Cell Proliferation

*Cell Prolif.*, 2015, **48**, 600–610 doi: 10.1111/cpr.12208

# Analysis of proliferation and apoptotic induction by 20 steroid glycosides in 143B osteosarcoma cells *in vitro*

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*Received 28 April 2015; revision accepted 4 July 2015*

## Abstract

*Objectives*: Osteosarcoma is the most common type of malignant bone tumour in children and adolescents; it has poor prognosis, is highly metastatic and is resistant to current therapeutic approaches. In this study, different herbal extracts used in phytotherapy have been screened after searching innovative natural anti-cancer components.

*Materials and methods*: Twenty steroid glycosides were examined for accordance to their potential of inhibiting cell proliferation and inducing apoptosis in the osteosarcoma cell line 143B. Cell proliferation was examined using a CASY counter. Effects of cardiac glycosides on induction of apoptosis were evaluated by Annexin V-APC and flow cytometry, caspase activity assay and measurement of mitochondrial membrane potential.

*Results*: The study revealed that various steroid glycosides suppress cell proliferation in a concentration-dependent manner. Further investigations indicated apoptotic induction by 17 of the 20 tested cardenolides and bufadienolides. Bufadienolide proscillaridin A, arenobufagin, and cardenolides evomonoside, convallatoxol and ouabain waged strongest apoptotic induction, associated with breakdown of mitochondrial membrane potential and activation of caspases -8 and -9. In contrast, the bufadienolide resibufogenin and cardenolide uzarin had no effect on proliferation inhibition, apoptotic induction or change in mitochondrial membrane potential.

*Conclusion*: These results indicate that bufadienolides proscillaridin A and arenobufagin and cardenolide evomonoside, or related natural compounds might be promising new starting points for development of novel anti-cancer agents for treatment of osteosarcoma.

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## Introduction

Osteosarcoma is the most common primary malignant bone tumour, mainly occurring in children and adolescents (1). The most challenging problem facing orthopaedic oncologists is to counter multidrug resistance (MDR) induced by classic chemotherapeutic agents such as methotrexate, adriamycin and cisplatin. In the order of 50% of osteosarcoma cases are resistant to chemotherapy or acquire resistance during treatment (2,3). Thus, there is great need for exploring mechanisms of chemotherapeutic resistance and developing new or additional treatment regimens for chemotherapy-resistant osteosarcoma. Novel strategies in cancer therapy based on steroid glycosides are discussed as possibilities for counteracting multidrug resistance by using cardiac glycosides.

Steroid glycosides have been used in the treatment of congestive heart failure, arrhythmia (4,5) and malignant diseases in the past (6,7). They are a category of compounds isolated from plants or animals (mainly *bufo*) from which their names have originated (8). Chemically, they can be categorized into two groups. On the one hand are the cardenolides, characterized by having an unsaturated butyrolactone ring of five members at C-17, plus C-23 steroids, and on the other hand the bufadienolides, which have a six-membered double unsaturated pyrone ring (9). Both groups are able to

bind to and inhibit the $Na^+/K^+$-ATPase pump, which results in activation of different signal cascades involved in the mechanism of proliferation, induction of apoptosis or gene expression (10–15). For example, for 19-hydroxy-2-oxovoruscharine anti-cancer activity in chemosensitive versus MDR, cancer and apoptosis-resistant cells, has been described (16–18).

However, over the few last decades, several studies have demonstrated anti-cancer properties of different steroid glycosides, for example digoxin and ouabain in human breast cancer cell lines (19,20). McConkey *et al.* demonstrated apoptotic induction by steroid glycosides such as oleandrin, ouabain and digoxin in human prostate cancer cell lines *in vitro* (14). Digoxin and digitoxin have already been reported in clinical studies to afford therapeutic effectiveness in breast cancer and prostate cancers and leukaemia (21–23). For ouabain and digoxin, apoptotic induction has been described in human acute T-cell lines (24,25). Lopez-Lazaro *et al.* reported that digitoxin inhibited cell proliferation in cancer cell lines at concentrations commonly found in the plasma of cardiac patients (26). Of the bufadienolides, bufalin and cinobufagin, an anti-proliferative effect and anti-cancer properties in prostate cancer and human hepatocellular carcinoma cells have already been published (27,28).

The aim of this study was to analyse growth inhibitory effect and induction of apoptosis in 20 structurally different steroid glycosides, in the 143B osteosarcoma cell line, hopefully to discover new agents to negotiate a way around multidrug resistance.

## Materials and methods

### *Steroid glycosides*

A library of cardioactive steroids was obtained from Prof. Brigitte Kopp (Department of Pharmacognosy, University of Vienna, Austria). Their purities were >95% determined by HPLC/UV analysis. The cardenolides desglucocheirotoxin, strophanolloside, lokundjoside, convallatoxol and the bufadienolide proscillaridin A as well as the bufadienolide aglycons arenobufagin, resibufogenin, bufarenogin, bovogenin A, gamabufogenin, bufotaline and the cardenolide evomonoside, were isolated from *Convallaria majalis* L. toad venoms and *Urginea* species. Their chemical structures were elucidated by NMR spectroscopy and mass spectrometry (MS) as described in more detail previously (29–35).

