

Cardiac Glycosides Stimulate Ca^{2+} Increases and Apoptosis in Androgen-independent, Metastatic Human Prostate Adenocarcinoma Cells

David J. McConkey,¹ Yun Lin, Leta K. Nutt, Huseyin Z. Ozel, and Robert A. Newman

Departments of Cancer Biology [D. J. M., L. K. N.] and Experimental Therapeutics [Y. L., R. A. N.], University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Ozelle Pharmaceuticals, Inc., San Antonio, Texas 78230 [H. Z. O.]

ABSTRACT

Cardiac glycosides are used clinically to increase contractile force in patients with cardiac disorders. Their mechanism of action is well established and involves inhibition of the plasma membrane Na^+/K^+ -ATPase, leading to alterations in intracellular K^+ and Ca^{2+} levels. Here, we report that the cardiac glycosides oleandrin, ouabain, and digoxin induce apoptosis in androgen-independent human prostate cancer cell lines *in vitro*. Cell death was associated with early release of cytochrome *c* from mitochondria, followed by proteolytic processing of caspases 8 and 3. Oleandrin also promoted caspase activation, detected by cleavage poly(ADP-ribose) polymerase and hydrolysis of a peptide substrate (DEVD-pNA). Comparison of the rates of apoptosis in poorly metastatic PC3 M-Pro4 and highly metastatic PC3 M-LN4 subclones demonstrated that cell death was delayed in the latter because of a delay in mitochondrial cytochrome *c* release. Single-cell imaging of intracellular Ca^{2+} fluxes demonstrated that the proapoptotic effects of the cardiac glycosides were linked to their abilities to induce sustained Ca^{2+} increases in the cells. Our results define a novel activity for cardiac glycosides that could prove relevant to the treatment of metastatic prostate cancer.

INTRODUCTION

Cardiac glycosides are a class of natural products that are used to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias (1). The most familiar are digoxin/digitoxin and ouabain, which are derived from the plant genera *Digitalis* and *Strophanthus gratus*, respectively. Their mechanisms of action in the heart are well known and involve inhibition of the plasma membrane Na^+/K^+ -ATPase (2), leading to increased intracellular Na^+ and Ca^{2+} and decreased intracellular K^+ (3). The increased intracellular Ca^{2+} promotes muscle contraction and cardiac contractile force (2).

Oleandrin and oleandrinigenin are cardiac glycosides derived from oleander (*Nerium oleander*) that have been used in the treatment of cardiac abnormalities in Russia and China for years (4). Interestingly, anecdotal evidence has emerged from this experience suggesting that they may produce beneficial side effects in patients with leiomyosarcoma, Ewing's sarcoma, prostate cancer, and breast cancer. Indeed, there are also scattered reports of breast tumor regression in Scandinavian patients taking other cardiac glycosides (5). Therefore, there is growing interest in evaluating the oleander products and possibly other cardiac glycosides as antineoplastic agents. The first of these therapies to be developed in the United States is a patented, water-soluble oleander extract called Anvirezol. Preclinical studies have demonstrated that the extract has excellent activity against a variety of human solid tumor cell lines. In preparation for clinical trials within the United States, an Investigational New Drug application for Anvirezol has been submitted recently for Food and Drug Administration approval.²

Received 11/24/99; accepted 5/17/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed, at Department of Cancer Biology, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-8591; Fax: (713) 792-8747; E-mail: dmconcke@mdanderson.org.

² R. A. Newman, personal communication.

