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Triflavin, an Arg-Gly-Asp-containing peptide, prevents platelet plug formation in in vivo experiments

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Abstract

Triflavin, an Arg-Gly-Asp-containing peptide from *Trimeresurus flavoviridis* snake venom (M_r of 7500 Da) inhibits platelet aggregation through the blockade of fibrinogen binding to activated platelets. The present study demonstrated that the intravenous injection of triflavin (0.1 and 0.25 mg/kg) significantly prolonged the bleeding time about 1.8- to 2.4-fold as compared with control (normal saline) of severed mesenteric arteries in rats, whereas the injection of Gly-Arg-Gly-Asp-Ser (GRGDS) (2–8 mg/kg) failed to increase the bleeding time in this model. Continuous infusion of triflavin (0.08 mg/kg/min) significantly increased the bleeding time about 2.6-fold, and the bleeding time returned to normal within 20 min after the cessation of triflavin infusion. Triflavin (10–20 mg/kg) significantly prolonged the occlusion time of platelet plug formation induced by irradiation of mesenteric venules of fluorescein sodium-pretreated mice. In contrast, trigramin (10–20 mg/kg) and GRGDS (500 and 1000 mg/kg) showed no significant effect. These results suggest that triflavin has an effective antiplatelet effect in vivo and this peptide may be a useful therapeutic agent for arterial thrombosis.

Keywords: Triflavin; RGD (Arg-Gly-Asp)-containing peptide; Fibrinogen receptor antagonist; Bleeding time; Mesenteric artery; Thrombosis

1. Introduction

Adhesion of platelets to extracellular matrices and platelet activation are crucial events in thrombosis and hemostasis. Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. In normal circulation, platelets cannot aggregate by themselves. However, 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 (Bennett and Vilaire, 1979; Bennett et al., 1981; Plow and Marguerie, 1980; Morinelli et al., 1983). The agonist binds to its specific platelet membrane receptor, resulting in the exposure of the cryptic fibrinogen receptor. It is now well established that the binding of fibrinogen to its receptor associated with a Ca^{2+}-dependent glycoprotein IIb/IIIa complex is the common mechanism of platelet aggregation stimulated by these agonists (Phillips et al., 1988). The platelet membrane glycoprotein IIb/IIIa complex, a member of the family of Arg-Gly-Asp (RGD)-binding adhesive protein receptors serves as an activation-dependent receptor for plasma adhesive proteins, i.e., fibrinogen, fibronectin, and von Willebrand factor (Pytela et al., 1986). It is known that the platelet receptor recognition site on human fibrinogen involves the Arg-Gly-Asp sequence in the α (A) chain and dodecapeptide (residues γ 400–411) at the carboxy terminal segment of the γ chain (Gartner and Bennett, 1985; Kloczewiak et al., 1984; Lam et al., 1987). The Arg-Gly-Asp sequence is also present in two other proteins which mediate the platelet-adhesive reaction, i.e., fibronectin and von Willebrand factor (Titani et al., 1980; Doolittle, 1984). Therefore, pep-

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tides containing the Arg-Gly-Asp sequence may partially or fully inhibit fibrinogen binding to its specific receptor associated with the glycoprotein IIb/IIIa complex (Kloczewiak et al., 1984; Ginsberg et al., 1985). On the other hand, monoclonal antibodies directed against the glycoprotein IIb/IIIa complex have been demonstrated to be potent inhibitors of platelet aggregation in vivo (Coller et al., 1986).

Snake venoms affect platelet function in various ways. Some components induce aggregation and release reactions (Seegers and Ouyang, 1979; Ouyang et al., 1982, 1987), whereas some other components inhibit these reactions. We have previously reported that there are three kinds of antiplatelet proteins derived from hemorrhagic snake venoms, including ADPase, α-fibrinogenase (Ouyang and Huang, 1983a, 1986) and trigramin-like peptides, which have been reported to inhibit competitively fibrinogen binding to glycoprotein IIb/IIIa complex on platelet surfaces (Ouyang and Huang, 1983b; Huang et al., 1987, 1989). It has been established that fibrinogen binding to the platelet integrin IIb/IIIa complex is essential for platelet aggregation regardless of the agonists or the diverse signal transduction pathways responsible for platelet activation (Coller et al., 1983).

Recently, many trigramin-like antiplatelet peptides (termed 'disintegrin') have been described (Gould et al., 1990; Huang et al., 1991a,b). Trigramin, a single-chain (M_r ~ 7500), cysteine-rich peptide purified from the venom of the *Trimeresurus gramineus* snake venom contains the Arg-Gly-Asp sequence and is a highly specific fibrinogen receptor antagonist with binding affinity (K_d, 20 nM) for the activated platelet fibrinogen receptor (Huang et al., 1987,1989). These peptides all contain Arg-Gly-Asp, are rich in cysteine, and bind with high affinity to integrin on the surface of platelets and other cells (Gould et al., 1990; Sheu et al., 1992a,b, 1993, 1994; Knudsen et al., 1988). Triflavin, a trigramin-like antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom (Huang et al., 1991c,d) is more potent than trigramin. Its primary structure consists of 70 amino acid residues including 12 cysteines with the Arg-Gly-Asp sequence at position 49–51 (Huang et al., 1991e). Triflavin inhibits human platelet aggregation stimulated by thrombin, collagen, ADP and U46619, not only in washed human platelets but also in platelet-rich plasma and whole blood (Huang et al., 1991c). We previously reported that triflavin inhibited platelet aggregation by interfering with the interaction of fibrinogen with its specific receptor associated with the glycoprotein IIb/IIIa complex (Huang et al., 1991e; Sheu et al., 1992c).

