Biochimica et Biophysica Acta 1840 (2014) 153–159

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Functional characterization of a slow and tight-binding inhibitor of plasmin isolated from Russell's viper venom

An-Chun Cheng [a,1], Inn-Ho Tsai [a,b,*]

[a] Institute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan
[b] Institute of Biochemical Sciences, National Taiwan University, Taipei 10617, Taiwan

ARTICLE INFO

Article history:
Received 17 June 2013
Received in revised form 22 August 2013
Accepted 26 August 2013
Available online 30 August 2013

Keywords:
Kunitz-type protease inhibitor
Plasmin
Russell's viper venom
Tight-binding inhibition
Time-dependent inhibition
Antifibrinolytic agent

ABSTRACT

Background: Snake venoms are rich in Kunitz-type protease inhibitors that may have therapeutic applications. However, apart from trypsin or chymotrypsin inhibition, the functions of most of these inhibitors have not been elucidated. A detailed functional characterization of these inhibitors may lead to valuable drug candidates.
Methods: A Kunitz-type protease inhibitor, named DrKIn-II, was tested for its ability to inhibit plasmin using various approaches such as far western blotting, kinetic analyses, fibrin plate assay and euglobulin clot lysis assay. In addition, the antifibrinolytic activity of DrKIn-II was demonstrated in vivo.
Results: DrKIn-II potently decreased the amidolytic activity of plasmin in a dose-dependent manner, with a global inhibition constant of 0.2 nM. Inhibition kinetics demonstrated that the initial binding of DrKIn-II causes the enzyme to isomerize, leading to the formation of a much tighter enzyme-inhibitor complex. DrKIn-II also demonstrated antifibrinolytic activity in fibrin plate assay and significantly prolonged the lysis of the euglobulin clot. Screening of DrKIn-II against a panel of serine proteases indicated that plasmin is the preferential target of DrKIn-II. Furthermore, DrKIn-II treatment prevented the increase of FDP in coagulation-stimulated mice and significantly reduced the bleeding time in a murine tail bleeding model.
Conclusion: DrKIn-II is a potent, slow and tight-binding plasmin inhibitor that demonstrates antifibrinolytic activity both *in vitro* and *in vivo*.
General significance: This is the first in-depth functional characterization of a plasmin inhibitor from a viperid snake. The potent antifibrinolytic activity of DrKIn-II makes it a potential candidate for the development of novel antifibrinolytic agents.

1. Introduction

Plasmin is a serine protease that plays a pivotal role in maintaining the patency of blood vessels by degrading the fibrin clots in blood, a process known as fibrinolysis [1]. It has been established that plasmin inhibition, either directly or through the inhibition of plasminogen activation, has the clinical advantage of reducing blood loss in cardiac surgeries that involve cardiopulmonary bypass or organ transplantation [2]. One of the antifibrinolytic agents used under such conditions was aprotinin, a Kunitz-type protease inhibitor that inhibits plasmin. However, due to the various negative reports concerning the safety of aprotinin, the drug was no longer prescribed [3,4]. A new plasmin inhibitor is therefore needed, and peptides from snake venoms may represent a useful pool of therapeutic agents to replace the old drug [5].

Viperidae and *Elapidae* snake venoms are generally rich in serine protease inhibitors belonging to the Kunitz/BPTI (bovine pancreatic trypsin inhibitor) family [6–12]. These Kunitz-type protease inhibitors are approximately 60 amino acids long and are characterized by the presence of a canonical binding loop which interacts specifically with the enzyme and six conserved cysteine residues that form three pairs of disulfide bonds to stabilize the overall structure [13]. Most of these venom-derived protease inhibitors have been classified into trypsin or chymotrypsin inhibitors, based on whether the P1 site contains a basic or a non-basic residue [14]. The best studied examples of snake venom Kunitz type protease inhibitors are the potassium channel blockers from *Dendroaspis* species [15,16] and the plasmin inhibitors from *Pseudonaja textilis* [11]. However, for the majority of all the other inhibitors from snake venoms, apart from trypsin or chymotrypsin inhibition, their functional roles remain largely unknown.

The aim of this study was to elucidate the functions of a Kunitz-type protease inhibitor, named DrKIn-II (*Daboia russelii* Kunitz Inhibitor-II), that was recently isolated from the venom of *Daboia russelii russelii*

Abbreviations: DrKIn-II, *Daboia russelii* Kunitz Inhibitor-II; BPTI, bovine pancreatic trypsin inhibitor; HPLC, high performance liquid chromatography; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; APTT, activated partial thromboplastin time; PT, prothrombin time; RVV, Russell's viper venom; FDP, fibrin/fibrinogen degradation product

* Corresponding author at: Institute of Biological Chemistry, Academia Sinica, No. 128 Academia Road Section 2, Nan-Kang, Taipei 11529, Taiwan. Tel.: +886 2 23665521; fax: +886 2 23635038.
E-mail addresses: adamcheng@gate.sinica.edu.tw (A.-C. Cheng), bc201@gate.sinica.edu.tw (I.-H. Tsai).
[1] Postal address: No. 128 Academia Road Section 2, Nan-Kang, Taipei 11529, Taiwan.

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.08.019

[17]. It is a viperid snake that is responsible for a large proportion of mortality and morbidity in many parts of Southeast Asia and India, and many of the patients envenomed by this snake suffer from spontaneous bleeding and incoagulable blood [18,19]. Based on these symptoms of coagulopathy, it was speculated that this venom could contain both potent and specific antifibrinolytic agents, like the plasmin inhibitors from *P. textilis*. Here, we describe the physiological target, kinetics, binding mechanism and functions of this Kunitz inhibitor. In addition, its therapeutic implication is discussed.

2. Materials and methods

2.1. Materials

Lyophilized *Daboia russelii russelii* venom (Pakistan) was purchased from Latoxan (Valence, France). DrKIn-II was purified from the venom by gel filtration followed by reversed-phase HPLC as described previously [17]. Purified human plasmin, activated protein C (APC), factor XIIa (FXIIa), factor XIa, factor Xa (FXa), factor IXa (FIXa), factor VIIa (FVIIa), factor Va (FVa), thrombin, and plasma kallikrein were purchased from Haematologic Technologies Inc. (VT, USA). Urokinase plasminogen activator (uPA) was a gift from Polyamine Corp. (Taiwan). Trypsin and tissue plasminogen activator (tPA) were obtained from Merck Chemicals (Darmstadt, Germany). Chromogenic substrates S-2222, S-2303, S2366, S-2288 and S-2251 were purchased from Chromogenix (Milano, Italy), while Spectrozyme PCa, Spectrozyme tPA and Spectrozyme FIXa were from American Diagnostica (CT, USA). Citrated human plasma was purchased from HYPHEN Biomed (Andresy, France), and human fibrinogen was from Sigma Aldrich (MO, USA).