Disruption of intracellular Ca^{2+} homeostasis results in the induction of apoptosis in diverse cell types (reviewed in Ref. 6). The mechanisms involved have been particularly well studied in prostate cells. In the normal prostate, androgen withdrawal leads to a rapid wave of apoptosis (7) associated with Ca^{2+} influx (8) and Ca^{2+} -dependent endonuclease activation (9, 10). In addition, agents that directly stimulate an intracellular Ca^{2+} increase (Ca^{2+} ionophores, thapsigargin) trigger apoptosis in androgen-sensitive and -insensitive prostate cancer cells (11). Given that cardiac glycosides cause increases in cytosolic Ca^{2+} , we wondered whether Anvirezol, oleandrin, and/or other cardiac glycosides would induce apoptosis in prostate cancer cells. Such an effect might, in part, explain the antitumor effects of the drug in cancer patients.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The PC-3 human prostatic adenocarcinoma line was obtained from American Type Culture Collection (Rockville, MD). A metastatic subclone of PC-3 (PC-3 M) was isolated from a liver metastasis produced in nude mice after intrasplenic injection of PC-3 cells (12). PC-3 M cells were then used to derive both PC-3 M-Pro4 and PC-3 M-LN4 by orthotopic "recycling" in nude mice (13). Both subclones are highly tumorigenic and metastatic to lymph nodes, but the LN4 cells display increased metastasis to distant sites (13). The cells were maintained in RPMI 1640 supplemented with 10% FCS, antibiotics, sodium pyruvate, and 10 mM HEPES (pH 7.4). Cell survival/cytostasis were quantified at 72 h using the tetrazolium dye MTT³ as described previously (14). Each experiment was performed in triplicate.

DNA Fragmentation Analysis. DNA fragmentation was quantified by propidium iodide staining and FACS analysis as described previously (15). Cells were resuspended in PBS containing 50 $\mu\text{g}/\text{ml}$ propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16 h and vortexed prior to FACS analysis (Becton Dickinson FACScan, Mountain View, CA; FL-3 channel). For qualitative analysis of oligonucleosomal DNA fragmentation (DNA laddering), cells were lysed in a hypotonic buffer containing 0.5% Triton X-100, 20 mM EDTA, and 25 mM Tris (pH 8.0) and centrifuged for 20 min at 12,000 $\times g$, and DNA fragments in the supernatants were precipitated with isopropanol (overnight at -20°C). The DNA fragments were treated with proteinase K (0.2 mg/ml for 1 h at 37°C) and resolved by electrophoresis for 1 h at 75 V on 1.5% agarose gels preimpregnated with ethidium bromide.

PARP Cleavage. Extraction of PARP and detection of specific M_r 89,000 caspase-derived cleavage product by immunoblotting was conducted as described previously (16). Cells (1×10^6) were resuspended in 50 μl ice-cold PBS and mixed with 200 μl of a buffer containing 6 M urea, 2% SDS, 10% glycerol, 5 mM EDTA, 5% 2-mercaptoethanol, and 100 mM Tris (pH 6.8). Samples were disrupted by pipetting through a 1-ml tip and sonicated for 20 s at high power. Lysates were then incubated for 15 min at 65°C and resolved on 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (0.2 μm ; Schleicher & Scheull, Keene, NH), and PARP was detected with a mouse monoclonal antibody (C2-10; generously provided by Dr. Scott H. Kaufmann, Department of Oncology Research, Mayo Clinic, Rochester, MN). Immunoreactive bands were detected by enhanced chemiluminescence (West Pico; Pierce, Inc., Rockford, IL).

³ The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorter; PARP, poly(ADP-ribose) polymerase.

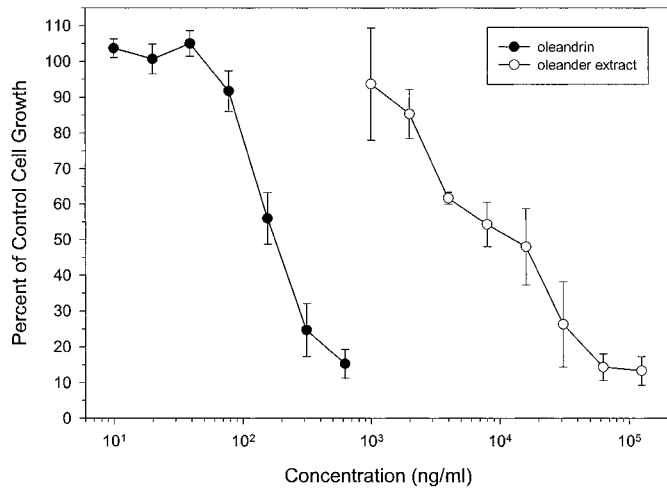


Fig. 1. Effects of Anvizrzel and oleandrin on cell survival. Cells were exposed to the indicated concentrations of each drug for 48 h. Cells were transferred to medium without drug, and inhibition of cell growth (measured as decreased MTT reduction) was determined 24 h later. Data are reported as means from three experiments; bars, SD.

