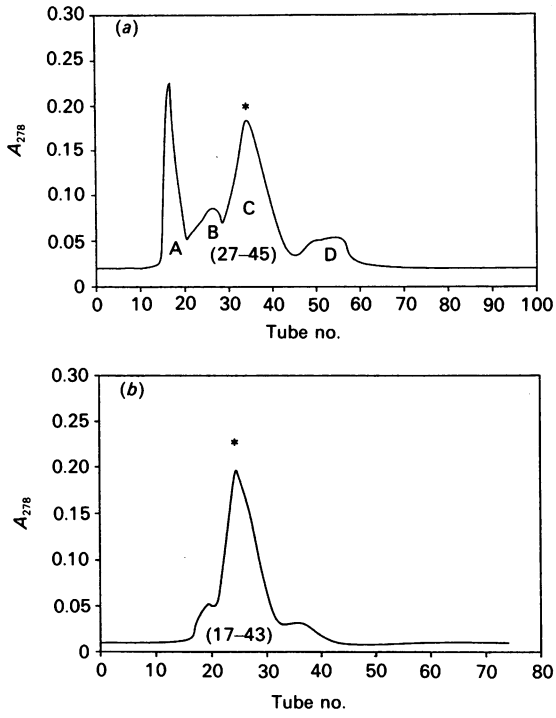

Fig. 3. Sephadex G-75 chromatography of subfraction 3 and Sephadex G-50 chromatography of sub-subfraction C chromatography

(*a*) Re-fractionation of subfraction 3 (obtained from CM-Sephadex C-50 column chromatography; see Fig. 2) on a Sephadex G-75 column (2.8 cm × 100 cm). Ammonium bicarbonate (0.01 M) was used as eluent. The flow rate was adjusted to 25 ml/h and fractions (3 ml/tube) were collected. (*b*) The antiplatelet fraction (C*, tube nos. 27–45) was further purified by Sephadex G-50 chromatography (bed vol. 180 ml). The column was eluted with 0.01 M-ammonium bicarbonate. The flow rate was adjusted to 20 ml/h and fractions (3 ml/tube) were collected. The eluate was monitored continuously at 278 nm at 5 °C with an LKB Uvicord instrument. The antiplatelet fraction (*, tube nos. 17–43) was further purified by reverse-phase h.p.l.c.

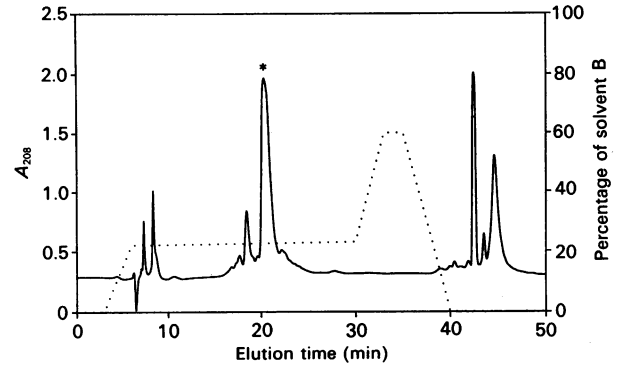

Fig. 4. Purification of triflavin by using reverse-phase h.p.l.c.

A sample (200–400 μl; 1 mg/ml) of crude triflavin (obtained from Sephadex G-50 column chromatography; see Fig. 3*b*) was injected on to a column (4 mm × 300 mm) of μBondapak C_{18} equilibrated in 0.1 % trifluoroacetic acid. The chromatography was carried out with a two-solvent gradient (see the Materials and methods section for details) as indicated (· · · ·), at a flow rate of 1.0 ml/min. The eluate was monitored continuously at 208 nm with a Waters 490 E programmable multiwavelength detector. One peak with antiplatelet activity was eluted at about 21 min (*).

Table 1. Amino acid composition of triflavin, a platelet-aggregation inhibitor, from *Trimeresurus flavoviridis* venom

Minimum molecular mass was 7584 Da. Abbreviations: N.D., not determined.

