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Assessment of metalloproteinase inhibitors clodronate and doxycycline in the neutralization of hemorrhage and coagulopathy induced by *Bothrops asper* snake venom

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ABSTRACT

Snake venom metalloproteinases (SVMPs) play a prominent role in the local and systemic manifestations of viperid snakebite envenomations. Thus, the possibility of using metalloproteinase inhibitors in the treatment of these envenomations is a promising therapeutic alternative. This study assessed the ability of two metalloproteinase inhibitors, the biphosphonate clodronate and the tetracycline doxycycline, to inhibit proteolytic, hemorrhagic, coagulant and defibrinogenating effects of *Bothrops asper* venom. Both compounds were able to inhibit these activities, at concentrations in the mM range, when incubated with venom prior to testing. However, when inhibition of hemorrhage was assessed in assays involving independent injection of venom and drug, inhibition was poor, even when these compounds were injected immediately after envenomation. These findings support the concept that the effectiveness of compounds, such as clodronate and doxycycline, whose inhibitory action on SVMPs is based on zinc chelation alone, is limited, and stress the view that more specific molecules are required for an effective inhibition of SVMPs in vivo.

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1. Introduction

Envenomations by snake species of the family Viperidae are characterized by local pathological effects that develop rapidly after the bite and, depending on the severity of the case, may result in prominent tissue damage and sequelae. Such local pathological events include hemorrhage, edema, myonecrosis, dermonecrosis and blistering (Gutiérrez and Lomonte, 2003; Warrell, 2004). In addition, viperid snakebite envenomations often concur with systemic alterations, i.e. coagulopathy and bleeding, which may lead to hemodynamic disturbances and shock (Otero et al., 2002; Warrell, 2004). Many of these local and pathological effects are due to the action of snake venom metalloproteinases (SVMPs), which are zinc-dependent endopeptidases that belong to the 'reprolysin' group of metalloproteinases (Fox and Serrano, 2005). SVMPs are abundant in viperid snake venoms and, on the basis of their domain composition, are classified into groups P-I to P-IV. In general, SVMPs comprising domains additional to the basic catalytic domain, especially those belonging to group P-III, exert potent hemorrhagic or clotting effects (Fox and Serrano, 2005; Gutiérrez et al., 2005).

The key role played by SVMPs in the pathophysiology of viperid snake envenomations has prompted the search for inhibitors of these enzymes, with the rationale that abrogation of SVMP-induced effects may represent an effective therapeutic strategy which could complement antivenom administration in the management of these envenomations (Gutiérrez et al., 1999, 2007). There are abundant inhibitors

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developed by pharmaceutical research against endogenous mammalian metalloproteinases, especially matrix metalloproteinases (MMPs), which are known to play key roles in the pathogenesis of many diseases, from cancer to inflammatory conditions (Folgueras et al., 2004; Liu et al., 2006; Ganea et al., 2007), being also involved in dermonecrosis induced by *Loxosceles* sp. spider envenomation (Paixão-Cavalcante et al., 2007). MMPs are grouped together with astacins, reprolysins and serralysins, within the superfamily of 'metzincins', characterized by similar structural features at the catalytic site (Bode et al., 1993). Such similarity between MMPs and SVMPs opens the possibility that inhibitors developed against MMPs may also be effective against SVMPs. Such hypothesis has been demonstrated for several synthetic inhibitors which have been shown to effectively neutralize enzymatic and toxic activities of SVMPs (Escalante et al., 2000; Howes et al., 2007).

Two groups of molecules that constitute effective MMP inhibitors are biphosphonates and tetracyclines. Biphosphonates, such as clodronate, are synthetic compounds that show high affinity for the hydroxyapatite crystals of bone. They inhibit various MMPs by sequestering the zinc present at the catalytic site of these enzymes, in addition to other actions related with MMP expression and activation (Teronen et al., 1999). Clodronate has been used therapeutically for preventing metastasis of osteolytic tumors, and also against osteoporosis and multiple myeloma (Heikkilä et al., 2002). On the other hand, the tetracycline doxycycline is an effective MMP inhibitor with diverse clinical applications which do not depend on its antibiotic effect (Acharya et al., 2004). Its mechanism of action is based on a combination of zinc chelation and inhibition of MMP expression (Acharya et al., 2004). The demonstrated effectiveness of clodronate and doxycycline against MMPs, together with the fact that they are being used at the clinical setting, prompted us to evaluate their capacity to inhibit local and systemic activities of the venom of the snake *Bothrops asper* which are known to depend on the action of SVMPs.

