A Novel Heparin-dependent Inhibitor of Activated Protein C That Potentiates Consumptive Coagulopathy in Russell’s Viper Envenomation*

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Background: The reason behind the development of consumptive coagulopathies in Russell’s viper bite patients is still elusive.
Results: An inhibitor of activated protein C (a major physiological anticoagulant) synergizes with the venom’s procoagulating enzymes.
Conclusion: Down-regulation of activated protein C in Russell’s viper envenomation is associated with consumptive coagulation.
Significance: The discovery provides new insights into the pathogenesis of Russell’s viper venom-induced coagulopathies.

The activation of coagulation factors V and X by Russell’s viper venom (RVV) has been implicated in the development of consumptive coagulopathies in severely envenomed patients. However, factor Va is prone to inactivation by activated protein C (APC), an important serine protease that negatively regulates blood coagulation. It is therefore hypothesized that APC may be down-regulated by some of the venom components. In this study, we managed to isolate a potent Kunitz-type APC inhibitor, named DrKIn-I. Using chromogenic substrate, DrKIn-I dose-dependently inhibited the activity of APC. Heparin potentiated the inhibition and reduced the IC50 of DrKIn-I by 25-fold. DrKIn-I, together with heparin, also protected factor Va from APC-mediated inactivation. Using surface plasmon resonance, DrKIn-I exhibited fast binding kinetics with APC (association rate constant = 1.7 × 107 M−1 s−1). Direct binding assays and kinetic studies revealed that this inhibition (Ki = 53 pM) is due to the tight binding interactions of DrKIn-I with both heparin and APC. DrKIn-I also effectively reversed the anticoagulant activity of APC and completely restored the thrombin generation in APC-containing plasma. Furthermore, although the injection of either DrKIn-I or RVV-X (the venom factor X-activator) into ICR mice did not significantly deplete the plasma fibrinogen concentration, co-administration of DrKIn-I with RVV-X resulted in complete fibrinogen consumption and the deposition of fibrin thrombi in the glomerular capillaries. Our results provide new insights into the pathogenesis of RVV-induced coagulopathies and indicate that DrKIn-I is a novel APC inhibitor that is associated with potentially fatal thrombotic complications in Russell’s viper envenomation.

Envenomation by Russell’s vipers has long been a serious health threat in Pakistan, India, Bangladesh, Sri Lanka, and many parts of Southeast Asia (1, 2). The most striking features of these envenomed patients are spontaneous bleeding and incoagulable blood, which result from disseminated intravascular coagulation (DIC)2 or consumptive coagulopathy induced by procoagulant factors in Russell’s viper venom (RVV). Thrombocytopenia and hypofibrinogenemia are therefore evident in systemically envenomed patients (3, 4).

Despite the fact that the procoagulant nature of RVV has been recognized for decades, our knowledge of the responsible components in this venom still lingers on factor X-activating enzyme (RVV-X) and factor V-activating enzyme (RVV-V) (3). The activated factors X (FXa) and V (FVa), in the presence of calcium and phospholipids, form a prothrombinase complex that catalyzes the conversion of prothrombin to α-thrombin (5). It is, however, difficult for coagulations to spread uncontrollably because there are physiological anticoagulant mechanisms that oppose the widespread formation of α-thrombin (6). Furthermore, animal studies have shown that although RVV-X is strongly procoagulant in vitro, it failed to effectively decrease the plasma fibrinogen concentrations in vivo (7). It is, therefore, unlikely that RVV-X and RVV-V are solely responsible for the coagulopathies seen in Russell’s viper envenomed patients. Based on the severity of bleeding disorders seen in these patients, we hypothesized that RVV may contain proteins that interfere with the negative regulations of blood coagulation.

Significance: The discovery provides new insights into the pathogenesis of Russell’s viper venom-induced coagulopathies.

