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Glycolic acid inhibits enzymatic, hemorrhagic and edema-inducing activities of BaP1, a P–I metalloproteinase from *Bothrops asper* snake venom: Insights from docking and molecular modeling

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ABSTRACT

Glycolic acid (GA) (2-Hydroxyethanoic acid) is widely used as chemical peeling agent in Dermatology and, more recently, as a therapeutic and cosmetic compound in the field of skin care and disease treatment. In this work we tested the inhibitory ability of glycolic acid on the enzymatic, hemorrhagic and edema-inducing activities of BaP1, a P–I metalloproteinase from *Bothrops asper* venom, which induces a variety of toxic actions. Glycolic acid inhibited the proteolytic activity of BaP1 on azocasein, with an IC_{50} of 1.67 mM. The compound was also effective at inhibiting the hemorrhagic activity of BaP1 in skin and muscle in experiments involving preincubation of enzyme and inhibitor prior to injection. When BaP1 was injected i.m. and then, at the same site, different concentrations of glycolic acid were administered at either 0 or 5 min, 7 mM solutions of the inhibitor partially abrogated hemorrhagic activity when administered at 0 min. Moreover, glycolic acid inhibited, in a concentration-dependent manner, edema-forming activity of BaP1 in the footpad. In order to have insights on the mode of action of glycolic acid, UV–vis and intrinsic fluorescence studies were performed. Results of these assays suggest that glycolic acid interacts directly with BaP1 and chelates the Zn^{2+} ion at the active site. These findings were supported by molecular docking results, which suggested that glycolic acid forms hydrogen bonds with residues Glu143, Arg110 and Ala111 of the enzyme. Additionally, molecular modeling results suggest that the inhibitor chelates Zn^{2+}, with a distance of 3.58 Å, and may occupy part of substrate binding cleft of BaP1. Our results suggest that glycolic acid is a candidate for the development of inhibitors to be used in snakebite envenomation.

1. Introduction

Envenomations by snakebites are a relevant public health issue in many regions of the world, particularly in tropical and subtropical countries of Africa, Asia, Latin America and Oceania (Gutiérrez et al., 2010). The pathophysiological effects observed in these envenomations combine the action of several enzymes, proteins and peptides, which include phospholipases A_2, hemorrhagic metalloproteinases and other proteolytic enzymes, coagulant components, neurotoxins, cytotoxins and cardiotoxins, among others (Markland, 1997).

Snake venom metalloproteinases (SVMPs) are one of the main components in snake venoms, and they belong to the reprolysin subgroup of metalloproteinases. SVMPs have

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been shown to participate in the hemorrhagic process by proteolytic degradation of endothelial cell surface proteins and extracellular matrix components involved in the maintenance of capillary structure and integrity, leading to disruption of capillary networks, edema and hemorrhage (Escalante et al., 2006; Fox and Serrano, 2005). SVMPs classification is based on their different domain structures, presented as follows: P–I (SVMPs comprised by single metalloproteinase domain), P-IIa to P-IIe (containing metalloproteinase and disintegrin domains), P-IIIa to P-IIIc (containing metalloproteinase, disintegrin-like and cysteine-rich domains), and P-IIId, formerly known as P-IV (containing the P-III structure and two C-type lectin-like domains connected by disulfide bonds to the cysteine-rich domain) (Fox and Serrano, 2008).

Bothrops asper is responsible for 50–80% of snakebites, and 60–90% of deaths secondary to snakebites in Central America and northern South America (Otero-Patiño, 2009). Envenomation by this species induces marked local tissue damage that includes pain, edema, hemorrhage, and myonecrosis, mediated predominantly by venom phospholipases A_2 and SVMP (Gutiérrez et al., 2009; Otero-Patiño, 2009). Several SVMPs have been isolated from *B. asper* venom (Angulo and Lomonte, 2009). The most abundant one is BaP1, a 22.7 kDa P–I SVMP comprising a single chain of 202 amino acids with a high sequence identity with SVMPs isolated from other Crotalinae snake venoms. In addition, the amino acid sequence and the crystal structure of this enzyme have been described (Watanabe et al., 2003). BaP1 exerts multiple tissue-damaging activities, including hemorrhage, myonecrosis, dermonecrosis, blistering, and edema (Gutiérrez et al., 1995; Jiménez et al., 2008; Rucavado et al., 1995, 1998).

