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Effectiveness of Batimastat, a Synthetic Inhibitor of Matrix Metalloproteinases, in Neutralizing Local Tissue Damage Induced by BaP1, a Hemorrhagic Metalloproteinase from the Venom of the Snake *Bothrops asper*

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ABSTRACT. Batimastat (BB-94), a synthetic hydroxamate peptidomimetic matrix metalloproteinase inhibitor, was tested for its ability to inhibit proteolytic and toxic effects induced by BaP1, a 24-kDa hemorrhagic metalloproteinase isolated from the venom of *Bothrops asper*, the medically most important snake species in Central America and southern Mexico. Batimastat inhibited proteolytic activity on biotinylated casein, with an IC_{50} of 80 nM. In addition, batimastat was effective in inhibiting hemorrhagic, dermonecrotic, and edema-forming activities of this metalloproteinase if incubated with the enzyme prior to the assays. When the inhibitor was administered i.m. at the site of the toxin injection without preincubation, rapidly after metalloproteinase administration, it totally abrogated the hemorrhagic and dermonecrotic effects of BaP1. Inhibition was less effective as the time lapse between toxin and batimastat injection increased, due to the extremely rapid development of BaP1-induced local tissue damage in this experimental model. On the other hand, batimastat was ineffective if administered by the i.p. route immediately after toxin injection. It is concluded that batimastat, and probably other synthetic metalloproteinase inhibitors, may become useful therapeutic tools aimed at the *in situ* inhibition of venom metalloproteinases, when injected at the site of the bite rapidly after envenomation. BIOCHEM PHARMACOL **60**;2:269–274, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. matrix metalloproteinases; metalloproteinase inhibitors; batimastat; snake venom; hydroxamates; hemorrhage; venom metalloproteinases; *Bothrops asper*

Envenomations by pit vipers (family Viperidae, subfamily Crotalinae) constitute a public health hazard in many regions of the world [1]. They are characterized by prominent local tissue damage, i.e. necrosis, hemorrhage, and edema, owing to the action of various toxins [2–4]. In addition, systemic alterations such as hemorrhage, coagulopathy, shock, and acute renal failure may occur in these patients [5–7]. Local tissue damage develops very rapidly after venom injection, and, as a consequence, permanent tissue loss or dysfunction often ensues if treatment is delayed, as frequently occurs in tropical regions of Africa, Asia, and Latin America [1, 8].

Intravenous administration of equine or ovine-derived antivenoms constitutes the mainstay in treating snakebite envenomations [6]. However, due to the rapid development of local tissue damage [3], and since antivenoms are available mainly in health facilities, these products usually are administered when local effects have developed to some extent. Therefore, their neutralization is achieved only partially [9]. Consequently, there is a need to develop alternative approaches that may be applied in the field, complementing antivenom therapy in the neutralization of venom-induced local effects.

The pathogenesis of myonecrosis, hemorrhage, and edema has been investigated in detail in the case of the venom of *Bothrops asper*, the most important venomous snake in Central America and southern Mexico [10–15]. Several studies have demonstrated that zinc-dependent metalloproteinases play a relevant role in the pathogenesis of various local tissue alterations induced by this venom, such as hemorrhage [16–18], myonecrosis [18, 19], edema [20], and blister formation [15]. Therefore, it can be hypothesized that inhibition of venom metalloproteinases may result in a significant reduction of overall local tissue damage in these envenomations.