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The abbreviations used are: DIC, disseminated intravascular coagulation; RVV-Russell’s viper venom; FXa, factor Xa; FVa, factor Va; PC, protein C; APC, activated protein C; FVIIIa, factor VIIIa; BPTI, bovine pancreatic trypsin inhibitor; DrKIn-I, D. russelli Kunitz inhibitor-I; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phosphoserine; SPR, surface plasmon resonance; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; APTT, activated partial thromboplastin time.
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The protein C (PC) pathway, which becomes activated by the thrombin-thrombomodulin complex, represents a major physiological anticoagulant component in which the activated protein C (APC) functions by proteolytically inactivating activated coagulation factors V (FVa) and VIII (FVIIIa) (8). Although there are several other physiological anticoagulants such as antithrombin III, heparin cofactor II and tissue factor pathway inhibitor that either inhibit thrombin directly or prevent the activation of prothrombin (9–11), APC remains the only serine protease that is involved in anticoagulation. Since Viperidae snake venoms are rich in serine protease inhibitors belonging to the Kunitz/bovine pancreatic trypsin inhibitor (BPTI) family (12), it is tempting to speculate that some members of this little understood protein family in RVV might target APC to promote the extensive coagulations seen in severely envenomed patients.

In this study, we describe the isolation and kinetic characterization of a Kunitz-type protease inhibitor named DrKIn-I (Daboia russelii Kunitz Inhibitor-I) that possesses strong inhibitory activity against APC in the presence of heparin, in both purified system and in plasma. We demonstrate that it binds tightly to both APC and heparin. Moreover, we show that the presence of this inhibitor greatly exacerbates coagulation and hypofibrinogenemia induced by RVV-X in mice. These findings may necessitate the use of APC or protein C concentrates in Russell’s viper bite patients, and should offer insights into better treatments for these patients.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized venom of Daboia russelii russelii (Pakistan) was purchased from Latoxan. Purified human activated protein C, protein S, factor XIIa (FXaIIa), factor XIa (FXia), factor Xa (FXa), factor IXa (FIXa), factor VIIa (FIXia), factor Va (FVa), thrombin, plasma kalliurein, and plasmin were obtained from Hematologic Technologies. Trypsin and tissue plasminogen activator (tPA) were from Merck Chemicals. Urokinase plasminogen activator (uPA) was a kind gift from Polyamine Corp. Synthetic chromogenic substrates Spectrozyme PCa, Spectrozyme tPA, and Spectrozyme FXa were purchased from American Diagnostica, while S-2222, S-2302, S-2366, S-2288, and S-2251 were from Chromogenix. T-1637 was from Sigma-Aldrich. RVV-X was prepared from our laboratory according to the method provided by Chen et al. (13). Unfractionated heparin and heparan sulfate were from Sigma-Aldrich, while heparan sulfate dimers, trimers, hexamers and octamers were gifts from Dr. Hung Shang-Cheng (Genomics Research Center, Academia Sinica, Taiwan). Synthetic phospholipids 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were bought from Avanti Polar Lipids. Normal coagulation control plasma and antithrombin/heparin cofactor II immune-depleted plasma were from HYPHEN Biomed and Enzyme Research Laboratories, respectively. The CM5 sensor chip for surface plasmon resonance (SPR) analysis was purchased from GE Healthcare. ICR mice were purchased from BioLASCO and housed in a pathogen free environment. All animal experiments were approved by the Academia Sinica Institutional Animal Care and Utilization Committee.

In Vitro Assays for the Inhibition of APC by DrKIn-I—All inhibition assays were performed in 96-wells microtitrater plates in 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM CaCl2, and 5 mg/ml BSA. For comparison between DrKIn-I and DrKIn-II, the amidolytic activity of 10 nM APC, with or without 0.1 units/ml heparin, was assayed in the presence or absence of equal molar concentrations of DrKIn-I or DrKIn-II. Immediately after the addition of APC, Spectrozyme PCa was added and the rates of p-nitroaniline release were monitored at 405 nm for 10 min at 37 °C. For dose response curves, APC was mixed with different concentrations of DrKIn-I in the presence or absence of heparin. The final concentrations were as follows: APC (10 nM), heparin (0.1 U/ml) and DrKIn-I (0–100 nM in the presence of heparin and 0–12800 nM in the absence of heparin). Dose-response curves were fitted using GraphPad Prism (GraphPad Software). In other inhibition experiments, varying concentrations of heparin (0–1000 mU/ml) or different lengths of heparan sulfate chains (10 μg/ml) were added to equimolar concentrations of APC and DrKIn-I (20 nM each). In all the inhibition experiments, Spectrozyme PCa was added to a final concentration of 0.2 mM. Changes in absorbance were measured using SpectraMax M2* Microplate Reader (Molecular Devices).

