

Oleandrin-mediated inhibition of human tumor cell proliferation: Importance of Na,K-ATPase α 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 $\alpha 3$ subunit protein expression. Human pancreatic cancer cell lines were found to differentially express varying levels of $\alpha 3$ protein, but rodent cancer cells lacked discernable expression of this Na,K-ATPase isoform. A correlation was observed between the ratio of $\alpha 3$ to $\alpha 1$ isoforms and the level of oleandrin uptake during inhibition of cell growth and initiation of cell death; the higher the $\alpha 3$ expression relative to $\alpha 1$ 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 $\alpha 3$ was decreased by knocking down the expression of $\alpha 3$ isoform with $\alpha 3$ siRNA or increasing expression of the $\alpha 1$ isoform through transient transfection of $\alpha 1$ cDNA to the cells. Our data suggest that the relative lack of $\alpha 3$ (relative to $\alpha 1$) in rodent and some human tumor cells may explain their unresponsiveness to cardiac glycosides. In conclusion, the

relatively higher expression of $\alpha 3$ with the limited expression of $\alpha 1$ may help predict which human tumors are likely to be responsive to treatment with potent lipid-soluble cardiac glycosides such as oleandrin. [Mol Cancer Ther 2009;8(8):2319–28]

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 oleandrogenin inhibited proliferation and induced apoptosis due to an increase in intracellular Ca^{2+} via inhibition of Na,K-ATPase (5). Oleandrin and oleandrogenin also inhibited the export of fibroblast growth factor-2 through membrane interaction and inhibition of Na,K-ATPase activity (11). In addition, we reported 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 digitoxin 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 tumor necrosis factor- α /nuclear factor κB signaling pathway by blocking tumor necrosis factor- α -dependent TNFR1/TRADD complex formation (14). Oleandrin has also been shown to induce apoptosis in human leukemia cells by dephosphorylation of Akt, expression of FasL, 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 the mechanisms that might be responsible for the selective anticancer activity of this potent cardiac glycoside and the compounds related to it.

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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 uses ATP to provide the free energy necessary for driving K⁺ influx and Na⁺ efflux across the plasma membrane against electrochemical gradients (for a review, see ref. 15). 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. Its binding partner, the β -subunit, functions to stabilize the catalytic α -subunit and may also have regulatory activity. Four different α isoforms (α 1, α 2, α 3, and α 4) and three different β isoforms (β 1, β 2, and β 3) 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 α 1 binding affinity being low and the α 2 and α 3 isoforms being as much as 100-fold higher (25, 26). In species other than rodents, isoform differences to ouabain sensitivity are less marked yet are still found to vary under different physiologic conditions. At physiologic K⁺, the cardiotonic steroid may predominantly target α 2 and α 3 isoforms (25). Thus, cell sensitivity to oleandrin and other cardiac glycosides seems to relate to the Na,K-ATPase α isoform expression pattern.

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 by human cancer cells may 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 close correlation between specific subunit

content 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 acetoxyethyl 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 Mia-Paca), human colon cancer cell lines (CaCO-2, DOD-1, HCT-116, HT29, RKO, and LST174), 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 Stehlin 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 μ g/mL streptomycin, and 2 mmol/L of L-glutamine from Life Technologies (Invitrogen).

Cytotoxicity Determination

Cells were grown at a density of 1×10^4 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, inhibition 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.

Cellular Uptake of Cardiac Glycosides

Uptake of oleandrin in Panc-1 (highest expression of α 3 with poor expression of α 1 isoforms) cells and BxPC3 cells (poor expression of α 3 relative to α 1) 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 μ mol/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

Table 1. Description of human cancer cell phenotype and cell culture medium

Name of cells	Phenotype description	Cell culture medium
PANC-1	Human pancreatic carcinoma	DMEM/10% FBS
BXPC3	Human pancreatic adenocarcinoma	RPMI 1640/10% FBS/NaP
MiaPaca	Human pancreatic adenocarcinoma	DMEM/10% FBS/2% equine serum
MDA 231	Breast cancer	DMEM/10% FBS
SUM 149	Breast cancer	F12/5% FBS/HEPES/insulin/hydrocortisone
CaCO2	Colon carcinoma	RPMI 1640/10% FBS
DOD-1	Colon carcinoma	RPMI 1640/10% FBS
HCT 116	Colon carcinoma	RPMI 1640/10% FBS
HT 29	Colon carcinoma	RPMI 1640/10% FBS
LIS-174t	Colon carcinoma	MEM/10% FBS/NaP/NEAA
BRO	Human melanoma	MEM/10% FBS/NaP
SCC-9	Oral squamous cell carcinoma	DMEM/10% FBS
CAL27	Oral squamous cell carcinoma	DMEM/10% FBS
MCF-7	Breast cancer	MEM/10% FBS/insulin/hydrocortisone/EGF

