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Triacontyl *p*-coumarate: An inhibitor of snake venom metalloproteinases

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ABSTRACT

Snake venom metalloproteinases (SVMPs) participate in a number of important biological, physiological and pathophysiological processes and are primarily responsible for the local tissue damage characteristic of viperid snake envenomations. The use of medicinal plant extracts as antidotes against animal venoms is an old practice, especially against snake envenomations. Such plants are sources of many pharmacologically active compounds and have been shown to antagonize the effects of some venoms and toxins. The present study explores the activity of triacontyl *p*-coumarate (PCT), an active compound isolated from root bark of *Bombacopsis glabra* vegetal extract (*Bg*), against harmful effects of *Bothropoides pauloensis* snake venom and isolated toxins (SVMPs or phospholipase A_2). Before inhibition assays, *Bg* or PCT was incubated with venom or toxins at ratios of 1:1 and 1:5 (w/w; venom or isolated toxins/PCT) for 30 min at 37 °C. Treatment conditions were also assayed to simulate snakebite with PCT inoculated at either the same venom or toxin site. PCT neutralized fibrinogenolytic activity and plasmatic fibrinogen depletion induced by *B. pauloensis* venom or isolated toxin. PCT also efficiently inhibited the hemorrhagic (3MDH – minimum hemorrhagic dose injected i.d into mice) and myotoxic activities induced by Jararhagin, a metalloproteinase from *B. jararaca* at 1:5 ratio (toxin: inhibitor, w/w) when it was previously incubated with PCT and injected into mice or when PCT was administered after toxin injection. Docking simulations using data on a metalloproteinase (Neuwiedase) structure suggest that the binding between the protein and the inhibitor occurs mainly in the active site region causing blockade of the enzymatic reaction by displacement of catalytic water. Steric hindrance may also play a role in the mechanism since the PCT hydrophobic tail was found to interact with the loop associated with substrate anchorage. Thus, PCT may provide a alternative to complement ophidian envenomation treatments.

1. Introduction

Snake venoms possess components that are used as offensive weapons for immobilizing, killing and digesting their prey (Sajevic et al., 2011). Bothropic and bothropoic snakebites in humans can cause acute medical emergencies involving intense pain (Costa et al., 2008), local tissue damage that can cause permanent disability and may result in limb amputation (Escalante et al., 2009; Gutiérrez and Rucavado, 2000; Gutiérrez et al., 2009), as well as engendering, bleeding disorders due mainly to alterations in the hemostatic system (Sajevic et al., 2011). Snake venoms are composed of a complex mixture of peptides and proteins whose primary constituents are disintegrins, phospholipases A_2, phosphodiesterases, phosphomonoesterases, L-amino acid oxidases, hyaluronidases, serine proteinases and metalloproteinases (Serrano and Maroun, 2005; Kini, 2005) that are involved in a complex series of events dependent on the synergist action of these molecules.

The snake venom metalloproteinases (SVMPs) (EC 3.4.24) are among the most widely studied animal toxins. Most of them display a large proteolytic diversity and fibrinogen, as well as extracellular matrix components such as collagen type IV, laminin, fibronectin, proteoglycan, are their main substrates (Gutiérrez and Rucavado, 2000; Ramos and Selistrede Araujo, 2006; Hati

Abbreviations: Bg, *Bombacopsis glabra*; *Bleuc*MP, PI metalloproteinase; BnSP-6, Lys49 PLA2; *Bp*, *Bothropoides pauloensis*; Jar, Jararhagin; MS, mass spectrometry; Neu, PI metalloproteinase (Neuwiedase); NMR, nuclear magnetic resonance; PLA_2, phospholipase A_2; PCT, triacontyl *p*-coumarate; SVMP, snake venom metalloproteinases.

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et al., 1999; Inoue, 1989; Moura-da Silva et al., 2008; Baldo et al., 2010).

