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Ex-vivo and In-vivo Antithrombotic Effect of Triflavin, an RGD-containing Peptide

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Abstract—Triflavin, an Arg-Gly-Asp-containing snake venom peptide, inhibits platelet aggregation through the blockade of fibrinogen binding to the activated platelets. It binds to fibrinogen receptors associated with the glycoprotein IIb/IIIa complex with a K_d value of 7×10^{-8} M. In this study, we found that ^{125}I-triflavin reached the maximal binding to human platelets within 5 min at 25°C. In addition, when triflavin was intravenously administered at 1·0 mg kg^{-1} to rabbits, it reversibly impaired the platelet aggregation of platelet-rich plasma caused by ADP (20 μM) ex-vivo over 30 min. The platelet counts of the experimental rabbits remained unchanged. Triflavin was effective in reducing the mortality of ADP-induced acute pulmonary thromboembolism in mice when administered intravenously at a dose of 2 $\mu g\ g^{-1}$. Therefore, triflavin was proven to be an effective antithrombotic agent in preventing ADP-induced acute pulmonary thromboembolism in mice and impairing reversibly the platelet function of rabbits when given intravenously.

Platelet membrane glycoprotein IIb and IIIa form a calcium-dependent heterodimeric complex of 256 kDa in stimulated and unstimulated platelets (Marguerie et al 1979; Shattil et al 1985). When platelets are activated by aggregation agonists such as thrombin, ADP, and adrenaline (Ginsberg et al 1988), platelets initially undergo shape change and subsequently express cryptic fibrinogen receptor on their membrane surface. Binding of fibrinogen to its specific platelet receptor associated with the glycoprotein IIb/IIIa complex appears to be the final common pathway for platelet aggregation. Human fibrinogen composed of three pairs of non-identical polypeptide chains, αA, βB, and r, serves as a substrate for thrombin. It is now well established that the platelet receptor recognition site on human fibrinogen involves the Arg-Gly-Asp sequence in the αA chain and a dodecapeptide of the carboxy-terminal segment of the r chains (Gartner & Bennett 1985; Lam et al 1987). The Arg-Gly-Asp sequence is also present in two other proteins which mediate platelet-adhesive reactions, fibrinogen and von Willebrand factor (Titani et al 1980; Pierschbacher & Ruoslahti 1984). Therefore, peptides containing Arg-Gly-Asp sequence or corresponding to C-terminal residues of the fibrinogen r chain with the sequence His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val can inhibit fibrinogen binding to its specific receptor associated with the glycoprotein IIb/IIIa complex (Kloczewiak et al 1982; Plow et al 1985).

Snake venoms affect platelet function in various ways; some components induce aggregation and release reaction, whereas others inhibit these reactions (Teng & Huang 1991). We have previously reported that there are three kinds of antiplatelet proteins derived from haemorrhagic snake venoms, including ADPase, α-fibrinogenase and trigramin-like peptides, which have been reported to inhibit competitively fibrinogen binding to the glycoprotein IIb/IIIa complex on platelet surfaces (Huang et al 1987, 1989).

Triflavin, a potent platelet aggregation inhibitor purified from venom of *Trimeresurus flavoviridis*, is a polypeptide composed of 70 amino acid residues, containing the Arg-Gly-Asp sequence. 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 1991b). Its IC50 value for inhibiting platelet aggregation is about one-third that of trigramin, an Arg-Gly-Asp-containing peptide from *T. gramineus* venom (Huang et al 1987, 1989). Trigramin is a specific fibrinogen receptor antagonist with a high binding affinity (K_d 10 nM) comparable with those of monoclonal antibodies against the glycoprotein IIb/IIIa complex (Gan et al 1988). Recently, many trigramin-like antiplatelet peptides have been discovered and their physicochemical properties, amino acid sequence, and antithrombotic effects have been revealed (Huang et al 1987, 1989, 1991a, e; Chao et al 1989; Gan et al 1988; Shebuski et al 1989). We previously reported that triflavin acts like trigramin, inhibiting platelet aggregation by interfering with fibrinogen binding to its specific receptor associated with the glycoprotein IIb/IIIa complex on the platelet surface membrane (Huang et al 1991c, d; Sheu et al 1992).

