Comparison of Textilinin-1 with Aprotinin as Serine Protease Inhibitors and as Antifibrinolytic Agents

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Key Words
Textilinin · Aprotinin · Thrombelastography · Fibrinolysis · Enzyme inhibition · Plasmin · Kallikrein

Abstract
Textilinin-1 (Q8008) was isolated from the venom of the \textit{Pseudonaja textilis} and has a 47% sequence identity to the antihemorrhagic therapeutic agent aprotinin. When equimolar concentrations of enzyme and aprotinin were pre-incubated, plasmin was inhibited 100%, plasma kallikrein 58%, and tissue kallikrein 99%. Under the same conditions, textilinin-1 inhibited plasmin 98%, plasma kallikrein 16% and tissue kallikrein 17%. Whole blood clot lysis was inhibited strongly by both aprotinin and textilinin-1, as shown by thrombelastography. At 2 \textmu M inhibitor lysis initiated by t-PA was greater than 99% inhibited by aprotinin (LY60 = 0.4 ± 0.1) whereas textilinin-1, inhibited lysis by 91% (LY60 = 8.9 ± 0.7). The same trend was found with the lysis of euglobulin fractions. From these data textilinin-1 appears to be a more specific plasmin inhibitor than aprotinin but aprotinin inhibits clot lysis to a greater extent.

Introduction
Reducing blood loss during surgery is a clinical imperative due to shortages in donor blood supply and the risk of introducing blood borne diseases such as hepatitis, human immunodeficiency virus and variant Creutzfeldt-Jakob disease from replacement blood.

Textilinin-1 is a 6.7 kDa peptide serine protease inhibitor isolated from the venom of \textit{Pseudonaja textilis} \cite{1}. It has a similar sequence (47% identity) to the 6.5 kDa peptide aprotinin, isolated from bovine lung, which is widely used as an antibleeding agent. Aprotinin (marketed as Trasylol\textsuperscript{®}) was identified independently in the 1930s by Kraut et al. \cite{2} and Kunitz and Northrop \cite{3} and was first used for treatment of pancreatitis and severe head injury. Aprotinin is a potent inhibitor of trypsin and the high resolution structure of the aprotinin-trypsin complex has been determined \cite{4, 5}. As well as trypsin, aprotinin inhibits other serine proteases including several involved in haemostasis \cite{6, 7}.

The use of aprotinin to reduce blood loss is now common during cardiac surgery \cite{8}. The mechanism by which aprotinin acts are thought to be primarily through its direct inhibition of plasmin \cite{9–11}. The preservation of platelet activity is also thought to contribute to the reduction in blood loss \cite{12–14}.
More recently aprotinin has been found to be an effective treatment for other conditions such as tendonitis [15], tumours [16] and sepsis [17]. Not all of the observed effects of aprotinin are caused directly by enzyme inhibition and effects on platelet function; some involve more complex interactions, which affect coagulation [18, 19] and inflammation [20].

Textilinin-1 inhibits plasmin and has been shown to reduce bleeding in a small animal model [1]. However, the specificity of textilinin-1 for other clinically relevant serine proteases and its action as an antifibrinolytic agent in vitro has not been previously reported.

This paper describes experiments measuring the inhibition of plasmin, plasma kallikrein, tissue kallikrein and several other serine proteases as well as examining the effect of textilinin-1 on lysis of clots formed in whole blood and plasma in vitro. These results are used to discuss potential advantages and disadvantages of textilinin-1 over aprotinin as a therapeutic antibleeding agent.

Materials and Methods

Reagents
All enzyme activities were measured using an appropriate chromogenic substrate purchased from Chromogenix (Sweden). The substrate codes used for the enzymes were as follows: S-2222 (factor Xa), S-2251 (plasmin), S-2302 (tissue and plasma kallikrein), S-2288 (t-PA), S-2238 (thrombin) and S-2765 (trypsin and trypptase). All other reagents were purchased from Sigma Aldrich (USA) unless otherwise specified.

Enzymes
Human plasma was purchased from Laboratoire Choay (France) and reconstituted to 4.6 U/mg in 50 mM Tris-HCl (pH 7.4) containing 50% glycerol. Plasma kallikrein was purchased from Calbiochem (Germany) at a concentration of 20.4 U/mg. Porcine tissue kallikrein was purchased from Sigma Aldrich at a concentration of 26 U/mg. Trypsin was purchased from Sigma Aldrich and reconstituted to a concentration of 140,000 U/mg. Recombinant tissue plasminogen activator (rt-PA) was obtained from Genentech (USA) as Actilyse®. Thrombin, factor Xa and trypptase were purchased from Sigma Aldrich.

