Intraspecies differences in hemostatic venom activities of the South American rattlesnakes, *Crotalus durissus cumanensis*, as revealed by a range of protease inhibitors

Ana M. Salazar[a], Irma Aguilar[b], Belsy Guerrero[a], María E. Girón[b], Sara Lucena[a], Elda E. Sánchez[c] and Alexis Rodríguez-Acosta[b]

Crotalus durissus cumanensis is an endemic rattlesnake found in Venezuela and Colombia. In this study, a comparative analysis of hemorrhagic, coagulation and fibrino(geno)lytic activities in the presence or absence of protease inhibitors was performed with venoms of the same species _Crotalus durissus cumanensis_, from seven geographical regions of Venezuela (Lagunetica, Santa Teresa, Carrizales, Guarenas, Anzoátegui, Margarita and Maracay). Lagunetica, Carrizales and Anzoátegui venoms induced hemorrhagic activity. All venoms, except that of snakes from the Carrizales region presented thrombin-like activity, which was inhibited completely by phenylmethanesulfonyl fluoride and ethylene glycol-bis-_N_, _N_,_N′_,_N′_-tetraacetic acid. This effect of the latter could be explained by the high chelant calcium effect, which is a cofactor for the fibrin polymerization process. Soybean trypsin inhibitor was effective on Santa Teresa venom. Antithrombin III/Hep complex and phenantroline partially inhibited this activity in all venoms except Margarita and Anzoátegui, respectively, which were not inhibited. Serine protease inhibitors were more effective against thrombin, kallikrein and plasmin-like amidolytic activities. Additionally, metalloprotease inhibitors significantly inhibited the t-PA-like amidolytic activity and completely the hemorrhagic and fibrino(geno)lytic activities. In conclusion, the thrombin-like activity observed in these venoms was partially reduced by serine protease inhibitors, indicating the possible presence of catalytic domains in those enzymes that do not interact with these inhibitors. Using protease inhibitors on venom hemostatic activities could contribute to our understanding of the active components of snake venom on the hemostatic system, and further reveal the intraspecies variation of venoms, which is important in the treatment of envenomation, and in addition represents an interesting model for the study of venom in hemostasis. _Blood Coagul Fibrinolysis_ 19:525–530 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Blood Coagulation and Fibrinolysis 2008, **19**:525–530

Keywords: coagulation, _Crotalus durissus cumanensis_, fibrino(geno)lytic, hemorrhage, hemostasis, protease inhibitors, rattlesnakes

[a]Physiopathology Laboratory, Experimental Medicine Center, Venezuelan Institute of Scientific Research (IVIC), [b]Immunochemistry Section, Tropical Medicine Institute of the Central University of Venezuela, Caracas, Venezuela and [c]Natural Toxins Research Center, Texas A&M University-Kingsville, Kingsville, Texas, USA

Correspondence to Alexis Rodríguez-Acosta, MD, PhD, Apartado 47423, Caracas 1041, Venezuela
Tel: +58 212 6053505; e-mail: rodriguezacosta1946@yahoo.es

Received 3 October 2007 **Revised** 13 March 2008
Accepted 15 April 2008

Introduction

Snake venoms have a vast variety of components that display enzymatic and nonenzymatic activities, leading to important changes in hemostasis, which are seen after snakebites [1–3]. Snake venom toxins that influence hemostasis have been categorized by many effects such as anticoagulant, coagulant (prothrombin activators, thrombin-like enzymes), fibrino(geno)lytic, hemorrhagic and antiplatelet activities. Several investigators have analyzed many of these activities [4–9]; however, the study of the effects of protease inhibitors on snake venom serine and metalloproteases are scarce, and has not been fully studied.

The biochemical and biological actions of venoms may diverge among families, genera and even within a given species. Venom components are found to vary in compliance with ontogenetic, ecobiology and individual factors [10]. For instance, venom intraspecies variability has been demonstrated in *Bothrops atrox* [9] and *Crotalus durissus cumanensis* [11]. In the current work, we compared venom hemorrhagic, coagulant and fibrino(geno)lytic activities in the presence of protease inhibitors to further reveal intra-populational variability in *C. durissus cumanensis* snakes, and to further elucidate the types of components found in snake venom that affect the hemostatic system.