In this study, we have further characterized the kinetic binding properties of triflavin toward platelets, and demonstrated its ex-vivo antiplatelet activity in experimental rabbits and the antithrombotic activity in experimental acute pulmonary thrombosis of mice, revealing the potential use of triflavin as an antithrombotic agent.

Materials and Methods

Purification of triflavin and trigramin

Triflavin and trigramin were purified from venom of *T. flavoviridis* and *T. gramineus*, respectively, as described previously (Huang et al 1987, 1991b). In brief, the procedure for triflavin 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 on a reverse-phase HPLC

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C18 column. The purified triflavin and trigramin migrate as a single band and their molecular weights were estimated to be about the same-7500 Da on SDS-PAGE (20% gel).

Labelling of triflavin

The iodination of triflavin was performed as previously described with the reagent of Bolton & Hunter (1973) (Amersham, sp. act. 2000 Ci mmol^{-1}). The specific activity of [^{125}I]triflavin was 480000 counts min^{-1} μg^{-1}.

Preparation of anti-triflavin polyclonal antibody

Polyclonal antibody was produced as described previously using a multiple intradermal administration method (Hurn & Chantler 1980). In brief, 300 μg triflavin was dissolved in 1 mL physiological saline and an equal volume of Freund's complete adjuvant, and the protein emulsion injected at multiple intradermal sites on rabbit back; booster injection was performed three weeks later. Antibody was prepared by precipitation with Rivanol and ammonium sulphate from serum obtained six weeks after the initial injection. The immunoprecipitability of the polyclonal antibody with triflavin was estimated to be 86%.

Preparation of human washed-platelet suspension

Human washed-platelet suspension was prepared by the method of Mustard et al (1972) and Kornecki et al (1981). Blood was collected from healthy volunteers who had not taken any drugs within the previous two weeks. Blood was mixed with acid citrate dextrose (9:1, v/v), then centrifuged for 10 min at 120 *g* at room temperature (25°C). The supernatant (platelet-rich plasma) was supplemented with prostaglandin E_1 (PGE_1) (0·5 μM) and heparin (6·4 units mL^{-1}), incubated for 10 min at 37°C and centrifuged at 500 *g* for 10 min. After discarding the supernatant, the platelet pellets were suspended in 5 mL Tyrode solution (in mM: NaCl 11·9, KCl 2·7, $MgCl_2$ 2·1, NaH_2PO_4 0·4, $NaHCO_3$ 11·9, glucose 11·1, pH 7·5), then apyrase (1 unit mL^{-1}), PGE_1 (0·5 μM), and heparin (6·4 units mL^{-1}) were added and the whole was incubated for 10 min at 37°C. Following centrifugation of the suspension at 500 *g* for 10 min, the washing procedure was repeated. The washed platelets were finally suspended in Tyrode solution containing bovine serum albumin (3·5 mg mL^{-1}) and adjusted to about $4·5 \times 10^8$ platelets mL^{-1}.

Binding of ^{125}I-triflavin to platelets

The experiments were performed by the previously described method (Niewiarowski et al 1981) with slight modification. In brief, 400 μL human washed-platelet suspension ($4·5 \times 10^8$ cells mL^{-1}) was preincubated with 10 μL ^{125}I-triflavin following addition of ADP (20 μM), then 10 mM EDTA or anti-triflavin antibody (150 μg mL^{-1}) was added at the indicated time intervals to inhibit ^{125}I-triflavin binding. This mixture was gently shaken and incubated for another 5 min at 25°C before centrifugation. Four hundred microlitres of platelet suspension mixture was overlayed on sucrose solution (15%, w/v) and centrifuged for 10 min at 14000 rev min^{-1} and 25°C (Eppendorf centrifuge). The radioactivities of the cut-off tips containing the platelet pellet and the supernatant were measured by using a γ-counter (LKB).