Cardenolides digitoxin, gitoxin, lanatosid C, digoxin, ouabain, k-strophanthin and helveticoside were purchased from Sigma-Aldrich (Munich, Germany) and uzarin was provided by ChromaDex (Irvine, CA, USA). Ouabain, k-strophanthin, uzarin and helveticoside were dissolved in phosphate-buffered saline (PBS, Gibco Life technologies, Darmstadt, Germany) to constitute 5 mM stock solutions, and stored at room temperature. Remaining substances were dissolved in DMSO from Sigma-Aldrich for stock solutions (5 mM). Bufarenogin, uzarin and resibufogenin stock solutions were diluted in PBS to a final concentration of 100 μM, the remaining substances to a 20 μM/PBS working solution, before use. Highest DMSO concentration did not exceed 0.04 % (v/v).

### *Cell culture*

Human 143B and U-2OS osteosarcoma cell lines were obtained from the ATCC (American Type Culture Collection, Rockwille, MD, USA). 143B was cultured in RPMI 1640 medium, and U-2OS in McCoys medium supplemented with 10% heat inactivated foetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% $CO_2$. Both cell lines were transferred to six-well plates at $4 \times 10^5$/well 18 h before assay set-up. Cells were treated at indicated concentrations and duration with different cardiac glycosides, and then subjected to analysis. RPMI 1640 and McCoys medium base and penicillin/streptomycin stock solution were purchased from Gibco, Life Technologies.

Effects of cardiac glycosides on cell viability were measured after 4 h using a Cytotoxicity Detection Kit Plus (Roche, Grenzach-Wyhlen, Germany) that quantifies lactate dehydrogenase (LDH) release, according to the manufacturer's protocol.

### *Propidium iodide and annexin V binding assay*

Cells were incubated for 24 h with described cardiac glycosides and concentrations. After incubation, apoptotic level was analysed as described previously (30). Results were evaluated with FlowJo Software (TreeStar, Ashland, OR, USA).

### *Analysis of mitochondrial membrane potential ($\Delta\Psi m$)*

Mitochondrial membrane potential was assessed using 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Sigma-Aldrich) and flow cytometric analysis, as described previously (36). Cells were analysed after 24 h incubation or depicted time points. Depolarizing carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich) was used as reference, cells being treated with 50 μM CCCP (final concentration) for 30 min.

*Measurement of caspase activity*

Activity of caspases -8 and -9 was measured by fluorescent caspase staining kit according to the manufacturer's protocol (Promokine, Heidelberg, Germany). For caspase inhibitor assay, cells were pre-incubated with 100 μM z-VAD-fmk for 1 h. After additional incubation with depicted cardiac glycosides for 24 h, induction of apoptosis was determined using annexin V-APC/PI with FACS analyses. DMSO was added to extracts as solvent control.

*Statistical analyses*

Student's *t*-test was applied to determine differences between glycoside-treated cells and controls and significance level was set at $P \leq 0.05$ for all tests.

## Results

*Steroid glycosides affected proliferation of 143B cells*

Anti-proliferative effect of 20 steroid glycosides (Table 1) was evaluated using the CASY© Counter system, after incubation with increasing concentrations of reagents (range 0.05-1 μM) for 24 h. Cell population growth was inhibited in a dose-dependent manner in 17 of the 20 steroid glycoside-treated cells (Fig. 1). Classical digitalis glycosides ouabain and digitoxin, were able to inhibit cell proliferation within a range of 0.1–0.15 μM (50% cell population growth inhibition). Data in Fig. 1 demonstrate that most efficient anti-proliferative effect was obtained by cardenolides convallatoxol and evomonoside and bufadienolide proscillaridin A, as well as ouabain and bufadienolide aglycon arenobufagin A. In contrast, neither uzarin nor resibufagenin altered cell proliferation after 24 h incubation.

*Steroid glycosides induced apoptosis in a concentration-dependent manner*

To assess possible effects of steroid glycosides on apoptotic induction, 143B cells were incubated with increasing doses of identical steroid glycosides as used before. Figure 2 displays apoptotic induction after 24 h. Glycosides evomonoside, ouabain and arenobufagenin caused 50% apoptotic induction in a range of 0.05–0.15 μM, whereas bufotalin, bovogenin A, lokundjoside, desglucocheirotoxin and strophanallosid induced 50% apoptosis in a range of 0.2–1 μM. Treatment with bufadienolide proscillaridin A showed strongest apoptotic effect between 0.05 and 0.1 μM.

*Steroid glycosides induced apoptosis via the mitochondrial pathway*

To determine whether apoptotic induction by the most effective cardiac glycosides (IC50 range between 0.05 and 0.2 μM) was attributable to breakdown of mitochondrial membrane potential (ΔΨm/MMP), cells were incubated under the same conditions as before and analysed after JC-1 staining, by flow cytometry. Data in Fig. 3 reveal dose-dependent breakdown of ΔΨm for all tested substances.

