

## RESEARCH ARTICLE

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# Carbon monoxide attenuates the effects of snake venoms containing metalloproteinases with fibrinogenase or thrombin-like activity on plasmatic coagulation†

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Exposure of plasma to iron and carbon monoxide (CO) renders fibrinogen resistant to fibrinogenolytic or thrombin-like activity contained in pit viper venom. However, the direct effects of iron/CO on venom activity are unknown. Thus, we assessed if four different, metalloproteinase containing snake venoms exposed to iron/CO or CO alone could attenuate their fibrinogenolytic or thrombin-like activity. Venom (0–500  $\mu\text{g mL}^{-1}$ ) was exposed to 0–10  $\mu\text{M}$   $\text{FeCl}_3$  and/or 0–100  $\mu\text{M}$  carbon monoxide releasing molecule-2 (CORM-2), or inactivated CORM-2 (iCORM-2) for 3 min at room temperature. Venom solution (0–8  $\mu\text{g mL}^{-1}$  final concentration) was then placed in citrated human plasma containing tissue factor, followed by  $\text{CaCl}_2$  addition for commencement of coagulation. Data were determined with thrombelastography for 10–15 min at 37  $^\circ\text{C}$ . Iron had no effect on the first venom tested, so only CO was investigated subsequently. Exposure of venom to CO attenuated fibrinogenolytic or thrombin-like activity, and iCORM-2 did not affect the venom activities. Further investigation of the effect of CO exposure on similar venoms is justified.

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## Introduction

Envenomation by vipers with hemostatically active venom continues to be a major health problem worldwide. Systemic hypofibrinogenemia or afibrinogenemia can occur by the action of fibrinogenolytic enzymes that cleave fibrinogen such that it cannot be recognized and polymerized by thrombin, or by the action of thrombin-like or prothrombin activating enzymes that cause intravascular coagulation and consumption of available fibrinogen. Specifically, snake venom metalloproteinases (SVMP) may interact with the plasmatic coagulation several ways by acting as fibrinogenases, as thrombin-like enzymes, or as a prothrombin activators.<sup>1–5</sup> Similarly, snake venom serine proteases (SVSP) also can affect coagulation as thrombin-like enzymes, or as activators of factor V.<sup>6–8</sup> For example, *Crotalus* and *Agkistrodon* pit viper species possess metalloproteinase-containing venoms that can be fibrinogenolytic,<sup>9–11</sup> leading to consequent hypofibrinogenemia and hemorrhage after envenomation. Of interest, it has been demonstrated that pre-exposure of human plasma to small concentrations of iron and carbon monoxide (CO) causes plasma to be resistant to degradation of coagulation by *Crotalus* and *Agkistrodon* venoms containing either SVMP or SVSP.<sup>12–15</sup> The putative mechanism for iron/CO mediated

protection was that these agents change the configuration of fibrinogen, making molecular recognition of catalytic sites by venom less favorable,<sup>12–15</sup> based on the changes in ultrastructure of plasma clots mediated by iron/CO.<sup>16</sup> In sum, the addition of iron/CO to plasma appears to indirectly attenuate the effects of fibrinogenase activity from a variety of species of venomous snakes on coagulation.

Of interest, the direct effects of iron/CO on snake venom in isolation were not determined in these previous works.<sup>12–15</sup> Thus, when preliminary experiments to verify as a negative control that iron/CO would not inhibit the hemostatic effects of SVMP containing venom demonstrated the opposite effect, the decision to formally test this serendipitous finding was made. Thus, the purpose of this investigation was to determine if exposure of isolated SVMP containing venoms to iron and/or CO diminish fibrinogenolysis or abnormal procoagulation in human plasma.

## Materials and methods

### Materials

Lyophilized venom containing SVMP obtained from four different species of snakes were analyzed. The venoms and SVMP are displayed in Table 1. *Crotalus atrox* (western diamondback rattlesnake) and *Agkistrodon contortrix mokasen* (northern copperhead) venoms were obtained from the National Natural Toxins Research Center at Texas A&M

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† The authors declare no competing interests.