Abbreviations: NaP, sodium pyruvate; FBS, fetal bovine serum; NEAA, nonessential amino acids; EGF, epidermal growth factor.

was achieved using a Quantix charged coupled device camera and IP Labs software (Scanalytics, Inc.). Alteration of oleandrin uptake in wild-type and Panc-1 cells transfected with $\alpha 3$ 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 μ mol/L) in phenol red-free DMEM for 15 min at 37°C. Cells were simultaneously incubated with MitoTracker orange CM-H₂XRos (1 μ mol/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

Cancer cells were established as monolayers on laminin-coated coverslips to perform immunofluorescence studies. Cells were treated with 1 μ mol/L of oleandrin for 72 h, then fixed in 1% paraformaldehyde prior to processing for immunofluorescence studies. Coverslips were immunolabeled with rabbit anti- $\alpha 3$ (Santa Cruz Biotechnology), and Alexa488-labeled secondary antibody (*green*; Molecular Probes) followed by counterstaining to detect DNA with DAPI (*blue*) and actin (Alexa 594-phalloidin, *red*). Slides were then image-analyzed as described above.

Determination of Na,K-ATPase Isoform $\alpha 3$ and $\alpha 1$ Expression

Cells were washed with cold PBS and scraped free in the presence of lysis buffer (20 mmol/L MOPS, 2 mmol/L

EGTA, 5 mmol/L EDTA, 30 mmol/L NaF, 40 mmol/L β -glycerophosphate, 20 mmol/L sodium pyruvate, 0.5% Triton X-100, and 1 mmol/L sodium orthovanadate with protease inhibitor cocktail). 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 \times g (10 min at 4°C). Protein levels were quantified via the Bio-Rad Dc protein assay (Bio-Rad, Inc.). Equal levels of protein (50 μ g) were applied to Bio-Rad precast gels or NuPAGE Novex precast bis-tris mini-gels (Invitrogen) and then 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 $\alpha 3$ (Affinity Bioreagents) and $\alpha 1$ (Upstate) isoforms diluted 1:2,000 in blocking buffer. Protein bands were visualized via chemiluminescence using the ECL+ detection kit and hyper-film (Amersham Biosciences). Equal loading of samples was illustrated 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 $\alpha 3$ siRNA

Panc-1 cells were plated in 6-well and 48-well plates and allowed to attach overnight. Transient transfection of non-specific siRNA (control siRNA) and $\alpha 3$ siRNA molecules was carried out using siPORT Amine Transfection Agent (Ambion) and $\alpha 3$ silencing RNA (30–75 nmol/L; Santa Cruz Biotechnology) following the instructions of the manufacturer. Twenty-four hours after transfection, cells were treated with 10 to 50 nmol/L of oleandrin, bufalin, and digoxin for 48 h. Protein was collected from the six-well plates after 72 h of transfection for Western blot analysis, and the assessment of cell viability affected by different treatments was carried out by Calcein AM staining.

Transfection of Panc-1 Cells with $\alpha 1$ cDNA

Panc-1 cells were plated in 6-well and 48-well plates and allowed to attach overnight. Alpha 1 cDNA (NM-00701.6) was purchased from Origene. DNA was prepared using Pure Link miniprep kit (Invitrogen). DNA was transfected

into Panc-1 cells. Briefly, 5 μ g of DNA was transfected using LipofectAMINE 2000 transfection agent (Invitrogen) according to the protocols of the manufacturer. Twenty-four hours after transfection, cells were treated with oleandrin at various concentrations. Cell death was quantified by calcein acetoxymethyl assay as described previously.

Statistical Analysis

Student's *t* test was used to determine the statistical differences between various experimental groups; *P* < 0.05 was considered to be significant.

