Oleandro. Oleandrin, glicosídeo cardíaco, inibe bomba de Na+ K+ ATPase e diminui a proliferação do câncer de pâncreas

10/04/11


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Nerium oleander

Oleandrin-mediated inhibition of human tumor cell proliferation: importance of Na,K-ATPase alpha subunits as drug targets.
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Abstract
Cardiac glycosides such as oleandrin are known to inhibit the Na,K-ATPase pump, resulting in a consequent increase in calcium influx in heart muscle. Here, we investigated the effect of oleandrin on the growth of human and mouse cancer cells in relation to Na,K-ATPase subunits. Oleandrin treatment resulted in selective inhibition of human cancer cell growth but not rodent cell proliferation, which corresponded to the relative level of Na,K-ATPase alpha3 subunit protein expression. Human pancreatic cancer cell lines were found to differentially express varying levels of alpha3 protein, but rodent cancer cells lacked discernable expression of this Na,K-ATPase isoform. A correlation was observed between the ratio of alpha3 to alpha1 isoforms and the level of oleandrin uptake during inhibition of cell growth and initiation of cell death; the higher the alpha3 expression relative to alpha1 expression, the more sensitive the cell was to treatment with oleandrin. Inhibition of proliferation of Panc-1 cells by oleandrin was significantly reduced when the relative expression of alpha3 was decreased by knocking down the expression of alpha3 isoform with alpha3 siRNA or increasing expression of the alpha1 isoform through transient transfection of alpha1 cDNA to the cells. Our data suggest that the relative lack of alpha3 (relative to alpha1) in rodent and some human tumor cells may explain their unresponsiveness to cardiac glycosides. In conclusion, the relatively higher expression of alpha3 with the limited expression of alpha1 may help predict which human tumors are likely to be responsive to treatment with potent lipid-soluble cardiac glycosides such as oleandrin.
PMID: 19671733

Introduction
Cardiac glycosides are a class of compounds used to treat congestive heart failure by increasing myocardial contractile force (1). Oleandrin is a cardiac glycoside derived from Nerium oleander, which has been used for many years in Russia and China for this purpose. In contrast to its use for the treatment of heart failure, preclinical and retrospective patient data suggest that cardiac glycosides (e.g.,
digoxin, digitoxin, ouabain, and oleandrin), may reduce the growth of various cancers including breast, lung, prostate, and leukemia (2–7). Recent work from our laboratory and others has shown that these compounds induced selective cell death in certain human but not murine tumor cells (8, 9) or normal human cells (10). Previously, we reported that oleandrin and oleandrigenin inhibited proliferation and induced apoptosis due to an increase in intracellular Ca2+ via inhibition of Na+,K-ATPase (5). Oleandrigenin and oleandrin also inhibited the export of fibroblast growth factor-2 through membrane interaction and inhibition of Na+,K-ATPase activity (11). In addition, we have shown that oleandrin inhibits the growth of human melanoma BRO cells due, in part, to the generation of reactive oxygen species that caused mitochondrial injury (12). Other investigators have reported that cardiac glycoside drugs, such as digoxin and oleandrin, inhibit the constitutive hypersecretion of nuclear factor κB-dependent proinflammatory cytokine interleukin 8 from cystic fibrosis lung epithelial cells (13). These investigators also observed that oleandrin, as well as digoxin, suppressed the tumour necrosis factor-α/nuclear factor κB signaling pathway by blocking tumor necrosis factor-α-dependent TNFR1/TRADD complex formation (14). Oleandrin has been shown to induce apoptosis in human leukemic cells by dephosphorylation of Akt, expression of Fas, as well as alteration of membrane fluidity (9). More interestingly, this study also showed that oleandrin binds to the plasma membrane of human lymphoma U937 cells but does not bind to murine NIH3T3 cells (9). Although plausible mechanisms associated with oleandrin-induced cell growth suppression in various cancer cells have been proposed, there is only a poor understanding at best of the differential response of human and rodent cancer cells to this and other related cardiac glycosides. In this study, we sought to better understand these mechanisms that might be responsible for the selective anticancer activity of this potent cardiac glycoside and the compounds related to it.