Caspase Processing and Activation. Cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 25 mM Tris (pH 7.5), and protease inhibitors (Complete Mini tablet; Boehringer-Mannheim). Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes, and caspases were detected by immunoblotting using polyclonal antibodies to human caspase-3, caspase-7, caspase-8, or caspase-9 (PharMingen, San Diego, CA). Caspase activation was measured in cytosolic extracts using the peptide substrate, DEVD-pNA (Alexis Biochemicals, San Diego, TX; Ref. 17). Cells were lysed in a buffer containing 150 mM NaCl, 25 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM DTT, and 10 μ M digitonin for 15 min on ice. Samples were centrifuged for 10 min at 12,000 \times g, and protein content in the supernatants was measured using the Bradford reagent (purchased from Bio-Rad, Hercules, CA). Extracts were diluted to 1 mg/ml in lysis buffer and incubated in triplicate for 60 min at 37°C with 50 μ M peptide. Blanks were prepared by incubating peptide in lysis buffer alone. Absorbance was measured at 405 nm on a microtiter plate reader. Results are expressed as absorbance units/ μ g protein.

Cytochrome c Release Measurements. Release of cytochrome c from mitochondria was measured by immunoblotting essentially as described previously (18). Cells were gently lysed (30 s) in an ice-cold buffer containing 250 mM sucrose, 1 mM EDTA, 0.1% digitonin, and 25 mM Tris (pH 6.8). Lysates were centrifuged for 2 min at 12,000 \times g, supernatants were mixed with 2 \times Laemmli's reducing SDS-PAGE sample buffer, and extracts from equal numbers of cells (10–20 \times 10⁶) were resolved by 15% SDS-PAGE. Polypeptides were transferred to nitrocellulose membranes, and cytochrome c was detected by immunoblotting with a monoclonal antibody (clone 7H8.2C12; PharMingen, San Diego, CA).

Quantification of Intracellular Ca²⁺ in Prostate Cancer Cells. Cells were plated on a 22 \times 30-mm glass coverslip. On culture day 2, cells were loaded with fura-2 acetoxymethyl ester (10 μ M; Molecular Probes) for 1 h at 37°C with 5% CO₂. The coverslips were washed thoroughly afterward with PBS and mounted on a 1.5-ml volume chamber (cells facing upward). The chamber was placed on an epifluorescence/phase contrast microscope for Ca²⁺ imaging and quantification. Cells were bathed in 1 ml of HBSS with 1 mM Ca²⁺ at room temperature. After a baseline [Ca²⁺]_i was established, cells were then treated with oleandrin (10 μ g/ml), ouabain (1 μ M), or thapsigargin (5 μ M). An INCA workstation (Intracellular Imaging, Inc.) was used to measure [Ca²⁺]_i levels. Fluorescence was monitored using a \times 20 fluorescence objective. Cells were illuminated alternately at excitation wavelengths of 340 and 380 nm using a xenon arc lamp. The emitted fluorescence was monitored at 511 nm with a video camera, and the calculated free [Ca²⁺]_i was determined using the cell-free calibration curve. The data were collected with INCA software (Win 3.1 version).

RESULTS

The human PC-3 prostate adenocarcinoma line was derived from a lytic bone metastasis and is representative of advanced disease. The cells lack androgen receptor and p53 expression and do not produce prostate-specific antigen. *In vivo* selection of the cells by serial intra-prostatic injection resulted in the isolation of two variants, designated PC-3 M-Pro4 (Pro4) and PC-3 M-LN4 (LN4), which exhibit low and high metastatic potential, respectively, when grown as xenograft orthotopic tumors in nude mice (13). These cells were used in the experiments that follow.