Results

The Relationship of Oleandrin-Mediated Inhibition of Cell Proliferation to Na,K-ATPase Subunit Composition

Oleandrin differentially inhibited the proliferation of rodent (mouse Panc-02) and 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 IC_{50} of 5.6 nmol/L, whereas, mouse Panc-02 cells were the least sensitive (IC_{50} > 500 nmol/L). Among the four pancreatic cancer cell lines tested, the relative sensitivity to oleandrin based on the IC_{50} values was Panc-1 > MiaPaca > BxPC3 \gg Panc-02 (murine). We hypothesized that the relative expression of Na,K-ATPase subunits led to a differential cellular response to treatment with cardiac glycosides. To test this hypothesis, the protein expression of Na, K-ATPase α 3 and α 1 subunits in the four pancreatic cancer cell lines listed above was determined. Mouse pancreatic cancer Panc-02 cells were found to express only the α 1 iso-

form, whereas human pancreatic cancer Panc-1 and MiaPaca expressed high levels of α 3 with minimal expression of α 1. In contrast to Panc-1 and MiaPaca cells, the level of α 1 was much higher than α 3 in BxPC3 cells (Fig. 1B). Panc-1 and MiaPaca cells were more sensitive to oleandrin than either BxPC3 or Panc-02 cells, suggesting that oleandrin-mediated cytotoxicity correlated with the relative expression of α 3 to α 1 subunits. Similar results were also observed in human and rodent melanoma cells (Supplementary Fig. S1A). In order to confirm the validity of our assay on expression of α 3, the expression of α 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 α 1 protein, but no α 3. In contrast, the α 3 subunit protein was detected in mouse brain tissues, which has been reported previously (25).

We also examined the Na,K-ATPase subunit composition relative to oleandrin cytotoxicity in multiple types of human cancer cells, including those derived from colon (HT29, CaCo-2, RKO, HCT116, LS174T, and DOD1), breast (SUM149, MCF-7, and MDA-231), oral (SCC-9 and CAL-27), and lung (A549 and H1299) cells. Regardless of the tissue origin of the cell line, the higher the α 3 expression relative to α 1 expression, the more sensitive they were to growth inhibition by oleandrin. When the α 3 expression was relatively less than α 1 expression in any particular cell line regardless of the origin of cancer, the IC_{50} value of oleandrin was 150 ± 50 nmol/L. In comparison, when the relative expression of α 3 was higher than α 1 expression, oleandrin inhibition of cell proliferation was much stronger

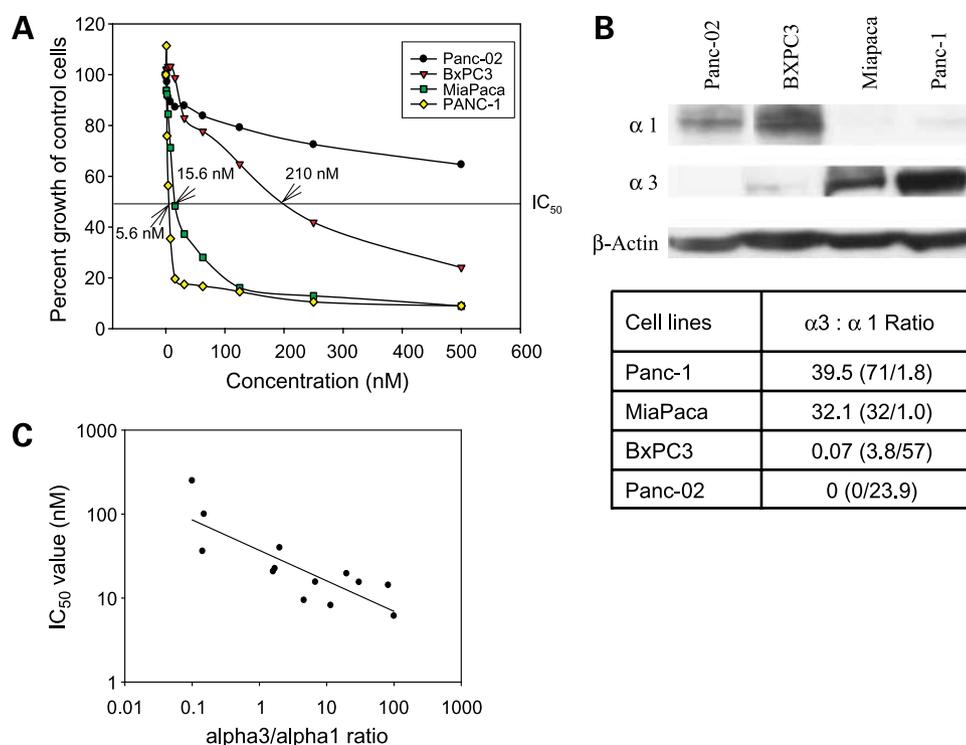
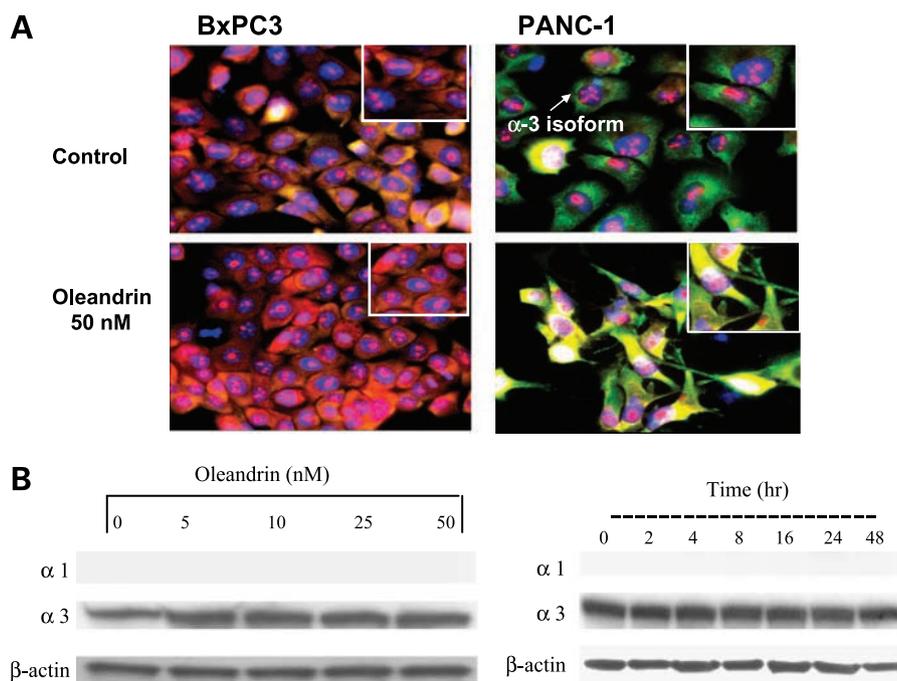