Functionally, Na+,K-ATPase is a transmembrane protein that catalyzes the active transport of Na+ and K+. Its presence in the heart is well-established as a pharmacologic receptor for cardiac glycosides. The enzyme utilizes ATP to provide the free energy necessary for driving Na+/K+ exchange. We reviewed the physiological and biochemical properties of Na+,K-ATPase as a model system for a review (see ref. 10). In failing cardiac muscle fibers, cardiac glycosides bind to the Na pump, thereby inhibiting its activity, which results in an increase in intracellular Na+. The resulting intracellular increase in Na+ produces a concomitant increase in cytosolic Ca2+ and an enhancement of contractile force (16). In addition to acting as an ion pump, Na+,K-ATPase may also engage in the assembly of signal transduction complexes that transmit signals to different intracellular compartments (17). Based on current knowledge, the functional properties of Na+,K-ATPases seem to rely heavily on the structural characteristics of this family of molecules. It is only recently, however, that Na+,K-ATPase has been proposed as a novel target for anticancer therapy (refs. 18, 19, Review articles).

Structurally, Na+,K-ATPase exists as a heterodimer that contains a catalytic α-subunit and a glycosylated β-subunit. The existence of a γ-subunit was also reported but is not well understood. The α-subunit has binding sites for ATP, Na+, K+, and cardiac glycosides. It's binding partner, the β-subunit, functions to stabilize the catalytic α-subunit and may also have regulatory activity. Four different α isoforms have been identified in mammalian cells. The expression of α isoforms is tissue type-specific and varies among rodent and human tissues (20). Variation in the expression of these isoforms occurs in human cancers (e.g., renal, lung, hepatocellular, and colon) and contrasts with corresponding normal tissues (21–24). The binding affinity of cardiac glycosides varies depending on the α isoform present with α1 binding affinity being low and the α2 and α3 isoforms being as much as 100-fold higher (25, 26). In species other than rodents, isoform differences to ouabain sensitivity are less marked and tend to vary in rodent tissues (for a review, see ref. 24). The binding of cardiac glycosides to the Na pump is thought to affect the activity of the β-subunit (25), the channel activity (26), and the ATPase activity (27). The catalytic α2 and α3 isoforms seem to rely heavily on the structural characteristics of this family of molecules. It is only recently, however, that Na+,K-ATPase has been proposed as a novel target for anticancer therapy (refs. 18, 19, Review articles).

Collectively, our work and that of others led us to hypothesize that the differential cytotoxicity of oleandrin to human and murine cancer cells may be associated with differences in expression of the α isoforms. Extending this hypothesis, we thought that differential expression of the α isoforms of cancer cell lines might be a key determinant of oleandrin-induced cell growth inhibition. The current report examines the selective effect of oleandrin on growth inhibition of human and mouse pancreatic cancer cells that were found to express different levels of the various Na+,K-ATPase α isoforms. Modulation of the relative expression of Na+,K-ATPase subunit expression through use of siRNA or transient transfection of the cDNA for specific subunits suggests that there is a closer correlation between specific subunit expression and sensitivity to anticancer effects of lipid-soluble cardiac glycosides such as oleandrin.

Materials and Methods

Materials and Reagents

Oleandrin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma Chemical, Co. BODIPY-oleandrin, Mito-Tracker Red CM-H2XRos, Mito-Tracker Orange CM-H2XRos (PFA fixation stable), calcein acetoxymethyl ester, and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes-Invitrogen Corporation. Anti–β-actin antibody was also purchased from Sigma.

