Tokaracetin, a new platelet antagonist that binds to platelet glycoprotein Ib and inhibits von Willebrand factor-dependent shear-induced platelet aggregation

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A new platelet antagonist, tokaracetin, was isolated from the venom of *Trimeresurus tokarensis* by ion-exchange chromatography, heparin–Sepharose chromatography and hydrophobic HPLC. The purified protein showed an apparent molecular mass on SDS/PAGE of 28.9 kDa under non-reducing conditions. On reduction, 16.1 and 15.4 kDa subunits were observed, suggesting that the molecule is a heterodimer. Tokaracetin inhibited the binding of ^{125}I-labelled bovine von Willebrand factor (vWF) and ^{125}I-labelled human vWF in the presence of botrocetin to fixed human platelets. It did not block ADP-, collagen- or thrombin receptor agonist peptide-induced platelet aggregation in human platelet-rich plasma (PRP), or induce platelet agglutination in PRP. On reduction, tokaracetin lost its inhibitory activity on the agglutination of fixed human platelets by bovine vWF. ^{125}I-Tokaracetin specifically bound to washed human platelets with high affinity (K_d 3.9 ± 1.4 nM) at 47440 ± 2780 binding sites per platelet. Binding of tokaracetin to fixed human platelets was reversible, and was inhibited by monoclonal antibody GUR83-35, which is directed against the N-terminal vWF-binding domain of human glycoprotein Ib (GPIb). Tokaracetin completely inhibited vWF-dependent shear-induced platelet aggregation in PRP at 3 μg/ml. The N-terminal amino acid sequences of tokaracetin subunits showed a high degree of identity with those of alboaggregin-B. These results suggest that this new platelet antagonist may be a useful tool in the development of specific inhibitors of the vWF–GPIb interaction.

INTRODUCTION

The interaction between platelet glycoprotein Ib (GPIb) and von Willebrand factor (vWF) plays a key role in the initial contact adhesion of platelets to exposed subendothelium [1]. The importance of vWF binding to GPIb has been demonstrated in studies in patients with Bernard–Soulier syndrome, who lack GPIb–IX complex [2], and with von Willebrand disease [3], and confirmed using monoclonal antibodies against GPIb [4] and vWF [5]. Since platelet GPIb does not normally bind to intact vWF, some abnormal circumstance(s), such as high shear stress caused by atherosclerotic stenosis, is believed to be necessary before the interaction of GPIb with vWF can occur. Binding of human vWF to platelets *in vitro* occurs in the presence of exogenous agonists such as the antibiotic ristocetin [6] and the snake venom botrocetin [7], whereas bovine vWF induces human platelet agglutination directly [8]. Bovine and human vWF are thought to bind to the same region on platelet GPIb [9]. vWF-mediated platelet adhesion and aggregation are likely to be involved in arterial thrombus formation. Because human platelets are aggregated in the high-shear field by large human plasma vWF multimers without the addition of exogenous agonists, high-shear-stress-induced platelet aggregation (h-SIPA) may be a model of *in vivo* arterial thrombosis [10]. It follows that substances which specifically inhibit the binding of vWF to platelet GPIb and h-SIPA may interrupt the formation of thromboses.

Peng et al. [11,12] have isolated a new protein, alboaggregin-B (AL-B), from the venom of *Trimeresurus albolabris* and characterized its function. This protein binds directly to GPIb and inhibits the binding of bovine vWF to GPIb. However, it also induces the agglutination of fixed human platelets without the need for any cofactor. These authors later reported that echicetin, purified from the venom of *Echis carinatus*, inhibits the binding of AL-B and vWF to GPIb without inducing platelet agglutination [13]. However, the effects of echicetin on h-SIPA and its N-terminal amino acid sequence have not been reported.

In the present study we have characterized a new protein, tokaracetin, which specifically and reversibly binds to a site on platelet GPIb close to or identical with the site for vWF binding. We also determined the N-terminal amino acid sequence of this new platelet antagonist and investigated its effect on h-SIPA.

MATERIALS AND METHODS

Materials

Trimeresurus tokarensis venom was prepared as follows: the mouth of the snake was held down on a cone cup for collection of crude venom; extracted venoms were diluted with distilled water, and the supernatants were freeze-dried after centrifugation. Ristocetin, ADP and equine collagen (type I) were purchased from Cosmo-Bio (Tokyo, Japan). Thrombin receptor agonist peptide (TRAP) with the sequence SFLLRN was pur-

Abbreviations used: AL-B, alboaggregin-B; DTT, dithiothreitol; GPIb, glycoprotein Ib; PRP, platelet-rich plasma; h-SIPA, high-shear-stress-induced platelet aggregation; IAA, iodoacetamide; I-SIPA, low-shear-stress-induced platelet aggregation; PE, pyridylethyl; RP-HPLC, reversed-phase HPLC; TRAP, thrombin receptor agonist peptide; vWF, von Willebrand factor.

