Steroids 152 (2019) 108490

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

New bufadienolides extracted from *Rhinella marina* inhibit Na,K-ATPase and induce apoptosis by activating caspases 3 and 9 in human breast and ovarian cancer cells

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ARTICLE INFO

Keywords:
Bufadienolides
Apoptosis
Na,K-ATPase
Caspase 3 and 9

ABSTRACT

Bufadienolide compounds have been used for growth inhibition and apoptosis induction in tumor cells. Those families of cardiotonic steroids can bind the Na,K-ATPase, causing its inhibition. The use of bufadienolides is widely described in the literature as an anticancer function. The aim of this study was to evaluate the effects of bufadienolides and alkaloid isolated from venom samples from *R. marina* on tumor cells. We performed cytotoxicity assay in MDA-MB-231 and TOV-21G cells and evaluated the activity of Caspases (3 and 9), Na, K-ATPase, PMCA and SERCA. Four compounds were extrated from the venom of *R. marina*. The compound 1 showed higher cytotoxicity in MDA-MB-231cells. Compound 1 also showed activation of Caspase 3 and 9. This compound caused an inhibition of the activity and expression of Na, K-ATPase, and also showed activation of both caspase-9 and caspase-3 in MDA-MB-231 cells. We also observed that Compound 1 had a direct effect on some ATPases, such as Na, K-ATPase, PMCA and SERCA. Compound 1 was able to inhibit the activity of the purified Na, K-ATPase enzyme from the concentration of 5 μM. It also caused inhibition of PMCA at all concentrations tested (1 nM-30 μM). However, the compound 1 led to an increase of the activity of purified SERCA between the concentrations of 7.5–30 μM. Thus, we present a Na, K-ATPase and PMCA inhibitor, which may lead to the activation of caspases 3 and 9, causing the cells to enter into apoptosis. Our study suggests that compound 1 may be an interesting molecule as an anticancer agent.

1. Introduction

In 1957, J.C, Skou described the Na,K-ATPase, an enzyme transmembrane of the family P-ATPase, responsible for asymmetrically transports tree Na^+ out of the cell and two K^+ into the cell, and is responsible for maintaining the electrochemical gradient at the expense of one ATP hydrolyzed per transport cycle. This enzyme is also a receptor protein for a class of molecules known as Cardiotonic steroids (CTS) [1]. The CTS bind to the α-subunit of the Na,K-ATPase, causing its inhibition. Within therapeutic doses, the partial inhibition of the Na,K-ATPase causes an increase in intracellular Na^+ which dissipates the gradient necessary for the proper function of the Na^+/Ca^{2+} exchanger, leading to increased levels of intracellular Ca^{2+}. This rise in the intracellular Ca^{2+} leads to the activation of myosin II and actin, causing the increased muscle contraction observed in response to CTS [2].

The CTS can also activation of intracellular signaling pathways using the Na,K-ATPase as a hormone receptor has been extensively

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https://doi.org/10.1016/j.steroids.2019.108490
Received 22 January 2019; Received in revised form 26 August 2019; Accepted 27 August 2019
Available online 06 September 2019
0039-128X/

investigated in recent year [3–6]. The use of CTS is widely described in the literature as an anticancer effect [7,8]. *In vitro* and *ex vivo* studies have shown that some CTSs, such as digitoxin, digoxin, ouabain, and bufalin, induce anticancer effects, acting as potent inhibitors of tumor cell growth [9–11].

The CTS include cardenolides and bufadienolides, known collectively as digitalis, which inhibit the Na,K-ATPase. Cardenolides, such as cardiac glycosides and digoxin, possess a steroid skeleton bearing a butenolide ring at position C17β and one or more sugar groups at C34. Bufadienolides, which have been isolated from many animals and plants, consist of a steroid skeleton bearing a pentadienolide ring at C17β and there are glycosides or aglycones in plants but only aglycones in animals [8,12].

Members of the CTS superfamily are bufadienolides, which are been isolated from amphibians [13]. Bufadienolides compounds have been used as growth inhibition and apoptosis induction in tumor cells [14]. Indeed, those families of CTS are well-studied against a wide spectrum of cancer cell lines, as leukemia, breast, prostate, gastric and liver cancer [15–18].

The main sources of bufadienolides are the frog venom obtained from members of Bufonidae family. In Brazil, this family is represented by seven genera, and Rhinella is the most representative one, with approximately 40 species [19,20]. The frogs of the species *R. marina* (popularly known “sapo-cururu”) are one of the most studies species and they are found in the Amazon regions of Brazil, Bolivia, Colombia, Peru, Suriname, Guiana and Venezuela. Recent studies have shown that their poison presents mainly the cytotoxic and/ or anticancer activity [21,22].

Breast and ovarian cancer are considered one of the most leading causes of cancer death in women worlrwide [23]. About two million of new breast cancer cases were diagnosed only in 2018, and more than 1.8 million of ovarian cases [24].

The most breast cancer types express estrogen receptor α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/ERBB2). In these cases, treatment with anti-estrogens and HER2-targeted compounds improve patient rate survival [25]. However, the resistance to treatment is common in breast cancer cases classified as triple-negative breast cancer (TNBC). TNBCs do not express ERα, PR and HER2/ERBB2, limiting the therapeutic options [26]. In ovarian cancer, the resistance to chemotherapy treatment is associated with a different set of histological subtypes as serous carcinoma, endometrioid carcinoma and clear cell carcinoma [27].