Figure 1. Oleandrin-mediated inhibition of cell proliferation association with Na^+, K^+ -ATPase expression patterns. **A**, human and murine pancreatic cancer cell lines were exposed to oleandrin (72 h) followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis. **B**, Western blot analysis of total cell lysates for $\alpha 3$ and $\alpha 1$ isoform expression patterns as well as the ratio of $\alpha 3$ to $\alpha 1$ isoforms. **C**, cumulative expression pattern in a series of 19 human pancreatic, colon, breast, oral, and ovarian tumor cell lines represented as a ratio of $\alpha 3$ to $\alpha 1$ isoforms as a function of oleandrin sensitivity. Points, mean \pm SD of the relative $\alpha 3$ to $\alpha 1$ ratio as a function of the cellular sensitivity to oleandrin (IC_{50} value).

Figure 2. Expression of $\alpha 3$ 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 $\alpha 3$ immunostaining, cells were counterstained with DAPI to detect nuclei (*blue*). BxPC3 cells contain low $\alpha 3$ levels whereas Panc-1 cells exhibit high $\alpha 3$ 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 $\alpha 3$ protein for at least 48 h at concentrations up to 50 nmol/L.



(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 $\alpha 3$ Subunits

The relative presence and distribution of the $\alpha 3$ isoform was then examined in Panc-1 and BxPC3 cell lines. The expression of $\alpha 3$ protein (*green*) fluorescence was scarcely detectable in BxPC3 cells, whereas in Panc-1 cells, there was marked $\alpha 3$ protein present as evidenced by abundant (*green*) fluorescence staining (Fig. 2A). At higher magnifications, it became apparent that the $\alpha 3$ 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 $\alpha 3$ subunit distribution in Panc-1 cells. Interestingly, $\alpha 3$ 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.

Changes in Expression of $\alpha 3$ or $\alpha 1$ 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 $\alpha 3$ -expressing Panc-1 cells, whereas in BxPC3 cells that lack $\alpha 3$, 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, $\alpha 3$ -containing Panc-1 cells exhibited morphologic changes that included an elongated phenotype which was not apparent in the more epithelioid BxPC3 cells lacking $\alpha 3$.

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 $\alpha 3$ -bearing Panc-1 cells within 2 hours but not in BxPC3 cells that lacked $\alpha 3$. After 24 hours of exposure to BODIPY-oleandrin, the fluorescence intensity was greater in $\alpha 3$ -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 $\alpha 3$ -expressing Panc-1 cells compared with treated BxPC3 cells lacking $\alpha 3$. This apparent colocalization of mitochondria with BODIPY-oleandrin was most striking in focal perinuclear deposits (see insets).