2.2. Biotinylation of DrKIn-II

DrKIn-II was biotinylated using the Sulfo-NHS-LC-Biotin labeling kit (Pierce, IL, USA) according to the manufacturer's protocol. Briefly, 180 μg of DrKIn-II dissolved in PBS was mixed with 50 μl of 10 mM biotin reagent and incubated on ice for 2 h. DrKIn-II was then purified from the excess biotin reagent by Zebra Desalt Spin Column (Pierce, IL, USA) and stored at −20 °C until use.

2.3. Far-western blotting

Purified human plasmin, tPA and uPA were subjected to SDS-PAGE under non-reducing conditions and blotted onto a PVDF membrane. The membrane was blocked with 3% skim milk dissolved in TBST–Ca buffer (TBS containing 0.05% Triton X-100 and 2.5 mM $CaCl_2$). Subsequently, the membrane was incubated with 1 μg/ml of biotinylated DrKIn-II for 1 h at room temperature in TBST–Ca containing 0.3% BSA. After three 5 min washes with TBST–Ca, ExtrAvidin Peroxidase (Sigma Aldrich, MO, USA), diluted 5000-fold in TBST–Ca containing 0.3% BSA, was added and incubated for 1 h at room temperature. The bound DrKIn-II was then visualized by ECL reagent.

2.4. Kinetics of plasmin inhibition by DrKIn-II

2.4.1. Tight binding kinetics

Plasmin (20 nM) was incubated with different concentrations of DrKIn-II for 40 min in a buffer containing 25 mM Tris HCl, 0.15 M NaCl, 2.5 mM $CaCl_2$ and 0.1% PEG (pH 8.0). Chromogenic substrate S-2251 was then added to a final concentration of 0.2 mM and the rate of *p*-nitroaniline release was monitored at 405 nm for 10 min at 37 °C using SpectraMax M2^e Microplate Reader (Molecular Devices, CA, USA). Dose response curve was fitted by non-linear regression using GraphPad Prism (GraphPad Software, CA, USA). Fractional velocities were plotted against inhibitor concentrations and the values were fitted to Morrison's tight binding equation [20]:

$$\frac{V_i}{V_0} = 1 - \frac{\left([E] + [I] + K_i^{*}{}_{app}\right) - \sqrt{\left([E] + [I] + K_i^{*}{}_{app}\right)^2 - 4[E][I]}}{2[E]} \quad (1)$$

where V_i is the steady state velocity in the presence of inhibitor and V_0 is the uninhibited velocity. [*E*] is the total enzyme concentration and [*I*] is the total inhibitor concentration. *$K^*_{i\,app}$ is the apparent inhibition constant and is related to the true inhibition constant (K^*_i) by the following equation:*

$$K_i^{*}{}_{app} = K_i^{*}\left(1 + \frac{[S]}{Km}\right) \quad (2)$$

where [S] is the substrate concentration and *Km* is the Michaelis–Menten constant for S-2251.

2.4.2. Slow binding kinetics

Plasmin (20 nM) was added to a mixture containing S-2251 (1 mM) and varying concentrations of DrKIn-II in a buffer containing 25 mM Tris HCl, 0.15 M NaCl, 2.5 mM $CaCl_2$ and 0.1% PEG (pH 8.0) and the rate of product formation was measured at 37 °C using Spectrophotometer U-3200 (Hitachi, Japan). The progress curves were analyzed by the following equation for time dependent inhibition [21]:

$$[P] = v_s t + \frac{v_i - v_s}{k_{obs}}[1 - \exp(-k_{obs} t)] \quad (3)$$

where [*P*] is the amount of *p*-nitroaniline released from substrate hydrolysis, v_i and v_s are the initial and steady state velocities, respectively, in the presence of inhibitor, and k_{obs} is the apparent first order rate constant for the interconversion between v_i and v_s.

For inhibitors that bind to the enzyme rapidly and cause the enzyme to isomerize slowly, the reaction mechanism can be described by the following scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*I \quad \text{(Scheme1)}$$

where EI is the initial loose complex and E*I is the final isomerized complex. In this mechanism, k_{obs} is related to k_2 and k_{-2} and to the equilibrium dissociation constant of the initial loose complex ($K_i = k_{-1}/k_1$) by Eq. (4):

$$k_{obs} = k_{-2} + \frac{k_2[I]}{[I] + K_i(1 + [S]/Km)}. \quad (4)$$

The values of k_2, k_{-2} and K_i were determined by computer fitting to Eq. (4). These values were then used to determine the overall inhibition constant (K_i^*) *according to Eq. (5):*

$$K_i^{*} = \frac{K_i k_{-2}}{k_2 + k_{-2}}. \quad (5)$$

All computer fittings were performed using GraphPad Prism (GraphPad Software, CA, USA).

2.5. Fibrin plate assay

The assay was performed with 10 ml of purified human fibrinogen (0.5%) clotted with 3 units of α-thrombin in a 9 cm Petri dish. After 30 min, 10 μl of plasmin (0.05 μg/μl), with or without DrKIn-II (0.05 μg/μl), was dropped onto the fibrin plate and incubated at 37 °C for various time periods. Fibrinolytic activity was determined by the formation of clear area on the fibrin plate.

2.6. Euglobulin clot lysis assay

For the preparation of euglobulin fraction, 2 ml of normal plasma was added to 36 ml of acetic acid (0.016% v/v) and incubated on ice for 20 min. After centrifuging the solution at 1100 g for 15 min, the pellet was resuspended in 2 ml of 2.6 mM sodium borate solution containing 0.15 M NaCl, pH 9.0. The solution was placed in a 37 °C water bath for 90 s, mixed and placed back into the water bath for another 90 s. The euglobulin fraction (125 μl) was then mixed with the indicated concentrations of DrKIn-II or aprotinin in a flat bottom microtiter plate (Nalge Nunc International, NY, USA). Finally, $CaCl_2$ was added to a final concentration of 12.5 mM to initiate clotting. The microtiter plate was kept at 37 °C and the absorbance at 340 nm of each well was read every 10 min for 20 h. Lysis time was defined as the time point at which the absorbance values returned to baseline.