Cell Lines

Human pancreatic cancer cells (Panc-1, BxPC3, and MiaPaca), human colon cancer cell lines (CaCO-2, DOD-1, HCT-116, HT29, RKO, and LS174), rodent melanoma B16 cells, human breast cancer cells (SUM149, MCF-7, and MDA231), human oral cancer cells (SCC9 and CAL-27), human ovarian cancer (ES3, TOV1120, and SKOV cells), and human non-small cell lung cancer (A549 and H1299 cells) were obtained from American Type Culture Collection and maintained in a humidified atmosphere containing 5% CO2 at 37°C. Human melanoma BRO cells were a kind gift from the Stelkin Foundation (Houston, TX). Murine pancreatic cancer Panc-02 cells were a kind gift from Dr. David Chang (The University of Texas, M. D. Anderson Cancer Center, Houston, TX). Cell lines derived from different epithelial origins were routinely cultured in tissue culture media (Invitrogen Corp.; Table 1) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories Inc.), 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2 mmol/L of L-glutamine from Life Technologies.

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Table 1

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<th>Cell lines grown to a density of 1 × 104 cells per well in their relevant media as indicated in Table 1. After a 24-h incubation period, cells were treated with various concentrations of oleandrin (1–500 nmol/L). After an additional 72 h incubation, the efficacy of cellular proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (27). Absorbance was read at a wavelength of 570 nm and a reference wavelength of 650 nm using a V-Max Micro-plate Reader by Molecular Devices, Inc.</th>
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Cellular Uptake of Cardiac Glycosides

Uptake in Panc-1 (highest expression of α3 with poor expression of α1 isoforms) cells and BxPC3 cells (poor expression of α3 relative to α1) was determined after treatment with BODIPY-oleandrin, a fluorescent analogue of oleandrin by fluorescence microscopy. Cells in 96-well plates were treated with 0, 5, 20, and 50 nmol/L oleandrin for 2 or 24 h. Treatments were done in 0.5% fetal bovine calf serum in DMEM/F12 medium. Cells were simultaneously incubated with MitoTracker Red CM-H2XRos (1 µmol/L), and DAPI (1 ng/mL), a selective nuclear dye (Molecular Probes). Nuclear morphology, DNA, and mitochondria dye uptake were assessed by fluorescence microscopy using an Olympus IX-70 inverted microscope. Image acquisition was achieved using a Quantix charged coupled device camera and IPLabs software (Scion, Inc.). Alteration of oleandrin uptake in wild-type and Panc-1 cells transfected with α3 siRNA was determined in cells cultured on laminin-coated coverslips and treated with BODIPY-oleandrin for 1 h.

Analysis of Cell Viability, Apoptosis, and Mitochondrial Distribution by Fluorescence Microscopy

Apoptosis and nuclear morphology, DNA dye uptake, and cellular staining were assessed by fluorescence microscopy. Cells were plated in 96-well plates and treated with 50 nmol/L of oleandrin for 6, 24, or 72 h. Treatments were done in 0.5% fetal bovine calf serum in DMEM/F12 medium. Cell viability was determined at each time point by staining with vital dye calcein acetoxymethyl ester (2 µmol/L) in
phenol red-free DMEM for 15 min at 37°C. Cells were simultaneously incubated with MitoTracker orange CM-H2XRos (1 µmol/L) and DAPI (1 ng/mL; Molecular Probes). Nuclear morphology, DNA dye uptake, and cellular staining were assessed by fluorescence using an Olympus IX-70 inverted microscope. Image acquisition was achieved using a Quantix charged coupled device camera and IP Labs software (Scanalytics) on a Macintosh computer (Apple Computer Corporation).