2. Materials and methods

2.1. Venom and inhibitors

The venom of *B. asper* was a pool obtained from adult specimens collected in the Pacific region of Costa Rica. Venom was lyophilized and stored at −40 °C. Clodronate and doxycycline were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Experimental animals

Mice of the strain CD-1 were used throughout the study. All experimental protocols involving mice were approved by the Committee for the Use and Care of Laboratory Animals (CICUA) of the University of Costa Rica.

2.3. Inhibition of proteolytic activity

Proteolytic activity was tested on azocasein (Wang et al., 2004; Escalante et al., 2006). Venom (12 μg) dissolved in 10 μL of 25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 was incubated with 100 μL of a 5-mg/mL azocasein solution (Sigma–Aldrich). After incubation at 37 °C for 90 min, the reaction was stopped by the addition of 200 μL of 5% trichloroacetic acid. After centrifugation at 100 × g, 100 μL of supernatant was diluted with 100 μL of 0.5 M NaOH, and the absorbance at 450 nm was recorded. The absorbances of samples of azocasein incubated with buffer alone were subtracted from the values of absorbances of samples incubated with venom. For inhibition experiments, various concentrations of clodronate (5–200 mM) or doxycycline (5–80 mM) were incubated with a fixed concentration of venom for 30 min at 22–25 °C. Then, proteolytic activity was determined as described above. Controls included venom incubated without inhibitors, inhibitors incubated without venom, and buffer alone.

2.4. Inhibition of hemorrhagic activity

2.4.1. Experiments with preincubation

Mixtures containing a fixed amount of *B. asper* venom and various concentrations of either clodronate (from 25 to 200 mM) or doxycycline (from 12.5 to 200 mM) were prepared, using PBS as diluent. Controls included mixtures of venom and PBS, and inhibitors and PBS. After 30 min of incubation at 22–25 °C, aliquots of 100 μL of the mixtures, containing 15 μg venom (which corresponds to 10 minimum hemorrhagic doses, Gutiérrez et al., 1985), were injected intradermally, in the ventral abdominal region, into groups of four mice (18–20 g). One hour after injection, mice were sacrificed by CO_2 inhalation, their skins were removed and the hemorrhagic area determined (Gutiérrez et al., 1985).

2.4.2. Experiments with independent injection

Groups of mice (18–20 g) were injected intramuscularly (i.m.) in the right thigh with 50 μg venom, dissolved in 50 μL PBS. Immediately, 50 μL of PBS containing various concentrations of either clodronate or doxycycline (from 25 to 200 mM), were administered i.m. in the same region where venom had been injected. Controls included mice injected with venom and then PBS, and mice injected with PBS and then with the inhibitors. In some experiments, injections were performed in the gastrocnemius muscle. One hour after injection, mice were sacrificed by CO_2 inhalation and the injected muscles were dissected out, weighed, cut with a razor blade into small pieces and placed in 1.5 mL of Drabkin solution (Rucavado et al., 2000). After an overnight incubation at 4 °C, aliquots of 1.2 mL each sample were centrifuged at 2000 × g for 5 min, and then 1 mL of supernatant was diluted with 1 mL Drabkin, and the absorbances at 540 nm were recorded. Hemoglobin concentration was estimated against a standard curve, and expressed as mg hemoglobin per g tissue. Hemorrhagic activity was expressed as percentage, considering 100% the hemoglobin content in muscle injected with venom and no inhibitor.