**Table 1.** Overview of used cardiac glycosides

| Drug | Originated from | Family |
|---|---|---|
| Convallatoxol | *Convallaria majalis* L. | Cardenolide glycoside |
| Desglucocheirotoxin | *Convallaria majalis* L. | Cardenolide glycoside |
| Digitoxin | *Digitalis purpurea* L. | Cardenolide glycoside |
| Digoxin | *Digitalis lanata* Ehrh. | Cardenolide glycoside |
| Evomonoside | *Euonymus europaeus* L. | Cardenolide glycoside |
| Gitoxin | *Digitalis purpurea* L | Cardenolide glycoside |
| Helveticoside | *Erysimum cheiranthoides* L. | Cardenolide glycoside |
| k-strophanthin | *Strophanthus kombé* Oliv. | Cardenolide glycoside |
| Lanatosid C | *Digitalis lanata* Ehrh. | Cardenolide glycoside |
| Lokundjoside | *Convallaria majalis* L. | Cardenolide glycoside |
| Strophanollosid | *Convallaria majalis* L. | Cardenolide glycoside |
| Uzarin | *Xysmalobium undulatum* (L.) R.Br. | Cardenolide glycoside |
| Ouabain | *Strophanthus gratus* (Wall. et Hook. Ex Benth.) Baill. | Cardenolide glycoside |
| Proscillaridin A | *Drimia maritima* (L.) Stearn | Bufadienolide glycoside |
| Arenobufagin | *Bufo melanostictus* Schneider, 1799 | Bufadienolide aglycon |
| Bovogenin A | *Bufo melanostictus* Schneider, 1799 | Bufadienolide aglycon |
| Bufarenogin | *Bufo melanostictus* Schneider, 1799 | Bufadienolide aglycon |
| Bufotaline | *Bufo melanostictus* Schneider, 1799 | Bufadienolide aglycon |
| Gamabufotalin | *Bufo melanostictus* Schneider, 1799 | Bufadienolide aglycon |
| Resibufogenin | *Bufo melanostictus* Schneider, 1799 | Bufadienolide aglycon |

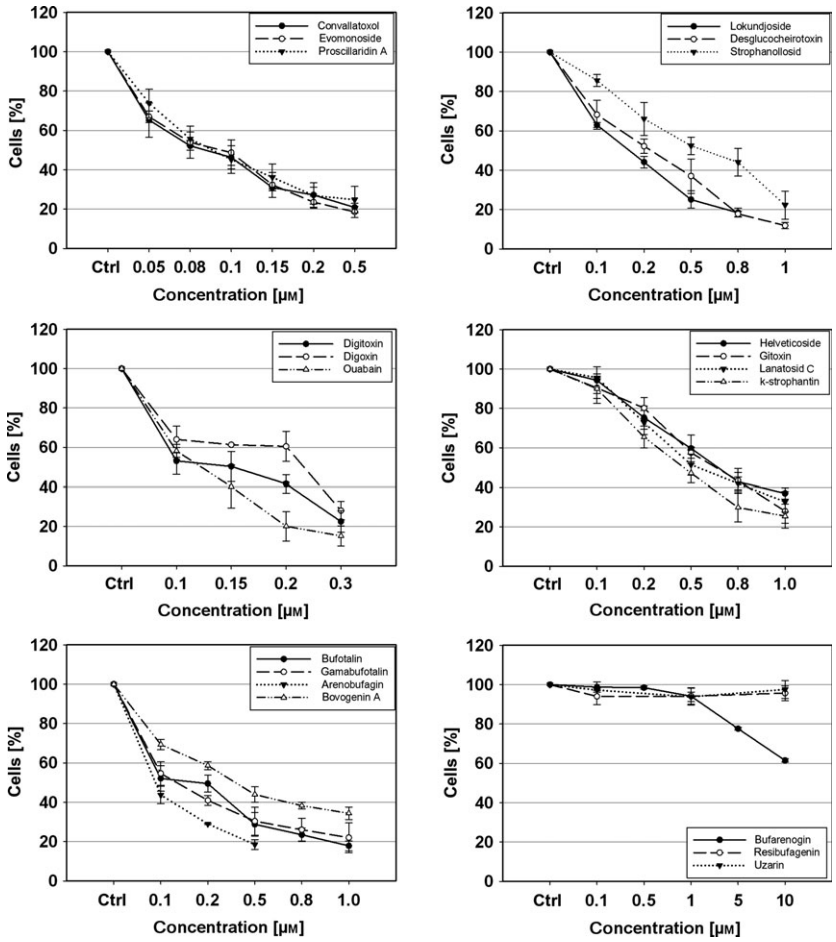


**Figure 1. Inhibition of cell population growth of steroid glycoside-treated cells.** 143B cells were treated with a range of steroid glycoside concentrations (0.05–10 µM) for 24 h. Cell counts were analysed *via* CASY® Counter. Eighteen of 20 steroid glycosides were able to inhibit cell proliferation in a concentration-dependent manner ($n$ = 3).