Hemorrhage is the main effect induced by several SVMPs. These enzymes can cause degradation and disruption of the capillary basement membrane (Baramova et al., 1989; Harris, 2003; Gutiérrez et al., 2005; Pérez et al., 2007; Baldo et al., 2010), subsequently leading to edema, shock, myonecrosis and reduced ability to regenerate muscle tissue (Pithayanukul et al., 2009; Lopes et al., 2009).

Several SVMPs have been isolated from snake venoms and their structure and mechanisms of action have been shown. These toxins are zinc-dependent proteins that belong to the family Metzincin. They are classified into the groups PI to PIII, according to their structural domains (Fox and Serrano, 2008). In their mature form, the PI metalloproteinases are composed of only one catalytic metalloprotein domain and are either weakly or are not hemorrhagic. *Bleuc*MP and Neuwiedase, two SVMPs used in the present work, are non-hemorrhagic metalloproteinases isolated from *Bothrops leucurus* and *Bothrops neuwiedi* venoms, respectively (Gomes et al., 2011; Rodrigues et al., 2000). These toxins are capable of degrading fibrinogen, extracellular matrix proteins, and of causing muscle necrosis. Jarharagin, another SVMP presented in this work, is a strongly hemorrhagic 52 kDa PIII metalloproteinase isolated from *Bothrops jararaca* venom (Paine et al., 1992). This toxin possesses the metalloproteinase, disintegrin-like and cysteine-rich domains (Bjarnason and Fox, 1994; Fox and Serrano, 2008), a characteristic that may explain its diverse functions that include disturbance of hemostasis (Laing and da Silva, 2005), pro-inflammatory activity (Clissa et al., 2006), platelet aggregation inhibition (Kamiguti et al., 1996), and pro-apoptotic activity inhibition in endothelial cells (Tanjoni et al., 2003).

SVMPs participate in a number of important biological, physiological and pathophysiological processes and are primarily responsible for the local tissue damage characteristic of viperid snake envenomations. The inhibition of SVMPs and other components may result in a significant overall reduction in local tissue damage following the envenomation; therefore, the search for SVMP inhibitors has become an important research target. Some studies, for example, are concerned with the isolation and structural features of plant secondary metabolites that show antiophidian properties (Vale et al., 2011).

In recent years, several plants have been studied in relation to their antiophidic potential, such as *Cordia verbenacea* (Ticli et al., 2005), *Musa paradisiaca* (Borges et al., 2005), *Casearia sylvestris* (Cavalcante et al., 2007), *Azadirachta indica* (Mukherjee et al., 2008), *Areca catechu* and *Quercus infectoria* (Leanpolchareanchai et al., 2009), *Magifera indica* (Pithayanukul et al., 2009), *Camellia sianesis* (Pithayanukul et al., 2010), *Hibiscus aethiopicus* (Hasson et al., 2010) and *Schizolobium parahyba* (Mendes et al., 2008; Vale et al., 2008, 2011).

Bombacopsis glabra or "Chestnut of Maranhão" is a native species that exists in northeast and southeast Brazil (Lorenzi, 1992) and contains compounds able to neutralize some harmful venom effects. From its root bark were isolated four active compounds or secondary metabolites: 5-hydroxy-3,7,4-trimethoxyflavone (**1**), 5-hydroxy-3,6,7,4-tetramethoxyflavone (**2**), isohemigossypolone (**3**) and triacontyl *p*-coumarate (**4**) (PCT) (Figure 1) (Paula et al., 2006). PCT (**4**) is a phenolic compound derived from *p*-coumaric acid, and its biosynthesis in plants occurs via deamination and hydroxylation of cinnamic acid (Simões, 2003; Aung et al., 2011). Some cinnamic acid analogues were also previously examined against snake venoms, with an example being rosmarinic acid which is present in several plant species (Aung et al., 2010, 2011; Ticli et al., 2005)

In the present work, the ability of the *B. glabra* vegetal extract and PCT to inhibit the harmful effects induced by *Bothropoides pauloensis* snake venom (*Bp*) and isolated toxins (Jararhagin (Jar), *Bleuc*MP as well as the PLA_2 BnSP-6) on clotting disturbances, hemorrhage and myonecrosis, was evaluated. Furthermore, a PI metalloproteinase (Neuwiedase) was used in order to investigate possible interactions between the metalloproteinase domain and PCT by molecular docking studies.

