Textilinin-1, an alternative anti-bleeding agent to aprotinin: Importance of plasmin inhibition in controlling blood loss

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Summary

Aprotinin has been used widely in surgery as an anti-bleeding agent but is associated with a number of side effects. We report that textilinin-1, a serine protease inhibitor from *Pseudonaja textilis* venom with sequence relatedness to aprotinin, is a potent but reversible plasmin inhibitor and has a narrower range of protease inhibition compared to aprotinin. Like aprotinin, textilinin-1 at 5 μmol/l gave almost complete inhibition of tissue plasminogen activator-induced fibrinolysis of whole blood clots. The activated partial thromboplastin time for plasma was markedly increased by aprotinin but unaffected by textilinin-1. In a mouse tail-vein bleeding model, intravenous textilinin-1 and aprotinin caused similar decreases in blood loss but time to haemostasis in the textilinin-treated animals was significantly shorter than in aprotinin-treated mice. Based on these data, textilinin-1 merits further investigation as a therapeutic alternative to aprotinin.

Keywords: textilinin-1, aprotinin, serine protease inhibitor, anti-bleeding, anti-fibrinolytic.

The use of aprotinin for cardiac surgery has been associated with an increased incidence of myocardial infarction, vein graft occlusion and anaphylactic shock (Wuthrich *et al*, 1992; Cohen *et al*, 1999; Beierlein *et al*, 2005; Engles, 2005; Karkouti & Beattie, 2006; Mangano *et al*, 2006; Sodha *et al*, 2006). In a large-scale observational study, Mangano *et al* (2006) found that the use of aprotinin was associated with an increased risk of adverse renal and non-renal events in aprotinin-treated groups of cardiac surgery patients when compared with an untreated group. A multicentre study from the Blood Conservation using Antifibrinolytics in a Randomized Trial (BART) revealed a strong and consistent negative mortality trend associated with the use of aprotinin compared to the lysine analogues, precluding its use in high risk cardiac surgery (Fergusson *et al*, 2008).

The serine protease inhibitor, textilinin-1, from the venom of the Australian eastern brown snake, *Pseudonaja textilis* is related in sequence to aprotinin and exhibits a three-dimensional structure typical of Kunitz/BPTI-type inhibitors (Masci *et al*, 2000; Filippovich *et al*, 2002; Millers *et al*, 2006). In preliminary studies, textilinin-1 was found to strongly inhibit plasmin and trypsin (Flight *et al*, 2005) and to reduce blood loss in an animal model (Masci *et al*, 2000). We describe here an expanded kinetic study of the inhibition of serine proteases involved in haemostasis by textilinin-1 and aprotinin. We also report a comparison of the effects of these two inhibitors on the clotting of plasma and whole blood, on lysis of whole blood clots, and on blood loss in an animal model.

Materials and methods

Enzyme reaction rates were obtained by continuous monitoring of *p*-nitroaniline production at 405 nm from specific peptide substrates. Inhibitor dissociation constants (K_i) were determined by non-linear regression analysis of progress curves using the two-step model and equations of Morrison and Walsh (1988), and commercial software (SIGMAPLOT 8; Systat Software Inc., CA, USA). Activated

partial thromboplastin time (aPTT) and prothrombin time (PT) were measured as described (Austen & Rhymes, 1975). Thromboelastography of citrated whole blood was performed using a Thrombelastograph® (TEG®; Haemoscope Corporation Pty Ltd, Niles, IL, USA). The reaction time (R) is the time from initiation to the formation of the first detectable clot. The percentages of clot lysis at 60 and 180 min after maximum amplitude (MA) are the LY60 and LY180 values.

