Autophagic Cell Death of Human Pancreatic Tumor Cells Mediated by Oleandrin, a Lipid-Soluble Cardiac Glycoside

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Lipid-soluble cardiac glycosides such as bufalin, oleandrin, and digitoxin have been suggested as potent agents that might be useful as anticancer agents. Past research with oleandrin, a principle cardiac glycoside in *Nerium oleander* L. (Apocynaceae), has been shown to induce cell death through induction of apoptosis. In PANC-1 cells, a human pancreatic cancer cell line, cell death occurs not through apoptosis but rather through autophagy. Oleandrin at low nanomolar concentrations potently inhibited cell proliferation associated with induction of a profound G_2/M cell cycle arrest. Inhibition of cell cycle was not accompanied by any significant sub G1 accumulation of cells, suggesting a nonapoptotic mechanism. Oleandrin-treated cells exhibited time- and concentration-dependent staining with acridine orange, a lysosomal stain. Subcellular changes within PANC-1 cells included mitochondrial condensation and translocation to a perinuclear position accompanied by vacuoles. Use of a fluorescent oleandrin analog (BODIPY-oleandrin) revealed co-localization of the drug within cell mitochondria. Damaged mitochondria were found within autophagosome structures. Formation of autophagosomes was confirmed through electron microscopy and detection of green fluorescent protein–labeled light chain 3 association with autophagosome membranes. Also observed was a drug-mediated inhibition of pAkt formation and up-regulation of pERK. Transfection of Akt into PANC-1 cells or inhibition of pERK activation by MAPK inhibitor abrogated oleandrin-mediated inhibition of cell growth, suggesting that the reduction of pAkt and increased pERK are important to oleandrin's ability to inhibit tumor cell proliferation. The data provide insight into the mechanisms and role of a potent, lipid-soluble cardiac glycoside (oleandrin) in control of human pancreatic cancer proliferation.

Keywords: *autophagy; oleandrin; autophagosome; mitochondria; pancreatic cancer;* Nerium oleander; *cardiac glycoside*

The use of cardiac glycosides in the treatment of human malignant disease may provide an interesting as well as novel form of therapy.[1-3] For example, oleandrin (Figure 1), the principal cytotoxic component of *Nerium oleander*, has been shown by several laboratory groups, including our own, to mediate cell death in human but not murine cell lines.[4,5] The mechanisms by which oleandrin selectively controls malignant but not normal cell proliferation may be related to a preferential decreased activation of transcription factors such as nuclear transcription factor-κB (NF-κB) and activator protein-1,[6] alteration of membrane potential and fluidity,[5,7] activation of MAPK and JNK pathways,[7] increased calcineurin content with subsequent FasL expression,[8] up-regulation of death receptors 4 and 5,[9] and induction of reactive oxygen species (ROS) and oxidative stress[10] in tumor cells. Collectively, these mechanisms have been associated with induction of apoptosis and cell death in a wide variety of human tumor cell lines (eg, Jurkat, U-937, HL-60, HeLa, PC3, and MCF-7). Although we also have reported[11] oleandrin-mediated apoptosis in a human prostate cell line (PC3), our recent research with human pancreatic cancer cells has strongly suggested that oleandrin mediates cell death through an alternate cell death pathway. The purpose of the present series of studies was to investigate the mechanisms of human tumor pancreatic cancer cell death produced by a lipid-soluble cardiac glycoside, oleandrin.

Initial examination of the subcellular morphology of a series of malignant human cell lines suggested that degradation pathways common to autophagy might be involved. For example, oleandrin-mediated early mitochondrial condensation associated with ROS production in human melanoma cells indicated a cellular

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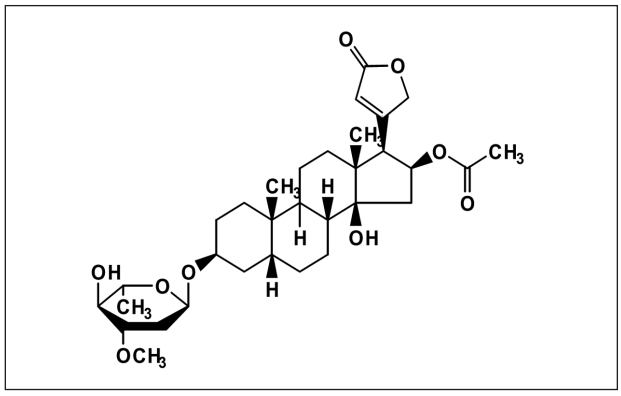

Figure 1 Structure of oleandrin.

attempt to clear these dysfunctional organelles.[10] This was particularly apparent after examination of a number of human pancreatic cancer cell lines following exposure to this lipid-soluble cardiac glycoside. Research presented here demonstrates potent oleandrin-mediated G_2/M cell cycle arrest and lack of accumulation of human pancreatic cancer cells in the sub G1 population, mitochondrial condensation and disaggregation, formation of autophagosomes, and the clear presence of light chain 3 (LC3)-II, a hallmark autophagy related protein. In addition, we demonstrate that oleandrin-mediated cell injury and autophagy in PANC-1 cells may be mediated through a potent decrease in the Akt/mammalian target of rapamycin (mTOR) pathway as evidenced by inhibition of phosphorylation of Akt and coordinate increased expression of the pERK of Raf-1/MEK1/2/ERK1/2-related pathway. Finally, the involvement of PI3kinase and MAPK pathways in oleandrin-induced autophagic cell death is evidenced by selective blocking of oleandrin-mediated changes in these cell signal transduction pathways that significantly reduces the ability of oleandrin to mediate tumor cell death. These data provide important insights into mechanisms of action of this potent cardiac glycoside as an antitumor agent.

Materials and Methods

Antibodies and Reagents

Anti-Akt and anti-phospho-Akt at Ser^{473} were purchased from Cell Signaling Technology (Beverly, Mass). Anti-ERK and anti-phosphorylated ERK at Thr^{202}/Tyr^{204}were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). LC3 antibodies were provided by Yasuko Kondo's laboratory (M. D. Anderson Cancer Center, Houston, Tex). acridine orange, Adriamycin, oleandrin, and anti-β-actin were purchased from Sigma Chemical Co (St. Louis, Mo). The purity of oleandrin was confirmed to be >99% through analyses using both high-performance liquid chromatography–electrospray tandem mass spectrometry and Micromass Q-Tof time of flight mass spectrometry determinations. The fluorescent oleandrin product, BODIPY-oleandrin, was custom synthesized for us by Molecular Probes (Carlsbad, Calif).