Immunofluorescence Analysis
Cellular monolayers were established on coverslips to perform immunofluorescence analysis. Cells were treated with 1 µmol/L of oleandrin for 72 h, then fixed in 1% paraformaldehyde prior to performing for immunofluorescence analysis. Coverslips were immersed in cold PBS and stored at room temperature for 1 min, incubated in a 5% CO2 incubator for additional 10 min at 4°C prior to centrifugation at 14,000 × g for 10 min at 4°C. Cell lysates were then sonicated on ice for 3 min, incubated for an additional 10 min at 4°C prior to centrifugation at 14,000 × g for 10 min at 4°C. Protein levels were quantified via the Bio-Rad Dc protein assay (Bio-Rad, Inc.). Levels of protein (50 µg) were applied to Bio-Rad precast gels or NuPAGE Novex precast mini-gels (Invitrogen) and were transferred onto polyvinylidene difluoride membranes, according to standard methods. Following a 1-h to 2-h incubation period in 5% nonfat dry milk blocking buffer prepared in TBS with 0.1% Tween 20, membranes were probed with primary antibodies to α3 (Affinity Bioreagents) and α1 (Upstate) isoforms diluted 1:2,000 in blocking buffer. Protein bands were visualized via chemiluminescence using the ECL+ detection kit and hyperfilm (Amersham Biosciences). Equal loading of samples was confirmed by Western blotting for the presence of β-actin. Protein bands were quantified using Alpha DigiDoc 1000 software (Alpha Innotech, Corp.).

Transfection of Panc-1 Cells with α3 siRNA
Panc-1 cells were plated in 6-well and 48-well plates and allowed to attach overnight. Transient transfection of nonspecific siRNA (control siRNA) and α3 siRNA molecules was carried out using siPORT Amine Transfection Agent (Ambion) and α3 silencing RNA (30–75 nmol/L; Santa Cruz Biotechnology) following the instructions of the manufacturer. Twenty-four hours after transfection, cells were trypsinized and counted. Transfection efficiency was assessed by fluorescent microscopy. The percent transfection was calculated as follows: transfection efficiency (%) = (number of transfected cells/the total number of cells) × 100.

Results
The Relationship of Oleandrin-Mediated Inhibition of Cell Proliferation to Na,K-ATPase Subunit Composition
Oleandrin differentially inhibited the proliferation of rodent or human pancreatic cancer (Panc-1, MiaPaca, and BxPC3) cell lines (Fig. 1A). Panc-1 cells were the most sensitive cell line to oleandrin treatment with an IC50 of 5.6 mmol/L, whereas, mouse Panc-02 cells were the least sensitive (IC50 > 500 mmol/L). Among the four pancreatic cancer cell lines tested, the relative sensitivity of oleandrin to each cell line was determined. Mouse pancreatic cancer Panc-02 cells were found to express only the α1 isoform, whereas human pancreatic cancer Panc-1 and MiaPaca expressed high levels of α3 with minimal expression of α1. In contrast to Panc-1 and MiaPaca cells, the level of α1 was highest in mouse Panc-02 cells (Fig. 1B). Panc-1 and MiaPaca cells were more sensitive to oleandrin than either BxPC3 or Panc-02 cells. Similar results were obtained in human and rodent melanoma cells (Supplementary Fig. S1A). In order to confirm the validity of our assay on expression of α3, the expression of a3 isoforms was examined in several mouse cancer cell lines as well as mouse brain tissue. As shown in Supplementary Fig. S1B, all mouse cancer cells tested expressed α3 protein, but no α3. In contrast, the a3 subunit protein was detected in mouse brain tissue, which has been reported previously (25).
comparison, when the relative expression of α3 was higher than α1 expression, oleandrin inhibition of cell proliferation was much stronger (IC50 values of 12.5 ± 6.4 nmol/L; Fig. 1C). These additional data lend further support to the notion that the antiproliferative effects of oleandrin correlates with relative expression of Na,K-ATPase α subunits.

Localization of Na,K-ATPase α3 Subunits

The relative presence and distribution of the α3 isoform was then examined in Panc-1 and BxPC3 cell lines. The expression of α3 protein (green) fluorescence was scarcely detectable in BxPC3 cells, whereas in Panc-1 cells, there was marked α3 protein present as evidenced by abundant (green) fluorescence staining (Fig. 2A). At higher magnifications, it became apparent that the α3 subunit protein distribution was in the cell cytoplasm as well as colocalized within the cell membrane. Treatment with oleandrin (50 nmol/L) did not alter the α3 subunit distribution in Panc-1 cells. Interestingly, α3 isoform expression remained unaltered in Panc-1 cells when exposed to oleandrin in either a concentration-dependent or time-dependent basis (Fig. 2B). Upon closer examination, however, exposure of Panc-1 cells to oleandrin caused obvious changes in cell morphology, denoting a shift to a more differentiated cell phenotype.

