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Inhibitory efficacy of bufadienolides on Na^+,K^+-pump activity versus cell proliferation

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ABSTRACT

Bufadienolides are cytotoxic drugs that may form the basis for anticancer agents. Due to structural and functional similarity to cardiotonic glycosides, application is restricted. We, therefore, investigated correlation of their putative anticancer effects with inhibition of Na^+,K^+pumps. The natural bufalin and three derivatives were tested. The anticancer effects of the drugs were checked by observing their inhibitory effects on proliferation of rat liver cancer cells using MTT assay. Inhibition of Na^+,K^+-pump was determined by measuring pump-mediated current of rat α1/β1 and α2/β1 Na^+,K^+pumps expressed in *Xenopus* oocytes.

All tested bufadienolides inhibited cell proliferation and Na^+,K^+pump activity. An activity coefficient $A = 100 x IC_{50}^{Na,K\ pump}/IC_{50}^{proliferation}$ was used to describe drug effectivity as anticancer drug. Natural bufalin exhibited lowest effectivity on cell proliferation, and also the *A* value for rat α1 isoform was the lowest (0.08), the α2 isoform was much less sensitive ($A = 1.00$). The highest *A* values were obtained for the BF238 derivative with $A = 0.88$ and 2.64 for the α1 and α2 isoforms, respectively. Therefore, we suggest that search for bufalin derivatives with high anticancer effect and low affinity for both Na^+,K^+pump isoforms may be a promising strategy for development of anticancer drugs.

1. Introduction

Bufadienolides are natural steroids originally extracted from Chan Su, a traditional Chinese remedy found in skin and parotid venom glands of toads (*Bufo*) as well as in plants [1]. The drugs have been recognized as effective antitumor agents [1–4]. Because of some structural similarity to digitalis glycosides, they also act as potent cardiotonic steroids [5,6]. The application of bufadienolides in cancer treatment is, therefore, restricted by their side effects on the Na^+,K^+pump (also known as Na^+,K^+-ATPase or Na^+ pump), the most essential membrane protein of nearly all animal cells.

Bufalin is the major active component of the *Bufo* venum and exhibits cytotoxic effects against a large variety of tumor cells (see e.g. [7,8]) and has also been introduced in clinical cancer therapy [9,10]. The main problem for its clinical application is the high cardiac toxicity of this drug [5,11]. To minimize such side effects, bufalin-loaded carriers have been developed for tissue-specific targeting [12,13]. Another strategy might be to develop derivatives of the natural bufalin with relatively weaker inhibition on the Na^+,K^+pump and stronger inhibition on cell proliferation.

In our experiments we used bufalin and several representative bufalin derivatives to answer the question whether the cytotoxic effects are correlated with the effects on Na^+,K^+pumps, or whether there exists a drug with high anticancer but comparably low cardiotonic activity, which may then form the basis for the development of new anticancer drugs.

We investigated the effects of the naturally derived bufalin. In addition, we screened three synthesized bufalin derivatives with respect to their inhibitory effect on proliferation of rat cancer cells and inhibition of rat Na^+,K^+pumps:

Bufalin-3-yl [3-(1h-imidazol-1-yl)propyl]carbamate(BF238),

Abbreviations: BAP, Bufalin-3-MeON-arabinopyranoside; BF238, Bufalin-3-Yl [3-(1h-imidazol-1-Yl)propyl]carbamate; BF601, Bufalin-3-Yl [3-(methylamino)propyl] carbamate; MTT, 3,[4,5-dimethylthiazol-2-Yl-] diphenyltetrazolium bromide; ORi, Oocyte Ringer's (solution); Rα1/β1, rat Na^+,K^+pump formed by A1 and B1 subunits; Rα2/β1, rat Na^+,K^+pump formed by A2 and B1 subunits; TEA-Cl, Tetraethylammonium chloride

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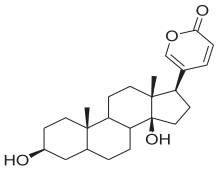
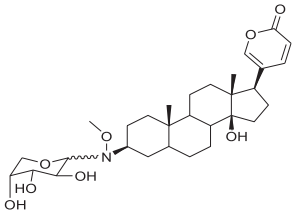
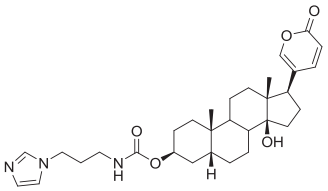
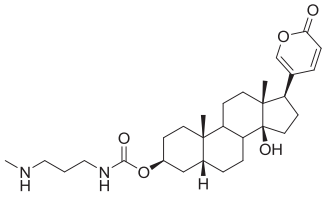
Table 1
Effect of bufadienolides on rat RH-35 and human HeLa cells described by IC_{50} values for 50% inhibition of cell proliferation. Data represent averages of 3 independent experiments ± SEM.