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chased from Peptide Institute (Osaka, Japan). Two-chain botrocetin was purified from *Bothrops jararaca* venom (Sigma) [7], while human and bovine vWF were obtained from cryoprecipitates of human and bovine plasma respectively, as described previously [14,15]. AL-B was purified from *Trimeresurus albolabris* venom (Sigma) as described previously [16]. SP-Sepharose Fast Flow and heparin–Sepharose CL-6B were from Pharmacia-LKB (Uppsala, Sweden). TSKgel Phenyl-5PW column for HPLC was from Tosoh (Tokyo, Japan). Dithiothreitol (DTT), iodoacetamide (IAA) and other chemicals were purchased from Sigma and Wako Pure Chemical (Osaka, Japan).

Determination of protein concentration

Protein concentration was determined by a photometric method using a dye binding assay kit (Bio-Rad) and BSA as standard.

Preparation of platelet-rich plasma (PRP), washed platelets and formalin-fixed platelets

PRP was obtained from fresh human blood collected into a one-tenth volume of 3.8% sodium citrate by centrifugation at 200 *g* for 15 min at room temperature. Washed platelets were prepared according to the method of Katagiri et al. [17]. Fixed human platelets were prepared according to MacFarlane et al. [18] and suspended in Tyrode's solution (pH 7.35).

Platelet aggregation assay

Bovine vWF-mediated and ristocetin- or botrocetin-induced human vWF-mediated aggregation of fixed human platelets were studied using an aggregometer (NBS Hematracer 801; Niko Bioscience, Tokyo, Japan) at a final platelet count of 3×10^8/ml. Ristocetin- or botrocetin-induced, as well as ADP-, collagen- or TRAP-induced, platelet aggregation in PRP was also investigated using the same method. Inhibitory activity was calculated by dividing the maximum rate of decrease in absorbance of a mixture containing the test sample by the maximum rate obtained in the presence of buffer alone. Three separate measurements were performed.

Platelet agglutination

Agglutination of PRP without any cofactor was assayed at 37 °C using an aggregometer, and the extent of light transmission change was measured for 5 min. Three separate measurements were performed.

Purification of tokaracetin

Lyophilized *Trimeresurus tokarensis* venom (2 g) was dissolved in 200 ml of 0.05 M Tris/HCl (pH 7.5) containing 0.05% NaN_3 (buffer A). After centrifugation at 2500 *g* for 30 min, the supernatant was applied to an SP-Sepharose Fast Flow column (1.6 cm × 20 cm) equilibrated with buffer A, and eluted with a linear gradient of NaCl (0–0.4 M) in buffer A over 400 min at a flow rate of 1 ml/min. Fractions were assayed for their inhibitory activity on bovine vWF-induced aggregation of fixed platelets. Active fractions were concentrated by ultrafiltration using an Amicon stir cell with a YM10 membrane, and dialysed against 0.1 M Tris/HCl buffer (pH 7.4) containing 0.05% NaN_3 (buffer B). The retained material was loaded on to a heparin–Sepharose CL-6B column (2.6 cm × 20 cm) equilibrated with buffer B, and eluted with a linear gradient of NaCl (0–0.4 M) in buffer B over 700 min at a flow rate of 1 ml/min. Active fractions were pooled and concentrated by dialysis against 0.05 M sodium acetate buffer (pH 6.0) containing 1.5 M ammonium sulphate (buffer C). The dialysate was applied to a TSKgel Phenyl-5PW column (0.75 cm × 7.5 cm) equilibrated with buffer C, and eluted with the same buffer at a flow rate of 1 ml/min. Fractions thereby obtained were pooled, and purified tokaracetin was extensively dialysed against 0.05 M Hepes/0.15 M NaCl buffer (pH 7.4) and stored at −70 °C.

SDS/PAGE of tokaracetin

The apparent molecular mass of tokaracetin was determined by SDS/16%-PAGE under reducing and non-reducing conditions as described previously [19]. The gels were run using a Bio-Rad minigel system and stained with Coomassie Blue R250.

Reduction and S-alkylation

Tokaracetin was reduced in the presence of various concentrations of DTT and then alkylated with an excess of IAA. Reduction was confirmed by SDS/PAGE. The inhibitory effect of reduced tokaracetin on bovine vWF-induced platelet agglutination of fixed platelets was examined.

Iodination of proteins

Purified human and bovine vWF and tokaracetin were labelled with $Na^{125}I$ (Amersham International, Amersham, Bucks., U.K.) by the Iodo-Gen method [20]. Specific radioactivities were between 2 and 3.4 μCi/μg.

Binding of ^{125}I-labelled human vWF in the presence of botrocetin and of ^{125}I-labelled bovine vWF to fixed human platelets

The inhibitory effect of tokaracetin on the binding of ^{125}I-(human vWF) in the presence of botrocetin and ^{125}I-(bovine vWF) to fixed human platelets was determined as described previously [5]. Non-specific binding was estimated under identical conditions in the presence of an excess of unlabelled human or bovine vWF. All measurements were performed in duplicate.

Binding of ^{125}I-tokaracetin to platelets and other blood cells

Binding of tokaracetin to washed platelets

Binding of ^{125}I-tokaracetin to washed platelets was measured in duplicate as described above. The final concentration of platelets was 2.5×10^7/ml. Non-specific binding was estimated in the presence of an excess of unlabelled tokaracetin. Three separate measurements were performed for Scatchard plot analysis.

