Antiproliferative activity and chemical composition of the venom from the Amazonian toad *Rhinella marina* (Anura: Bufonidae)

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ABSTRACT

Little is known on the composition of Peruvian Amazon toad venoms. The large toad *Rhinella marina* is common in the cleared tropical forests of the Iquitos region and is regarded as poisonous. The venom from two different populations of *R. marina* was collected in the Departamento de Loreto, Perú. The samples were assessed for antiproliferative effect and composition. Some 29 compounds were identified or tentatively identified from the venom by spectroscopic and spectrometric means. The main free bufadienolide was marinobufagin **7** while marinobufotoxin **15** and bufalitoxin **9** were the main bufadienolide argininyl diacid derivatives. The alkaloids dehydrobufotenin **28** and bufotenidin **29** were present in both venoms. The main difference in the venoms was the relative ratio of argininyl diacids from bufadienolides to free bufadienolides. The argininyl diacids included derivatives from bufalin, marinobufagin, telocinobufagin, hellebrigenin, resibufogenin and bufotalinin. Four compounds, including undecadienoyl aginine **6** and three argininyl diacids from bufadienolides were tentatively identified for the first time in the samples. The venom showed a strong antiproliferative effect towards MRC-5 normal human lung fibroblasts (0.063—0.247 µg/mL), AGS human gastric adenocarcinoma cells (0.076—0.272 µg/mL), SK-MES-1 human lung cancer cells (0.154—0.296 µg/mL), J82 human bladder carcinoma cells (0.169—0.212 µg/mL), and HL-60 human promyelocytic leukemia (0.071—0.283 µg/mL). The antiproliferative effect is mediated by ROS production and cell cycle arrest in human breast cancer cells (MCF7 and MDA-MB-231). This is the first report on the composition of *R. marina* venom from the Peruvian Amazon pointing out the need to include different venom samples to get a better picture from the activity and composition of South American toad defense substances.

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1. Introduction

Toad venoms are bioactive substances produced by amphibians that play a relevant role as defense compounds. The venom is located either in the parotid glands or distributed in the skin. Studies on the toxic skin secretions from South American frogs include the classic work by Daly on Dendrobatidae and Bufonidae frogs (Daly, 1998; Daly et al., 2008), leading to the isolation of several alkaloids that were associated with the toad diet. Frog secretions were also used for better hunting by the Amazon Indians living in the border of Peru and Brazil. The skin secretion used by the Peruvian Matses and Brazilian Mayoruna Indians is obtained by gently scrapping the skin secretions of the hylid *Phyllomedusa bicolor* and contain bioactive peptides inducing first illness and then a euphoric state (Daly et al., 1992).

The use of toad skins as psychoactive drug has been described for Central American cultures, including the Olmec and K'iché group of Maya (Carod-Artal, 2015). The hallucinogenic compounds include bufotenin and 5-methoxy-*N,N*-dimethyltryptamine. The toxicity, chemical composition and mechanisms of action of toad venoms is an active research subject. The aqueous extract from the

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skin and defense glands of the Chinese toad *Bufo bufo gargarizans*, known as Chan Su is used in traditional Chinese medicine to treat cancer (Qi et al., 2010). The crude drug contains bufadienolides, peptides and alkaloids and show antitumor activity (Qi et al., 2011).

The South American toad *Rhinella marina* (Linnaeus 1758) (formerly *Bufo marinus*) is known as cane toad or giant toad and, recently, its distributional range has been restricted to the populations from the eastern Andes of Peru, Brazil, Colombia and Venezuela (Fig. 1) and the populations from the western Andes have been assigned to a cryptic species, *Rhinella horribilis* (Wiegmann, 1833), which is genetically and morphologically different to *R. marina* (Acevedo et al., 2016). In the distribution range of *R. marina*, the giant toad is considered poisonous. It affects mainly dogs when the toads are biten or hold in the mouth and the toxin is released (Peterson and Roberts, 2013). Human population living in places where the large *Rhinella* toads occurs, avoid getting in contact with the amphibian and take care of children. It is known that while the toad is useful controlling insect pests, their milky secretion is poisonous and should be avoided to come in contact with the oral mucosa or eyes. It was also introduced to Australia where it became an invasive species (Hayes et al., 2009). The cane toad is now considered a threat to native fauna in Australia and has impact from the native anuran tadpoles (Crossland et al., 2008) to the large freshwater crocodiles (*Crocodylus johnstoni*) (Letnic et al., 2008). The toxin contained in the defense glands from the cane toad can be lethal to other animals and is also a potential danger to children and animal pets.

Little is known on the composition of Peruvian Amazon frog venoms, on spite of the biodiversity and ethnical richness of the area. The large toad *Rhinella marina* is common in the cleared tropical forests of the Iquitos region and is regarded as poisonous.

Following our studies on bioactive compounds from South American toad venoms, we now report the composition and antiproliferative effect of the defense substances from *R. marina* collected at two locations of the Departamento de Loreto, in the eastern Andes of Perú. To assess a possible mechanism of action, the effect of the venoms was investigated on ROS production and cell cycle arrest using two human breast cancer cell lines.

2. Materials and methods

2.1. Collection of the toad venom

The defense substances from the Peruvian Amazon giant toad *Rhinella marina* were collected by one of the authors (G. V-A) together with the taxonomist Luis Alberto Giussepe Gagliardi Urrutia, Programa de Investigación en Biodiversidad Amazonica, Instituto de Investigaciones de la Amazonia Peruana, Iquitos, Peru. The samples were from the Departamento de Loreto, Peru. The size and weight of the animals ranged between 8.2 and 13.2 cm for males and 16.1—18.0 cm for females, respectively. The weight ranged between 98 and 248 g for males and 295—450 g for females, respectively. The sample A1 was collected on July 17th (2013) and the sample A2 was obtained on August 6th (2013) from the outskirts of Unión Progreso (UP) (3°51′25.8″S; 73°20′25.8″W). The fresh venom (4.326 g for A1 and 4.209 g for A2) was resuspended in MeOH and sonicated for 5 min, filtered and taken to dryness under reduced pressure. After treating the solid with CHCl$_3$ and MeOH, the combined solubles were evaporated under reduced pressure to afford the MeOH-soluble extracts that was investigated. From A1, 720 mg was obtained while A2 yielded 667 mg. A third sample was collected at the Provincia Maynas, Departamento Loreto, Reserva Nacional Alpahuayo Mishana (RNAM) (3°58′0.9″S 73°25′5.94″W), on July 10th (2013). The venom (3.60 g) was obtained by gentle pressing the parotid glands from the toads. All animals were released after collecting the sample. The milky secretion was resuspended in MeOH and sonicated for 5 min, filtered and taken to dryness under reduced pressure. After treating the solid with CHCl$_3$ and MeOH, the combined solubles were evaporated under reduced pressure to afford the MeOH-soluble extracts (525 mg) that was
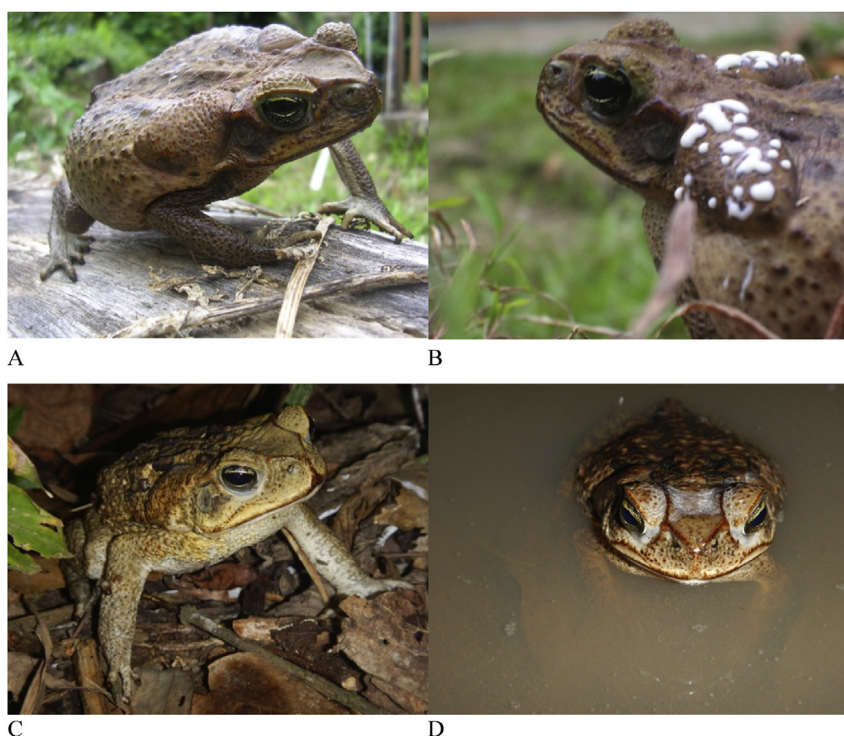

Fig. 1. The toad *Rhinella marina* from the Peruvian Amazon. A and B: females; C and D: males.

investigated. The w/w extraction yield of MeOH extract was as follows: Unión Progreso A1: 16.65%; Unión Progreso A2: 15.84%; Reserva Nacional Alpahuayo Mishana 14.56%.