Bufadienolide	Structure	Cell proliferation	
		RH-35 IC_{50} (μM)	HeLa IC_{50} (μM)
Bufalin		257.0 ± 17.5	23.3 ± 2.4 (10^{-3})
***Bufalin-3-MeON-arabinopyranoside* (BAP)**		67.2 ± 10.4	26.8 ± 2.3 (10^{-3})
***Bufalin-3-yl [3-(1h-imidazol-1-yl)propyl]carbamate* (BF238)**		37.4 ± 3.1	47.3 ± 2.4 (10^{-3})
***Bufalin-3-yl [3-(methylamino) propyl]carbamate* (BF601)**		30.8 ± 5.0	115.0 ± 4.4 (10^{-3})

Bufalin-3-MeON-arabinopyranoside (BAP), and
Bufalin-3-yl [3-(methylamino)propyl]carbamate(BF601).

For comparison, the structures of the bufadienolides we used in our investigation are shown in Table 1.

The catalytically functional subunit of Na^+,K^+pump is the α subunit, containing the binding sites for ATP, the transported cations and also cardiac steroids. The glycosylated auxiliary β subunit is needed for proper folding and insertion of the pump molecule into the cell membrane [14–19]. In rat cardiac muscle the α1 and α2 subunits are the dominating isoforms, the α3 subunit is only detected at the neonatal stage [20,21]. Pumps with α1 subunit are found ubiquitous and are considered as dominating Na^+,K^+pumps with housekeeping function. Inhibition of Na^+,K^+pump, particularly the α2/β1 pump, by cardiac steroids leads to prolongation of the cardiac action potential and an increase in intracellular Na^+, which in turn decreases the driving force for the Na^+,Ca^{2+}exchanger and results in elevation of intracellular Ca^{2+}. As a consequence, contractility of the working cardiac myocyte is strengthened [22–28]. To investigate to which extend the bufadienolides may exhibit cardiotonic effects, we compared the effects of the drugs on the activity of rat α1/β1 (Rα1/β1) and α2/β1 (Rα2/β1) Na^+,K^+pumps expressed in the model system *Xenopus* oocyte. Since the oocytes express endogenous Na^+ pumps, these pumps also had to be characterized with respect to their sensitivity to the drugs, and the respective signals had to be subtracted from the total pump-current signal. Pump activities were monitored by measuring the electrogenic current mediated by the pumps under voltage clamp [29–31].

The aim of our study was to find out whether cytotoxicity can be correlated to pump inhibition, or whether there exists a bufalin derivative with high anticancer effect, but relatively low effect on activity of pumps.

2. Materials and methods

2.1. Cell culture

The RH-35 rat liver cancer cell line was obtained from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, P.R. China). Cells were cultured in DMEM (Life Technologies, Gaithersburg, MD, USA) with 1.5 g/L sodium bicarbonate plus 10% fetal bovine serum. Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Karlsruhe, Germany).

2.2. MTT assay for inhibitory effects on cell proliferation

The inhibitory effects of bufalin and its derivatives on cancer cell proliferation was determined by MTT assay, a tetrazolium-based colorimetric assay, as reported before [32]. Briefly, cells were plated in 96-well flat-bottomed plates at density of 1×10^3 cells/well in the complete medium and incubated overnight. Then, media were changed to fresh media containing the various amounts of the respective drug for 72 h. At the end of the incubation period, 20 μL of the dye 3, [4,5-dimethylthiazol-2-yl-] diphenyltetrazolium bromide (MTT, 5 mg/mL), was added to each well, and the plates were incubated for 3 h at 37 °C. Thereafter, 100 μL of lysis buffer (20% sodium dodecyl sulfate [SDS] in 50% N, N-dimethylformamide, containing 0.5% [v:v] 80% acetic acid and

0.4% [v:v] 1N HCl) was added to each well and incubated overnight (16 h). Cell viability was evaluated by measuring the mitochondrial-dependent conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic-active cells. The optical density (proportional to the number of live cells) was assessed with a Microplate Reader Bio-Rad 550 (Bio-Rad Laboratories, Hercules, CA, USA) at 570 nm. Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis. IC_{50} values (half-maximum inhibitory concentration) were calculated by the Logit method [32].