The human breast cancer cell line MDA-MB-231 was used in all of the studies presented below. This estrogen receptor negative cell line was chosen to avoid the possibility of cardiotonic steroids interaction with estrogen receptor [28,29]. The human ovarian adenocarcinoma line TOV-21g also is important in gynecological cancer studies, and like MDA-MB-231, is an estrogen receptor negative cell line [30]. This cellular model is also interesting because, the potent inhibitors of Na, K-ATPase, suche as ouabain and related digitalis could also act as estrogen receptor antagonists and could be developed as a group of potentially promising anti-breast cancer drugs [31]. Thus, the effects found in this work are exclusively related the Na, K-ATPase.

Thus, the aim of this study was to evaluate the effects of substances isolated from *R. marina* against a human breast adenocarcinoma cell line (MDA-MB-231) and a human ovarian adenocarcinoma line (TOV-21g).

2. Experimental methods

2.1. Isolation of the compounds

The animals were captured by the team of biologists from the Federal University of Mato Grosso (campus Sinop), under the coordination of Prof. Dr. Domingos de Jesus Rodrigues (IBAMA, SISBIO: 30034-1), which also carried out the process of extraction of the material secreted by the glands of the frog by manual compression. After this procedure, the animals were returned to nature, between December 2013 to February 2014, in the municipality of Sinop – MT. Voucher specimen (*Rhinella marina* – ABAMH 1262) was deposited in the Biological Collection of the Southern Amazon (Sinop, Mato Grosso, Brazil).

The venom samples from *R. marina* were dried in a desiccator with silica and after that triturated with mortar and pestle. The powder was extracted (3 × 20 mL) in methanol on ultrasonic sonicator (Eco-sonics – Ultronique) for 10 min at room temperature.

The extract was fractionated in a Sephadex LH-20 column, using methanol as eluent. Four fractions were obtained: CRV-6 (783.8 mg); CRV-28 (102.9 mg); CRV-52 (315.8 mg) and CRV-70 (394.14 mg). Fraction CRV-28 (102.9 mg) was analyzed by NMR and mass spectrometry and identified the compound 2.

The fraction CRV-6 was submitted to silica gel column with gradient of CHCl3/ MeOH and after to a Sephadex-LH-20 column using MeOH 100% as eluent, obtaining the compound 1 (35.4 mg). The fractions CRV-52 and CRV-70 were fractionated in silica gel column with the gradient CHCl3/ MeOH obtaining the compound 3 and the sub-fraction CRV-70–004 (80.7 mg), which was further analyzed by High Performance Liquid Chromatography (HPLC).

For HPLC analysis was used an analytical HPLC equipped with degasifier DGU-20A5R, pump LC-20AT, valve LPGE kit, injector SIL-20AHT, oven CTO-20A, detector DAD SPD-M20A and controller CBM20A from SHIMADZU corporation. The software used was Shimadzu Lab. Solutions developed by their own company. The chromatographic separation was performed in a column Phenomenex Luna C-18 (250 mm × 4.6 mm; 5 μm). For the isolation was used a Shimadzu HPLC equipped with a LC-6AD pump, manual injector, SPD-20a UV detector, controller CBM20A semi-preparative Phenomenex Luna C-18 column (250 mm × 10 mm; 5 μm). The mobile phase consisted in purified water in Milli-q system (eluent A) and acetonitrile (B) with gradient elution of 31% acetonitrile (0 min), 62% acetonitrile (12 min) and 62% acetonitrile (20 min), using a flow rate of 1 mL min^{-1}, optimized by Kerkhoff et al (2016). The injection volume was 20 μL, the sample concentration of 2.5 mg mL^{-1} and UV detection was monitored at wavelength range of 190–900 nm [32].

For the isolation of the chemical constituents of this fraction in semipreparative HPLC, the mobile phase used was the same as the other, but using isocratic programation with 60% of acetonitrile. The flow rate was 4 mL min^{-1}, the sample concentration was 20 mg mL^{-1}, and the injection volume was 100 μL, consisting in the isolation of two substances: 3 (22.6 mg) and 4 (7.9 mg).

2.2. Cell culture

The human breast adenocarcinoma cell line, MDA-MB-231 (ATCC# HTB-26); human ovarian adenocarcinoma line TOV-21g (ATCC# CRL11730), and human lung fibroblast line, WI-26VA4 (ATCC# CCL-95.1) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and gentamicin (100 μg/mL). All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2. All experiments described were performed at least three times using cells in the exponential growth phase.

2.3. Cytotoxicity assay

The cytotoxicity effect was assessed using a tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma®, St. Louis, MO, USA) colorimetric method. Briefly, the cells were plated in 96-well plates ($1x10^5$ cells/well) and incubated for 24 h at 37 °C in humid atmosphere with 5% CO_2 until adhesion. After this period, the wells were washed with culture medium and incubated for 48 h with the compounds at different concentrations. After the incubation, the plates were treated with MTT. The reading was performed in a

microplate reader Spectramax M5e (Molecular Devices, Sunnyvale, CA, USA) at 550 nm. Cytotoxicity was scored as the percentage of absorbance reduction when compared to untreated control cultures. All experiments were performed in triplicate. The results were expressed as the means of IC50 (the drug concentration that reduced cell viability to 50%). The selectivity index (SI) of cytotoxicity was calculated based on the IC50 values obtained for each compound as follows:

$$SI = IC50\ Y/IC50\ X,$$

where IC50 X is the IC50 value obtained for MDA-MB-231 and TOV-21g cell lines and IC50 Y is the IC50 value obtained for WI-26VA4, the human non-tumor cell line used as a reference.