In assays involving FVα, 20 nM purified FVα was incubated at 37 °C with a mixture containing 1 nM or 5 nM APC, 20 nM protein S, 20 μM DOPC/DOPS (75:25) and 5–250 nM DrKIn-I...
in the presence or absence of 0.1 U/ml heparin. At specific time intervals, 5 μl aliquots were removed and mixed with 50 μl of FV-deficient plasma. The residual FVa activities were quantified in a standard prothrombin time-based assay using a calibration curve obtained by adding variable amounts of FVa to FV-deficient plasma. All concentrations given were final concentrations.

**Heparin Binding Assay**—A 5 ml HiTrap Heparin HP column (GE Healthcare) that had been pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0) was loaded with 70 μg of DrKln-I. After washing with 5 ml of equilibrating buffer, a 50 ml gradient from 0.0–1.0 M NaCl was applied at a flow rate of 5 ml/min and the salt concentration corresponding to the protein peak was determined as a measure of its heparin binding affinity.

**Surface Plasmon Resonance Analysis**—Biacore T200 (GE Healthcare) was used for analysis. APC or PC dissolved in 10 mM acetate buffer (pH 5.0) was immobilized on a CM5 sensor chip to a response unit (RU) of 1000 with an amine coupling kit. Associations and dissociations of DrKln-I were performed in 10 mM HEPE (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.05% P20 lo f2 0 mM CaCl2. Measurements were taken at 1 min intervals on a coagulometer (Hemostasis Analyzer KC-1; Sigma Diagnostics).

**Thrombin Generation Assay**—The procoagulant function of DrKln-I in heparin-containing plasma was assessed by thrombin generation assay. Briefly, 80 μl of antithrombin/heparin cofactor II-deficient plasma containing 4 μM corn trypsin inhibitor, 0.1 units/ml heparin and 30 mM DOPC/DOPS (75:25) was incubated with 20 μl of 500-fold diluted tissue factor solution (Innovin; Dade Behring) in the presence or absence of APC and/or DrKln-I (20 nM each). Following incubation for 3 min at 37 °C, thrombin generation was initiated by the dispensation of 20 μl of 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC.HCl) dissolved in 0.15 M NaCl, 60 mg/ml BSA, and 100 mM CaCl2. Measurements were taken at 1 min intervals on a SpectroMax M2 Microplate Reader using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Results were evaluated using the Technothrombin TGA software (Technoclone).

**In Vivo Assays**—8–10-week-old ICR mice were used in our experiments. RVV-X and/or DrKln-I were administered intravenously at the indicated concentrations. 3 h after injection, plasma was collected and analyzed for fibrinogen concentrations. Briefly, 100 μl of 10-fold diluted plasma was mixed with 50 μl of 75 NIH units/ml thrombin (Trinity Biotech) and the time required for clot formation was measured. Fibrinogen levels were determined based on a calibration curve prepared from a fibrinogen reference (HYPHEN Biomed).

For histopathological examinations, mice were injected intravenously with 0.005 μg/g RVV-X and/or 0.04 μg/g DrKln-I. The mice were anesthetized after 3 h with pentobarbital, and the kidneys were extracted. Fixed and paraffin-embedded kidneys were sectioned at 6 μm and subjected to hematoxylin-eosin staining. The slides were scanned by ScanScope CS System (Aperio Technologies) with a 20×/0.75 Plan APO objective, and images were analyzed using the Aperio Imagescope software (version 9.1.19.1569).