Changes in the Relative Expression of $\alpha 3$ or $\alpha 1$ Subunit Alter the Sensitivity of Cells to Oleandrin

Panc-1 cells were transfected with Na,K-ATPase $\alpha 3$ siRNA to suppress the expression of $\alpha 3$ protein. Decreased expression of $\alpha 3$ (51.2 ± 12.4%; bar graph represents

densitometry-derived data) was observed in $\alpha 3$ siRNA-transfected cells compared with either untransfected controls or nonspecific siRNA-transfected cells (Fig. 4A). The $\alpha 3$ protein expression decreased in a concentration-dependent manner with increasing $\alpha 3$ siRNA concentrations (Fig. 4A, *bottom*). In contrast, the expression of $\alpha 1$ protein was not affected by transfection of $\alpha 3$ siRNA (Fig. 4A, *bottom*). The $\alpha 3$ siRNA-mediated reduction in $\alpha 3$ protein decreased BODIPY-oleandrin uptake (Fig. 4B). When $\alpha 3$ siRNA-transfected, control siRNA-transfected, and untransfected cells were treated with increasing concentrations of unlabeled oleandrin (5–25 nmol/L), the knock-down of $\alpha 3$ protein rescued cells from death as evidenced by differences in IC_{50} levels (IC_{50} 50 nmol/L, $\alpha 3$ siRNA-transfected versus IC_{50} 17.2 and 7.5 nmol/L, control siRNA-transfected and untransfected cells, respectively; Fig. 4C). Additionally, similar observations have been made in cells treated with other lipid-soluble cardiac glycosides such as bufalin and digoxin (Supplementary Fig. S2). These data suggest that the relative expression of $\alpha 3$ protein significantly influences cell sensitivity to oleandrin treatment.

To further confirm the important role of $\alpha 3$ protein in relation to $\alpha 1$ in cardiac glycoside-induced cell growth suppression, Panc-1 cells were transfected with $\alpha 1$ cDNA and then treated with oleandrin. Results in Fig. 5A show that $\alpha 1$ cDNA-transfected Panc-1 cells did express the Na,K-ATPase subunit $\alpha 1$ compared with that of nontrans-

fected Panc-1 cells. The relative expression of $\alpha 3$ in $\alpha 1$ -transfected Panc-1 cells was reduced by ~80% in comparison with that of nontransfected Panc-1 cells. The antiproliferative activity of oleandrin in Panc-1 cells transfected with $\alpha 1$ cDNA was reduced as evidenced by the >10-fold increase in IC_{50} values from 4.7 nmol/L against nontransfected Panc-1 cells to >50 nmol/L in $\alpha 1$ cDNA-transfected Panc-1 cells. However, the trend of inhibition of cell proliferation in both $\alpha 1$ -transfected and nontransfected cells was similar, suggesting that $\alpha 1$ protein expression is less important than that of $\alpha 3$ for oleandrin-induced cell death in this particular cell line.

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). 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 binding capability of α