2.3. Oocytes

Females of the clawed toad *Xenopus laevis* (purchased from Maosheng Bio-Technology Com., Shanghai, China) were anaesthetized in a bath medium containing 1 g/L tricaine (Sandoz, Basel, Switzerland) and kept on ice. Parts of ovary were removed and treated with 0.5 or 0.25 mg/mL collagenase (Sigma) for 2–4 h, or overnight, respectively. Full-grown prophase-arrested oocytes were selected for experiment. For expression of rat pumps, cRNA encoding rat α1 or α2 isoform of the Na^+,K^+pump together with rat β1 subunit were injected into oocytes. To minimize complexation of endogenous *Xenopus* α subunit with rat ß1 subunit (see [33] cRNA of rat α and ß1 subunits was injected at a weight ratio of 2/1(20 ng in total). Uninjected cells served as controls. The oocytes were incubated for expression for 2 days at 20 ± 1 °C in oocyte Ringer's solution (ORi, see 2.7 solutions) with added 70 mg/L gentamycin. Experiments were performed at room temperature (24–26 °C).

2.4. cRNA preparation

Constructs encoding full-length rat α1 (P06685) and α2 (P06686), as well as ß1 (P07340) subunits of the Na^+,K^+pump were kindly provided by Dr. G. Schmalzing and propagated in JM109 bacteria. Capped cRNAs were synthesized in vitro using the mMESSAGE mMACHINE SP6 kit (Ambion, USA). A Nanophotometer P-Class (Implen, Germany) was used to check the quality and quantity of cRNA.

2.5. Voltage-clamp experiments

Electrophysiological experiments were performed by conventional two-electrode voltage clamp using Turbo TEC 03X with CellWorks software (NPI electronic, Tamm, Germany). For detecting Na^+,K^+pump-mediated currents, oocytes were preloaded with Na^+ [31] by incubation in Na^+-loading solution for 30 min (see solutions); before experiment the cells were kept at least for 20 min in post-loading solution (see solutions). Steady-state currents were recorded from the Na^+-loaded oocytes at the end of 200-ms voltage pulses from −150 up to +30 mV in 10-mV increments that were applied from a holding potential of −60 mV. The difference between the steady-state currents in the presence of extracellular $K^+$and in its absence was used as a measure of the pump-mediated currents [31]. The external test solutions were similar to ORi solution (see 2.7 Solutions) but contained $BaCl_2$ and tetraethylammonium chloride (TEA-Cl) to reduce other K^+-sensitive currents. A remaining contribution of non-pump-mediated current (about 10%) was subtracted for final data analysis.

2.6. Bufadienolides

Bufalin was isolated and purified from Chan Su as reported before [34]. Derivatives of bufalin such as BAP, BF238 and BF601 were obtained by structure modification of bufalin. The structures of bufalin and its derivatives are shown in Table 1.

Stock solutions of 1 or 100 mM were prepared in DMSO and diluted to final concentration in the test solution. DMSO concentrations in the test solution were always below 0.1% which was without effect on the pump-mediated currents.

2.7. Solutions

The standard ORi (Oocyte Ringer's) solution was composed of (in mM): 90 NaCl, 2 KCl, 2 $CaCl_2$ and 5 MOPS (adjusted to pH 7.4 with Tris). Test solutions contained (in mM) 0 or 2 KCl, and 90 NaCl, 5 $BaCl_2$, 20 TEA-Cl and 5 MOPS (adjusted to pH 7.4 with Tris). Na^+-loading solution was composed of (in mM) 110 NaCl, 2.5 Na^+-citrate and 5 MOPS; and post-loading solution contained (in mM) 100 NaCl, 1 $CaCl_2$, 5 MOPS, 5 $BaCl_2$ and 20 TEA-Cl (both solutions adjusted to pH 7.6 with Tris).

2.8. Data analysis

All measured data are represented as averages ± SEM (standard error of the mean) of N experiments. To estimate dependency of cell viability and Na^+,K^+-pump activity on drug concentration Hill equation was fitted to the normalized data using Origin software:

$$Y = \frac{IC_{50}^{n}}{IC_{50}{}^{n} + [drug]^{n}} \quad (1)$$

with drug concentration [drug], IC_{50} the drug concentration for 50% inhibition, and Hill coefficient *n*.