2.7. Selectivity profile of DrKIn-II

DrKIn-II was screened for its inhibitory activity against trypsin, activated protein C and also against serine proteases in the coagulation cascade (FXIIa, FXIa, FXa, FIXa, thrombin and kallikrein) and in the fibrinolytic system (plasmin, tPA and uPA). The amidolytic activities of these proteases were determined in the presence or absence of equimolar concentrations of DrKIn-II using their respective chromogenic substrates.

2.8. Coagulation tests

All coagulation tests were performed with a coagulometer (Hemostasis Analyzer KC-1, Sigma Diagnostics) at 37 °C.

2.8.1. Activated partial thromboplastin time (APTT)

Normal coagulation control plasma (50 μl) was mixed with the indicated concentrations of DrKIn-II. After incubation for 1 min at 37 °C, 50 μl of activated partial thromboplastin reagent (HYPHEN Biomed) was added and further incubated for 1 min. Clotting was initiated by the addition of 50 μl of 20 mM $CaCl_2$ and the coagulation time was recorded.

2.8.2. Prothrombin time (PT)

Normal coagulation control plasma (50 μl) was mixed with the indicated concentrations of DrKIn-II. After incubation for 1 min at 37 °C, 100 μl of thromboplastin reagent (Helena Laboratories, TX, USA) was added to trigger the coagulation and the time required for clot formation was measured.

2.9. In vivo assays

All animal experiments were approved by Academia Sinica Institutional Animal Care and Utilization Committee.

2.9.1. Determination of FDP concentrations

ICR mice (~35 g) were injected intravenously with RVV-X (0.01 μg/g) or RVV-X (0.01 μg/g) plus DrKIn-II (0.1 μg/g). 8 h after injection, plasma was obtained by cardiac puncture and FDP levels were quantitated using NANOPIA P-FDP kit (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's protocol.

2.9.2. Murine tail bleeding model

DrKIn-II (100 μl) was injected into the tail veins of adult ICR mice (30–33 g) that had been anesthetized with 60 μg/g of sodium pentobarbital to achieve a DrKIn-II concentration of 0.1 μg/g. After 30 min, the distal 4 mm of the tail was transected and the tail was immediately placed in PBS at 37 °C. An equal volume of PBS was injected into control animals. Bleeding time was defined as the time point of first cessation of bleeding. All experiments were carried out on coded animals by personnel who were blinded to experimental groups.

2.10. Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, CA, USA). Paired t-tests were used to compare between two different treatment groups in our in vivo assays.

3. Results

3.1. Sequence alignment of DrKIn-II with textilinin-1 and aprotinin

In order to determine the function of DrKIn-II, the primary sequence of DrKIn-II was first compared with Kunitz inhibitors that had been well characterized, namely textilinin-1, a potent plasmin inhibitor from *P. textilis* [22], and aprotinin, a classical trypsin, plasmin and kallikrein inhibitor derived from bovine lungs [23,24]. Sequence alignment immediately reveals that DrKIn-II shares the common Kunitz type scaffold, with six conserved cysteine residues and a P1 site (Fig. 1A). The presence of a basic P1 arginine suggests that DrKIn-II, like textilinin-1, belongs to the family of venom trypsin inhibitors according to the classification by Yuan et al. [25]. Since DrKIn-II shares a sequence identity of 48% and 40% with textilinin-1 and aprotinin, respectively, DrKIn-II was tested for its antifibrinolytic potential.

3.2. Far western blotting

The interactions of DrKIn-II with plasmin and other fibrinolytic serine proteases, namely tPA and uPA, were probed using far western blotting. Purified human plasmin, tPA and uPA were blotted onto a PVDF membrane and incubated with biotinylated DrKIn-II. As shown in Fig. 1B, DrKIn-II bound to plasmin, but not tPA and uPA.

3.3. DrKIn-II is a tight binding and time-dependent inhibitor of plasmin

The ability of DrKIn-II to inhibit plasmin was assayed with a synthetic chromogenic substrate, S-2251. As shown in Fig. 2A, DrKIn-II potently inhibited the amidolytic activity of plasmin in a dose-dependent manner. The Hill coefficient, which represents the steepness of the dose–response curve, was estimated to be 2.2, indicating the tight binding nature of inhibition. A non-linear regression fit of the fractional velocity data to Morrison's competitive tight binding equation yielded an inhibition constant (K_i^*) of 0.19 ± 0.04 nM for DrKIn-II (Fig. 2B). Since the progress curves for DrKIn-II-mediated plasmin inhibition displayed a quasi-linear relationship with time (Fig. 2C), the effect of DrKIn-II concentration on the apparent first-order rate constant k_{obs} was determined. As shown in Fig. 2D, k_{obs} varied as a hyperbolic function of DrKIn-II concentration in accordance with the mechanism shown in Scheme 1. This indicates that the initial rapid binding of DrKIn-II to plasmin causes the enzyme to isomerize slowly which leads to a much tighter binding between the enzyme and the inhibitor. The values of k_2 and k_{-2}, obtained from the plot of k_{obs} versus DrKIn-II concentration, were 0.043 ± 0.003 s^{-1} and 0.0022 ± 0.0010 s^{-1}, respectively. The equilibrium dissociation constant of the initial loose enzyme-inhibitor complex (K_i) was found to be 6.7 ± 1.5 nM, which was higher than the overall inhibition constant (K_i^*) of 0.3 ± 0.1 nM determined from the values of k_2, k_{-2} and K_i. The value of K_i^* also compared very well with that obtained through the fit of steady-state data to Morrison's equation.