The therapy for snakebite envenomations has been based on the intravenous administration of antivenoms (Bon, 1996). However, it has been demonstrated that antivenoms have a limited efficacy against the local tissue damaging activities of venoms (Gutiérrez et al., 1998). Thus, it is important to search for alternative venom inhibitors, either synthetic or natural, that would complement the action of antivenoms, particularly regarding neutralization of local tissue damage.

α-Hydroxy acids (αHAs) are organic acids with one hydroxy group attached to the α-position of the carboxylic acid moiety. They are non-toxic and occur naturally in plants, animals and human skin. αHAs have been used as chemical peeling agents in Dermatology and, more recently, there has been considerable interest in the therapeutic and cosmetic uses in the field of skin care and disease treatment (Green et al., 2009; Yu and Van Scott, 2004). The simplest αHA is glycolic acid (GA) (2-Hydroxyethanoic acid). GA has shown photo-protective, anti-inflammatory and anti-oxidant effects on UVB-irradiated skin (Ditre et al., 1996; Morreale and Livrea, 1997; Perricone and DiNardo, 1996). In addition, GA has also shown a preventive effect on the UV-induced skin tumor development and is efficacious in the treatment of acne (Hong et al., 2001; Perić et al., 2011).

In recent studies, it have been demonstrated the importance of hydroxyl groups to inhibit SVMPs, since these moieties could interact with Zn^{2+} ion on the active site of these enzymes (Lingott et al., 2009; Pithayanukul et al., 2009). In order to find novel SVMPs inhibitors, in this work we describe the inhibitory ability of GA on proteolytic activity and hemorrhagic effect of BaP1. Moreover, the mode of action of GA is explored using molecular modeling approaches.

2. Materials and methods

2.1. Purification of BaP1

BaP1 was isolated from a venom pool collected from adult specimens of *B. asper* of the Pacific regions of Costa Rica. After extraction, venom was lyophilized and stored at −20 °C. BaP1 was purified following established methods (Rucavado et al., 1998; Gutiérrez et al., 1995). Briefly, initial purification was done via ion-exchange chromatography on a CM Sephadex C-25 column (Pharmacia), followed by an affinity chromatography on Affi Gel Blue column (Bio-Rad). After BaP1 was judged to be pure by SDS-PAGE (Laemmli, 1970), it was lyophilized and stored at −20 °C until its use.

2.2. Chemicals and reagents

Glycolic acid was purchased from Sigma–Aldrich Co, USA. Other reagents used in this work were of the highest purity available from Sigma and Merck.

2.3. Animals

Swiss Webster mice, 18–20 g body weight, were used for the in vivo assays. All experiments were conducted in accordance with guidelines of the Universidad de Antioquia Ethics Committee (Medellín, Colombia).

2.4. Inhibition of proteolytic activity

Proteolytic activity was tested on azocasein (Wang et al., 2004). BaP1 (10 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 10 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 BaP1. For inhibition experiments, various concentrations of glycolic acid (0.219–7 mM) were incubated with a fixed concentration of BaP1 for 30 min at 37 °C. Then, proteolytic activity was determined as described above. Controls included BaP1 incubated without inhibitor, inhibitor incubated without BaP1, and buffer alone.

2.5. Inhibition of hemorrhagic activity

2.5.1. Experiments with preincubation

Mixtures containing a fixed amount of BaP1 (40 μg) and various concentrations of glycolic acid (from 0.7 to 7 mM)