Materials and methods

Reagents

Chromogenic substrates and antithrombin III were obtained from Chromogenix AB (Milano, Italy). Molecular mass standards for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad Laboratories Ltd. (Richmond, California, USA). Human fibrinogen (11% w/w of plasminogen as contaminant), bovine alpha thrombin and single chains u-PA (scu-PA) were obtained from American Diagnostica Inc. (Greenwich, Connecticut, USA). Heparin, leupeptin,

0957-5235 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

aprotinin, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenantroline, benzamidine/HCl, ethylene glycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and other reagents used in this study were obtained from Sigma Chemical Co (St. Louis, Missouri, USA).

Animals and venoms

Albino Swiss NIH strain male mice between 18 and 22 g were obtained from the Instituto Nacional de Higiene 'Rafael Rangel', Caracas, Venezuela. The investigation complies with the bioethical norms taken from the guide 'Principles of laboratory animal care' [12].

Crotalus durissus cumanensis venoms from adult snakes were obtained from Lagunetica, Santa Teresa, Carrizales, and Guarenas (Miranda state); Anzoátegui (Anzoátegui state); Margarita (Nueva Esparta state); Maracay (Aragua state) Venezuelan geographical locations. All venoms were pooled samples (six individual adults) of the same geographical location.

Protein concentration determination

The protein concentration was determined by the method of Lowry *et al.* [13].

Hemorrhagic activity

The method of Omori-Satoh *et al.* [14] was used to establish the minimal hemorrhagic dose (MHD) for crude venoms. MHD is defined as the amount of venom protein that causes a 10 mm hemorrhagic spot.

Coagulant activity on plasma

The coagulant activity of crude venoms and fractions was assayed by a modified method of Austen and Rhymes [15]. Briefly, 0.1 ml of fresh citrate human plasma was incubated in a borosilicate tube at 37°C for 1 min. Then 0.1 ml of 0.05 mol/l Tris–HCl buffer (coagulation buffer, pH 7.4) and 0.1 ml venom sample (diluted in coagulation buffer) or 0.1 ml thrombin were added to the plasma or fibrinogen. Time-of-clot formation was recorded. The thrombin-like activity was determined by plotting the clotting times against a calibration curve prepared with a standard thrombin (National Institute for Biological Standards and Control, London, England).

Amidolytic activity

Amidolytic activity of crude venoms was measured by a micromethod standardized in our laboratory [16]. Chromogenic substrates 0.6 mmol/l S-2238, 0.8 mmol/l S-2222, 0.8 mmol/l S-2251, 1.2 mmol/l S-2288, 0.3 mmol/l S-2444 and 0.16 mmol/l S-2586 (final concentrations) were assayed.

Fibrinolytic activity

Fibrinolytic activity of crude venoms was studied by the fibrin plate method as proposed by Marsh and Arocha-Piñango [17]. Fibrinolytic activity was expressed as the diameter of the lysed area per microgram of protein (mm^2/μg).

Fibrinogenolytic activity

The effect on fibrinogen molecule of proteases presented in Carrizales, Anzoátegui and Lagunetica crude venoms was evaluated. Briefly, fibrinogen was incubated with venom at a 2.5 μg venom/100 μg fibrinogen ratio, for 4 h at 37°C. The degradation of the fibrinogen molecule was visualized by SDS-PAGE on a 5% gel under non-reduced conditions by Tris–Tricine-system [18].

Effect of inhibitors

The inhibition of all the activities evaluated was performed by using a pool of serine protease inhibitors at 50 μg/ml soybean trypsin inhibitor (SBTI), 10 mmol/l PMSF, 10 mmol/l benzamidine/HCl, and 100 IU/ml aprotinine (final concentrations), except the plasmin activity that was assayed in the presence of 50 μg/ml SBTI, 100 IU/ml aprotinine and 10 mmol/l leupeptin. Metalloprotease inhibitors were used as a mixture of 10 mmol/l EGTA-Na and 10 mmol/l 1,10-phenantroline (final concentrations). Inhibition of venom thrombin-like coagulant activity (4 IU/ml) also was performed by incubation at 37°C for 30 min with a mixture of antithrombin III (2 IU/ml) and Heparin (50 IU/ml) [9,19].