2. Results and discussion

Several vegetal extracts contain compounds capable of neutralizing the pro-coagulant and anticoagulant activities of snake venoms (Borges et al., 2000, 2005; Biondo et al., 2003; Mendes et al., 2008; Vale et al., 2011). In this work, the inhibition of coagulant activity on bovine plasma induced by the *B. pauloensis* snake venom by the *Bg* extract and PCT was demonstrated. The isolated compound PCT, when incubated at the highest ratio of 1:5 (*B. pauloensis* venom, w/w), inhibited approximately 45% of this activity (results not shown).

Snake venom toxins display a large proteolytic diversity with fibrinogen being one of their main substrates. The fibrinogenases may be classified according to their structural domains as hemorrhagic metalloproteinases or coagulant serine proteinases. Several fibrinogenases can show specific proteolytic activity that favors the Aα-chain in relation to their somewhat lower action towards the Bβ-chain (Gomes et al., 2009; Rodrigues et al., 2000). *Bp* venom, Jar and *Bleuc*MP metalloproteinases showed proteolytic activity preferentially to the Aα-chain of fibrinogen, according to the experimental conditions showed in Fig. 2A–C.

Partial inhibition was observed when venom and the *Bg* vegetal extract were incubated at a ratio 1:10 (w/w). Degradation of the Aα-chain was partially inhibited by the *Bg* extract, thus indicating that the latter contains components capable of neutralizing this activity (Figure 2A). Fig. 2B shows the action of PCT (**4**) against the Jararhagin toxin. PCT (**4**) at a ratio 1:1 (w/w) was very efficient in protecting against degradation of fibrinogen chains. Fig. 2C shows protection by PCT (**4**) against the fibrinogen degradation that would otherwise be induced by the toxin *Bleuc*MP. However, higher concentrations of PCT (**4**) were needed than those used to neutralize Jararhagin toxin (Figure 2B and C).

Another very common effect after envenomation is the blood incoagulation. This action can be caused by some snake venom components that are able to activate blood-clotting factors, causing the consumption of fibrinogen (Costa et al., 2009, 2010; Gomes et al., 2009, 2011). In this manner, the inhibitory effect of PCT (**4**) was demonstrated on decreasing the plasma fibrinogen level induced by *B. pauloensis* venom and Jararhagin. PCT (**4**) inhibited reduction in plasma fibrinogen concentrations induced by *Bp* venom and Jararhagin at a 1:1 ratio (venom or toxin:PCT (**4**), w/w) by 53% and 26%, respectively (Figure 2D and E). This inhibition was also observed when PCT (**4**) was inoculated 15 min after either venom or toxin inoculation (treatment condition).

Another approach in the present study was evaluation of the inhibitory effect of PCT (**4**) on muscle tissue damage induced by *Bp* and isolated toxins. Muscle tissue damage (myonecrosis) is the most dramatic and severe characteristic of envenomations by bothropic and bothropoic snakes (Gutiérrez et al., 2010). The proteins that cause myonecrosis are primarily phospholipases A_2 and metalloproteinases (Gutiérrez et al., 2009). These myotoxic PLA_2s induce damage in the muscle fibers and cause an important medical complication of snakebites, leading sometimes to such drastic sequelae as permanent disability or amputation (Gutiérrez et al., 2010; Lomonte et al., 2003; Otero et al., 2002). Myotoxic PLA_2s provoke drastic perturbation of the plasma membrane or sarcolemma, resulting in a loss in the control of calcium permeability, and consequently, causing disruption of this membrane, and