The mouse tail-vein bleeding model was adapted from that of Tanabe *et al* (1999). Textilinin-1 or aprotinin was injected into the tail veins of adult B57/BL mice (20–30 g) at a concentration of 50 µmol/l to give an expected circulating concentration of approximately 5 µmol/l (assuming a plasma volume of 1 ml and exclusion from red cells). The mice were anaesthetized (isofluorane, 2·5%, 1·5 l/min) and blood loss from the excised tail was then adsorbed onto a filter paper chad and haemoglobin measured by the method of Shaw *et al* (1972). Ethical approval was obtained from the Animal Care Committee of the Queensland Institute of Medical Research and the University of Queensland Animals Ethics Committee and protocols were compliant with National Health and Medical Research Council Animal Experimental Ethics Committee guidelines.

Statistical analysis was performed with the programs SAS, version for Windows 9·1 (SAS Institute, Cary, NC, USA) and STATA/SE 9·2 for Windows (Stata Corp LP, College Station, TX, USA). Analysis of variance (ANOVA) was used to compare average blood loss in the treatment groups. Average blood loss was log-transformed prior to analysis to remove positive skew and Tukey's studentized range test for multiple comparisons was used to compare treatment groups. In all cases significance was defined as $P < 0·05$.

Results

Inhibition of serine proteases involved in coagulation and fibrinolysis

Aprotinin proved to be a more potent inhibitor than textilinin-1 of several different serine proteases, as shown quantitatively by the K_i ratios (Table I). Based on these results, at therapeutic doses used for aprotinin (2 and 5 µmol/l) both textilinin-1 and aprotinin would be expected to produce complete or almost complete inhibition of plasmin. Aprotinin would also cause extensive inhibition of plasma kallikrein, whereas textilinin-1 would have little or no effect. For activated protein C (APC), neutrophil elastase and tissue kallikrein, aprotinin would be expected from the K_i values to cause significant inhibition at therapeutic doses, whereas textilinin-1 would have little or no effect. Aprotinin is also a better inhibitor of tissue plasminogen activator (t-PA) and urokinase, but the K_i values for these enzymes are in excess of the therapeutic dose. No significant inhibition by either inhibitor was detected for α and β factor XIIa, VIIa, Xa, or thrombin. Table I also includes

Table I. K_i data for inhibition of serine proteases by textilinin-1 and aprotinin.

Enzyme	K_i (textilinin-1) (nmol/l)	K_i (aprotinin) (nmol/l)	K_i ratio§
t-PA	>500 × 10³	(30 ± 5) × 10³	>17
Urokinase	>100 × 10³	(30 ± 8) × 10³ 8 × 10³*	>3
APC	>100 × 10³	(5 ± 1) × 10³ 1·35 × 10³†	>20
Elastase	>100 × 10³	(2·6 ± 0·5) × 10³ 3·5 × 10³*	>38
Plasma kallikrein	4830 ± 610	16·4 ± 0·7¶	295
Tissue kallikrein	(12·9 ± 1·1) × 10³	1·3 ± 0·1 1·0*	9·9 × 10³
Plasmin	3·5 ± 0·3‡	0·053 ± 0·004¶	66
Trypsin	0·76 ± 0·02	6·0 × 10⁻⁵*	1·3 × 10⁴

*Engles, 2005.
†Taby *et al*, 1990.
‡Masci *et al*, 2000.
§K_i (textilinin-1)/K_i (aprotinin).
¶Time dependent inhibition. Values quoted are for K_i^* (Morrison & Walsh, 1988).
Results are means ± standard deviation ($n \geq 5$).
t-PA, tissue plasminogen activator; APC, activated protein C.

previously published K_i values for human plasmin and bovine trypsin.

Effects of textilinin-1 and aprotinin on the extrinsic coagulation pathway

Effectors of the extrinsic coagulation pathway can be identified using the prothrombin time (PT) assay (Colman, 2006). Consistent with the failure to observe inhibition of factor VIIa, factor Xa, and thrombin, concentrations of textilinin-1 and aprotinin up to 12 µmol/l had no effect on PT (measured PT at 0 and 12 µmol/l was 12 ± 2 s). Thus, neither textilinin-1 nor aprotinin appeared to have any effect on the extrinsic pathway of blood coagulation at concentrations up to and exceeding those found in plasma during the high-dose Hammersmith regimen in cardiac surgery (Royston *et al*, 1987).