Binding of fibrinogen to the glycoprotein IIb/IIIa complex results in platelet aggregation. Substances that have been found to inhibit this interaction include synthetic peptides, like Arg-Gly-Asp-Ser (Gartner and Bennett, 1985) and the carboxy-terminal dodecapeptide of the fibrinogen γ chain (Kloczewiak et al., 1984), and monoclonal antibody raised against glycoprotein IIb/IIIa complex in mice (Pidard et al., 1983). Infusion of the $F(ab')_2$ fragments of the 7E3, a monoclonal antiglycoprotein IIb/IIIa antibody, can prevent the re-occlusion of coronary arteries after thrombolysis with recombinant tissue plasminogen activator (Yasuda et al., 1988). A monoclonal antiglycoprotein IIb/IIIa antibody has been shown to prolong bleeding time, inhibit platelet aggregation in vivo, and inhibit the deposition of platelets on Dacron vascular grafts in baboons (Hanson et al., 1988; Torem et al., 1988). Weiss et al. (1987) demonstrated that platelet adhesion to the subendothelium of rabbit aorta and thrombus formation were inhibited by two synthetic peptides known to interact with the glycoprotein IIb/IIIa complex.

In this study, we further evaluated the in vivo antiplatelet activity of triflavin in two in vivo models: hemostatic bleeding time in rat mesenteric arteries, and irradiation of the mesenteric microvessels in fluorescein sodium-pretreated mice. Concurrently, we compared the effect of triflavin with that of other Arg-Gly-Asp-containing peptides, trigramin and the synthetic peptide, Gly-Arg-Gly-Asp-Ser (GRGDS). It has been reported that platelet thrombi were induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium (Sato and Ohshima, 1984, 1986), and the platelet thrombi thus obtained were localized to the irradiated region in arteriolar or venular walls. We used these two models to evaluate the in vivo antiplatelet activity of triflavin.

2. Materials and methods

2.1. Materials

T. flavoviridis and *T. gramineus* venoms were purchased from Latoxan, France and a local merchant, respectively, and stored at −20°C. GRGDS was purchased from Peninsula Laboratories, USA. Heparin, indomethacin and fluorescein sodium were purchased from Sigma Chemical Co., St. Louis, MO, USA. Rats (Sprague-Dawley strain) and mice (ICR strain) were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection.

2.2. Purification of triflavin

Triflavin was purified from *T. flavoviridis* venom as previously described (Huang et al., 1991e). In brief, the procedure consisted of Fractogel TSK HW-50 gel filtration, CM-Sephadex C-50 column chromatography and gel filtration on Sephadex G-75 and G-50 columns. The last step of purification was accomplished by re-

verse-phase high performance liquid chromatography (HPLC) on a C18 column. The purified triflavin migrates as a single band and its molecular mass was estimated to be 7500 Da on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20% gel). Trigramin was purified from *Trimeresurus gramineus* venom as described by Huang et al. (1987, 1989).

2.3. Measurement of bleeding time in mesenteric arteries of rats

The bleeding time of severed mesenteric arteries was measured according to a modification of the method of Zawilska et al. (1982). We performed these experiments on rats (150–200 g of body weight) that were maintained on food and water ad libitum before investigation. After the administration of sodium pentobarbital (50 mg/kg i.p), the rats were shaved in preparation for surgery. The trachea was cannulated with PE-100 (polyethylene tubing, Intramedic, Becton Dickinson&Co.) to facilitate spontaneous breathing. Both femoral artery and vein were cannulated with PE-50 tubing to monitor blood pressure and drug administration, respectively. The body temperature was maintained at 37.5°C with a heating pad and monitored with a rectal thermometer. Blood pressure was measured with a pressure transducer (Statham P23D) via a polyethylene cannula placed in the right femoral artery, and data were recorded on a polygraph (Grass model 7B).