Incubation of Pro4 cells with oleander extract (Anvizrzel) or purified oleandrin resulted in concentration-dependent growth arrest, measured by mitochondrial reduction of MTT (Fig. 1). Comparison of the IC₅₀ concentrations of Anvizrzel and oleandrin demonstrated that oleandrin was at least 50-fold more potent than Anvizrzel.

Visual examination revealed that Anvizrzel and oleandrin caused them to bleb and detach from the tissue culture plates, consistent with involvement of apoptosis. To measure apoptosis directly, cells were incubated with various concentrations of Anvizrzel or oleandrin, and apoptosis was quantified by propidium iodide staining and FACS analysis. This technique measures cells with fragmented DNA, which appear as a population with a hypodiploid DNA content (Fig. 2A). Both Anvizrzel and oleandrin induced concentration-dependent DNA fragmentation in the Pro4 and LN4 cells (Fig. 2B). Chromatin cleavage was first detectable at 24 h and reached a maximum at 48 h (Fig. 2B). The nonmetastatic Pro4 cells were significantly (*P* < 0.01) more

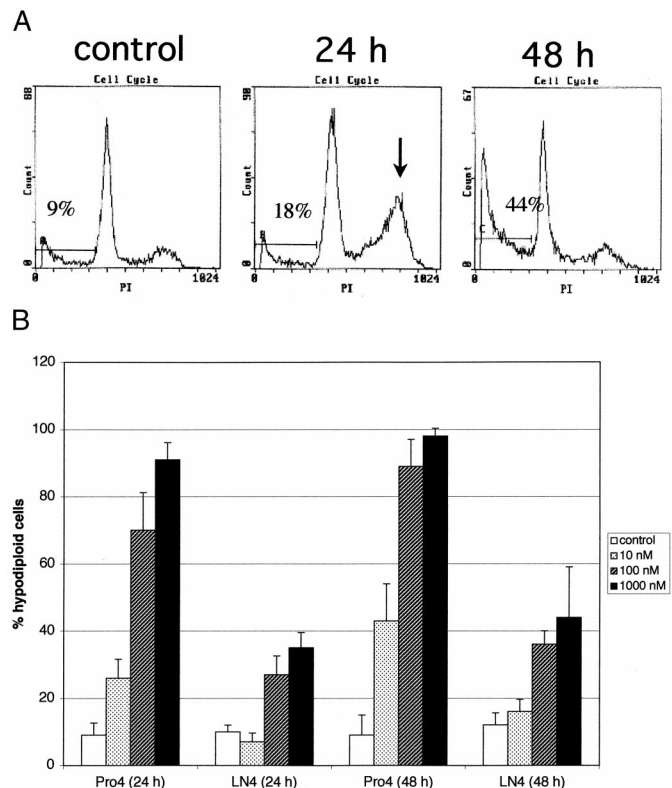


Fig. 2. Oleandrin induces apoptosis. A, representative FACS histogram of Pro4 cells exposed to 50 nM oleandrin for 24 and 48 h. Hypodiploid, apoptotic cells are indicated by the gate (left); numbers above the gate correspond to percentages of cells within the gate. Note accumulation of cells in G₂-M (arrow) at 24 h. B, dose-response analysis of oleandrin-induced apoptosis in Pro4 and LN4 cells. Cells were treated as indicated prior to quantification of hypodiploid cells by propidium iodide staining and FACS analysis. Data are means; bars, SD; n = 3. Responses were significantly lower in LN4 cells compared with Pro4 cells at every concentration level and time point tested (*P* < 0.01 by either paired Student's *t* test or ANOVA).

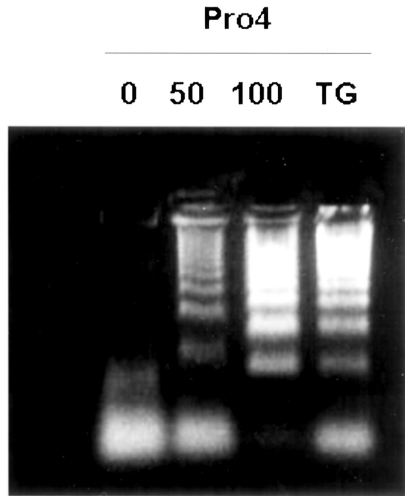


Fig. 3. Oleandrin stimulates endogenous endonuclease activation. Pro4 cells were treated with the indicated concentrations of oleandrin or 1 μ M thapsigargin (TG; positive control) for 48 h. Oligonucleosome-length DNA fragments (DNA ladders) were extracted from $12,000 \times g$ supernatants and resolved by 1% agarose gel electrophoresis as outlined in "Materials and Methods."