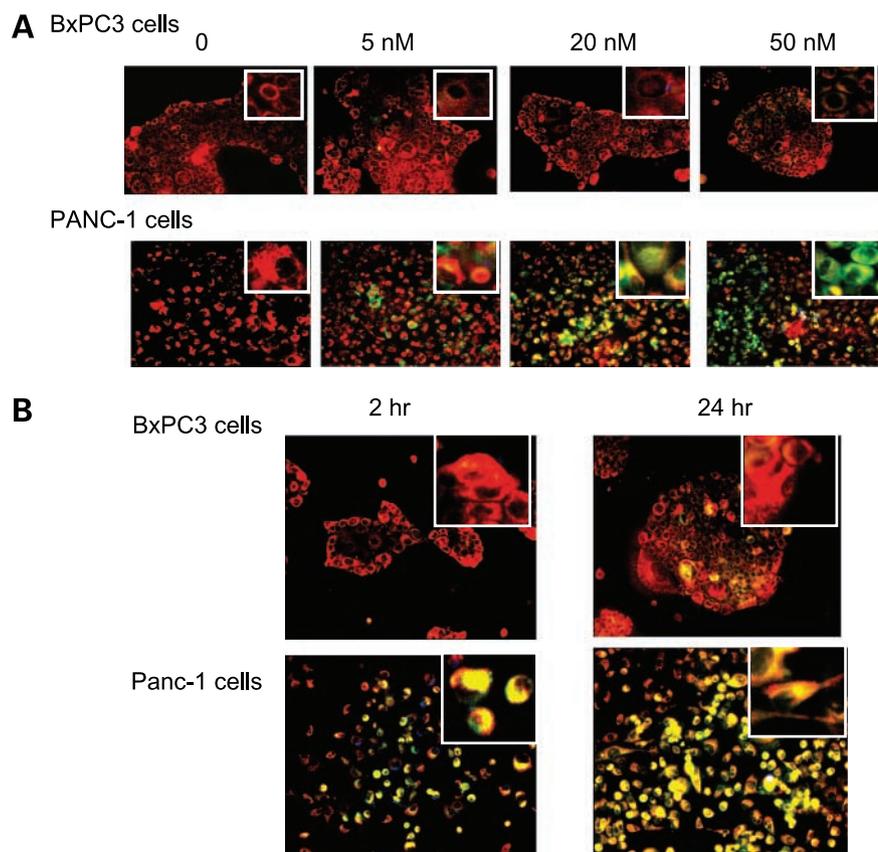
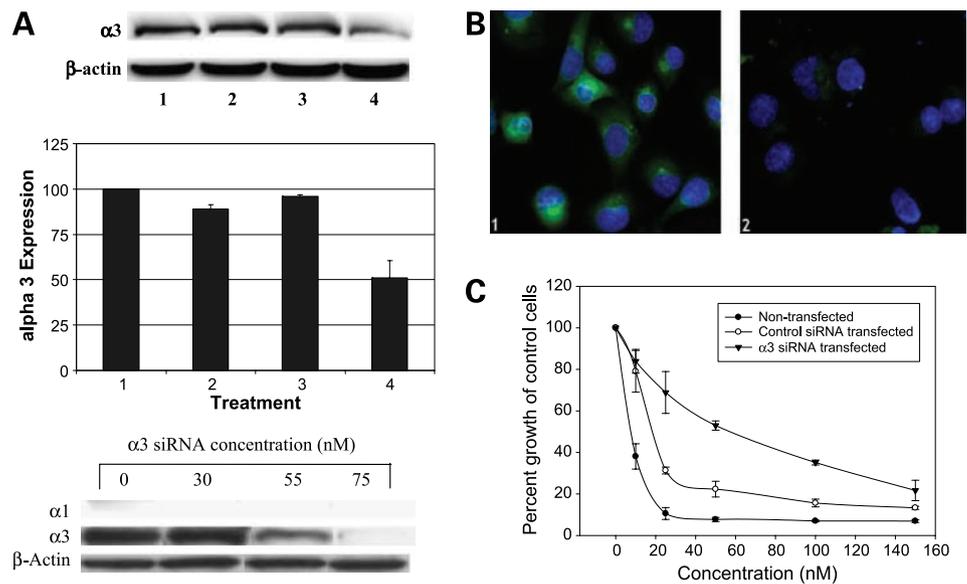


Figure 3. Uptake of fluorescent oleandrin analogue (BODIPY-oleandrin) by living cells. **A**, concentration-dependent uptake of oleandrin within BxPC-3 human pancreatic cells was observed at concentrations as high as 50 nmol/L over the 24 h observation period. In contrast, drug uptake was easily noted 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.

Figure 4. Decreasing the expression of $\alpha 3$ protein in Panc-1 cells affects cell sensitivity to oleandrin. **A**, cells were transfected for 24 h with either control siRNA or $\alpha 3$ siRNA. As shown, this resulted in an approximately 50% reduction of $\alpha 3$ protein. The extent of the reduction was dependent on the concentration of the $\alpha 3$ siRNA agent. 1, nontransfected control; 2, cells transfected with transfection agent alone; 3, control siRNA-transfected cells; 4, $\alpha 3$ siRNA-transfected cells. **B**, oleandrin uptake or association with Panc-1 cells is clearly evident in the nontransfected cells (1) yet significantly reduced in the siRNA-treated cells (2). **C**, the cells exhibiting knockdown of $\alpha 3$ protein were less sensitive to oleandrin implying that the $\alpha 3$ target is necessary to retain sensitivity to this cardiac glycoside.