Cell Culture and Cytotoxicity Determination

PANC-1 human pancreatic cancer cells were purchased from the American Type Culture Collection (Manassas, Va). Cells were cultured in Dulbecco's modified eagle media supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, Calif), 50 IU/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine from GIBCO (Invitrogen) at 37°C in 5% CO_2. Relative inhibition of cell proliferation by oleandrin and Adriamycin was determined after 72 hours of continuous drug exposure of a series of concentrations of the drugs of interest. The MTT assay was used as previously described[10] to assess cell growth relative to untreated PANC-1 cell proliferation.

Cell Signal Transduction

PANC-1 cells were treated in serum-free conditions with 5, 10, 25, and 50 nM oleandrin for 24 hours. Cells were then washed with cold phosphate-buffered saline (PBS) and scraped with a lysis buffer, containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM b-glycerophosphate, 20 mM sodium pyruvate, 0.5% triton X-100, and 1 mM sodium orthovanadate with 1× protease inhibitor cocktail (Sigma, St. Louis, Mo). Lysates were then sonicated on ice for 3 minutes, incubated for 10 minutes, and centrifuged at (14 000 rpm) for 10 minutes at 4°C. Protein levels were quantified via the BioRad Dc protein assay (BioRad, Inc, Hercules, Calif). Equal levels of protein (50 μg) were fractionated on precast gels (BioRad) and then transferred on polyvinylidene difluoride membranes, according to standard methods. Following a 1- to 2-hour incubation in 5% nonfat dry milk blocking buffer prepared in Tris-buffered saline with 0.1% Tween 20 (TBS-T), membranes were probed with primary antibodies diluted 1:2000 in blocking buffer. Protein bands were visualized via chemiluminescence, using the ECL+ detection kit and hyper-film (Amersham Biosciences, Piscataway, NJ). Equal loading of samples was illustrated by Western blotting for β-actin content.

Cell Cycle Analysis

Cells (5×10^5) grown in 60-mm dishes were treated with oleandrin (0, 20, and 40 nM) for 24 hours. Cells were trypsinized, washed in PBS, and fixed overnight in 70% ethanol at 4°C. They were then washed and resuspended in PBS containing 50 μg/mL of propidium iodide (PI) and 20 μg/mL of deoxyribonuclease-free ribonuclease. Cells were then incubated in the dark for

30 minutes at room temperature before analysis by fluorescence-activated cell-sorting analysis (FACS) using a Coulter Epics-XL (Coulter Corp, Fullerton, Calif). The percentage of cells in each phase of the cell cycle was then estimated from the DNA histogram content.

Transmission Electron Microscopy

To detect the induction of autophagy morphologically in oleandrin-treated cells, cellular structural alteration of PANC-1 cells was examined by transmission electron microscopy (TEM). Cells were grown on glass coverslips for 24 hours, where they reached about 70% confluence before treatment with 20 nM oleandrin for 8 hours. Cells were washed with cold PBS and prepared for electron microscopy. This included fixation with a solution of 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 hour. Samples were then postfixed in 1% OsO_4 in the same buffer for 1 hour and then subjected to electron microscopic analysis. Representative areas were chosen for ultrathin sectioning and viewed with a JEM 1010 transmission electron microscope (JEOL, Peabody, Mass) at an accelerating voltage of 80 kV. Digital images were obtained with an AMT imaging system (Advanced Microscopy Techniques, Danvers, Mass).

Determination of Autophagy

Examination of autophagic cell death induced by oleandrin was carried out by acridine orange staining and determination of alteration of microtube-associated protein 1 LC3-I and -II proteins as previously described.[12] Paglin et al[13] described autophagy as a process of sequestering cytoplasmic proteins into the lytic component, which is characterized by the formation and promotion of acidic vesicular organelles (AVOs). To detect AVOs, a hallmark of autophagy, cells were treated with 20 nM oleandrin for various periods of time and then stained with acridine orange (1 μg/mL) for 15 minutes, collected by trypsinization, and then subjected to analysis using a FACScan flow cytometer and Cell Quest software (Beckton Dickson, San Jose, Calif).

Expression of LC3-II (as derived from LC3-I) is closely associated with the membranes of autophagosomes.[14] For detection of LC3, soluble proteins were isolated from oleandrin-treated and untreated PANC-1 cells. Equal amounts of protein were separated by 10% SDS-PAGE gel (Bio-Rad, Richmond, Calif) and then transferred on polyvinylidene difluoride membranes, according to standard methods. The membranes were treated with primary antibodies to LC3-I and II (courtesy of Yasuko Kondo, M. D. Anderson Cancer Center, Houston, Tex) overnight at 4°C and incubated for 1 hour with a horseradish peroxidase conjugated anti-mouse secondary antibody (1:3000 dilution; GE Healthcare, Piscataway, NJ) at room temperature for 1 hour. Anti-LC3 antibody against a synthetic peptide corresponding to the N-terminal 14 amino acids of isoforms B LC3 and an additional cysteine (PSEKTFKQRRTFEQC) was prepared by immunization of rabbit and was affinity purified on an immobilized peptide-Sepharose column (Covance, Denver, Pa). Bound antibody complexes were detected using an enhanced chemiluminescence reagent (GE Healthcare) according to the manufacturer's instructions.

The green fluorescent protein (GFP)-tagged microtubule-associated protein 1 LC3 expression vector was kindly provided by Dr Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). LC3 is recruited to the autophagosome membrane during autophagy.[15] Therefore, GFP-tagged LC3-expressing cells have been used to demonstrate induction of autophagy.[16,17] GFP-LC3 cells present a diffuse distribution under control conditions, whereas a punctuate pattern of GFP-LC3 expression is indicative of autophagy. Cells were transiently transfected with the GFP-LC3 vector using Fugene 6 transfection reagent (Roche, Alameda, Calif). After overnight culture, cells were treated with 20 nM oleandrin for 72 hours, fixed with 4% paraformaldehyde, and examined under a fluorescence microscope. To quantify autophagic cells after oleandrin treatment, the number of autophagic cells demonstrating punctuate GFP-LC3 staining among 200 cells was determined.