Amino acid	Composition (residues/ molecule)
Lysine	4
Histidine	0
Arginine	7
Aspartic acid	10
Threonine	3
Serine	3
Glutamic acid	6
Proline	6
Glycine	7
Alanine	7
Half-cystine	10
Valine	1
Methionine	0
Isoleucine	2
Leucine	3
Tyrosine	0
Tryptophan	N.D.
Phenylalanine	2
Total	71

Effect of triflavin on platelet aggregation of human platelet-rich plasma

As shown in Fig. 5, triflavin (0.1–0.8 μg/ml) inhibited in a dose-dependent way ADP (20 μM)-, adrenaline (10 μM)- and collagen (10 μg/ml)-induced platelet aggregation in human platelet-rich plasma. The IC_{50} values for platelet aggregation induced by ADP, adrenaline and collagen were estimated to be 0.35 μg/ml (46 nM), 0.31 μg/ml (41 nM) and 0.49 μg/ml (64 nM) respectively. At 0.8 μg/ml (130 nM), triflavin almost completely abolished aggregation stimulated by ADP and adrenaline. However, even at 1.0 μg/ml, it did not completely inhibit platelet aggregation induced by collagen (10 μg/ml). In addition, triflavin

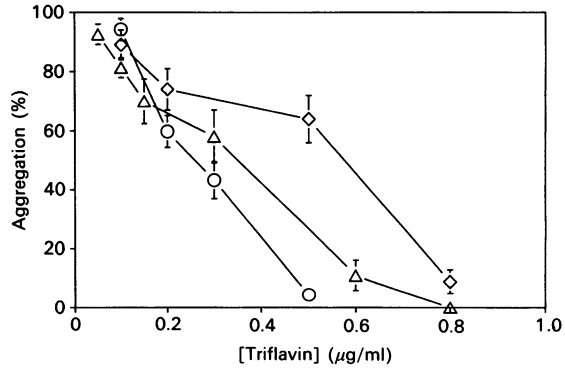

Fig. 5. Dose–inhibition curve of triflavin on adrenaline (10 μM, ○)-, collagen (10 μg/ml, ◇)- and ADP (20 μM, △)-induced platelet aggregation of human platelet-rich plasma

Platelets (3.6×10^8 ml) were preincubated with triflavin (0.1–0.8 μg/ml) and stirred for 1 min at 37 °C, then aggregation agonist was added to trigger aggregation. Aggregation was measured by a turbidimetric method (changes in transmission, ΔT). Data are presented as percentage of control (means ± S.E.M., $n = 4$–5).

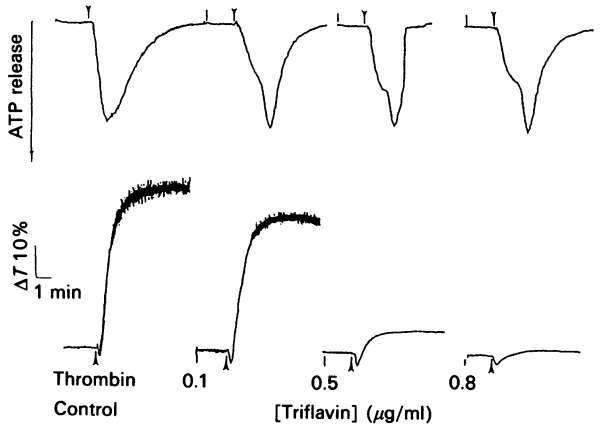

Fig. 6. Typical patterns of antiplatelet effect of triflavin on thrombin (0.1 unit/ml)-induced aggregation and ATP release of washed human platelet suspension

Platelets (4.5×10^8/ml) were preincubated with triflavin (0.1, 0.5 or 0.8 μg/ml) at 37 °C for 1 min, then thrombin (0.1 unit/ml) was added to trigger aggregation (upward tracing) and ATP release (downward tracing). Luciferin/luciferase mixture (20 μl) was added 2 min before the agonist in order to measure the ATP-release reaction.

