INHIBITION OF LOCAL HEMORRHAGE AND DERMONECROSIS INDUCED BY BOTHROPS ASPER SNAKE VENOM: EFFECTIVENESS OF EARLY IN SITU ADMINISTRATION OF THE PEPTIDOMIMETIC METALLOPROTEINASE INHIBITOR BATIMASTAT AND THE CHELATING AGENT CaNa₂EDTA

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Abstract. The effectiveness of the chelating agent CaNa₂EDTA and the peptidomimetic matrix metalloproteinase inhibitor batimastat (BB-94) to inhibit local tissue damage induced by Bothrops asper snake venom was studied in mice. Both compounds totally inhibited proteolytic, hemorrhagic, and dermonecrotic effects, and partially reduced edema-forming activity, when incubated with venom prior to injection. Much lower concentrations of batimastat than of CaNa₂EDTA were required to inhibit these effects. In addition, batimastat, but not CaNa₂EDTA, partially reduced myotoxic activity of the venom. When the inhibitors were administered at various time intervals after envenomation at the same site of venom injection, both compounds were effective in neutralizing local hemorrhage and dermonecrosis if administered rapidly after venom. Inhibition was not as effective as the time lapse between venom and inhibitor injections increased. Owing to the relevance of metalloproteinases in the pathogenesis of local tissue damage induced by B. asper and other pit viper venoms, it is suggested that administration of peptidomimetic metalloproteinase inhibitors or CaNa₂EDTA at the site of venom injection may represent a useful alternative to complement antivenoms in the neutralization of venom-induced local tissue damage.

INTRODUCTION

Snakebite envenomations constitute a medical hazard in many regions of the world.1–2 Pit vipers (family Viperidae, subfamily Crotalinae) are responsible for the majority of the cases in the Americas.3–8 These envenomations are characterized by prominent local tissue damage, i.e., necrosis of skeletal muscle, edema, hemorrhage, dermonecrosis, and blistering, as well as by systemic alterations such as hemorrhage, cardiovascular shock, acute renal failure, and coagulopathies.1–4,10 Antivenoms are highly effective in the neutralization of systemic alterations in snakebite envenomations. However, neutralization of venom-induced local effects is difficult owing to the rapid development of tissue damage after envenomation, and to the delay to reach health centers where antivenoms are available.6 As a consequence, permanent sequelae develop in a number of snakebitten patients.9,10 Thus, there is an urgent need to find therapies that would complement antivenoms in the neutralization of local tissue damage.

Bothrops asper (terciopelo or fer-de-lance) is responsible for the majority of snakebite cases in southern Mexico and Central America.4,11 It is widely distributed in tropical rain forests and lowland agricultural areas.12 Prominent local tissue damage is commonly observed in B. asper envenomations.4,13 The pathogenesis of local myonecrosis, dermonecrosis, hemorrhage, and edema induced by B. asper venom has been investigated.14–19 Moreover, various muscle-damaging toxins and hemorrhagic toxins have been isolated and characterized from this venom.20,21 Hemorrhagic metalloproteinases play a relevant role in the pathogenesis of local tissue damage in pit viper envenomations, since besides hemorrhage22–24 they induce edema,25 myonecrosis,24,26–28 and skin damage.19 Therefore, inhibition of venom metalloproteinases may significantly reduce the extent of pit viper-induced local tissue damage.

Hemorrhagic toxins are zinc-dependent endopeptidases belonging to the “metzincin” group of metalloproteinases.29 Their proteolytic and hemorrhagic activities are inhibited by chelating compounds such as EDTA salts and o-phenanthroline.30 Previous studies have shown that CaNa₂EDTA, a chelating agent currently used in the treatment of lead poisoning,18–20 is effective in the inhibition of hemorrhagic and dermonecrotic activities of B. asper venom when preincubated with venom before injection in experimental animals.33,34 Moreover, batimastat, a synthetic hydroxamate peptidomimetic matrix metalloproteinase inhibitor, is highly effective in the inhibition of BaP1, a hemorrhagic and dermonecrotic toxin from B. asper venom.35 The possibility of inhibiting venom metalloproteinases in situ, by locally injecting CaNa₂EDTA or batimastat at the site of venom injection, was evaluated in this study. Results indicate that local injection of these compounds rapidly after envenomation significantly reduces hemorrhage and dermonecrosis induced by B. asper venom.