**RESULTS**

**Purification and Cloning of Kunitz-type Protease Inhibitors**—In view of the fact that Kunitz-type protease inhibitors are relatively small with a length of only ~60 amino acids (12), the crude venom of *Daboia russelii russelii* was first separated into several fractions based on their molecular sizes by gel filtration. The fifth fraction (indicated by a horizontal bar in Fig. 1A) was...
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A, 20 mg of Daboia russelli russelli crude venom was dissolved in 0.1 M ammonium acetate buffer (pH 6.5) and fractionated by gel filtration using FPLC. The fractions containing Kunitz-type protease inhibitors (indicated by a horizontal bar) were pooled together and lyophilized. B, subsequent fractionation using reversed-phase HPLC. A linear gradient of 20–50% acetonitrile was applied over a period of 60 min. The protein peaks corresponding to DrKIn-I and DrKIn-II were indicated. C, amino acid sequences of DrKIn-I and DrKIn-II. Non-identical amino acids are denoted in bold letters.

then subjected to a second purification step using reversed-phase HPLC. The first two proteins that were eluted (designated DrKIn-I and DrKIn-II) had approximate yields of 1.7% (w/w) and 2.6% (w/w), respectively (Fig. 1B). The masses of these two proteins were determined by MALDI-TOF analysis, which gave an m/z signal of 7548.9 Da for DrKIn-I and an m/z signal of 6940.3 Da for DrKIn-II. The molecular weights of these two proteins were exactly identical to that of the two signal of 6940.3 Da for DrKIn-II. The molecular weights of 

Daboia russelii russelii

identical amino acids are denoted in bold letters. 

Then both inhibitors exhibited little inhibitory activity against APC. However, in the presence of 0.1 units/ml heparin (180 units/mg), DrKIn-I decreased the activity of APC by 100%, while DrKIn-II decreased the activity by only ~20%. Of the two Kunitz-type protease inhibitors purified, only DrKIn-I showed a potent inhibitory activity against APC. Dose-response curve of DrKIn-I obtained in the presence of heparin showed that the increase in inhibition occurred over a very narrow range of DrKIn-I concentration, as denoted by a large Hill slope of ~3.64 ± 0.30 (Fig. 2B). This indicates that DrKIn-I is a tight binding inhibitor of APC in the presence of heparin where the K_d is much lower than the enzyme concentration (16). Furthermore, complete inhibition was achieved for equimolar concentrations of APC and DrKIn-I (Fig. 2B). In contrast, the dose-response curve obtained in the absence of heparin was less steep, with a Hill slope of ~0.85 ± 0.02 (Fig. 2B). The IC_{50} values in the presence and absence of heparin were 3.5 ± 0.2 nm and 88.9 ± 1.0 nm, respectively.

To determine the concentration of heparin required for the potentiation of APC inhibition, the enzyme-inhibitor mixture was spiked with varying concentrations of heparin. 0.01 units/ml of heparin potentiated the inhibition by more than 70% (Fig. 2C), and at 0.1 units/ml, no APC activity was detectable, suggesting that only low concentrations of heparin are required for APC inhibition.

Apart from heparin, we also tested the ability of heparan sulfate to potentiate DrKIn-I-mediated APC inhibition, since heparan sulfate is structurally similar to heparin, and is abundant as part of proteoglycans on the surface of endothelial cells (17). As shown in Fig. 2D, heparan sulfate can also act as a cofactor for APC inhibition. Furthermore, while heparan sulfate dimers and tetramers enhanced the inhibition by only ~10 and ~25%, respectively, heparan sulfate hexamers enhanced the inhibition by ~80%, suggesting that heparan sulfate chains should be at least 6 units long for sufficient potentiation of APC inhibition (Fig. 2D).