*Time-dependent analysis of apoptotic induction by the most potent substances, evomonoside, proscillaridin A and arenobufagin*

Next, level of apoptosis and breakdown of MMP were analysed at different time points for the three most effective glycosides (proscillaridin A, arenobufagin, evomonoside). Data in Fig. 4 reveal that drug-induced apoptosis occurred after 6 h and that there was no significant difference in apoptotic level and loss of mitochondrial membrane potential between 16 and 24 h. To exclude unwanted cytotoxic effects such as necrosis, LDH release was measured after 4 h incubation with proscillaridin A, arenobufagin and evomonoside. As shown in Fig. 4b, no significant LDH release was measurable for different doses of the three tested drugs.

Furthermore, effectiveness of proscillaridin, evomonoside and arenobufagin was analysed in a secondary osteosarcoma cell line U-2OS. In that, these glycosides were also able to induce apoptosis in a concentration-dependent manner accompanied by loss of $\Delta\Psi m$ (Fig. S1), but were less sensitive to the glycosides than line 143B.

*Cardiac glycosides affected caspase activity of both intrinsic and extrinsic apoptotic pathways*

For further characterization of the molecular pathways involved in apoptosis, activation of caspases -8 and -9 were assessed in 143B cells. Both caspases are upstream initiator proteases, which play key roles in the extrinsic (caspase-8) or the intrinsic (caspase-9) apoptotic pathway. Data in Fig. 5a reveal similar activation of both caspases by proscillaridin, evomonoside and arenobufagin in a concentration-dependent manner.

To analyse the role of caspases, 143B cells were treated in the absence or presence of the broad range caspase inhibitor z-VAD-fmk. In all three treated cell types, z-VAD-fmk treatment reduced apoptotic induction between 30% and 40%.

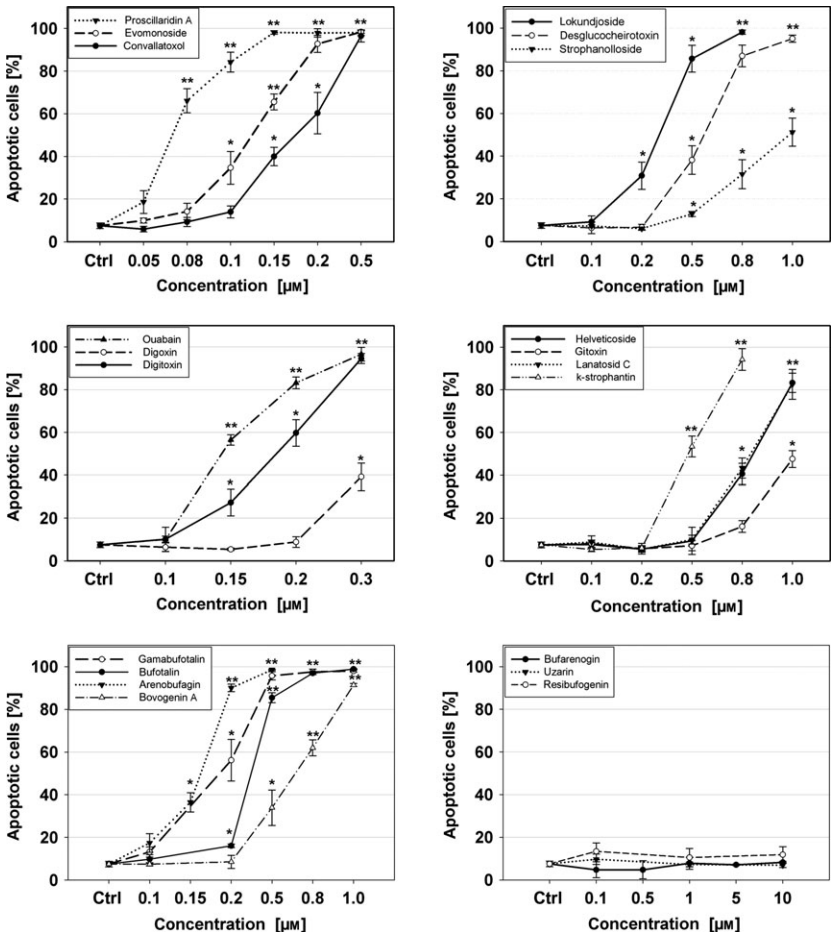


**Figure 2. Apoptotic induction in 143B cells by steroid glycosides.** Cells were incubated with depicted concentrations of steroid glycosides for 24 h. After treatment, induction of apoptosis was analysed using annexin V-APC and propidium iodide (PI). Values are given as percentages of annexin-positive cells ±SD. Seventeen of the 20 cardiac glycosides increased percentages of annexin V-positive cells in a dose-dependent manner ($n = 3$). Asterisk represents statistically significant differences compared to control group ($*P < 0.05$, $**P < 0.001$).