MATERIALS AND METHODS

Venom, batimastat, and EDTA. Bothrops asper venom was a pool obtained from more than 40 adult specimens collected in the Pacific region of Costa Rica. It was lyophilized and stored at −40°C. CaNa₂EDTA was obtained from Sigma (St. Louis, MO). Batimastat (a generic name, the inhibitor is also known as BB-94; [4-(N-hydroxiamino)-2R-isobutyl-3S-(thienyl thiomethyl)-succinyl]-L-phenylalanine-N-methylamide; Mr, 478) was provided by British Biotech Pharmaceuticals, Ltd. (Cowley, Oxford, United Kingdom).

Local toxicity of CaNa₂EDTA and batimastat. To assess
if CaNa₂EDTA or batimastat exert local toxic activities, various concentrations of these inhibitors were tested for hemorrhagic, myotoxic, dermonecrotic, and edema-forming effects, using the methodologies described below. In addition, the hyperalgesic effect of CaNa₂EDTA was assessed in adult male Wistar rats (150–180 grams). Briefly, rats were injected with 100 µl of either PBS or various concentrations of CaNa₂EDTA dissolved in PBS into the subplantar hindpaw region. The pain threshold was determined at different time intervals after injection with an Ugo-Basile pressure apparatus, essentially as described by Randall and Selitto.36

Inhibition of proteolytic activity. The procedure of Rinderknecht and others,37 using hide powder azure (Sigma) as substrate, was followed. Briefly, solutions containing either B. asper venom, or mixtures of venom and various amounts of either CaNa₂EDTA or batimastat, were incubated at 37°C for 30 min. Aliquots containing 50 µg of venom were added to tubes containing 5 mg of hide powder azure in 1.25 ml of Tris 0.05 M, pH 8.0, buffer. Incubations were carried out for 1 hr at 37°C. Then tubes were centrifuged and the absorbances of the supernatants at 595 nm were recorded.

Inhibition of pharmacologic activities. Inhibition of various pharmacologic activities of B. asper venom was assessed by two different types of assays. In some cases, venom was incubated with either CaNa₂EDTA or batimastat for 30 min at 37°C before testing them in Swiss-Webster mice (18–20 grams). In other experiments, venom was injected intramuscularly in mice and then, at various time intervals, CaNa₂EDTA or batimastat were administered at the site of venom injection. In both types of assays the dose of venom was selected after dose-response studies for each particular effect, and corresponded to a linear portion of the curve. These types of assays have been previously used and validated in the study of the neutralizing ability of antivenoms and inhibitors.38,39

Inhibition of hemorrhagic activity. A constant amount of venom was incubated with various concentrations of either CaNa₂EDTA or batimastat for 30 min at 37°C. Aliquots of the mixtures, containing 20 µg of venom in a total volume of 100 µl, were then injected intradermally (id) into groups of four mice (18–20 grams). The following control groups were included: 1) mice injected with venom alone dissolved in PBS; 2) mice injected with PBS; 3) mice injected with CaNa₂EDTA dissolved in PBS; 4) mice injected with venom alone dissolved in PBS-Tween; and 5) mice injected with PBS-Tween. Animals were killed 2 hr after injection, their skin was removed, and the diameter of the hemorrhagic area in the inner side of the skin was measured.40,41 In another series of experiments, mixtures of venom and CaNa₂EDTA or batimastat were prepared and incubated as described, and aliquots containing 50 µg venom were injected intramuscularly (im) in the right thigh of mice. The same controls described above were included. One hour after injection, the hemoglobin content of muscle tissue was assessed by a modification of the method of Ownby and others.42 Briefly, mice were killed and the injected muscles were dissected out, weighed, cut into pieces, and placed into 1.5 ml of Drabkin solution. After an overnight incubation at 4°C, an aliquot of the solution was withdrawn, centrifuged at 10,000 rpm for 10 min, and diluted 1:2 with Drabkin solution. The absorbances at 540 nm were recorded using Drabkin solution as a blank. Hemoglobin concentration was estimated from a calibration curve, and results are expressed as milligrams of hemoglobin per gram of muscle wet weight.

In experiments with independent injection of venom and CaNa₂EDTA or batimastat, groups of five mice were injected im with 50–µg of venom dissolved in 50 µl of PBS in the right footpad. At various time intervals, the mice then received an injection of either a 1.5 M CaNa₂EDTA solution or a 500-µM batimastat suspension at the same site where venom had been administered. These doses of inhibitors was selected on the basis of dose-response studies in which inhibitors were administered immediately after envenomation. Hemorrhage was assessed by determining the hemoglobin concentration in muscle tissue, as described. The same controls described above were included.