2.2. Isolation of compounds

The MeOH soluble extract of A2 (0.666 g) was dissolved in 1 mL of MeOH and loaded in a Sephadex LH-20 column (column length, 70 cm; i.d., 3 cm). The column was eluted with MeOH and 60 fractions of 2 mL each were obtained. After TLC comparison (silica gel, EtOAc/isopropanol/NH₄OH 9:7:4 as the mobile phase, detection under UV light and after exposure to iodine), fractions with similar TLC patterns were pooled as follows: 1–18 (26.2 mg), 19–25 (288 mg), 26–30 (74.6 mg), 31–37 (138.6 mg), 38–43 (24.4 mg) and 44–60 (33.0 mg). The fraction pools were analyzed by ^{1}H-NMR and QTOF-MS to identify the main constituents.

The fraction pool 1–18 contained a mixture of argininyl diacids with fatty acids and was no further investigated. The fraction pool 19–25 showed in the ^{1}H NMR spectrum the typical signals of the α-pyrone ring of a main and a minor bufadienolide argininyl diacid derivative as well as the H-3 protons at δ 5.22 and 5.15 of two main compounds, the br s at 4.28 of the H-14 proton, the broad signals of the $(CH_2)_n$ belonging to the diacid moiety and the angular methyl groups from the bufadienolides. In addition, the aromatic protons and N-methyl groups of an alkaloid suggest the presence of compound **28**. QTOF-MS analysis allowed to identify the constituents as marinobufotoxin **15**, bufalitoxin **9**, the argininyl diacids **3** and **4** and the alkaloid **28**. The spectroscopic and spectrometric data are in agreement with that reported for *R. schneideri* by Schmeda-Hirschmann et al. (2014).

^{1}H NMR and QTOF-MS analysis of the fraction pool 26–30 showed dehydrobufotenin (**28**) as main compound and a mixture of argininyl diacids of bufadienolides. The identity of the argininyl diacids was established as **2**, **3** and **4** by QTOF-MS spectrometry while the main bufadienolide derivatives were marinobufotoxin **15** with bufalitoxin **9**, 3-(*N*-pimeloyl argininyl) marinobufagin **14** and 3-(*N*-suberoyl argininyl) telocinobufagin (telocinobufatoxin) **20** as minor constituents. The spectrometric data were in agreement with the study on *R. schneideri* (Schmeda-Hirschmann et al., 2014) and the reports of Gao et al. (2010) and Hu et al. (2011). The fraction pool 31–37 showed in the NMR spectrum typical signals of a main bufadienolide with a dd at δ 7.88 (1H, J = 9.6 and 2.4 Hz), a d at δ 7.41 (1H, J = 2.4 Hz) and a d at a δ 6.26 (1H, J = 9.6 Hz) for the α-pyrone ring, a br s at δ 4.26 (1H, H-3), a s at δ 3.59 (1H, H-14) and two angular methyl groups at δ 0.79 and 0.98 ppm, in agreement with marinobufagin **7** (Schmeda-Hirschmann et al., 2014; Córdova et al., 2016). The alkaloid bufotenidin **29** appeared as a minor compound and was identified on the basis of the NMR signals and QTOF-MS analysis. The fraction was acetylated (acetic anhydride/pyridine, room temperature, 24 h) and the acetate mixture was chromatographed on 30 g silica gel using an PE:EtOAc 1:1 mixture. Fractions of 3 ml each were collected and pooled together according to the TLC pattern. Fractions 26–42 contained a pure compound (Rf 0.21; silica gel, PE:EtOAc 1:1), identified as marinobufagin acetate in full agreement with the spectroscopic data reported by Schmeda-Hirschmann et al. (2014) and Córdova et al. (2016). The fraction pool 38–43 contained a mixture of marinobufagin **7** and the alkaloid **29** while the fraction pool 44–60 yielded degraded products and was discarded.

2.3. HPLC analysis

The HPLC system used for DAD analysis of extracts was 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 18-5 μm (250 × 4.6 mm) 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 a 0.45 μm PTFE filter (Waters) and submitted to HPLC-DAD analysis. The compounds were monitored at 295 nm. The HPLC analysis were performed using a linear gradient solvent system consisting of 1% formic acid in water (A) and acetonitrile (B) as follows: 92% A to 46% A over 12 min, followed by 46% A to 46% A from 12 to 30 min, 46% A to 92% A from 30 to 31 min. The flow rate was 1 mL/min. Detection: UV, 295 nm. The volume injected was 20 μL.

2.4. HPLC-MS-MS analysis

Mass spectra were recorded using an Agilent 1100 (Agilent Technologies Inc, CA-USA) liquid chromatography system connected through a split to an Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltoniks, Germany). Ionization was performed at 3000 V assisted by nitrogen as nebulizing gas at 60 psi and as drying gas at 365 °C and a flow rate of 10 L/min. Positive and negative ions were detected using full scan (m/z 20–2200) and normal resolution (scan speed 10,300 m/z/s; peak with 0.6 FWHM/m/z). The trap parameters were set in ion charge control (ICC) using manufacturer default parameters, and maximum accumulation time of 200 ms. The mass spectrometric conditions for positive and negative ion mode were: electrospray needle, 4000 V; end plate offset, −500 V; skimmer 1, 56.0 V; skimmer 2, 6.0 V; capillary exit offset, 84.6 V; capillary exit, 140.6 V.

Collision induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.00 V (MS/MS) using helium as the collision gas and was automatically controlled through SmartFrag option.

2.5. QTOF MS analysis

ESI-MS-MS analyses were conducted in a Micromass (Manchester, UK) Q-TOF micro instrument. The samples were directly infused at a flow rate of 10.0 μL/min using a syringe pump (Harvard Apparatus, Holliston, United States of America). ESI mass and tandem mass spectra were acquired in the positive ion mode. The following operating conditions were used: 3.0 kV capillary voltage, 40 V cone voltage and desolvation gas temperature of 100 °C. Tandem ESI-MS-MS spectra were collected by causing collision-induced dissociation (CID) of the mass-selected protonated molecules using argon as the buffer gas and collision energies from 5 to 45 eV. Mass-selection was performed by Quadrupole 1 using a unitary m/z window, and collisions were performed in the rf-only quadrupole collision cell, followed by Time of Flight (TOF) mass analysis. ESI-MS were acquired over a m/z range of 80–1000 amu.

2.6. NMR analysis

The NMR spectra were recorded on a Bruker Avance 400 (Bruker, Rheinstetten, Germany) spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C in CDCl₃ or MeOH-d4. Chemical shifts are given in ppm with residual chloroform or methanol as the internal standard.