3.4. DrKIn-II exhibits antifibrinolytic activity

The antifibrinolytic activity of DrKIn-II was assayed on a fibrin plate (Fig. 3A). In the absence of DrKIn-II, plasmin degraded the fibrin clot in a time-dependent manner. The addition of DrKIn-II significantly inhibited the formation of clear area on the fibrin plate, confirming

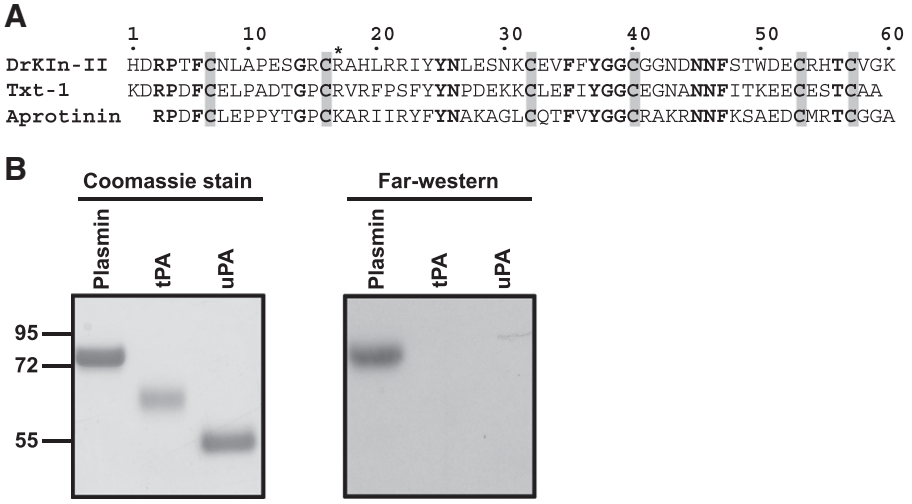

Fig. 1. A. Amino acid sequence alignment of DrKIn-II with textilinin-1 and aprotinin. Bold letters denote identical amino acids. Conserved cysteine residues are highlighted in gray, and the P1 position is marked by an asterisk. B. Analysis of binding between DrKIn-II and plasmin. Left: Coomassie staining of plasmin, tPA and uPA (2 μg each) after SDS-PAGE under non-reducing conditions. Right: the protein bands were blotted onto a PVDF membrane and probed with 1 μg/ml of biotinylated DrKIn-II. The interaction was detected with the ExtrAvidin Peroxidase system.

the antifibrinolytic property of DrKIn-II. Next, we performed euglobulin clot lysis assay in the presence of different concentrations of DrKIn-II and compared the antifibrinolytic effect of DrKIn-II with aprotinin. As expected, DrKIn-II prolonged the clot lysis time in a dose-dependent manner (Fig. 3B). DrKIn-II at a concentration of 100 nM prolonged the clot lysis time by almost 10 h, and at a concentration of 500 nM, DrKIn-II completely abrogated the lysis of the euglobulin clot. At room temperature, no lysis was observed even after 48 h when 500 nM of DrKIn-II was added (data not shown). However, at all inhibitor concentrations, aprotinin exhibited better antifibrinolytic activity compared to DrKIn-II.

3.5. Specificity of DrKIn-II

In order to determine the specificity of DrKIn-II, the inhibitory activity of DrKIn-II was screened against trypsin and also against serine proteases in the coagulation and fibrinolytic systems using their respective chromogenic substrates. As shown in Fig. 4A, DrKIn-II at the same molar

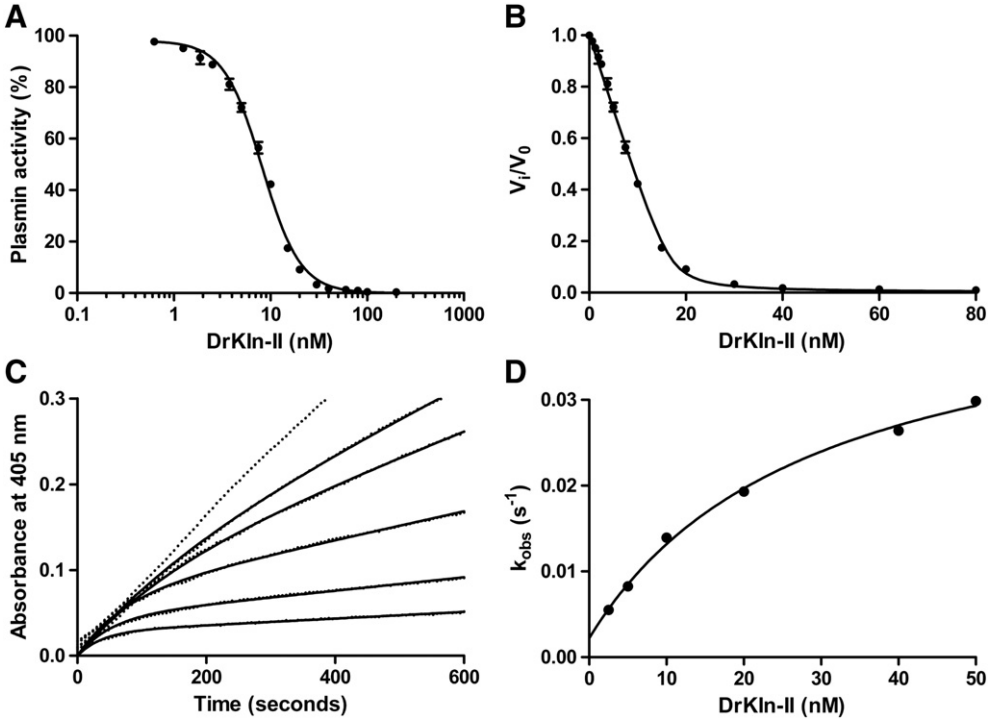

Fig. 2. Kinetic analyses of DrKIn-II. A. Dose–response curve of plasmin inhibition by DrKIn-II. Initial velocities of plasmin (20 nM) were measured using S-2251 (0.2 mM) as substrate in the presence of increasing concentrations of DrKIn-II (0–200 nM). B. Non-linear regression fit of the same fractional velocity data to Morrison's competitive tight binding equation, yielding an inhibition constant (K_i^*) of 0.19 ± 0.04 nM. C. Progress curves for the inhibition of plasmin. The enzyme (20 nM) was added to a mixture containing S-2251 (1 mM) and different concentrations of DrKIn-II (0, 2.5, 5, 10, 20 and 40 nM). Black traces represent the best fits to Eq. (3) from which the apparent first-order rate constants (k_{obs}) were obtained. D. Plot of k_{obs} as a function of DrKIn-II concentration. The black line represents the best fit to Eq. (4) according to the mechanism shown in Scheme 1.