Snake venom metalloproteinases belong to a large family of zinc-dependent enzymes having either a single metalloproteinase domain or a multi-domain organization [21]. They have strong similarities with MMPs,† as both groups belong to the family of "metzincins" [22]. MMPs play a

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† *Abbreviation*: MMPs, matrix metalloproteinases.

relevant role in a variety of pathologies such as rheumatoid arthritis, osteoarthritis, cancer, periodontal disease, and various inflammatory diseases [23]. Thus, there is a growing effort to develop synthetic inhibitors of MMPs [23, 24]. The most successful advances are related to the design of peptidomimetics that mimic the conserved C-terminal triplet of the collagenase-mediated cleavage site of collagen, incorporating a zinc ligand in the place of the scissile amide bond [24]. Batimastat, also known as BB-94, is a matrix metalloproteinase inhibitor currently being tested in clinical trials [25], which has a hydroxamate group as the zinc ligand [24]. Due to the similarities in the zinc-binding motif and in the catalytic mechanism between MMPs and venom metalloproteinases, this study was designed to assess the ability of batimastat to inhibit BaP1, a tissue-damaging metalloproteinase from the venom of *B. asper*.

MATERIALS AND METHODS

Metalloproteinase and Inhibitor

BaP1 was isolated from a venom pool of more than 40 adult specimens of *B. asper* collected in the Pacific region of Costa Rica. Purification was performed by ion-exchange chromatography on Carboxymethyl Sephadex C-50, gel filtration in Sephacryl S-200, and affinity chromatography on Affi-Gel Blue, as previously described [15, 20]. Homogeneity was demonstrated by SDS–PAGE run under reducing conditions [26], using 12% acrylamide gels. Batimastat (also known as BB-94; [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(thienylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide]; M_r 478) was provided by British Biotech Pharmaceuticals Ltd. Batimastat suspension was prepared by sonication in phosphate-buffered saline solution, pH 7.2 (PBS), containing 0.01% Tween 80 (PBS–Tween).

Inhibition of Proteolytic Activity

The modification described by Franceschi *et al.* [27] of the highly sensitive assay of Koritsas and Atkinson [28] was used, with biotinylated *N,N*-dimethylcasein as substrate. Microtiter plates (Immulon 2, Dynatech) were coated with 200 ng of biotinylated casein, dissolved in coating buffer. After 24 hr of incubation at room temperature, plates were washed with PBS–Tween 0.05% (v/v), and the free sites in the plastic wells were blocked with 100 μL of a 2 g/dL BSA solution (in PBS). After five additional washings with PBS–Tween, 100-μL aliquots of solutions, containing either 0.5 μg BaP1 or mixtures of BaP1 and various concentrations of batimastat that had been incubated for 30 min at 37°, were added and incubated for 24 hr at 37°. Plates were washed with PBS–Tween, 100 μL of avidin–peroxidase conjugate (Sigma), diluted 1:4000 with PBS, was added to each well, and the plates were incubated for 30 min at 25°. After five additional washings, 100 μL of substrate solution (2 mg/mL of *O*-phenylenediamine, 0.012% H_2O_2 in 0.1 M sodium citrate, pH 5.0) was added, and the plates were incubated for 3 min at room temperature. The reaction was stopped by adding 50 μL of 2 M HCl, and absorbances were recorded at 492 nm in a Dynatech MR 5000 microplate reader. All samples were run in triplicate.

Inhibition Studies of Biological Activities

Two types of assays were performed: (a) assays where metalloproteinase and inhibitor were incubated prior to injection, and (b) assays in which metalloproteinase was injected and then, at various time intervals, the inhibitor was administered. In both types of assays, the dose of BaP1 was selected after dose–response studies for each particular effect, and corresponded to the linear portion of the curve. These types of assays have been used and validated in the study of the neutralizing ability of antivenoms [9, 29].