2.4. Caspase-3 and 9 colorimetric assays

Enzymatic activities of caspase-3 and −9 were assessed using the colorimetric assays from R&D Systems (Wiesbaden-Nordenstadt, Germany) according to the manufacturer's protocol. Briefly, approximately 2×10^5 MDA-MB-231 and TOV-21G cells were inoculated in 24-well plates and the plates were incubated at 37 °C in a humidified 5% CO_2 incubator; after 24 h, the medium was replaced and the cells were treated with the compounds (at their respective IC50 values) for 48 h. The cells were collected, centrifuged, and washed twice with phosphate-buffered saline (PBS). Samples containing 1×10^6 cells were harvested and lysed in 50 µL Lysis Buffer (Triton X-100 and DTT solution, according Safety Data Sheet of the manufacturer) on ice for 10 min and then homogenates were centrifuged at 10,000 × g for 1 min. After centrifugation, the supernatant was incubated with caspase-3 and −9 substrate (DEVD-pNA and LEHD-pNA, respectively) in Reaction Buffer. Samples were incubated in 96-well flat bottom microplates at 37 °C for 2 h. The absorbance of each sample was recorded using amicroplate reader (Bio-Rad, Hercules, California, USA) at 405 nm and the caspase-3 and −9 activity was directly proportional to the color reaction.

2.5. Determination of the inorganic phosphate (Pi) released by ATP hydrolysis by Na,K-ATPase, SERCA and PMCA

For the quantification of the phosphate originated by the ATP hydrolysis, the Fiske colorimetric method was used for the ATPases enzymes. After the reactions were stopped by 1% SDS, was added 100 µL of 5:1 ammonium molybdate solution/Fiske reagent was added. Fifteen minutes after the addition of the solution, the ELISA plate was read in a spectrophotometer at a wavelength of 660 nm [33].

2.6. Determination of the activity of purified pig kidney Na,K-ATPase

The purified Na,K-ATPase was courtesy of the Laboratory of Structure and Regulation of P-ATPase of the Institute of Medical Biochemistry of the Federal University of Rio de Janeiro (UFRJ), and was obtained according Cortes et al. (2006) [34]. The same amount of protein was placed in each well of the ELISA plate. The sample was incubated for 20 min with compound 1 at different concentrations.

The determination of the Na,K-ATPase activity was determined through the quantification of the inorganic phosphate (Pi) released by ATP hydrolysis by Na,K-ATPase, using the Fiske's colorimetric method [35]. The assay was performed in reaction medium containing: 40 mM Tris-Hcl (pH 7.4), 120 mM NaCl, 20 mM KCl, and 2 mM $MgCl_2$. The same amount of protein from each sample (10 µg) and reaction medium were added to each well and the reaction was initiated by the addition of 3 mM ATP. The plate was incubated at 37 °C for 20 min and the reaction was stopped with the addition of 100 µL of 1% SDS. Data was expressed as percentage in relation of control.

2.7. Determination of the activity of the plasma membrane Ca^{2+}-ATPase (PMCA)

The Erythrocyte plasma membrane preparation (GHOST) was courtesy of the Laboratory of Structure and Regulation of P-ATPase of the Institute of Medical Biochemistry of the Federal University of Rio de Janeiro (UFRJ), and was obtained according Oliveira et al., 2008 [36]. The same amount of protein was placed in each well of the ELISA plate.

The same amount of protein was placed in each well of the ELISA plate. The sample was incubated for 20 min with compounds 1 at different concentrations. The PMCA activity was obtained measuring inorganic phosphate from ATP hydrolysis.

The determination of the PMCA activity was determined through the quantification of the inorganic phosphate (Pi) released by ATP hydrolysis by PMCA, using the Fiske's colorimetric method [33]. The assay was performed in reaction medium containing: Tris-HCl 10 mM, pH 7.4, KCl 80 mM, MgCl2 0.5 mM, EGTA 0.2 mM, CaCl2 0.2 mM, ouabain 1 mM, in the presence or absence of Calmodulin (2 µg/ml) [37]. The same amount of protein from each sample (10 µg) and reaction medium were added to each well and the reaction was initiated by the addition of 3 mM ATP. The plate was incubated at 37 °C for 60 min and the reaction was stopped with the addition of 100 µL of 1% SDS.

2.8. Determination of the purified sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity of rat striated muscle

The purified SERCA was courtesy of the Laboratory of Bioquímica Celular of the Federal University of São João del Rei (UFSJ), and was obtained according [38]. The same amount of protein was placed in each well of the ELISA plate. The sample was incubated for 20 min with compounds 1 at different concentrations.

The same amount of protein was placed in each well of the ELISA plate. The sample was incubated for 20 min with compounds 1 at different concentrations. The SERCA activity was obtained measuring inorganic phosphate from ATP hydrolysis.

The determination of the SERCA activity was determined through the quantification of the inorganic phosphate (Pi) released by ATP hydrolysis by SERCA, using the Fiske's colorimetric method. The assay was performed in reaction medium containing: Tris-HCl 50 mM, pH 7.4, KCl 40 mM, $MgCl_2$ 2 mM, $CaCl_2$ 0.05 mM, ouabain 1 mM. The same amount of protein from each sample (10 µg) and reaction medium were added to each well and the reaction was initiated by the addition of 3 mM ATP. The plate was incubated at 37 °C for 60 min and the reaction was stopped with the addition of 100 µL of 1% SDS.

2.9. Preparation of cell membrane MDA-MB-231

MDA-MB-231 cells were culture in 75 cm^2 culture bottles in RPMI medium with 10% FBS, where the cells grew until they reached 80% confluence. Subsequently, RPMI culture medium was removed and cells were treated (with RPMI 1% FBS) for 48 h with compound 1 at concentrations of 1.5 µM, 15 µM and a control was maintained untreated. Non-treated cell membrane preparations were also performed to obtain the membrane isolated for further treatment with a concentration curve of 1 in the Na, K-ATPase activity experiment.