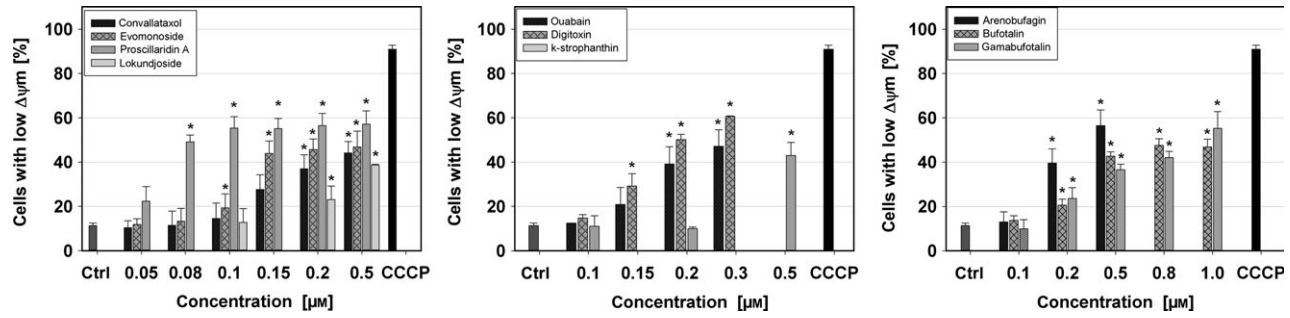


**Figure 3. Steroid glycosides induced mitochondrial membrane depolarization.** Cells were treated with the most effective steroid glycosides for 24 h and mitochondrial membrane potential was evaluated using fluorescent dye JC-1 and flow cytometry. Values of mitochondrial permeability transition are given as percentages of cells with low MMP ±SD ($n = 3$). Asterisk represents statistically significant differences compared to control group ($*P < 0.05$).

These results indicate that both extrinsic and intrinsic signalling pathways play a role in cardiac glycoside-induced apoptosis in osteosarcoma *in vitro.*

## Discussion

Previous studies have shown anti-proliferative and anti-tumour properties for some analysed steroid glycosides in various cancer cell lines, although little has been known concerning their effects on osteosarcoma cells. To our knowledge, this is the first time that an anti-proliferative effect and apoptotic induction have been analysed for evomonoside, convallatoxol, lokundjoside, desglucocheirotoxin and strophanolloside in cancer cells. These five cardenolides have the ability to inhibit cell proliferation and exert induction of apoptosis in a