Competition between tokaracetin and bovine vWF for binding to fixed human platelets

To determine whether the binding of tokaracetin and bovine vWF to platelets is competitive, either ^{125}I-tokaracetin (0.5 μg/ml) or ^{125}I-(bovine vWF) (0.5 μg/ml) was mixed with various concentrations of unlabelled proteins. At 30 min after incubation, mixtures were centrifuged to determine the amount of radioactivity bound to platelets. Non-specific binding was measured in the presence of an excess of unlabelled protein (60 μg/ml). All measurements were performed in duplicate.

Reversibility of binding of tokaracetin to platelets

Fixed platelets (1×10^8/ml) were incubated with ^{125}I-tokaracetin for different periods of time. Unlabelled tokaracetin was added after 5 min of incubation with ^{125}I-tokaracetin.

Specificity of binding of tokaracetin to platelets

Blood after the collection of PRP was placed on MONO-POLY resolving medium (ICN Biomedicals, Asse-Relegem, Belgium) and centrifuged at 400 *g* for 30 min, and human erythrocytes and neutrophils were isolated. The erythrocytes were used as a 3% suspension in Tyrode's buffer. The concentration of neutrophils and platelets was 2.6×10^7/ml and 1.0×10^8/ml respectively. Binding of ^{125}I-tokaracetin to erythrocytes, neutrophils and platelets was compared in the presence and absence of an excess of unlabelled tokaracetin.

Effect of monoclonal antibody on the binding of ^{125}I-tokaracetin to platelets

The inhibitory effect of an anti-GPIb monoclonal antibody on the binding of ^{125}I-tokaracetin to fixed human platelets was studied. GUR83-35 is a mouse monoclonal antibody (IgG1) raised against a crude fraction of sonicated human washed platelets in an aqueous solution which bound to wheat-germ-agglutinin-linked affinity gels (Sigma) as described previously [21,22]. Initial screening of hybridomas was performed by an enzyme-linked immunoassay using 96-well microtitration plates to which washed human platelets had been fixed. Purified IgG was prepared from mouse ascitic fluid using a Protein A-linked Sepharose CL-4B gel (Sigma). The antibody completely inhibits ristocetin-induced platelet aggregation in PRP at concentrations around 2–5 μg/ml. It also inhibits ristocetin- or botrocetin-induced binding of human vWF to fixed human platelets with an IC_{50} value of 3 μg/ml. The epitope of the antibody has been localized to the N-terminal 45 kDa peptide of the GPIbα chain by the method reported previously [23]. GUR83-35 at various concentrations (0.3–30 μg/ml) was incubated with fixed platelets (1×10^8/ml) prior to mixing with ^{125}I-tokaracetin (0.5 μg/ml). The binding of ^{125}I-tokaracetin was compared in the presence and absence of the antibody.

Effect of tokaracetin on SIPA in PRP

The inhibitory effect of tokaracetin on SIPA was measured as described previously [10]. A 40 μl sample of tokaracetin at various concentratious was added to 360 μl of PRP (3×10^8/ml). A shear stress gradient [6×10^{-5} to 1.08×10^{-3} N/cm^2 (6–108 dyn/cm^2)] was then applied to the PRP mixture. After exposure to 6×10^5 N/cm^2 for an initial 15 s, shear stress was increased to 1.2×10^{-4} N/cm^2 over the next 90 s period (low shear stress). Stress was then increased linearly from 1.2×10^{-4} to 1.08×10^{-3} N/cm^2 over the following 120 s, and finally kept constant at 1.08×10^{-3} N/cm^2 for the last 125 s (high shear stress). Aggregation was monitored continuously by recording the intensity of the light transmitted through the platelet suspension from the beginning of application of the shear forces. Inhibitory activity was calculated by dividing the maximum rate of decrease in absorbance of a mixture containing the test sample by the maximum rate obtained in the presence of buffer. PRPs from three volunteers were used separately in this study.

Effects of tokaracetin on the platelet count in mice *in vivo*

Male ICR mice weighing 20–25 g (Japan SLC Inc., Hamamatsu, Japan) were anaesthetized with diethyl ether. A solution of saline or various concentrations of tokaracetin in a total volume of 0.2 ml was injected intravenously through a lateral tail vein. At 5 min after injection of tokaracetin, blood (0.5 ml) was obtained from the abdominal vena cava and collected in a sample cup with EDTA-2K. Haematological parameters (red blood cells, white blood cells, platelets, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration) were counted using an automatic cell counter (MEK-5158; Nihon Kohden, Tokyo, Japan).

Sequence determination

A 300 μg sample of purified tokaracetin was reduced with 20 μl (81 mM) of tri-n-butylphosphine [24] and then S-pyridylethylated with 10 μl (94 mM) of 4-vinylpyridine [25] in 1 ml of 0.3 M Tris buffer (pH 8.3) containing 6 M guanidine hydrochloride at room temperature for 3 h. The S-pyridylethylated α (PE-α) and β (PE-β) subunits were separated by reversed-phase (RP) HPLC on a SynChropak RP-8 column (4.1 mm × 250 mm; SynChrome) as described previously [26]. N-terminal amino acid sequencing of the PE-α and PE-β subunits was performed using an Applied Biosystems Model 470A protein sequencer connected on-line to a Model 120A PTH analyser.