Ex-vivo platelet aggregability of platelet-rich plasma from rabbits

Triflavin (1·0 mg kg^{-1}) was administered to rabbits (New Zealand White) as a single intravenous bolus and blood samples (5 mL) were drawn through marginal ear veins and collected into plastic tubes containing 3·8% sodium citrate (9:1) before and 5, 10, 15, 20, 25, 30, 40 and 60 min after triflavin administration. Platelet-rich plasma was prepared by rapid centrifugation of blood at 120 *g* for 9 min at 25°C. Platelet aggregation was measured with a Lumi-Aggregometer (Chrono-Log) by a turbidimetric method (Born & Gross 1963). Results are expressed as percent aggregation of platelet-rich plasma caused by ADP (20 μM), compared with that of control (before the administration of triflavin).

Effect of triflavin on ADP-induced acute pulmonary thrombosis in mice

Acute pulmonary thromboembolism was induced according to the previously described method (Nordoy & Chandler 1964). ICR strain mice, 20–25 g, were used. Various doses of triflavin and trigramin or saline (all in 50 μL) were administered by injection into the tail vein. Four minutes later, ADP (0·6 mg g^{-1}) was injected into a different vein in the tail. The mortality of mice in each group after injection was determined within 10 min.

Materials

T. flavoviridis and *T. gramineus* snake venoms were purchased from Latoxan, Rosans, France, and from a local merchant, respectively, and stored at −20°C. ADP, PGE_1, apyrase, heparin, and EDTA were obtained from Sigma Chemical Co. (St Louis, MO).

Results

Kinetic binding property of [^{125}I]triflavin toward platelets

In our previous report (Huang et al 1991d), ^{125}I-triflavin was shown to bind to human platelets in a specific and saturable manner. This binding was blocked by EDTA (>96%),

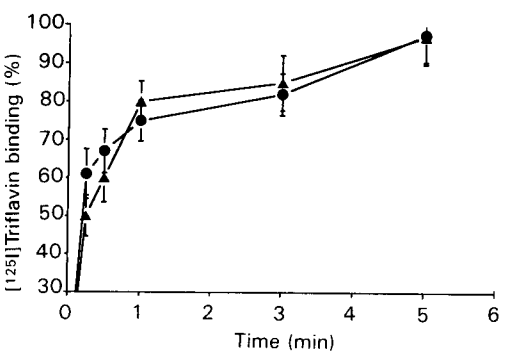

FIG. 1. Inhibition of [^{125}I]triflavin binding to platelets by EDTA or anti-triflavin antibody. Human platelet suspension ($4·5 \times 10^8$ cells mL^{-1}) was preincubated with [^{125}I]triflavin followed immediately by addition of ADP (20 μM), then 10 mM EDTA (▲) or anti-triflavin antibody (150 μg mL^{-1}; ●) was added at the indicated time intervals to inhibit [^{125}I]triflavin binding. This mixture was further incubated at 25°C for 5 min before centrifugation. The amount of [^{125}I]triflavin bound to the platelet pellet was measured after centrifugation for 10 min. In the absence of EDTA, the radioactivity of [^{125}I]triflavin binding to platelets 5 min after addition of ADP (20 μM) was taken as control (100%). Data are presented as percentage of control as mean ±s.e.m. (n = 5).