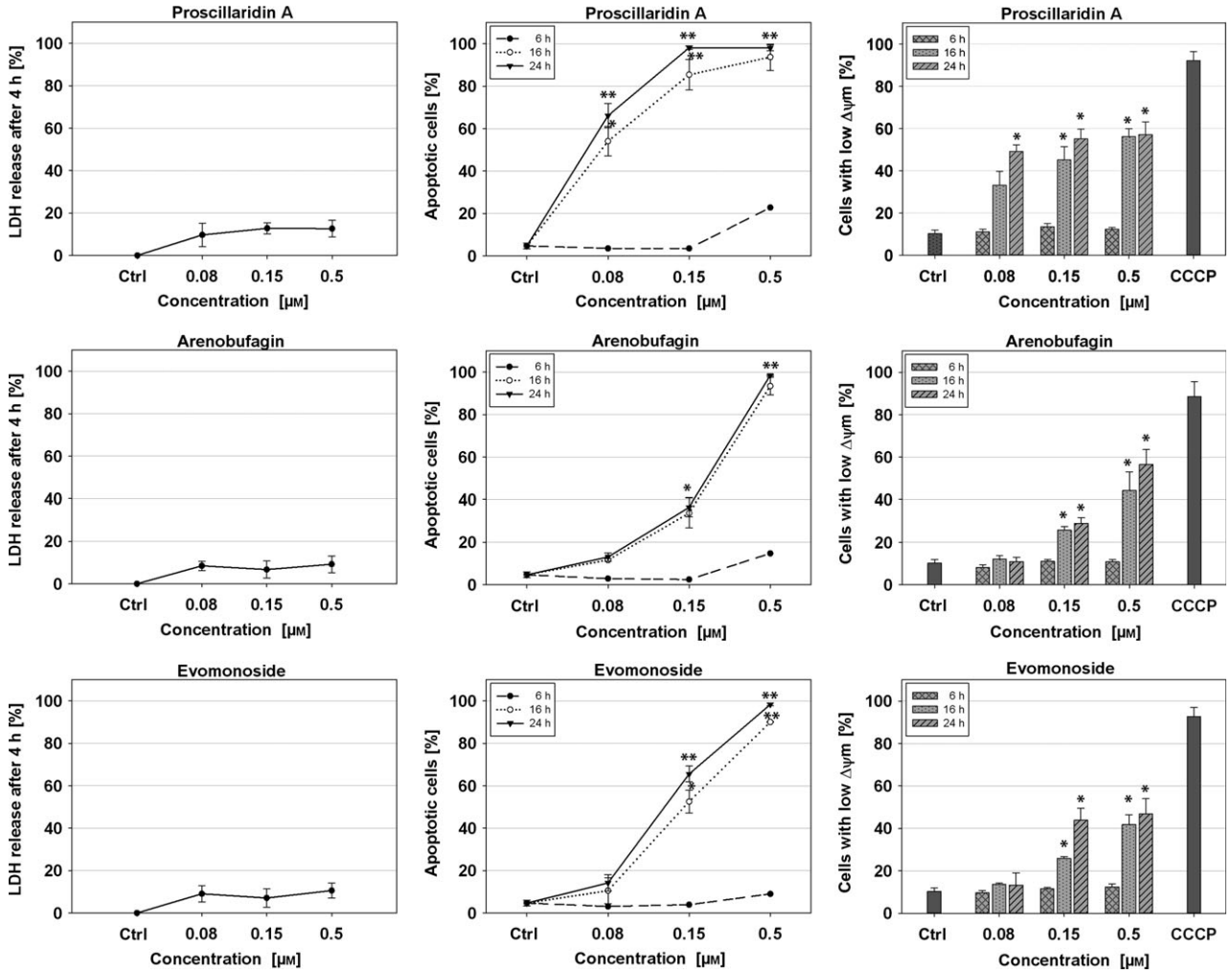


**Figure 4. Apoptotic induction took place after 6 h incubation.** Cells were incubated with described concentrations of proscillaridin A, evomonoside and arenobufagin for 6, 16 or 24 h. After incubation, apoptotic level and loss of mitochondrial membrane potential were analysed as shown previously. Early cytotoxicity was measured after 4 h incubation, by LDH release assay. There was no relevant induction of LDH release after 4 h by the three steroid glycosides. ($n$ = 3). Asterisk represents statistically significant differences compared to control group (*$P$ < 0.05, **$P$ < 0.001).

concentration range in the order of 0.080–1 μM. Here, evomonoside had the strongest effect within 0.08–0.15 μM. In the literature, we found no data to be available on an anti-cancer effect of this cardenolide.

By comparison of all 20 steroid glycosides analysed here, the bufadienolide proscillaridin A displayed the strongest effect in causing apoptosis and inhibition of cell proliferation. Johansson *et al.* have shown apoptotic induction in 9 of 10 analysed human cancer cell lines at concentration of 0.06 – 0.67 μM (37). In our study, concentration ranged between 0.05 and 0.1 μM. Bielawski *et al.* demonstrated an anti-proliferative effects of proscillaridin A, digitoxin and ouabain at nanomolar drug concentrations, whereas proscillaridin A was much more effective at lower concentrations (0.03 μM for proscillaridin A, 0.1 μM for digitoxin and ouabain) in breast cancer cells (38). In glioblastoma, proscillaridin A causes cytotoxicity *in vitro* (IC50 2–4 nM), reduces tumour weight and improves survival of tumour bearing mice *in vivo* (39). Recent studies by Cerella *et al.* have validated Mcl-1 as a common target of cardenolides (40), with down-regulation of Mcl-1 before apoptotic induction in U937 cells, not only for the hemi-synthetic cardenolide UNBS1450 but also for proscillaridin, oubain, digoxin and digitoxin. These data are consistent with the results obtained by other groups at the level of protein expression under requirement of proteasome degradation (41,42).

Anti-proliferative effects of 27 bufadienolides have been reported in six human and two mouse cancer cell