RESULTS

Purification of tokaracetin

Chromatography of *Trimeresurus tokarensis* venom was performed first on an SP-Sepharose Fast Flow column. Three major

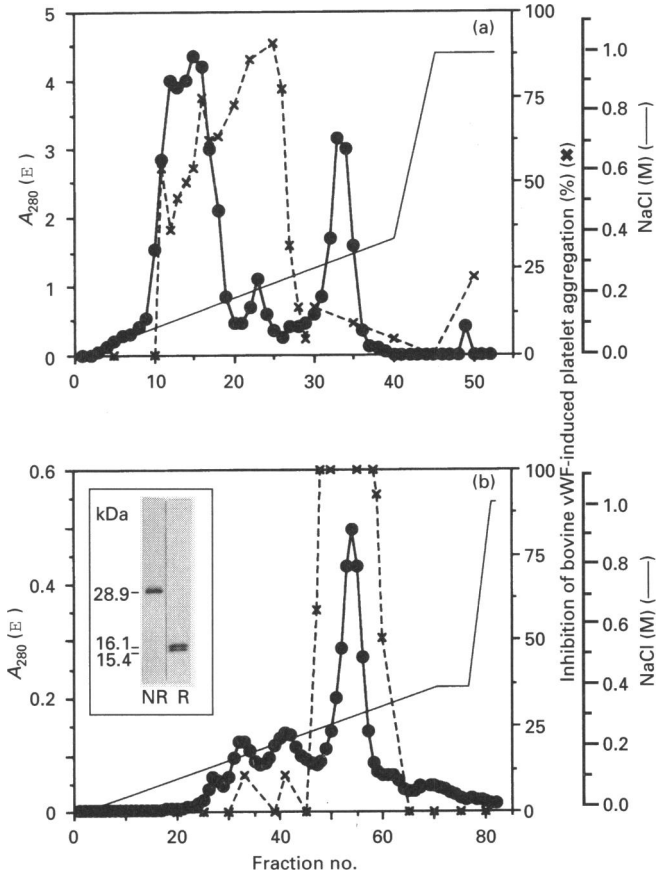

Figure 1 Purification of tokaracetin

(**a**) Chromatography on an SP-Sepharose FF column. Elution was performed with a linear gradient of NaCl (0–0.4 M) over 400 min in 0.05 M Tris buffer (pH 7.5). (**b**) Chromatography on a heparin–Sepharose CL-6B column. Elution was performed with a linear gradient of NaCl (0–0.4 M) over 700 min in 0.1 M Tris buffer (pH 7.4). Fractions were assayed for their inhibitory activity on bovine vWF-induced aggregation of fixed human platelets. Inset: SDS/PAGE of purified tokaracetin under non-reduced (NR) and reduced (R) conditions.

Table 1 Effect of tokaracetin on platelet aggregation

The IC_{50} value is the concentration necessary to inhibit total platelet aggregation to 50% of the control value.

Inducer	IC_{50} (μg/ml)	
	Fixed platelets	PRP
Ristocetin (1.5 mg/ml)	0.25	0.50
Botrocetin (3 μg/ml)	0.48	0.30
Bovine vWF (3 μg/ml)	0.58	
ADP (10 μM)		> 30
Collagen (5 μg/ml)		> 30
TRAP (50 μM)		> 30

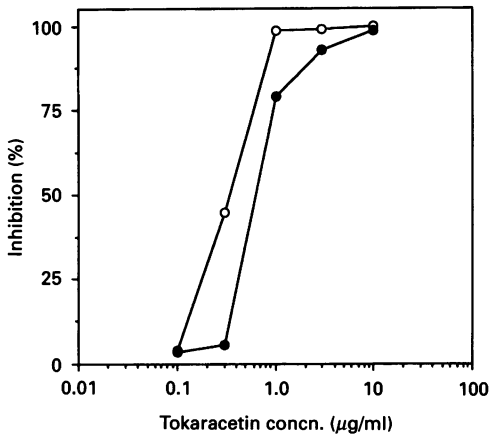

Figure 2 Effect of tokaracetin on botrocetin-induced ^{125}I-(human vWF) (●) and ^{125}I-(bovine vWF) (○) binding to fixed human platelets

At 30 min after incubation of ^{125}I-(human vWF) (0.87 μg/ml) in the presence of botrocetin (10 μg/ml), or of ^{125}I-(bovine vWF) (1.2 μg/ml), with various concentrations of tokaracetin and fixed human platelets (1×10^8 platelets/ml) in Eppendorf tubes, specific binding was measured. Platelet-bound and free vWF were separated by centrifuging the platelets through a 20% sucrose layer, and platelet-bound radioactivity was measured in a γ-counter. Non-specific binding was estimated in the presence of a 50-fold excess of unlabelled human or bovine vWF. Specific binding was obtained after subtraction of non-specific binding from the total. Results are represented as percentage inhibition compared with control measurements using buffer alone. Points are averages of duplicate determinations.