After the treatment period on treated cells or the period in which the untreated cells reached confluence, the culture medium was discarded and the cells were washed three times with cold PBS and scrapped from the culture bottle with a rubber policeman with 3 mL of membrane preparation buffer (6 mM Tris (pH 6.8), 20 mM imidazole, 0.25 M sucrose, 0.01% SDS, 3 mM EDTA and 1% Protease Inhibitor Cocktail (sigma cod. P2714). The cells were homogenized thirty times in a potter in ice and subjected to ultracentrifugation at 10,000 g for 20 min at 4 °C. The pellet was discarded and the supernatant ultracentrifuged at 70,000 g for 1 h at 4 °C. The supernatant was discarded and the pellet resuspended with 200 µL of membrane preparation

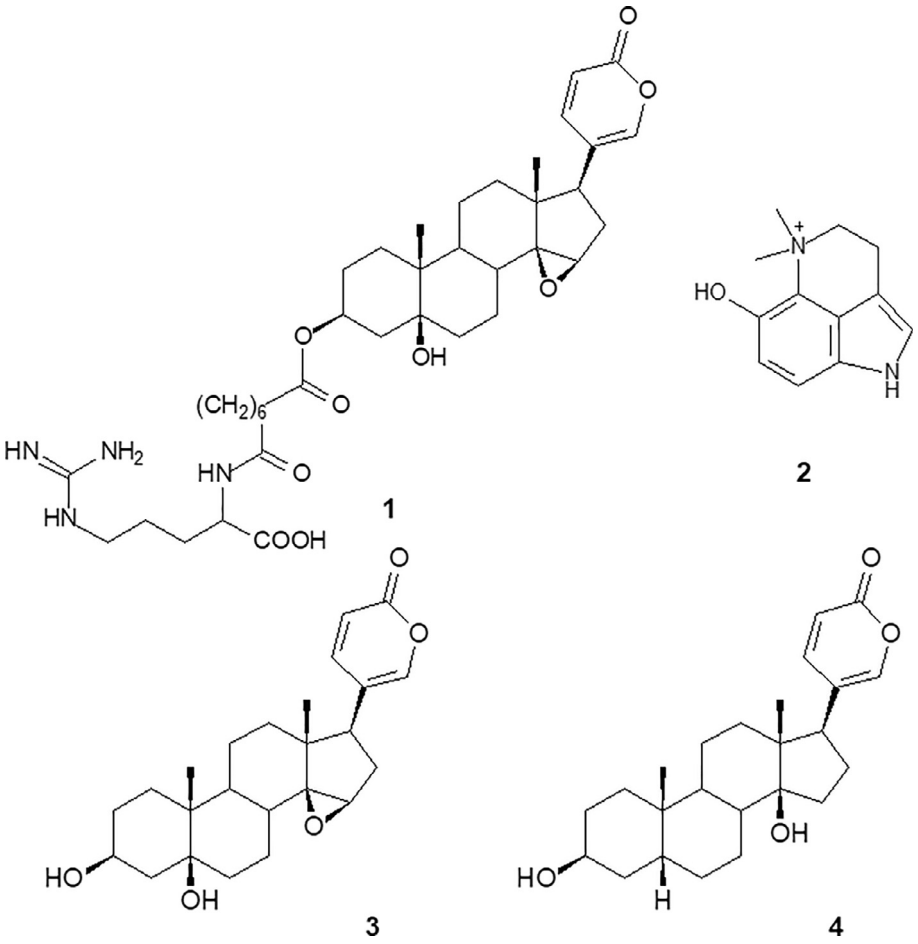

Fig. 1. Chemical structures of extract compounds from *Rhinella marina*. NMR data are presented in the Suplemmentary material.

buffer. The final content was sonicated for 10 s at 25% power until complete dispersion of the membrane.

2.10. Determination of Na,K-ATPase activity in MDA-MB-231 cells

After 48 h treatment the membranes were obtained from MDA-MB-231 cells, and the determination of the Na,K-ATPase activity was determined through the quantification of the inorganic phosphate (Pi) released by ATP hydrolysis by Na,K-ATPase, using the Fiske's colorimetric method [35]. The assay was performed in reaction medium containing: 40 mM Tris-Hcl (pH 7.4), 120 mM NaCl, 20 mM KCl, and 2 mM $MgCl_2$. The same amount of protein from each sample (10 μg) and reaction medium were added to each well and the reaction was initiated by the addition of 3 mM ATP. The plate was incubated at 37 °C for 20 min and the reaction was stopped with the addition of 100 μL of 1% SDS.

2.11. Determination of Na,K-ATPase and PMCA activities in MDA-MB-231 cells membrane

MDA-MD-231 cells membranes without treatment were obtained for the determination of the direct effect of the compounds on the ATPases activities. The same amount of protein was placed in each well of the ELISA plate. The sample was incubated for 60 min with compound 1 at different concentrations. The determination of the Na,K-ATPase and PMCA activities was determined as described in itens 2.6 and 2.7, respectively.

2.12. Protein determination

The protein content of the sample was determined by the Bradford method using BSA as the standard [39].

2.13. Western blot

The membrane proteins from MDA-MB-231 cells were submitted to 10% SDS-PAGE and lysates from MDA-MB-231 cells were submitted to 12.5% on a Bio-Rad mini-Protean III apparatus (Bio-Rad, Hercules, CA, USA) running at 90 V (90 min). The proteins were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and incubated with the specific antibodies for α1-Na,K-ATPase (sc-21712, Santa Cruz Biotechnology, Santa Cruz, CA, USA), PMCA4 (ab2783, Abcam plc), Capase-3 (sc-56053, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Caspase-3 claved (D175, Cell Signalling technology), β-actina (sc-81178, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse (sc-516102, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-rabbit (sc-2357, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The Ponceau red method was used to ensure equal protein loading to immunoblots. Proteins recognized by antibodies were revealed by an equimolar solution mix (Solution 1: 100 mM Tris (pH = 8.5), 2.5 mM Luminol and 0.396 mM p-cumaric acid; Solution 2: 100 mM Tris (pH = 8.5) and 0.06% H_2O_2). Immunoblots were quantified using the ImageJ software (http://rsb.info.nih.gov/ij). Several exposure times were analyzed to ensure the linearity of the band intensities.