Inhibition of dermonecrotic activity. A constant amount of venom was incubated with various concentrations of the inhibitors at 37°C for 30 min. Aliquots of the mixtures containing 200 µg of venom in a volume of 100 µl, were then injected id into groups of four mice (18–20 grams). The same control groups described for inhibition of hemorrhagic activity were included. After 72 hr, the mice were killed and the necrotic lesion on the inner side of the skin was measured.43 Dermonecrotic activity was also assessed in experiments with independent injection of venom and CaNa₂EDTA or batimastat. In this case, 100 µg of venom was injected in the right gastrocnemius, and the inhibitors were injected at the same site various time intervals afterwards. Dermonecrosis was evaluated macroscopically on the outer side of the skin covering the injected hindlimb at various time intervals after envenomation.

Inhibition of edema-forming activity. A constant amount of venom was incubated with various concentrations of CaNa₂EDTA or batimastat for 30 min at 37°C. Aliquots of the mixtures containing 5 µg of venom in a volume of 50 µl were then injected subcutaneously (sc) in the right footpad of mice (18–20 grams). The same controls described above were included. Edema was evaluated at various time intervals by measuring the increase in footpad thickness with a low-pressure spring caliper.44

Inhibition of myotoxic activity. A constant amount of venom was incubated with either CaNa₂EDTA (50 mM) or batimastat (20 µM), as described. Aliquots of the mixtures, containing 50 µg of venom in a volume of 100 µl, were injected im in the right gastrocnemius of mice (n = 4). The same control groups described above were included. Three hours after injection blood samples were collected from the tail into heparinized capillary tubes. Plasma creatine kinase (CK) activity was quantitated by the Sigma kit 47-UV. The CK activity was expressed in units/L, with one unit defined as the amount of enzyme that produces one µmole of NADH under the conditions of the assay. In some experiments, the ability of the inhibitors to neutralize myotoxicity induced by B. asper myotoxin III was evaluated. Myotoxin III, an Asp-49 phospholipase A₂,45 was isolated by ion-exchange chromatography on CM-Sephadex (Sigma).46

Statistical analyses. Results are expressed as mean ± SEM. The significance of the differences between mean values of two different experimental groups was determined by the Student’s t-test.
FIGURE 1. Inhibition of proteolytic activity of Bothrops asper venom by A, CaNa₂EDTA and B, batimastat. Solutions of venom and each inhibitor were incubated for 30 min at 37°C and then tested for proteolytic activity on hide powder azure. After 1 hr of incubation at 37°C, the tubes were centrifuged and the absorbances of the supernatants at 595 nm were recorded. Results are presented as the mean ± SEM (n = 3).

FIGURE 2. Inhibition of hemorrhagic activity of Bothrops asper venom by A, CaNa₂EDTA and B, batimastat. Venom was incubated with various concentrations of either inhibitor for 30 min at 37°C. Aliquots of the mixtures, containing 20 µg of venom, were injected intradermally in mice. After 2 hr, the animals were killed, their skin was removed, and the diameters of hemorrhagic lesions were assessed in the inner side of the skin. Results are presented as the mean ± SEM (n = 4).

RESULTS

Local toxicity of CaNa₂EDTA and batimastat. Injections of CaNa₂EDTA or batimastat im in the right gastrocnemius muscle and id in the abdominal region did not induce hemorrhage nor dermonecrosis even at concentrations of 2 M and 50 µM, respectively. No increments in plasma CK activity were observed 3 hr after im injection of 1 M CaNa₂EDTA or 50 µM batimastat in the gastrocnemius muscle. Subcutaneous injections of CaNa₂EDTA or batimastat in the footpads of mice induced a mild edematogenic response (see below), which peaked 1 hr after injection. Finally, the sc intraplantar injection of CaNa₂EDTA solutions at concentrations of up to 0.3 M in rats did not cause a significant increase in the sensitivity to pain because no hyperalgesic response was observed throughout the 5-hr observation period.

Inhibition of proteolytic activity. CaNa₂EDTA and batimastat inhibited proteolytic activity of venom on hide powder azure, with 50% inhibitory concentrations (IC₅₀) of 4.3 mM and 0.39 µM, respectively (Figure 1), indicating that metalloproteinases are responsible for the majority of proteolytic activity of B. asper venom on this substrate.