Inhibitors
Aprotinin was purchased as Trasylol® from Bayer Pharmaceuticals (Germany) as a 50 ml sterile aqueous solution consisting of 1.4 mg/ml protein (10,000 KIU/ml) in NaCl 450 mg, NaOH q.s. HCl q.s. Recombinant textilinin-1 (textilinin-1), coded Q8008 and produced by BresaGen Ltd. (Australia) has the same amino acid sequence as native textilinin-1 [1]. Q8008 is the product code for the recombinant textilinin-1 designated by QRx Pharma Pty Ltd. Textilinin-1 was stored in 25 mM Tris–HCl (pH 7.8), 50 mg/ml mannitol, 0.01% Tween-20 at 0.8 mg/ml at 4°C.

Comparison of Textilinin-1 with Aprotinin

Molecular Modelling of Textilinin-1
A model of textilinin-1 based on the crystal structure of an aprotinin mutant (K15R, A16L, M52L) in complex with bovine β-trypsin (PDB ID 1ejm) was created using the modeler module in Insight II (Biosym).

Chromogenic Enzyme Assays
Each 1 ml assay was formulated in a plastic cuvette and contained 150 μM of the relevant substrate. The concentration of uninhibited enzyme used for each test was that which resulted in a change of ~0.1 absorbance units in 10 min. Inhibition experiments were performed by pre-incubating the enzyme and inhibitor at equimolar concentrations for 5 min at 22°C. Each test was monitored at 405 nm for 10 min and performed in triplicate. The results are expressed as percentage inhibition of the control by the inhibitor-containing tests.

Thromboelastography
Thromboelastography was performed using a Thrombelastograph® (TEG®, Haemscope Corporation Pty Ltd, USA). The TEG® measures the elastic properties of the whole blood clot during its formation and lysis through impedance. The basic principles of thromboelastography are described by Salojoa and Perry [21]. The reaction time (R) is the time from the start to the formation of the first significant levels of a detectable clot. This is the point at which most traditional plasma clotting assays reach their end point. The amplitude (A) is a measurement of the stiffness or strength (shear modulus) of the clot. The maximum amplitude (MA) is the point where the clot is at its strongest. The LY60 value is the percentage lysis 60 min after MA is reached.

Each clot formation/lysis assay contained 320 μl of a citrated whole blood, bovine thrombin (1 U), rt-PA (33 IU), CaCl2 (20 mM) and textilinin-1 or aprotinin (2 μM) to make a total assay volume of 360 μl. This level of rt-PA (33 IU/assay or ~14 nM) was chosen as it resulted in the production of plasmin to result in almost full clot lysis over 90 min, allowing the effects of the plasmin inhibitors to be monitored. The concentration of inhibitor chosen (2 μM) is similar to the plasma concentration after the dose of aprotinin received in the Hammersmith (low-dose) regime [22]. Each experiment was performed for at least 60 min after the maximal amplitude was reached to establish the LY60 value. TEG LY60 data are presented in tabular form as averages. Errors are the standard error of the mean and the significance between data sets was determined using the Student t test (paired, 2-tailed distribution). p values <0.05 were considered statistically significant.

Euglobulin Clot Lysis Time
Euglobulin clot lysis time was performed using a method similar to that described by Lassen [23]. Citrated human plasma was diluted with H2O at 4°C (9 ml of H2O to 1 ml of plasma) and adjusted with 1% acetic acid to pH 5.9 then incubated at 4°C for 1 h. The plasma was transferred to test tubes (10 ml per tube) and centrifuged (10,000 g for 20 min). The supernatant was decanted, discarded and the tubes inverted for 15 min. The sides of the tubes were dried with swabs and each pellet was resuspended in 80 mM barbitone buffer pH 8.6. Inhibitors (0.4 nM) were added as appropriate. Bovine thrombin (1 U) was added to clot the euglobulin fractions. Tubes were incubated at 37°C and lysis monitored. All clots were weighed when full lysis of the control was first evident.
Results

Enzyme Inhibition by Textilinin-1 and Aprotinin

Table 1 illustrates the percentage inhibition of trypsin and three enzymes important in haemostasis and the inflammatory response by equimolar concentrations of textilinin-1 and aprotinin. Both compounds were potent inhibitors of trypsin and plasmin at equimolar concentrations of enzyme and inhibitor. Aprotinin was also a strong inhibitor of tissue and plasma kallikrein whereas textilinin-1 was a much weaker inhibitor of these two enzymes. At equimolar concentrations the activity of t-PA, thrombin, fXa and tryptase was not significantly affected by textilinin-1 or aprotinin.