Statistical analysis

The activities were statistically described according to their mean and standard deviation. Variance analysis was examined by a Kruskal–Wallis one-way analysis of variance (ANOVA) followed by Dunńs post test, and Student's *t* test. Results represent mean ± SD ($n = 4$). Values of $P = 0.05$ or less were considered statistically significant (GraftPad Prism, San Diego, California, USA).

Results

Coagulant activity

The serine protease and metalloprotease inhibitors and antithrombin III/heparin complex (ATIII/Hep) on thrombin-like coagulant activity was evaluated using citrated human plasma. In this study, 16% of 3.68 IU/ml standard thrombin was inhibited by SBTI. The results indicated that thrombin-like venom activity (4 IU/ml) was completely abolished by phenylmethanesulphonyl-fluoride or phenylmethylsulphonyl fluoride (PMSF) and EGTA; in contrast, the ATIII/Hep inhibited this activity between 20 and 31%, except in Margarita venom, where the activity was not significantly altered. Phenantroline inhibited this activity between 13 and 52%, except in Anzoátegui venom, where the activity was not significantly altered. This activity was partially inhibited by SBTI (30%) only in Santa Teresa venom (Table 1).

Fibrinolytic activity

The fibrinolytic activity evaluated by rich fibrin plate-plasminogen method demonstrated that Lagunetica and

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Table 1 Coagulant activity of *Crotalus durissus cumanensis* venoms in the presence of inhibitors, using purified human fibrinogen as substrate

	Coagulant activity (% inhibition)							
Venoms locality	ATIII/Hep		PMSF	SBTI		Phen		EGTA
Lagunetica	30.5 ± 0.1	$^{*}P < 0.01$	Inc.	0		28.1 ± 0.6		Inc.
Santa Teresa	19.6 ± 0.2		Inc.	30.0 ± 0.2	$^{*}P < 0.01$	51.5 ± 0.5	$^{**}P < 0.005$	Inc.
Guarenas	23.5 ± 0.4		Inc.	0		47.9 ± 0.5		Inc.
Anzoátegui	22.9 ± 0.1		Inc.	0		3.0 ± 0.5		Inc.
Aragua	21.4 ± 0.4		Inc.	6.4 ± 0.1		37.6 ± 0.1		Inc.
Carrizales	ND		ND	ND		ND		ND
Margarita	1.4 ± 0.2		Inc.	3.3 ± 0.1		12.6 ± 0.2		Inc.

Data are expressed as mean ± SD ($n = 4$). Coagulant activity was determined by the Austen and Rhymes method [15]. The results are expressed in percentage inhibition comparing the residual activity with initial activity (4 IU-ml). Venoms were preincubated with ATIII/Hep, PMSF, SBTI, phenantroline and EGTA for 30 min at 37°C. EGTA, ethylene glycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid; Inc., incoagulable; ND, not determined; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor. $^{*}P < 0.01$; $^{**}P < 0.05$: the most inhibited venom.

Carrizales venoms presented the highest activity, whereas Santa Teresa and Margarita venoms were the less active ones. The results obtained in the presence of inhibitors demonstrate that the fibrinolytic activity (a dose that gives a lysis area of 150 mm^2) was significantly neutralized by metalloprotease inhibitors in all venoms. Additionally, the serine protease inhibitors abolished this activity in Santa Teresa, Aragua and Margarita venoms (Table 2).

Amidolytic activity

All venoms showed thrombin-like amidolytic activity. In addition, the venoms did not present factor Xa-like amidolytic activity. Venoms also showed a t-PA, kallikrein and plasmin-like activities. The urokinase-like activity was insignificant. Carrizales presented the lowest amidolytic activities on all substrates. In contrast, Santa Teresa and Margarita had the highest amidolytic activities.