3. Results

3.1. Inhibitory effect on cancer cell proliferation

To determine inhibitory effects of the bufadienolides on cancer cell proliferation, a colorimetric tetrazolium (MTT) assay was applied to the rat liver cancer cell line RH-35. Fig. 1 shows the dependency of inhibition of cell proliferation on concentration of bufalin and the bufalin derivative BF238. The IC_{50} value for bufalin was with 257.0 μM nearly one order of magnitude higher than the value for BF238 with 37.4 μM.

IC_{50} values were also estimated for BF601, with about 30.8 μM

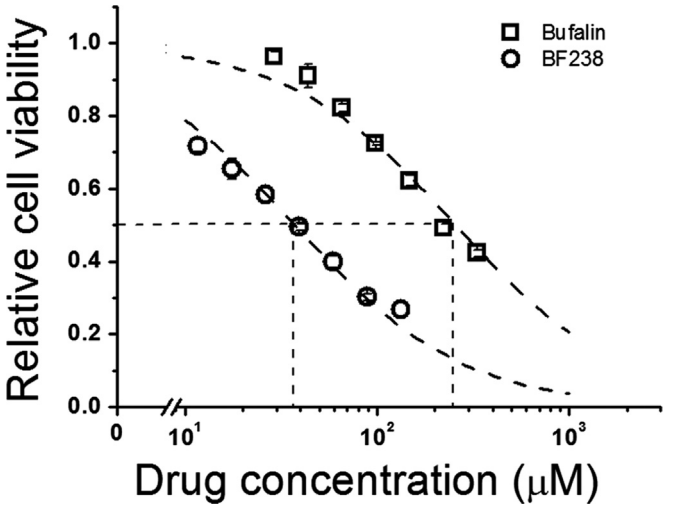


Fig. 1. Dependence of viability of RH-35 cells on concentration of bufalin (open squares) and BF238 (open circles). Data represent averages ± SEM (N=3). Dashed lines are approximations of euq. 1 with n=1, Y_0=1, yielding IC_{50} values of 257.0 and 37.4 μM for bufalin and BF238, respectively.

similar to the value for BF238, and for BAP with about 67.2 μM. The results are summarized in Table 1, listed from top to bottom from the lowest to highest inhibitory effect on cell proliferation.

Table 1 also lists corresponding IC_{50} value for inhibition of cell proliferation of the human HeLa cancer cell line. HeLa cells are by about 3 orders of magnitude more sensitive than the rat RH-35 cells.

3.2. Expression of Rα1/β1 and Rα2/β1 in Xenopus oocytes

To extract pump-mediated current from total membrane current, we blocked K^+-selective channels, so the current, which can be activated by extracellular K^+ under these conditions, can be considered to represent predominantly pump-mediated current [31]. The voltage dependencies of K^+-sensitive current in oocytes injected with the respective cRNA are shown together with those of uninjected cells in Supplemental Fig. 1. The increase in pump current reflects the additional activity of the exogenous rat pumps. Exogenous pump current is about 3–4 times larger than the endogenous pump current.

3.3. Sensitivity of endogenous pump to bufadienolides

Though our focus was on the effects of bufadienolides on the rat Rα1/β1 and Rα2/β1 pumps, we first investigated the effects on the endogenous pumps of *Xenopus* oocytes, which was necessary for calculating the current generated by the expressed rat pumps.

Endogenous pump-mediated current was determined as K^+-sensitive current in uninjected control oocytes. As for inhibition of cancer cell proliferation, we analyzed in detail the effects of bufalin and BF238. Fig. 2A shows an example of averaged current-voltage dependencies from oocytes in the absence and presence of 0.05 μM bufalin, which gave nearly 50% inhibition of the pump current by this natural product of the *Bufo* venom over the entire voltage range. A more detailed analysis revealed only slight voltage dependency. At the same concentration of 0.05 μM, BF238 exhibited slightly more inhibition (Fig. 2B). The dependency of pump inhibition on bufalin and BF238 concentration is illustrated for the pump current at −60 mV in Fig. 2C. The IC_{50} values for 50% inhibition could be estimated to about 0.06 μM and 0.02 μM, respectively, by fitting Eq. (1) to the data. Because the other drugs also exhibited no significant voltage dependency of current inhibition, we screened the concentration dependence of the two other drugs, BAP and BF601, also at −60 mV. The inhibition could be described by IC_{50} values of about 0.05 and 0.10 μM for BAP and BF601, respectively (Table 2). The BF238 derivative seems to be slightly more effective in inhibiting the endogenous Na^+,K^+ pump than the natural bufalin as well as the two other derivatives (see Table 2).