2.7. Cell lines

Human cell lines were purchased from the American Type Culture Collection (ATCC, Manasas, VA, USA). Normal lung MRC-5 fibroblasts (CCL-171), SK-MES-1 lung cancer cells (HTB-58) and J82 bladder carcinoma cells (HTB-1) were grown as monolayers in

minimum essential Eagle medium (MEM) with Earles's salts, 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. Gastric epithelial AGS cells (CRL-1739) were grown as monolayers in Ham F-12 medium containing 1 mM L-glutamine and 1.5 g/L sodium bicarbonate. Promyelocytic leukemia HL-60 cells (CCL-240) were grown in suspension in RPMI medium containing 1 mM sodium pyruvate and 2.0 g/L sodium bicarbonate. Breast cancer MCF7 (HTB-22) and MDA-MB-231 (HTB-26) cells were grown in DMEM high glucose medium containing 4 mM L-glutamine and 3.7 g/L sodium bicarbonate. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin G and 100 μg/mL streptomycin. Breast epithelial MCF10A cells (CRL-10317) were grown in DMEM/F12 medium (1:1) supplemented with 5% horse serum, bovine insuline (10 μg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/mL). Cells were grown in a humidified incubator with 5% CO_2 in air at 37C.

2.8. Antiproliferative assay

The antiproliferative effect was determined by means of 3–4,5-dimethythiazol-2,5 biphenyl tetrazolium bromide (MTT, Sigma-AldrichCorp., St.Louis, MO,USA) reduction assay. Adherent cells (MRC-5, SK-MES-1, J82, AGS, MCF10A, MCF7 and MDA-MB-231) were plated at a density of 5×10^4 cells/mL and non-adherent HL-60 cells at 30×10^4 cells/mL. Cells were seeded in 96-well plates (100 μL/well). One day after seeding, cells were treated with medium containing the venoms at concentrations ranging from 0 up to 10 μg/mL during 72 h. The fractions were first dissolved in DMSO (0.5% final concentration) and complete medium. Untreated cells (medium containing 0.5% DMSO) served as 100% viability controls. Etoposide (98% purity, Sigma-Aldrich, St. Louis, MO, USA) was used as reference compound. Each concentration was tested in sextuplicate and experiments were repeated twice. Cell viability was determined by means of the MTT reduction assay at the end of the incubation. The results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves (Schmeda-Hirschmann et al., 2014).

2.9. Cell morphology and intracellular ROS levels

Changes in the cell morphology were analyzed by flow cytometry, obtaining parameters related to the size (FSC channel) and granularity (SSC channel) after 24 h of exposition to *Rhinella* venoms. The ROS levels were determined using the CellROX Green probe according to the manufacturer's specifications (Thermo Fisher Scientific, USA). MCF7 cells were incubated with DMSO (control) or 0.1 μg/mL *R. marina* venoms for 24 h. Then, cells were washed with PBS and incubated with 2.5 μM CellROX for 30 min. Cells were collected, washed, re-suspended and fluorescence was detected using a BD FACSAria III flow cytometer.

2.10. Cell cycle analysis and cell count

To estimate cell cycle distribution, cellular DNA contents were measured by flow cytometry as previously described (Urra et al., 2016). MCF7 cells were incubated with DMSO (control) or 0.1 μg/mL *R. marina* venoms for 48 h. All samples were analyzed for cell cycle distribution using a FACS Calibur flow cytometer and the Becton-Dickinson CellQuest Acquisition software (San Jose, CA, USA). Data were reported as percentage of sub-population cells in each phase of the cell cycle respect to total. To evaluate the cell number, MCF7 cells (25.000 cells) were seeded into 24-well plates and incubated for 24 h. Then, the cells were exposed to DMSO (control) or 0.1 μg/mL venoms from Unión Progreso (UP1 and UP2) and Reserva Nacional Alpahuayo Mishana (RNAM) for 72 h. After

treatment, MCF7 cells were counted as described (Urra et al., 2016).

2.11. Statistics

The statistical analyses for cell count, morphology and cell cycle were performed using Graph Pad Prism 4.03 (GraphPad Software, San Diego, California USA). The data are expressed as mean ± standard deviation (SD) of three independent experiments, each performed in triplicate. Statistical analysis was performed using one-way ANOVA with Bonferroni's post test for pairwise comparisons. The data were considered statistically significant when $p < 0.05$. For the antiproliferative assay, results are expressed as IC_{50} values (μg/mL ± SD) of two independent experiments tested in sextuplicate.

3. Results

The chemical composition of the parotid glands venom from Peruvian Amazon *Rhinella marina* toad was investigated by spectroscopic and spectrometric means including NMR and HPLC-MS-MS^n. The HPLC chromatogram of the venom is presented in Fig. 2 and the structures of the compounds isolated and/or tentatively identified in the venom are shown in Fig. 3. The assignment of the venom constituents is summarized in Tables 1 and 2.

After permeation in Sephadex LH-20, the main constituents of the venom were identified by NMR. Marinobufagin was the main bufadienolide while bufalitoxin and marinobufotoxin were the major bufadienolides argininyl diacid derivatives. QTOF-MS analysis allowed to confirm the identity of different argininyl diacids in mixtures. The main compounds isolated and fully characterized by spectroscopic and spectrometric means were used as reference for the analysis of the different fractions by QTOF-MS as well as by HPLC-MS-MS^n.

3.1. HPLC-MS-MS^n analysis

The composition of the Peruvian Amazon giant toad venoms was assessed by negative and positive mode ionization HPLC-MS-MS^n. Selected chromatograms are shown in Fig. 2 and the results are summarized in Tables 1 and 2. The venom constituents were identified by comparison with the main compounds isolated from *R. marina* and *R. schneideri* or tentatively identified by HPLC-MS-MS^n. A search in SciFinder (accessed on April 19, 2016) indicate that the compounds **6**, **18**, **25** and **26** identified as minor constituents from *R. marina*, are reported for the first time.

3.1.1. Agininyl diacid derivatives

Compounds **1**–**6** were detected in the negative and positive ion mode. The six compounds showed the diacid loss leading to the arginine moiety and were identified as adipyl (C6 diacid), pimeloyl (C7 diacid), suberoyl-(C8 diacid), azelayl (C9 diacid), sebacyl (C10 diacid) and undecadienoyl (C11 diacid) arginine, respectively. Compounds **1**–**5** were reported recently from the Pantanal toad *Rhinella schneideri* (Schmeda-Hirschmann et al., 2014).

3.1.2. Bufalin argininyl diacids

The compounds **8** and **9**, detected in the negative ion mode and the compounds **9**–**11**, detected in the positive ion mode, showed a common loss a 369 amu fragment, in agreement with bufalin $[M + H–H_2O]^+$ leading to the argininyl diacid ion. The compounds differ in the diacid moiety and were identified as 3-*N*-pimeloyl-, 3-*N*-suberoyl-, 3-*N*-azelayl- and 3-*N*-sebacyl argininyl bufalin, respectively. The compounds **9**–**11** were reported from *R. schneideri* from the Brazilian Pantanal (Schmeda-Hirschmann et al., 2014).
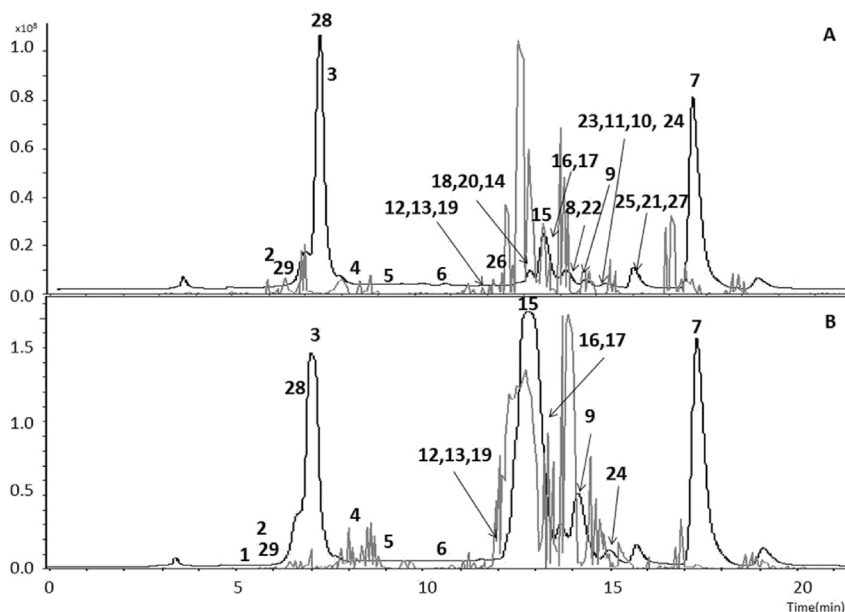
Sample origin: Panel A: Unión Progreso; Panel B: Reserva Nacional Alpahuayo Mishana.