**Table 1** Characteristics of metalloprotease containing snake venoms assessed

Species	Metalloproteinase/serine protease	Ref.
<i>Crotalus atrox</i>	Metalloproteinase with fibrinogenase activity	2
<i>Agkistrodon contortrix mokasen</i>	Metalloproteinase with fibrinogenase activity	3
<i>Daboia russelli</i>	Factor X activating metalloproteinase	4, 6 and 8
	Prothrombin activating metalloproteinases	
	Serine protease with thrombin-like activity	
	Factor V activating serine protease	
<i>Bothrops asper</i>	Prothrombin activating metalloproteinase	5 and 7
	Serine protease with thrombin-like activity	

University-Kingsville, TX, USA. *Daboia russelli* (Russell's viper) venom was obtained from Sigma-Aldrich, Saint Louis, MO, USA. *Bothrops asper* (Fer-de-lance) venom was a generous gift from Sergio Bárcenas-Arriaga, graduate student of Alejandro Alagón of the Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México. Venoms were reconstituted in calcium-free phosphate buffered saline (PBS, Sigma-Aldrich) at concentrations of 20–50 mg ml<sup>-1</sup>, and stored at -80 °C. Pooled human normal plasma anticoagulated with sodium citrate was obtained from George King Bio-Medical, Overland Park, KS, USA. Ferric chloride (FeCl<sub>3</sub>, 99.9% pure), tricarbonyldichlororuthenium(II) dimer (CORM-2), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. The final concentrations of FeCl<sub>3</sub> (dissolved in PBS) and CORM-2 (dissolved in DMSO) were 0–10 μM and 0–100 μM, respectively; these were the concentrations used in previous works involving the effects of modifying fibrinogen prior to venom exposure in plasma.<sup>12–15</sup> To isolate the effects of CO from the carrier molecule of CORM-2, a 100 μM solution of CORM-2 was placed in a sealed plastic tube and incubated in a 37 °C water bath for 18 hours in order to release CO and become inactive (iCORM-2) as has been previously described.<sup>17</sup>

#### Isolated fibrinogenase containing venom exposure experiments

To increase the detection of effects of iron and/or CO on venom activity, the concentration of *C. atrox* and *A. c. mokasen* venoms chosen for isolated exposure and for addition to plasma was twice the amount expected to eliminate or enhance coagulation based on previous works.<sup>12,13</sup> In the case of *C. atrox* venom, to test the effects of iron and CO (together and separately) exposure on SVMP fibrinogenase activity, a 1 ml quantity of a 200 μg ml<sup>-1</sup> solution of venom in PBS was prepared for each condition subsequently presented. The conditions were: 1) 1% addition (v/v) of PBS and DMSO to PBS without venom; 2) 1% addition (v/v) of PBS and DMSO to PBS with venom; 3) 1% addition of FeCl<sub>3</sub> and CORM-2 to PBS without venom; 4) 1% addition of FeCl<sub>3</sub> and CORM-2 to PBS with venom; 5) 1% addition of FeCl<sub>3</sub> and DMSO to PBS with venom; 6) 1% addition of CORM-2 to PBS with venom; and lastly, 7) 1% addition of PBS and iCORM-2 to PBS with venom. As this first series of experiments demonstrated a significant difference in *C. atrox* venom effects on

coagulation after CO exposure, only CO exposure experiments were conducted with the remaining venoms. Thus, in the case of *A. c. mokasen* venom, the effects of CORM-2 and iCORM-2 addition to 1 ml of a 500 μg ml<sup>-1</sup> solution of venom in PBS were tested and compared to the aforementioned conditions 1 and 2. After 3 min of incubation at room temperature, 3.6 μl of solution from one of these conditions was placed into 326 μl of plasma and 10 μl of tissue factor reagent (0.1% final concentration in distilled water; Diagnostica Stago S.A.S., Asnieres sur Seine, France) in a disposable cup in a computer-controlled thrombelastograph® hemostasis system (Model 5000, Haemonetics Inc., Braintree, MA, USA). After test solution addition, 20 μl of 200 mM CaCl<sub>2</sub> was immediately added to initiate clotting. Data were collected at 37 °C for 15 min. The following parameters previously described<sup>12–15</sup> were determined: time to maximum rate of thrombus generation (TMRTG): this is the time interval (min) observed prior to maximum speed of clot growth; maximum rate of thrombus generation (MRTG): this is the maximum velocity of clot growth observed (dynes per cm<sup>-2</sup> s<sup>-1</sup>); and total thrombus generation (TTG, dynes per cm<sup>-2</sup>), the final viscoelastic resistance observed after clot formation.