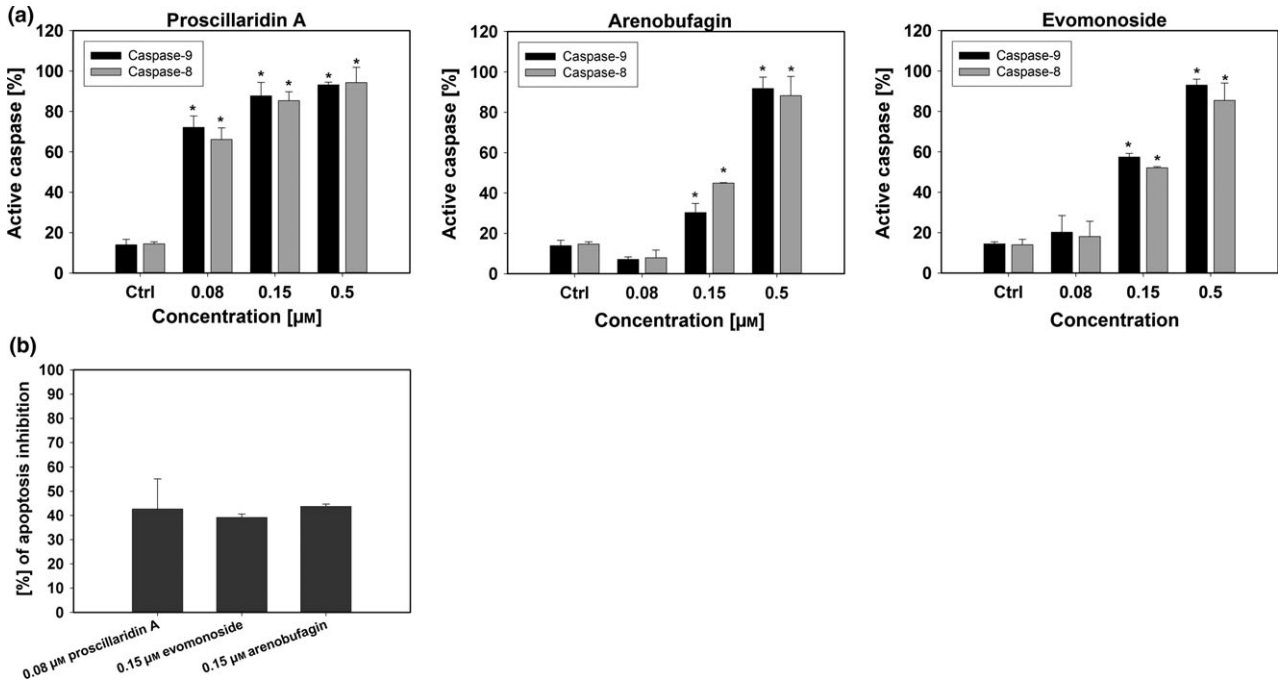


**Figure 5. Activation of caspases -8 and -9 in glycoside-induced apoptosis**. (a) Cells were treated with depicted glycosides and activity of caspases -8 and -9 were analysed by FITC-LEHD and FITC-IETD and flow cytometry after 24 h; ±SD, $n = 3$. (b) Cells were treated with proscillaridin, evomonoside or arenobufagin for 24 h in the presence or absence of 100 μM Z-VAD-fmk. Effects of caspase inhibitor were analysed using annexin V/propidium iodide staining and flow cytometry, ±SD, $n = 3$. Asterisk represents statistically significant differences compared to control group (*$P < 0.05$).

lines in comparison to ouabain and digoxin. Moreno *et al.* showed that several bufadienolides, for example gamabufotalin rhamnoside (2–8 nM), bufotalin (9–74 nM) and hellebrin (7–68 nM), displayed higher rates of proliferation inhibition than digoxin (40–266 nM) and ouabain (28–211 nm) after 3 days culture (32).

In our study, strongest effect in cell population growth inhibition and apoptotic induction among the group of cardiac glycosides, was observed for the common cardiac glycosides ouabain > digitoxin > digoxin > k-strophantin. For these glycosides, apoptotic level has been shown *in vitro* and *in vivo* (43,44).

For the cardiotonic steroid ouabain, an anti-proliferative effect was observed, at the very high concentrations of 1-10 μM, in three different prostate and breast cancer and leukaemia cell lines (20,45). Moreover, activity of caspases -3 and -9 was described in HeLa, neuroblastoma SH-SY5Y and human fibroblasts (46). Furthermore, ouabain triggered cytochrome c release from mitochondria in neuroblastoma and HeLa cells in correlation with down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-XL (43,46). Recent studies have proposed caspase-independent autophagic cell death *via* JNK activation and Bcl-2 reduction, for oubain, in non-small cell lung cancer (concentration 50–100 nM) (44). Pezzani *et al.* were able to show an anti-proliferative effect in adrenocortical cell lines, whereas concentrations from 1 to 1000 nM did not induce apoptosis after 24 h (47). In our study, we observed both inhibition of cell population growth and induction of apoptosis, at clearly lower concentrations than described previously. Overall, these data indicate that the ouabain-induced apoptotic mechanism might be dependent on the particular cancer under consideration. Hiyoshi *et al.* have reported that ouabain significantly reduced tumour growth in a xenografted neuroblastoma mouse model *in vivo* without significant toxicity to the host (48). These data were confirmed by two other groups with an AML and prostate cancer xenograft mouse models (49,50).

For digitoxin, Stenkvist *et al.* reported epidemiological studies in which women with breast cancer on digitalis therapy developed more benign forms of breast tumour and succumbed to levels death (6%) compared to control patients (51–53). Furthermore, the recurrence rate of patients with digitalis treatment was 9.6-times lower than that of control patients.

Within the group of bufadienolide aglycons in our study, arenobufagin A was the most effective glycoside. Effectiveness of bufadienolide aglycons in order of

rank are: arenobufagin A >gamabufotalin > bufotalin > bovogenin A. Arenobufagin A is listed as a key cardiotonic steroid of toad venom, but only a small number of studies on arenobufagin and cancer, seem to have been published. We observed induction of apoptosis, accompanied by loss of mitochondrial membrane potential and activation of caspases -8 and -9, between 0.1 and 0.2 μM of arenobufagin A. Additionally, arenobufagin A markedly inhibited population growth of 143B cells. In accord with our results, Zhang *et al.* reported for the first time that arenobufagin A induced apoptosis *via* caspase 3- and -9 activation and autophagy through inhibition of the phosphatidyl inositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway in hepatocellular carcinoma (HCC) cells, in a concentration range between 0.2 and 0.5 μM (54). Moreover it has been reported that arenobufagin is able to block the $Na^+/K^+$-ATPase pump (55) and vascular endothelial growth factor-mediated angiogenesis, in several human cancer cell lines, *in vitro* and *in vivo* (56).

