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Bufadienolides from parotoid gland secretions of Cuban toad *Peltophryne fustiger* (Bufonidae): Inhibition of human kidney Na^+/K^+-ATPase activity

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ABSTRACT

Parotoid gland secretions of toad species are a vast reservoir of bioactive molecules with a wide range of biological properties. Herein, for the first time, it is described the isolation by preparative reversed-phase HPLC and the structure elucidation by NMR spectroscopy and/or mass spectrometry of nine major bufadienolides from parotoid gland secretions of the Cuban endemic toad *Peltophryne fustiger*: ψ-bufarenogin, gamabufotalin, bufarenogin, arenobufagin, 3-(*N*-suberoylargininyl) marinobufagin, bufotalinin, telocinobufagin, marinobufagin and bufalin. In addition, the secretion was analyzed by UPLC-MS/MS which also allowed the identification of azelayl arginine. The effect of arenobufagin, bufalin and ψ-bufarenogin on Na^+/K^+-ATPase activity in a human kidney preparation was evaluated. These bufadienolides fully inhibited the Na^+/K^+-ATPase in a concentration-dependent manner, although arenobufagin (IC_{50} = 28.3 nM) and bufalin (IC_{50} = 28.7 nM) were 100 times more potent than ψ-bufarenogin (IC_{50} = 3020 nM). These results provided evidence about the importance of the hydroxylation at position C-14 in the bufadienolide skeleton for the inhibitory activity on the Na^+/K^+-ATPase.

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1. Introduction

Isolation of bioactive compounds from venoms represents an extremely valuable pharmacological approach for the development of animal toxin-based drugs with high specificity and potency towards their molecular targets mainly located in the cardiovascular and nervous systems. As an example, the analgesic properties of the alkaloid epibatidine, isolated from *Epipedobates anthonyi* (Daly, 1998; Fitch et al., 2010), is the result of its binding to nicotinic and muscarinic acetylcholine receptors, while "Chan Su", a mixture of bufadienolides from *Bufo gargarizans* and *Duttaphrynus melanostictus* (also found in literature as *Rhinella gargarizans* and *Rhinella melanostictus*) (Steyn and Heerden, 1998; Chen et al., 2015) has been widely used as a therapeutic agent in China for stimulation of myocardial contraction or the treatment of tonsillitis, sore throat, and palpitations. This traditional crude mixture has also been tested against tumor cell lines and in cancer models (Chan et al., 1995; Steyn and Heerden, 1998; Takai et al., 2012; Lee et al., 2014).

Toad venoms are found in skin secretions (Li et al., 2015) and have been isolated mainly from parotoid glands (Ferreira et al., 2013; Jing et al., 2013; Sciani et al., 2013) of species from the Bufonidae family (Daly et al., 2004), although they have not been detected in the *Melanophryniscus* genus (Mebs et al., 2007). Usually, these secretions contain a wide range of molecules, like biogenic amines, alkaloids, steroids, peptides and high molecular weight proteins (Rash et al., 2011; Tian et al., 2013; Schmeda-Hirschmann

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et al., 2014). These molecules play an important physiological role for anti-microbial defense and against predator attacks (Toledo and Jared, 1995; Jared et al., 2009; Mailho-Fontana et al., 2014). Therefore, parotoid gland venoms are considered a promising reservoir for unexplored active molecules. Among them, cardiac bufadienolides comprise one of the most interesting groups of bioactive substances from secretions of amphibians (Kamboj et al., 2013). They have also been found in reptiles such as the Asian snake *Rhabdophis tigrinus* (Hutchinson et al., 2013) as well as in several plant families (Steyn and Heerden, 1998; Evans, 2002; Mulholland et al., 2009; Kamboj et al., 2013; Zhang et al., 2014). Bufadienolides are C-24 steroids having a 2-pyrone ring linked at C-17 (β-oriented) and a *cis* fusion for both the A/B and C/D ring junctions, and a *trans* fusion for the A/B junction. They are usually characterized by the presence of a 14-β hydroxyl group. Some bufadienolides have been pharmacologically assayed in different *in vitro* and *in vivo* models (Slingerland et al., 2013; Schmeda-Hirschmann et al., 2014). They have shown antitumoral and antiproliferative activity (Tian et al., 2013; Moreno et al., 2013; Ferreira et al., 2013; Schmeda-Hirschmann et al., 2014; Wang and Bi, 2014). Some studies have demonstrated antimicrobial (Cunha-Filho et al., 2010), anti-leishmanial and antitrypanosomal activity (Tempone et al., 2008). The pharmacological effects of bufadienolides were reviewed by Cunha-Filho et al. (2010) and included antiangiogenic, hypertensive or anti-hypertensive, immunosupression, anti-endometriosis and positive ionotropic actions. However, the most relevant pharmacological effect for these steroids is their specific inhibition of Na^+/K^+-ATPase activity (Bagrov et al., 1998; Touza et al., 2011). Some studies have been conducted to establish the structure-activity relationship of bufadienolides as Na^+/K^+-ATPase inhibitors with recent advances comprising the description of the bufalin-Na^+/K^+-ATPase complex using purified porcine kidney enzyme (Tian et al., 2013; Laursen et al., 2015).