Table 2
IC_{50} values for inhibition of Na^+,K^+ pump current at −60 mV. The values are given ± errors of the fitted IC_{50} values to the respective set of data presenting averages of 5–6 oocytes were obtained by least-squares fits of Eq. (1) to the data shown in Figs. 2, 3, and 4, respectively.

Bufadienolide	Endogenous pump IC_{50} (μM)	Rα1/β1 pump IC_{50} (μM)	Rα2/β1 pump IC_{50} (μM)
Bufalin	0.063 ± 0.012	0.215 ± 0.069	2.558 ± 1.154
BAP	0.047 ± 0.007	0.063 ± 0.015	0.110 ± 0.015
BF238	0.021 ± 0.005	0.329 ± 0.083	0.987 ± 0.322
BF601	0.101 ± 0.026	0.107 ± 0.027	0.283 ± 0.045

To evaluate the effect of bufadienolides on the rat pumps, the contribution of endogenous pumps to total pump-mediated current was always subtracted using the above data for the results illustrated below.

3.4. Sensitivity of Rα1/β1 pump to bufadienolides

Rα1/β1 is known to be quite insensitive to cardiac glycosides [35]. Our data also show that sensitivity of the Rα1/β1 pump to bufadienolides is indeed significantly lower than that of the endogenous *Xenopus* pump isoform. At 0.05 μM bufalin the endogenous pump was inhibited by 50%, but Rα1/β1 activity was less affected at this concentration (compare Figs. 2A and 3A). The BF238 even hardly affected Rα1/β1 pump current at 0.05 μM, and only 0.50 μM exhibited slightly more than 50% inhibition (see Fig.3B). For analyzing IC_{50} values we subtracted the contribution of endogenous current component from total pump-mediated current before calculating the efficiency of inhibition by bufadienolides for the expressed Rα1/β1 pump. Fig. 3C shows the dependency of the Rα1/β1 pump current at −60 mV on bufalin concentration, which was with an IC_{50} value of 0.215 μM by a factor of 3 less effective than for the endogenous *Xenopus* pump; BF238 was by more than an order of magnitude less effective with an IC_{50} value of 0.329 μM (Fig. 3B, compare Table 2). The other two screened drugs, BF601 (IC_{50}=0.107 μM) and BAP (IC_{50}=0.063 μM) were also less effective in inhibiting the Rα1/β1 pump than the endogenous pump, but more effective than the natural bufalin and BF238 (see Table 2).

3.5. Sensitivity of Rα2/β1 pump to bufadienolides

Also for the Rα2/β1 pump concentrations of bufalin and BF238 in the 0.05 μM range were not sufficient to produce clear inhibition, and 0.50 μM needed to be applied (see Fig. 4A and B). For