In addition to using the synthetic tripeptide (Spectrozyme PCa) as the substrate of APC, we also tested the inhibitory activity of DrKIn-I using FVα, APC’s natural substrate. In the absence of heparin, APC (1 nm) progressively degraded FVα (20 nm) over a period of 10 min (Fig. 2E). The addition of DrKIn-I (5 nm) alone had relatively no effect on APC activity. However, in the presence of heparin, DrKIn-I (5 nm) protected 100% of FVα from inactivation (Fig. 2F). Without heparin, the addition of a 50-fold molar excess of DrKIn-I (250 nm) protected only less than 20% of FVα from inactivation (Fig. 2F), confirming that heparin is absolutely essential for DrKIn-I-mediated APC inhibition. Regardless of the type of substrate used, heparin alone at 0.1 units/ml did not alter the activity of APC (Fig. 2A and F).

Physical Interactions of DrKIn-I with Heparin and APC—Because it has been reported that APC possesses a heparin-binding site that allows it to physically interact with heparin (18), what remains to be characterized is the binding of DrKIn-I with both heparin and APC. The binding of DrKIn-I to heparin was assessed using a heparin-Sepharose column. As expected, DrKIn-I bound to the heparin column with a very high affinity (Fig. 3A). The inhibitor eluted at 0.95 M NaCl, which was three times higher than that required for APC elution (19).
Next, we investigated the physical interactions between DrKIn-I and APC using surface plasmon resonance. DrKIn-I concentrations between 0.78 and 6.25 nM were flowed across an APC-coated CM5 sensor chip. DrKIn-I bound to immobilized APC with a $K_d$ of $2.6 \pm 0.8$ nM (Fig. 3B). The association rate constant was determined to be $1.3 \pm 0.8 \times 10^{7} \text{M}^{-1} \text{s}^{-1}$, while the dissociation rate constant was found to be $3.4 \pm 2.2 \times 10^{-2} \text{s}^{-1}$. Interestingly, no binding was observed between DrKIn-I and the immobilized protein Czymogen (Fig. 3B, inset).

**Determination of the Inhibition Constant of DrKIn-I**—Although DrKIn-I binds to APC in the absence of heparin, its effect on APC-mediated FVa degradation was negligible. The inhibition constant ($K_i$) of DrKIn-I was therefore determined only in the presence of heparin. By fitting the inhibition curves globally to Morrison’s competitive tight binding equation, DrKIn-I was found to inhibit APC with a $K_i$ of $53 \pm 39$ pM (Fig. 4). Although the plot of fractional velocity against inhibitor concentration showed overlapping inhibition curves for all the substrate concentrations tested (0.025–0.4 mM) (Fig. 4 inset), addition of a very high substrate concentration (3.3 mM) diminished...
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The procoagulant nature of DrKIn-I in the presence of heparin was further confirmed by thrombin generation assay. As the absence of heparin, APC (40 nM) prolonged the clotting time of normal plasma by ~7-fold (Fig. 6A). Although DrKIn-I attenuated the prolongation, its effect was relatively small. However, when exogenous heparin was added, the clotting time was effectively shortened (Fig. 6A). To exclude the synergistic effect of antithrombin and heparin cofactor II on heparin-induced prolongation of clotting time, we also tested the APC inhibitory activity of DrKIn-I in antithrombin/heparin cofactor II-deficient plasma. Similar to the results obtained with normal plasma, DrKIn-I restored the clotting time to that of the control level only when heparin was added (Fig. 6B).

The procoagulant nature of DrKIn-I in the presence of heparin was further confirmed by thrombin generation assay. As
expected, although DrKIn-I itself did not alter the thrombin generation profile, it completely restored the generation of thrombin in APC-containing plasma (Fig. 6C). Combined, these results suggest that DrKIn-I is able to specifically target APC in plasma, an environment where dozens of other serine proteases exist.

**DrKIn-I Aggravates Hypofibrinogenemia and Coagulation Induced by RVV-X in Mice**—Patients severely envenomed by RVV often develop bleeding disorders due to the consumption of coagulation factors, the most prominent of which is fibrinogen. We therefore speculated that DrKIn-I, being a potent inhibitor of APC, should potentiate the procoagulant effect of RVV-X. In line with our hypothesis, DrKIn-I did not decrease the plasma fibrinogen level when the stimulus (RVV-X) was excluded (Fig. 7A). However, DrKIn-I significantly and dose-dependently decreased the level of fibrinogen in mice when RVV-X (0.02 μg/g) was co-injected with the inhibitor (Fig. 7A). Furthermore, co-injection of RVV-X with higher doses of DrKIn-I (≥ 0.08 μg/g) led to immediate death (data not shown).