To determine the relationship of the MAPK pathway to oleandrin-induced autophagic cell death, PANC-1 cells were treated with ERK activation inhibitor peptide I (ERK I, 10 μM) and 25 μM PD98059, a potent MAPK inhibitor (Calbiochem, La Jolla, Calif). Fifteen minutes following addition of the inhibitors, oleandrin (20 nM) was added and cells were incubated for 24, 48, or 72 hours. LC3 protein expression was assessed according to the method described above. To determine whether the inhibition of MAPK pathway abrogated the autophagic cell death induced by oleandrin, cell viability was also assessed by calcein AM (CAM) assay as described previously.[18] Briefly, PANC-1 cells (1×10^4) were plated on 96-well plates. At 24 hours, serum-free medium was added. Cells were then pretreated with 10 μM ERK activation inhibitor peptide I (ERK I) and 25 μM PD98059, respectively, followed by the addition of oleandrin (5-50 nM). Twenty-four hours after drug treatment, 1 μM calcein AM (Molecular Probes, Eugene, Ore) was added, and samples were incubated at 25°C for 15 minutes. Fluorescence intensity was then read with an FLX 800 Fluorescence Plate Reader (Bio-Tek Instruments Inc, Winooski, Vt) with excitation

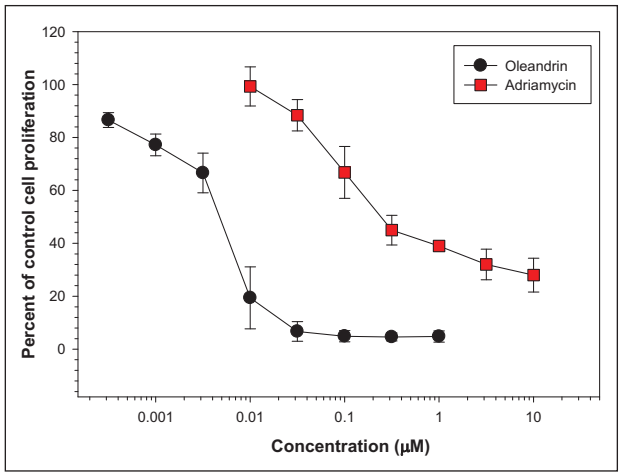

Figure 2. Oleandrin-mediated inhibition of PANC-1 cell proliferation. PANC-1 cells (1×10^4) were allowed to attach overnight and then were treated with oleandrin and Adriamycin at indicated concentrations. The antiproliferative activity was determined with MTT assay. Data are presented as mean ± SD of 3 independent experiments.

and emission wavelengths of 485 nm and 528 nm, respectively.

Active Akt Transfection

AKT DNA constructs were kindly provided by Dr Yiling Lu of M. D. Anderson Cancer Center Houston, Texas. DNA was purified from *Escherichia coli*–transformed AKT constructs using the Purelink plasmid miniprep kit (Invitrogen, Carlsbad, Calif). Plasmid DNAs were then transfected into PANC-1 cells using lipofectamine (Invitrogen), according to the manufacture's protocol. Twenty-four hours after transfection, cells were treated with varying concentrations of oleandrin (10-20 nM) for 48 hours. Cell viability of transfected cells versus nontransfected cells was compared using the CAM assay as described previously. The status of Akt and phosphorylation of Akt in the transfected cells was also assessed by Western blot analysis.

Results

Oleandrin-Mediated Inhibition of PANC-1 Cell Proliferation

We reported previously that oleandrin is a potent inhibitor of growth of human melanoma BRO cells with an IC_{50} of 0.03 μM.[10] Here we examined the cytotoxic effect of oleandrin and Adriamycin against human pancreatic cancer PANC-1 cells. As shown in Figure 2, oleandrin markedly inhibited the growth of PANC-1 cells with an IC_{50} value 0.005 μM, which is comparable to what we reported with BRO cells. Interestingly, the antiproliferative activity of oleandrin is about 60-fold greater than that of Adriamycin (IC_{50}, 0.3 μM).

Effect of Oleandrin on Cell Cycle

To investigate whether oleandrin-induced cell death was mediated through cell cycle alteration or apoptosis, DNA flowmetric analysis was performed. As shown in Figure 3A, oleandrin treatment (10 nM) increased the population of the G_2/M phase by 2-fold compared with that of control; this effect was concentration dependent. Inhibition of the cell cycle occurs without any apparent induction of apoptosis as evidenced by the lack of cells in sub-G_1. These results indicate that the cytotoxic effect of oleandrin is not attributable to the induction of apoptosis.

Induction of Autophagy in Oleandrin-Treated PANC-1 Cells

The potent inhibition of proliferation of oleandrin in PANC-1 cells without accompanying apoptosis suggested that an alternate cell death pathway could be involved. Here we examined the role of autophagy in PANC-1 cells treated with oleandrin. To identify the development of AVO, a characteristic of autophagy, we used acridine orange, a dye known to concentrate in acidic lysosomal vesicles as well as autophagic vacuoles. As shown in Figure 3B, there was a clear time-dependent increase in acridine orange uptake, presumably into lysosomes and acidic vacuoles within the cells reaching a maximum of 39% of the cell population by 72 hours. This was a nearly 6-fold increase in acridine orange staining above baseline levels.

Oleandrin-Mediated Subcellular Changes

Exposure of human tumor cells to oleandrin previously has been shown to shrink and destroy mitochondria, most notably by mitochondrial condensation.[10] Similar changes were noted in PANC-1 cells in this study. As seen in Figure 4, the normal architecture and distribution of mitochondria within cells (A) are followed by marked drug-mediated (exposure to 20 nM oleandrin for 8 hours) mitochondria condensation (subsets to panels A-C) as well as distinct mitochondrial relocation to a perinuclear location (B and C). In addition, incubation of cells with oleandrin (20 nM for 8 hours) indicated a marked increase in cell vacuole formation. These data indicate a role of mitochondria in oleandrin-mediated cellular injury.

