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Inhibitory effect of pinostrobin from *Renealmia alpinia*, on the enzymatic and biological activities of a PLA_2

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ABSTRACT

Pinostrobin is a flavanone isolated from *Renealmia alpinia*, a plant used in folk medicine to treat snakebites. We tested the inhibitory ability of pinostrobin on the enzymatic, anticoagulant, myotoxic and edema-inducing activities of a PLA_2 isolated from *Crotalus durissus cumanensis* venom. The compound displayed IC_{50} values of 1.76 mM and 1.85 mM (95% Confidence intervals: 1.34–2.18 and 1.21–2.45) on the PLA_2 enzymatic activity, when either aggregated or monodispersed substrates were used, respectively. When mice were injected with PLA_2 preincubated with 0.4, 2.0 and 4.0 mM of pinostrobin, myotoxic activity induced by the PLA_2 was inhibited up to 87%. Nevertheless, these values decreased up to 56% when the pinostrobin was injected into muscle after PLA_2. Pinostrobin inhibited edema-forming and anticoagulant activities of the PLA_2. In order to have insights on the mode of action of pinostrobin, intrinsic fluorescence and ultraviolet studies were performed. Results suggest that pinostrobin interacts directly with the PLA_2. These findings were supported by molecular docking results, which suggested that pinostrobin forms hydrogen bonds with residues His48 and Asp49 of PLA_2, besides, a π-π stacking interactions with those of residues Phe5 and Trp31, and rings C of flavanone and Tyr52 of the toxin.

1. Introduction

Envenomation by snakebites is a relevant public health issue in many regions of the world, particularly in tropical and subtropical countries of Africa, Asia, Latin America and Oceania [1]. In the case of snakes of the family Viperidae, which inflict the vast majority of accidents in the Americas, the pathophysiology of envenomation includes both local and systemic manifestations associated with hemorrhage, necrosis, edema, hypovolemia, nephrotoxicity, coagulopathy and cardiovascular shock [2]. This complex clinical picture is the result of the action of various venom components, predominantly proteinases, both metallo- and serine proteinases, phospholipases A_2, C-type lectin-like proteins, and other minor components [3]. The most important and abundant muscle-damaging components in snake venoms are phospholipases A_2 (PLA_2; EC 3.1.1.4). These enzymes hydrolyze the *sn*-2 ester bond of glycerophospholipids, releasing a fatty acid and a lysophospholipid [4]. In addition, PLA_2 can also induce several pharmacological effects such as edema, modulation of platelet aggregation, as well as neurotoxicity, myotoxicity and anticoagulation [4].

The therapy for snakebite envenomations has been based on the intravenous administration of antivenoms [5,6]. However, it has been demonstrated that antivenoms have a limited efficacy against the local tissue damaging activities of venoms [7,8]. Thus, it is important to search for alternative sources of venom inhibitors, either synthetic or natural, that would complement the action of antivenoms, particularly regarding neutralization of local tissue damage.

Medicinal plants represent a vital source of novel bioactive compounds with several pharmacological activities, and constitute possible alternatives for inhibiting venom components which, eventually, might complement the therapeutic action of conventional antivenom therapy [9,10]. *Renealmia alpinia* (Rottb.) Maas (Zingiberaceae) is known as guaiporé, pinturanegra, jazmín demonte, matandrea or achira de monte [11], and it has been used to treat snakebites in the northwest region of Colombia [12]. In addition, extracts of this plant have been effective to neutralize edema-forming, hemorrhagic, lethal, and defibrinating activities of *Bothrops asper* venom [13–15]. It also has reduced the enzymatic and biological effects of isolated both metallo- and serine proteinases [16]. These findings constitute this plant in a valuable source of inhibitory substances. In this direction, Gómez-Betancur

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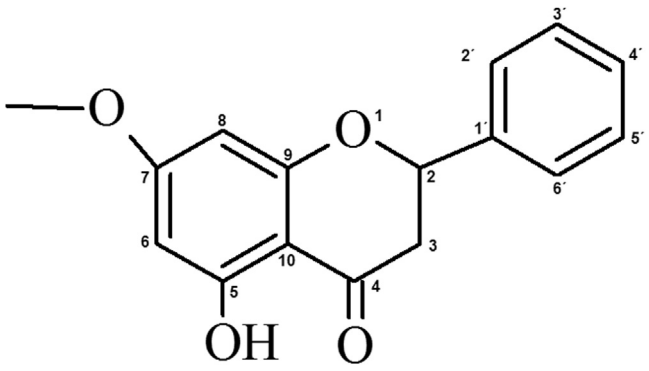

Fig. 1. Structure of pinostrobin ((2*S*)-5-hydroxy-7-methoxy-2-phenyl-2,3-dihydrochromen-4-one). The structure was built using the program Avogadro 1.1.0 [18].

et al., isolated and identified pinostrobin (Fig. 1) from *R. alpinia*, a flavanone, which inhibited the proteolytic, indirect hemolytic, coagulant and hemorrhagic activities of *B. asper* venom [17].

In our ongoing efforts to search for snake venom PLA_2 inhibitors, in this work pinostrobin was purified from *R. alpinia* extract of dichloromethane, and tested in its capacity to inhibit enzymatic and toxic activities of a PLA_2 isolated from the venom of Colombian *C. durissus cumanensis* rattlesnake. Moreover, we explored the mode of action of pinostrobin using a variety of techniques.