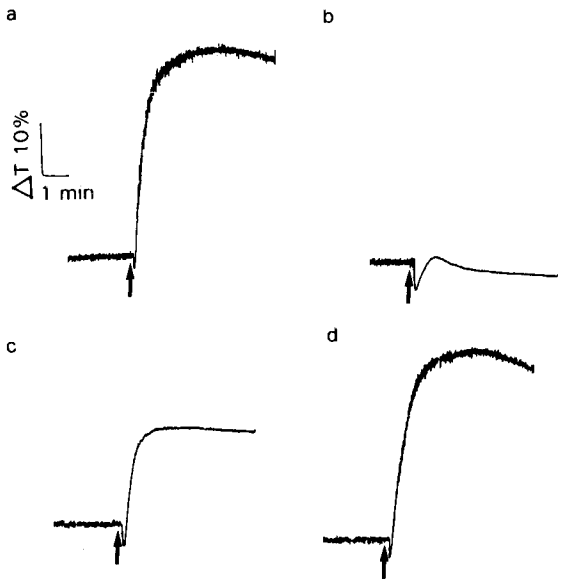

FIG. 2. Inhibition of ADP (20 μM)-induced platelet aggregation before (a), and 5 (b), 20 (c) and 30 min (d) after intravenous administration of triflavin (1·0 mg kg^{-1}) to rabbits.

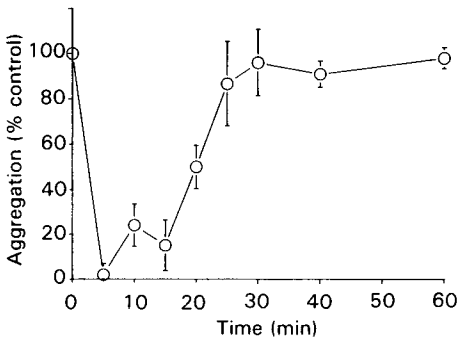

FIG. 3. Inhibition of platelet aggregation (ex-vivo) after intravenous administration of triflavin to rabbits. Results are expressed as percent aggregation caused by ADP (20 μM), compared with that of control (before the administration of triflavin). Data are presented as mean ± s.e.m.

indicating that the binding activity, like that of fibrinogen, is divalent cation-dependent. In this study, we found the binding of ^{125}I-triflavin to platelets was time-dependent at 25°C. After 15 s incubation, the amounts of [^{125}I]triflavin bound to platelets corresponded to 50% of the maximal amount of triflavin bound at 5 min incubation under optimal conditions (Fig. 1). However, if 10 mM EDTA or anti-triflavin polyclonal antibody was added 5 min after addition of [^{125}I]triflavin, EDTA and anti-triflavin polyclonal antibody did not inhibit [^{125}I]triflavin binding to platelets. This result suggested that the binding of triflavin reached a plateau after 5 min incubation, and the binding was reversible only during the initial incubation period; after a longer incubation time (≥5 min), triflavin became irreversibly bound to platelets.

Ex-vivo platelet aggregability of experimental rabbits

Triflavin was administered as a single bolus intravenous injection (1·0 mg kg^{-1}) and platelet aggregation of platelet-rich plasma obtained at various time intervals measured (Figs 2, 3). ADP (20 μM)-induced aggregation was completely impaired within 5 min of triflavin administration, and platelet aggregability was inhibited by 50% 20 min after injection, returning to control values within 30 min. Platelet count remained constant throughout the experiment. This result demonstrated that triflavin inhibits platelet aggregation both in-vitro (Huang et al 1991b) and ex-vivo.

Table 1. Dose-response of trigramin and triflavin on the mortality of acute pulmonary thrombosis caused by intravenous injection of ADP in experimental mice.

Control	Death number (total number)
ADP 0·6 mg g^{-1}	43 (50)
ADP (0·6 mg g^{-1}) + trigramin	
6 μg g^{-1}	7 (12)
8 μg g^{-1}	5 (12)
12 μg g^{-1}	2 (8)
ADP (0·6 mg g^{-1}) + triflavin	
2 μg g^{-1}	4 (12)
3 μg g^{-1}	3 (13)

Effect of triflavin on ADP-induced acute pulmonary thrombosis in mice

From the results summarized in Table 1, we compared the effect of triflavin with trigramin in preventing acute pulmonary embolism death in mice. Triflavin and trigramin significantly lowered the mortality of mice challenged with ADP in a dose-related manner. Trigramin reduced the mortality from 86% (control) to 25% when administered at 12 μg g^{-1}; triflavin exhibited a similar effect (86 vs 23%) when administered at 3 μg mL^{-1}. Thus, compared on a molar basis, triflavin (mol. wt 7573) was more potent than trigramin (mol. wt 7507) in reducing mortality in this model. On the other hand, neither aspirin nor indomethacin had a protective effect at 200 μg g^{-1} (data not shown).