Because renal failure is the most common complication in envenomed patients (22), a histopathological examination of the kidneys was conducted, and renal glomeruli, the basic blood filtration units which determine the overall function of the kid-

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**FIGURE 6.** DrKIn-I nullifies the effect of APC in heparin-containing plasma. Purified APC (40 mM) was added to normal plasma (A) or antithrombin III and heparin cofactor II-deficient plasma (B) containing varying concentrations of DrKIn-I (40, 80, 160 mM) in the presence or absence of heparin (0.1 units/ml). After incubation for 1 min at 37 °C, plasma APTT was measured. Clotting times were compared with the control experiments performed in the absence of DrKIn-I and heparin. Results shown are means ± S.D. of three experiments. C, effect of 40 mM DrKIn-I and/or 40 mM APC on thrombin generation was assessed in the presence of 0.1 units/ml heparin as described in “Experimental Procedures.” Representative thrombin generation curves are shown.

**FIGURE 7.** DrKIn-I potentiates the procoagulant activity of RVV-X. A, ICR mice were injected intravenously with the indicated concentrations of RVV-X and/or DrKIn-I. Plasma fibrinogen concentrations were determined 3 h after injection. Each error bar represents S.E. about the mean (n = 4). B, histological sections of hematoxylin-and-eosin stained kidneys of mice administered with normal saline (i), 0.04 μg/g DrKIn-I (ii), 0.005 μg/g RVV-X (iii), or 0.04 μg/g DrKIn-I plus 0.005 μg/g RVV-X (iv). Images were obtained using the ScanScope CS System. Original magnification × 400.
DISCUSSION

Russell’s vipers are responsible for most of the snakebite incidents in many parts of South and Southeast Asia where the majority of the patients often suffer from mild to severe coagulopathies, depending on the extent of envenomation (2). It is, therefore, important to fully understand the causes of these coagulopathies so that effective treatments could be given. In this study, we have demonstrated that besides having RVV-X and RVV-V (proteases that activate FX and FV, respectively) as the main procoagulating components in the venom, RVV also contains DrKIn-I, a heparin-dependent tight binding inhibitor of APC that effectively potentiates coagulation in RVV-X-stimulated mice.

DrKIn-I, a member of the snake Kunitz/BPTI family, consists of 66 amino acids, with three conserved disulphide linkages to stabilize the overall structure (12). DrKIn-I is unique among all the other Kunitz-type protease inhibitors in that it is extraordinarily basic (predicted pI = 9.6), with two putative heparin-binding motifs in its C-terminal region (49TRKKCRQ55 and 40PRKGRPS25) (23, 24). The presence of these -XBBXXBX- and -XBBXBX- regions (where X represents uncharged amino acids and B represents basic amino acids) probably contributes to the high affinity of DrKIn-I toward heparin and allows heparin to potentiate the inhibition of APC by DrKIn-I. This is supported by our findings that although DrKIn-I and DrKIn-II are highly identical, with a percent identity of 71%, DrKIn-II, which lacks the heparin-binding motifs, showed no affinity toward heparin column (data not shown). Consequently, heparin was unable to enhance the inhibition of APC by DrKIn-II.

Although the exact mechanism for DrKIn-I-mediated APC inhibition in the presence of heparin has not been elucidated, the absence of a bell-shaped response curve in the plot of APC activity versus heparin concentration (Fig. 2C) suggests that DrKIn-I does not employ the typical template mechanism whereby both the protease and the inhibitor bind to a heparin molecule in proximity to each other (25). Furthermore, DrKIn-I only requires a length of 6 saccharide units to enhance the inhibition of APC by ~80% (Fig. 2D). This is in contrast with the typical template mechanism which requires heparin molecules to be at least 18 saccharide units long (26). The ability of heparan sulfate hexamers to act as cofactors for APC inhibition also suggests a non-template based mechanism, since 6 saccharide units would be insufficient to bridge both the protease and the inhibitor.