2. Materials and methods

2.1. Plant material

The plant material used in this study, *R. alpinia* was collected at the San Rafael (Antioquia – Colombia), during February 2011, with permission of CORNARE (The environmental authority of the collection zone). A specimen has been deposited at the University of Antioquia Herbarium under voucher number 176395. The dried leaves (1500 g) of *R. alpinia* were extracted with dichloromethane (3 L × 3) at room temperature and dichloromethane was removed using rotary evaporator and the extract (74.0 g) was kept in 4 °C until needed.

2.2. Extraction and isolation

Vacuum Liquid Chromatography (VLC) was performed with dichloromethane extract (20 g) of the wild material using different solvent mixtures, eluting sequentially with hexane-dichloromethane (3:1, 1:1, 1:3), dichloromethane-acetone (3:1, 1:1), and acetone- methanol (3:1, 1:1, 1:3) gradients (1 L each), successively, to obtain fractions 1–8: 1 (0.125 g), 2 (0.471 g), 3 (1.9 g), 4 (3.0 g), 5 (1.0 g), 6 (7.0 g), 7 (1.6 g), 8 (1.6 g). Fraction 4 (3.0 g) was applied to a silica gel column, eluting with hexane–dichloromethane (6:4) to obtain subfractions 4–1 to 4–4. Subfraction 4–3 (1.8 g) was recrystallized using ethyl acetate-methanol affording colorless crystals of pinostrobin (1300 mg) (Fig. 1), ^1H and ^{13}C NMR, COSY, HMQC, HMBC were measured on Fourier-300 spectrometers. The compound was identified by comparison of its spectral data with those reported in the literature, structure elucidation was performed by 1D and 2D NMR, EIMS, and by comparison with data from the literature [19,20]. The absolute stereochemistry for the pinostrobin, was found to be 2*S* by comparing the specific rotation value of our isolate (−48.7°; *c* 0.41, $CHCl_3$) with the value reported by Kinghorn (−57.5°; *c* 0.80, $CHCl_3$) [20,21].

2.3. Purification of the PLA_2

C. durissus cumanensis venom was obtained from four specimens coming from Meta, in the south east region of Colombia, and kept in captivity at the Serpentarium of the Universidad de Antioquia (Medellín, Colombia). PLA_2 was purified by molecular exclusion chromatography on Sephadex G-75, followed by reverse-phase HPLC on C-18 column eluted at 1.0 mL/min with a gradient from 0 to 100% acetonitrile in 0.1% trifluoroacetic acid (v/v). Absorbance in effluent solution was recorded at 280 nm [22]. Snake venom PLA_2s are enzymes that are able to resist extreme chemical conditions, such as low pH, higher ion concentrations and temperature, among others [22,23]. In addition, several studies have demonstrated that snake venom PLA_2s can be purified by HPLC using acetonitrile without changes in their enzyme and biological activities [22,24].

2.4. Animals

Swiss Webster male mice, 18–20 g body weight, were used for the in vivo assays. Animals were maintained according to the conditions contained in the Guidelines of the Canadian Council for the care of experimental animals and the Guide for the Care and Use Laboratory Animals of the National Institutes of Health in the United States [25]. All experiments were performed in accordance with guidelines of the Universidad de Antioquia Ethics Committee (Medellín, Colombia), which approved the protocol used in this study on its Acta number 65 (September 29th, 2010). Mice were placed in transparent cages propylene, the temperature in the experimental room was 22 ± 3 °C with a relative humidity of 60–70% and artificial lighting and alternating cycles of 12 h light and 12 h of darkness. Animals were fasted 6 h before the experiment and provided “ad libitum” water.

Indicators of pain in mice were evaluated as described Morton and Griffiths [26] and Carstens and Moberg [27]. Breifly, if an animal presented weight loss 15% body weight, hunched posture, rough haircoat, and/or inability to eat or drink, they were killed prior to the planned experiment termination with an over exposition to Isoflurane vapors. Each parameter had a scale from 0 through 5. Then, total score was recorded and the severity of pain was classified as follow: 0–5: normal; 6–10: monitor carefully, consider analgesics; 11–15: suffering, provide relief, observe regularly; and 16–20 Severe pain, sacrifice animal. For all injection, measurement and blood collection procedures, the animals were anesthetized with isoflurane.

2.5. Inhibition of PLA_2 activity using phosphatidylcholine as aggregated substrate

This activity was assayed according to the method reported by Dole [28], with titration of free fatty acids (FA) released from phosphatidylcholine (from dried egg yolk, Sigma) suspended in 1% Triton X-100, 0.1 M Tris-HCl, 0.01 M $CaCl_2$, pH 8.5 buffer, using 20 μg/10 μL of PLA_2. The time of reaction was 15 min at 37 °C. The amount of protein was selected from the linear region of activity curves. For inhibition experiments, several concentrations of pinostrobin or quercetin were pre-incubated with the enzyme for 30 min at 37 °C before PLA_2 activity determination. The results are indicated as inhibition percentages, and these values were fitted to a linear regression curve to determine the IC50 value, which is the pinostrobin concentration that inhibits 50% of activity.