Discussion

In previous studies, we reported that triflavin competitively interferes with the interaction of fibrinogen binding with its specific receptor associated with the glycoprotein IIb/IIIa complex (Huang et al 1991d; Sheu et al 1992). The binding of [^{125}I]triflavin to human platelets was inhibited by EDTA, monoclonal antibodies raised against the glycoprotein IIb/IIIa complex, and by synthetic peptide GRGDS (Huang et al 1991d). However, the binding sites and dissociation constant (K_d) of [^{125}I]triflavin towards unactivated- and activated-platelets showed no significant difference (Huang et al 1991d). Moreover, triflavin did not significantly bind to platelets of patients with Glanzmann's thrombasthenia, deficient in the glycoprotein IIb/IIIa complex (data not shown). In this study, after a prolonged incubation period of triflavin with platelets (>5 min), its binding becomes irreversible. We found the ex-vivo antiplatelet effect of

triflavin is rapid in onset, short in duration and fully reversible (< 30 min), in contrast to the prolonged effect of other agents, such as aspirin (Harker & Gent 1987) or monoclonal antibodies raised against glycoprotein IIb/IIIa (Coller 1985). This transient effect may result from rapid clearance or the proteolytic degradation of the active peptide by plasma factors in the circulation. This may offer an advantage for the monitoring of antithrombotic therapy, and it may be advantageously utilized for the prevention of thrombosis formation, such as in extracorporeal circulation during cardiopulmonary bypass. After rapid injection with ADP, the sudden death caused by a pulmonary thromboembolism was associated with occlusion of the pulmonary microcirculation with platelet aggregates (Nordoy & Chandler 1964). Since platelet aggregation was intimately involved in this experimental thrombosis, Arg-Gly-Asp-containing antiplatelet peptides, trigramin and triflavin, were effective in preventing the ADP-induced thromboembolic death as expected, since trigramin and triflavin inhibit platelet aggregation through the blockade of fibrinogen to its receptor associated with the glycoprotein IIb/IIIa complex of the activated platelets. On the other hand, aspirin and indomethacin did not significantly reduce the mortality caused by ADP. Wang et al (1985) also reported that aspirin was ineffective against ADP-induced thromboembolism. It may be that aspirin only blocks thromboxane-related reactions whereas triflavin blocks the common step of platelet aggregation, i.e. fibrinogen binding to platelets. Moreover, it was observed that triflavin was more potent than trigramin in this model. Plow et al (1987) have reported that synthetic peptide Arg-Gly-Asp-Phe was 4- to 5-fold as potent as Arg-Gly-Asp-Ser in inhibiting fibrinogen binding. Triflavin (containing Arg-Gly-Asp-Phe) was 3-fold as potent as trigramin (containing Arg-Gly-Asp-Asp) in inhibiting platelet aggregation (Huang et al 1991d). Therefore, this in-vivo effect was correlated with the potency of the antiplatelet activity in-vitro. Since the binding of triflavin reached a plateau 5 min after the addition of [^{125}I]triflavin, we treated the mice 4 min before ADP injection in the pulmonary thromboembolic study.

In conclusion, the unique property of triflavin in inhibiting 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, triflavin exhibits antiplatelet and antithrombotic effect ex-vivo and in-vivo in the experimental models. Recently, we also found that triflavin was also effective in prolonging the lag-time period of inducing platelet plug formation of mouse mesenteric venules in-vivo caused by sodium fluoresceine administration and irradiation at 510 nm wavelength (unpublished data). From the above results, we suggest that triflavin, the Arg-Gly-Asp-containing snake venom peptide, may be an ideal antithrombotic agent in certain clinical situations.

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