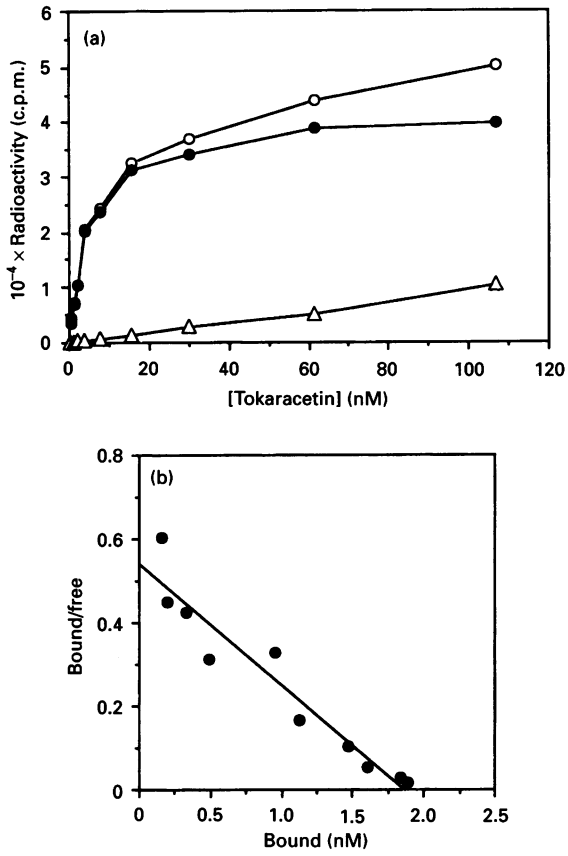

Figure 3 Saturable binding of ^{125}I-tokaracetin to washed human platelets

(**a**) Labelled tokaracetin was diluted to various concentrations with Tris buffer. At 30 min after various concentrations of tokaracetin were mixed with washed human platelets (2.5×10^7 platelets/ml) in Eppendorf tubes, the platelet mixtures were centrifuged to determine the amount of tokaracetin bound. ○, Total binding; ●, specific binding; △, non-specific binding. (**b**) Scatchard plot of tokaracetin binding to washed human platelets. Platelet-bound and free tokaracetin were separated by centrifuging the platelets through a 20% sucrose layer, and platelet-bound radioactivity was measured in a γ-counter. Data have been corrected for non-specific binding. Points are averages of duplicate determinations. The results are representative of three separate experiments.

peaks were obtained (Figure 1a), the second of which (fraction nos. 20–26) showed the strongest inhibitory activity against aggregation of fixed platelets induced by bovine vWF. Chromatography was then carried out on a heparin–Sepharose CL-6B column (Figure 1b). The most active fractions (fraction nos. 48–59) were further purified on a TSKgel Phenyl-5PW column. Inhibitory activity coincided with the major peak (results not shown). Tokaracetin comprised about 0.36% of the total venom protein prior to purification. As shown in the inset of Figure 1b, purified tokaracetin gave a single band with an apparent molecular mass of 28.9 kDa on SDS/PAGE under non-reducing conditions. After reduction, two bands of 16.1 and 15.4 kDa were observed, suggesting that the molecule is a heterodimer.

Effect of tokaracetin on platelet agglutination or aggregation

Tokaracetin at 30 μg/ml did not induce platelet agglutination in PRP, whereas AL-B at 5 μg/ml clearly induced agglutination in PRP (results not shown). Table 1 shows the IC_{50} values for the effects of tokaracetin on platelet aggregation in fixed human platelets or PRP. Tokaracetin at 1 μg/ml completely inhibited ristocetin- or botrocetin-induced platelet aggregation in both. In contrast, however, tokaracetin at 30 μg/ml had no effect on ADP (10 μM)-, collagen (5 μg/ml)- or TRAP (50 μM)- induced platelet aggregation in PRP. Tokaracetin also inhibited bovine vWF-mediated aggregation of fixed human platelets in a dose-dependent manner, with an IC_{50} value of 0.58 μg/ml. The inhibitory potency of tokaracetin on vWF-mediated platelet aggregation in PRP was comparable with that in fixed human platelets.

Effects of tokaracetin on binding of ^{125}I-(human vWF) in the presence of botrocetin and ^{125}I-(bovine vWF) to fixed platelets

Figure 2 shows the inhibitory effects of tokaracetin on the binding of ^{125}I-(human vWF), in the presence of botrocetin, and of ^{125}I-(bovine vWF) to fixed human platelets. Tokaracetin dose-dependently inhibited the binding of ^{125}I-(human vWF), in the

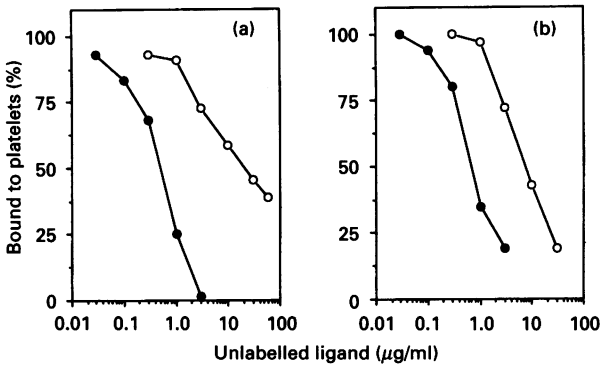

Figure 4 Competition between tokaracetin and bovine vWF for binding to fixed human platelets