sensitive to oleandrin compared with the metastatic LN4 cells at each concentration of oleandrin tested. Qualitative analysis of DNA fragmentation by agarose gel electrophoresis confirmed the presence of oligonucleosomal DNA fragments (DNA ladders) characteristic of apoptosis (Fig. 3). Oleandrin was at least 50-fold more potent than Anvirlzel in promoting DNA fragmentation (data not shown), consistent with the MTT analyses. Oleandrin also caused marked accumulation of cells in the G₂-M phase of the cell cycle (Fig. 2A, arrow), indicating that cell cycle arrest also contributes to the response. Importantly, the extent of cell cycle arrest did not appear to vary significantly in the Pro4 and LN4 cells.

To investigate whether the effects of oleandrin were shared by other cardiac glycosides, we treated cells with various concentrations of digoxin or ouabain and measured DNA fragmentation by propidium iodide staining and FACS analysis. The results confirmed that both drugs induced high levels of apoptosis in the Pro4 cells and lower levels in the LN4 cells (Fig. 4). Concentration-response analyses demonstrated that 100 nM concentrations of ouabain and digoxin stimulated maximal responses in both cell lines. In this respect, all of the cardiac glycosides were very similar (compare Figs. 2B and 4).

Caspases are a family of aspartate-specific cysteine proteases that are thought to be obligatory components of the apoptotic pathway (19). The most familiar caspase substrate is PARP, an enzyme that regulates chromatin structure during differentiation and DNA repair. The M_r 116,000 native PARP protein is specifically cleaved at an NH₂-terminal DEVD motif by caspase-3 and caspase-7 to yield an M_r 89,000 fragment that can be detected by immunoblotting (16). To confirm the involvement of caspase activation in oleandrin-induced apoptosis, we treated cells with increasing concentrations of oleandrin and measured PARP cleavage at 24 h. The Pro4 cells displayed prominent PARP cleavage at all dose levels, whereas the LN4 cells did not demonstrate substantial cleavage at this time point (Fig. 5A). Direct measurement of caspase activation using a synthetic peptide substrate (DEVD-pNA) confirmed that oleandrin stimulated protease activation more rapidly in the Pro4 cells (Fig. 5B). These data are consistent with the DNA fragmentation results and indicate that the LN4 cells are refractory to oleandrin-mediated caspase activation.

Caspases exist as inactive zymogens in resting cells and undergo proteolytic processing upon activation. Therefore, involvement of particular caspase(s) in an apoptotic response can be directly deter-

mined by measuring procaspase expression in dying cells by immunoblotting (19); activation of a particular caspase will result in a reduction in its proenzyme form (20). Oleandrin treatment resulted in the processing of procaspase-8 and procaspase-3 in the Pro4 cells (Fig. 6). Processing of procaspase-8 was also detectable in the LN4 cells by 36 h (Fig. 6A). Interestingly, processing of procaspase-8 appeared to precede processing of procaspase-3 in the Pro4 cells (Fig. 6). Furthermore, procaspase-8 appeared as a doublet, and levels of