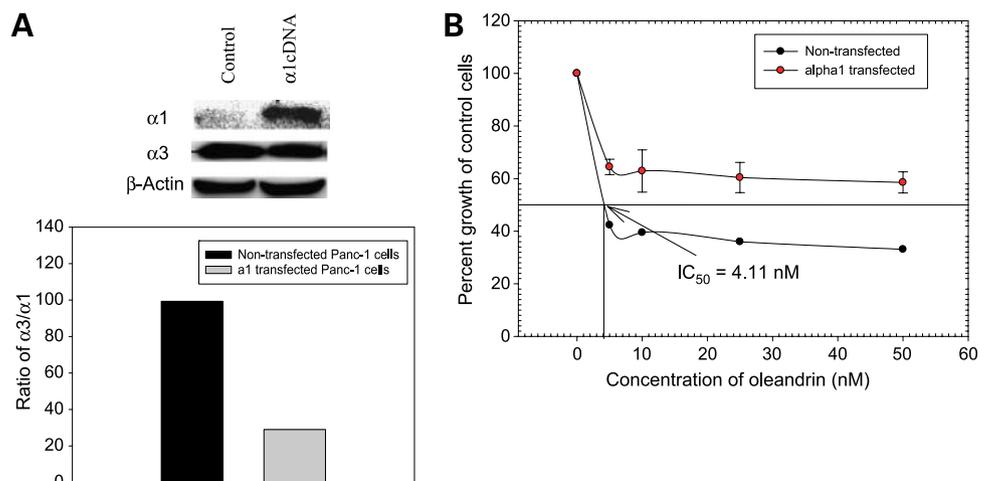
isoforms to cardiac glycosides varies considerably under different physiologic conditions (25, 26). These findings prompted us to determine if the differential anticancer activity of oleandrin between human and rodent cancer cells or among specific human cancer cell lines is associated with a particular 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.

We report here that pancreatic cells exhibit distinct differential sensitivities to oleandrin treatment. Additionally, evidence is provided that rodent tumor cells express the Na,K-ATPase $\alpha 1$ isoform, but not the $\alpha 3$ isoform, whereas a majority of human cancer cells we have examined expressed both $\alpha 1$ and $\alpha 3$ isoforms. Although the relative ex-

pression of $\alpha 3$ to $\alpha 1$ isoforms falls into a wide range, the values are remarkably higher in human cancer cells than that in rodent tumor cells. The higher the $\alpha 3$ expression in human tumor cells, the more sensitive they are to oleandrin treatment as evidenced by lower IC_{50} values. When expression of $\alpha 3$ isoform was reduced by transfecting Panc-1 cells with $\alpha 3$ siRNA, the inhibitory effect of oleandrin was decreased. Likewise, in Panc-1 cells transfected with $\alpha 1$ cDNA (altering the ratio of $\alpha 1/\alpha 3$) the inhibition of cell proliferation by oleandrin was reduced. These results suggest an important role of $\alpha 3$ in anticancer activity of oleandrin in human cancer cells.

The α subunit is a multispanning membrane protein that catalyzes ion transport and contains binding sites for cations, ATP, and cardiac glycosides (25–28). The four α isoforms of Na,K-ATPase ($\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 4$), are each derived from separate genes. Across species, the degree of

Figure 5. The antiproliferative activity of oleandrin was reduced in Panc-1 cells transfected with $\alpha 1$ subunits. By transfecting Na, K-ATPase $\alpha 1$ subunit to Panc-1 cells (which normally lack $\alpha 1$ expression), the relative expression of $\alpha 3$ to $\alpha 1$ 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 IC_{50} value of oleandrin from 4.7 nmol/L of nontransfected Panc-1 cells to >50 nmol/L in $\alpha 1$ -transfected cells.



homology for the $\alpha 1$ and $\alpha 2$ isoforms has been reported to be 92%, and is >96% for $\alpha 3$. One obvious difference among the isoforms is in the response to cardiac glycosides. In the rat, the $\alpha 1$ isoform was reported to be 100-fold more resistant to ouabain than either $\alpha 2$ or $\alpha 3$ (19, 25, 29, 30). In contrast, in other species, including humans, the affinity of $\alpha 1$ isoforms to cardiac glycosides is higher, causing a greater sensitivity to ouabain (31–33). Additionally, the rodent $\alpha 1$ isoform is almost 1,000 times less sensitive to cardiac glycosides than that in the human due to a double mutation in the first extracellular loop (18). After the assessment of adenocarcinomas and squamous cell carcinoma tissues from 59 patients with lung cancer, Mijatovic et al. suggested that the $\alpha 1$ isoform of Na,K-ATPase may be a useful target for cardiac glycosides in the treatment of non-small cell lung cancer cells (34). They reported that cardiac glycosides such as ouabain, digoxin, and UNBS1450 showed the strongest inhibition of the rat Na,K-ATPase $\alpha 3\beta 1$ isoform followed by $\alpha 2\beta 1$ and $\alpha 1\beta 1$ isoforms growing in sf9-insect cells. Their UNBS1450 compound inhibited Na,K-ATPase $\alpha 1\beta 1$ isoforms 200 times more strongly than did either ouabain or digoxin. Seligson et al. also reported that even though Na,K-ATPase $\alpha 1$ isoform and $\beta 1$ 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 $\alpha 1$ 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 $\alpha 1$ expression and even a minimum expression of $\alpha 3$ are more sensitive to oleandrin than are cells that completely lack $\alpha 3$ expression (comparison between BxPC3 and Panc-02; Fig. 1). Upon examining $\alpha 1$ and $\alpha 3$ isoform protein expression in relation to oleandrin cytotoxicity using a variety of human cancer cells, those cells that expressed $\alpha 3$ protein were all more sensitive than were either rodent or human cells which did not express $\alpha 3$ protein. This relationship was also found within a given human tissue type when the relative expression of $\alpha 3$ was higher than $\alpha 1$ cells which exhibited a much greater sensitivity to oleandrin compared with cells that had relatively lower expression of $\alpha 3$. These results suggest the importance of $\alpha 3$ relative to $\alpha 1$ on the sensitivity of human cancer cells to oleandrin treatment. This notion is supported following genetic manipulation in which $\alpha 3$ protein was knocked down with $\alpha 3$ siRNA or when $\alpha 1$ protein was restored with transfection of $\alpha 1$ cDNA to the cells that caused a corresponding loss in sensitivity to oleandrin.