(**a**) ^{125}I-Tokaracetin (0.5 μg/ml) was added 10 min after incubation of various concentrations of either unlabelled tokaracetin (●) or bovine vWF (○) with fixed human platelets (1×10^8 platelets/ml). (**b**) ^{125}I-labelled bovine vWF (0.5 μg/ml) was added 10 min after incubation of various concentrations of either unlabelled tokaracetin (●) or bovine vWF (○) with fixed human platelets (1×10^8 platelets/ml). At 30 min after the addition of radiolabelled ligand, samples were centrifuged through a 20% sucrose layer to determine the amount of radioactivity attached to platelets. Non-specific binding was measured in the presence of an excess of unlabelled ligand (60 μg/ml). Data are expressed as a percentage of the control value determined in the absence of unlabelled protein. Points are averages of duplicate determinations.

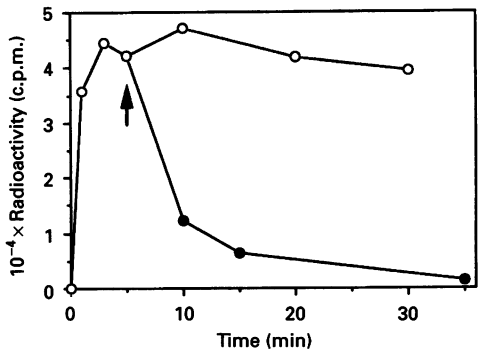

Figure 5 Reversibility of binding of tokaracetin to fixed human platelets

At 5 min after incubation of ^{125}I-tokaracetin (0.95 μg/ml) with fixed human platelets (1×10^8 platelets/ml), an excess of unlabelled tokaracetin (40 μg/ml) was added to suspensions (●). Platelet-bound and free tokaracetin were separated by centrifuging the platelets through a 20% sucrose layer, and platelet-bound radioactivity was measured in a γ-counter. Total binding was determined in the absence of unlabelled tokaracetin (○). Points are averages of duplicate determinations.

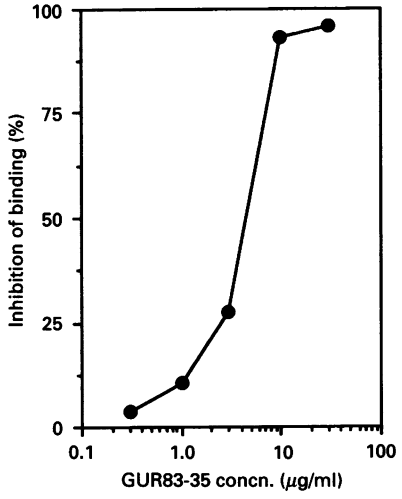

Figure 6 Effect of monoclonal antibody GUR83-35 on the binding of ^{125}I-tokaracetin to fixed human platelets

GUR83-35 at various concentrations (0.3–30 μg/ml) was preincubated with fixed human platelets (1×10^8 platelets/ml) just before mixing with ^{125}I-tokaracetin (0.5 μg/ml). At 30 min after incubation at 22 °C, the platelet mixture was centrifuged through a 20% sucrose layer to determine the amount of tokaracetin bound. Non-specific binding was measured in the presence of an excess of unlabelled tokaracetin (30 μg/ml). Data have been corrected for non-specific binding. Points are averages of duplicate determinations.

presence of botrocetin, and of ^{125}I-(bovine vWF) to fixed platelets, with IC_{50} values of 0.60 and 0.32 μg/ml respectively.

Effect of reduced tokaracetin on platelet aggregation

Purified tokaracetin (300 μg/ml) was incubated with DTT (10 mM) in 50 mM Tris buffer, pH 8.6, at 37 °C for 30 min. An 8-fold concentration of IAA in 50 mM Tris buffer, pH 8.6, was added to this mixture under conditions of darkness, and the mixture was allowed to stand for 30 min at 22 °C. Non-reduced tokaracetin (300 μg/ml) was incubated with IAA (80 mM) before DTT (10 mM) as a control experiment. Samples were electrophoresed on a SDS/16%-PAGE under non-reducing conditions, to confirm that the molecule was completely reduced. Reduction of tokaracetin (at 3 μg/ml) abolished its inhibitory activity on bovine vWF-mediated aggregation of fixed platelets (results not shown).

Binding of ^{125}I-tokaracetin to platelets

Labelling of tokaracetin with ^{125}I did not result in loss of its inhibitory activity on aggregation of fixed human platelets induced by bovine vWF (results not shown). Binding of ^{125}I-tokaracetin to washed platelets was saturable (Figure 3a). Non-specific binding was 5–13% of total binding. Scatchard binding analysis revealed that tokaracetin has one class of binding sites for washed platelets (Figure 3b). Calculated K_d and $B_{max.}$ values from three comparable experiments were 3.9 ± 1.4 nM and 47440 ± 2780 binding sites per platelet respectively (means ± S.E.M.). Results were obtained from three comparable experiments.