Inhibition of Hemorrhagic Activity

A constant amount of BaP1 was incubated with various concentrations of batimastat for 30 min at 37°. Then, aliquots of the mixtures, containing 15 μg BaP1 in a total volume of 100 μL, were injected intradermally into groups of four Swiss–Webster mice (18–20 g). The following control groups were included: (a) mice injected with BaP1 alone dissolved in PBS, (b) mice injected with BaP1 alone dissolved in PBS–0.01% Tween, (c) mice injected with PBS, (d) mice injected with PBS–Tween, and (e) mice injected with batimastat dissolved in PBS–Tween. Mice 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 [30, 31]. In another series of experiments, mixtures of BaP1 and batimastat were prepared and incubated as described, and aliquots containing 60 μg BaP1 were injected i.m. in the right thighs of mice. The same controls described above were included. One hour after injection, the mice were killed, and the injected muscle was dissected out and weighed. It was homogenized immediately in 1.5 mL of Drabkin solution [32] using a Brinkmann homogenizer PT 10/35 (Polytron). After centrifugation, 1 mL of the supernatant was added to 2 mL of Drabkin solution, and the absorbance at 540 nm was recorded as a quantitative index of the amount of hemoglobin present in the tissue [32, 33]. Results were expressed as milligrams hemoglobin per gram of muscle wet weight.

In experiments with independent injection of metalloproteinase and inhibitor, groups of 5 mice were injected i.m. with 60 μg BaP1, dissolved in 50 μL PBS, in the right thigh. Then, at various time intervals (0, 1, 3, 5, and 15 min), mice received an injection of batimastat (50 μL of a 200 μM suspension) at the same site where BaP1 had been administered. This dose was selected on the basis of the results obtained in experiments with preincubation of BaP1 and the inhibitor. Hemorrhage was assessed by determining the hemoglobin in muscle tissue, as described. The same controls described above were included. In another series of experiments, BaP1 was injected i.m. as described and then, immediately after toxin injection, mice received an i.p.

administration of 0.5 mL batimastat (30 mg/kg). Animals were killed after 1 hr, and hemorrhage in muscle was quantitated. This i.p. dose of batimastat has been used in previous studies dealing with the effect of this inhibitor in experimental cancer models [34].

Inhibition of Dermonecrotic Activity

A constant amount of BaP1 was incubated with various concentrations of batimastat for 30 min at 37°. Then, aliquots of the mixtures, containing 60 μg BaP1 in 100 μL, were injected intradermally in groups of 4 mice (18–20 g). The same control groups described for inhibition of hemorrhagic activity were included. After 72 hr, mice were killed, and the necrotic lesion in the inner side of the skin was measured [35].

Inhibition of Edema-Forming Activity

A constant amount of BaP1 was incubated with various concentrations of batimastat for 30 min at 37°. Then, aliquots of the mixtures, containing 5 μg BaP1 in 50 μL, were injected s.c. into the right footpads of mice (18–20 g). The same controls described above were included. Edema was assessed at various time intervals by measuring the footpad thickness with a low-pressure spring caliper [36], and expressed as the increment in footpad thickness.

RESULTS

Assays with Preincubation of BaP1 and Batimastat

Batimastat inhibited, in a concentration-dependent way, the proteolytic activity of BaP1 on biotinylated casein, with an IC_{50} of 80 nM (Fig. 1). A concentration of 400 nM batimastat totally inhibited caseinolytic activity. Moreover, batimastat was effective in inhibiting the hemorrhagic activity of BaP1 in skin and muscle (Fig. 2). When hemorrhage inhibition was assessed in the skin, the IC_{50} was 1.95 μM. Batimastat concentrations of 5.71 and 12.6 μM abrogated hemorrhagic activity totally in skin and muscle, respectively. Mice injected in the skin with batimastat alone, dissolved in PBS–Tween, presented a hyperemic region in the area of injection, whereas no macroscopic alterations were observed in muscle tissue injected with PBS, PBS–Tween, or batimastat alone. Batimastat was devoid of necrotic activity in skin and muscle and induced only a mild edema when administered into the footpads of mice (Fig. 3). On the other hand, batimastat inhibited, in a concentration-dependent fashion, edema-forming activity of BaP1 in the footpad (Fig. 3). In addition, a 25.3 μM (12.1 μg/mL) suspension of batimastat abrogated the dermonecrotic activity of BaP1, as no necrotic lesions were observed in the skin 72 hr after injection of the BaP1–batimastat mixture, whereas mice injected with BaP1 dissolved in PBS–Tween showed a necrotic lesion of 5-mm diameter.