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Figure 2.
Expression of α3 isoform in BxPC3 and Panc-1 human pancreatic cancer cell lines. Cell lines were exposed to diluent (Control) or oleandrin (50 nmol/L) and MitoTracker Orange CM-H2XRos (red-orange) to detect the presence and location of mitochondria for 2 h prior to fixation. After processing for α3 immunostaining, cells were counterstained with DAPI to detect nuclei (blue). BxPC3 cells contain low α3 levels whereas Panc-1 cells exhibit high α3 content. Also shown is a change in cell morphology to a more differentiated morphology upon exposure of Panc-1 cells to oleandrin. Insets, higher magnification of representative cells. B, exposure of Panc-1 cells to oleandrin did not affect the expression of α3 protein for at least 48 h at concentrations up to 50 nmol/L. Changes in Expression of α3 or α1 Correlate with Oleandrin Uptake

The relative importance of Na,K-ATPase isoform content on pancreatic cancer cell sensitivity to oleandrin was examined. A fluorescent oleandrin analogue was prepared by attaching a BODIPY moiety to the oleandrin molecule. In this study, we also combined the use of BODIPY-oleandrin with a dye to selectively stain mitochondria (MitoTracker Red CM-H2XRos) and DAPI to label nuclear DNA (blue). Both Panc-1 and BxPC3 cells were treated with BODIPY-oleandrin (5–50 nmol/L) for 24 hours and the uptake and location of oleandrin were recorded with a fluorescence microscope. Figure 3A shows that BODIPY-oleandrin (5 nmol/L) was easily detected in α3-expressing Panc-1 cells, whereas in BxPC3 cells that lack α3, only minimal fluorescence was observed even at a BODIPY-oleandrin concentration of 50 nmol/L. The uptake of oleandrin by Panc-1 cells was concentration-dependent. Counterstaining with MitoTracker Red CM-H2XRos suggested that BODIPY-oleandrin associated with mitochondria, and the exclusion of DAPI indicated that the plasma membrane remained intact. Moreover, α3-containing Panc-1 cells exhibited morphologic changes that included an elongated phenotype which was not apparent in the more epithelioid BxPC3 cells lacking α3.

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Figure 3.
Uptake of fluorescent oleandrin analogue (BODIPY-oleandrin) by living cells. A, concentration-dependent uptake of oleandrin within BxPC-3 human pancreatic cancer cells was observed at concentrations as high as 50 nmol/L over the 24 h observation period. In contrast, drug uptake was easily noted within Panc-1 cells with concentrations as low as 5 nmol/L. B, time-dependent uptake of oleandrin was evident in Panc-1 cells as early as 2 h, whereas at 24 h, nearly all cells contained marked concentrations of the drug. In contrast, no drug is observed in BxPC3 cells. Living cells were counterstained with MitoTracker orange CM-H2XRos (red) to detect viable mitochondria and DAPI, which is excluded from viable cells with intact plasma membranes. More yellow signals indicate where mitochondria and BODIPY-oleandrin colocalize. DAPI uptake was minimal over this 2-h timeframe. Insets, a higher magnification of representative cells. BODIPY-Oleandrin Uptake is Time-Dependent