Compounds identification: **1**: Adipyl arginine; **2**: Pimeloyl arginine. **3**: Suberoyl arginine; **4**: Azelayl arginine; **5**: Sebacyl arginine; **6**: Undecadienoyl arginine; **7**: Marinobufagin;

8: 3-(*N*-pimeloyl argininyl)-bufalin; **9**: 3-(*N*-suberoyl argininyl)-bufalin (Bufalitoxin); **10**: 3-(*N*-azelayl argininyl)-bufalin; **11**: 3-(*N*-sebacyl argininyl)-bufalin; **12**: 3-(*N*-glutaryl argininyl)-marinobufagin; **13**: 3-(*N*-adipoyl argininyl) marinobufagin; **14**: 3-(*N*-pimeloyl argininyl) marinobufagin; **15**: 3-(*N*-suberoyl argininyl) marinobufagin (Marinobufotoxin); **16**: 3-(*N*-azelayl argininyl) marinobufagin; **17**: 3-(*N*-sebacyl argininyl) marinobufagin; **18**: 3-(*N*-adipoyl argininyl) telocinobufagin; **19**: 3-(*N*-pimeloyl argininyl) telocinobufagin; **20**: 3-(*N*-suberoyl argininyl) telocinobufagin (Telocinobufatoxin); **21**: 3-(*N*-suberoyl argininyl) hellebrigenin; **22**: 3-(*N*-adipoyl argininyl) resibufogenin; **23**: 3-(*N*-pimeloyl argininyl) resibufogenin; **24**: 3-(*N*-suberoyl argininyl) resibufogenin; **25**: 3-(*N*-azelayl argininyl) resibufogenin; **26**: 3-(*N*-suberoyl argininyl) bufotalinin; **27**: Scillaridin A; **28**: Dehydrobufotenin; **29**: Bufotenidin. x-axis: retention time (minutes); y-axis: relative intensity.

Fig. 2. HPLC chromatogram of *Rhinella marina* venom from Iquitos, Peruvian Amazon. UV chromatogram at 295 nm and total ionic current (TIC) in positive mode. Sample origin: A: Unión Progreso; B: Reserva Nacional Alpahuayo Mishana. *x*-axis: retention time (minutes); *y*-axis: relative intensity.

3.1.3. Marinobufagin argininyl diacids

The marinobufagin argininyl diacids were detected either in the negative ion mode (compounds **13**–**17**) or in the positive ion mode (compounds **12**–**17**). The compounds are derivatives of marinobufagin **7**, identified as the main bufadienolide in the venom by NMR analysis. In the positive ion mode, compounds **12**–**17** loss a 383 amu fragment, in agreement with marinobufagin $[M + H-H_2O]^+$ leading to the argininyl diacid ion. As the argininyl ion has a *m/z* of 175 amu, the compounds differ in the length of the diacid linker. The compounds **12**–**17**, differing in the diacid moiety were assigned as 3-*N*-glutaryl-, 3-*N*-adipoyl-, 3-*N*-pimeloyl-, 3-*N*-suberoyl-, 3-*N*-azelayl- and 3-*N*-sebacyl argininyl marinobufagin, respectively. The compounds **13**–**17** were also described for *Rhinella schneideri* (Schmeda-Hirschmann et al., 2014).

3.1.4. Telocinobufagin argininyl diacids

The compounds **18**–**20** were detected both in the negative and in the positive ion mode. The derivatives **18**–**20** showed a common loss of 384 amu, in agreement with telocinobufagin $[M + H-H_2O]^+$

leading to the argininyl diacid ion. In the positive ion mode, the difference with the arginine ion at *m/z* 175 amu allowed the identification of the diacid as adipic-, pimelic- and suberic acid, respectively. The compound **19** was a minor constituent from a sample of *B. marinus* venom from Australia (Hayes et al., 2009). Telocinobufotoxin **20** was previously reported from *Bufo marinus*, Venenum Bufonis (Hu et al., 2011) as well as from *R. schneideri*.

3.1.5. Hellebrigenin argininyl diacids

The compound **21** was detected in the negative ion mode and showed the loss of 398 amu, in agreement with 3-(*N*-suberoyl argininyl) hellebrigenin, reported from *R. schneideri*.

3.1.6. Resibufogenin argininyl diacids

Compounds **22**–**25** were detected in the positive and negative ion mode. The derivatives **22**–**25** showed a common loss of 366 amu, in agreement with resibufogenin $[M + H-H_2O]^+$ leading to the argininyl diacid ion. The difference with the arginine ion allowed the assignation of the compounds as 3-*N*-adipoyl-, 3-*N*-
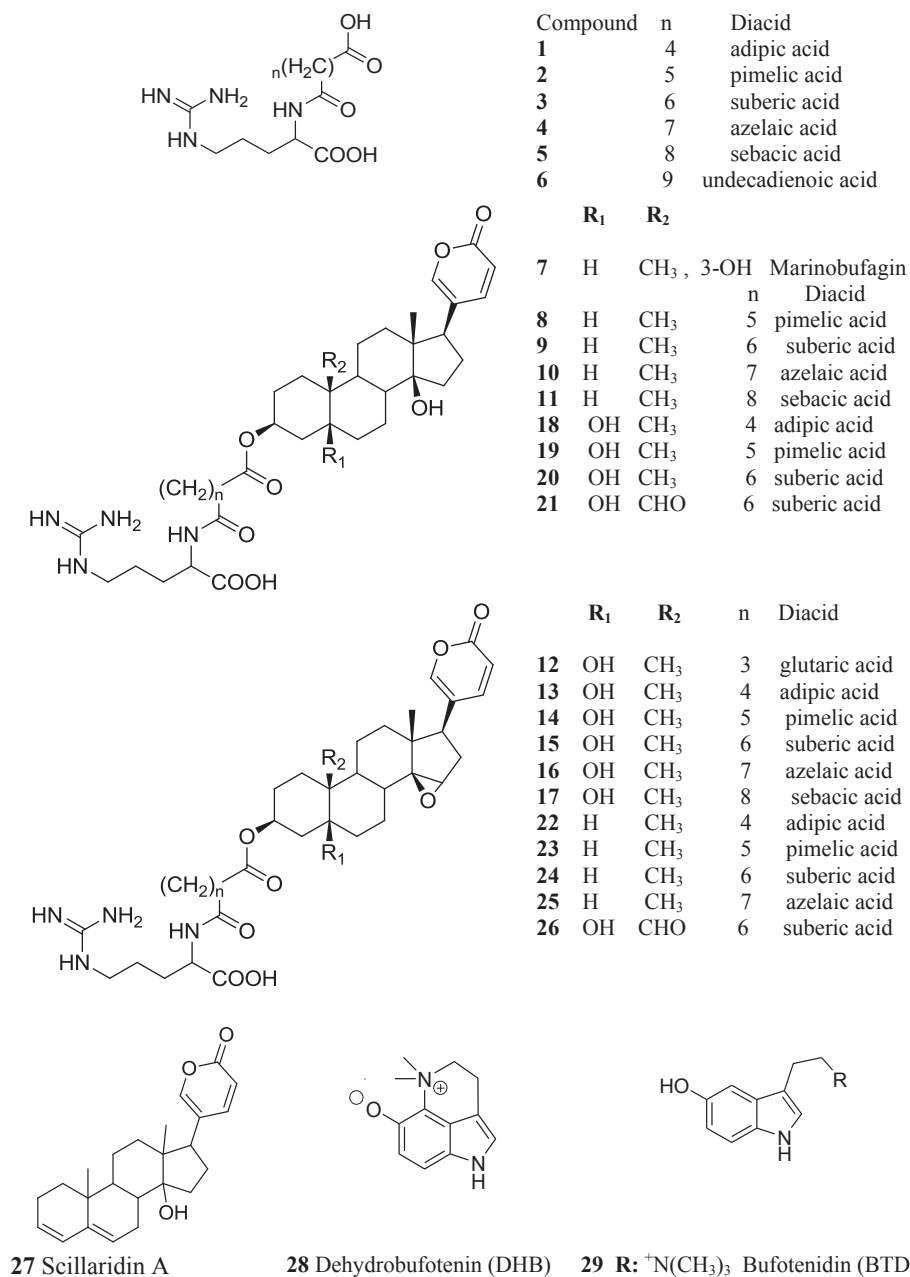
Compound	n	Diacid
1	4	adipic acid
2	5	pimelic acid
3	6	suberic acid
4	7	azelaic acid
5	8	sebacic acid
6	9	undecadienoic acid