Inhibition of hemorrhagic activity. Intradermal injection of 20 µg of B. asper venom, dissolved in either PBS or PBS-Tween, induced hemorrhagic lesions of 17.5 mm and 18.5 mm diameter, respectively, 2 hr after injection. Hemorrhagic activity of venom was abrogated by CaNa₂EDTA and batimastat, with IC₅₀ of 16.9 mM and 0.74 µM, respectively (Figure 2). Preincubation with the inhibitors also abrogated hemorrhagic activity of venom in thigh muscle. In another set of experiments, venom was injected im in the right thigh and the inhibitors were administered im at various time intervals at the same site of venom injection. When CaNa₂EDTA and batimastat were injected rapidly after venom, they were able to inhibit hemorrhagic activity, at concentrations of 1.5 M and 500 µM, respectively (Figure 3). However, when time lapse between venom injection and administration of inhibitors increased, hemorrhage inhibition was only partial (Figure 3). A time-course of the development of local hemorrhage by venom alone was performed. Muscle injected with PBS had 0.70 ± 0.07 mg of hemoglobin/g wet weight (mean ± SEM, n = 4). A slight increment in hemoglobin (2.5 ± 0.36 mg of hemoglobin/g wet weight) was already observed when muscles were excised immediately after venom injection. Hemoglobin in muscle increased afterwards, reaching values of 2.52 ± 0.05, 3.72 ± 0.42,
Inhibition of hemorrhagic activity induced by Bothrops asper venom in muscle tissue. Venom (50 μg in 50 μl) was injected intramuscularly in the right thigh of mice and then, at various time intervals (immediately, 1 min, 3 min, and 5 min), mice were injected at the same site of envenomation with 50 μl of either A, a 1.5 M CaNa₂EDTA solution (in phosphate-buffered saline [PBS]) or B, a 500 μM batimastat suspension (in PBS-Tween). Controls were injected with venom alone (Ven.), PBS alone (PBS), CaNa₂EDTA alone (EDTA), and batimastat alone (bati.). One hour after venom injection, the mice were killed, their thigh muscles were removed and weighed, cut into pieces, and placed in Drabkin solution. After an overnight incubation at 4°C and a further dilution in Drabkin solution, samples were centrifuged and the absorbances of the supernatants at 540 nm were recorded. Hemoglobin concentration was then estimated from a calibration curve. Results are presented as milligrams of hemoglobin/gram of tissue (mean ± SEM, n = 5). Both inhibitors significantly reduced the extent of hemorrhage when compared with venom alone (*P < 0.05).

Inhibition of edema-forming activity of Bothrops asper venom by A, batimastat and B, CaNa₂EDTA. Venom was incubated with various concentrations of either inhibitor for 30 min at 37°C. Aliquots of the mixtures, containing 5 μg of venom in a volume of 50 μl, were injected subcutaneously in the right footpad of mice. Controls included mice injected with venom alone and with the inhibitors alone. The increment in footpad thickness was quantified with a low-pressure spring caliper at various time intervals, as an index of edema. Results are presented as the mean ± SEM (n = 4). Edema was significantly reduced (P < 0.05) by both inhibitors at all time intervals, with the exception of 6 hr with CaNa₂EDTA.

Inhibition of dermonecrotic and edema-forming activities. Intradermal injection of 200 μg of B. asper venom induced a necrotic lesion of 7 mm diameter 72 hr after injection. CaNa₂EDTA and batimastat completely inhibited dermonecrotic activity of venom, with IC₅₀ of 100 mM and 11 μM, respectively, in experiments involving preincubation of venom and inhibitors. In addition, when the inhibitors were administered locally immediately after venom injection in the thigh muscle, total inhibition of dermonecrosis was observed. Inhibition of dermonecrosis was only partial when CaNa₂EDTA or batimastat injections were delayed 15 and 30 min after envenomation. On the other hand, CaNa₂EDTA and batimastat significantly reduced edema-forming activity of B. asper venom when preincubated before injection (Figure 4). Reduction of edema was evident at various time intervals after venom and inhibitor administration.

Inhibition of myotoxic activity. CaNa₂EDTA was ineffective at reducing the extent of myonecrosis induced by B. asper venom at a concentration of 50 mM (Figure 5). In contrast, batimastat (20 μM) caused a small but significant reduction in plasma CK activity when incubated with venom prior to injection (Figure 5). Neither of the inhibitors reduced myotoxic activity of B. asper myotoxin III. Plasma CK activity of mice injected with myotoxin III (50 μg), myotoxin (50 μg) + CaNa₂EDTA (100 mM), and myotoxin (50 μg) + batimastat (20 μM) were 4,051 ± 268 U/L, 3,940 ± 5.61 ± 1.09, and 7.08 ± 1.12 mg of hemoglobin/g wet weight at 1, 3, 5, and 15 min, respectively.
asper metalloproteinase BaP1, highly relevant in counteracting the local toxicity of viper venom. To reduce hemorrhagic activity of crotaline snake venoms, EDTA or batimastat may represent a useful therapeutic alternative to confront the difficult task of neutralizing crotaline venom-induced local tissue damage and to complement the neutralizing effect of antivenoms.