2.5. Inhibition of coagulant activity

The minimum coagulant dose (MCD) of *B. asper* venom on human citrated plasma was estimated as described

(Gené et al., 1989). For inhibition studies, a constant amount of venom (1.2 μg, corresponding to 2 MCDs), dissolved in PBS, was incubated with various concentrations of the inhibitors (from 10 to 100 mM), for 30 min at 22–25 °C. Then, 100 μL of the mixtures were added to 200 μL of citrated human plasma, previously incubated at 37 °C and clotting times were recorded. Controls included venom incubated without inhibitors, inhibitors incubated without venom and PBS alone. On the basis of these results, a different experimental setting was performed. A fixed concentration of inhibitor, selected on the basis of the previous experiments, was incubated with varying concentrations of venom, for 30 min at 22–25 °C. Then, 100 μL of the mixtures were added to 200 μL of human plasma, and clotting times recorded. The MCD was then estimated as described above.

2.6. Inhibition of defibrinogenating activity

The minimum defibrinogenating dose (MDD) of *B. asper* venom was determined as described by Gené et al. (1989). Mixtures containing a fixed amount of venom, and two concentrations of either clodronate or doxycycline (25 and 50 mM), were prepared in PBS. Controls included venom without inhibitors, inhibitors without venom and PBS alone. After 30 min of incubation at 22–25 °C, 200 μL of the mixtures, containing 10 μg venom (2 MDD), were injected intravenously (i.v.) in the tail vein of groups of mice (18–20 g). After 2 h, mice were bled from the retroorbital venous plexus, blood was placed into glass tubes and clotting times were recorded.

2.7. Statistical analysis

The significance of the differences of the mean values of several experimental groups was determined by ANOVA, followed by Tukey test. When only two groups were compared, the Student's *t* test was used.

3. Results

3.1. Inhibition of proteolytic activity

Clodronate and doxycycline were effective at inhibiting proteolytic activity of *B. asper* venom on azocasein. IC_{50}s for these inhibitors were 69 and 16 mM, respectively. A clodronate concentration of 200 mM completely abrogated proteolytic activity. In the case of doxycycline, when using concentrations higher than 25 mM, absorbances at 540 nm increased due to the color of the inhibitor; therefore, concentration achieving 100% inhibition could not be determined.

3.2. Inhibition of hemorrhagic activity

3.2.1. Assays involving preincubation of venom and inhibitors

Clodronate and doxycycline inhibited, in a dose-dependent fashion, hemorrhagic activity of *B. asper* venom in the intradermal test (Fig. 1). Estimated IC_{50}s of these drugs for this effect were 63 and 14 mM, respectively. Control mice receiving 200 mM clodronate and 100 mM doxycycline showed hyperemic areas of <3 mm diameter; moreover, 200 mM doxycycline induced a lesion in the skin and, therefore, inhibition could not be tested at this concentration.

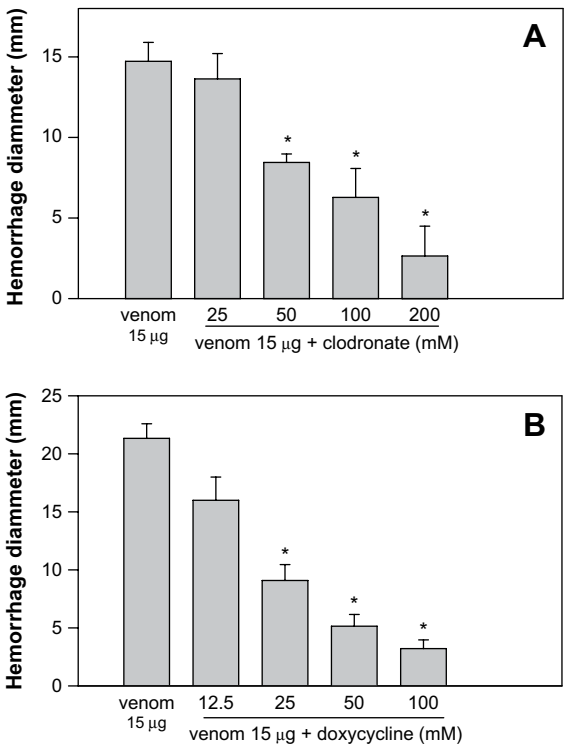

Fig. 1. Inhibition of hemorrhagic activity of *B. asper* venom by clodronate (A) and doxycycline (B). Venom (15 μg) was incubated either with PBS or with various concentrations of the inhibitors. Then, aliquots were injected i.d. in mice and the diameter of hemorrhagic lesions in the skin was assessed after 1 h, as described in Section 2. Controls injected with either 200 mM clodronate alone or 100 mM doxycycline alone were included. Results are presented as mean ± SD ($n = 4$). $^*p < 0.05$ when compared with hemorrhagic diameters of mice injected with venom alone.