When both BxPC3 and Panc-1 cells were treated with 20 nmol/L of oleandrin for 2 and 24 hours, the drug was detected in α3-bearing Panc-1 cells within 2 hours but not in BxPC3 cells that lacked α3. After 24 hours of exposure to BODIPY-oleandrin, the fluorescence intensity was greater in α3-expressing Panc-1 cells than treated BxPC3 cells, and the overall increase in intensity illustrated that this process was time-dependent (Fig. 3B). Mitochondrial association with BODIPY-oleandrin was more apparent in α3-expressing Panc-1 cells compared with treated BxPC3 cells lacking α3. This apparent colocalization of mitochondria with BODIPY-oleandrin was most
The α subunit is a multispanning membrane protein that catalyzes ion transport and contains binding sites for cations, ATP, and cardiac α3 in anticancer activity of oleandrin in human cancer cells. Binding capability of α isoforms to cardiac glycosides varies considerably under different physiologic conditions (25, 26). These findings prompted us to determine if the differential anticancer activity of oleandrin between human and rodent cancer cells or among specific human epithelial cells, peripheral blood mononuclear cells, or neutrophils (8–10). Mechanistically, cardiac glycosides bind to the Na,K-ATPase ion exchange pump inhibiting enzymatic activity. The enzyme contains two types of subunits, α and β. Four α isoforms and two β isoforms are known and the relative expression of each type is markedly altered in normal and disease states. Additionally, the Na,K-ATPase ion exchange pump inhibiting enzymatic activity. The enzyme contains two types of subunits, α and β. Four α isoforms and two β isoforms are known and the relative expression of each type is markedly altered in normal and disease states.

We report here that pancreatic cells exhibit distinct differential sensitivities to oleandrin treatment. Additionally, evidence is provided that mechanisms associated with the differential inhibitory effect of oleandrin in sensitive and nonsensitive cell lines would be important in understanding the antiproliferative activity of oleandrin was reduced in Panc-1 cells transfected with α1 subunits. By transfecting Na,K-ATPase α1 subunit to Panc-1 cells (which normally lack α1 expression), the relative expression of α3 to α1 was reduced in the transfected Panc-1 cells. As a result, the sensitivity of these cells to oleandrin treatment was reduced as evidenced by the shift of IC50 value of oleandrin from 4.7 nmol/L of nontransfected Panc-1 cells to >50 nmol/L in α1 cDNA–transfected Panc-1 cells. However, the trend of inhibition of cell proliferation in both a1-transfected and nontransfected cells was similar, suggesting that α1 protein expression is less important than that of α3 for oleandrin-induced cell death in this particular cell line.