#### Isolated thrombin-like activity containing venom exposure experiments

In the case of the two thrombin-like activity containing venoms, a final concentration of 2 μg ml<sup>-1</sup> was used to produce a vigorous procoagulant response based on unpublished, preliminary work. The effects on *D. russelli* and *B. asper* venoms of 1% additions of solutions of PBS/DMSO, CORM-2 and iCORM-2 to 1 ml of a 500 μg ml<sup>-1</sup> solution of venom in PBS were tested and compared to each other and to PBS/DMSO solution addition to PBS without venom present as outlined previously. After 3 min of incubation at room temperature, 3.6 μl of solution from one of these conditions was placed into 336 μl of plasma in a disposable cup in a computer-controlled thrombelastograph® hemostasis system (Model 5000, Haemonetics Inc.). After test solution addition, 20 μl of 200 mM CaCl<sub>2</sub> was immediately added to initiate clotting. Thrombelastographic parameter data were collected at 37 °C for 10 min as previously described. The rationale for using only a 10 min time period to collect data was that at least 13 to 15 min are required for contact activation of cup and pin with recalcified plasma is required to generate



thrombin and generate measurable coagulation kinetic phenomena in our system.

### Determination of CORM-2 concentration SVMP activity relationship

In order to determine the kinetic nature of SVMP response to various concentrations of CO, we chose to focus on CO mediated modulation of *C. atrox* fibrinogenase activity. The six conditions ( $n = 4$  per condition) tested were addition of 1% (v/v) of 0, 10, 20, 30, 40, or 50  $\mu\text{M}$  CORM-2 to PBS with venom that was incubated for 3 min at room temperature as in the previous experiment series. Thereafter, 3.6  $\mu\text{l}$  of solution from one of these six conditions was placed into 326  $\mu\text{l}$  of plasma and 10  $\mu\text{l}$  of tissue factor reagent (0.1% final concentration; Diagnostica Stago S.A.S.) in a disposable cup in a computer-controlled thrombelastograph® hemostasis system (Model 5000, Haemonetics Inc.). Subsequently, 20  $\mu\text{l}$  of 200 mM  $\text{CaCl}_2$  was added to initiate clotting. Thrombelastographic data were collected at 37 °C for 15 min.

### Statistical analyses

Data are presented as median (1st–3rd quartiles) or as raw data with kinetic modeling. All conditions were represented by  $n = 6$  replicates. This number of replicates were used as a statistical power  $>0.8$  has been observed with similar analyses with this *in vitro* model.<sup>9,10</sup> If no coagulation occurred, the sample observed was assigned a TMRTG value of 10 or 15 min depending on the experimental series, with values for MRTG and TTG designated as 0. Thus, as the data were not normally distributed, Kruskal–Wallis one-way analysis of variance followed by Student–Newman–Keuls *post hoc* test were used for analyses (SigmaStat 3.1, Systat Software, Inc., San Jose, CA, USA). With regard to coagulation kinetic modeling of the *C. atrox* CORM-2 concentration fibrinogenase activity response relationship, data were analyzed with and graphed

with a commercial graphics program (OriginPro 7.5, OriginLab Corporation, Northampton, MA, USA).  $P < 0.05$  was considered significant.

## Results and discussion

Coagulation kinetic data for the four venoms tested are displayed in Tables 2–5. As seen in Table 2, compared to plasma with addition of PBS/DMSO, the further addition of *C. atrox* venom resulted in a significant, and essentially complete lack of coagulation. Addition of PBS containing  $\text{FeCl}_3$ /CORM-2 resulted in coagulation kinetic parameters not significantly different from plasma exposed to PBS/DMSO, but significantly different from samples exposed to *C. atrox* venom. Similarly, addition of PBS with *C. atrox* venom exposed to  $\text{FeCl}_3$ /CORM-2 resulted in coagulation kinetic parameters not significantly different from plasma exposed to PBS/DMSO, but significantly different from samples exposed to *C. atrox* venom. When PBS with *C. atrox* venom exposed to  $\text{FeCl}_3$  was tested, absence of coagulation not different from samples exposed to venom alone was observed. In contrast, PBS containing *C. atrox* venom exposed to CORM-2 added to plasma produced coagulation kinetic values not different from PBS/DMSO exposed plasma, but significantly different from plasma mixed with venom with PBS/DMSO exposure. Lastly, addition of PBS with *C. atrox* venom exposed to iCORM-2 to plasma resulted in no discernable coagulation, significantly different from the results observed with PBS/DMSO exposed plasma but not different from plasma samples containing *C. atrox* venom with PBS/DMSO exposure.