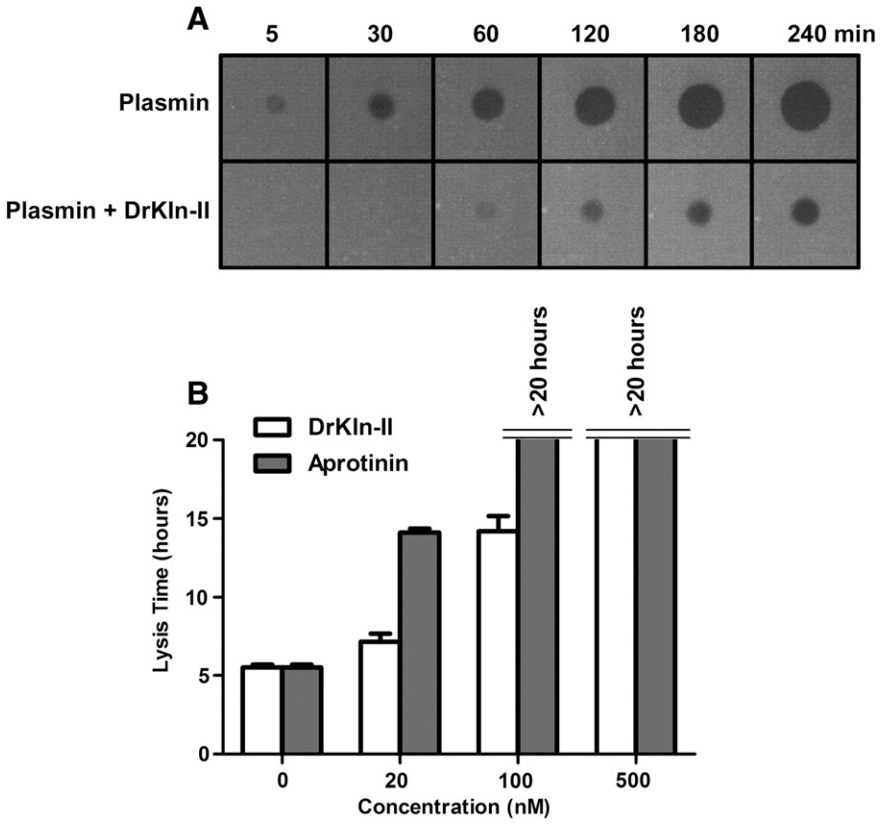

Fig. 3. DrKIn-II exhibits antifibrinolytic activity. A. The degradation of fibrin by plasmin (0.5 μg) was monitored on a fibrin plate for various periods of time at 37 °C in the absence or presence of DrKIn-II (0.5 μg). B. Different concentrations of DrKIn-II or aprotinin were added to the euglobulin fraction before coagulation was initiated with 25 mM $CaCl_2$. Clot lysis was measured spectrophotometrically at 340 nm. Results shown are means ± S.D. of three experiments.

concentration as the enzyme active site inhibited primarily plasmin, with a percent inhibition of approximately 90%. Apart from plasmin, DrKIn-II also inhibited the amidolytic activities of trypsin (70% inhibition), FXIa (37% inhibition) and FXa (20% inhibition). While DrKIn-II at a final concentration of 1 μM prolonged the activated partial thromboplastin time by 32 s, DrKIn-II failed to prolong the prothrombin time at all the concentrations tested (Fig. 4B), implying that DrKIn-II, even at high concentrations, has absolutely no inhibitory effect on serine proteases in the extrinsic pathway of coagulation. These results suggest that plasmin is the primary physiological target of DrKIn-II.

3.6. Effect of DrKIn-II on FDP levels in mice

Since FDP is produced through the degradation of fibrinogen or fibrin by plasmin, FDP should be a good indicator of fibrinolysis *in vivo*. In order to induce fibrinolysis, a sub-lethal dose of FX activator from Russell's viper venom (RVV-X) was injected into ICR mice. As shown in Fig. 5A, there was a 3-fold increase in FDP level after RVV-X injection. However, with the co-injection of DrKIn-II, the rise in FDP level was abolished.

3.7. Effect of DrKIn-II on tail bleeding time

Given that DrKIn-II exhibits antifibrinolytic activities *in vivo*, we tested the ability of DrKIn-II to reduce blood loss in a murine tail bleeding model. As expected, DrKIn-II treatment significantly reduced the median bleeding time from 180 s to 43 s (Fig. 5B). Combined, our results demonstrate that DrKIn-II is a potent antifibrinolytic agent *in vivo* and has the potential to reduce massive blood loss in clinical settings.

4. Discussion

DrKIn-II is not the only plasmin inhibitor isolated from Russell's viper venom. In 1974, Takahashi *et al.* described a very similar Kunitz-type protease inhibitor that differed from DrKIn-II by seven amino acid substitutions, namely G18A, K33E, A44D, E48S, R50W, E55H and G58V [26]. However, apart from testing the effects of this inhibitor on the amidolytic activities of plasmin and other enzymes [27], no additional assays were performed. More recently, a trypsin inhibitor was purified from the venom of *Daboia russelii siamensis* that differed from DrKIn-II by only one threonine to serine substitution at position 49 [8]. However, its functional role has not been determined. This is therefore the first time that a plasmin inhibitor from Russell's viper venom has been characterized in relation to its binding mechanism and therapeutic potential, both *in vitro* and *in vivo*.

Our kinetic analyses indicate that DrKIn-II is a slow and tight binding inhibitor of plasmin. Equimolar concentration of DrKIn-II inhibited the amidolytic activity of plasmin by approximately 90%. The K_i^* value of DrKIn-II for plasmin inhibition (~0.2 nM) is at least 10-fold lower than that of textilinin-1 ($K_i^* = 3.5$ nM) [28]. DrKIn-II is also more potent than those Kunitz inhibitors derived from invertebrates like *Bombus ignitus* (bumblebee) and *Araneus ventricosus* (spider) [29,30]. To observe a slow onset of inhibition, the overall inhibition constant K_i^* must be less than the dissociation constant of the initial loose enzyme-inhibitor complex K_i [21]. In our experiment, the K_i^* value was at least 20 times lower than the K_i value. The low values of k_2 and k_{-2} (0.043 s^{-1} and 0.0022 s^{-1}, respectively) also confirmed the slow transition from EI to $E*I$ (the final isomerized complex). In fact, textilinin-1 also displayed a similar mode of inhibition [11]. This type of time-dependent inhibition probably gives these venom-derived Kunitz inhibitors the potency comparable to that of irreversible suicide inhibitors and increases their efficacy by extending the dissociative half-