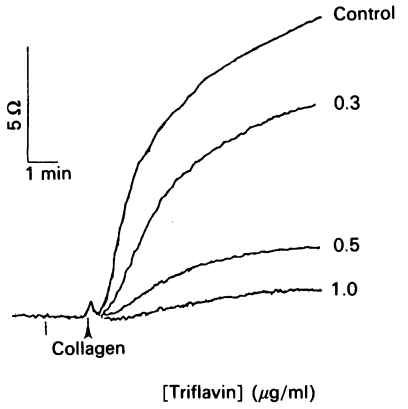

Fig. 8. Effect of triflavin on collagen-induced platelet aggregation in human whole blood

Triflavin (0.3, 0.5 or 1.0 μg/ml) was added 1.5 min before collagen (10 μg/ml). This experiment is a representative one of four similar experiments. Platelet aggregation was monitored by the impedance method with a whole-blood aggregometer (Chrono-Log).

also inhibited dose-dependently platelet aggregation induced by the prostaglandin endoperoxide analogue compound U46619 (1 μM) (results not shown). On the other hand, at a concentration of 1.0 μg/ml, it did not affect the initial shape change (assessed by the initial decrease in light transmission) induced by collagen and ADP in the presence of EDTA (5 μM).

Effect of triflavin on platelet aggregation in human platelet suspension

Triflavin inhibited in a dose-dependent manner platelet aggregation stimulated by a variety of aggregation inducers in human washed platelet suspension (Figs. 6 and 7). It similarly inhibited fibrinogen (200 μg/ml)-induced aggregation of ADP (20 μM)-stimulated platelets. It also dose-dependently inhibited platelet aggregation induced by thrombin (0.1 unit/ml), compound U46619 (1 μM) and collagen (10 μg/ml). However, triflavin did not affect the ATP-release reaction and the initial platelet-shape change when the platelets were stimulated by these agonists (Fig. 6). The IC_{50} values for platelet aggregation induced by ADP (20 μM), thrombin (0.1 unit/ml), compound U46619 (1 μM) and collagen (10 μg/ml) were estimated to be 0.40 μg/ml (53 nM), 0.47 μg/ml (62 nM), 0.59 μg/ml (78 nM) and 0.64 μg/ml (84 nM) respectively (Fig. 7).

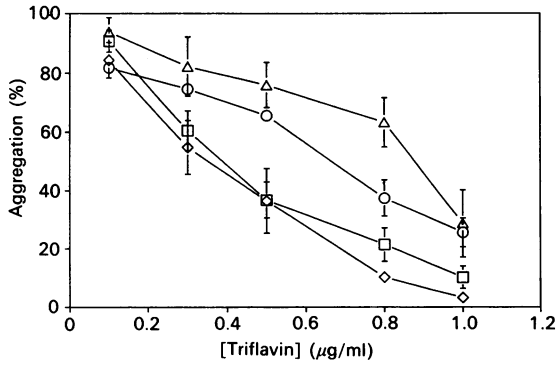

Fig. 7. Dose-inhibition curve of triflavin on compound U46619 (1 μM, △)-, ADP (20 μM, ◇)-, thrombin (0.1 unit/ml, □)- and collagen (10 μM, ○)-induced aggregation of washed human platelets

Data are presented as percentage of control (means ± S.E.M., $n = 4$–5).

Effect of triflavin on platelet aggregation in human whole blood

Platelet aggregation in whole blood is a more physiological condition compared with that in platelet-rich plasma or platelet suspension. We tested whether triflavin can inhibit platelet aggregation in this preparation. As measured by the impedance method, triflavin did inhibit in a dose-dependent manner platelet aggregation in whole blood stimulated by collagen (Fig. 8). The IC_{50} for platelet aggregation induced by collagen (10 μg/ml) was estimated to be 0.58 μg/ml (76 nM), indicating that triflavin is apparently not easily inactivated or strongly bound to other blood cells or plasma proteins.