3.2.2. Assays involving independent injection of venom and inhibitors

When clodronate or doxycycline was administered i.m., at the site of venom injection, only a partial reduction of hemorrhage was achieved when administering the inhibitors immediately after venom injection (Fig. 2A and B).

3.3. Inhibition of coagulant activity

The minimum coagulant dose of *B. asper* venom was 0.6 μg. Clodronate inhibited the coagulant activity of *B. asper* venom in plasma (Fig. 3). The effect of doxycycline could not be tested because a precipitate formed when this inhibitor was mixed with venom and plasma, precluding the proper observation of clot formation. When the coagulant activity of venom on human plasma was determined in the presence of 100 mM clodronate, the MCD was 49 ± 2 μg, corresponding to 82 times the MCD of venom alone (which was 0.6 ± 0.01 μg).

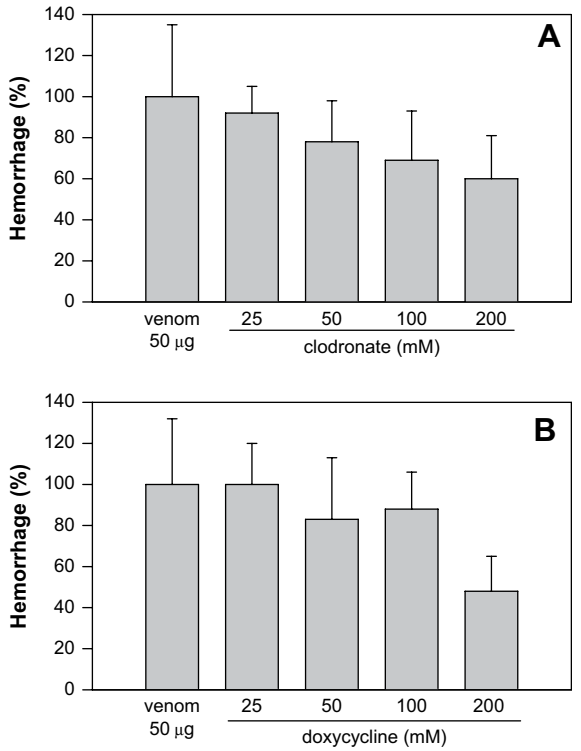

Fig. 2. Inhibition of hemorrhagic activity of *B. asper* venom by clodronate (A) or doxycycline (B) in assays involving independent injection of venom and inhibitors. Mice were injected i.m., with 50 μg venom, in 50 μL PBS. Immediately after venom injection, various concentrations of either clodronate or doxycycline, in 50 μL PBS, were injected at the same location where venom had been administered. Controls included mice injected with venom and then with PBS without inhibitors. The amount of hemoglobin present in the injected muscles was determined as described in Section 2, and hemorrhagic activity was expressed as percentage, considering 100% the amount of hemoglobin present in muscle injected with venom alone. Results are presented as mean ± SD ($n = 4$). There was a partial, albeit not significant ($p > 0.05$) reduction in the extent of local hemorrhage with both inhibitors.

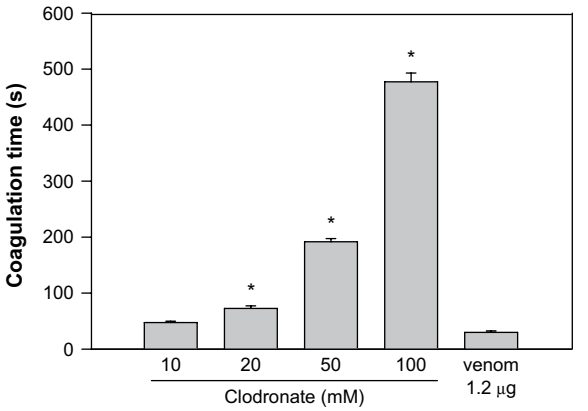

Fig. 3. Inhibition of coagulant activity of *B. asper* venom by clodronate. Venom was incubated with either PBS or various concentrations of clodronate. Then, aliquots of the mixtures were added to citrated human plasma, and coagulation times were recorded. A dose-dependent inhibition of coagulant activity was observed. Results correspond to mean ± SD ($n = 4$). *$p < 0.05$ when compared with coagulation time of plasma incubated with venom without inhibitors.