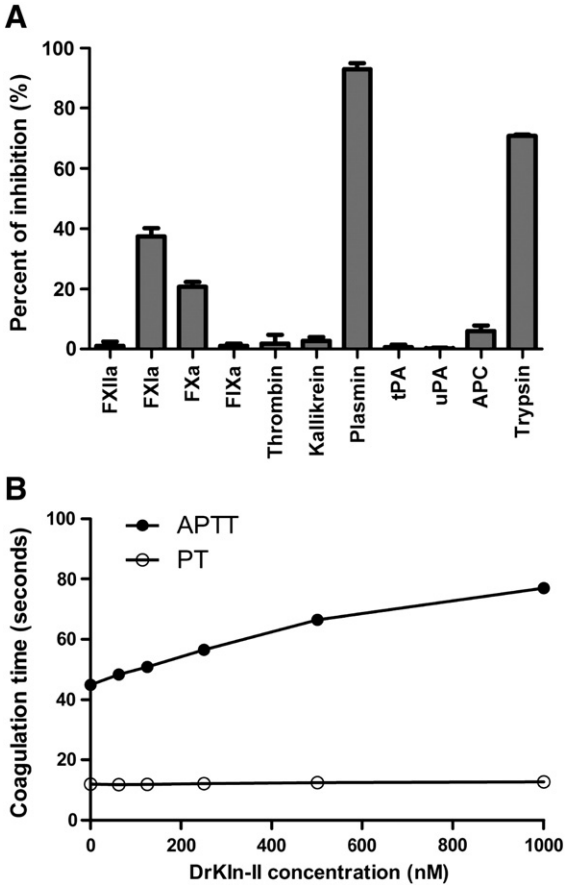

Fig. 4. Selectivity analyses of DrKIn-II. A. The inhibitory activity of DrKIn-II was screened against trypsin and also against serine proteases in the coagulation and fibrinolytic systems. In each test, equal molar concentrations of protease active site and DrKIn-II were mixed. The final concentrations of these proteases and their respective substrates were as follows: FXIIa (20 nM)/S-2302 (0.2 mM), FXIa (2.5 nM)/S-2366 (0.2 mM), FXa (10 nM)/S-2222 (1.3 mM), FIXa (200 nM)/Spectrozyme FIXa (1.3 mM), thrombin (5 nM)/T-1637 (0.2 mM), kallikrein (5 nM)/S-2302 (0.2 mM), plasmin (20 nM)/S-2251 (0.2 mM), tPA (80 nM)/Spectrozyme tPA (0.2 mM), uPA (100 nM)/S-2288 (1.3 mM), APC (10 nM)/Spectrozyme PCa (0.2 mM) and trypsin (5 nM)/S-2222 (0.2 mM). Values are means ± S.D. of triplicate determinations. B. APTT (●) and PT (○) of normal plasma in the presence of the indicated concentrations of DrKIn-II.

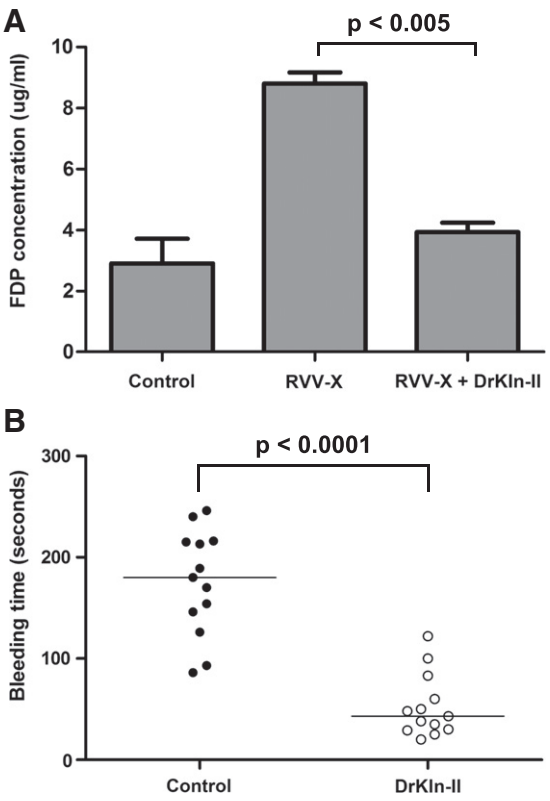

Fig. 5. DrKIn-II demonstrates antifibrinolytic activities in vivo. A. Animals were injected with RVV-X (0.01 μg/g) alone or together with DrKIn-II (0.1 μg/g) and the FDP levels were determined 8 h after injection. Results shown are means ± SEM (n = 5 for each group). B. Effect of DrKIn-II (0.1 μg/g) injection on murine tail bleeding time. Control animals received an equal volume of PBS. Horizontal lines represent the median values (n = 13 for each group).

life of the enzyme-inhibitor complex [31,32]. As a potential therapeutic agent, this two stage mode of inhibition also has the added advantage of longer binary complex residence time, the period for which the enzyme is occupied by an inhibitor [32].

Besides inhibiting the amidolytic activity of plasmin, DrKIn-II also bound to plasmin (Fig. 1B) and demonstrated antifibrinolytic activity in fibrin plate assay and euglobulin clot lysis assay (Fig. 3). Although aprotinin showed better antifibrinolytic activity compared with DrKIn-II, aprotinin was withdrawn from the market in 2008 due to its treatment associated side effects such as acute renal failure, myocardial infarction, stroke, encephalopathy and increased risk of death [3,4,33]. As suggested by Millers *et al.*, the negative side effects of aprotinin may stem from its overall lack of specificity for target proteases and its virtually irreversible inhibition of plasmin [34]. The increased incidence of postoperative thrombosis has also been attributed to the inhibition of nitric oxide synthesis and the impairment of endothelial function [35]. Currently, the only therapeutic antifibrinolytic agents are the lysine analogs, aminocaproic acid and tranexamic acid, which form reversible complexes with plasminogens and displace them from the fibrin surfaces [36]. However, these lysine analogs inhibit only the activation of plasmin and have no inhibitory effect on plasmin itself. DrKIn-II may therefore be viewed as a potential candidate to replace aprotinin as an antifibrinolytic agent. However, whether DrKIn-II has any therapeutic advantages over aprotinin remains to be elucidated. Recently, a recombinant form of textilinin-1, named Q8008, has gone into preclinical development [37]. Q8008 has the added benefit of fewer off-target effects and is at least as effective as aprotinin in reducing blood loss *in vivo*. The addition of DrKIn-II to the list of Kunitz-type plasmin inhibitors should further aid in the design of drugs that have greater potency and better pharmacological profiles.