	R_1	R_2		
7	H	CH_3, 3-OH		Marinobufagin

			n	Diacid
8	H	CH_3	5	pimelic acid
9	H	CH_3	6	suberic acid
10	H	CH_3	7	azelaic acid
11	H	CH_3	8	sebacic acid
18	OH	CH_3	4	adipic acid
19	OH	CH_3	5	pimelic acid
20	OH	CH_3	6	suberic acid
21	OH	CHO	6	suberic acid

	R_1	R_2	n	Diacid
12	OH	CH_3	3	glutaric acid
13	OH	CH_3	4	adipic acid
14	OH	CH_3	5	pimelic acid
15	OH	CH_3	6	suberic acid
16	OH	CH_3	7	azelaic acid
17	OH	CH_3	8	sebacic acid
22	H	CH_3	4	adipic acid
23	H	CH_3	5	pimelic acid
24	H	CH_3	6	suberic acid
25	H	CH_3	7	azelaic acid
26	OH	CHO	6	suberic acid

27 Scillaridin A   **28** Dehydrobufotenin (DHB)   **29** **R:** $^+N(CH_3)_3$ Bufotenidin (BTD)

Fig. 3. Compounds identified/tentatively identified in *Rhinella marina* toad venom from the Peruvian Amazon basin.

pimeloyl-, 3-*N*-suberoyl- and 3-*N*-azelayl argininyl resibufogenin, respectively. Compounds **22**–**24** were previously isolated from toad venom (Krenn and Kopp, 1998; Dictionary of Natural Products on DVD, 2016).

3.1.7. Bufotalinin argininyl diacids

The compound **26**, detected in the positive ion mode showed the loss of 396 amu, corresponding to bufotalinin $[M + H-H_2O]^+$ leading to the argininyl diacid ion as base peak. The compound was assigned as 3-*N*-suberoyl-argininyl bufotalinin.

3.1.8. Scillaridin A

The compound **27** with a $[M-H]^-$ ion at 367.3 amu shows the loss of water leading to a base peak at 349 amu. The compound was tentatively assigned as scillaridin A, which has a molecular formula

of $C_{24}H_{30}O_3$. Scillaridin A was previously reported from *Urginea epigea* (Koorbanally et al., 2004; Dictionary of Natural Products on DVD, 2016).

3.1.9. Alkaloids

The alkaloids **28** and **29** were detected in the positive ion mode and are in agreement with dehydrobufotenin and bufotenidin, previously reported from *R. schneideri* (Schmeda-Hirschmann et al., 2014).

3.2. Antiproliferative activity

The venom was assessed for antiproliferative activity against MCF10A normal breast epithelial cells, MRC-5 normal human lung fibroblasts, AGS human gastric adenocarcinoma cells, SK-MES-1

Table 1
Identification of the main constituents and tentative identification of minor compounds from *Rhinella marina* venom by HPLC-MS-MS in the positive ion mode. *Identified by NMR analysis.

Rt (min)	[M+H]$^+$	MS/MS	Tentative identification
5.1	303.3	249.9 (100), 175 (36)	Adipyl arginine **1**
5.7	632.8	317.2 (100)	[2M + H]$^+$ pimeloyl arginine **2**
5.8	218.8	204 (100); 159.5 (26)	Bufotenidin **29***
6.9	202.9	187.7 (100)	Dehydrobufotenin **28**
7.0	660.8	331.2	Suberoyl arginine **3** [2M + H]$^+$ (*m/z* 331)
8.1–8.7	688.5	345.3 (100)	Azelayl arginine **4** [2M + H]$^+$ (*m/z* 345)
9.0	359.5	344	Sebacyl arginine **5**
10.6	373.3	175	Undecadienoyl arginine **6**
11.9	671.7	653.5 (63), 289.1 (100)	3-(*N*-glutaryl argininyl) marinobufagin **12**
12.2	685.7	667.5 (77), 303.1 (100)	3-(*N*-adipoyl argininyl) marinobufagin **13**
12.2	701.6	683.6 (85), 317.2 (100)	3-(*N*-pimeloyl argininyl) telocinobufagin **19**
12.3	727.6	709.8 (100), 331.3 (93), 278.1 (7)	3-(*N*-suberoyl argininyl) bufotalinin **26**
12.3	687.7	669.6 (81), 303.2 (100)	3-(*N*-adipoyl argininyl) telocinobufagin **18**
12.5	715.6	697.6 (100), 331.2 (85)	3-(*N*-suberoyl argininyl) telocinobufagin **20**
12.7	700.1	681.5 (98), 317.2 (100)	3-(*N*-pimeloyl argininyl) marinobufagin **14**
13.1	713.2	695.6 (76), 331.2 (100)	3-(*N*-suberoyl argininyl) marinobufagin (Marinobufotoxin) **15***
13.4	742.0	723.7 (75), 359.3 (100), 253.0 (23)	3-(*N*-sebacyl argininyl) marinobufagin **17**
13.5	727.7	709.6 (71), 345.3 (100)	3-(*N*-azelayl argininyl) marinobufagin **16**
13.6	686	667.6 (100), 317 (13)	3-(*N*-pimeloyl argininyl) bufalin **8**
13.8	669.7	651.5 (72), 518.5 (14), 303.1 (100)	3-(*N*-adipoyl argininyl) resibufogenin **22**
14.0	699.7	681.6 (100), 331.2 (13)	3-(*N*-suberoyl argininyl) bufalin (Bufalitoxin) **9***
14.4	683.7	665.5 (50), 532.6 (17), 317.2 (100), 264 (26)	3-(*N*-pimeloyl argininyl) resibufogenin **23**
14.5	727.7	709.6 (100), 359.3 (12), 253.0 (15)	3-(*N*-sebacyl argininyl) bufalin **11**
14.6	713.8	695.7 (100), 345.3 (15)	3-(*N*-azelayl argininyl) bufalin **10**
14.8	697.7	679.5 (95), 331.2 (100), 278.0 (33)	3-(*N*-suberoyl argininyl) resibufogenin **24**
15.5	711.8	693.6 (90), 345.4 (100)	3-(*N*-azelayl argininyl) resibufogenin **25**
15.6	729.2	709.8 (100), 331.3 (43)	3-(N-suberoyl argininyl) hellebrigenin **21**
15.7	367.3	349.2 (100), 295.2 (74), 277.1 (50)	Scillaridin A **27**
17.0	401.3	383 (41), 365 (100)	Marinobufagin **7***

Table 2
Identification of the main constituents and tentative identification of minor compounds from *Rhinella marina* venom by HPLC-MS-MS in the negative ion mode. *Identified by NMR analysis.