The abdomen was opened by a mid-line incision and a portion of the small intestine was brought out to display the mesenteric artery. The mesentery was draped over a plastic plate and exposed tissue was kept moist by continuous superfusion or was rinsed by means of a dropper with warmed normal saline. Experimental solutions were infused into the right femoral vein at 0.2 ml/min for a 10-min period or were given as a bolus injection. An arterial vessel (external diameter 125–200 μm) located at the junction of the small intestinal wall and the mesentery was incised at 4 min after the start of the 10-min infusion or immediately after the bolus injection. Blood was flushed away by the superfusion system. Bleeding was observed through a dissecting microscope ($\times$100), and bleeding time was recorded from the start of incising until the bleeding was arrested by a hemostatic plug formation. Each animal was used as its own control with bleeding time determined during the infusion of both saline and the selected experimental drug. Repeated measurements were made by selecting sequential vessels of the same diameter along the small intestine mesentery. To ensure similar blood flow characteristics for each test, once a vessel had been severed and a plug had formed, it was not used for additional determination of bleeding time. Five rats were evaluated with a normal saline infusion (0.2 ml/min for 10 min) to ensure that repeated measurements did not influence the subsequent bleeding time response. In other experiments, immediately before and at the end of the infusion, blood (1 ml) was collected from the femoral artery of rats and mixed with heparin (0.2 U/ml) for determinations of platelet counts and basic hematological parameters (i.e., red blood cell, white blood cell, platelet, hematocrit, hemoglobin) with an automatic cell counter (Sebia, France).

2.4. Fluorescene dye-induced platelet thrombus in mesenteric microvessels of mice

A method modified from previous reports (Sato and Ohshima, 1984,1986) was used. Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p). After a tracheotomy was performed, an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (i.v. bolus), another tubing was cannulated through the femoral artery for monitoring blood pressure. A segment of the small intestine attached with its mesentery was exteriorized loosely through a midline incision on the abdominal wall and was placed onto a transparent culture dish for microscopic observation. Frequent rinsing of the mesentery with warm saline solution kept at 37 $\pm$ 0.5°C was performed to prevent the mesentery from drying. Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules with diameter 30–40 μm were selected to be irradiated to produce a microthrombus. In the epi-illumination system, the light from a 100-W mercury lamp was excited by a filter (B-2A, Nikon) with a dichromic mirror (DM 510, Nikon, Co., Tokyo, Japan). This filtered light which eliminates wavelengths below 520 nm irradiated a microvessel (the area of the irradiation was about 100 μm in diameter on the focal plane) through an objective lens ($\times$20). The doses of fluorescein sodium used were 0.01 and 0.02 mg/kg. The injected volume of test solution or normal saline (control) was smaller than 50 μl. Five minutes after the administration of dye, the irradiation by filtered light and the video timer were started simultaneously and platelet aggregates were observed on a TV monitor. The time lapse for inducing thrombus formation leading to ceasing of blood flow was measured and the image of the microvascular bed was recorded by a video recorder. The elapsed time for inducing platelet plug formation was measured repeatedly every 5 min on irradiation of venules.

2.5. Statistical analysis of data

Each experiment was repeated several times as indicated (n) using different rats and mice, a mean and

Table 1
Comparison of the effects of triflavin and GRGDS on bleeding time in mesenteric artery of rats

Dose (mg/kg)		Bleeding time (min)	n
Normal saline		3.05 ± 0.25	4
GRGDS	2	3.80 ± 0.42	2
GRGDS	8	4.21 ± 0.47	4
Triflavin	0.05	3.66 ± 0.31	4
	0.1	5.49 ± 0.58 [a]	4
	0.25	7.32 ± 1.30 [a]	4

Values are presented as means ± S.E.M. (n). [a] $P < 0.01$ as compared with the control (normal saline).

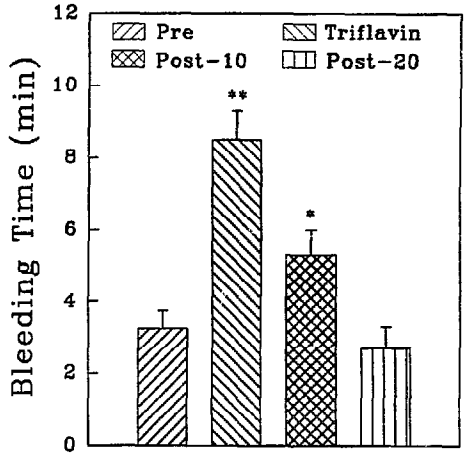

Fig. 1. Effect of continuous infusion of triflavin (0.008 mg/kg/min) for 10 min on bleeding time of rat mesenteric arteries. Pre, bleeding time of rats during infusion of normal saline; Triflavin, bleeding time during continuous infusion of triflavin; Post-10, bleeding time measured 10 min after cessation of triflavin infusion; Post-20, bleeding time measured 20 min after cessation of triflavin infusion. Data are presented as means ± S.E.M. ($n = 5$). * $P < 0.05$; ** $P < 0.01$ as compared with control (normal saline).

standard error mean thus obtained. Mean blood pressure was expressed as [(systolic pressure − diastolic pressure)/3 + diastolic pressure]. Statistical significance was assessed by paired Student's t-test and P values less than 0.05 are considered significant.