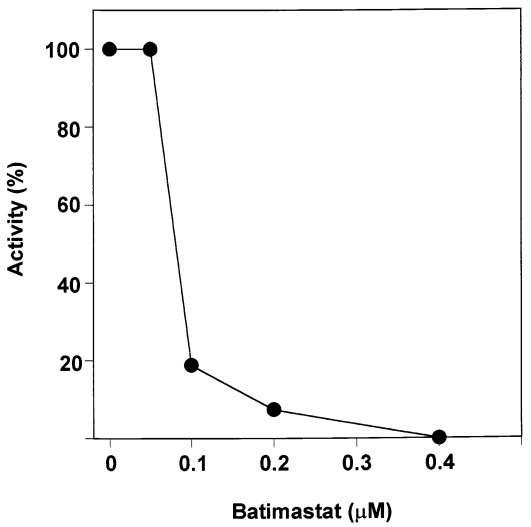

FIG. 1. Inhibition of proteolytic activity of BaP1 by batimastat. A constant amount of BaP1 (0.5 μg) was incubated, for 30 min at 37° with either PBS–Tween or various concentrations of batimastat, and the proteolytic activity of the mixtures was assessed on biotinylated casein, as described in Materials and Methods. Proteolytic activity was expressed as a percentage, 100% corresponding to the activity of BaP1 incubated without the inhibitor. One hundred percent activity corresponds to a change in absorbance (at 490 nm) of 0.74, under the conditions described in Materials and Methods. Experiments were performed in triplicate.

Assays with Independent Injection of BaP1 and Batimastat

When BaP1 was injected i.m. and then, at various time intervals, batimastat was administered at the same site, the inhibitor totally abrogated hemorrhagic activity when ad-

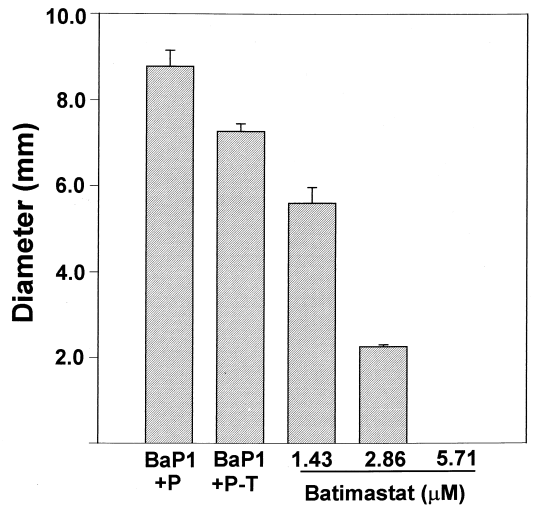

FIG. 2. Inhibition of hemorrhagic activity of BaP1 by batimastat. A constant amount of BaP1 was incubated for 30 min at 37° with either PBS (P), PBS–Tween (P-T), or various concentrations of batimastat in PBS–Tween. Aliquots of the mixtures, containing 15 μg BaP1, were injected intradermally into mice, and the diameter of hemorrhagic lesions was assessed after 2 hr. Results are presented as means ± SD (N = 4).