3.4. Inhibition of defibrinogenating activity

The minimum defibrinogenating dose of *B. asper* venom was 5 μg. Incubation of two MDDs of *B. asper* venom with doxycycline (25 and 50 mM) completely inhibited the defibrinogenating activity of the venom (Table 1). A solution of 100 mM doxycycline induced lethality in all mice injected. In the case of clodronate, a concentration of 50 mM was ineffective at inhibiting defibrinogenation. Concentrations of clodronate of 100 and 200 mM were toxic to mice upon i.v. injection, inducing lethality within the first minutes of injection.

4. Discussion

SVMPs play a key role in the local and systemic effects induced by viperid snake venoms (Markland, 1998; Gutiérrez and Rucavado, 2000; Kamiguti, 2005). Therefore, the development of therapeutic strategies aimed at inhibiting SVMPs constitutes a relevant step forward for improving the treatment of snakebite envenomations (Gutiérrez et al., 2007). In the case of local tissue damage, the possibility of using enzyme inhibitors in the field, rapidly after the onset of envenomation, has been raised (Borkow et al., 1997; Rucavado et al., 2000; Gutiérrez et al., 1999, 2007), since such intervention would abrogate the action of these enzymes before significant tissue damage has been inflicted, and before these toxins are distributed systemically. Such rapid inhibition would be followed by the administration of antivenoms in health centers which usually occur several hours after the bite (Arroyo et al., 1999). The fact that many inhibitors for MMPs have been developed and tested in the clinical setting for a number of diseases allows for the assessment of such inhibitors against SVMPs. Clodronate and doxycycline are well-known MMP inhibitors used in various diseases and whose activity is based, among other suggested mechanisms, on the chelation of the zinc atom present at the catalytic center of metalloproteinases (Teronen et al., 1999; Acharya et al., 2004). Thus, these drugs are of potential value for

Table 1
Inhibition of defibrinogenating activity of *B. asper* venom by clodronate and doxycycline[a]

Treatment	Coagulation time (s)
PBS	89 ± 2
Venom (10 μg)	>900[b]
Doxycycline (25 mM)	83 ± 1
Doxycycline (50 mM)	78 ± 1
Venom (10 μg) + doxycycline (25 mM)	85 ± 1
Venom (10 μg) + doxycycline (50 mM)	67 ± 2
Clodronate (50 mM)	75 ± 1
Venom (10 μg) + clodronate (50 mM)	>900

[a] Solutions of *B. asper* venom were incubated with PBS, doxycycline or clodronate. Aliquots of the mixtures were injected i.v. in mice and, after 1 h, animals were bled and clotting times recorded. Controls received PBS, doxycycline or clodronate alone. Results are presented as mean ± SD ($n = 4$).

[b] In these cases, blood did not clot after 900 s of observation.

snakebite envenomings, especially since they are readily accessible and relatively cheap.

Proteolytic activity of *B. asper* venom on azocasein was inhibited by clodronate and doxycycline, thus confirming previous observations with batimastat and $CaNa_2EDTA$ (Rucavado et al., 2000) and indicating that SVMPs are greatly responsible for proteolysis by this venom. Moreover, when incubated with venom before injection, these inhibitors are effective in the neutralization of hemorrhagic activity of *B. asper* venom, which is known to depend on the action of SVMPs (Bjarnason and Fox, 1994; Gutiérrez et al., 2005). A number of hemorrhagic SVMPs have been isolated from *B. asper* venom. The most abundant SVMP is the P-I enzyme BaP1 (Gutiérrez et al., 1995; Watanabe et al., 2003), although the most potent hemorrhagic enzymes are the high molecular mass P-III SVMPs BaH1 and BaH4 (Borkow et al., 1993; Franceschi et al., 2000). Moreover, the effectiveness of clodronate and doxycycline to inhibit coagulant activity of *B. asper* venom on plasma and defibrinogenating activity (in the case of doxycycline) can also be explained by the observation that SVMPs are the most important coagulant components in this venom (Rucavado et al., 2004). A prothrombin-activating SVMP, named basparin A, has been characterized from this venom (Loría et al., 2003). Thrombin-like serine proteinases are also present in *B. asper* venom (Aragón and Gubensek, 1978; Pérez et al., 2008), but they seem to play a secondary role in the coagulopathy inflicted by this venom (Rucavado et al., 2004, 2005), probably due to their low concentration in the venom (Pérez et al., 2008).