3. Results

3.1. Effect of triflavin on bleeding time in mesenteric artery in rats

The reproducibility of the bleeding time was verified in control experiments. In control rats, normal saline was injected into the circulation and the bleeding time measured in mesenteric arteries was about 3.05 ± 0.25 min. Table 1 shows that triflavin administered as bolus to rats markedly increased the bleeding time in a dose-dependent manner. At 0.05 mg/kg, triflavin showed no significant effect on bleeding time. At 0.1 mg/kg, triflavin significantly increased the bleeding time about 1.8-fold as compared with the control (5.49 ± 0.58 vs. 3.05 ± 0.25 min). At 0.25 mg/kg, triflavin exhibited a maximal effect, prolonging bleeding time about 2.4-fold (Table 1); however, the bleeding time did not increase further even at a higher dose of triflavin (0.35 mg/kg) (data not shown). A comparison of the effect of triflavin and the synthetic peptide, GRGDS, on bleeding time is shown in Table 1. This result indicated that triflavin at doses ranging from 0.1 to 0.25 mg/kg caused a significant prolongation of bleeding time, whereas GRGDS was ineffective at the doses of 2–8 mg/kg. The effect of the continuous infusion of triflavin (0.008 mg/kg/min) on the prolongation of bleeding time is shown in Fig. 1. This result demonstrated that the bleeding time of the severed mesenteric artery was prolonged about 2.6-fold (3.25 ± 0.50 vs. 8.52 ± 0.81 min) during the infusion of triflavin, and was also significantly prolonged about 1.6-fold (3.25 ± 0.50 vs. 5.32 ± 0.71 min) within 10 min after the termination of the triflavin infusion but returned to its control value within 20 min after the completion of the triflavin infusion in all experiments.

3.2. Effect of triflavin on blood pressure and hematological parameters in rats

The pressure response to intravenous injection of triflavin in anesthetized rats is depicted in Table 2. The mean arterial pressure of rats (with intact mesenteric blood vessels) was continuously monitored during the infusion of triflavin or the same volume of normal saline. The mean arterial pressure for the control rats was 89.5 ± 13.6 mm Hg. The baseline blood pressure was not significantly altered during the infusion of triflavin (0.01 mg/kg/min). The steady-state and post-triflavin values of the basic systemic and hematological parameters are also presented in Table 2. There are no significant differences in the hematological parameters,

Table 2
Blood pressure and hematological parameters after the 10-min i.v. infusion of normal saline (control) or triflavin (0.01 mg/kg/min) in rats

Parameters	Control	Triflavin (0.01 mg/kg/min)
Mean blood pressure (mm Hg)	89.5 ± 13.6 (3)	82.7 ± 12.8 (3)
White blood cell (10^3/mm^3)	7.2 ± 0.6 (4)	5.3 ± 0.7 (4)
Red blood cell (10^6/mm^3)	6.9 ± 0.7 (4)	7.1 ± 0.9 (4)
Platelet (10^5/mm^3)	9.5 ± 1.2 (4)	6.8 ± 0.8 (4)
Hemoglobin (g/dl)	12.5 ± 1.3 (4)	11.5 ± 1.0 (4)
Hematocrit (%)	43.7 ± 5.8 (4)	40.0 ± 4.6 (4)

Data are presented as means ± S.E.M. (n).

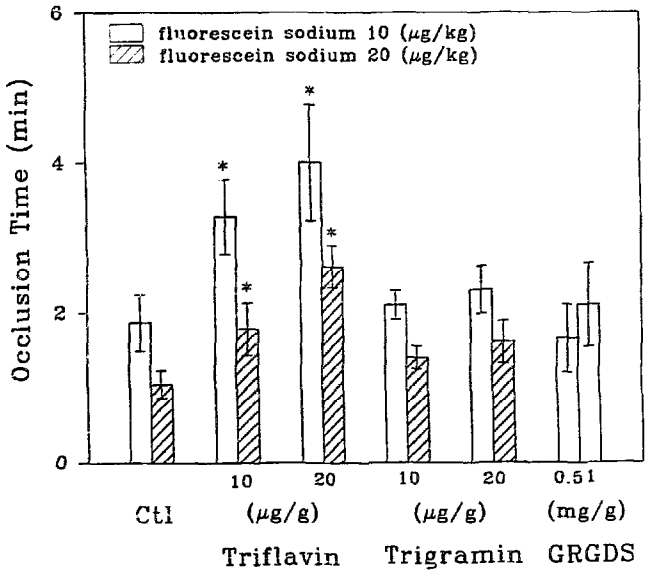

Fig. 2. Effect of triflavin (10 and 20 mg/kg), trigramin (10 and 20 mg/kg) and GRGDS (500 mg/kg and 1000 mg/kg) on the occlusion time for inducing thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (0.01 or 0.02 mg/kg). Data are presented as occlusion time (in min) of platelet plug formation, means ± S.E.M. ($n = 4$).

including hemoglobin, hematocrit, numbers of red blood cells, platelets, and white blood cells, between the control (normal saline treatment) and post-triflavin values.