Effect of inhibitors on the intrinsic coagulation pathway

Plasma kallikrein is a component of the intrinsic pathway and its inhibition would be expected to result in the inhibition of coagulation by this pathway. The aPTT measures the clotting time resulting from the activation of factor XII by plasma kallikrein formed from prekallikrein by the action of kinin. The aPTT was prolonged by aprotinin in a dose-dependent manner whereas textilinin-1 had no significant effect (Fig 1A). These differential effects were reinforced by the thromboelastograph® reaction time for aprotinin, which was significantly larger than the control, while that for textilinin-1 was not (Fig 1B and C). Thus, clot formation in whole blood by the
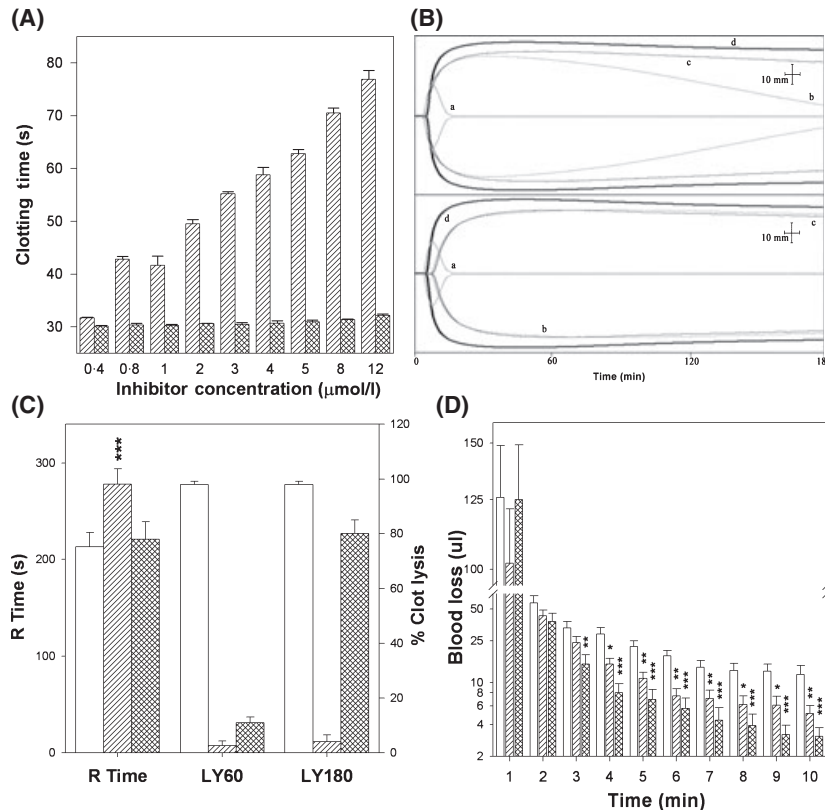
Fig 1. Effect of aprotinin (hatched bars) and textilinin-1 (cross-hatched bars) compared with controls (open bars) on clotting of citrated plasma and whole blood by re-calcification. (A) Activated partial thromboplastin time (aPTT) of pooled human plasma in the presence of inhibitors (0·4–12 μmol/l, $n = 3$). (B) Representative thromboelastograms illustrating the formation and lysis of whole blood clots in the presence of calcium, kaolin, t-PA (92 IU/ml), and either textilinin-1 (upper panel) or aprotinin (lower panel) at concentrations of 0 (trace a), 2 (trace b) and 5 μmol/l (trace c). Trace (d) shows clotting and fibrinolysis without the addition of t-PA or inhibitor. (C) TEG parameters: reaction time R (time from initiation to formation of the first detectable clot) in the absence of t-PA ($n = 6$); LY60 and LY180 (% clot lysis at 60 and 180 min after maximum amplitude respectively) in the presence of inhibitors (2 μmol/l) and t-PA ($n = 12$). (D) Effect of saline ($n = 19$), aprotinin ($n = 15$) and textilinin-1 ($n = 26$) on blood loss in a mouse tail vein bleeding model. Inhibitors were injected into the tail vein, to give plasma inhibitor concentration of approximately 5 μmol/l. Average blood loss was log-transformed prior to analysis to remove positive skew. Dunnett's t-test for multiple comparisons was used to compare experimental treatment groups with the control (saline injection) group and follow up time points with the baseline result at time = 0 min. Results are shown as mean ± SE, and significant differences from controls by asterisks (* $P < 0·05$, ** $P < 0·025$, *** $P < 0·001$).