The reason for the presence of APC inhibitors in the D. russelli species is not hard to understand, as it is known that uncomplexed FVa (those that are not bound to FXa or prothrombin) are prone to enzymatic degradation by APC (27, 28). It is, therefore, reasonable from the evolutionary point of view for RVV to not only activate FV, but also to protect the activated cofactor (FVa) from APC-mediated inactivation. The presence of DrKIn-I ensures that there is always a constant supply of FVa for the formation of prothrombinase complexes with FXa. Although APC inactivates both FVa and FVIIIa, we have not tested whether DrKIn-I protects FVIIIa from degradation. This is because RVV induces coagulations primarily through the common pathway. In addition, factor IX (the protease that complexes with FVIIIa) is rarely activated or consumed in envenomed patients (3), suggesting that relative to FVa, FVIIIa plays only a minor role in RVV-induced coagulopathies seen in these patients.

Over the years, several plasma APC inhibitors belonging to the serpin family have been found, including protein C inhibitor and α1-antitrypsin (29, 30). In the absence of heparin, both serpins inhibit APC slowly, with second order rate constants of $2.5 \times 10^7 M^{-1} s^{-1}$ and $1.0 \times 10^4 M^{-1} s^{-1}$, respectively. DrKIn-I, however, is the first APC inhibitor discovered that belongs to the Kunitz/BPTI family. It differs from the serpin-type APC inhibitors in that it is not a slow-binding inhibitor. Using the synthetic substrate, DrKIn-I inhibited the amidolytic activity of APC as soon as it was added to the enzyme. While heparin enhances the second order rate constant of protein C inhibitor by 30–230-fold (29, 31), the binding between APC and DrKIn-I is intrinsically fast, with an association rate constant of ~1.3 $\times 10^7 M^{-1} s^{-1}$. These differences suggest that DrKIn-I is the only inhibitor discovered that exhibits fast-binding kinetics with APC. The low $K_i$ of ~53 ± 39 pm suggests that DrKIn-I, in the presence of heparin, may be the most potent APC inhibitor found to date.

The selectivity profile of DrKIn-I suggests that besides APC, the inhibitor may also target FXIa and plasmin. However our kinetic analyses indicate that the $K_i$ for APC inhibition is at least 25-fold lower than that for FXIa and plasmin inhibition. Although high concentrations of DrKIn-I would invariably prolong the APTT clotting time (probably due to the inhibition of FXIa), our plasma experiments indicate that at concentrations below 160 nM, the effect of DrKIn-I on APTT was negligible (Fig. 6A). Furthermore, we have also performed euglobulin clot lysis assays on both DrKIn-I and aprotinin (a well known plasmin inhibitor) (32) to assess the plasmin inhibitory activity of DrKIn-I. Whereas 20 nM aprotinin prolonged the euglobulin clot lysis time by ~9 h, 20 nM of DrKIn-I failed to prolong the clot lysis time, either in the presence or absence of heparin (data not shown). At a concentration of 100 nM, aprotinin prolonged the clot lysis time by more than 16 h, while DrKIn-I only prolonged the clot lysis time by approximately an hour. Combined, these data support our hypothesis that among all the serine proteases tested, APC is the preferential target of DrKIn-I.

In our experiments, we have demonstrated that both heparin and heparan sulfates can act as cofactors for APC inhibition. It is, however, uncertain which of the two plays a more important role. Since heparan sulfates are abundant on the surface of endothelial cells (17), it is speculated that heparan sulfates may play a more important role compared with heparin. Although the endogenous heparin level in plasma has been reported to be in the range of 1.0–2.4 μg/ml or 0.1–0.2 units/ml (33, 34), these heparin molecules are already bound with other plasma
proteins and are therefore unavailable to act as cofactors for APC inhibition (33). The need to add heparin to our plasma experiments (Fig. 6, A and B) also indicates that most of these heparin molecules are already in a complexed state with other proteins. However, it has also been documented that mast cells can degranulate and release heparin when stimulated with RVV (35), and that these heparin molecules can enhance the activity of RVV-X, causing an increase in the activation of FV by nearly 4-fold (36). Furthermore, endogenous heparin levels have been shown to increase in response to hemorrhagic shock (37). Metz et al. also recently showed that mast cells do degranulate and release carboxypeptidase A in response to snake and honeybee venom exposure (38). These references therefore suggest that besides heparan sulfate, heparin molecules secreted by mast cells also play an important role in the pathogenesis of Russell’s viper envenomation.