Inhibitory concentrations of clodronate and doxycycline when tested against *B. asper* venom are in the mM range, similarly to the inhibitory concentrations of other chelating compounds such as N_4EDTA and $CaNa_2EDTA$ (Ownby et al., 1975; Borkow et al., 1997; Rucavado et al., 2000). In contrast, more specific metalloproteinase inhibitors, such as the peptidomimetic hydroxamate batimastat and other synthetic compounds, are effective SVMP inhibitors in the μM range (Escalante et al., 2000; Rucavado et al., 2000; Howes et al., 2007). Clearly, inhibition based on zinc-chelating action alone requires higher concentration than more specific inhibitors, to block venom enzymes. Moreover, since most chelating agents also sequester other cations such as Ca^{2+}, they are toxic when administered in high concentrations. This was observed in the present study when using concentrations of clodronate and doxycycline higher than 100 mM. An exception is $CaNa_2EDTA$, which is of low toxicity (Rucavado et al., 2000) owing to its inability to chelate Ca^{2+}. This EDTA salt is used in the treatment of lead poisoning (Klaassen, 1990; Tandon et al., 1998).

When inhibition assays were performed with a protocol that reproduces the actual circumstances of snakebite, i.e. when venom is injected and the inhibitor is administered immediately after envenomation at the site of venom injection, inhibition of hemorrhagic activity in muscle is achieved only to a partial extent. Similar findings were described with $CaNa_2EDTA$ (Rucavado et al., 2000). It is suggested that such inability to completely abrogate local hemorrhage is due to the non-specificity of the mechanism of inhibition by chelating agents. Thus, when injected in vivo, it is likely that chelating molecules are rapidly diluted and bind to cations in the tissues, thus precluding the interaction of these compounds with the zinc at the catalytic site of SVMPs. In these circumstances, their inhibitory potential against venom enzymes is greatly diminished, even when injected rapidly after envenomation. In contrast, a more effective inhibition of local hemorrhage is achieved when the peptidomimetic hydroxamate batimastat was tested (Escalante et al., 2000; Rucavado et al., 2000). This molecule mimics the cleavage site of collagen, thus highly increasing its affinity for the catalytic site of metalloproteinases (Bottomley et al., 1998). Since the hydroxamate moiety of batimastat chelates the catalytic zinc, this combination of structural features greatly enhances the inhibitory potential of this type of inhibitor, when compared with inhibitors based on ion chelation only. Accordingly, batimastat, and probably other similar peptidomimetic inhibitors, is highly effective at abrogating hemorrhagic activity of SVMPs even when injected after envenomation (Escalante et al., 2000; Rucavado et al., 2000).

In conclusion, our observations demonstrate that the biphosphonate clodronate inhibits proteolytic, hemorrhagic and coagulant activities of *B. asper* venom, and the tetracycline doxycycline is effective in the inhibition of proteolytic, hemorrhagic and defibrinogenating activities, when incubated with venom before testing, albeit requiring high, i.e. mM concentrations to exert their action. Such low activity might hamper their potential usefulness in snakebite envenomations as shown hereby in experiments involving independent injection of venom and inhibitor. It is suggested that the search for SVMP inhibitors that could be tested for viperid snakebite envenomations in the clinical setting should be centered on more specific molecules, such as peptidomimetics, which are able to inhibit SVMPs in the nM or μM ranges, and which have a good safety profile.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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