In all venoms, serine protease inhibitors significantly ($P < 0.05$) reduced the kallikrein-like (0.1 UA/min per μg), thrombin-like (4 IU/ml), t-PA-like (5×10^3 IU/ml) and plasmin-like (7 nkat/ml) amidolytic activities between 64 and 78%, 70 and 91%, 36 and 67% and 87 and 94%, respectively. However, metalloprotease inhibitors did not significantly affect these amidolytic activities, except the t-PA-activity, which reduced between 84 and 96% (Table 3).

Table 2 Effect of inhibitors on fibrinolytic activity observed in *Crotalus durissus cumanensis* venoms

	Fibrinolytic activity on fibrin plate[a] mm^2/10 μg	Percentage inhibition	
Venom locality	Buffer	SPI[b]	MPI[c]
Lagunetica	41.3 ± 2.3	8.0 ± 0.5	100
Santa Teresa	11.7 ± 0.9	100	100
Guarenas	18.7 ± 1.6	15.0 ± 0.7	68.0 ± 2.8
Anzoátegui	20.5 ± 1.6	0	100
Aragua	30.0 ± 2.0	100	100
Carrizales	49.8 ± 2.6	4.0 ± 0.4	100
Margarita	5.7 ± 1.0	100	60.0 ± 2.3

Data are expressed as mean ± SD ($n = 4$). MPI, metalloprotease inhibitors; SPI, serine protease inhibitors. [a] Fibrinolytic activity was evaluated on plasminogen rich-fibrin plates using Marsh and Arocha-Piñango method [17]. [b] Venoms preincubated with SPI. [c] Venoms preincubated with MPI.

Fibrinogenolytic activity

Studies performed with the purified human fibrinogen treated with Lagunetica or Carrizales venoms (noncoagulant activity), under nonreduced conditions showed an increase of the fibrinogen molecule electrophoretic mobility with a well defined degradation fragment between 66 and 205 kDa. Other fragment traces were observed to be between 29 and 14 kDa. The Anzoátegui venom (with the lowest thrombin-like activity) produced a lower electrophoretic mobility alteration, and fragments of higher molecular mass were absent. The degradation of fibrinogen molecule was completely inhibited by metalloprotease inhibitors (Fig. 1). Serine protease inhibitors did not modify the degradation pattern (data not shown).

Hemorrhagic activity

MHD present in Lagunetica, Anzoátegui and Carrizales venoms were of 4.1, 16.2 and 14.3 μg/mouse, respectively. In contrast, Santa Teresa, Guarenas, Aragua, and Margarita did not present hemorrhagic activity. Metalloprotease inhibitors completely neutralized this hemorrhagic activity, whereas the serine protease inhibitors were not significantly effective in neutralizing this activity (Fig. 2).

Discussion

Differences in habitat, age, diet and amount of venom production, and venom activities are common among snakes [10,20] and are accompanied by significant differences in the relative activity of venom components on their natural and synthetic substrata [9,11].

Crotalus venoms contain serine and metalloproteases, several of which participate in the pathogenesis of tissue alterations in the bitten site and hemorrhages in tissues far from the site of the bite, such as kidneys, heart, lungs and the central nervous system [21,22]. In the current

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Table 3 Effects of inhibitors on amidolytic activity observed in *Crotalus durissus cumanensis* venoms

