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Research Paper

Antiproliferative activity and new argininyl bufadienolide esters from the "cururú" toad Rhinella (Bufo) schneideri

Guillermo Schmeda-Hirschmann [a,*], Cristina Quispe [a], Cristina Theoduloz [b], Paulo Teixeira de Sousa Jr.[c,d], Carlos Parizotto [c,d]

[a] Laboratorio de Química de Productos Naturales, Instituto de Química de Recursos Naturales, Universidad de Talca, Casilla 747, 3460000 Talca, Chile
[b] Laboratorio de Cultivo Celular, Facultad de Ciencias de la Salud, Universidad de Talca, Casilla 747, 3460000 Talca, Chile
[c] Laboratório de Pesquisa em Química de Produtos Naturais, Departamento de Química, Universidade Federal de Mato Grosso, Av. Fernando C. da Costa s/n, 78060-900 Cuiabá, Mato Grosso, Brazil
[d] Instituto Nacional de Ciência e Tecnologia em Áreas Úmidas (INAU), Rua Nove, 305, Boa Esperanca, 78068-410 Cuiabá, Mato Grosso, Brazil

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ABSTRACT

Ethnopharmacological relevance: Toads known as "cururú" (*Rhinella schneideri*) have been used in the Brazilian Pantanal and Paraguayan Chaco wetlands to treat erysipelas and cancer. The aim of the study was to assess the antiproliferative effect of the venom obtained from *Rhinella schneideri* and to identify its constituents by spectroscopic and spectrometric methods.
Materials and methods: The venom was obtained by gentle pressing the parotid glands of the toads. The dry crude drug was analyzed by HPLC–MS–MS and chromatographed on Sephadex LH-20 to obtain purified compounds and fractions for spectroscopic analysis. The venom and fractions were evaluated for antiproliferative activity towards normal human lung fibroblasts (MRC-5) and four human cancer cell lines: gastric epithelial adenocarcinoma (AGS), lung cancer (SK–MES-1), bladder carcinoma (J82) and promyelocytic leukemia (HL-60).
Results: From the *Rhinella schneideri* venom, 29 compounds were isolated and/or identified by spectroscopic and spectrometric means. Three known alkaloids and five argininyl diacids were identified in the complex mixture by HPLC–MS–MS. Nine out of fifteen argininyl diacid derivatives of the bufadienolides bufalin, marinobufagin and telocinobufagin are reported for the first time and four argininyl diacids are described for the first time as natural products. The venom and the fractions 9–13 showed a remarkable antiproliferative effect, with IC_{50} values in the range 0.019–0.022, 0.035–0.040, 0.028–0.064, 0.042–0.056 and 0.044–0.052 μg/mL for MRC-5, AGS, SK-MES-1, J82 and HL-60 cell lines, respectively. Under the same experimental conditions, IC_{50} values of the reference compound etoposide were 2.296, 0.277, 1.295, 1.884 and 1.059 μg/mL towards MRC-5, AGS, SK-MES-1, J82 and HL-60 cells, respectively.
Conclusions: The venom showed a strong antiproliferative effect towards human cancer cells and presented a high chemical diversity in its constituents, supporting its use as anticancer agent. These findings encourage further work on the chemistry and bioactivity of South American toad venoms.

1. Introduction

Frogs have played a relevant role in South American cultures since prehistoric times. They were represented in material goods such as earthen ware, pottery, stone sculpture and gold portrayals (Wassen, 1934a) as well as in myths (Wassen, 1934b) all over the South American continent. Dendrobatid frogs were used to poison arrows and darts in the Amazon basin (Bisset, 1989) and the large toad *Bufo alvarius* (now *Rhinella alvarius*) was described as a powerful hallucinogen Weil, Davis, 1994. The structure of several groups of alkaloids discovered in South American amphibians has been revised and show a remarkable chemical diversity (Daly, 1998; Daly et al., 2008). In the Guarani tradition of Paraguay and the Brazilian Pantanal, "cururú", the large frog belonging to genus *Rhinella*, is linked to firemyths and to the obtention of fire by the Guarani culture[1].