Figure 4 shows the competition between tokaracetin and bovine vWF for binding to fixed human platelets. Unlabelled tokaracetin dose-dependently inhibited the binding of ^{125}I-tokaracetin or ^{125}I-(bovine vWF) to fixed human platelets, with IC_{50} values of 0.44 and 0.74 μg/ml respectively. In contrast, unlabelled bovine vWF weakly inhibited the binding of ^{125}I-tokaracetin to fixed human platelets with an IC_{50} value of 22 μg/ml.

Figure 5 shows the time course of ^{125}I-tokaracetin binding to fixed platelets. Binding reached a plateau at 3–5 min after the start of incubation. An excess of unlabelled tokaracetin was added to the suspensions after 5 min of incubation with ^{125}I-tokaracetin. The excess unlabelled tokaracetin was exchanged rapidly with the previously bound tokaracetin, suggesting that the binding was reversible.

Binding of ^{125}I-tokaracetin to erythrocytes, neutrophils and

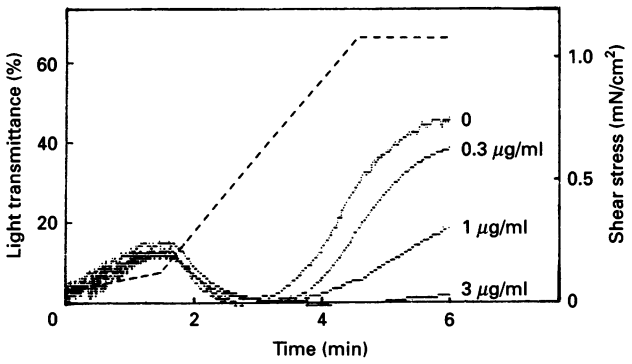

Figure 7 Effect of tokaracetin on SIPA

The broken line indicates the change in the level of shear stress. PRP was exposed to a gradient of 6×10^{-5} to 1.08×10^{-3} N/cm^2 for 6 min at 22 °C. After an initial 15 s at 6×10^{-5} N/cm^2, the gradient increased from 6×10^{-5} to 1.2×10^{-4} N/cm^2 over 90 s, from 1.2×10^{-4} to 1.08×10^{-3} N/cm^2 over the next 120 s, and remained constant at 1.08×10^{-3} N/cm^2 for the last 90 s. Aggregation was measured as described in the Materials and methods section. The results are representative of six separate experiments. The tokaracetin concentrations used are indicated.

Table 2 Effect of tokaracetin on platelet counts in mice

Platelet counts were carried out 5 min after an intravenous bolus injection of three doses of tokaracetin ($n = 3$) or saline ($n = 6$). Data are means ± S.E.M.; * $P < 0.005$ (unpaired t test).

n	Platelet count (cells/μl)
Saline	$(71.4 \pm 3.9) \times 10^4$
Tokaracetin	
1 μg/kg	$(68.5 \pm 8.7) \times 10^4$
10 μg/kg	$(60.4 \pm 4.1) \times 10^4$
100 μg/kg	$(9.2 \pm 2.7) \times 10^{4*}$

platelets was 0, 0.49 and 16.1 ng/cell respectively. These results suggest that the binding of tokaracetin to platelets is highly specific.

Figure 6 shows the inhibition by monoclonal antibody GUR83-35 of the binding of ^{125}I-tokaracetin to fixed human platelets. GUR83-35 dose-dependently inhibited the binding of ^{125}I-tokaracetin to fixed platelets with an IC_{50} value of 4.5 μg/ml.

Effect of tokaracetin on SIPA in PRP

Tokaracetin at 3 μg/ml completely inhibited h-SIPA, without affecting low-shear-stress-induced platelet aggregation (l-SIPA). Figure 7 shows a representative of six experiments.

Effects of tokaracetin on platelet count in mice *in vivo*

Platelet counts showed a dose-dependent decrease at 5 min after an intravenous bolus injection of tokaracetin (Table 2). At a dose of 100 μg/kg, platelet counts were significantly decreased to 13% of those in the saline group. Other haematological parameters (red blood cells, white blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration) did not change (results not shown).

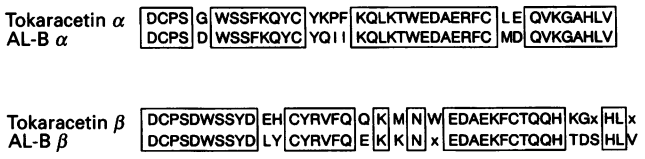

Figure 8 N-terminal amino acid sequences of α- and β-subunits from reduced and S-pyridylethylated tokaracetin separated by RP-HPLC

The sequences of the α- and β-subunits of AL-B corresponded to those of peak 2 and peak 1 subunits [16]. x, unidentified. Identical residues between the two proteins are boxed.

N-terminal amino acid sequences of tokaracetin

Reduced and S-pyridylethylated tokaracetin was separated into two major peaks by RP-HPLC (results not shown). Figure 8 shows the N-terminal 40-residue sequences for the α- and β-subunits. These two sequences showed a high degree of sequence similarity to each other (58% identity). Moreover, the α- and β-subunits of tokaracetin had a high degree of similarity to those of AL-B [16], with identities of 83% and 78% respectively.