	Amidolytic activity (% inhibition)					
	S-2238 (Thrombin)		S-2302 (Kallikrein)	S-2251 (Plasmin)		S-2288 (t-PA)
Inhibitors substrates	[a]ATIII/Hep	[b]SPI	[b]SPI	[b]SPI	[b]SPI	[c]MPI
Venom locality						
Lagunetica	51.4 ± 0.4	70.2 ± 3.3	65.1 ± 1.5	94.3 ± 4.5 *$P < 0.01$	43.1 ± 3.5	85.6 ± 4.0
Santa Teresa	58.9 ± 1.3	85.6 ± 2.4	75.8 ± 0.5	88.1 ± 2.5	42.9 ± 2.2	84.5 ± 5.9
Guarenas	57.9 ± 1.3	83.4 ± 0.7	77.4 ± 0.6	89.9 ± 3.0	37.8 ± 2.8	87.7 ± 6.1
Anzoátegui	56.2 ± 0.3	91.1 ± 0.5 **$P < 0.01$	74.2 ± 1.0	87.4 ± 2.9	35.8 ± 2.5	86.2 ± 5.7
Aragua	50.7 ± 0.3 *$P < 0.01$	82.7 ± 3.1	76.8 ± 0.7	88.5 ± 3.5	37.9 ± 2.1	89.1 ± 6.0
Carrizales	52.3 ± 0.5	83.3 ± 1.0	77.5 ± 0.8 **$P < 0.05$	91.5 ± 3.1	39.6 ± 3.7	92.7 ± 5.5
Margarita	62.3 ± 2.8	89.9 ± 0.8	64.2 ± 0.6	91.1 ± 3.5	67.2 ± 5.7 *$P < 0.01$	96.4 ± 5.6 *$P < 0.01$

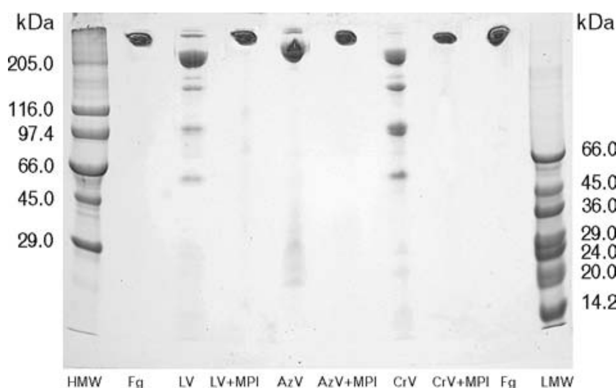
Data are expressed as mean ± SD ($n = 4$). MPI, metalloprotease inhibitors; SPI, serine protease inhibitors. [a] Venoms preincubated with antithrombin III/heparin. [b] Venoms preincubated with SPI. [c] Venoms preincubated with MPI. * $P < 0.01$; ** $P < 0.05$: most inhibited venom.

work, we evaluated the coagulant, fibrinolytic and hemorrhagic activities of seven *C. durissus cumanensis* venoms in the presence and absence of protease inhibitors. The use of protease inhibitors against snake venom active components could demonstrate the different biochemical behavior of individual venoms of the same species, which would reveal that the intraspecific venom differences exist among individual snakes. Venom differences among and within the same species are important in research for antivenom production.

The venoms contain metalloproteases, which produce severe bleeding by interfering with hemostatic processes or by degrading the basement membrane or extracellular matrix components of blood vessels [4]. The evaluation of the fibrinolytic and hemorrhagic activities of venoms (Table 2 and Fig. 2), demonstrated that Santa Teresa, Margarita, Aragua and Guarenas venoms presented fibrinolytic activity but no hemorrhagic activity. Furthermore, Lagunetica and Carrizales (with high fibrinolytic activity) and Anzoátegui (with moderate fibrinolytic activity) contained hemorrhagic activity. Metalloprotease inhibitors (MPIs) neutralized hemorrhagic activity in all venoms (EGTA > 1,10-phenantroline), indicating that the hemorrhagins belonged to the metalloproteases family.

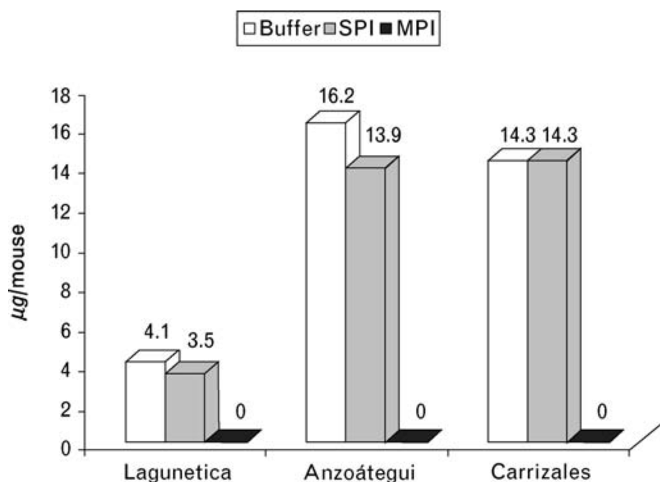
The majority of the venom metalloproteases, either hemorrhagic or nonhemorrhagic, are fibrino(geno)lytic enzymes [3]. Fibrino(geno)lytic and hemorrhagic