To assess whether oleandrin was directly associated with mitochondria within cells, a fluorescent analog of oleandrin (BODIBY-oleandrin) was prepared and incubated with cells. As seen in Figure 4B, the green oleandrin analog (subpanel A) co-localized with Mitotracker, a selective orange mitochondrial dye (subpanel B). Little drug is apparent in cell nuclei

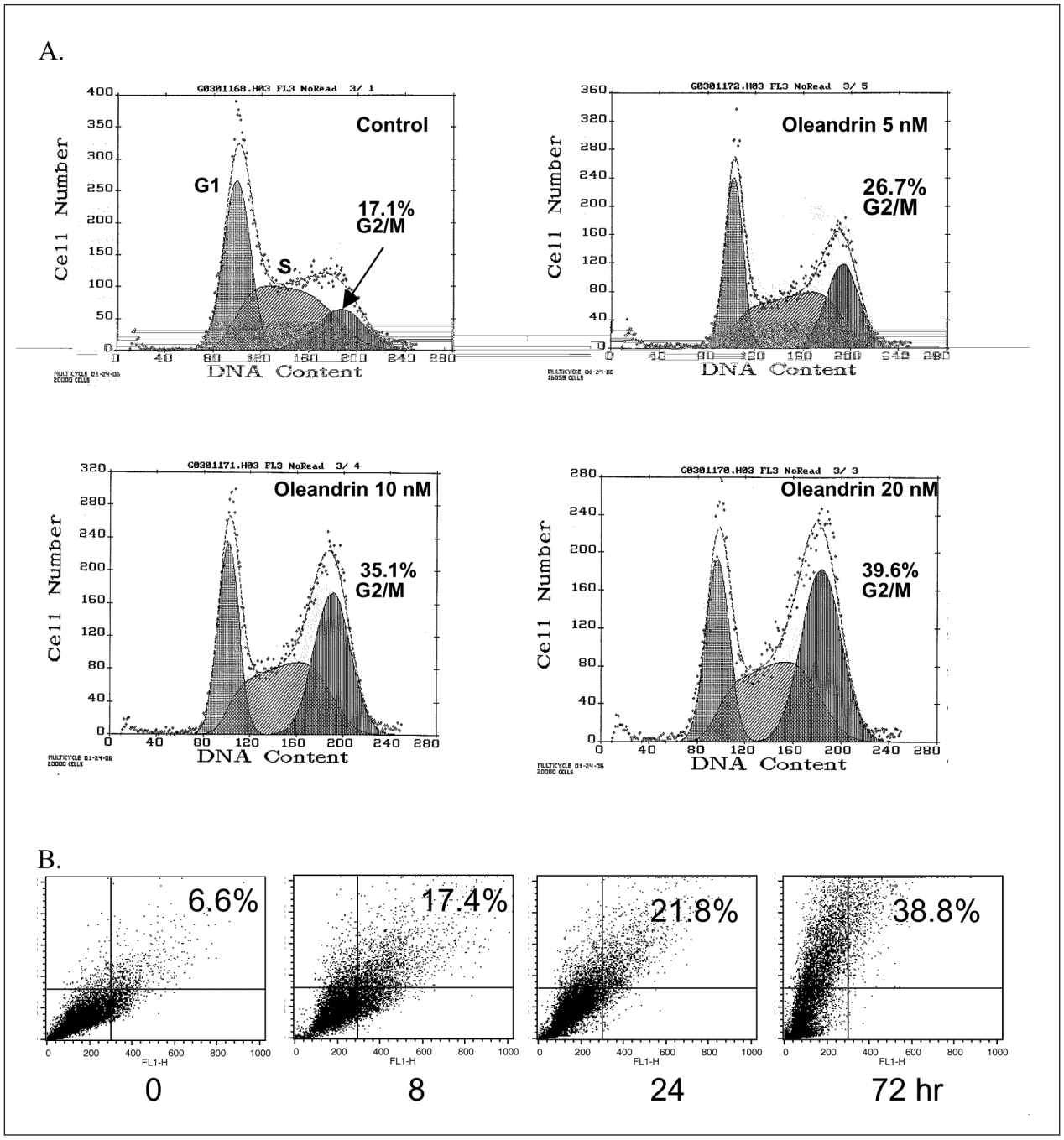

Figure 3. Cell cycle and staining with acridine orange to detect acidic vesicular organelles (AVO) after treatment with oleandrin. (A) Cell cycle analysis. Cells were treated with oleandrin (5-20 nM) for 24 hours and subjected to cell cycle analysis. Cell cycle showing G_2/M arrest with lack of sub G1 cell population indicating little evidence of apoptosis. The results shown are representative of 3 separate experiments. (B) Time-dependent acridine orange staining of AVO. Cells were treated with 20 nM oleandrin and subjected to acridine orange staining for the indicated length of time.

stained blue in subpanel C. Analyses of the Mitotracker and fluorescent oleandrin analog dyes suggest a similar distribution and hence an association of drug with this cellular organelle.

Direct examination of PANC-1 cells exposed to oleandrin by TEM revealed injured mitochondria as evidenced at an early stage by atypical "whirled" organelle structures. The appearance of numerous autophagosome structures, some with remnants of what appear to be mitochondria and other cellular membranous debris, is apparent in Figure 4C.