Table 1
Peaks purity of Compound in the chromatogram.

Compound	Purity (%)
Marinobufotoxin (**1**)	90.0
Dehydrobufotenin (**2**)	92.6
Marinobufagin (**3**)	99.9
Bufalin (**4**)	87.8

2.14. Statistical analysis

All the results are expressed as mean ± SEM. The results were tested by one-way analysis of variance (ANOVA), followed by the Newman-Keuls post hoc test. All analyses were performed using the Prism 5 software package (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered to reflect a statistically significant difference.

3. Results

3.1. Compound identification

The structure of the isolated compounds marinobufotoxin (1), dehydrobufotenin (2), marinobufagin (3) and bufalin (4) are showed in Fig. 1. Based on chromatographic and NMR analisys the purity of the compounds is summarized in Table 1, and all presented high peak purity, in according with NMR spectra (supplementary data, S1), that are in agreement with previously studies [40,41]. Compounds 1, 3 and 4 are bufadienolides and the compound 2 is an alkaloid.

3.2. Cytotoxicity assay

First, in order to assess the cytotoxic activity of the compounds, MTT assays were performed on the human cells lines MDA-MB-231 and TOV-21G (tumor cell lines), and WI-26VA4 (non-tumor cell line), using doxorubicin as reference cytotoxic compounds.

The IC_{50} values obtained for the compounds tested *in vitro* (Table 2) showed that all compounds were cytotoxic. It is noteworthy that compound **1** was more active than the other compounds against the tumor cell lines MDA-MB-231 and TOV-21G, presenting IC_{50} values of 13.14 and 19.29 μM, respectively, after 48 h.

3.3. Detection of caspase activation

Based on the cytotoxic experiment, we performed an assay of Caspase-3 and −9 activities to verify the apoptosis process. As indicated in Fig. 2, the activity of caspases-3 and −9 significantly increased after treatment of the MDA-MB-231 and TOV-21G cells with compound **1**. Maximum enzymatic activity was detected after 48 h of exposure.

To confirm whether there was an increase in caspase 3 cleavage, we performed the western blot assay (Fig. 3). We observed an increase in the caspase-3 cleavage with treatment of the MDA-MB-231 at concentrations 7.5 μM and 15 μM with compound **1**.

One of the mechanisms of activation of caspase 3 and 9 by cardiotonic steroids is its inhibition of Na,K-ATPase membrane enzyme. Thus, we sought to evaluate the effect of compound **1** on the activity of Na,K-ATPase.

3.4. Effects of compound 1 on the activity of ATPases

We evaluated if compound **1** may had a direct effect on the enzyme. For this it was used of purified pig kidney Na,K-ATPase was incubated with different concentrations of the compound. After 20 min treatment we observed a significant inhibition dose-dependent from the 5 μM concentration. We also observed an IC50 inhibition of Na,K-ATPase at the concentration of 7.28 ± 0.05 μM ($p < 0.05$) (Fig. 4A). Compound **1** was also able to inhibit Na,K-ATPase activity when directly incubated (20 min) with fraction of membrane from MDA-MB-231 cell control (without-treatment) from the concentration of 100 nM ($p < 0.05$) (Fig. 4B).

After that, we performed the treatment of the compound **1** on MDA-MB-231 cells in concentrations of 1.5 and 15 μM for 48 h. In Fig. 4C, we showed that both caused significantly decreased Na,K-ATPase activity (approximately 54% and 90%, respectively) in comparison of without-treatment ($p < 0.05$).

In evaluating the effect of compound **1** against PMCA activity in ghost, it can be seen that when incubated directly with ghost membranes all concentrations tested were able to inhibit PMCA activity ($p < 0.05$) (Fig. 4D). In Fig. 4E, we showed the effect of compound **1** on the PMCA in MDA-MB-231 cells membrane, when we incubated directly for 20 min, there is not effects under PMCA activity of those cells. Finaly, the effect of compound **1** on purified SERCA activity of rat striated muscle was evaluated, the compound was found to provide a significant increase at all concentrations tested ($p < 0.05$) (Fig. 4F).

3.5. Effects of compound 1 on the Na,K-ATPase and PMCA4 from MDA-MB-231 cells

After 48 h treatment with compound **1** we evaluated the expression of the α1-Na,K-ATPase isoform from cell membranes. We notice that compound **1** was able to significantly decrease the expression of the Na,K-ATPase in MDA-MB-231 cells ($p < 0.05$) (Fig. 5A). There is also a decrease of the expression of PMCA4 in the cellular lysates of those cells after 48 h of treatment (Fig. 5B).

4. Discussion

We evaluated the cellular viability of all compounds against the non-tumor cell line WI-26VA4, as show on Table 2. All compounds were considered cytotoxic. Although, compound **1** was the most active of the series against the tumor cell lines MDA-MB-231 and TOV-21G. Bufadienolides has been used to treat patients with various types of cancer in Chinese tradicional medicine (Chansu tea), including hepatoma, gallbladder carcinoma and lung cancer. Even that in our work the compounds had a cytotoxic effect on WI-26VA4, others bufadienolide

Table 2
IC_{50} values obtained *in vitro* assays with MDA-MB-231, TOV-21G, WI-26VA4 cell lines.