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Discussion

Oleandrin is a lipid-soluble cardiac glycoside that selectively inhibits the proliferation of various cancer cells. Our group and others have observed that human melanoma and leukemia cells were 100-fold more sensitive to oleandrin than were murine tumor cells, normal human epithelial cells, peripheral blood mononuclear cells, or neutrophils (8–10). Mechanically, cardiac glycosides bind to the Na,K-ATPase ion exchange pump inhibiting enzymatic activity. The enzyme contains two types of subunits, α and β. Four α isoforms and two β isoforms are known and the relative expression of each type is markedly altered in normal and disease states. Additionally, the binding capability of α isoforms to cardiac glycosides varies considerably under different physiologic conditions (25, 26). These findings prompted us to determine if the differential anticancer activity of oleandrin between human and rodent cancer cells or among specific human cancer cell lines is associated with a particular expression of the isoforms of Na,K-ATPase in those cells. Understanding the mechanisms associated with the differential inhibitory effect of oleandrin in sensitive and nonsensitive cell lines would be important in defining the appropriate target for the use of this promising anticancer agent.
The four α isoforms of Na,K-ATPase, α1, α2, α3, and α4, are each derived from separate genes. Across species, the degree of homology for the α1 and α2 isoforms has been reported to be 92%, and is ~96% for α3. One obvious difference among the isoforms is in the response to cardiac glycides. In the rat, the α1 isoform was reported to be 100-fold more resistant to ouabain than either α2 or α3 (19, 25, 29, 30). In contrast, in other species, including humans, the affinity of α1 isoforms to cardiac glycides is higher, causing a greater sensitivity to ouabain (31–33). Additionally, the rodent α1 isoform is almost 1,000 times less sensitive to cardiac glycides than that in the human due to a double-loop motif (18). After the assessment of adenocarcinomas and squamous cell carcinoma tissues from 59 patients with lung cancer, Mijatovic et al. suggested that the α1 isoform of Na,K-ATPase may be a useful target for cardiac glycides in the treatment of non–small cell lung cancer cells (34). They reported that cardiac glycides such as ouabain, digixon, and UNBS1450 showed the strongest inhibition of the rat Na,K-ATPase α3β1 isoform followed by α2β1 and α1β1 isoforms growing in s9 insect cells. Their UNBS1450 compound inhibited Na,K-ATPase α1β1 isoform more 20 times more strongly than did either ouabain or digixon. Seligson et al. also reported that even though Na,K-ATPase α1 isoform and β1 levels were decreased in renal clear cell carcinoma compared with those in matched, morphologically normal proximal tubules, there was a tendency toward increased expression of these isoforms in high-grade tumors. Additionally, the higher expression of the α1 isoform in these patients seemed to correlate with a higher risk for disease-specific death (35). Our results do not seem to fully agree with this study, with one possible explanation being that the isoform expression profile is tissue-specific. Our data show that human pancreatic cancer cells with α1 expression and even a decrease from the expression of α3 or more sensitive to oleandrin than are cells that completely lack α3 expression (comparison between BxPC3 and Panc-2; Fig. 1). Upon examining α1 and α3 isoform protein expression in relation to oleandrin cytotoxicity using a variety of human cancer cells, those cells that expressed α3 protein were all more sensitive than were either rodent or human cells which did not express α3 protein. This relationship was also found within a given human tumor cell line; the relative expression of α3 was higher in cells which exhibited a much greater sensitivity to oleandrin compared with cells that had relatively lower expression of α3. These results suggest the importance of α3 relative to α1 on the sensitivity of human cancer cells to oleandrin treatment. This notion is supported following genetic manipulation in which α3 protein was knocked down with α3 siRNA or when α1 protein was restored with transfection of α1 cDNA to the cells that caused a corresponding loss in sensitivity to oleandrin.

Cardiac glycides bind to a given molecule and inhibit Na,K-ATPase activity. The expression of α3 relative to α1 was much lower in BxPC3 cells than in Panc-1 cells. Our data suggest that the uptake of oleandrin in these two different cell lines is likely to account for different antiproliferative activity. This is consistent with the study reported recently that the human leukemia cell line U-937 is capable of binding oleandrin in the cell membrane, whereas mouse NIH-3T3 cells did not bind oleandrin at all (9). We also examined the expression of α3 isoform protein in Panc-1 cells treated with oleandrin but did not observe any change in protein expression. The cardiac glycides activity in the oleandrin-resistant cell line was not altered either during in vitro culturing or during the development of disease (37–42). Arystarkhova and Sweadner reported that in rat heart, there was a postnatal switch in the expression of Na,K-ATPase isoforms. When rat cardiomyocytes were cultured in serum-free medium, the cardiomyocytes expressed both α1 and α3. In contrast, the α3 expression was gradually decreased and α2 was significantly increased when the cardiomyocytes were cultured in the medium supplemented with dexamethasone and thyroid hormones (37). It seems that the expression of Na,K-ATPase isoforms is posttranscriptionally regulated. The freshly isolated skeletal muscle from newborn rats expresses both α1 and α2, whereas primarily α1 from this tissue 1 day after culturing expression only the α1 isoform (38). In consideration of the fact that expression of Na,K-ATPase may be regulated by hormone or other growth factors, we have tested the expression of α3 in the tumor tissues derived from Panc-1 tumor–bearing mice. It was intriguing that the expression of α3 proteins were similar in both Panc-1 cells in culture as well as the Panc-1 xenograft tumor tissues, suggesting that the expression of Na,K-ATPase α3 isoform was not altered under different microenvironments and exhibited a much greater sensitivity to oleandrin.