The importance of APC in maintaining the patency of blood vessels has been well documented (39). It has been suggested that APC is particularly important in regulating coagulations in the microcirculation, as small blood vessels and capillaries have higher concentrations of thrombomodulin compared with larger vessels (39). Homozygous PC deficiency often results in DIC and microvascular thrombosis (purpura fulminans) in newborn infants (40). Furthermore, in a primate sepsis model, it was found that PC depletion resulted in complete fibrinogen consumption, organ failure, and death when the animals were stimulated with an otherwise nonlethal dose of E. coli (41). In view of the above references, it is not surprising that DrKIn-I can induce fibrinogen consumption and the formation of fibrin thrombi in the glomerular capillaries when co-administered with low doses of RVV-X (Fig. 7). Consistent with our findings, Rapaport et al. reported that administration of RVV-X alone into normal rabbits did not result in significant fibrinogen depletion (7). Although he demonstrated that fibrinogen consumption could only be achieved by RVV-X in antithrombin-immunodepleted rabbits, antithrombin was found to be the only anticoagulant that was within the normal range in Russell’s viper bite patients (3). Our results therefore help to explain why severely envenomed patients often develop bleeding complications and suggest that APC, and not antithrombin, is the major anticoagulant that is down-regulated in these patients.

Histopathologically, fibrin thrombi were observed in the glomerular capillaries of mice that received both DrKIn-I and RVV-X. The presence of these glomerular thrombi corroborated with the depletion of fibrinogen, suggesting that these mice might be experiencing DIC-like symptoms (42). Since APC deficiency is associated with microvascular thrombosis and that DIC is always manifested with renal dysfunction (42), it is speculated that the occlusion of glomerular capillaries induced by DrKIn-I-mediated APC inhibition may be the cause of acute renal failure. Although it has been documented that RVV-induced renal failure may be due to the direct nephrotoxic components in the venom (43), other studies have shown that intravascular clotting and hemolysis may be the main cause of renal failures in these patients (44, 45). In support of our hypothesis, it was found that none of the envenomed patients who did not develop DIC suffered from renal failures (46). Furthermore, Gupta et al. demonstrated using a rat model of endotoxemia that APC could significantly improve the renal blood flow in rats with acute renal failure (47). These evidences suggest that DrKIn-I, through the inhibition of APC, may intensify the kidney damage induced by the procoagulating enzymes in RVV.

The present study stresses on the importance of APC in RVV-induced coagulopathies because this physiological anticoagulant is inhibited by DrKIn-I from the same venom with lethal consequences. From our results, we can imply that if APC is replenished in Russell’s viper bite patients, the concentrations of RVV-X and RVV-V needed for the induction of consumptive coagulopathy would be much higher. Since severely envenomed patients develop bleeding disorders (fibrinogen consumption and microvascular occlusion with fibrin thrombi) similar to that seen in patients with severe sepsis and homozgyous PC deficiency (40, 41), administration of PC concentrate (Ceprotin) or recombinant APC (Drotrecogin α-activated or Xigris) before the consumption of coagulation factors should be able to pacify, if not prevent, the development of DIC or DIC-like symptoms in envenomed patients (48, 49). This, in addition to treatments with antivenom, should reduce fibrin depositions in the glomerular capillaries, and should subsequently ameliorate the damage done to the kidneys. The discovery of a fast and tight binding inhibitor of APC therefore provides new insights into the pathogenesis of RVV-induced coagulopathies, which may form a basis for the administration of APC or PC concentrates in Russell’s viper bite patients.

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REFERENCES
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