3.3. Effect of triflavin on platelet plug formation in the microvessels of fluorescein-pretreated mice

The blood flow rate of the irradiated venule was slower than that of the nonirradiated venules, because the platelet plug was becoming apparent upon irradiation of venules. The latent period for inducing platelet plug formation was shortened as the administered dose of fluorescein sodium was increased (Fig. 2). When fluorescein sodium was given at 0.01 mg/kg and 0.02 mg/kg, the occlusion time was 1.9 ± 0.4 and 1.1 ± 0.2 min, respectively. When triflavin was given intravenously at 10 and 20 mg/kg, it significantly prolonged the occlusion time of venules (3.3 ± 0.5, 4.0 ± 0.8 min, for mice receiving 0.01 mg/kg of dye and 1.8 ± 0.4, 2.6 ± 0.3, for mice receiving 0.02 mg/kg of dye), respectively. Triflavin also exhibited its antithrombic effect in arterioles (data not shown). However, as arterioles sometimes showed slight vasoconstriction, venules were chosen for induction of platelet plug formation in this study. On the other hand, the effect of RGD-containing snake venom peptide, trigramin (10 and 20 mg/kg) (Huang et al., 1987,1989) and GRGDS (500 and 1000 mg/kg) was examined at the same time. Trigramin and GRGDS showed no significant effect to prolong the occlusion time (Fig. 2). Heparin (1.5 U/kg) and indomethacin (200 mg/kg) also showed no significant effect in prolonging the occlusion time (data not shown).

4. Discussion

Triflavin, a potent platelet aggregation inhibitor purified from *T. flavoviridis* venom inhibits aggregation of platelets by acting as a specific fibrinogen receptor antagonist. Triflavin contains Arg-Gly-Asp residues at position 49–51 (Huang et al., 1991e). This tripeptide has been demonstrated to mediate the interaction of adhesive proteins, such as fibrinogen, von Willebrand factor, and vitronectin with their respective receptors on the platelet surface membrane (Pierschbacher and Ruoslahti, 1984; Titani et al., 1981; Doolittle, 1984). Our previous studies showed that triflavin interferes competitively with the interaction of fibrinogen binding with its specific receptor associated with the glycoprotein IIb/IIIa complex (Huang et al., 1991e; Sheu et al., 1992c). The binding of ^{125}I-triflavin to human platelets is inhibited by EDTA, monoclonal antibodies raised against the glycoprotein IIb/IIIa complex, and by the synthetic peptide, GRGDS (Huang et al., 1991e). Moreover, triflavin did not significantly bind to thrombasthenic platelets, lacking the glycoprotein IIb/IIIa complex (unpublished observation).

In this study, we demonstrated that triflavin is a potent inhibitor of the formation of hemostatic platelet plugs in rat mesenteric arterioles. The prolongation of bleeding time was seen in all experimental rats receiving triflavin. Animal studies with glycoprotein IIb/IIIa antagonists have shown a prolongation of the bleeding time (Cook et al., 1994). However, based on a careful analysis of bleeding time, it has been recently suggested that a prolongation of the bleeding time in man does not predict the risk of hemorrhage or surgical bleeding, thus calling into question the rationale behind the use in the clinical evaluation of antiplatelet compounds (Rodgers and Levin, 1990; Lind, 1991). Rosenblum and El-Sabban (1977) have previously reported that thrombus formation induced by fluorescein sodium was mainly composed of the activated platelet masses, which showed pseudopods or degranulated zones as observed with the electron microscope. In a fluorescein dye-induced thrombotic platelet plug study, triflavin exhibited a marked antithrombotic effect. It prolonged the occlusion time of thrombus formation induced by irradiation of the mesenteric venules in fluorescein sodium-pretreated mice. In this study, the mesenteric venules of mice were continuously irradiated by filtered light during the experimental period. However, a single incision of the mesenteric arteriole was made for the rat bleeding time measurement. Also, the animal species used are different in hemostatic

(rat) and thrombotic platelet plug (mouse) studies. This may partly explain why triflavin prolonged the occlusion time of thrombus formation in mice at a dose 100-fold higher than that employed for the hemostatic bleeding time in rats. Nevertheless, other mechanisms may be involved in these two experimental models and this remains to be elucidated. Trigramin has been previously demonstrated to significantly prolong the bleeding time of mesenteric artery in hamster (Cook et al., 1989), but in the fluorescein dye-induced thrombotic platelet plug model, trigramin (20 mg/kg) did not significantly prolong the occlusion time (Fig. 2). The different profile of triflavin and trigramin in preventing platelet plug formation induced by fluorescein dye may be due to a higher potency of triflavin (3- to 5-fold). However, the exact mechanism is still unclear and needs further characterization. On the other hand, prostaglandin E_1, a potent inhibitor of platelet aggregation and a powerful vasodilator (Nowak and Wennmalm, 1978), was tested at the same time. However, it (0.04 mg/kg/min i.v. infusion) also showed no significant effect on occlusion time induced by fluorescein dye (data not shown). In this system, the occlusion time is related to blood flow rate, microvessel diameter and dose of fluorescein dye. It has been reported that the kinetics of intravascular thrombus formation are related to blood flow conditions (Sato and Ohshima, 1984). Furthermore, thrombus volume may correlate well with platelet aggregation. However, it is rather difficult to measure accurately the volume of a thrombus, even on the TV screen.