Peltophryne fustiger Schwartz, is one of the eight endemic toads from the Cuban archipelago and it is the biggest species among Antillean toads of the family Bufonidae (Alonso et al., 2014). It has a broad distribution in the lowlands and mountains of Western Cuba (Fig. 1A) from the Guanahacabibes Peninsula to the borders of the "Llanura de Zapata", below 390 m elevation (Estrada and Ruibal, 1999). It is a common species that normally inhabits undisturbed areas such as moist broadleaf forests along stream banks in mesic situations and coastal tickets, but it is also found in degraded forest and rural gardens (Díaz and Cádiz, 2008; Henderson and Powell, 2009). Females reach 198.0 mm of snout-vent length, whereas males are smaller with average of 129.5 mm (Schwartz, 1960). Both sexes have large and conspicuous parotoid glands located transversally in a postorbital position on the head (Fig. 1B).

The chemistry of the paratoid gland secretions from Cuban toads has remained unstudied until now. In this study, we focused on the isolation and structural elucidation of the major bufadienolides from the parotoid gland secretions of the *P. fustiger* and the evaluation of the inhibitory effect of three of them on human kidney Na^+/K^+-ATPase activity.

2. Materials and methods

2.1. Secretion collection and extraction

Parotoid gland secretions were collected from seven adult male specimens of *P. fustiger* in Santo Tomás stream, El Moncada, Viñales, Pinar del Río, Cuba (22°33′00.8″N 83°50′14.6″W) on March, 29, 2014. The secretions were obtained by mechanical compression of both glands from living individuals. These yellowish and doughy secretions were collected on a surface of a watch glass. After this procedure, the individuals were released; only one specimen was preserved as a voucher and deposited in the Herpetological Collection of the Museum of Natural History "Felipe Poey", Faculty of Biology, University of Havana, Cuba (MFP 11.599).

Fresh secretions (1.45 g) were extracted three times with 80 mL MeOH by shaking at room temperature. Extract solutions were pooled, filtered and evaporated under reduced pressure to afford 266 mg of a pale yellowish crude extract.

2.2. HPLC analyses

2.2.1. Analytical conditions

20 μL (2 mg/mL) of crude extract were injected in a RP C-18 Purospher, Merck Millipore (250 × 4 mm, 5 μm) column at 30 °C. The elution was performed using gradient of 0.1% trifluoroacetic acid (A) and acetonitrile (B) as follows: 0–10 min, 5% B; 10–20 min, 5–40% B; 20–25 min, 40% B; 25–60 min, 40–70% B. The flow rate was set at 1 mL/min and the chromatogram was analyzed mainly at 300 nm.

2.2.2. Preparative conditions

250 mg of crude extracts were dissolved in 8 mL of MeOH and filtered through 0.45 μm Minisart RC 4 filters. 300 μL of the sample were successively subjected to preparative HPLC Dionex Ultimate 3000 on a Phenomenex Luna RP-C18 (250 mm × 21.2 mm, 10 μm) by using gradient of elution as follows: 0–10 min, 5% B; 10–20 min,

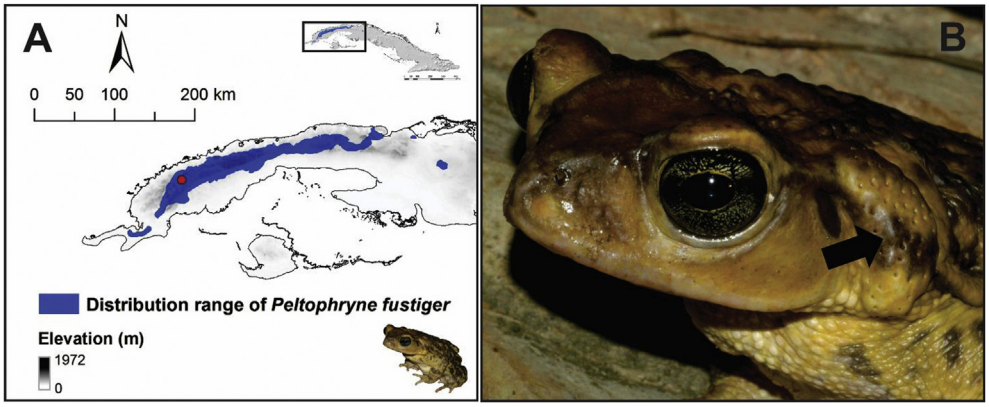

Fig. 1. (A) Shaded area represents the approximate distribution range of *Peltophryne fustiger* in Western Cuba. Red dot indicates the collection locality (B) Details of the head of an adult male of this species, with the arrow indicating the large parotoid gland. Photograph courtesy of J. Bosch. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)