Fig. 1



Fibrinogenolytic activity of *C. cumanensis* venom. Fibrinogen incubated with Lagunetica, Anzoátegui and Carrizales venoms. A 5 μg venom/100 μg fluorescein monoglucuronide ratio was used in the presence or absence of metalloprotease inhibitors during 240 min at 37°C. Samples (50 μg) were electrophoresed under nonreducing conditions in the presence of sodium dodecyl sulfate on a 5% gel and stained with Coomassie Blue. AzV, Anzoátegui; CrV, Carrizales; HMW, high molecular weight; LMW, low molecular weight, LV, Lagunetica; MPI, metalloprotease inhibitors.

Fig. 2



MHD of three specimens of *Crotalus durissus cumanensis* venoms, in the presence or absence of protease inhibitors. Data are expressed as mean ($n = 4$). Minimum hemorrhagic dose was determined by Omori-Satoh *et al.* [14]. Venoms preincubated with serine protease inhibitors. Venoms preincubated with metalloprotease inhibitors. MHD, minimum hemorrhagic dose; SPI, serine protease inhibitors.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

activities emerge to be characterized by separate, yet strongly related, proteolytic enzymes [23]. In all venoms, the fibrinolytic activity in fibrin plates was completely inhibited by MPIs. In Santa Teresa, Aragua and Margarita venoms, the serine protease inhibitors also abolished this activity. These results indicate that the fibrin lysis observed in Lagunetica, Guarenas, Anzoátegui and Carrizales could be dependent of metalloproteases. For instance, in Santa Teresa, Aragua and Margarita venoms this activity could be due to both metalloproteases and serine proteases.

Venom fibrinogenolytic enzymes may be classified as being either α-chain fibrin(ogen)ases or β-chain fibrin(ogen)ases [2]. Most fibrinolytic metalloproteases are α-fibrinogenases and are clearly inhibited by EDTA and β-mercaptoethanol, whereas *β*-fibrinogenases are typically serine proteases that are inhibited by PMSF. Many of the serine proteases are both fibrinogenolytic and fibrinolytic; however, less are fibrinolytic [24,25].

The fibrinogenolytic activity was assayed with Lagunetica, Carrizales and Anzoátegui venoms. Our results showed that Lagunetica and Carrizales venoms (without coagulant activity) induced fibrinogen molecule degradation and formed fragments with varying weights. In Anzoátegui (with lowest thrombin-like and fibrinolytic activities), the fibrinolytic effect was low.

The fibrino(geno)lytic activity was neutralized by MPIs. These results indicated that this fibrinogenolytic activity might be due to metalloproteases. These fibrino(geno)lytic *C. durissus cumanensis* venom enzymes, once purified, may have important therapeutic applications in thrombotic vascular diseases.

Procoagulant enzymes are classified as factor V, X or prothrombin activators, as well as thrombin-like enzymes, which can be metalloproteases or serine proteases [3]. In the present study, when the procoagulant activity was tested by the amidolytic method, a direct thrombin-like activity in all *C. durissus cumanensis* venoms was present.

Thrombin-like enzymes are inhibited by classical low molecular weight serine protease inhibitors but not by thrombin inhibitors such as antithrombin III and hirudin [26,27]. The results obtained in the presence of protease inhibitors showed that the ATIII/heparin, a physiological inhibitor complex of thrombin, partially reduced the thrombin-like coagulant activity present in the venoms, with the exception of Margarita venom, which was not affected. This thrombin-like activity was completely inhibited by PMSF and EGTA. In contrast, SBTI, a selective inhibitor of factor Xa [28], slightly (33%) inhibited the standard thrombin activity (data not shown) and partially inhibited the thrombin-like activity present in Santa Teresa venom (30%), but did not inhibit this activity present in other venoms.