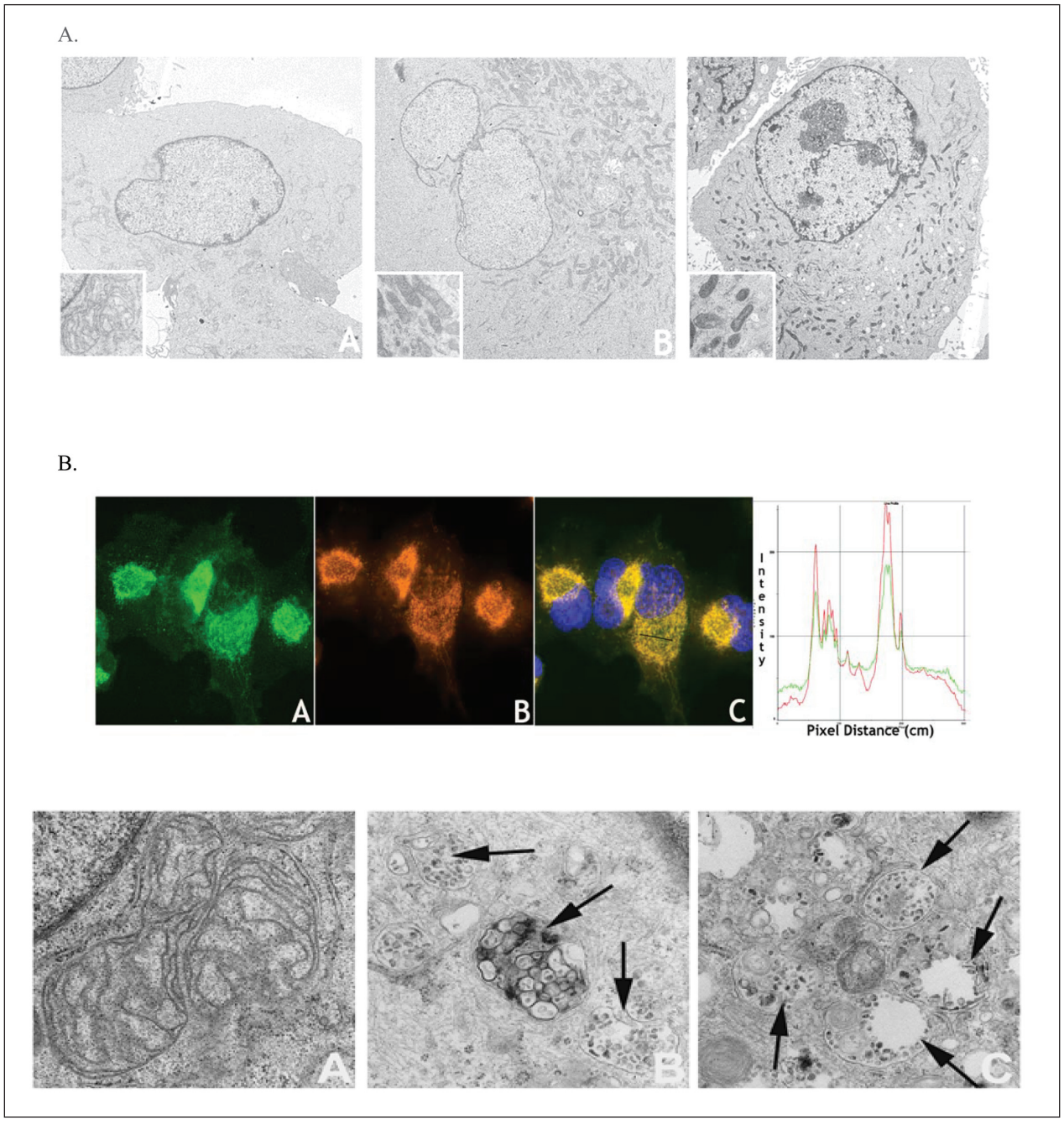

Figure 4. Oleandrin-mediated cell and subcellular changes. (A) Transmission electron micrographs of PANC-1 cells showing condensed mitochondria with perinuclear position. Cells were treated with 20 nM oleandrin for 8 hours. Typical morphology of PANC-1 cells is shown in subpanel A. After 1 hour of treatment of oleandrin (20 nM), PANC-1 cells showed an abnormal perinuclear mitochondrial distribution (subpanel B). Cells shown in subpanel C were treated with oleandrin (50 nM) and demonstrate a marked mitochondrial condensation. All of the photomicrographs were taken at a magnification of ×5000. (B) Intracellular localization of oleandrin (BODIPY-oleandrin) within PANC-1 cells. Cells were exposed to BODIPY-oleandrin (25 nM) for 2 hours before counterstaining with Mitotracker-orange to determine the presence and location of mitochondria. (subpanel A) Determination of the presence of BODIPY-oleandrin alone; (subpanel B) detection of Mitotracker-orange only; (subpanel C) merged images of A and B to determine co-localization of drug within intracellular organelles clearly show that oleandrin is present and associated with mitochondria. The line profile plot demonstrates that at each pixel distance along the x-axis, the 2 discrete wavelengths of light used to determine the BODIPY and Mitotracker dyes occupy the same physical location and relative intensity value. That is, the drug co-localizes with mitochondria within the cells. (C) Determination of autophagy in PANC-1 cells by TEM. (subpanel A) Morphology of control PANC-1 cells (×25 000). (subpanels B and C) PANC-1 cells treated with 20 nM oleandrin for 8 hours showed extensive autophagic vacuolization and autophagosome content as indicated by arrows (subpanel B). Arrows in subpanel C indicate autophagosomes and lamellar structure and "whirling" of the membranes. Subpanels B and C × 50 000.

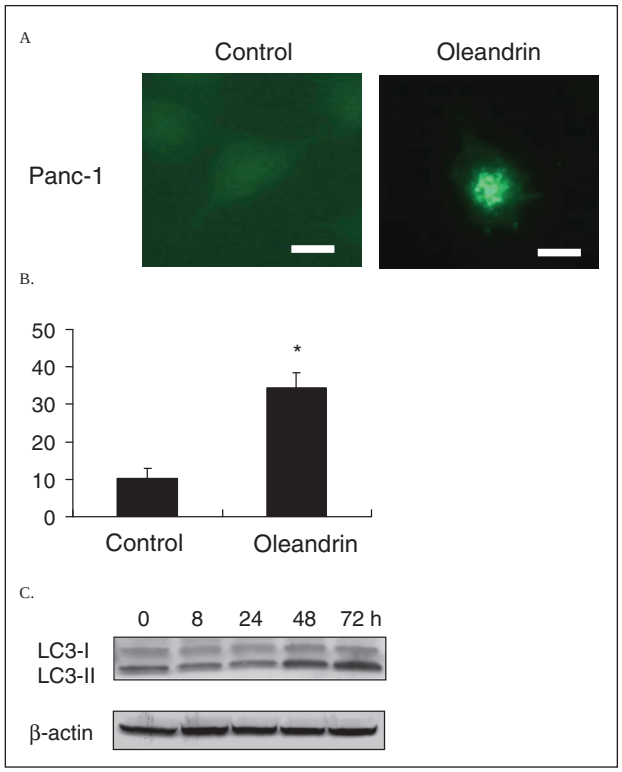

*Figure 5. Involvement of LC3 in oleandrin-induced autophagy. (A) Representative micrographs of PANC-1 cells showing green fluorescent protein light chain 3 (GFP-LC3) localization. Cells were transiently transfected with GFP-LC3 plasmid for 24 hours and treated with dimethyl sulfoxide (control) or 20 nM oleandrin for 72 hours. (B) Quantitative determination of GFP-LC3 protein in control and oleandrin-treated (24 hours, 20 nM oleandrin) PANC-1 cells transfected with GFP-LC3 plasmid. The level of LC3 in oleandrin-treated cells was 3.5-fold higher than that of control cells. (C) Western blot analysis of LC3-I and LC3-II protein. Cells were treated with 20 nM of oleandrin for indicated times (24-72 hours). β-actin was used as loading control. * P < .05 vs. control.*

Involvement of LC3 in Oleandrin-Mediated Autophagy

As shown in Figure 5A, PANC-1 cells were transiently transfected with GFP-LC3 plasmid for 24 hours and then treated with either dimethyl sulfoxide (DMSO) (control) or 20 nM oleandrin for 4 hours. In untreated (not exposed to oleandrin) transfected PANC-1 cells, GFP-LC3 fluorescence was diffusely distributed, whereas in cells treated with oleandrin there is clear evidence of GFP-LC3 localization within the cells. Exposure of cells to oleandrin led to a 3.5-fold increase in cellular fluorescence levels compared with untreated control cells (Figure 5B).