Effect on fibrinogen-induced aggregation of elastase-treated platelets

When platelets are treated with various proteinases such as Pronase, α-chymotrypsin or elastase, their fibrinogen receptors are exposed; this mediates platelet aggregation in the presence of fibrinogen through the formation of the fibrinogen–fibrinogen receptor complex [19–21]. Therefore, as expected, the addition of fibrinogen caused aggregation of elastase-treated platelets (Fig. 9), and this aggregation was not inhibited by prostaglandin E_1 (results not shown), indicating that fibrinogen-induced aggregation was indeed mediated by the binding of fibrinogen to its surface receptors on the platelets. Triflavin dose-dependently inhibited fibrinogen (200 μg/ml)-induced aggregation (Fig. 9*a*). At 0.5 μg/ml, it completely inhibited aggregation in this preparation, indicating that it may directly interrupt the association of fibrinogen with its receptors on platelet surfaces. Comparing Fig. 9(*b*) with Fig. 9(*a*), triflavin not only prevented fibrinogen-induced aggregation but also disaggregated the platelet plug formed when triflavin was added 1 min (B), 3 min (C) or 5 min (D) after the addition of fibrinogen (200 μg/ml). This result shows that the binding of fibrinogen to its receptor is reversible and triflavin effectively displaces fibrinogen from its binding sites on the platelet membrane surface in this preparation. The IC_{50} was estimated to be 0.29 μg/ml (38 nM).

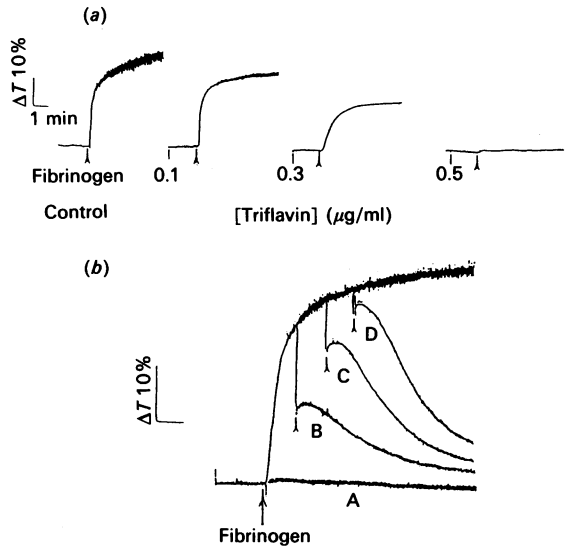

Fig. 9. Effect of triflavin on fibrinogen (200 μg/ml)-induced platelet aggregation of elastase-treated human platelets

(*a*) The elastase-treated human platelet suspension (1.25 units of elastase/10^8 platelets) was preincubated with triflavin (0.1, 0.3 or 0.5 μg/ml) 1 min before the addition of fibrinogen (200 μg/ml). (*b*) Triflavin (0.5 μg/ml) was added to the same platelet suspension 1 min (A) before or 1 min (B), 3 min (C), 5 min (D) after the addition of fibrinogen.

Table 2. Effect of triflavin on ^{125}I-fibrinogen binding to ADP-activated platelets

The incubation mixture was composed of 420 μl of platelet suspension (5×10^8 platelets/ml), 10 μl of ADP (20 μM), 10 μl of ^{125}I-fibrinogen (100 μg/ml) and 10 μl of Tyrode's solution or various concentrations of triflavin. The specific fibrinogen binding was defined as the difference between radioactivities of total binding and non-specific binding (in the presence of 10 mM-EDTA). For details see the Materials and methods section. These data are presented as means ± S.E.M. ($n = 4$).

Concn. of triflavin (μg/ml)	Specific ^{125}I-fibrinogen binding (ng/10^8 platelets)	Inhibition (%)
0	108.9±22.0	0
0.1	53.3±6.8	51.1±3.7
0.3	43.3±8.6	65.4±7.7
0.5	21.0±8.8	84.6±6.0
0.8	13.4±7.5	91.8±4.3
1.0	10.0±7.0	93.8±3.8

Effect of triflavin on ^{125}I-fibrinogen binding to ADP-activated platelets

As shown in Table 2, triflavin (0.1–1.0 μg/ml) dose-dependently inhibited ^{125}I-fibrinogen binding to ADP (20 μM)-activated platelets. The IC_{50} was around 0.1 μg/ml, which is comparable with the IC_{50} for inhibition of fibrinogen (200 μg/ml)-induced aggregation of ADP (0.4 μg/ml)-activated platelets.