The results obtained with amidolytic method in the presence and absence of inhibitors showed that the ATIII/heparin complex partially inhibits the thrombin-like venom activity. This would explain the lack of identity of exosites in thrombin-like molecules that participate in the binding with these physiological inhibitors. Serine protease inhibitors eliminated 70–91% of this activity, PMSF being the most effective (data not shown). In contrast, the metalloprotease inhibitors did not have a significant effect. In the presence of protease inhibitors it was observed that a mixture of leupeptin, SBTI and aprotinin reduced the plasmin-like amidolytic activity between 94 and 87% in all venoms, whereas the metalloprotease inhibitors did not have such a strong effect on this activity. In contrast, the t-PA-like amidolytic activity was more sensitive to metalloprotease inhibitors (85–96% inhibition), than serine protease inhibitors (36–67%).

Serine protease inhibitors neutralized between 64 and 78% of the kallikrein-like amidolytic activity. In contrast, the metalloprotease inhibitors increased the kallikrein-like activity (Table 3). This result could be due to the presence of high metal concentrations (like Zn) in the venoms, which could be explained by either three-dimensional structures in which the active site or the domains involved in the catalysis of these proteases are hidden or the metal chelants neutralize the proteolytic effects that these metalloproteases are exerting. This enhanced effect unravels a new and interesting subject for investigation.

The thrombin-like activity observed in these venoms, which can induce disseminated intravascular coagulation, was partially reduced by serine protease inhibitors, indicating the possible presence of catalytic domains in the thrombin-like enzymes that do not interact with these inhibitors. On the other hand, the inhibition of the thrombin coagulant activity by EGTA could be explained by the high chelant calcium effect, which is a cofactor for the process of fibrin polymerization.

The comparative study of snake venom components on hemostatic factors evaluated in this work also demonstrated the presence of geographic differences in *C.durissus cumanensis* venoms influencing the potency of these venoms and the production of antivenoms, which is important in the treatment of patients.

Acknowledgements

Financial support was obtained from the Science and Technology Fund (FONACIT) Programme (PG-2005000400 and FC-2005000212) and IVIC grant (Venezuela). We also wish to thank Amparo Gil and Luis Navarrete for their technical assistance.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