COMPOUND	IC50 (μM) ± SD[a]			Selective Index (SI)	
	MDA-MB-231	TOV-21G	WI-26 VA4	MDA-MB-231	TOV-21G
1	13.14 ± 2.53	19.29 ± 6.03	8.89 ± 2.66	0.68	0.46
2	82.11 ± 1.52	69.4 ± 16.74	235.76 ± 4.03	2.86	3.4
3	37.45 ± 2.39	29.5 ± 3.29	3.04 ± 0.25	0.08	0.1
4	70.72 ± 4.66	51.29 ± 3.37	25.9 ± 7.04	0.37	0.5
Doxorubicin	2.33 ± 0.052	4.37 ± 1.06	1.96 ± 0.9	0.84	0.45

[a] Standard Deviation.

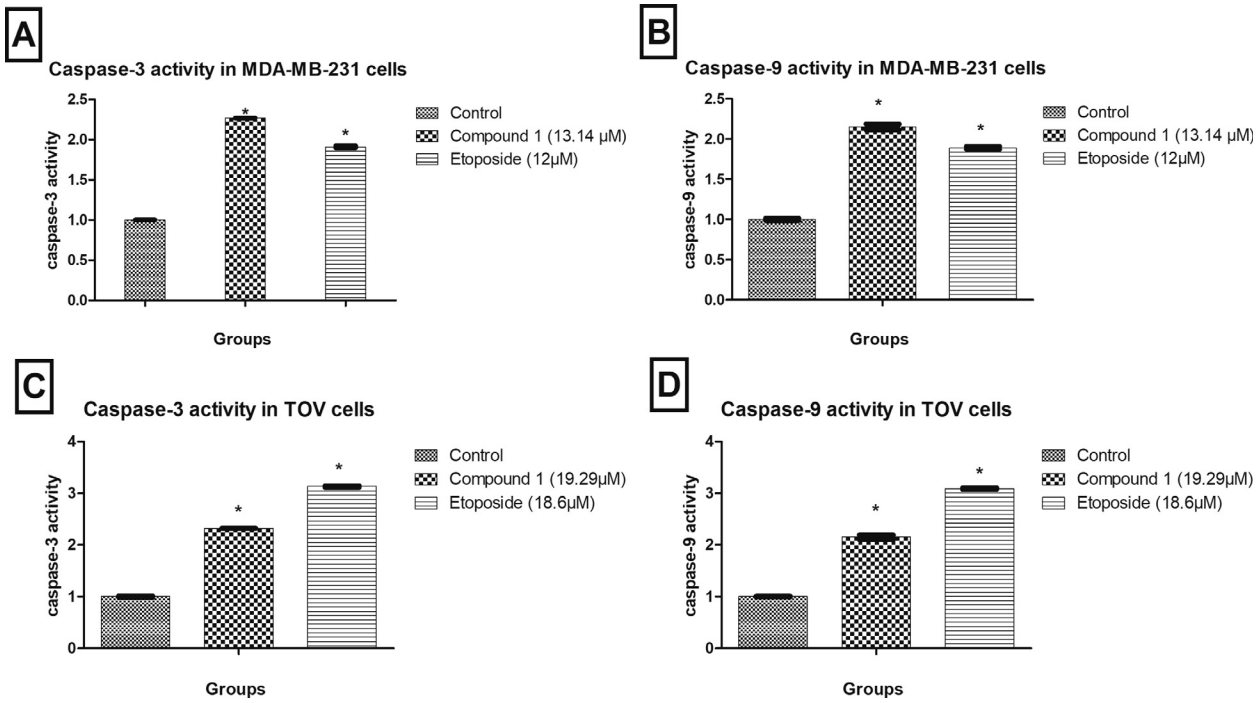

Fig. 2. . Relative quantification of caspase-3 and caspase 9 by colorimetric assay in MDA-MB-231 and TOV-21G cell lines. A) Caspase-3 activity in MDA-MB-231 cell. B) Caspase-9 activity in MDA-MB-231 cell. C) Caspase-9 activity in TOV-21G cell lines. D) Caspase-9 activity in TOV-21G cell lines. Data are presented as the mean ± SEM of three independent experiments. Data were statistically evaluated by one-way ANOVA followed by Newman-Keuls post hoc test, with $P < 0.05$ considered statistically significant. * Significantly different from without-treatment, $P < 0.05$.

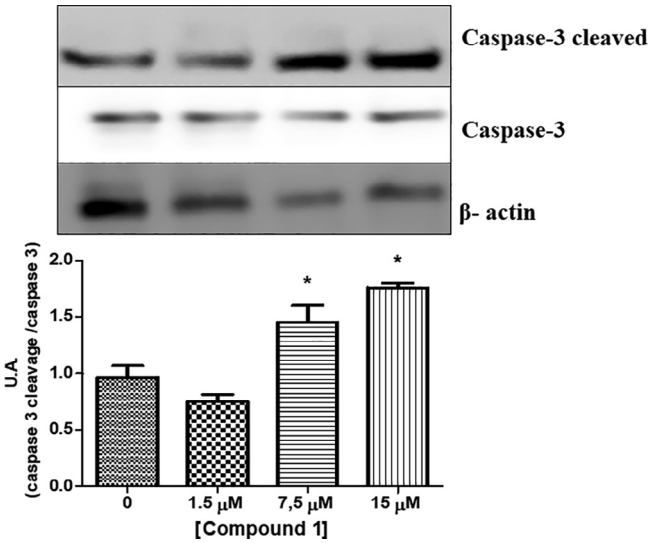

Fig. 3. . Expression of caspase-3 cleaved in MBA-MB-231. Data are presented as the mean ± SEM of three independent experiments. Data were statistically evaluated by one-way ANOVA followed by Newman-Keuls post hoc test, with $P < 0.05$ considered statistically significant. * Significantly different from without-treatment, $P < 0.05$.

compounds exhibit selective cytocidal effects against intractable cancer cells, including in glioblastoma, but minimal effects on normal peripheral blood mononuclear cells (PBMCs) prepared from healthy volunteers [42].