Weidemann hypothesized that alterations in the metabolism of digitals-like steroid hormones and their interactions with Na,K-ATPase may influence malignancy (43). Support for this hypothesis is based on the various Na,K-ATPase isoforms exhibiting altered expression patterns in malignant versus normal cells. For example, β isoform (B1 and/or B2) expression patterns are down-regulated in certain human cancers, such as renal, lung, hepatocellular, gastric, and bladder cancers (21–23, 44). In contrast, the expression patterns of the α3 isoform of Na,K-ATPase isoforms is up-regulated in malignant tissues. For example, it has been reported that cardiac glycides such as digitoxin, digoxin, and UNBS1450 showed the strongest inhibition of the rat Na,K-ATPase α3β1 isoform followed by α2β1 and α1β1 isoforms growing in s9 insect cells. Their UNBS1450 compound inhibited Na,K-ATPase α1β1 isoform more 20 times more strongly than did either ouabain or diginoxin. Seligson et al. also reported that even though Na,K-ATPase α1 isoform and β1 levels were decreased in renal clear cell carcinoma compared with those in matched, morphologically normal proximal tubules, there was a tendency toward increased expression of these isoforms in high-grade tumors. Additionally, the higher expression of the α1 isoform in these patients seemed to correlate with a higher risk for disease-specific death (35). Our results do not seem to fully agree with this study, with one possible explanation being that the isoform expression profile is tissue-specific. Our data show that human pancreatic cancer cells with α1 expression and even a decrease from the expression of α3 or more sensitive to oleandrin than are cells that completely lack α3 expression (comparison between BxPC3 and Panc-2; Fig. 1). Upon examining α1 and α3 isoform protein expression in relation to oleandrin cytotoxicity using a variety of human cancer cells, those cells that expressed α3 protein were all more sensitive than were either rodent or human cells which did not express α3 protein. This relationship was also found within a given human tumor cell line; the relative expression of α3 was higher in cells which exhibited a much greater sensitivity to oleandrin compared with cells that had relatively lower expression of α3. These results suggest the importance of α3 relative to α1 on the sensitivity of human cancer cells to oleandrin treatment. This notion is supported following genetic manipulation in which α3 protein was knocked down with α3 siRNA or when α1 protein was restored with transfection of α1 cDNA to the cells that caused a corresponding loss in sensitivity to oleandrin.

Cardiac glycides bind to a given molecule and inhibit Na,K-ATPase activity. The expression of α3 relative to α1 was much lower in BxPC3 cells than in Panc-1 cells. Our data suggest that the uptake of oleandrin in these two different cell lines is likely to account for different antiproliferative activity. This is consistent with the study reported recently that the human leukemia cell line U-937 is capable of binding oleandrin in the cell membrane, whereas mouse NIH-3T3 cells did not bind oleandrin at all (9). We also examined the expression of α3 isoform protein in Panc-1 cells treated with oleandrin but did not observe any change in protein expression. The cardiac glycides activity in the oleandrin-resistant cell line was not altered either during in vitro culturing or during the development of disease (37–42). Arystarkhova and Sweadner reported that in rat heart, there was a postnatal switch in the expression of Na,K-ATPase isoforms. When rat cardiomyocytes were cultured in serum-free medium, the cardiomyocytes expressed both α1 and α3. In contrast, the α3 expression was gradually decreased and α2 was significantly increased when the cardiomyocytes were cultured in the medium supplemented with dexamethasone and thyroid hormones (37). It seems that the expression of Na,K-ATPase isoforms is posttranscriptionally regulated. The freshly isolated skeletal muscle from newborn rats expresses both α1 and α2, whereas primarily α1 from this tissue 1 day after culturing expression only the α1 isoform (38). In consideration of the fact that expression of Na,K-ATPase may be regulated by hormone or other growth factors, we have tested the expression of α3 in the tumor tissues derived from Panc-1 tumor–bearing mice. It was intriguing that the expression of α3 proteins were similar in both Panc-1 cells in culture as well as the Panc-1 xenograft tumor tissues, suggesting that the expression of Na,K-ATPase α3 isoform was not altered under different microenvironments and exhibited a much greater sensitivity to oleandrin.