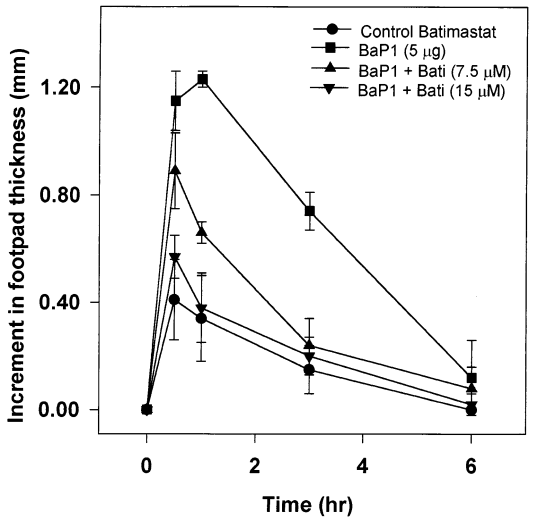

FIG. 3. Inhibition of edema-forming activity of BaP1 by batimastat. A constant amount of BaP1 was incubated for 30 min at 37° with either PBS–Tween or various concentrations of batimastat. Then aliquots of 50 μL of the mixtures, containing 5 μg BaP1, were injected s.c. into the right footpads of mice. Controls included mice injected with batimastat alone (Control batimastat). Edema was assessed at various time intervals by measuring the increase in footpad thickness with a low-pressure spring caliper. Results are presented as means ± SD (N = 4). Inhibition by 7.5 and 15 μM batimastat was significant at 0.5, 1, and 3 hr ($P < 0.05$).

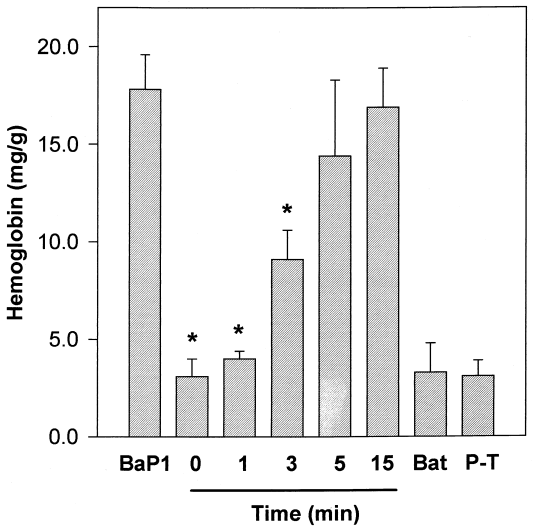

FIG. 4. Batimastat inhibition of hemorrhagic activity induced by BaP1 in muscle tissue. BaP1 (60 μg) was injected i.m. into the right thighs of mice and then, at various time intervals (0, 1, 3, 5, and 15 min), 50 μL of a 200 μM batimastat suspension was injected at the same site of toxin administration. Controls included injections of BaP1 alone (BaP1), PBS–Tween (P-T), and batimastat in PBS–Tween (Bat). Hemoglobin concentration in the injected muscle was determined after 1 hr by recording the absorbance at 540 nm of muscle homogenate supernatants, as a quantitative index of hemorrhage. Hemoglobin was expressed as milligrams of hemoglobin per gram of muscle wet weight. Results are presented as means ± SD (N = 5). Key: (*) $P < 0.05$ when compared with hemorrhage induced by BaP1 alone.

ministered at 0 and 1 min after BaP1 injection (Fig. 4). When the time lapse between metalloproteinase injection and batimastat administration increased, inhibition was only partial or absent (Fig. 4). Furthermore, immediate injection of batimastat prevented the development of blisters and dermonecrotic lesions, which typically develop after i.m. injection of BaP1 [15]. Thus, batimastat was effective in counteracting hemorrhagic and skin-damaging activities of BaP1 when administered rapidly after metalloproteinase injection. On the other hand, when batimastat was administered i.p. at a dose of 30 mg/kg, the extent of hemorrhage in muscle tissue was not reduced, even when the treatment was performed immediately after toxin injection.

DISCUSSION

Venom metalloproteinases play a relevant role in the pathogenesis of local tissue damage characteristic of pit viper envenomations [3, 21]. In the case of *B. asper* venom, metalloproteinases induce hemorrhage [17, 27], myonecrosis [18, 19], dermonecrosis, blister formation [15], and edema [20]. Neutralization of these activities by antivenoms is a difficult task, due to the time lapse between envenomation and antivenom administration in hospitals [9]. The use of synthetic metalloproteinase inhibitors that could be administered in the field directly at the site of venom injection may represent a valid alternative to confront this difficult problem.