The mechanism of the triflavin inhibition of the formation of a hemostatic and thrombotic plug in experimental animals is probably through the inhibition of fibrinogen and von Willebrand factor binding to the glycoprotein IIb/IIIa complex on the surface of the platelets. However, GRGDS did not show any significant effect in this in vivo model. Although we used a dose of GRGDS much greater than that of triflavin, the bleeding time was not different from the control. We previously reported that Arg-Gly-Asp represents the active site of triflavin (Huang et al., 1991e); in vitro studies, however, showed that triflavin is at least 1000 times more potent to inhibit platelet aggregation and fibrinogen binding to platelets than GRGDS (Huang et al., 1991c,e). Therefore, the negative result with GRGDS in this in vivo model may be related to an insufficient dosage being given or to its non-specific binding property, resulting in the lack of an effective concentration at the local irradiated vessel region. The ex vivo antiplatelet effect of triflavin was rapid in onset, and short in duration in rabbits (Sheu and Huang, 1994) in contrast to the prolonged effect of monoclonal antibodies raised against the glycoprotein IIb/IIIa complex (Coller, 1985), which can prevent thrombus formation in partially stenosed coronary arteries of the dog and prevent re-occlusion of coronary arteries after thrombolysis with tissue plasminogen activator (Coller et al., 1986; Gold et al., 1988; Yasuda et al., 1988). Therefore, our data suggest that the inhibitory effect of triflavin on the hemostatic and thrombotic function of platelets is more transient than that of antiglycoprotein IIb/IIIa monoclonal antibodies. For instance, the antiplatelet effect of 7E3, a monoclonal antibody raised against glycoprotein IIb/IIIa complex in vivo may last for several days (Gold et al., 1988). Similarly, the prolongation of bleeding time in baboons injected with AP_2, an antiglycoprotein IIb/IIIa antibody, has been shown to last for at least 2 days (Hanson et al., 1988). Thus the transient effect of triflavin may result from rapid clearance or from the proteolytic degradation of the active peptide by plasma factors in the circulation. Alternatively, this may offer an advantage for the monitoring of antithrombotic therapy, and may be utilized for the prevention of thrombosis formation, such as in extracorporeal circulation during cardiopulmonary bypass (Musial et al., 1990).

In conclusion, the property of triflavin to inhibit platelet aggregation caused by a wide variety of aggregating agents is advantageous in the treatment of thrombotic disorders which may be caused by different factors in different clinical situations. In this study, we demonstrated that triflavin inhibits the formation of platelet plugs at the site of the irradiated mesenteric venules in mice. Under the same conditions, trigramin and GRGDS, even at much higher doses, appear ineffective. We suggest that triflavin has a novel antithrombotic activity and this peptide may be a useful therapeutic agent for the treatment of arterial thromboembolism.

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References

Bennett, J.S. and G.J. Vilaire, 1979, Exposure of platelet fibrinogen receptors by ADP and epinephrine, J. Clin. Invest. 76, 1790.

Bennett, J.S., G. Vilaire and J.W. Burch, 1981, A role for prostaglandins and thromboxanes in the exposure of platelet fibrinogen receptors, J. Clin. Invest. 68, 981.

Coller, B.S., 1985, A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenviroment of the platelet glycoprotein IIb/IIIa complex, J. Clin. Invest. 76, 1018.

Coller, B.S., E.I. Pearschke, L.E. Scudder and C.A. Sullivan, 1983, A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produce a thrombasthenic-like state in

normal platelets and binds to glycoprotein IIb/IIIa. J. Clin. Invest. 72, 325.

Coller, B.S., J.D. Fokts, L.E. Scudder and S.R. Smith, 1986, Antithrombotic effect of a monoclonal antibody to the platelet glycoprotein IIb/IIIa receptor in an experimental animal model. Blood 68, 783.

Cook, J.J., T.F. Huang, B. Rucinski, M. Strzyzewski, R.F. Tuma, J.A. Williams and S. Niewiarowski, 1989, Inhibition of platelet hemostatic plug formation by trigramin, a nocel RGD-peptide. Am. J. Physiol. 256, H1038.

Cook, N.S., K. Georg and H.-G. Zerwes, 1994, Platelet glycoprotein IIb/IIIa antagonist. Drugs Future 19, 135.

Doolittle, R.F. 1984, Fibrinogen and fibrin. Annu. Rev. Biochem. 53, 195.

Gartner, T.K. and J.S. Bennett, 1985, The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. J. Biol. Chem. 260, 11891.

Ginsberg, M., M.D. Pierschbacher, E. Ruoslahti, G. Marguerie and E.F. Plow, 1985, Inhibition of fibronectin binding to platelets by proteolytic fragments and synthetic peptide which support fibroblast adhesion. J. Biol. Chem. 260, 3931.

Gold, H.K., B.S. Coller, T. Yasuda, T. Saito, J.T. Fallon, J.L. Guerrero, C.R. Leinbach, A.A. Ziskind and D. Collen, 1988, Rapid and sustained coronary artery recanalization with combined bolus injection of recombinant tissue-type plasminogen activator and monoclonal anti-platelet GP IIb/IIIa antibody in a canine preparation. Circulation 77, 670.

Gould, R.J., M.A. Polokoff, P.A. Friedman, T.F. Huang, J.C. Holt, J.J. Cook and S. Niewiarowski, 1990, Disintegrin: a family of integrin inhibitory proteins from viper venom. Proc. Soc. Exp. Biol. Med. 195, 169.