Table 1. Enzyme inhibition by textilinin-1 and aprotinin at equimolar concentrations

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration of enzyme and inhibitor (nM)</th>
<th>Percentage inhibitiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>textilinin-1</td>
<td>aprotinin</td>
</tr>
<tr>
<td>Trypsin</td>
<td>18</td>
<td>96 ± 6</td>
</tr>
<tr>
<td>Plasmin</td>
<td>130</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>Plasma kallikrein</td>
<td>45</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Tissue kallikrein</td>
<td>49</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

a The rates of substrate hydrolysis by four enzymes pre-incubated with equimolar concentrations of textilinin-1 or aprotinin are presented as percentage inhibition of the uninhibited rate. Each value is the average of the mean of triplicate measurements ± standard deviation.

No inhibition of thrombin (270 nM), fXa (25 nM), t-PA (73 nM) or tryptase (0.33 nM) by either inhibitor was detected under the conditions used for this experiment.

Molecular Modelling of Textilinin-1

Textilinin-1 and aprotinin are both Kunitz-type serine protease inhibitors of 59 and 58 amino acid residues respectively, each with three disulfide bonds in equivalent positions as shown in figure 1.

In an effort to understand the differences in inhibition on a molecular basis, we have modeled textilinin-1 based on the crystal structure of an aprotinin mutant (K15R, A16L, M52L) in complex with bovine β-trypsin (PDB ID 1ejm) (see fig. 2). The modeling showed that textilinin-1 could adopt a similar structure to aprotinin. The primary interactions between textilinin-1 and the protease are between the canonical loop of the inhibitor (residues 15–20) and the active site of the enzyme. The positively charged side chain of the P1 residue, Arg in textilinin-1 and Lys in aprotinin, respectively, interacts with the negatively charged Asp189 in the enzyme’s specificity pocket. The biggest difference between textilinin-1 and aprotinin within the canonical loop lies at residue P1’, a bulky Val in textilinin-1, but an Ala in aprotinin. The structure of trypsin in complex with the aprotinin (K15R, A16L, M52L) mutant suggests that these differences may affect the tightness of binding and possibly the protease specificity of the inhibitors. Work is continuing in our laboratory to determine the structure of the textilinin-1-trypsin complex.

Effect of Inhibitors on Fibrinolysis of Clots Formed in Whole Blood and Plasma

Thromboelastography was performed to analyse the effect of textilinin-1 and aprotinin on the t-PA-stimulated lysis of clots formed in whole blood by addition of thrombin. Figure 3 illustrates examples of the TEG® traces obtained and table 2 lists the LY60 data so generated. With no t-PA or inhibitor added (control, no t-PA) no clot lysis
could be detected after 180 min and the LY60 values were <0.1%. In reactions containing t-PA but no inhibitor, clots were fully lysed after 90 min, with a mean LY60 value of 96.3 ± 4.4%. 60 min after the MA, both inhibitors caused a dramatic reduction of fibrinolysis (LY60 = 0.4 ± 0.1% aprotinin and LY60 = 8.9 ± 0.7% textilinin-1). The reactions were monitored for 180 min and at this time the amplitude of the t-PA + textilinin-1

Table 2. Data generated from the thromboelastography illustrated in figure 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>LY60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no t-PA)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Control (added t-PA)</td>
<td>96.3 ± 4.4</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Textilinin-1</td>
<td>8.9 ± 0.7</td>
</tr>
</tbody>
</table>

Each measurement represents the mean ± SE of triplicate measurements each using a pool of whole blood from 9 patients. Errors are the standard error of the mean. p-Value for the control (no t-PA) and aprotinin is 0.04 and for aprotinin and textilinin-1 p = 0.001.

Fig. 2. Ribbon representation of trypsin (light grey) in complex with the model of textilinin-1 (dark grey). The P1 residue is labeled.