Batimastat is a hydroxamate peptidomimetic being tested in experimental and clinical trials for various pathologies in which MMPs are involved [24, 34, 37]. Due to the relative lack of specificity of this inhibitor towards different MMPs [24], the possibility was considered that batimastat may also inhibit snake venom metalloproteinases, as they share with MMPs a similar zinc-binding motif [22]. The interaction of batimastat with atrolysin C, a hemorrhagic metalloproteinase from *Crotalus atrox* venom, has been reported [38]. Our observations clearly corroborate this hypothesis, as batimastat was highly effective in neutralizing the proteolytic, hemorrhagic, dermonecrotic, and edema-forming activities of metalloproteinase BaP1. These findings strongly suggest that all these pharmacological activities of BaP1 depend on the proteolytic degradation of tissue components, since batimastat specifically inhibits enzymatic activity.

Due to its poor oral bioavailability and low solubility in physiological solutions, batimastat usually is suspended in PBS–Tween through sonication and administered i.p. to experimental animals, to achieve sustained levels in serum [34, 37]. However, since snakebite envenomations are characterized by a direct and rapid injection of venom into muscle tissue, we modified this protocol to administer batimastat directly at the site of toxin injection, in an attempt to inhibit metalloproteinases already present in the tissue. As evidenced by the lack of inhibition observed in

our results with i.p. injection of batimastat, systemic administration of the inhibitor would not be effective, since its concentration at the site of toxin injection would be low owing to the dilution of batimastat in various body compartments. Additionally, it takes several hours for batimastat to reach peak levels in the circulation after i.p. injection. Therefore, we tested the hypothesis that a local and rapid administration of batimastat would inhibit metalloproteinases present in the tissue before they cause extensive local tissue damage and become distributed systemically. Since crotaline snakes inject their venoms mainly into muscle tissue, we used an experimental approach in which both BaP1 and inhibitor are administered i.m., as this represents a more adequate model of actual envenomations. Our results with independent injection of BaP1 and inhibitor are encouraging, since total inhibition of hemorrhage and skin damage was achieved when batimastat was administered locally immediately after toxin injection.

Previous studies have shown that hemorrhage develops very rapidly after either *B. asper* venom [10] or BaP1 injections [18]. This is the cause of poor neutralization when antivenom is administered i.v. [31, 39]. Moreover, the use of small antibody fragments, $F(ab')_2$ and Fab, does not improve neutralization, probably due to the rapid onset of local tissue damage [9, 40, 41]. Administration of antivenom directly at the site of venom injection does not improve neutralization either [39]. Thus, even though antivenom administration constitutes the central element in treating snakebite envenomations, new alternatives are needed to complement antivenoms and to inhibit locally acting toxins. Our results suggest that a rapid administration of metalloproteinase inhibitors at the site of venom injection might be effective in neutralizing venom metalloproteinases and, consequently, in reducing the extent of local tissue damage. In our experimental protocol, inhibition of hemorrhage was complete only if batimastat administration was performed rapidly after metalloproteinase injection, due to the fast development of local bleeding after BaP1 injection [18]. Nevertheless, observations in mice should not be extrapolated simplistically 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 batimastat injection may be beneficial is likely to be more prolonged, a hypothesis that needs to be addressed in clinical studies.

It has been shown that venom metalloproteinases participate in the pathogenesis of local tissue damage in two additional ways: (a) by releasing tumor necrosis factor-α from its membrane-bound precursor [42], contributing to the generation of a cascade of inflammatory mediators, which may induce further tissue damage, and (b) by inducing the synthesis of MMPs in affected tissues [15]. Thus, batimastat and other metalloproteinase inhibitors may mitigate venom-induced local tissue damage not only by directly inhibiting venom metalloproteinases, but also by modulating cytokine production and extracellular matrix degradation induced by MMPs synthesized as part of the inflammatory response. Since metalloproteinases are common in many medically relevant snake venoms [21], our findings support the hypothesis that administration of metalloproteinase inhibitors directly at the site of venom injection rapidly after envenomation may be an effective alternative to counteract venom-induced local tissue damage.

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