Hanson, S.R., F.J. Pareti, Z.M. Ruggeri, U.M. Marzec, T.J. Kunicki, R.R. Montgomery, T.S. Zimmerman and L.A. Harker, 1988, Effects of monoclonal antibodies against the platelet glycoprotein IIb/IIIa complex on thrombosis and hemostasis in the baboon. J. Clin. Invest. 81, 149.

Huang, T.F., J.C. Holt, H. Lukasiewcz and S. Niewiarowski, 1987, Trigramin, a low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb/IIIa complex. J. Biol. Chem. 262, 16157.

Huang, T.F., J.C. Holt, E.P. Kirby and S. Niewiarowski, 1989, Trigramin: primary structure and its inhibition of von Willebrand factor binding to glycoprotein IIb/IIIa complex on human platelets. Biochemistry 28, 661.

Huang, T.F., W.J. Wang, C.M. Teng and C. Ouyang, 1991a, Mechanism of action of the antiplatelet peptide, arietin, from *Bitis arietans* venom. Biochim. Biophys. Acta 1074, 144.

Huang, T.F., C.Z. Liu, C. Ouyang and C.M. Teng, 1991b, Halysin, an antiplatelet Arg-Gly-Asp-containing snake venom peptide, as fibrinogen receptor antagonist. Biochem. Pharmacol. 42, 1209.

Huang, T.F., J.R. Sheu and C.M. Teng, 1991c, A potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom. Biochem. J. 277, 351.

Huang, T.F., J.R. Sheu and C.M. Teng, 1991d, Mechanism of action of a potent antiplatelet peptide, triflavin from *Trimeresurus flavoviridis* snake venom. Thromb. Haemost. 66, 489.

Huang, T.F., J.R. Sheu, C.M. Teng, S.W. Chen and C.S. Liu, 1991e, Triflavin, an antiplatelet Arg-Gly-Asp-containing peptide, is a specific antagonist of platelet membrane glycoprotein IIb/IIIa complex. J. Biochem. 109, 328.

Kloczewiak, M., S. Timmons, T.J. Lukas and J. Hawiger, 1984, Platelet receptor recognition site on human fibrinogen. Synthesis and structure function relationship of peptides corresponding to the carboxy-terminal segment of the γ chain. Biochemstry 23, 1767.

Knudsen, K., G.P. Tuszynski, T.F. Huang and S. Niewiarowski, 1988 Trigramin, an RGD-containing peptide from snake venom, inhibits cell-substratum adhesion of human melanoma cells. Exp. Cell Res. 179, 42.

Lam, S.C.T., E.F. Plow, M.A. Smith, A. Andrieux, J.J. Ryckwaert, G. Marguerie and M.H. Ginsberg, 1987, Evidence that arginyl-glycyl-asparate peptides and fibrinogen γ chain peptides share a common binding site on platelets. J. Biol. Chem. 262, 947.

Lind, S.E., 1991, The bleeding time does not predict surgical bleeding. Blood 77, 2547.

Morinelli, T.A., S. Niewiarowski, E. Kornecki, W.R. Figures, Y. Wachtfogel and R.W. Colman, 1983, Platelet aggregation and exposure of fibrinogen receptors by prostaglandin endoperoxide analogues. Blood 61, 41.

Musial, J., S. Niewiarowski, B. Rucinski, G.J. Stewart, J.J. Cook and L.H. Edmunds, 1990, Inhibition of platelets adhesion to surfaces of extracorporeal circuits by disintegrins RGD-containing peptides from viper venoms. Circulation 82, 261.

Nowak, J. and A. Wennmalm, 1978, Influence of indomethacin and of prostaglandin E_1 on total and regional blood flow in man. Acta Physiol. Scand. 102, 484.

Ouyang, C. and T.F. Huang, 1983a, Inhibition of platelet aggregation by 5'-nucleotidase purified from *Trimeresurus gramineus* snake venom. Toxicon 21, 491.

Ouyang, C. and T.F. Huang, 1983b, Platelet aggregation inhibitors from *Trimeresurus gramineus* snake venom. Biochim. Biophys. Acta 757, 332.

Ouyang, C. and T.F. Huang, 1986, Platelet aggregation inhibitors from *Agkistorodon acutus* snake venom. Toxicon 24, 1099.

Ouyang, C., C.M. Teng and T.F. Huang, 1982, Characterization of the purified principles of Formosan snake venoms which affect blood coagulation and platelet aggregation. J. Formos. Med. Assoc. 81, 781.

Ouyang, C., C.M. Teng and T.F. Huang, 1987, Characterization of snake venom principles affecting blood coagulation and platelet aggregation. Asia Pac. J. Pharmacol. 2, 169.

Phillips, D.R., I.F. Charo, L.V. Parise and L.A. Fitzgerald, 1988, The platelet membrane glycoprotein IIb/IIIa complex. Blood 71, 831.