With regard to *A. c. mokasen* venom coagulation kinetic data, as seen in Table 3, venom significantly prolonged TMRTG, decreased MRTG and decreased TTG compared to PBS/DMSO exposed plasma. However, addition of *A. c. mokasen* venom exposed to CORM-2 to plasma resulted in coagulation kinetics not significantly different from plasma

**Table 2** Effects of  $\text{FeCl}_3$ , CORM-2 and iCORM-2 on *C. atrox* venom fibrinogenolysis in plasma

Effects of combinations of vehicle, venom and $\text{FeCl}_3$ + CORM-2 on coagulation			
Condition	TMRTG	MRTG	TTG
DMSO/PBS vehicle	3.7(3.2, 3.8)	4.7(4.1, 5.3)	168(153, 174)
Venom	15.0(15.0, 15.0) <sup>a</sup>	0.0(0.0, 0.0) <sup>a</sup>	0(0, 1) <sup>a</sup>
$\text{FeCl}_3$ + CORM-2	3.7(3.2, 3.8) <sup>b</sup>	5.0(4.8, 5.2) <sup>b</sup>	168(165, 176) <sup>b</sup>
Venom + $\text{FeCl}_3$ + CORM-2	3.2(3.0, 3.3) <sup>b</sup>	5.2(4.9, 5.6) <sup>b</sup>	176(169, 182) <sup>b</sup>
Effects of $\text{FeCl}_3$ exposed venom on coagulation			
Venom + $\text{FeCl}_3$	15.0(15.0, 15.0) <sup>a</sup>	0.0(0.0, 0.0) <sup>a</sup>	0(0, 0) <sup>a</sup>
Effects of CORM-2 or inactivated CORM-2 (iCORM-2) exposed venom on coagulation			
Venom + CORM-2	3.0(3.0, 3.1) <sup>a,b</sup>	5.9(5.7, 6.1) <sup>a,b</sup>	172(168, 178) <sup>b</sup>
Venom + iCORM-2	15.0(15.0, 15.0) <sup>a,c</sup>	0.0(0.0, 0.0) <sup>a,c</sup>	0.0(0.0, 0.0) <sup>a,c</sup>

Coagulation was activated with tissue factor addition to the samples in all conditions. DMSO/PBS vehicle = 1% addition of each of these vehicles to PBS solution without venom addition; venom = 2  $\mu\text{g ml}^{-1}$  final concentration of *C. atrox* venom in DMSO/PBS vehicle solution;  $\text{FeCl}_3$  + CORM-2 = PBS solution with 10  $\mu\text{M}$   $\text{FeCl}_3$  and 100  $\mu\text{M}$  CORM-2 final concentration; venom +  $\text{FeCl}_3$  + CORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration exposed to 10  $\mu\text{M}$   $\text{FeCl}_3$  and 100  $\mu\text{M}$  CORM-2; venom +  $\text{FeCl}_3$  = venom 2  $\mu\text{g ml}^{-1}$  final concentration exposed to 10  $\mu\text{M}$   $\text{FeCl}_3$ ; venom + CORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration exposed to 100  $\mu\text{M}$  CORM-2; venom + iCORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration exposed to 100  $\mu\text{M}$  iCORM-2. <sup>a</sup>  $P < 0.05$  vs. DMSO/PBS vehicle. <sup>b</sup>  $P < 0.05$  vs. venom. <sup>c</sup>  $P < 0.05$  vs. venom + CORM-2.