Additionally, we examined the expression of LC3-II using Western blot analysis. Expression of LC3-II (as derived from LC3-I) is closely associated with the membranes of autophagosomes.[10] Expression of LC3-II increased in PANC-1 cells treated with oleandrin in both a time-dependent (Figure 5C) and a concentration-dependent manner (data not shown). Collectively, these data indicate that oleandrin induces autophagy in PANC-1 cells.

Oleandrin-Mediated Alteration of Cell Signaling Pathways Associated With Autophagy

The Akt/mTOR/p70S6K pathway is now recognized as a main regulatory pathway leading to autophagy.[18-21] We therefore examined the effect of oleandrin on relative expression of Akt and phosphorylated Akt in PANC-1 cells. As shown in Figure 6A, oleandrin produced a concentration-dependent decrease in pAkt, which was clearly evident at 25 nM and 50 nM. Expression of Akt itself, however, was unaffected. Because the ERK pathway has been shown to positively regulate autophagy in cancer cells on starvation, we also examined the effect of oleandrin on this pathway. This cardiac glycoside produced a clear concentration-dependent increase in cellular pERK levels, which was evident at concentrations of 25 to 50 nM. Changes in relative expression of both pAkt and pERK were evident as soon as 8 hours after initial exposure (data not shown).

Introduction of pAkt Into PANC-1 Cells Abrogates Oleandrin-Mediated Inhibition of Cell Proliferation

To assess whether oleandrin-mediated inhibition of pAkt phosphorylation was directly associated with this drug's ability to inhibit proliferation of PANC-1 cells, active Akt was transiently transfected into PANC-1 cells. As shown in Figure 6B, pAkt was markedly increased in PANC-1 cells transfected with active Akt compared with nontransfected PANC-1 cells. Intriguingly, PANC-1 cells with transiently transfected active Akt were less sensitive to oleandrin compared with nontransfected cells. This was evident by a shift to the right of the concentration–response curve of cells transfected with active Akt (Figure 6C) compared with nontransfected cells. This indicates that increased levels of active Akt led to a partial diminution of oleandrin growth inhibitory activity, suggesting that inhibition of Akt phosphorylation by oleandrin might be associated with oleandrin-induced autophagy in PANC-1 cells.

Inhibition of ERK Pathway Reduced the Cytotoxic Effect of Oleandrin

It is well documented that the MAPK pathway, especially extracellular signal-regulated proteins, ERK1 and ERK2, are associated with autophagy in various

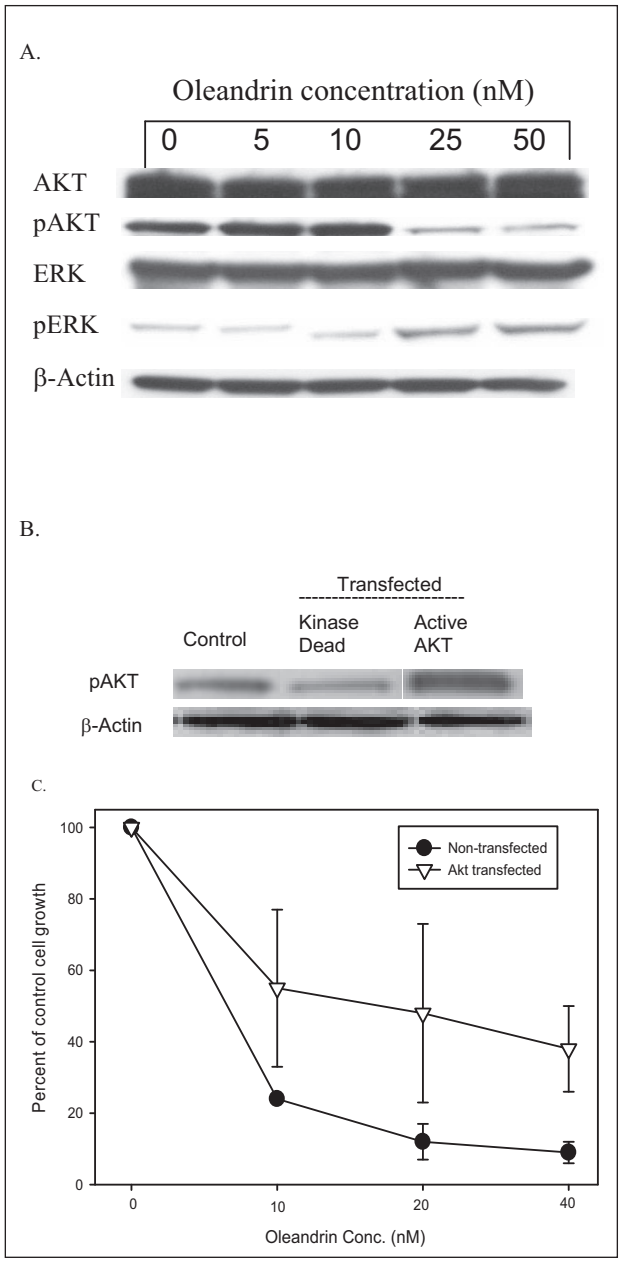

Figure 6. Oleandrin-mediated alteration of cell signaling. (A) Oleandrin inhibited the phosphorylation of Akt and increased phosphorylation of EKR. Cells were treated with oleandrin for 24 hours at indicated concentrations and subjected to Western blot analysis. The data suggest that oleandrin at concentrations as low as 5 nM markedly inhibited phosphorylation of Akt and increased pEKR. (B) Activation of the Akt pathway by transiently transfected active Akt in PANC-1 cells. Western blot showing that transfection led to an increased cellular content of pAkt. The data shown are representative of 2 separate experiments. (C) Activation of the Akt pathway by transiently transfected Akt diminished the oleandrin-mediated inhibition of proliferation. Nontransfected and transfected PANC-1 cells were treated with oleandrin (10-40 nM) for 72 hours. The cytotoxic effect of oleandrin was measured with CAM assay as described in the Methods and Materials section. Data are presented as mean ± SD of 3 separate experiments.

