Full Length Research Paper

Evaluation of antifungal activity of snake venoms from the Amazon forest

Marcelo dos Santos Neves¹, Diego Rayan Teixeira de Sousa³, Maria do Perpétuo Socorro Borges Carriço Ferreira², Maria Zeli Moreira Frota², João Vicente Braga Souza³* and Jorge Luis López Lozano¹

¹Universidade do Estado do Amazonas/Fundação de Medicina Tropical- Dr Heitor Vieira Dourado, Brazil.
²Federal University of Amazonas / UFAM, Brazil.
³Mycology Laboratory of the National Institute of Amazonian Research / INPA, Brazil.

Received 4 November, 2014; Accepted 30 March, 2015

In recent years, many antimicrobial peptides have been found in the venoms of animals from different sources and have been intensively studied to elucidate their ability to inhibit the growth of potential pathogenic microorganisms. The aim of this study was to characterize and evaluate the in vitro antifungal activity of crude venom from two amazonian snakes: Bothrops atrox and Crotalus durissus ruruima. The molecular profile of representative proteins from the venom samples was obtained by reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fungal inhibition was investigated by microdilution assays against two Candida albicans strains. Based on the chromatography and electrophoresis analyses, the venom from B. atrox and C. durissus ruruima were characterized. In addition, the venoms (400 µg/mL) were not able to cause significant inhibition (> 50%) of the growth of C. albicans KL-07, at only 9.09% (200 µg/mL) and 7.88% (400 µg/mL), respectively, and neither presented any influence on the growth of strain C. albicans ATCC 36232.

Key words: Antifungal, activity, snake and Amazon.

INTRODUCTION

The venoms of animals have evolved to generate a broad group of peptide toxins for capture and defense. These peptides are directed against a wide variety of pharmacological targets and represent sources of prototype drugs. Some of these peptides have been used for in vivo studies to prove their effectiveness, in preclinical or clinical trials, for developing treatments for pain, diabetes, multiple sclerosis and cardiovascular disease (Lewis and Garcia, 2003).

Most venoms comprise a mixture of highly complex peptides, usually showing diverse and selective pharmacology. Despite this diversity, venom peptides appear to have evolved from a relatively small number of structures that are particularly well adjusted to meet critical issues
of potency and stability (Lewis and Garcia, 2003). Studies have demonstrated that the peptides present in the venom from snakes, wasps, spiders and scorpions represent a new class of antifungal and antimicrobial proteins (Gomes et al., 2005; de Oliveira Junior et al., 2013).

In recent decades, an increasing number of human populations are becoming more susceptible to opportunistic fungal infections, which has led to a growing number of clinical cases involving emergent fungal species. Furthermore, the discovery of clinical isolates that exhibit inherent or developed resistance to drugs such as Amphotericin B, Clotrimazole, Econazole, Fluconazole, 5-Fluorocytosine, Itraconazole, Ketoconazole, Miconazole, and Nystatin presents a challenge when treating fungal infections. Therefore, studies aimed at the discovery of new antifungal drugs, particularly proteins derived from animal toxins, are fundamental and necessary to expand the therapeutic options, thus ensuring greater efficiency and control in the treatment of these infections (Arango et al., 2004).

Although it is important to evaluate the peptides from Brazilian snakes (Nunes et al. 2011; Okubo et al. 2012), studies on the antifungal activity and molecular characterization of proteins and peptides from snake venoms obtained from the Amazon as well as the in vitro growth inhibition of yeasts of clinical interest are scarce in the literature. This situation makes such work an important initiative for this line of research in the Amazon region (Núñez et al., 2009; Calvete et al., 2011). The objective of this study was to characterize and evaluate the in vitro antifungal activity of crude venom from two amazonian snakes: Bothrops atrox and Crotalus durissus ruruima.

MATERIALS AND METHODS

Venoms

The venom samples were obtained from adult specimens of B. atrox and C. durissus ruruima, species belonging to the Snakebite Center "Professor Paul Friedrich Bührnhein" from the Foundation of Tropical Medicine Hietor Viera Dourado (FMT-HVD). The venom was collected through manual pressure on the venom glands after anesthetization of the snakes with carbon dioxide (CO₂). The samples were centrifuged (5000 x g for 15 min), and the supernatant was filtered (0.45 µm), lyophilized and stored at -20°C.

Characterization of venom

The venoms from B. atrox and C. durissus ruruima were characterized by chromatographic and electrophoresis methods

Reversed-phase high-performance liquid chromatography: The protein fractions of the venoms used in inhibition tests were obtained using the conventional chromatographic method of reversed-phase fractionation using organic solvents and buffer solutions (López-Lozano et al. 2002). The detection of the molecular profile of the protein constituents of the venoms was achieved using reversed-phase high-performance liquid chromatography (RP-HPLC) with a semipreparative ODS column (Shim-Pack C18, 10 mm x 250 mm, 10 mM) equilibrated with 0.1% trifluoroacetic acid (TFA; solution A). The elution of the venom constituents was started with a continuous gradient flow of solution A for 10 min and 0.1% TFA in acetonitrile (solution B) from 0 to 60% in 70 minutes (min). The flow rate for the elution of the constituents was 2.5 ml/min using an analytical monitoring detector at 216 nm. For each chromatographic process, 4 µg of each sample was applied, with two chromatographic analyses each (Ali et al. 2010).

Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique described by Laemmli (1970) was used. The running gel concentration was 15%, and the stacking gel was 4%. The venoms were diluted (volume to volume) with sample buffer (0.125 M Tris, 2% SDS, 10% glycerol and 0.05% bromophenol blue, with or without β-mercaptoethanol), resulting in reduced and non-reduced fractions, respectively. The samples were heated for 5 min at 100°C (reduced) or 40°C for 30 min (non-reduced). B. atrox at a concentration of 0.2 µg/µl and C. durissus ruruima at a concentration of 0.4 µg/µl, along with two controls for race B. atrox and C. durissus ruruima, both at a concentration of 10 µg/µl, were electrophoresed at a constant current of 20 mA/gel using Tris-glycine, pH 8.3 (0.025 M Tris, 0.192 M glycine, 0.1% SDS) as the running buffer. The gels were then stained with silver nitrate, as described (Babaie et al., 2013).

Evaluation of antifungal activity of venoms

In vitro tests were used to evaluate the antifungal activity of the venoms. A Candida albicans strain, identified with code KL-07, isolated from a patient with a clinical and laboratory diagnosis of chronic or recurrent vulvovaginal candidiasis that showed clinical resistance to conventional therapy at the referral center for Diseases Sexually Transmitted Infections - Foundation of Tropical Dermatology and Venereology "Alfredo da Matta" (FUAM) was used. The standard strain of C. albicans, ATCC 32632, provided by the Mycology Laboratory of the National Institute for Amazonian Research (INPA) was also used.

The initial concentration (or stock) of B. atrox and C. durissus ruruima venoms used in the tests was 20 and 10 mg/ml, respectively. The MICs of the venoms were determined according to CLSI M27-A2. The final concentrations ranged from 400 to 0.8 µg/ml for both venoms, and 64 to 0.06 µg/ml ketoconazole was implemented as a control. Microdilution trays containing 100 µL of twofold serial dilutions of the antifungal in standard RPMI 1640 broth were inoculated with 100 µl of the fungi at 2.5 x 10⁵ CFU/ml and incubated in ambient air at 35°C for 24 to 48 h. Reference MICs were defined as the lowest drug concentration that showed 50% of growth inhibition compared with the control (Pfaller et al., 2013).

Statistical analysis

All experiments were performed in triplicate, and the data were used to calculate the average, median and standard deviation (SD) of the readings. In comparing the medians of different dilution levels, the test Non-parametric Kruskal-Wallis test with a significance level of 5% was used. Epi-Info 3.3 software for Windows, developed and distributed by the CDC (www.cdc.org / epinfo), was used in the analysis.

RESULTS

In order to characterize the venoms, HPLC and electro-
Figure 1. HPLC chromatographic profile of venom from *B. atrox* using a flow rate of 1 mL/minute. Solution A - 0.1% TFA; solution B - 0.1% TFA in acetonitrile; Column - C18 RP.

**DISCUSSION**

In recent years, many antimicrobial peptides have been found in the venoms of animals from different sources and have been intensively studied to elucidate their ability to inhibit the growth of potential pathogenic microorganisms (Liu et al., 2013; Bahar and Ren, 2013; He et al., 2013). The data presented in the present work constitute important preliminary information about the characteristic and antifungal activity of the venom from *B. atrox* and *C. durissus ruruima*.

Our characterization (chromatography and electrophoresis assays) demonstrated that the venoms have distinctive profiles of expected protein fractions in a snake venom (Liu et al., 2013; Bahar and Ren, 2013; He et al., 2013). The chromatograms and SDS-PAGE profiles presented here are important for comparisons with future works intended to investigate the characterization and/or biological function of these venoms.

By investigating the influence of the venoms on the growth of *C. albicans* KL-07, only slight activity was observed for both, with a poor correlation between the venom content and growth inhibition. Similar results were previously described in bioassays with venoms (Bustillo et al., 2008; Afc et al., 2010). One explanation for this situation is the existence of an optimal concentration for inhibition or that interactions between the venom compounds at different concentrations cause interference with growth inhibition (Haeberli et al., 2000; Kuhn-Nentwig et al., 2012; Ciscotto et al., 2009). Previous studies corroborate the last explanation in that the peptide...
fraction of the venom from *Bothrops jararaca* showed higher inhibitory activity on the growth of *Candida albicans* than the unfractionated venom (Gomes et al., 2005). According to the data obtained, the venoms may have also suffered from proteolysis by *C. albicans* proteases (Castro and Lima, 2012; Demitto et al., 2012).

The absence of inhibition against *C. albicans* ATCC 36232 demonstrated a difference between the two strains,
and mechanisms that prevent the binding of specific proteins to the fungal cell membrane is a possible explanation (Maróti et al., 2011; Barbosa et al., 2011).

Future works should avoid self-degradation caused by proteases from the venom (Schneiter and Di Pietro, 2013; Röhmm et al., 2013) because both venoms presented high concentrations of metalloproteases (Calvete et al., 2011).

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGMENTS

We thank CNPQ, CAPES and FAPEAM for funding this research.

REFERENCES