**Table 3** Effects of CORM-2 and iCORM-2 on *A. c. mokasen* venom fibrinogenolysis in plasma

Condition	TMRTG	MRTG	TTG
DMSO/PBS vehicle	3.7(3.2, 3.8)	4.7(4.1, 5.3)	168(153, 174)
Venom	7.6(7.1, 8.1) <sup>a</sup>	1.2(1.0, 1.4) <sup>a</sup>	100(88, 114) <sup>a</sup>
Venom + CORM-2	3.6(3.4, 3.6) <sup>b</sup>	4.6(4.2, 4.8) <sup>b</sup>	170(175, 186) <sup>b</sup>
Venom + iCORM-2	15.0(12.4, 15.0) <sup>a,b,c</sup>	0.0(0.0, 0.4) <sup>a,b,c</sup>	0(0, 26) <sup>a,b,c</sup>

Coagulation was activated with tissue factor addition to the samples in all conditions. DMSO/PBS vehicle = 1% addition of each of these vehicles to PBS solution without venom addition; venom = 8  $\mu\text{g ml}^{-1}$  final concentration of *A. c. mokasen* venom in DMSO/PBS vehicle solution; venom + CORM-2 = venom 8  $\mu\text{g ml}^{-1}$  final concentration of venom exposed to 100  $\mu\text{M}$  CORM-2; venom + iCORM-2 = venom 8  $\mu\text{g ml}^{-1}$  final concentration of venom exposed to 100  $\mu\text{M}$  iCORM-2. <sup>a</sup>  $P < 0.05$  vs. DMSO/PBS vehicle. <sup>b</sup>  $P < 0.05$  vs. venom. <sup>c</sup>  $P < 0.05$  vs. venom + CORM-2.

**Table 4** Effects of CORM-2 and iCORM-2 on *D. russelli* venom thrombin-like activity in plasma

Condition	TMRTG	MRTG	TTG
DMSO/PBS vehicle	10.0(10.0, 10.0)	0.0(0.0, 0.1)	1(0, 2)
Venom	1.2(1.2, 1.2) <sup>a</sup>	10.5(10.0, 11.0) <sup>a</sup>	179(172, 184) <sup>a</sup>
Venom + CORM-2	3.3(3.2, 3.6) <sup>a,b</sup>	4.8(4.4, 5.5) <sup>a,b</sup>	144(134, 152) <sup>a,b</sup>
Venom + iCORM-2	1.3(1.2, 1.3) <sup>a,c</sup>	10.0(9.5, 10.5) <sup>a,c</sup>	184(176, 187) <sup>a,c</sup>

DMSO/PBS vehicle = 1% addition of each of these vehicles to PBS solution without venom addition; venom = 2  $\mu\text{g ml}^{-1}$  final concentration of *D. russelli* venom in DMSO/PBS vehicle solution; venom + CORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration of venom exposed to 100  $\mu\text{M}$  CORM-2; venom + iCORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration of venom exposed to 100  $\mu\text{M}$  iCORM-2. <sup>a</sup>  $P < 0.05$  vs. DMSO/PBS vehicle. <sup>b</sup>  $P < 0.05$  vs. venom. <sup>c</sup>  $P < 0.05$  vs. venom + CORM-2.

**Table 5** Effects of CORM-2 and iCORM-2 on *B. asper* venom thrombin-like activity in plasma

Condition	TMRTG	MRTG	TTG
DMSO/PBS vehicle	10.0(10.0, 10.0)	0.0(0.0, 0.1)	1(0, 2)
Venom	1.2(1.2, 1.3) <sup>a</sup>	10.3(9.7, 11.0) <sup>a</sup>	178(168, 184) <sup>a</sup>
Venom + CORM-2	4.4(4.2, 4.8) <sup>a,b</sup>	4.2(4.0, 4.4) <sup>a,b</sup>	128(124, 131) <sup>a,b</sup>
Venom + iCORM-2	2.1(2.1, 2.2) <sup>a,b,c</sup>	9.0(8.7, 9.2) <sup>a,b,c</sup>	175(172, 182) <sup>a,c</sup>