human cancer cell lines. To confirm the activation of pERK on oleandrin-induced autophagic cell death, we examined the expression of a marker of autophagy, LC3-II protein, as well as cell viability in PANC-1 cells pretreated with MEK1 inhibitor, PD98058, and ERK activation inhibitor peptide I. ERK activation inhibitor peptide I (25 μM) partially blocked activation of pEKR induced by oleandrin treatment, as evidenced by the reduced expression of pERK in PANC-1 cells pretreated with ERK inhibitor followed by oleandrin treatment compared with the expression of cells treated with oleandrin alone (Figure 7A). When PANC-1 cells were pretreated with ERK inhibitor or PD 98059, the antiproliferative activity of oleandrin was reduced compared with treatment with oleandrin alone (Figure 7B). Furthermore, the data in Figure 7C show that expression of LC3-II protein was increased in oleandrin-treated cells and decreased to some extent in cells pretreated with either ERK inhibitor (25 μM). These results suggest that the ERK pathway is involved in oleandrin-induced autophagic cell death in PANC-1 cells.

Discussion

The ability of cardiac glycosides to block tumor cell proliferation in many types of human tumor cells but curiously not significantly injure normal human primary or rodent tumor cell lines is now well established[4,5,8] even if it is incompletely understood. Oleandrin, the principle cardiac glycoside within *Nerium oleander*, has, for example, been shown to selectively induce apoptosis in human tumor cells but not in neutrophils, oral epithelial cells, or peripheral blood mononuclear cells.[8] The presumed enzyme target, Na^+,K^+-ATPase, is inhibited by oleandrin and other cardiac glycosides leading to an imbalance in intracellular Ca^{2+} and Na^+ concentrations, evoking a positive inotropic effect. Na^+,K^+-ATPase is a heteromer consisting of α, β, and γ subunits. The enzyme α subunit is believed to contain the actual binding site for cardiac glycosides, although once it is bound to the membrane-associated enzyme its intracellular fate is largely unknown. Some have suggested an intracellular uptake and association with selected organelles,[22] although much work remains to be done to clarify this issue.

We have previously shown that the oleandrin-mediated increase of cellular calcium triggers the caspase cascade in human prostate tumor cells,[11] inhibits activation of the transcription factor NF-κB,[6] and can lead to mitochondrial condensation through activation of ROS pathways.[10] More recent studies from Sreenivasan et al[8] have indicated that oleandrin-mediated expression of Fas potentiates apoptosis in tumor cells. Research reported here and very recently reported by others[5] also demonstrates that

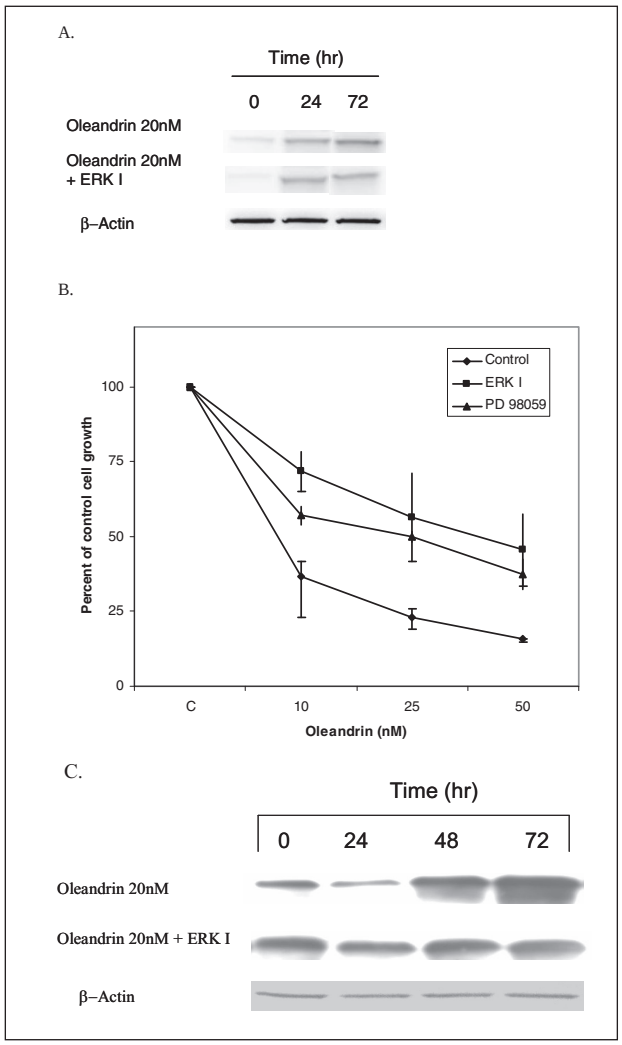

Figure 7. Inhibition of the ERK pathway by ERK activation inhibitor peptide I and PD 98059 partially inhibits oleandrin-induced autophagy and cell death. (A) Western blot analysis of the ERK pathways in cells pretreated with ERK activation inhibitor peptide I (ERK I). Cells were treated with 10 μM ERK I for 15 minutes and then treated with oleandrin (20 nM) for 24 or 72 hours. Expression of pERK was examined by Western blot analysis. Pretreatment with EKR I partially inhibited the phosphorylation of EKR induced by oleandrin, especially after 72 hours. (B) Cytotoxicity of oleandrin in PANC-1 cells pretreated with ERK I and PD 98059. Cells were treated with ERK I (10 μM) or PD 98059 (25 μM) for 15 minutes followed by oleandrin at indicated concentrations for 72 hours. Cytotoxicity effect was assessed with CAM assay as described in the Methods and Materials section. Data are mean ± SD of 3 independent experiments. (C) Western blot analysis of LC3-II protein. Cells were pretreated with ERK I as described previously for 15 minutes and then treated with 20 nM oleandrin for 24 to 72 hours. LC3-II protein expression was determined by Western blot analysis. The increases of LC3-II protein by oleandrin in PANC-1 cells were blocked by pretreatment of ERK I, suggesting that ERK activation is at least partially involved in oleandrin-induced autophagy in PANC-1 cells.

oleandrin is a potent inhibitor of the phosphorylation of Akt, a well-established biomarker responsible for activation of tumor cell proliferation, cell migration, invasion, and prevention of apoptosis.[19] It thus appears that oleandrin is a multimechanistic mediator of tumor cell death capable of selective killing of tumor but perhaps not normal cell types.