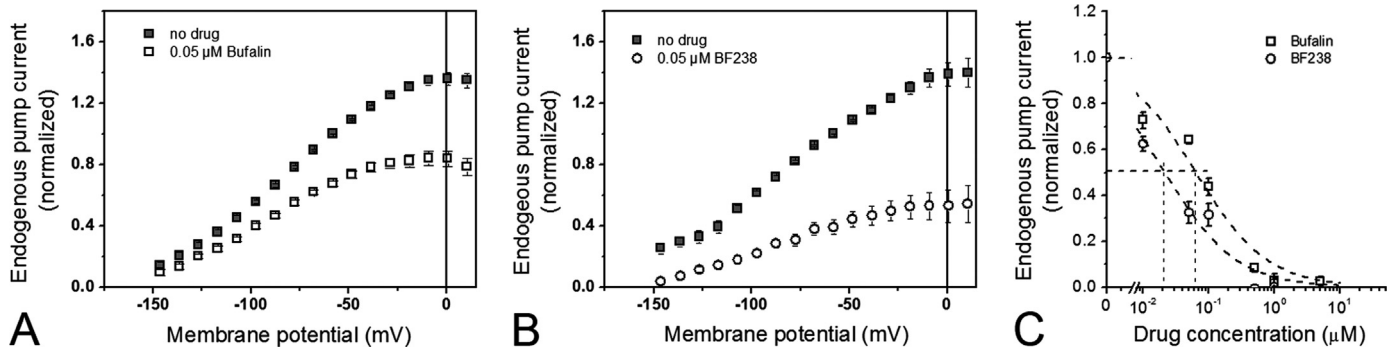


Fig. 2. Dependence of endogenous Na^+,K^+ pump current on concentration of bufadienolides. (A, B) Current-voltage dependencies in the absence (filled squares) and during application of 0.05 μM bufalin (open squares) and 0.05 μM BF238 (open circles), respectively. Data represent averages of N=5 oocytes ± SEM. (C) Concentration-dependent inhibition of endogenous Na^+,K^+ pump current by bufalin (open squares) and BF238 (open circles). Data represent averages of 5–10 measurements ± SEM. Dashed lines are fits of euq. 1 with n=0.81, yielding IC_{50} values of 0.063 and 0.021 μM, respectively (compare dashed lines).

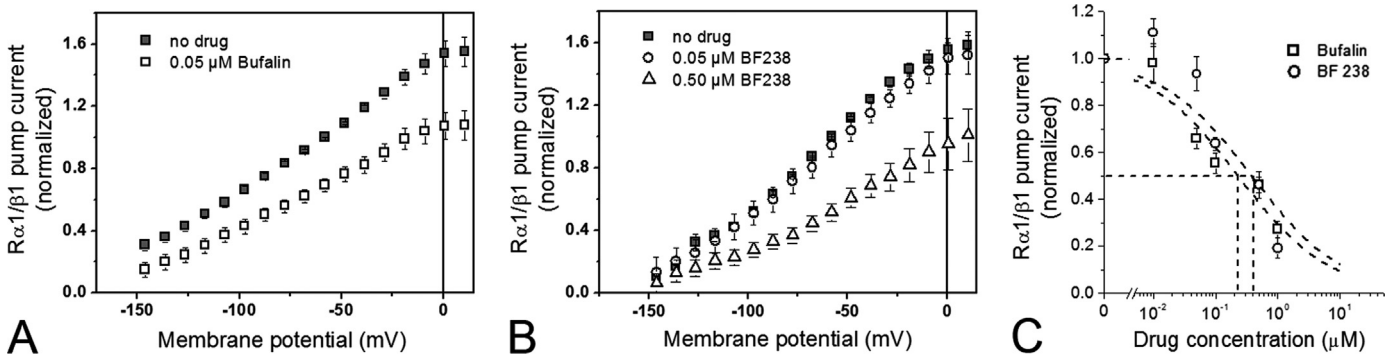


Fig. 3. Dependencies of Na^+,K^+pump current in oocytes with additionally expressed rat Rα1/ß1 pumps. (A, B) Current-voltage dependencies in the absence (filled squares) and during application of 0.05 μM bufalin (open squares) and 0.05 μM BF238 (open circles), respectively. Data represent averages of N=6 oocytes ± SEM. (C) Concentration-dependent inhibition of endogenous Na^+,K^+pump current by bufalin (open squares) and BF238 (open circles). Data represent averages of 5–10 measurements ± SEM. Solid lines are fits of euq. 1 with n=0.8, yielding IC_{50} values of 0.215 and 0.329 μM, respectively (compare dashed lines).

estimation of the IC_{50} values we again subtracted the contribution of the endogenous Na^+,K^+pump from total K^+-sensitive current as described above. The data are shown in Fig. 4C for the current at −60 mV. The calculated IC_{50} values are listed in Table 2 again together with the values for the two other screened drugs BAP and BF601. Except for BAP, all tested drugs were significantly less effective in inhibition of the Rα2/β1 pump than the Rα1/β1 pump. The IC_{50} value of bufalin for Rα2/β1 of more than 2 μM was by an order of magnitude higher than the value for Rα1/ß1. The estimated IC_{50} value with 0.11 μM for BAP was slight higher than the value for Rα1/ß1 (Table 2).

Table 3
Efficacy of bufadienolides as anticancer drugs expressed by the efficiency coefficient A determined by Eq. (2) for RH-35 cells and the respective rat Na^+,K^+pump from the data given in Tables 1 and 2.