Rt (min)	[M-H]$^-$	MS/MS	Tentative identification
5.2	603.0	300.9 (100)	[2M-H]$^-$ Adipyl arginine **1**
6.0	631.0	315.0 (100)	[2M-H]$^-$ Pimeloyl arginine **2**
7.1	659.0	329.1 (100)	[2M-H]$^-$ Suberoyl arginine **3**
8.6	687.2	343.0 (100)	[2M-H]$^-$ Azelayl arginine **4**
9.1	357.2	312.0 (100), 287.2 (33), 326.0 (23)	Sebacyl arginine **5**
10.5	371.7	329.1 (100), 297.1 (27), 172.5 (24), 130.5 (8)	Undecadienoyl arginine **6**
11.1	728.1	685.5 (58), 329.1 (100), 268.9 (15)	3-(N-suberoyl argininyl) hellebrigenin **21**
12.0	699.5	657.4 (100), 315.0 (98), 655.3 (39), 272.8 (55)	3-(*N*-pimeloyl argininyl) telocinobufagin **19**
12.1	685.8	643.4 (100), 401.2 (18), 300.9 (98), 282.9 (43), 240.8 (54)	3-(*N*-adipoyl argininyl) telocinobufagin **18**
12.1	684.0	641.4 (61), 301 (100)	3-(*N*-adipoyl argininyl) marinobufagin **13**
12.3	713.8	671.4 (57), 329.1 (100), 287.0 (35), 268.9 (34)	3-(*N*-suberoyl argininyl) telocinobufagin (Telocinobufatoxin) **20**
12.4–13.2	712.0	669.3 (25), 399.2 (4), 329.1 (100), 287.1 (30), 269.1 (32)	3-(*N*-suberoyl argininyl) marinobufagin (Marinobufotoxin) **15***
12.4	697.9	655.3 (47), 315.0 (100)	3-(*N*-pimeloyl argininyl) marinobufagin **14**
13.4	725.9	683.4 (16), 343.1 (100)	3-(*N*-azelayl argininyl) marinobufagin **16**
13.4	739.9	357.1 (100)	3-(*N*-sebacyl argininyl) marinobufagin **17**
13.5	684.1	641.5 (100), 315.1 (14)	3-(*N*-pimeloyl argininyl) bufalin **8**
13.8	668.0	625.0 (100), 300.9 (17)	3-(*N*-adipoyl argininyl) resibufogenin **22**
13.9	697.9	655.4 (100), 329.1 (18)	3-(*N*-suberoyl argininyl) bufalin (Bufalitoxin) **9***
14.3	682.2	639.3 (100), 315.0 (41)	3-(*N*-pimeloyl argininyl) resibufogenin **23**
14.7	696.0	635.5 (100), 329.0 (38)	3-(*N*-suberoyl argininyl) resibufogenin **24**
14.7	711.9	669.4 (100), 343.0 (24)	3-(*N*-azelayl argininyl) bufalin **10**
15.5	710.0	667.4 (100), 343.1 (62)	3-(*N*-azelayl argininyl) resibufogenin **25**
17.0	398.8	381.0 (100)	Marinobufagin **7***

human lung cancer cells, J82 human bladder carcinoma cells, MCF7 and MDA-MB-231 human breast cancer cells and HL-60 human promyelocytic leukemia. Results are summarized in Table 3. A strong antiproliferative effect was found towards MRC-5 fibroblasts (0.063–0.247 μg/mL), AGS cells (0.076–0.272 μg/mL), SK-MES-1 lung cancer cells (0.154–0.296 μg/mL), human bladder carcinoma (0.169–0.212 μg/mL) and HL-60 human promyelocytic leukemia (0.071–0.283 μg/mL). The most active venoms where those from Unión Progreso, showing more toxicity towards AGS and HL-60 cells. The venom from the Reserva Nacional Alpahuayo Mishana showed antiproliferative activity against all the cell lines assessed but the IC$_{50}$ values (μg/mL) were roughly twice higher than the Unón Progreso samples. Under the same experimental conditions, IC$_{50}$ values (μg/mL) of etoposide towards the different cell lines were: 3.931 (MRC-5), 0.368 (AGS), 2.553 (SK-MES-1), 2.832 (J82) and 0.814 (HL-60).

Table 3

Antiproliferative effect of *Rhinella marina* venoms from Unión Progreso (UP1 and UP2) and Reserva Nacional Alpahuayo Mishana (RNAM). Data are expressed as IC$_{50}$ values (μg/mL ± SD) of two independent experiments tested in sextuplicate.

Collection place	MCF10A	MRC-5	AGS	SK-MES-1	J82	HL-60	MCF7	MDA-MB-231
UP1	50.64 ± 1.342	0.091 ± 0.004	0.076 ± 0.003	0.296 ± 0.019	0.189 ± 0.008	0.071 ± 0.003	0.489 ± 0.044	0.774 ± 0.034
UP2	>100	0.063 ± 0.003	0.136 ± 0.007	0.154 ± 0.012	0.169 ± 0.011	0.131 ± 0.006	0.543 ± 0.032	1.309 ± 0.124
RNAM	>100	0.247 ± 0.021	0.272 ± 0.013	0.274 ± 0.015	0.212 ± 0.015	0.283 ± 0.019	0.912 ± 0.025	8.836 ± 0.032
Etoposide	27.06 ± 0.453	3.931 ± 0.157	0.368 ± 0.018	2.553 ± 0.151	2.832 ± 0.169	0.814 ± 0.032	7.48 ± 0.393	7.26 ± 0.245

3.3. Cell cycle analysis and ROS production in breast cancer cells

In order to determine a possible mechanism of the anti-proliferative effect, we exposed breast non-tumoral (MFC10A) and breast cancer (MCF7 and MDA-MB-231) cells to four concentrations of *Rhinella marina* venoms for 72 h. As shown in Fig. 4, all the venoms had different effects on the proliferation of the three cell lines. The non-tumoral MCF10A cells were significantly less affected compared to the breast cancer cells. In breast cancer MCF7 cells, the three *R. marina* venoms reduced cell proliferation (expressed as MTT reduction) in similar extents. Therefore, this cancer cell line was selected to perform the subsequent experiments using 0.1 μg/ml as the lowest concentration. After 24 h of exposition, *R. marina* venoms (0.1 μg/ml) produced changes in the cell morphology, involving reduced size and complexity (Fig. 5A–C) and an increase in the intracellular ROS levels (Fig. 5D). A high production of ROS can damage macromolecules and lipids, affecting the biosynthesis of mass required for cell proliferation (Hecht et al., 2016). Consistent with this, the effect of *R. marina* venoms was evaluated on the cell cycle progression in MCF7 cells. Venoms from UP and RNAM affected the cell cycle distribution, producing an arrest in S-phase (Fig. 5E). This fact indicates that the DNA replication is affected and duplication of cancer cells is inhibited. Accordingly, the number of cells was significantly reduced in presence of the venoms after 72 h of exposure (Fig. 5F). To confirm if the increased ROS production by *R. marina* venoms could be involved in the anti-proliferative effect, the anti-oxidant N-acetyl cysteine (NAC) and 0.1 μg/ml venoms were combined. As shown in Fig. 5E, 4 mM NAC had no significant effect on the cell number; however, the combination reduced strongly the effect of the venoms on the number of MCF7 cancer cells (Fig. 5F). All together, these results indicate that the *R. marina* venoms exhibit an antiproliferative effect mediated by an increased ROS production and producing an S-phase arrest in MCF7 cancer cells.