In contrast to the ability of oleandrin to induce apoptosis (type 1 cell death), the potential for lipid-soluble cardiac glycosides such as oleandrin to induce autophagy in human tumor cells has yet to be demonstrated. Macroautophagy or type 2 cell death involves sequestration of cytosol or cytoplasmic organelles within double membranes, thus creating autophagosomes (autophagic vacuoles). These subsequently fuse with endosomes and eventually with lysosomes to complete destruction of damaged organelles.[14,23,24] Our understanding of which agents mediate cell death through autophagy, recently referred to as a tumor suppressor mechanism, is limited, although Kondo and Kondo[23] recently showed a diverse number of cancer therapies that promote autophagy and autophagic cell death.

The apparent cell type selective production of autophagy by oleandrin is, we believe, a novel observation. As shown in the present studies, exposure of PANC-1 cells to oleandrin resulted in cell death associated with subcellular changes such as formation of autophagosomes that were abundant even at low nanomolar concentrations of this cardiac glycoside. Why oleandrin produces cell death through apoptotic mechanisms in human breast, prostate, and lung cancer cell lines, for example, yet preferentially produces cell injury and death through macroautophagy in human pancreatic tumor cell lines such as PANC-1 is not clear.

We have previously shown that incubation of human malignant melanoma cells with oleandrin leads to production of ROS, a decline in glutathione levels, and marked mitochondrial condensation.[10] The concentration-dependent loss of normal mitochondrial architecture was an early indication that oxidative stress–mediated organelle injury might trigger an autophagic process. Cell cycle analysis of the effect of oleandrin on human PANC-1 cells also revealed a concentration-dependent G_2/M arrest but without a significant indication of cells in a sub-G_1 state typically associated with apoptotic cell death. Because oleandrin was clearly producing cell death without apoptosis, the data suggested that autophagy might be involved. The potential role of autophagy was also suggested by staining of the cells with the lysosomotropic agent acridine orange, which can be used to follow

development of acidic vesicular organelles including autophagosomes.

Classically, electron microscopy has been used to demonstrate autophagosomes in cells. Both autophagosomes and "whirled" remnants of mitochondria are clearly evident in Figure 4. More recently, the autophagosome-associated protein microtubule-associated protein 1 LC3 has been used as a marker of autophagy.[25] Immediately after its synthesis as a precursor protein in the cytoplasm, the proform of LC3 is converted to LC3-I near the C-terminal end through removal of a glycine residue. The cytosolic form of LC3, LC3-I, is further converted to LC3-II, a membrane-bound form, by modification with a phospholipid such as phosphatidylethanolamine.[14,23] This membrane-bound form binds to the isolated autophagosome membranes, although a recent report indicates that LC3-II binding can also be incorporated into protein aggregates independent of autophagy.[25] Transfection with the GFP-LC3 chimeric plasmid is capable of showing the intracellular presence of LC3 in discrete intracellular areas of cells undergoing autophagy. This approach was used in the present study to demonstrate that not only does oleandrin result in a time-dependent increase in LC3-II, but the protein was also shown to be associated with intracellular autophagosomes as evidenced by punctuate localization of GFP-labeled LC3-II.

Despite unresolved questions about the regulation of autophagy in malignant cells, tumor suppressor genes acting in the mTOR signaling network are known to stimulate and even regulate autophagy.[12,26-29] Furthermore, an increase in the function of oncogenes acting in the mTOR signaling network (Akt, Ras) has been shown to have an inhibitory effect on autophagy.[20,21] We and others[8] have shown the ability of oleandrin to potently inhibit phosphorylation of Akt at nanomolar concentrations. Inhibition of Akt phosphorylation typically requires inhibition of growth factors such as epidermal growth factor (EGF), interleukin (IL-8), nerve growth factor (NGF), and fibroblast growth factor (FGF)-mediated signaling. Oleandrin has been shown to block IL-8-mediated cellular responses by down-regulation of IL-8 receptors.[6] Several growth factor (eg, NGF and EGF) mediated cellular responses have also been shown to be inhibited by oleandrin in human cells.[6] The exact mechanism that explains oleandrin-mediated inhibition of pAkt formation, however, remains to be determined. The relevance of the decrease in phosphorylation of Akt by oleandrin was examined. As presented in this report, transfection of active Akt into PANC-1 cells blocked oleandrin-mediated inhibition of cell growth, suggesting that this cell survival pathway is important in explaining, at least in part, oleandrin-mediated tumor cell death.

These observations imply a link between an oleandrin-mediated decrease in pAkt formation and autophagy. For example, Botti et al[24] suggested that there may be a relationship between cellular autophagic capacity and the activity of tumor suppressor and oncogenes (such as Akt) that are active in the mTOR signaling network. In addition, Takeuchi et al[29] showed that both Akt and mTOR regulate autophagy and cell death in human brain tumor cells. It has recently been suggested that the need of cancer cells to keep the level of autophagy low is probably an Achilles' heel that can be exploited to induce cell death.[14] As such, the precise role of oleandrin in altering Akt and the mTOR pathway in human tumor cells is considered important and is the subject of ongoing investigations.

We have shown that for the first time that oleandrin induces autophagy in the human pancreatic cancer PANC-1 cell. Mechanistically, both Akt and ERK pathways appear to be involved in autophagic cell death induced by oleandrin. In comparison with findings of apoptosis induced by oleandrin in other types of human cancer cell lines, the results from this study suggest that oleandrin-induced pancreatic tumor cell death could be mediated by targeted cell signaling pathways. Given the reported beneficial effects of cardiac glycosides, such as digoxin, in breast cancer patients, our study suggests that using oleandrin or plant extracts containing this potent cardiac glycoside as a new anticancer regimen for human pancreatic cancer warrants further consideration.

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