**DISCUSSION**

Due to the extremely rapid development of snake venom-induced local tissue damage, neutralization by antivenoms is often difficult to achieve, even if immunotherapy is performed using Fab fragments and Fab antibody fragments. Thus, the use of synthetic enzyme inhibitors of high diffusibility that could be applied locally in the field rapidly after envenomation is an alternative to overcome the limitations of antivenom therapy in the neutralization of venom-induced local tissue damage. Since snake venom metalloproteinases induce hemorrhage, edema, myonecrosis, blister formation, and dermonecrosis, inhibition of these enzymes would be highly relevant in counteracting the local toxicity of viper venoms. It has been previously shown that batimastat significantly reduces local pathological effects induced by *B. asper* metalloproteinase BaP1, and previous studies have demonstrated the ability of chelating agents such as EDTA and DTPA to reduce hemorrhagic activity of crotaline snake venoms.

In this study, we have demonstrated that local injection of the inhibitors directly at the site of venom injection rapidly after envenomation abrogates hemorrhage and dermonecrosis induced by *B. asper* venom. In addition, both inhibitors caused a partial reduction of venom-induced edema, whereas they were not effective in counteracting the effects of myotoxic phospholipases A₂, although batimastat was able to partially reduce myotoxicity induced by crude venom. Thus, since metalloproteinases play such a prominent role in local effects, their inhibition drastically reduces the overall extent of local tissue damage. When injection of CaNa₂EDTA and batimastat was delayed, inhibition was only partial or absent, due to the extremely rapid development of these effects after pit viper venom injection. Thus, a rapid application of inhibitors is required to neutralize venom enzymes before they diffuse in the tissue and significant damage occurs. Nevertheless, our observations in mice, concerning the need of an immediate administration of these inhibitors, should not be simplistically extrapolated to human cases. It is suggested that the time course of local tissue damage in humans is not as rapid as in rodents and, therefore, the time lapse in which EDTA or batimastat injection may be beneficial is likely to be more prolonged.

Local injection of metalloproteinase inhibitors in snakebite envenomation may have an additional benefit related to the observation that venom depots are formed at the site of injection in pit viper envenomations due to local necrosis and blood vessel disruption. Such deposits may not be reached by circulating antibodies after antivenom therapy, and venom may diffuse to the bloodstream causing tissue damage later on. In situ administration of inhibitors of high diffusibility may effectively inhibit metalloproteinases at the depot, precluding them of inducing further damage.

The concentration of CaNa₂EDTA required to inhibit proteolytic, hemorrhagic, and dermonecrotic activities of *B. asper* venom was higher than the inhibitory concentrations of batimastat. Moreover, when inhibitors were administered after envenomation, batimastat was more effective at reducing the extent of hemorrhage than CaNa₂EDTA. This is probably due to the fact that batimastat contains not only a hydroxamate moiety, but also a structure that mimics the conserved cleavage site of collagen, increasing its affinity for the enzyme active site. In contrast, CaNa₂EDTA exerts a chelating effect, but lacks specificity for the active site of metalloproteinases. Thus, the use of specific peptidomimetic metalloproteinase inhibitors in the treatment of local effects of snakebite envenomations seems to have greater potential than the use of compounds having only chelating activity.

CaNa₂EDTA is currently used in the treatment of lead poisoning, and atherosclerosis, and batimastat and related metalloproteinase inhibitors have been tested in several clinical trials. The fact that these compounds are being used clinically, together with the lack of local toxicity observed in our experiments in mice, suggest that clinical trials should be designed to evaluate their usefulness in *B. asper* and other crotaline snakebite envenomations.

In conclusion, our observations indicate that in situ injection of the chelating agent CaNa₂EDTA or the peptidomimetic inhibitor batimastat rapidly after venom injection abrogates hemorrhagic and dermonecrotic activities of *B. asper* venom. It is necessary to search for inhibitors of myotoxic phospholipases A₂ and edema-forming toxins to further reduce the extent of tissue damage. It is suggested that local injection of CaNa₂EDTA or batimastat may represent a useful therapeutic alternative to confront the difficult task of neutralizing crotaline venom-induced local tissue damage and to complement the neutralizing effect of antivenoms.
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REFERENCES