References

1 Rael ED, Martinez M, Molina O. Isolation of a fibrinolytic protease, M4, from venom of *Crotalus molossus molossus* (northern blacktail rattlesnake). *Haemostasis* 1992; **22**:41–49.
2 Kamiguti AS, Hay CR, Theakston RD, Zuzel M. Insights into the mechanism of haemorrhage caused by snake venom metalloproteinases. *Toxicon* 1996; **34**:627–642.
3 Markland FS. Snake venoms and the hemostatic system. *Toxicon* 1998; **36**:1749–1800.
4 Marsh N. Inventory of haemorrhagic factors from snake venoms. Registry of Exogenous Hemostatic Factors. Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost* 1994; **71**:793–797.
5 Markland FS Jr. Snake venoms. *Drugs* 1997; 4 (Suppl 3):1–10.
6 Braud S, Bon C, Wisner A. Snake venom proteins acting on hemostasis. *Biochimie* 2000; **82**:851–859.
7 Matsui T, Fujimura Y, Titani K. Snake venom proteases affecting hemostasis and thrombosis. *Biochim Biophys Acta* 2000; **1477**:146–156.
8 Sánchez EE, Galan JA, Powell RL, Reyes SR, Soto JG, Russell WK, *et al.* Disintegrin, hemorrhagic, and proteolytic activities of Mohave rattlesnake, Crotalus scutulatus scutulatus venoms lacking Mojave toxin. *Comp Biochem Physiol C Toxicol Pharmacol* 2005; **141**:124–132.
9 Salazar AM, Rodríguez-Acosta A, Girón ME, Aguilar I, Guerrero B. A comparative analysis of the clotting and fibrinolytic activities of the mapanare (*Bothrops atrox*) snake venom from different geographical areas in Venezuela. *Thromb Res* 2007; **120**:95–104.
10 Chippaux JP, Williams V, White J. Snake venom variability: methods of study, results and interpretation. *Toxicon* 1991; **29**:1279–1303.
11 Aguilar I, Guerrero B, Salazar AM, Girón ME, Pérez JC, Sánchez EE, Rodríguez-Acosta A. Individual venom variability in the South American rattlesnake *Crotalus durissus cumanensis*. *Toxicon* 2007; **50**:214–224.
12 Anonymous. *Principles of animal care.* Bethesda, Maryland, USA: National Institute of Health of United States; 1985. pp. 85–23: pp. 1–112.
13 Lowry OH, Rosembrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**:265–275.
14 Omori-Satoh T, Sadahiro S, Ohsaka A, Murata R. Purification and characterization of an antihemorrhagic factor in the serum of *Trimeresurus flavoviridis*, a crotalid. *Biochim Biophys Acta* 1972; **285**:414–426.
15 Austen D, Rhymes I. *A laboratory manual of blood coagulation.* Osney Mead, Oxford, London: Blackwell Scientific Publications; 1975.
16 Guerrero B, Arocha-Piñango CL. Activation of human prothrombin by the venom of *Lonomia achelous* (Cramer) caterpillars. *Thromb Res* 1992; **66**:169–177.
17 Marsh NA, Arocha-Piñango CL. Evaluation of the fibrin plate method for estimating plasminogen activators. *Thromb Diath Haemorrh* 1972; **28**:75–88.
18 Schägger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987; **166**:368–379.
19 Price NC, Johnson CM. Proteolysis of proteins for sequence analysis and peptide mapping. In: Beynon RJ and Bond JS, editors. Proteolytic enzymes: a practical approach. Oxford, UK: Oxford University Press; 1989.
20 Minton SA, Weinstein SA. Geographic and ontogenic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*). *Toxicon* 1986; **24**:71–80.
21 Rodríguez-Acosta A, Aguilar I, Girón M, Rodríguez-Pulido V. Haemorrhagic activity of neotropical rattlesnake (*Crotalus vegrandis* Klauber, 1941) venom. *Nat Toxins* 1998; **6**:15–18.
22 Girón ME, Pinto A, Finol HJ, Aguilar I, Rodríguez-Acosta A. Kidney structural and ultrastructural pathological changes induced by Uracoan rattlesnake (*Crotalus vegrandis* Klauber 1941) venom. *J Submicrosc Cytol Pathol* 2002; **34**:447–459.
23 Markland FS Jr. Snake venom fibrinogenolytic and fibrinolytic enzymes: an updated inventory. *Thromb Haemost* 1998; **79**:668–674.
24 Hung CC, Chiou SH. Isolation of multiple isoforms of alpha-fibrinogenase from the Western diamondback rattlesnake, *Crotalus atrox*: N-terminal sequence homology with ancrod, an antithrombotic agent from Malayan viper. *Biochem Biophys Res Commun* 1994; **201**:1414–1423.
25 Markland FS, Kettner C, Schiffman S, Shaw E, Bajwa SS, Reddy KN, *et al.* Kallikrein-like activity of crotalase, a snake venom enzyme that clots fibrinogen. *Proc Natl Acad Sci U S A* 1982; **79**:1688–1692.
26 Pirkle H, Theodor I, Miyada D, Simmons G. Thrombin-like enzyme from the venom of *Bitis gabonica*. Purification, properties, and coagulant actions. *J Biol Chem* 1986; **261**:8830–8835.
27 Magalhaes A, Da Fonseca BCB, Diniz DR, Gilroy J, Richardson M. The complete amino acid sequence of a thrombin-like enzyme/gyroxin analogue from venom of the bushmaster snake (*Lachesis muta muta*). *FEBS Lett* 1993; **329**:116–120.
28 Aronson DL, Mustafa AJ. The activation of human factor X in sodium citrate: the role of factor VII. *Thromb Haemost* 1976; **36**:104–114.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.