Bufadienolide	Rα1/β1 pump	Rα2/β1 pump
Bufalin	0.08	1.00
BAP	0.10	0.16
BF238	0.88	2.64
BF601	0.35	0.92

4. Discussion

Cardiotonic steroids, including bufalin, are used for many years in treatment of congestive heart failure by strengthening myocardial contractility [7,36]. These drugs have also been suggested as candidate for the development of anticancer drugs (see e.g. [37,38]). The aim of our work was to investigate whether anticancer effects can be correlated to Na^+,K^+pump inhibition, or whether a bufadienolide can be developed that is highly effective as anticancer agent, but is less potent as inhibitor of Na^+,K^+pump.

To express such effectivity, we determined efficiency coefficients *A* as the ratio of the IC_{50} values:

$$A = 100 * \frac{IC_{50}^{Na,Kpump}}{IC_{50}^{proliferation}} \quad (2)$$

The *A* values calculated on the basis of the data shown in Tables 1 and 2 are presented in Table 3. Interestingly, the Rα2/β1 pump was less sensitive to each of the tested drugs than the Rα1/β1 pump (Table 2). This is in contrast to the effect of the cardiotonic steroid ouabain, which is known to be highly insensitive to inhibit Rα1/β1 pump. Na^+,K^+pumps with the α1 subunit are found in nearly all animal cells, and seem to serve as the major housekeeping pump while pumps with α2 subunit exhibit tissue-specific distribution and are found predominately in muscle and nerve cells [26].

The pump with α1 subunit has been demonstrated to be involved in various signaling pathways [39] and is highly expressed in tumor tissue [40]. There are two pools of Na^+,K^+-ATPase within the plasma membrane: the classical pool of the enzyme acts as energy transducing Na^+,K^+pump; the other pool is restricted to the caveolae, forming the "Na^+/K^+-ATPase signalosomes" as signal-transducing enzyme [41]. Generally, the anti-cancer effects of cardiac steroids were thought to be mainly based on binding to the Na^+/K^+-ATPase signalosome [42]. The much lower IC_{50} values of inhibition of Na^+, K^+pump current may be attributed to the fact that inhibition of Na^+ pump current by bufadienolides is not directly associated with the binding to Na^+,K^+-ATPase

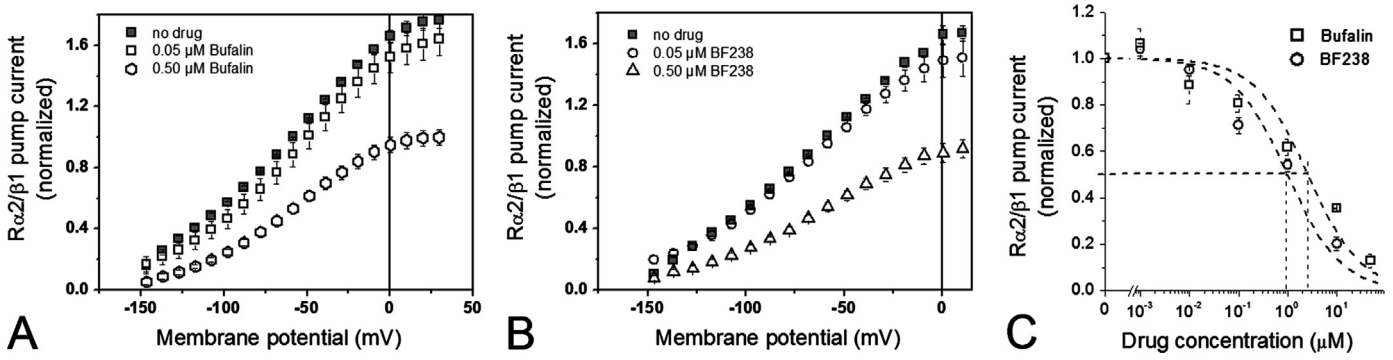


Fig. 4. Dependencies of Na^+,K^+pump current in oocytes with additionally expressed rat Rα2/ß1 pumps. (A, B) Current-voltage dependencies in the absence (filled squares) and during application of 0.05 and 0.5 μM bufalin (open squares and circles) and 0.05 and 0.5 μM BF 238 (open circles and rhombs). Data represent averages of N=6 oocytes ± SEM. (C) Concentration-dependent inhibition of endogenous Na^+,K^+pump current by bufalin (open squares) and BF238 (open circles). Data represent averages of 5–10 measurements ± SEM. Solid lines are fits of euq. 1 with n=0.8, yielding IC_{50} values of 2.558 and 0.987 μM, respectively (compare dashed lines).