DMSO/PBS vehicle = 1% addition of each of these vehicles to PBS solution without venom addition; venom = 2  $\mu\text{g ml}^{-1}$  final concentration of *B. asper* venom in DMSO/PBS vehicle solution; venom + CORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration of venom exposed to 100  $\mu\text{M}$  CORM-2; venom + iCORM-2 = venom 2  $\mu\text{g ml}^{-1}$  final concentration of venom exposed to 100  $\mu\text{M}$  iCORM-2. <sup>a</sup>  $P < 0.05$  vs. DMSO/PBS vehicle. <sup>b</sup>  $P < 0.05$  vs. venom. <sup>c</sup>  $P < 0.05$  vs. venom + CORM-2.

exposed to PBS/DMSO. Lastly, and surprisingly, addition of *A. c. mokasen* venom exposed to iCORM-2 to plasma resulted in essentially absent coagulation, with TMRTG, MRTG and TTG values significantly different from the other three conditions.

As for *D. russelli* venom with thrombin-like activity exposed to PBS/DMSO, as seen in Table 4, venom significantly decreased TMRTG, increased MRTG and increased TTG compared to PBS/DMSO exposed plasma, which was not capable of forming clot within 10 min. After exposing *D. russelli* venom to CORM-2, the venom induced a significantly longer TMRTG, smaller MRTG and smaller TTG values in plasma compared to venom not exposed to CORM-2. Lastly, after being exposed to iCORM-2, *D. russelli* venom displayed coagulation kinetics in plasma not significantly different from venom exposed to PBS/DMSO but significantly different from plasma with addition of venom exposed to CORM-2 or after addition of PBS/DMSO without venom.

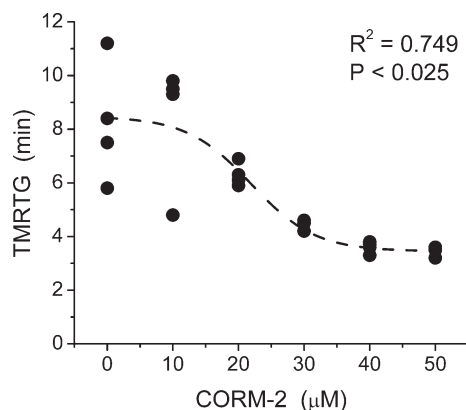
The data concerning the effects of exposure of *B. asper* venom to CORM-2 and iCORM-2 are displayed in Table 5. The thrombin-like activity of *B. asper* venom exposed to PBS/

DMSO demonstrates significantly smaller TMRTG, greater MRTG and greater TTG values that that observed with plasma without venom addition. Exposure of *B. asper* venom to CORM-2 resulted in significantly greater TMRTG, smaller MRTG and smaller TTG values in plasma compared to venom exposed to PBS/DMSO. Finally, exposure of *B. asper* venom to iCORM-2 resulted in TMRTG values significantly greater than venom exposed to PBS/DMSO but significantly smaller than values in plasma with venom exposed to CORM-2. However, MRTG and TTG values in plasma were not significantly different between iCORM-2 and PBS/DMSO exposed *B. asper* venom additions.

Lastly, with regard to the coagulation kinetic modeling of the *C. atrox* CORM-2 concentration fibrinogenase activity response relationship, the results of these experiments are displayed in Fig. 1–3. Exposure of *C. atrox* venom to progressively greater concentrations of CORM-2 resulted in significant, concentration-dependent decreases in TMRTG values, increases in MRTG values and increases in TTG values that were best described as sigmoidal relationships.