Our study showed that the compounds **1** was able to decreased the Na,K-ATPase activity both in purified enzyme (pig kidney membrane) and cell membrane of MDA-MB-231 cells (Fig. 4A and B). We demonstrated that those compound can modulate the Na,K-ATPase activity directly and their expression level in the cell (Figs. 4C and 5A). It also led to the activation of Caspase-3 and −9 (Fig. 2) as also increased of cleavage of Caspase-3 (Fig. 3) leading to cell death.

Studies report that the bufadienolides are inhibitors of the Na,K-ATPase activity [13,43,44]. The Na,K-ATPase inhibited makes it happen a decrease on electrochemical gradient that modulate NCX activity, leading reversal of its operation (promoting the influx Ca^{2+} and the efflux of Na^{+}). The Ca^{2+} excess is accumulated in endoplasmic reticulum through the SERCA activity [45,46]. We observed that the compound **1** was able to change PMCA and SERCA activities (Fig. 4D, E and 4F), suggesting that this compound also can modulate Ca^{2+} homeostasis.

Regarding PMCA, it is important to highlight the effect of the compound 1 on its activity. The compound 1 was able to inhibit the PMCA in the ghost membranes and decrease the expression levels after 48 h treatment of MDA-MB-231 cells. However, we did not find a direct effect of the compound 1 on the PMCA activity when MDA-MB-231 cell membranes were treated. This data could result because of differences of PMCA isoforms expressed in our system. PMCA4 is the major pump isoform expressed in erythrocytes, while MDA-MB-231 cells express PMCA1 and PMCA4 [47,48]. The PMCA activity assay was unable to determine the specific effect of each isoform of MDA-MB-231 cells, but through the western blotting and ghost experiments, we observed that the compound 1 had an effect on PMCA4.

Our data suggests an interesting effect of compound 1 in that this compound is not entirely selective in reducing Na, K-ATPase activity alone. However, further studies should be performed to confirm this hypothesis.

Compounds that can able to inhibit the Na,K-ATPase activity has potential anticancer effects, since there are studies suggesting that in cancer cells both activity and expression of the Na,K-ATPase were increased [7,49,50]. Among these compounds we can highlight cardiotonics steroids and/or their derivatives; they have already presented several anticancer effects [18,51–54].

The modulation of the Na,K-ATPase activity can activate signaling pathways, such as MAPK, ROS, phospholipase C, Src, and others