Fig. 3. Thrombelastogram illustrating the overlayed traces from four separate experiments under different conditions. All experiments contained bovine thrombin (1 U), CaCl2 (20 mM) and citrated whole blood (320 μl). 'Control (no t-PA or inhibitor)' contained no additional components, 't-PA only' contained 33 IU of added t-PA, 't-PA + aprotinin (2 μM)' contained 33 IU of t-PA and 2 μM aprotinin, 't-PA + textilinin-1 (2 μM)' contained 33 IU of t-PA and 2 μM textilinin-1. Experiments containing textilinin-1 and aprotinin were monitored for 180 min. In all experiments where no t-PA was added there was >98% lysis over 90 min and where no inhibitor was added there was <1% lysis over 180 min.
(2 μM) clots was approximately 50% of the MA. However, the amplitude of the t-PA + aprotinin (2 μM) clots was at least 90% of the MA. Since fibrinolysis in these experiments was induced by t-PA, the antifibrinolytic effects of aprotinin and textilinin-1 were due to inhibition of plasmin.

Euglobulin clot lysis time is a semi-quantitative measurement determined by visualising the time taken for a clot formed in a euglobulin fraction isolated from plasma to lyse (normally 2–4 h). In this experiment the effects of textilinin-1 and aprotinin (1.5 μM) on euglobulin clot lysis were determined by weighing the remaining clots in inhibitor-containing experiments when full lysis was first apparent in the control clots. Before incubation (at time zero) the clots weighed 0.88 ± 0.045 g. When full clot lysis was first apparent in the controls, the aprotinin and textilinin-1-containing clots weighed 0.75 ± 0.035 g and 0.37 ± 0.035 g respectively.

Discussion

Thromboelastography and euglobulin clot lysis were techniques to examine the comparative effects of textilinin-1 and aprotinin on the persistence of clots in vitro. The main differences between these two techniques are the source of t-PA (added in the thromboelastography and endogenous in the euglobulin clot lysis) and the presence of the cellular components of whole blood (absent from the euglobulin fraction derived from plasma). In both experiments the clots containing aprotinin were significantly more persistent than those containing textilinin-1. The less persistent textilinin-1-containing clots seen in these experiments indicate fibrinolysis had been inhibited to a lesser extent. This feature may reduce the risk of thrombosis associated with strong antifibrinolytics (such as aprotinin) making it a safer blood loss reduction therapeutic agent. Alternatively, the weaker inhibition of fibrinolysis could be perceived as a disadvantage in vivo possibly resulting in rapid clot lysis and loss of more blood at a comparable dosing range to aprotinin. However, because of its greater specificity for plasmin it may be possible to use higher doses of textilinin-1 without the risk of side effects associated with non-specific enzyme inhibition in a surgical setting. Earlier experiments have shown textilinin-1 to be as effective as aprotinin, at similar concentrations, at reducing blood loss from an arterial bleed in a small animal model [1] suggesting the less prolonged inhibition of fibrinolysis by textilinin-1 does not compromise its capacity to save blood.

There is evidence suggesting specific plasmin inhibitors such as aminocaproic acid and transamic acid may be less effect in reducing blood loss [24, 25] compared to the non-specific inhibitor aprotinin [26]. Recent data strongly suggests that kallikrein inhibition is an important target in reducing blood loss [27]. However, there is also a study showing that increasing the kallikrein inhibitory activity of an aprotinin-like inhibitor does not increase its efficacy in vivo [28]. Consequently the relative roles plasmin, kallikrein and other factors play in surgical bleeding are unknown.

Plasmin itself has broad substrate specificity and is intrinsic to many and varied functions in the body. Most plasmin-mediated effects such as cell invasion, chemotaxis, tissue remodelling, ovulation, migration of tumour and immune cells and wound healing are due to the proteolytic cleavage of extracellular proteins [29–33]. As plasmin is involved in these processes the therapeutic applications for plasmin inhibitors include treatment of the chronic inflammatory diseases such as joint diseases [34, 35], in particular rheumatoid arthritis [36, 37], along with arteriosclerosis [38] and restriction of cell proliferation during cancer [39].

Future research in our laboratory will include more detailed evaluation of textilinin-1 as an inhibitor of serine proteases whose inhibition may have therapeutic significance. Further in vitro studies will include examination of the effects of textilinin-1 in blood coagulation and fibrinolysis and studies using animal models.

Acknowledgement

This research was supported and funded by QRx Pharma Pty. Ltd.
References