4. Discussion

From the venom obtained from the parotid glands of the Amazonian giant toad *Rhinella marina*, 29 compounds were identified by spectroscopic and/or spectrometric means. Representative chromatograms of the samples of Unión Progreso (UP) and Reserva Nacional Alpahuayo Mishana (RNAM) are shown in Fig. 2. The chromatograms for both samples show three distinctive regions, associated with specific groups of toxins. The argininyl diacids and alkaloids elutes in the range of 5–10 min, while the argininyl diacids from bufadienolides elutes from 11 to 16 min, followed by the free bufadienolides at 17–18 min. The venom obtained from toads from the UP and RNAM shows large differences in the relative proportion of constituents (Fig. 2). While the main bufadienolide for the UP venom was the bufadienolide marinobufagin **7**, the main constituent of the RNAM toad venom was marinobufotoxin **15**. A further difference is the relative proportion of bufadienolide argininyl diacids to free bufadienolide, where the UP sample shows larger free bufadienolide content and the RNAM venom higher bufadienolide argininyl diacids content.
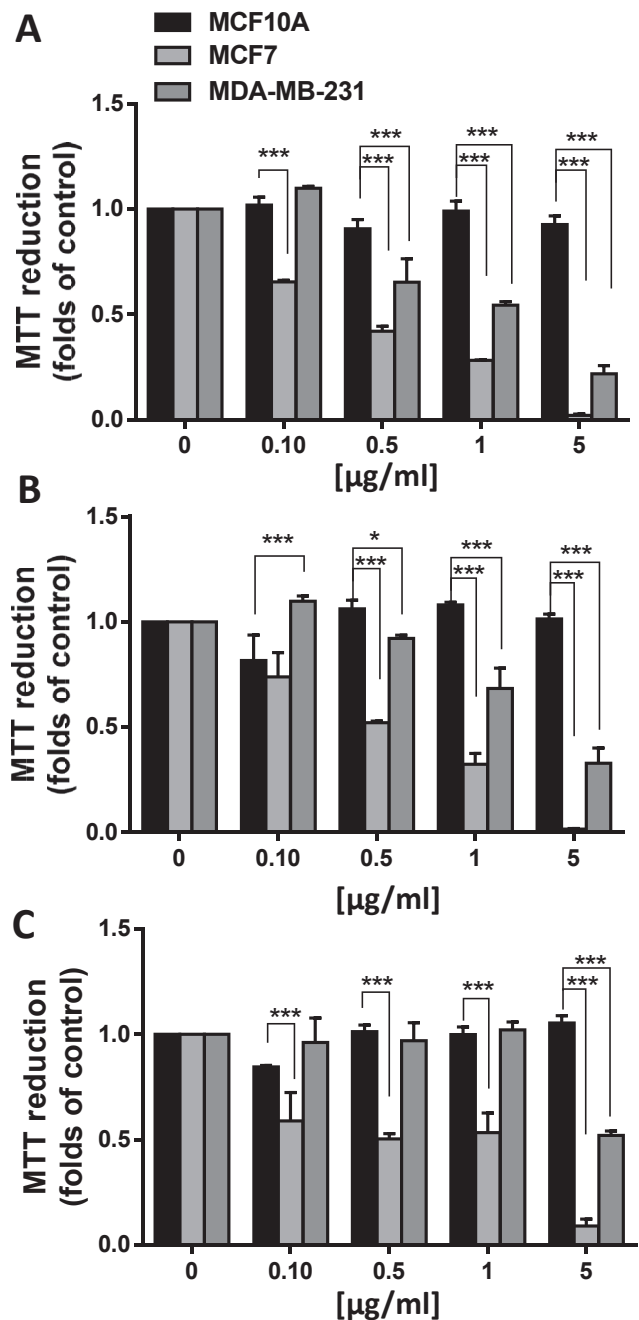

Fig. 4. Antiproliferative effect of *Rhinella marina* venoms on breast cancer cells. Effect of venoms from UP1 (A), UP2 (B) and RNAM (C) on non-tumoral breast MCF10A and breast cancer MCF7 and MDA-MB-231 cells after 72 h exposition was determined by MTT assay. Data shown are the mean ± SD of three independent experiments. ***$p < 0.001$ and *$p < 0.05$ vs. MCF10A cells.
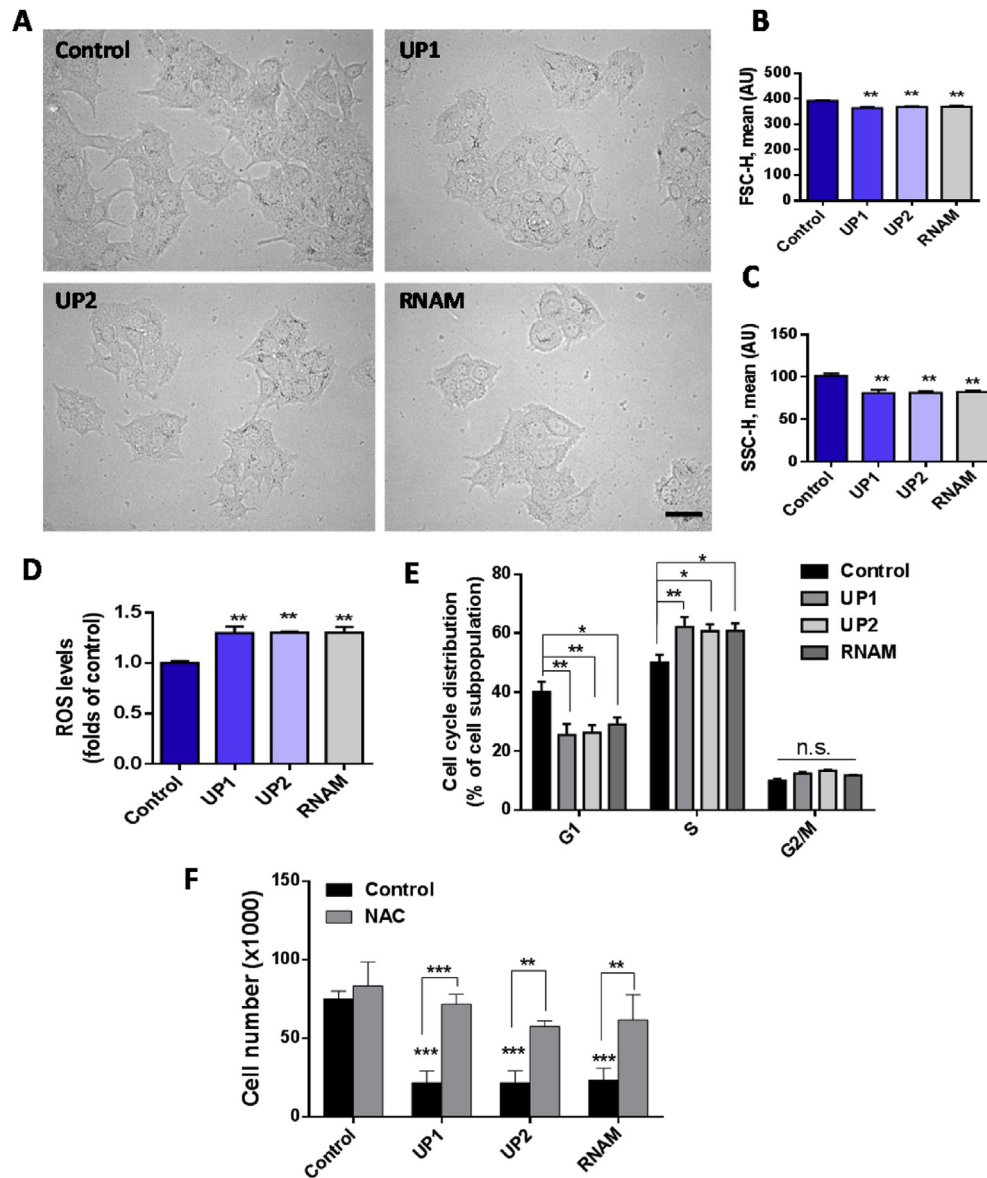
Fig. 5. *Rhinella marina* venoms increase ROS production and induce cell cycle arrest on breast cancer cells. (A) Effect of control (DMSO), venoms from UP1, UP2 and RNAM on morphology, (B) size, (C) granularity and (D) ROS production were obtained after 24 h of exposition by flow cytometry, using the FSC and SSC channels, and CellROX Green probe, respectively; (E) effect on cell cycle progression was evaluated after 48 h of exposition by flow cytometry and (F) effect on the number of breast cancer MCF7 cells was counted after 72 h of exposition, using trypan blue staining. Scale bar represents 150 μm. Data shown are the mean ± SD of three independent experiments. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. control (DMSO). AU: arbitrary units; n.s.: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The alkaloid composition from different Brazilian *Rhinella* species (described as *Bufo*) was revised by Maciel et al. (2003) using thin layer chromatography and mass spectrometry analysis. The main constituents were tryptophane derivatives with bufotenin, 5-hydroxytryptamin and dehydrobufotenin as main compounds.