From our results, it seems that DrKIn-II is not a very specific inhibitor of plasmin, since it inhibited the amidolytic activity of FXIa and prolonged the activated partial thromboplastin time. Textilinin-1, in contrast with DrKIn-II, does not prolong APTT [28]. The presence of a bulkier valine as the P1′ residue in textilinin-1, as opposed to the smaller alanine in DrKIn-II, probably contributes to the narrower specificity of textilinin-1 [38]. However, we can still propose that plasmin is the physiological target of DrKIn-II. The K_i of DrKIn-II for plasmin (~0.2 nM) is approximately 30-fold lower than that for FXIa (~6 nM, data not shown). Given that the physiological concentration of plasminogen (1.3 μM) is much higher than that of FXI (0.03 μM) [39,40], we can safely speculate that the vast majority of DrKIn-II in the blood stream would associate with plasmin and not FXIa. Even though DrKIn-II inhibited the amidolytic activity of FXa, it failed to even slightly prolong the prothrombin time in which FXa played a major role. This suggests that the interaction of serine proteases with each other and also with other plasma proteins and/or lipids may hinder the binding of DrKIn-II, making the inhibitor much more specific for plasmin than for any other enzymes in the blood stream.

The ability of DrKIn-II to prevent the increase in FDP concentration after coagulation induction with RVV-X indicates that DrKIn-II inhibits plasmin *in vivo*. It also suggests that DrKIn-II acts as a procoagulant in Russell's viper venom which also contains factor V and X activators [41]. DrKIn-II may also synergize with DrKIn-I, an APC inhibitor from

the same venom [17]. DrKIn-II, compared to saline injection, also effectively reduced the bleeding time in our murine tail bleeding model ($p < 0.0001$). Assuming that each mouse has a total blood volume of 2 ml, the final concentration of DrKIn-II injected should be approximately 200 nM. The low effective concentration of DrKIn-II for hemostasis indicates that DrKIn-II is a potent antifibrinolytic agent *in vivo*.

Taken together, we have discovered a Kunitz-type protease inhibitor from Russell's viper venom that acts as a slow and competitive tight binding inhibitor of plasmin. DrKIn-II employs the two stage mode of inhibition which involves the slow isomerization of the enzyme after binding to the inhibitor. Besides nullifying the activity of plasmin in fibrin plate assays, DrKIn-II also significantly prolonged the euglobulin clot lysis time and reduced the tail bleeding time in mice. Our *in vitro* and *in vivo* data thus open up further therapeutic possibilities for DrKIn-II as an antifibrinolytic agent.