The large toad *Rhinella schneideri* (Werner, 1894) (synonims: *Bufo paracnemis* A. Lutz, 1925 and *Bufo schneideri* Werner, 1894) is

* Corresponding author. Tel.: +56 71 2200288; fax: +56 71 2202484.
E-mail address: schmeda@utalca.cl (G. Schmeda-Hirschmann).

[1] (http://www.virtualberks.com/spanishasasecondlanguage/ElPrimerfuego_esen.html).

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common in the Paraguayan Chaco wetlands and in the Brazilian Pantanal[2] . The large frogs are poisonous when bitten by dogs or when the venom from the parotid glands came accidentally into contact with the mouth of children. In spite of their abundance, frogs were not eaten but sometimes used in healing practices. Alves, Alves (2011) reported the use of *Rhinella schneideri* (Werner, 1894) ("cururu" toad), to heal wounds, erysipelas, acne, cancer, to treat dental caries and to induce abortion in Brazil. The close related species *Rhinella jimii* (Stevaux, 2002) is used to treat erysipelas in northeastern Brazil (Alves et al., 2012). In the decade of 1960, a decoction of toad to fight cancer began to be known in Paraguay. This use seems to be a more recent practice in spite of some claims that it came from the Guayaki Indians from Eastern Paraguay. The "cururu" toad is rubbed in varicous wounds and skin lesions in Paraguayan rural medicine. According to the information received, the toad is used both to treat cancer, as an aqueous decoction, taken orally or locally applied to treat erysipelas. For the use against cancer, the toad is killed, skinned and the skin is placed in boiling water (about one liter). The decoction is bottled and stored in a cold place. The usual dose was a glass of about 100–150 ml of the decoction per day.

The toad venom has been used as a crude drug in Oriental medicine and traditional Chinese medicine for a very long time. It is recommended mainly as an anti-inflammatory (Ma et al., 2007) and anticancer (Meng et al., 2009) agent. According to The State Pharmacopoeia Commission of People´s Republic of China (2005), the crude drug and its aqueous extract (cinobufacini) are used as cardiotonic, antineoplastic, local anesthetic and antimicrobial agent. In spite of an increasing interest in the toad venoms as potential sources of bioactive agents, less is known on the use of frog secretions in South American traditional medicine.

Sciani et al. (2013) described the antiproliferative activity of crude secretions from Brazilian frogs, obtained by manual compression of the venom glands. Seven species of *Rhinella* (formerly *Bufo*) and one *Phyllomedusa* species were investigated. The venom of *Rhinella schneiderii* showed an IC_{50} value of 48 μg/mL towards the breast cancer cell MCF-7. However, no reference compound for comparison was included in this study. The extracts of the Brazilian frogs were investigated for the occurrence of already described molecules in the Chinese crude drug ChanSu by HPLC-MS. The alkaloids dehydrobufotenin and serotonin were found in all the secretions and were more abundant than the steroids (Sciani et al., 2013). Anjolette et al. (2011) reported two active subfractions from *Rhinella schneiderii* venom with effects on the complement system. In a congress abstract, Baldo et al. (2012a) describe the effect of different fractions from the *Rhinella schneideri* venom against seizures induced by the convulsant agents PTZ and NMDA, suggesting a neuroprotective effect of the toad venom molecules. In the same congress, Baldo et al. (2012b) report the cytotoxicity of the *Rhinella schneideri* poison towards murine melanoma cells, HL-60, HepG2, PC-12 and PBMC cells. The venom showed different effects on the cells, but IC_{50} values were not reported.