Bufadienolides are C-24 steroids of restricted distribution in nature (Krenn and Kopp, 1998). They have been reported from the venom of toads belonging to the family Bufonidae, from a snake and some arthropods as well as from the cardiac glycosides from higher plants (Krenn and Kopp, 1998). The bufadienolides telocinobufagin and hellebrigenin, isolated from the parotid glands from the Brazilian *Rhinella jimi* showed activity against the protozoa *Leishmania chagasi* and *Trypanosoma cruzi* (Tempone et al., 2008). Natural and semisynthetic bufadienolides from *R. schneideri*, including marinobufagin, bufalin, telocinobufagin and

hellebrigenin presented cytotoxic effect on a panel of human cancer cells (Cunha-Filho et al., 2010). Marinobufagin and telocinobufagin showed moderate activity as antimicrobials against *S. aureus* and *E. coli* (Cunha Filho et al., 2005). A recent work on *R. schneideri* reported the identification of 29 constituents and the antiproliferative activity of the venom on human cancer cells (Schmeda-Hirschmann et al., 2014).

The effect of *R. schneideri* venom on the complement system was reported by Anjolette et al. (2015). The most active fractions were shown to be proteins. The mechanisms of action of the *R. schneideri* venom on the cardiovascular system was described by Rostelato-Ferreira et al. (2014). The authors showed that the venom acts at the presynaptic level enhancing neurotransmitter release (Rostelato-Ferreira et al., 2014). Bufadienolides from the Cuban toad *Peltophryne fustiger* showed effect on Na^+/K^+-ATPase with

better effect for arenobufagin and bufalin (Córdova et al., 2016). The main constituents of the venom, according to the HPLC chromatogram, are bufadienolides with different oxidation patterns while the argininyl diacid derivatives are minor constituents.

A comparison of the constituents of *Bufo* venoms was reported by Gao et al. (2010) and the study was complemented by a report by Hu et al. (2011) on the constituents of Venenum Bufonis, using HPLC-ESI-MS/MS methods. In the samples analyzed by Hu et al. (2011), three groups of constituents were detected, namely free bufadienolides, bufadienolide conjugates with diacids and arginine (argininyl diacids of bufadienolides) and acetyl bufadienolides. The main compounds in Venenum Bufonis were the free bufadienolides, with higher content of resibufogenin, arenobufagin, telocinobufagin, bufalin and 17-hydroxybufalin. The main acetyl derivative was cinobufagin followed by bufotalin. Both cinobufagin and resibufogenin were the main bufadienolides from the venom. The diacid moiety in the argininyl conjugates included C2 to C10 diacids with higher content of the suberoyl and sebacyl derivatives. The argininyl diacids of bufadienolides were less abundant in the venom than the free bufadienolides (either acetylated or with free hydroxyl), being 3-(*N*-sebacyl argininyl)-bufalin and 3-(*N*-suberoyl argininyl) gamabufotalin the most common compounds from this group. According to Gao et al. (2010), relevant differences were found in chemical composition of the different samples of toad venoms investigated. However, three bufadienolides: marinobufagin, bufalin and telocinobufagin were found in all commercial samples. The compounds differ in the oxidation at C-5 and in the presence of either an epoxide at C14(15) for marinobufagin or a tertiary alcohol at C-14 for bufalin and telocinobufagin.

The antiproliferative effect of toad venoms has been reported by several authors working on Asia and South American samples. The extract of *R. marina* venom collected in the southern Brazilian Amazon was assessed for antiproliferative effect by Ferreira et al. (2013) while the antiproliferative and cytotoxic activity of frog extracts on breast cancer cells was described by Sciani et al. (2013). Meng et al. (2009) reported the results of a pilot study in hepatic cancer patients treated with the Chinese drug Huachansu, with favourable results. Single venom constituents have been investigated for their potential use to treat cancer, including hellebrigenin (Deng et al., 2014).

While the constituents and bioactivity of the Chinese crude drug Chan Su has been intensively investigated, much less is known on the venoms from the South American *Rhinella* species. In the *R. marina* venom from Iquitos, marinobufagin **7** was the main free bufadienolide. The suberoyl argininyl derivative **15** (marinobufotoxin) and the suberoyl argininyl bufalin **9** were the most abundant argininyl diacid conjugates. However, large differences in the constituent ratio were found for the two samples investigated and may reflect differences in the diet composition of the toads in both locations. When compared with the Brazilian Pantanal toad *R. schneideri*, 3-(*N*-suberoylargininyl) marinobufagin (marinobufotoxin) was the main compound followed by bufalitoxin while marinobufagin was the main free bufadienolide.

The South American *Rhinella* species investigated so far shows trypthophane-derived alkaloids such as dehydrobufotenin and bufotenidin but tryptamine alkaloids are not clearly associated with the Asian sources of Chan Su. However, more research work should be carried out to confirm this observation. A study on the venom of *R. marina* sampled in Australia showed marinobufagin as the main bufadienolide with telocinobufagin and bufalin as second group of main bufadienolides. The authors suggest that variation in chemical diversity of the venom constituents is mediated by bacterial biotransformation "in situ" (Hayes et al., 2009). However, the compounds reported as microbial products have been not reported so far as constituents from the South American venoms.

The Peruvian *R. marina* venoms showed antiproliferative effect but the activity was lower than that observed for the venom from the Brazilian Pantanal toad *R. schneideri* (Schmeda-Hirschmann et al. 2014). When compared with *R. schneideri*, similar activity was observed against AGS cells but the *R. marina* venoms were less active against lung, bladder and HL-60 cancer cells. The venoms from the Peruvian *R. marina* were more active as antiproliferative agents than the reference compound etoposide. The differences in the antiproliferative effect of the Peruvian samples can be related with the composition and different ratios of their chemical constituents. The main bufadienolide for the Unión Progreso (UP) venom was marinobufagin **7**, while the main constituent of the Reserva Nacional Alpahuayo Mishana (RNAM) was marinobufotoxin **15**. Additional differences can be found in the different ratios of bufadienolide argininyl diacids to free bufadienolide, where the UP sample shows larger free bufadienolide content and the RNAM venom higher bufadienolide argininyl diacids content.

The induction of cell cycle arrest in G2/M-phase and apoptosis have been described for venoms of several *Rhinella* species in breast cancer MCF7 cells (Sciani et al., 2013). Interestingly, we found that *R. marina* venoms produce inhibition of proliferation of MCF7 cells by a mechanism that involves increase in the intracellular ROS levels and cell cycle arrest in S-phase, without increase in apoptotic sub-G1 sub-population in the concentration tested (data not shown). Some components of *R. marina* venoms from the Peruvian Amazon basin may act on DNA replication and inhibit cell cycle progression like certain bufadienolides present in traditional Chinese medicine Chan Su. It has been reported that Chan Su produces disruption of cytoskeleton and cell cycle arrest in S-phase (Ma et al., 2012), reduces the activity and protein levels of topoisomerases (Hashimoto et al., 1997; Liu et al., 2015) and intercalates with DNA (Deng et al., 2015).

5. Conclusions

The present study shows differences in the composition of the venoms obtained from the South American *Rhinella* and the Asian *Bufo* that can be relevant for the identification of the venom source as well as for the possible use of South American toad venom for similar purposes as the Chinese drug Chan Su. Marinobufagin seem to be the main free bufadienolide in *R. marina* and *R. schneideri* and marinobufotoxin and bufalitoxin are the main argininyl diacid conjugates in both species. The main free bufadienolides in the *Bufo* venoms are close related compounds, including marinobufagin, bufalin and telocinobufagin. The antiproliferative effect of the venoms is mediated by the production of ROS and cell cycle arrest in human breast cancer cells. This is the first report on the composition of *R. marina* venom from the Peruvian Amazon basin. The results of our study on the Peruvian Amazon *R. marina* sets a reference for other South American frog venoms and encourages further work on the chemistry and bioactivity of *Rhinella* toads.

Ethical statement

On behalf of all the authors, GSH declares that the manuscript has not been published elsewhere and is not under editorial review for publication elsewhere. All the co-authors of this research approved this submission.

Conflict of interest

The authors declare that there are no conflict of interest.

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