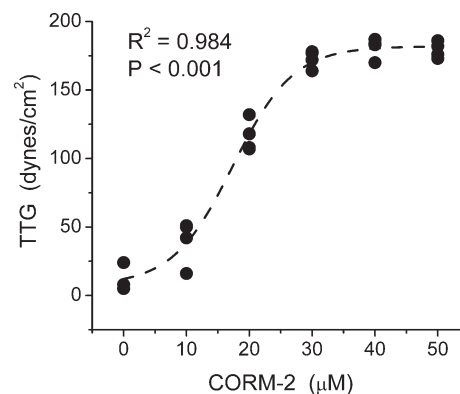






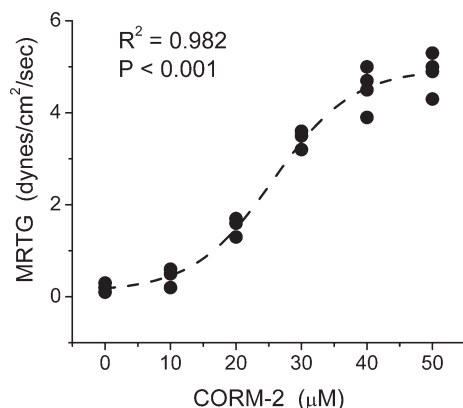
**Fig. 1** Relationship between TMRTG and CORM-2 after exposure of *C. atrox* venom to various concentrations of CORM-2. Raw data for six concentrations ( $n = 4/\text{concentration}$ ) of CORM-2 exposure of *C. atrox* venom are depicted. Dashed line represents kinetic modeled relationship, with coefficient of determination ( $R^2$ ) and statistical significance of the relationship displayed. The equation describing this relationship is as follows:

$$\text{TMRTG (min)} = \frac{8.47}{1 + e^{\left(\left[\text{CORM-2 } (\mu\text{M})\right]^{-21.9}/4.89\right)}}$$



**Fig. 3** Relationship between TTG and CORM-2 after exposure of *C. atrox* venom to various concentrations of CORM-2. Raw data for six concentrations ( $n = 4/\text{concentration}$ ) of CORM-2 exposure of *C. atrox* venom are depicted. Dashed line represents kinetic modeled relationship, with coefficient of determination ( $R^2$ ) and statistical significance of the relationship displayed. The equation describing this relationship is as follows:

$$\text{TTG (dynes per cm}^2\text{)} = \frac{356}{1 + e^{\left(\left[\text{CORM-2 } (\mu\text{M})\right]^{-17.4}/4.80\right)}}$$



**Fig. 2** Relationship between MRTG and CORM-2 after exposure of *C. atrox* venom to various concentrations of CORM-2. Raw data for six concentrations ( $n = 4/\text{concentration}$ ) of CORM-2 exposure of *C. atrox* venom are depicted. Dashed line represents kinetic modeled relationship, with coefficient of determination ( $R^2$ ) and statistical significance of the relationship displayed. The equation describing this relationship is as follows:

$$\text{MRTG (dynes per cm}^2\text{ per s)} = \frac{9.80}{1 + e^{\left(\left[\text{CORM-2 } (\mu\text{M})\right]^{-25.6}/6.10\right)}}$$

The primary finding of the present investigation was that CO derived from CORM-2 significantly inhibited the fibrinogenolytic and procoagulant effects of two

fibrinogenolytic metalloproteinase and two thrombin-like metalloproteinase containing venoms, respectively. As iron had no effect on *C. atrox* venom, we tested the effects of CO and the carrier molecule, iCORM-2. CO derived from CORM-2 has only once been demonstrated to directly inhibit the activity of metalloproteinases 1 and 2 in a human alveolar epithelial cell line.<sup>18</sup> These authors speculated that CO, being known to bind to transition metals,<sup>19,20</sup> could have bound to the  $\text{Zn}^{2+}$  metal located in the catalytic center of the enzyme as the mechanism of inhibition.<sup>18</sup> However,  $\text{Zn}^{2+}$  binds to CO with less avidity than other transition metals.<sup>21</sup> Of interest, CO has been shown to modulate protein function in an amino acid-dependent, heme-independent fashion;<sup>22</sup> thus, perhaps CO may inhibit SVMP by binding to amino acid residues within or near the catalytic site of the enzyme, displacing the  $\text{Zn}^{2+}$  ion entirely. While the precise molecular mechanism by which CO inhibits SVMP remains to be elucidated, it can be inferred that such enzyme inhibition is CO concentration-dependent and sigmoidal in nature in the case of *C. atrox* venom fibrinogenolytic activity, not unlike that seen with heme-containing proteins. Given that the  $\text{Zn}^{2+}$  ion contained in SVMP have been demonstrated to be critical to ligand binding *via* molecular dynamic simulation,<sup>23</sup> and that most of these enzymes possess  $\text{Zn}^{2+}$  containing catalytic centers,<sup>1</sup> the findings of the present study may have broad implications. For example, in addition to hemostatic effects, snake venom  $\text{Zn}^{2+}$  metalloproteinases also induce hemorrhage and circulatory failure.<sup>1</sup> If CO inactivates a significant number of such snake venom  $\text{Zn}^{2+}$  metalloproteinases, then a new potential therapeutic direction for the treatment of envenomation may be possible.