intrinsic pathway was significantly ($P < 0·001$) retarded by aprotinin, but unaffected by textilinin-1.

The effects of textilinin-1 and aprotinin on fibrinolysis of whole blood clots

Based on our *in vitro* kinetic measurements (Table I), it was predicted that both aprotinin and textilinin-1 would inhibit t-PA-induced fibrinolysis. The effect of the inhibitors on fibrinolysis of clots formed in citrated whole blood by activation of the intrinsic pathway was assessed by thromboelastography. t-PA was added to give a high rate of fibrinolysis in test samples. Overlaid TEG traces are shown in Fig 1B. Addition of calcium and kaolin resulted in the formation of a strong clot with minimal fibrinolysis during the test period (trace d). Addition of t-PA as well resulted in rapid fibrinolysis, preventing formation of a strong clot (trace a). Addition of either textilinin-1 or aprotinin as well as t-PA resulted in a dose-dependent inhibition of fibrinolysis for 2 and 5 μmol/l

inhibitor concentrations. At inhibitor concentrations of 5 μmol/l (as for aprotinin in the high dose Hammersmith regime, Royston *et al*, 1987), both inhibitors caused complete or almost complete inhibition of fibrinolysis (traces c Fig 1B). At 2 μmol/l inhibitor concentration (as in the low dose Hammersmith regime, Royston *et al*, 1988), aprotinin yielded 2·6% and 4·0% clot lysis at 60 and 180 min respectively compared with 11% and 80% for textilinin-1 treated clots for the corresponding time points (traces b in Fig 1B, and Fig 1C). These results for clot lysis suggest that textilinin-1 would be effective as an anti-bleeding agent *in vivo* and are consistent with the "66-fold" tighter binding of aprotinin to plasmin observed in K_i values listed in Table I.

Effects of textilinin-1 and aprotinin on blood loss in an animal model

In a mouse tail vein bleeding model both textilinin-1 and aprotinin had a marked effect on blood loss compared to

control (Fig 1D). The average total blood loss was reduced by 37% ($P = 0.019$) in textilinin-1 treated animals and by 33% ($P = 0.018$) in animals injected with aprotinin, compared with saline. After 3 min, there was a significant reduction in blood loss in the textilinin-1 treated animals compared to the saline group ($P = 0.022$) while in aprotinin-treated animals, a significant effect was only observed after 4 min ($P = 0.026$), suggesting a longer time to haemostasis in the aprotinin-treated animals. Focussing on the 3–5 min time period after injury, the blood loss of textilinin-1 treated animals (29.5 ± 33.0 μl) was significantly less than the blood loss from aprotinin-treated animals (49.7 ± 22.8 μl; $P = 0.01$). These results are consistent with significant inhibition of coagulation by aprotinin but not by textilinin-1 (see Fig 1A, B).

Discussion

The present study has shown that textilinin-1 is an effective inhibitor of plasmin-catalysed fibrinolysis of whole blood clots and reduces blood loss in an animal model, suggesting that it should be an effective anti-bleeding agent. Textilinin-1 was shown to be a very weak inhibitor of plasma kallikrein and, consistent with this observation, had a negligible effect on the aPTT of recalcified citrated plasma (Fig 1A). In agreement with previous results, aprotinin was found to be a potent inhibitor of plasmin and of fibrinolysis (Sodha *et al*, 2006). In contrast to textilinin-1, it is also an inhibitor of plasma and tissue kallikreins and of the intrinsic blood coagulation pathway, as shown by its effect on the aPTT (Fig 1A) and the R time (Fig 1C).