In Brazilian *Rhinella* species, telocinobufagin, hellebregenin and bufalin were identified as known cytotoxic/antitumoral compounds (Sciani et al., 2013). The main bufadienolides marinobufagin, bufalin, telocinobufagin, hellebrigenin, 20 S,21 R-epoxymarinobufagin and β-sitosterol were reported from *Rhinella schneideri* captured around Brasilia in the cerrado phytogeographic region (Cunha-Filho et al., 2010). The compounds and some semisynthetic derivatives were assessed for cytotoxicity using several cell lines.

The distribution range of *Rhinella schneideri* and the phylogenetic relationship of South American *Rhinella* species were reported by Maciel et al. (2010). The results of the study suggest diversification associated with geographic and environmental changes that could explain the current distribution of the different *Rhinella* species. The formation of the hydrological basins of the Amazon, Paraguay and Parana rivers in the late Tertiary and Quaternary, could have played a relevant evolutionary role in the genus speciation. The environmental conditions of the Chaco and Pantanal wetlands, associated with the Paraguay River basin, are very different from those of the Brazilian cerrado, suggesting the occurrence of distinctive bioactive compounds in the toad venoms. The aim of the present work was to verify the traditional indication of use of the Pantanal toad as an antiproliferative agent as well as to identify the toad venom components.

2. Materials and methods

2.1. Collection of the toad venom

The toad venom was collected by gentle pressing the parotid glands from free living individuals at SESC-Pantanal, Mato Grosso, Brazil, on April 26, 2013. The toad population surveyed gathers at the river harbor to feed on insects attracted by the town lights. About 30 individuals were captured and released after collecting the venom. The secretion was suspended/dissolved in MeOH for preservation until work-up at the laboratory. The toad was identified as *Rhinella (Bufo) schneideri* by Prof. Domingos de Jesus Rodrigues, Universidade Federal de Mato Grosso, Núcleo de Estudos da Biodiversidade da Amazônia Matogrossense (NEBAM), Instituto de Ciências Naturais, Humanas e Sociais, Cep 78557-267 Sinop, MT, Brazil.

2.2. Isolation

The freeze-dried solid (1.2 g) was treated with 3 × 20 mL of DCM:MeOH 1:1 under sonication (5 min each time), filtered and taken to dryness under reduced pressure to afford 256 mg of a yellowish oil. The solid remaining after DCM:MeOH extraction was sonicated with $CHCl_3$ (30 mL, 10 min) and MeOH (30 mL, 10 min) to obtain additional fractions (97 mg of $CHCl_3$ and 101 mg MeOH-solubles). After TLC comparison, the three combined extracts (453 mg) were pooled for chromatography. Some 440 mg of the combined extracts sample was loaded into a Sephadex LH-20 column (column length 40 cm, i.d. 3 cm, Sephadex load: 20 cm) equilibrated with DCM:MeOH 1:1 v/v to afford 25 fractions. After TLC comparison, the fractions were pooled as follows: 1–3 (160 mg), 4 and 5 (46 mg), 6–11 (35 mg), 12 and 13 (23 mg), 14–21 (10 mg), 22–25 (8 mg).

2.3. HPLC analysis

The HPLC system used for DAD analysis of extracts was a Shimadzu equipment (Shimadzu Corporation, Kyoto, Japan) consisting of a LC-20AT pump, a SPD-M20A UV diode array detector, CTO-20AC column oven and a LabSolution software. A MultoHigh 100 RP 18-5μ (250 × 4.6 mm^2) column (CS-Chromatographie Service GmbH- Germany) maintained at 25 °C was used. Approximately 5 mg of the extract obtained as explained above was dissolved in 1 mL MeOH, filtered through 0.45 μm PTFE filter (Waters) and submitted to HPLC-DAD analysis. The compounds were monitored at 295 nm. The HPLC analysis was performed using a linear gradient solvent system consisting of 1% formic acid (A) and acetonitrile (B) as follows: from 92% to 46% A over 45 min. The flow rate was 1 mL/min. The volume injected was 20 μL. The HPLC conditions were similar to those reported by Gao et al. (2010) for a comparison of venoms from different *Bufo* species.

[2] (http://www.iucnredlist.org/details/54755/0).

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