Pidard, P., R.R. Montgomery, J.S. Bennett and T.J. Kunicki, 1983, Interaction of AP_2 monoclonal antibody specific for the human platelet glycoprotein IIb/IIIa complex with intact platelets. J. Biol. Chem. 258, 12582.

Pierschbacher, M.D. and E. Ruoslahti, 1984, Cell attachment activity of fibrinogen can be duplicated by small synthetic fragments of the molecule. Nature 309, 30.

Plow, E.F. and G.A. Marguerie, 1980, Induction of the fibrinogen receptor on human platelets by epinephrine and the combination of epinephrine and ADP. J. Biol. Chem. 255, 10971.

Pytela, R., M.D. Pierschbacher, M.H. Ginsberg, E.F. Plow and E. Ruoslahti, 1986, Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science 231, 1559.

Rodgers, R.P.CX. and J. Levin, 1990, A critical reappraisal of the bleeding time. Semin. Thromb. Hemost. 16, 1.

Rosenblum, W.I. and F. El-Sabban, 1977, Platelet aggregation in the cerebral microcirculation: effect of aspirin and other agents. Circ. Res. 40, 320.

Sato, M. and N. Ohshima, 1984, Platelet thrombus induced in vivo by filtered light and fluorescent dye in mesenteric microvessels of the rats. Thromb. Res. 35, 319.

Sato, M. and N. Ohshima, 1986 Hemodynamic at stenoses formed by growing platelet thrombi in mesenteric microvasculature of rat. Microvasc. Res. 31, 66.

Seegers, W.H. and C. Ouyang, 1979, Snake venoms and blood coagulation, in: Snake Venoms, Handbook of Experimental Pharmacology, Vol. 52, ed. C.Y. Lee (Springer-Verlag, Berlin, Heidelberg, New York) p. 684.

Sheu, J.R. and T.F. Huang, 1994, Ex-vivo and in vivo antithrombotic

effect of triflavin, an RGD-containing peptide, J. Pharm. Pharmacol. 46, 58.

Sheu, J.R., C.H. Lin, J.L. Chang, C.M. Teng and T.F. Huang, 1992a, Triflavin, an Arg-Gly-Asp-containing antiplatelet peptide inhibits cell-substratum adhesion and melanoma cell-induced lung colonization, Jpn. J. Cancer Res. 83, 885.

Sheu, J.R., C.H. Lin, J.L. Chang, C.M. Teng and T.H. Huang, 1992b, Triflavin, an Arg-Gly-Asp-containing snake venom peptide, inhibits aggregation of human platelets induced by human hepatoma cell lines, Thromb. Res. 66, 679.

Sheu, J.R., C.M. Teng and T.F. Huang, 1992c, Triflavin, an RGD-containing antiplatelet peptide, binds to GP IIIa of ADP-stimulated platelets, Biochem. Biophys. Res. Commun. 189, 1236.

Sheu, J.R., C.H. Lin, H.C. Peng, C.M. Teng and T.H. Huang, 1993, Triflavin, an Arg-Gly-Asp-containing peptide, inhibits tumor cell-induced platelet aggregation, Jpn. J. Cancer Res. 84, 1062.

Sheu, J.R., C.H. Lin and T.F. Huang, 1994, Triflavin, an antiplatelet peptide inhibits tumor cell-extracellular matrix adhesion via an RGD-dependent mechanism, J. Lab. Clin. Med. 123, 256.

Titani, K., K. Takio, L.H. Ericsson, R.D. Wade, K. Ashida, S.A. Walsh, M.W. Chopek, J.E. Sadler and A. Fujikawa, 1980, Amino acid sequence of human von Willebrand factor, Biochemistry 25, 3171.

Titani, K., K. Takio, L.H. Ericsson, R.D. Wade, K. Ashida, S.A. Walsh, M.W. Chopek, J.E. Sadler and I.A. Fujikawa, 1981, Amino acid sequence of human von Willebrand factor, Biochemistry 25, 3171.

Torem, S., P.A. Schneider and S.R. Hanson, 1988, Monoclonal antibody-induced inhibition of platelet function: effect on hemostasis and vascular graft thrombosis in baboons, J. Vasc. Surg. 7, 172.

Weiss, H.J., J. Hawiger, Z.M. Ruggeri, V.T. Turitto and T. Hoffman, 1987, Evidence that von Willebrand factor, and not fibrinogen, is involved in glycoprotein IIb/IIIa mediated platelet adhesion and thrombus formation in subendothelium, Blood 70 (Suppl. 1), 1312.

Yasuda, T.H., H.K. Gold, J.T. Fallom, R.C. Leinbach, J.L. Guerrero, L.E. Scudder, M. Kanke, D. Shealy, M.J. Ross, D. Collen and B.S. Coller, 1988, A monoclonal antibody against the platelet GP IIb/IIIa receptor prevents coronary artery reocclusion following reperfusion with recombinant tissue type plasminogen activator in dogs, J. Clin. Invest. 81, 1284.

Zawilska, K.M., G.V.R. Born and N.A. Begent, 1982, Effect of ADP-utilizing enzymes on the arterial bleeding time in rats and rabbits, Br. J. Haematol. 50, 317.