The bufadienolide bufalin was the first identified bufadienolide to be analysed as a potential agent in different osteosarcoma cell lines *in vitro* and *in vivo* (57–62). Xie *et al.* demonstrated strong inhibition of tumour growth in a methotrexate-resistant cancer model *in vivo*, without reduction in host body weight (63). Other studies have shown caspase-independent apoptotic induction with involvement of autophagy-mediated cell death and JNK activation, after bufalin treatment (64,65).

Mechanisms of cardiac glycoside activity in cardiac disease treatment, with involvement of $Na^+/K^+$-ATPase are well characterized, whereas less is known regarding mechanisms of their anti-cancer effects. Correlation between binding to $Na^+/K^+$-ATPase and apoptotic induction plays an important role in the question of anti-cancer mechanisms. Binding to $Na^+/K^+$-ATPase leads to inhibition of ATPase, increase in intracellular $Na^+$ and $Ca^{2+}$, intracellular reduction in $K^+$ and inhibition of IL-8 production and the TNF-a/NF-κB pathway. Furthermore, DNA topoisomerase II was found to have been inhibited and the Src kinase pathway was activated (10,11,66,67). For digitoxin, it has already been described that apoptotic induction is connected to inhibition of $Na^+/K^+$-ATPase and increase in intracellular $Ca^{2+}$ (14,68). For human lung adenocarcinoma, anti-cancer effects of ouabain have been reported to regulate and inactivate Src-to-ezrin signalling, $Na^+/K^+$-ATPase subunits and proteins involved in focal adhesion (69). However, Akimova *et al.* reported that ouabain and other cardiac glycosides induced apoptosis in vascular endothelial cells and renal epithelial cells, $Na^+/K^+$ independently (70,71). One explanation for the different potency of apoptotic induction by the investigated steroidal glycosides could be different affinity to a $Na^+/K^+$-ATPase α-subunit, which is not primarily involved in ion efflux but in signal transduction, within the process of apoptotic induction (72). Further studies are warranted to examine whether inhibition of $Na^+/K^+$-ATPase is the main target structure for induction of apoptosis.

Moreover, several cardiac glycosides are known to be potent P-glycoprotein inhibitors with low resistance indices in multidrug resistant P-glycoprotein-overexpressing cells (37,73,74). Overexpression of the ABC transporter P-glycoprotein is responsible for classical MDR, by extruding chemotherapeutics to the outside of the cell. Thus, cardiac glycosides with the capability of modulating P-glycoprotein, represent a significant source of alternatives in cancer treatment. Mahringer *et al.* analysed the potential of P-glycoprotein inhibition by 57 compounds used in Chinese medicine combined with inhibition of cancer cell population growth. Among these, bufalin was identified as a potent inhibitor without any cross-resistance, whereas proscillaridin inhibited P-glycoprotein with low cross-resistance. Resibufogenin interestingly revealed collateral sensitivity in drug resistant CEM/ADR5000 cells (74). In that study, microRNA expression experimentation revealed an MDR-independent cytotoxic effect for the analysed bufadienolides, except for resibufogenin.

Some theories on the mechanism of inhibition are mentioned below. On the one hand cardiac glycosides can act as transport-independent inhibitors of P-glycoprotein-like cyclosporine A. On the other hand, it has been shown that they are able to inhibit stimulated P-glycoprotein ATPase activity slightly, despite acting as substrates for P-glycoprotein (73). To gain more insight into the details of the mechanisms and the role of P-glycoprotein for our compounds, further studies need to be performed.

The results from the *in vivo* studies of, for example ouabain and bufalin, and clinical trials with digitoxin and digoxin, support the idea that therapeutic potential of these drugs should be further evaluated in cancer patients. To counteract chemotherapeutic resistance, a combination of polychemotherapy with novel strategies is indispensable for osteosarcoma therapy. In our study, we provide a first hint for cardiac glycosides as anti-cancer drugs for osteosarcoma treatment. We have been able to show cell population growth inhibition, not only for common cardiac glycosides like ouabain, digoxin and digitoxin, but also for proscillaridin A, evomonoside and arenobufagin A. Our findings suggest that these cardiac glycosides are promising candidates for combined therapy with classical chemotherapeutic agents, to achieve potential synergism of effectiveness in treatment, and to overcome resistance to apoptosis in

patients currently with dismal prognoses. Thus, the therapeutic potential of steroid glycosides in combination with cytostatics must be further evaluated *in vivo*.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Apoptotic induction in U2OS cells. A. U2OS were incubated with depicted concentrations of steroid glycosides for 24 h. After treatment, apoptotic induction was analysed with annexin V-APC and propidium iodide (PI). Values are given as percentages of annexin-positive cells ±SD. B. Mitochondrial membrane potential was evaluated using fluorescent dye JC-1 and flow cytometry. Values of mitochondrial permeability transition are given as percentages of cells with low MMP ±SD ($n = 3$). Asterisk represents statistically significant differences compared to control group ($*P < 0.05$, $**P < 0.001$).