Textilinin-1, aprotinin and DX-88, a kallikrein inhibitor produced by Dyax Corporation, (Tanaka *et al*, 2004; http://www.dyax.com/clinical/cardiacsurg.html) are all Kunitz-type serine protease inhibitors capable of controlling blood loss and provide useful mechanistic comparisons. Textilinin-1 inhibits plasmin strongly and plasma kallikrein to a minimal extent. DX-88 inhibits plasma kallikrein strongly but not plasmin, while aprotinin is a potent inhibitor of both plasmin and plasma kallikrein. Aprotinin has been used successfully to reduce post-operative bleeding after cardiac surgery. DX-88, which is reported to have no anti-fibrinolytic activity (consistent with its inability to inhibit plasmin), has also been reported to reduce blood loss in cardiac surgery (Tanaka *et al*, 2004; http://www.dyax.com/clinical/cardiacsurg.html). Textilinin-1 was found to reduce blood loss when injected intravenously in an animal model (Fig 1D). In the present study, intravenous textilinin-1 and aprotinin both significantly reduced blood loss but textilinin-1 treated mice attained haemostasis more rapidly than aprotinin-treated mice.

The lysine analogs ε-aminocaproic acid and tranexamic acid have also been found to reduce blood loss in cardiac surgery (Hekmat *et al*, 2004; Levy *et al*, 2005). These compounds bind to lysine binding sites on plasminogen, in competition with fibrin (Colman, 2006). As t-PA activates plasminogen much more rapidly when it is bound to fibrin than when it is free, the effect of the lysine analogs is to decrease the rate of formation of plasmin and hence to inhibit fibrinolysis. Whether the ability of textilinin-1 and aprotinin to inhibit plasmin directly makes them superior to the lysine analogs for certain applications remains to be investigated. The current literature indicates that drugs which function by reducing plasmin activity (textilinin-1, lysine analogs and aprotinin) and drugs which inhibit plasma kallikrein (DX-88 and aprotinin), reduce blood loss. The relative importance of these two mechanisms in different surgical situations remains to be established.

Despite the 45% identity in amino acid sequence and identical positioning of the disulfide bonds, textilinin-1 and aprotinin have different specificity as protease inhibitors. The observed inhibition of various trypsin-like enzymes by aprotinin may enhance its effectiveness in cardiac surgery but may be responsible for adverse side effects. Inhibition of APC by aprotinin would enhance thrombin production and prolong coagulation events, which could cause clot extension (formation of additional clot by the action of fibrin bound thrombin), as suggested by Espana *et al* (2005). The present study has confirmed that aprotinin (but not textilinin-1) is a potent inhibitor of tissue kallikrein (urinary kallikrein). Consistent with the anti-bleeding results, the pattern of inhibition by aprotinin should lead to slower but more stable clot formation, whereas textilinin-1 would be expected to allow faster clot formation while showing potent but reversible anti-fibrinolytic properties.

At therapeutic doses, aprotinin is concentrated in the kidney where it selectively binds to the brush border of the proximal tubules, then enters the cytoplasm inhibiting protease secretion (Mangano *et al*, 2006). Inhibition of urinary kallikrein and/or other serine proteases in the kidney may account for the renal dysfunction reported as an adverse effect of aprotinin usage in cardiac surgery (Karkouti & Beattie, 2006; Mangano *et al*, 2006).

We have demonstrated here the efficacy of intravenous textilinin-1 in reducing surgical blood loss in an animal model. These results together with the *in vitro* whole blood coagulation and fibrinolysis, and the enzyme specificity data suggest that textilinin-1 has the potential to be a safer anti-bleeding agent than aprotinin. Furthermore, the availability of an immunologically distinct (Filippovich *et al*, 2002) alternative to aprotinin is desirable, given the risks inherent in repeated use of aprotinin. Because of the multiple roles of plasmin (Syrovets & Simmet, 2004), the availability of a selective plasmin inhibitor opens up further therapeutic possibilities.

Acknowledgements

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