In addition to the changes in SVMP activity made by carbon monoxide exposure, two of the venoms demonstrated modifications by exposure to iCORM-2. Specifically, iCORM-2 enhanced *A. c. mokasen* venom fibrinogenase activity (Table 3) and inhibited *B. asper* venom thrombin-like activity (Table 5). The ruthenium dimer may be interacting with  $\text{Zn}^{2+}$  in the catalytic site of the SVMP or binding elsewhere on the enzyme in the absence of CO presumably interacting with the  $\text{Zn}^{2+}$ . Our data indicate that in the presence of both released CO and residual iCORM-2, the predominant effect is CO mediated, and from a therapeutic standpoint there would not be an occasion to administer iCORM-2 alone instead of CORM-2. Of interest, iCORM-2 has been demonstrated to mediate effects similar to or opposite of CO *in vivo* and *in vitro* in various systems.<sup>24,25</sup>

Another issue of interest was that the exposure of venoms with prothrombin activating and thrombin-like activity to CO did not eliminate coagulation in plasma despite presumed inactivation of SVMP, unlike the inhibition observed in the case of *C. atrox* and *A. c. mokasen* venom fibrinogenase inactivation. This partial inhibition of prothrombin activating or thrombin-like activity in these two venoms may be in part secondary to incomplete inhibition of SVMP, but also possibly secondary to CO not interacting with the SVSP also present with thrombin-like activity (Table 1). In sum, the simplest explanation for the patterns of inhibition observed was interaction of CO with SVMP catalytic sites.

A potential concern about the use of CORM-2 or other CO releasing molecules to treat SVMP mediated coagulopathy is the likelihood of the envenomed individual experiencing CO poisoning and excessive carboxyhemoglobin concentrations. This fear may be unfounded, as hundreds of animal investigations involving administration of CO releasing molecules as a therapeutic intervention has not in either the acute or chronic setting resulted in an adverse outcome – instead, CO releasing molecules typically reduce the injury studied. As examples, in mouse<sup>26</sup> and rabbit<sup>27,28</sup> models involving the administration of CORM-3 (ref. 26) and CORM-2,<sup>27,28</sup> animals demonstrated no clinical signs of CO poisoning for days after administration of these compounds. Further, based on experiments that directly measured increases in carboxyhemoglobin concentrations in whole blood after CO releasing molecule addition, the concentration of carboxyhemoglobin would be expected to increase less than 1% in the presence of 100  $\mu\text{M}$  CORM-2.<sup>26</sup> To put this information into the clinical perspective, if an individual were to smoke two tobacco cigarettes within a 90 minute period, the average carboxyhemoglobin concentrations would be on average 5%, which is five times the normal concentration.<sup>29</sup> CO releasing molecules deliver CO in a site-directed manner, with even distribution throughout the solution in which they are added, permitting binding to heme or other molecular moieties on plasma proteins and within blood cells of all types. Thus, if an individual were to have systemic administration of CO equivalent to an instantaneous concentration of 100  $\mu\text{M}$  CORM-2, the expected increase in carboxyhemoglobin and

risk of CO poisoning would be less than if that individual consumed one cigarette. Therefore, based on the large amount of animal studies in existence without adverse outcomes after CO releasing molecule exposure and the expected small amount of increase in carboxyhemoglobin expected with the concentrations of CORM-2 used in the present investigation, we would anticipate that the clinical inhibition of SVMP with CO releasing molecules would not be associated with clinical CO poisoning.

In conclusion, the present study demonstrated that CO inhibits SVMP fibrinogenase, prothrombin activating and thrombin-like activity from four diverse viper species. Future investigations of the effects of CO on other snake venoms containing metalloproteinases are warranted.

## Authors' contributions

VGN designed and VGN and CMB conducted the experiments. VGN and CMB analyzed the data and wrote the manuscript. Both authors have reviewed and approve the final manuscript. This investigation was funded by the Department of Anesthesiology.

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