signalosomes, which is considered to form the basis for inhibition of cell proliferation. Experiments on genetically modified mice demonstrated that, instead of mediating inotropic effects (as expected for the α2 isoform of the pump), the α1 isoform may play a different role and mediate toxic effects [22]. In addition, elevated α1 expression has been reported in some malignant cells [43,44]. Such features may lead to higher sensitivity of cancerous cells to bufadienolides than that of normal cells; therefore, the strategy of screening bufadienolides as candidate for anti-tumor drugs is feasible. The α2/β1 pump is well known to govern the length of the cardiac action potential and controls Ca^{2+}influx through the Na^+,Ca^{2+}exchanger via intracellular Na^+ [25,27,28,45]. Therefore, the low sensitivity of α2/β1 pump against the bufadienolides makes them less harmful as cardiotonic drugs, but good candidates for developing anticancer drugs.

Among the tested drugs, the one with the lowest efficacy in inhibiting cell proliferation was the natural bufalin with an IC_{50} value of 257.0 μM (Table 1). Though the rat pumps were also quite insensitive to bufalin compared to the other drugs (see Table 2), the efficiency coefficients *A* for bufalin was with 0.08 the lowest for Rα1/β1, for Rα2/β1 the value turned out to be with 1.0 in an intermediate range making this natural bufadienolide suitable as a basis for developing anticancer drug. BAP exhibited the low efficiency for both isoforms, while BF601 was with A=0.35 and 0.92, for Rα1/β1 and Rα2/β1, respectively, quite effective (Table 3).

The inhibitory effect of BF238 on cancer cell proliferation, with an IC_{50} value of 37.4 μM is slightly less cytotoxic than BF601. However, both Rα1/β1 and Rα2/β1 are relatively insensitive to this drug, so the *A* value for BF238 turned out to be 0.88 for Rα1/β1 and 2.64 for Rα2/β1, which makes BF238 the best candidate for further research. Our results of MTT assay are not sufficient to prove the cytotoxicity effects and mechanism of action of the compounds. Unpublished data from our collaborators showed that the effects and mechanisms of cytotoxicity of bufadienolides might be similar. In cells treated with bufadienolides, apoptosis and change in signal pathway such as inhibition of PI3K/Akt could be observed (Feng et al. unpublished).

Our results indicate no direct correlation between inhibition of cell proliferation and pump inhibition. Therefore, an additional mechanism, like the interaction with the signalosome function, might favor the inhibitory effect of the BF238 on cancer cell proliferation. Interestingly, BF238 is characterized by an imidazole residue. A recent study [46] showed that imidazole can inhibit autophagy flux and induce apoptosis on HEC-1B, a human endometrial adenocarcinoma cell line, and provided the possible mechanisms of imidazole and its derivatives acting as antitumor drugs. Although we do not have data to prove it yet, the side chain imidazole group in BF238 may improve the inhibitory effect on cell proliferation.

Previous studies have shown that the Na pumps from different species vary in the sensitivity to cardiac glycosides [47,48], We also examined the inhibition of cell proliferation by the above bufadienolides on the human HeLa cell line, which seems to be much more sensitive to the bufadienolides than the rat cancer cells (preliminary data are added to Table 1). The difference in IC_{50} values of bufadienolides in inhibiting proliferation of RH-35 cells or HeLa cells may be related to different sensitivities of those two cell species [49]. The sensitivity of human HeLa cells to the bufadienolides with respect to cell proliferation was opposite to that of rat RH-35 cells though not as pronounced as in the rat cells. To judge on the applicability of bufadienolides as anticancer drugs in human, corresponding experiments on inhibition of human α1 andα2 pumps will be needed. Thus, high effectivity of BF238 on the rat system may not be readily transferred to human. However, our data indicate that proper modification of bufalin may increase anticancer effectivity of derivatives and, nevertheless, reduce their inhibition of Na^+, K^+pumps, which means having higher potential as anticancer agent with less side effects. Therefore, additional bufalin derivatives need to be screened in the future study, and the investigations need to be extended to human cancer cells and human Na^+,K^+pumps. We, in particular, suggest BF238 with an imidazole residue as a basis for further anticancer drug development.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.03.015.

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