Acknowledgements

This work was supported by grants from Academia Sinica and the National Science Council (NSC 99-2311-B-001-016-MYs).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] E.F. Plow, T. Herren, A. Redlitz, L.A. Miles, J.L. Hoover-Plow, The cell biology of the plasminogen system, FASEB J. 9 (1995) 939–945.
[2] S.E. Fremes, B.I. Wong, E. Lee, R. Mai, G.T. Christakis, R.F. McLean, B.S. Goldman, C.D. Naylor, Metaanalysis of prophylactic drug treatment in the prevention of postoperative bleeding, Ann. Thorac. Surg. 58 (1994) 1580–1588.
[3] D.T. Mangano, I.C. Tudor, C. Dietzel, The risk associated with aprotinin in cardiac surgery, N. Engl. J. Med. 354 (2006) 353–365.
[4] D.T. Mangano, Y. Miao, A. Vuylsteke, I.C. Tudor, R. Juneja, D. Filipescu, A. Hoeft, M.L. Fontes, Z. Hillel, E. Ott, T. Titov, C. Dietzel, J. Levin, Mortality associated with aprotinin during 5 years following coronary artery bypass graft surgery, JAMA 297 (2007) 471–479.
[5] R.J. Lewis, M.L. Garcia, Therapeutic potential of venom peptides, Nat. Rev. Drug Discov. 2 (2003) 790–802.
[6] A. Ritonja, V. Turk, F. Gubensek, Serine proteinase inhibitors from *Vipera ammodytes* venom. Isolation and kinetic studies, Eur. J. Biochem. 133 (1983) 427–432.
[7] L. Chang, C. Chung, H.B. Huang, S. Lin, Purification and characterization of a chymotrypsin inhibitor from the venom of *Ophiophagus hannah* (King Cobra), Biochem. Biophys. Res. Commun. 283 (2001) 862–867.
[8] C.T. Guo, S. McClean, C. Shaw, P.F. Rao, M.Y. Ye, A.J. Bjourson, Trypsin and chymotrypsin inhibitor peptides from the venom of Chinese *Daboia russellii siamensis*, Toxicon 63 (2012) 154–164.
[9] Y.Y. He, S.B. Liu, W.H. Lee, J.Q. Qian, Y. Zhang, Isolation, expression and characterization of a novel dual serine protease inhibitor, OH-TCI, from king cobra venom, Peptides 29 (2008) 1692–1699.
[10] J. Lu, H. Yang, H. Yu, W. Gao, R. Lai, J. Liu, X. Liang, A novel serine protease inhibitor from *Bungarus fasciatus* venom, Peptides 29 (2008) 369–374.
[11] P.P. Masci, A.N. Whitaker, L.G. Sparrow, J. de Jersey, D.J. Winzor, D.J. Watters, M.F. Lavin, P.J. Gaffney, Textilinins from *Pseudonaja textilis textilis*. Characterization of two plasmin inhibitors that reduce bleeding in an animal model, Blood Coagul. Fibrinolysis 11 (2000) 385–393.
[12] D.J. Strydom, Snake venom toxins. Purification and properties of low-molecular-weight polypeptides of *Dendroaspis polylepis polylepis* (black mamba) venom, Eur. J. Biochem. 69 (1976) 169–176.
[13] V. Zupunski, D. Kordis, F. Gubensek, Adaptive evolution in the snake venom Kunitz/BPTI protein family, FEBS Lett. 547 (2003) 131–136.
[14] M. Laskowski Jr., I. Kato, Protein inhibitors of proteinases, Annu. Rev. Biochem. 49 (1980) 593–626.
[15] E. Katoh, H. Nishio, T. Inui, Y. Nishiuchi, T. Kimura, S. Sakakibara, T. Yamazaki, Structural basis for the biological activity of dendrotoxin-I, a potent potassium channel blocker, Biopolymers 54 (2000) 44–57.
[16] S. Chaki, M. Muramatsu, Y. Ushiyama, S. Otomo, Purification and partial characterization of K^+ channel blockers from the venom of *Dendroaspis angusticeps*, Neurochem. Int. 20 (1992) 553–558.
[17] A.C. Cheng, H.L. Wu, G.Y. Shi, I.H. Tsai, A novel heparin-dependent inhibitor of activated protein C that potentiates consumptive coagulopathy in Russell's viper envenomation, J. Biol. Chem. 287 (2012) 15739–15748.
[18] D.A. Warrell, Snake venoms in science and clinical medicine. 1. Russell's viper: biology, venom and treatment of bites, Trans. R. Soc. Trop. Med. Hyg. 83 (1989) 732–740.
[19] A.K. Mukherjee, S.K. Ghosal, C.R. Maity, Some biochemical properties of Russell's viper (*Daboia russelli*) venom from Eastern India: correlation with clinico-pathological manifestation in Russell's viper bite, Toxicon 38 (2000) 163–175.
[20] R.A. Copeland, Tight binding inhibitors, in: R.A. Copeland (Ed.), Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, John Wiley & Sons, Inc., New York, 2000, pp. 305–317.
[21] R.A. Copeland, Time dependent inhibition, in: R.A. Copeland (Ed.), Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, John Wiley & Sons, Inc., New York, 2000, pp. 318–349.
[22] S. Flight, L. Johnson, M. Trabi, P. Gaffney, M. Lavin, J. de Jersey, P. Masci, Comparison of textilinin-1 with aprotinin as serine protease inhibitors and as antifibrinolytic agents, Pathophysiol. Haemost. Thromb. 34 (2005) 188–193.
[23] H. Fritz, G. Wunderer, Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs, Arzneimittelforschung 33 (1983) 479–494.
[24] H.M. Kang, M.H. Kalnoski, M. Frederick, W.L. Chandler, The kinetics of plasmin inhibition by aprotinin in vivo, Thromb. Res. 115 (2005) 327–340.
[25] C.H. Yuan, Q.Y. He, K. Peng, J.B. Diao, L.P. Jiang, X. Tang, S.P. Liang, Discovery of a distinct superfamily of Kunitz-type toxin (KTT) from tarantulas, PLoS One 3 (2008) e3414.
[26] H. Takahashi, S. Iwanaga, T. Kitagawa, Y. Hokama, T. Suzuki, Snake venom proteinase inhibitors. II. Chemical structure of inhibitor II isolated from the venom of Russell's viper (*Vipera russelli*), J. Biochem. 76 (1974) 721–733.
[27] H. Takahashi, S. Iwanaga, T. Suzuki, Snake venom proteinase inhibitors. I. Isolation and properties of two inhibitors of kallikrein, trypsin, plasmin, and alpha-chymotrypsin from the venom of Russell's viper (*Vipera russelli*), J. Biochem. 76 (1974) 709–719.
[28] S.M. Flight, L.A. Johnson, Q.S. Du, R.L. Warner, M. Trabi, P.J. Gaffney, M.F. Lavin, J. de Jersey, P.P. Masci, Textilinin-1, an alternative anti-bleeding agent to aprotinin: importance of plasmin inhibition in controlling blood loss, Br. J. Haematol. 145 (2009) 207–211.
[29] Y.M. Choo, K.S. Lee, H.J. Yoon, Y. Qiu, H. Wan, M.R. Sohn, H.D. Sohn, B.R. Jin, Antifibrinolytic role of a bee venom serine protease inhibitor that acts as a plasmin inhibitor, PLoS One 7 (2012) e32269.
[30] H. Wan, K.S. Lee, B.Y. Kim, F.M. Zou, H.J. Yoon, Y.H. Je, J. Li, B.R. Jin, A spider-derived Kunitz-type serine protease inhibitor that acts as a plasmin inhibitor and an elastase inhibitor, PLoS One 8 (2013) e53343.
[31] J. Singh, R.C. Petter, T.A. Baillie, A. Whitty, The resurgence of covalent drugs, Nat. Rev. Drug Discov. 10 (2011) 307–317.
[32] R.A. Copeland, D.L. Pompliano, T.D. Meek, Drug-target residence time and its implications for lead optimization, Nat. Rev. Drug Discov. 5 (2006) 730–739.
[33] A.D. Shaw, M. Stafford-Smith, W.D. White, B. Phillips-Bute, M. Swaminathan, C. Milano, I.J. Welsby, S. Aronson, J.P. Mathew, E.D. Peterson, M.F. Newman, The effect of aprotinin on outcome after coronary-artery bypass grafting, N. Engl. J. Med. 358 (2008) 784–793.
[34] E.K. Millers, L.A. Johnson, G.W. Birrell, P.P. Masci, M.F. Lavin, J. de Jersey, L.W. Guddat, The structure of human microplasmin in complex with textilinin-1, an aprotinin-like inhibitor from the Australian brown snake, PLoS One 8 (2013) e54104.
[35] S. Ulker, P.P. McKeown, U. Bayraktutan, Aprotinin impairs coronary endothelial function and down-regulates endothelial NOS in rat coronary microvascular endothelial cells, Cardiovasc. Res. 55 (2002) 830–837.
[36] M. Verstraete, Clinical application of inhibitors of fibrinolysis, Drugs 29 (1985) 236–261.
[37] S.T. Earl, P.P. Masci, J. de Jersey, M.F. Lavin, J. Dixon, Drug development from Australian elapid snake venoms and the Venomics pipeline of candidates for haemostasis: Textilinin-1 (Q8008), Haempatch (Q8009) and CoVase (V0801), Toxicon 59 (2012) 456–463.
[38] E.K. Millers, M. Trabi, P.P. Masci, M.F. Lavin, J. de Jersey, L.W. Guddat, Crystal structure of textilinin-1, a Kunitz-type serine protease inhibitor from the venom of the Australian common brown snake (*Pseudonaja textilis*), FEBS J. 276 (2009) 3163–3175.
[39] G. Leipnitz, C. Miyashita, M. Heiden, G. von Blohn, M. Kohler, E. Wenzel, Reference values and variability of plasminogen in healthy blood donors and its relation to parameters of the fibrinolytic system, Haemostasis 18 (Suppl. 1) (1988) 61–68.
[40] A.V. Hoffbrand, J.E. Pettit, P.A.H. Moss, Chapter 18: platelets, blood coagulation and haemostasis, Essential Haematology, Blackwell Science Ltd., 2001. 235–249.
[41] T. Than, R.A. Hutton, L. Myint, H. Khin Ei, S. Soe, S. Tin Nu, R.E. Phillips, D.A. Warrell, Haemostatic disturbances in patients bitten by Russell's viper (*Vipera russelli siamensis*) in Burma, Br. J. Haematol. 69 (1988) 513–520.