Cardiac glycosides bind to a given α molecule and inhibit Na,K-ATPase activity. The expression of $\alpha 3$ relative to $\alpha 1$ was much lower in BxPC3 cells than Panc-1 cells. Our data suggest that the uptake of oleandrin in these two different cell lines is likely to account for differential 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 $\alpha 3$ isoform protein in Panc-1 cells treated with oleandrin but did not observe any change in protein expression. The cardioglycoside ouabain increased the amount of $\alpha 3$, but not $\alpha 1$, in detergent-resistant membrane microdomain fractions prepared from the synaptic plasma membrane fraction of rat brain (36). The differential effect of $\alpha 3$ isoform protein expression by oleandrin and ouabain might result from the markedly different concentrations of oleandrin (20 nmol/L) versus ouabain (100 μ mol/L) used in the two different experiments.

The biological expression patterns of the various Na,K-ATPase isoforms are subject to developmental and hormonal cues that become 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 $\alpha 1$ and $\alpha 3$. In contrast, the $\alpha 3$ expression was gradually decreased and $\alpha 2$ 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 $\alpha 1$ and $\alpha 2$, whereas primary cells from this tissue 1 day after culturing express only the $\alpha 1$ 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 $\alpha 3$ in the tumor tissues derived from Panc-1 tumor-bearing mice. It was intriguing that the expression of $\alpha 3$ 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 $\alpha 3$ isoform was not altered under different microenvironments (Supplementary Fig. S3).

Weidemann hypothesized that alterations in the metabolism of digitalis-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 ($\beta 1$ and/or $\beta 2$) 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 $\alpha 1$ isoform seem to vary more in relation to the tissue site. For instance, certain of these same studies also reported no significant change in the expression patterns of $\alpha 1$ isoform associated with lung or renal cancers (21, 44), whereas other examples from these same studies reported decreased expression of the $\alpha 1$ isoform in human gastric and bladder cancers (23, 24). In the colon, the expression of $\alpha 1$ isoform was decreased in carcinoma compared with normal control samples, whereas the expression of $\alpha 3$ isoform protein was increased in 13 of 17 carcinomas (76%) compared with normal samples (45). Additionally, earlier reports showed that Na,K-ATPase activity was altered in malignant compared with normal cells (46–48). Thus, the

relative expression patterns of specific α subunits may play an important role in cardiac glycoside-induced tumor inhibition. The mechanism that drives the phenotypic switch in the expression of α isoforms during colon carcinogenesis requires further study.

As a whole, our studies suggest that $\alpha 3$ subunits of Na,K-ATPase may serve as a novel target for cardiac glycoside therapy of cancer. The synthesis of a cardiac glycoside analogue that inhibits tumor cell proliferation (or the selective extract of a plant containing a potent cardiac glycoside), whereas sparing normal heart tissue, is likely to result in a more targeted agent. To this end, a modified supercritical CO₂ extract of *N. oleander* is currently under clinical evaluation (clinical phase I trial, NCT00554268).

Disclosure of Potential Conflicts of Interest

P. Yang and R. Newman: paid consultants, Phoenix Biotechnology, Inc. R. Newman: employee, NewChapter, Inc.; consultant, Nerium Biotechnology. No other potential conflicts of interest were disclosed.

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