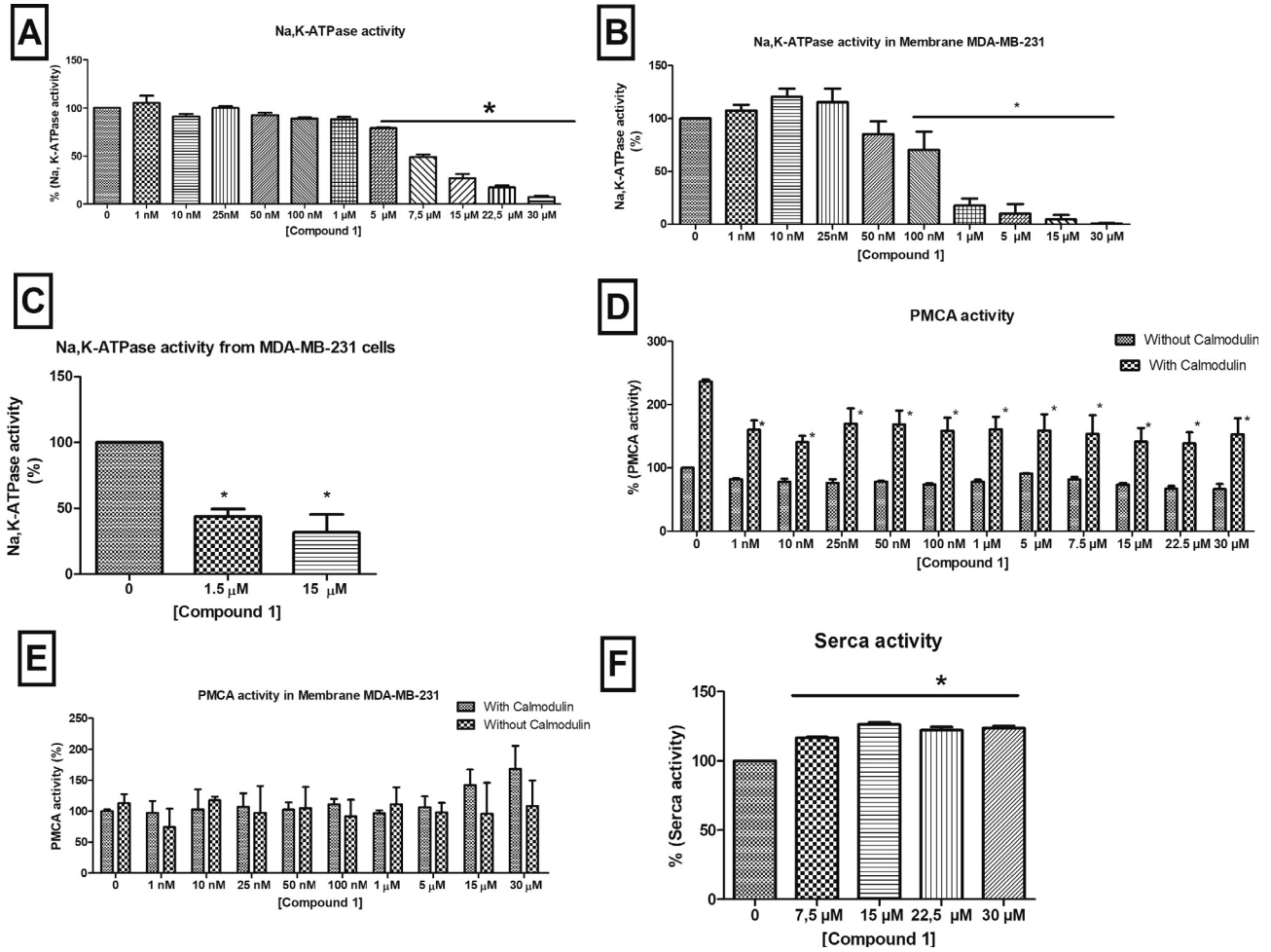

Fig. 4. . Effects of compound 1 on the activity of membrane enzymes. (A) Purified Na,K-ATPase activity from pig kidney preparation (20 min incubation). (B) The Na,K-ATPase activity in fraction of membrane from MDA-MB-231 cell control without-treatment (20 min incubation). (C) The Na,K-ATPase activity in MDA-MB-231 cells with 1.5 and 15 µM concentrations after 48 h treatment. (D) PMCA activity from GHOST preparation (20 min incubation). (E) The PMCA activity of membrane preparation from MDA-MB-231 cell control without-treatment (20 min incubation). (F) SERCA activity from rat striated muscle (20 min incubation). Data are presented as the mean ± SEM of three independent experiments. Data were statistically evaluated by one-way ANOVA followed by Newman-Keuls post hoc test, with $P < 0.05$ considered statistically significant. * Significantly different from without-treatment, $P < 0.05$.

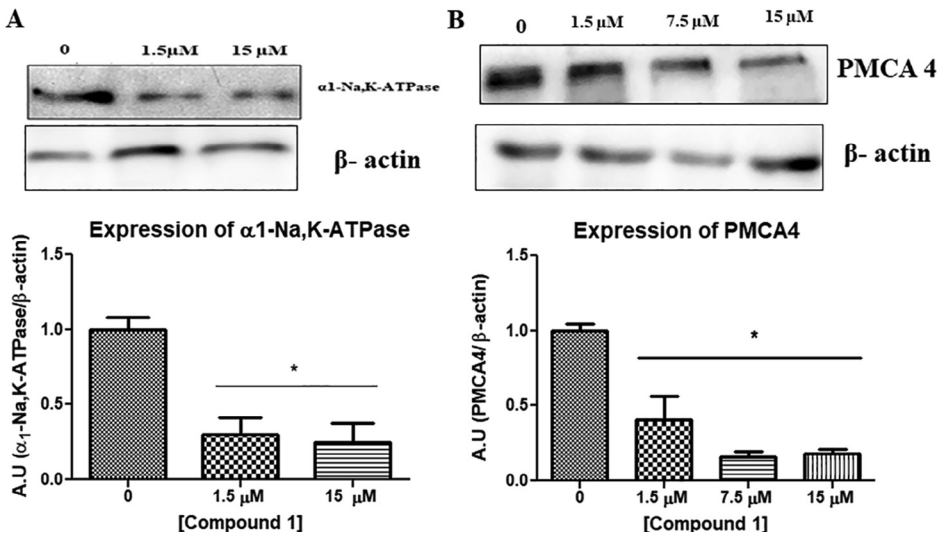

Fig. 5. Effects of compound 1 on expression from Na,K-ATPase and PMCA4 in MDA-MB-231 cells. (A) Expression of the α1-Na,K-ATPase in treated MDA-MB-231 cells in 1.5 and 15 µM concentrations for 48 h. (B) Expression of the PMCA4 in treated MDA-MB-231 cells. Data are presented as the mean ± SEM of three independent experiments. Data were statistically evaluated by one-way ANOVA followed by Newman-Keuls post hoc test, with $P < 0.05$ considered statistically significant. * Significantly different without-treatment, $P < 0.05$.

[55–57]. Studies about the cardiotonics steroids in tumors cells *in vitro* show effects such as influence cell proliferation, differentiation, and eventually cell death through the Na,K-ATPase [58,59].

Studies demonstrate that the use of cardiotonic steroids can induce apoptosis in human leukemia, HCC and prostate cancer mediated by inhibition of the Na,K-ATPase [60–62]. In relation to bufadienolides, they have anticancer activity mediated by the induction of apoptosis, antiproliferative behavior, cell cycle arrest, and autophagy in several tumours indicating its potential for use in anticancer therapy with effects similar to those of cardenolides [63–65].

It is important to note that the Compound **1** shows a change in the C3 scaffold region of bufadienolides which can be subsequently used for structural changes in that region to increase the selectivity to tumor cells using Na,K-ATPase and as a target [66], and thus, in a chemical biology point of view, could be used as synthesis start point for new drugs development.

5. Conclusion

We isolated four compounds from the venom of R. marina and found that compound 1 had the largest effect on inhibiting the viability of transformed human cell lines. Compound 1 inhibited Na,K-ATPase as well as PMCA activity and additionally led to elevated Caspase-3 and −9 activities, which caused the cells to enter into apoptosis. These data demonstrate that compound 1 may be an interesting molecule for anticancer properties. Future studies are needed to confirm the lack of compound 1 cytotoxicity in normal human cells. Based on the results of our study, the compound may affect the stability and/or expression of Na, K-ATPase, and future studies will address this issue concerning the mechanism of action of compound 1.

Acknowledgments

This work was funded by FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) APQ-00290-16 and APQ-01598-15, FAPEMAT (Fundação de Amparo à Pesquisa do Estado de Mato Grosso) 042/2016, INCT-BioNat (Intitutos Nacionais de Ciência e Tecnologia em Biodiversidade e Produtos Naturais), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) Financial Code 01 and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) 401914/2016-0, 305173/2018-9 and 314710/2018-3.

Conflict of interests

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2019.108490.

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