DISCUSSION

By means of gel-filtration, CM-Sephadex C-50 column chromatography and reverse-phase h.p.l.c., a potent platelet-aggregation inhibitor was purified from *Trimeresurus flavoviridis* snake venom. It is a single peptide chain, since its mobility on SDS/PAGE is the same both in the presence and in the absence of 2% 2-mercaptoethanol. The molecular mass estimated by electrophoresis is 7600 Da. It is composed of about 71 amino acid residues, rich in half-cystine, aspartic acid, arginine, glycine and alanine. The *N*-terminal sequence was shown to be Gly-Glu-Glu-Cys-Asp-Cys-Gly-Ser-Pro-Ser-Asn-Pro-Cys-Asp-Ala-Ala-Thr.... Although the action mechanisms of various platelet-aggregation inducers, such as thrombin, collagen, compound U46619, ADP and adrenaline, are different, triflavin inhibited platelet aggregation stimulated by all of them, not only in washed platelet suspension but also in platelet-rich plasma and whole blood. The IC_{50} values for these inducers were about 0.3–0.5 μg/ml (39–66 nM), about 3 times lower than that for trigramin [16,17]. At a concentration of 1.0 μg/ml (130 nM), triflavin showed a maximal effect on platelet aggregation caused by these inducers, implying that it may block a common step shared by these inducers. It also indicates that the site of action of triflavin is not at the receptor level. It is difficult to compare its potency based on the IC_{50} between the preparations of platelet suspension and whole blood, because the former was measured by a turbidimetric method and the latter by the impedance method. However, triflavin was considered not to be inactivated or highly bound to blood cells and plasma proteins other than platelets, because it was active in blocking aggregation of whole blood with a comparable IC_{50}.

Both initial shape change and ATP-release reactions induced by these compounds (i.e. collagen, thrombin, compound U46619 and ADP) were apparently not affected by triflavin (0.1–1.0 μg/ml), even though platelet aggregation was almost completely inhibited at the same concentration. Therefore it is inferred that triflavin did not affect Ca^{2+} release from intracellular Ca^{2+}-storage sites (e.g. dense tubular system or dense bodies) and this is in accord with the concept that intracellular Ca^{2+} release is responsible for the shape-change and ATP-release reactions [26,27]. Recently we have shown that triflavin did not affect the intracellular Ca^{2+} mobilization of Quin-2-loaded platelets stimulated by thrombin (0.1 unit/ml) (results not shown). It suggests that its inhibitory effect on platelet aggregation is not caused by its effect on the release reaction. Fibrinogen plays an important role in mediation of platelet aggregation [28,29]. Exposure of the fibrinogen receptor is believed to be a common step in platelet aggregation induced by several agonists [1–8]. As shown in Fig. 9, more direct evidence comes from the result that triflavin completely inhibited fibrinogen-induced aggregation of elastase-treated platelets and disaggregated platelet plug formation, indicating that triflavin interferes with the interaction between fibrinogen and the exposed fibrinogen receptors on platelet surfaces. Furthermore, triflavin was shown to inhibit ^{125}I-fibrinogen binding to ADP-activated platelets with an IC_{50} comparable with that for inhibition of fibrinogen-induced aggregation of ADP-activated platelets. Therefore we conclude that triflavin, acting probably in the same way as trigramin [16], inhibits platelet aggregation by interfering with the interaction between fibrinogen and its receptors on platelet surfaces. Its IC_{50} for blocking human platelet aggregation was about 3 times lower than that of trigramin (53 nM compared with 130 nM) [16], and therefore its detailed mechanism of action and binding properties towards platelets are explored in order to evaluate its potential as an antithrombotic agent [30].

After the submission of this paper triflavin was shown to be an Arg-Gly-Asp-containing peptide, composed of 70 amino acid residues [30].

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