DISCUSSION

Platelet adhesion to injured subendothelium induces secondary platelet responses, such as shape changes and release of granular constituents. These responses promote the formation of platelet aggregation and fibrin deposition. Recent research has been directed towards understanding the control of these secondary responses, particularly inhibition of the interaction of fibrinogen with the GPIIb/IIIa complex [27]. Because similar responses are seen in many types of cells, agents that alter secondary responses often lack specificity. Agents that inhibit platelet recognition of damaged endothelial surfaces and block platelet adhesion may therefore be more specific and effective in the control of some haemostatic and thrombotic processes.

To date, a number of GPIb-binding snake venom proteins have been reported, including AL-B [12] from *Trimeresurus albolabris*, CHH-B [28] from *Crotalus h horridus* and echicetin [13] from *Echis carinatus*. While AL-B induces agglutination of fixed human platelets, echicetin and CHH-B have no direct aggregability effect towards platelets. For none of these, however, has any description of an effect on h-SIPA been published. We present here a structural and functional characterization of tokaracetin, a GPIb-binding protein purified from the crude venom of *Trimeresurus tokarensis*.

The molecular mass of tokaracetin on SDS/PAGE under non-reducing and reducing conditions is closely similar to those of AL-B, CHH-B and echicetin. Tokaracetin is composed of a disulphide-linked heterodimer, consisting of an α-subunit with a molecular mass of 16.1 kDa and a β-subunit with a molecular mass of 15.4 kDa. Interestingly, the N-terminal amino acid sequence of each subunit was significantly similar to that of the respective subunit of AL-B [16]. Tokaracetin binds to platelet GPIb specifically and with high affinity; vWF-mediated platelet aggregation and binding of vWF to platelets were completely blocked at 1 μg/ml tokaracetin. Competitive inhibition by tokaracetin of ^{125}I-tokaracetin or ^{125}I-(bovine vWF) binding to fixed human platelets indicated that the binding domains for tokaracetin and bovine vWF on GPIb were the same or closely adjacent. Bovine vWF showed weak inhibition of the binding of ^{125}I-tokaracetin to fixed human platelets, suggesting that tokaracetin has higher affinity for platelets than does bovine vWF. The anti-GPIb monoclonal antibody GUR83-35, which is

directed against the N-terminal 45 kDa domain of GPIbα, inhibited the binding of tokaracetin to fixed human platelets. This finding suggests that the binding domain for tokaracetin on GPIb is close to or identical with that of vWF. The number of binding sites per platelet and the K_d value as calculated by Scatchard analysis were comparable with respective values for echicetin [13]. Previous studies using monoclonal antibody showed that vWF binding sites on the GPIbα chain number approx. 20000–25000 [4,29], fewer than for tokaracetin. A possible explanation for this is that tokaracetin may be able to bind to the vWF-binding site on GPIbα, for example GPIb in the open canalicular system, to which anti-GPIb monoclonal antibodies cannot bind because of their large size. Reduced and alkylated tokaracetin had no inhibitory effect on platelet aggregation induced by bovine vWF at 3 μg/ml. This result agrees with that for CHH-B [28]. In contrast, it has been reported that the biological activity of reduced echicetin is similar to that of its non-reduced form [13]. The individual biological activities of the subunits of tokaracetin with the intradisulphide bonds intact have yet to be determined.

Tokaracetin completely inhibited h-SIPA in human PRP at 3 μg/ml, without affecting l-SIPA. It has been reported that l-SIPA requires the interaction of fibrinogen with GPIIb/IIIa [10]. Because it does not require the presence of any agonist, SIPA at high shear stress is a clinically important phenomenon; this condition is commonly generated in the stenosed coronary artery [30].

Of critical importance in the clinical use of anti-thrombotic agents is the potential of the agent to induce thrombocytopenia. After intravenous injection of tokaracetin in mice, the platelet count decreased in a dose-dependent manner. Tokaracetin at 3 μg/ml also completely inhibited h-SIPA in mice PRP, comparable to the effect in human PRP. Similarly, tokaracetin reacts with platelets from rats, guinea pigs and rhesus monkeys. In this study we did not measure bleeding time in mice but, at the least, spontaneous bleeding from abdominal organs on dissection for blood sampling was not observed. The binding of tokaracetin to washed human platelets did not induce [^{14}C]hydroxytryptamine release from the platelets (results not shown), suggesting that its binding does not induce a release reaction. Further, it has been reported that the binding of AL-B to GPIb does not initiate the activation of the GPIIb/IIIa receptor for fibrinogen binding [16]. Although the mechanism of thrombocytopenia by tokaracetin remains unknown, similar thrombocytopenia has also been reported after injection of echicetin [13] and anti-GPIb monoclonal antibody [31]. In contrast, aurintricarboxylic acid, which binds to vWF multimers and inhibits vWF-mediated platelet aggregation *in vitro*, effectively prevents thrombus formation *in vivo* without decreasing the platelet count [32,33].

Since platelets and megakaryocytes are the only cells that have GPIb on their surface, studies on the relationship between the structure and function of tokaracetin could aid in the design of effective and specific peptide analogue inhibitors of platelet adhesion to injured subendothelium.

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