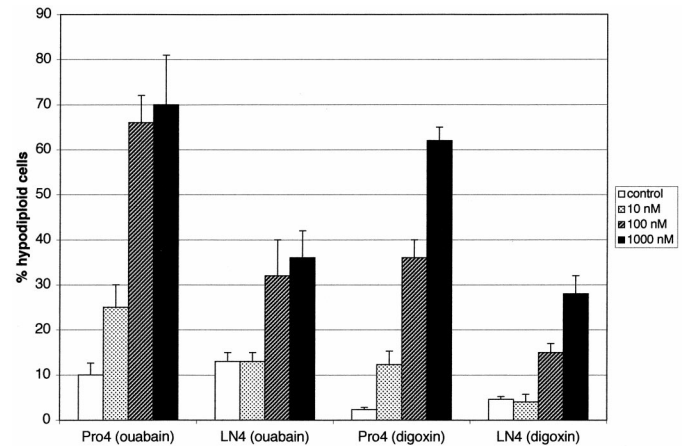


Fig. 4. Induction of apoptosis by other cardiac glycosides. Pro4 and LN4 cells were treated with the indicated concentrations of ouabain or digoxin for 48 h, and hypodiploid cells were quantified by propidium iodide staining and FACS analysis. Data are means; bars, SD; $n = 3$. Responses were significantly lower in LN4 cells compared with Pro4 cells at every concentration level tested ($P < 0.01$ by either paired Student's t test or ANOVA).

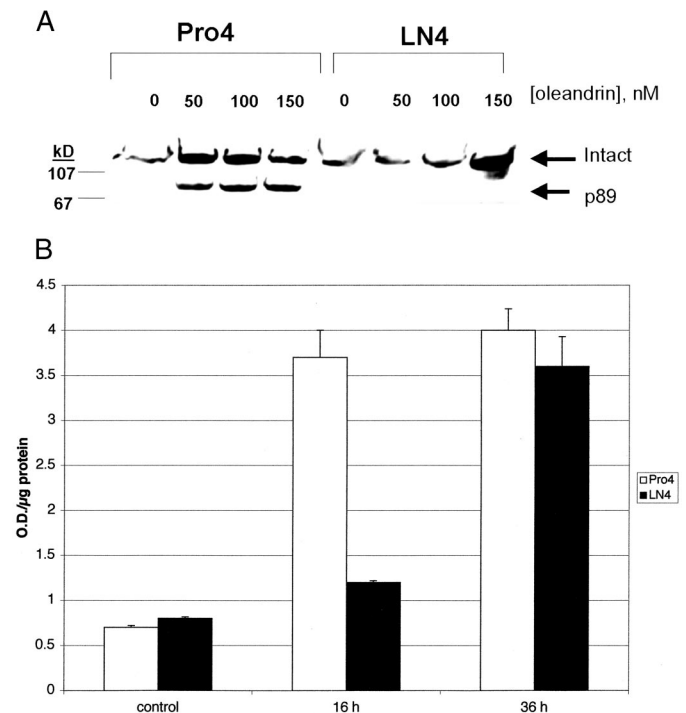


Fig. 5. Evidence for oleandrin-induced caspase activation. A, oleandrin-induced cleavage of PARP. Cells were incubated with the indicated concentrations of oleandrin for 24 h, and PARP cleavage was analyzed in urea cell extracts by immunoblotting. The characteristic (p89) caspase-generated cleavage product is indicated. B, oleandrin-induced caspase substrate hydrolysis. Caspase activity was measured in cytosolic extracts using the colorimetric substrate, DEVD-pNA. Data are means; bars, SD; $n = 3$. Caspase activity was significantly higher in Pro4 cells compared with LN4 cells at the 16-h time point ($P < 0.01$, paired Student's t test).

both species declined in oleandrin-treated cells. Although we do not have an explanation for this phenomenon at present, it could be attributable to posttranslational modification. The doublet can also be detected in human LNCaP prostate adenocarcinoma cells and a variety of human breast cancer cell lines (MCF-7, MDA-MB-468, and MDA-MB-435; data not shown), indicating that its presence is not specific to PC-3-derived cells. Caspase processing appeared to coincide with DNA fragmentation, consistent with work demonstrating that caspases can directly stimulate endonuclease activation (21). This was confirmed in experiments with a pan caspase inhibitor (zVAD-fmk), which blocked DNA fragmentation in Pro4 cells treated with the cardiac glycosides (Fig. 6C).

Mitochondrial alterations leading to the release of cytochrome *c* are thought to mediate caspase activation in many models of apoptosis. Consistent with this mechanism, treatment with oleandrin resulted in release of cytochrome *c* into cytosolic extracts of the Pro4 cells, first noted at ~18 h (Fig. 7). This release appeared to precede activation of

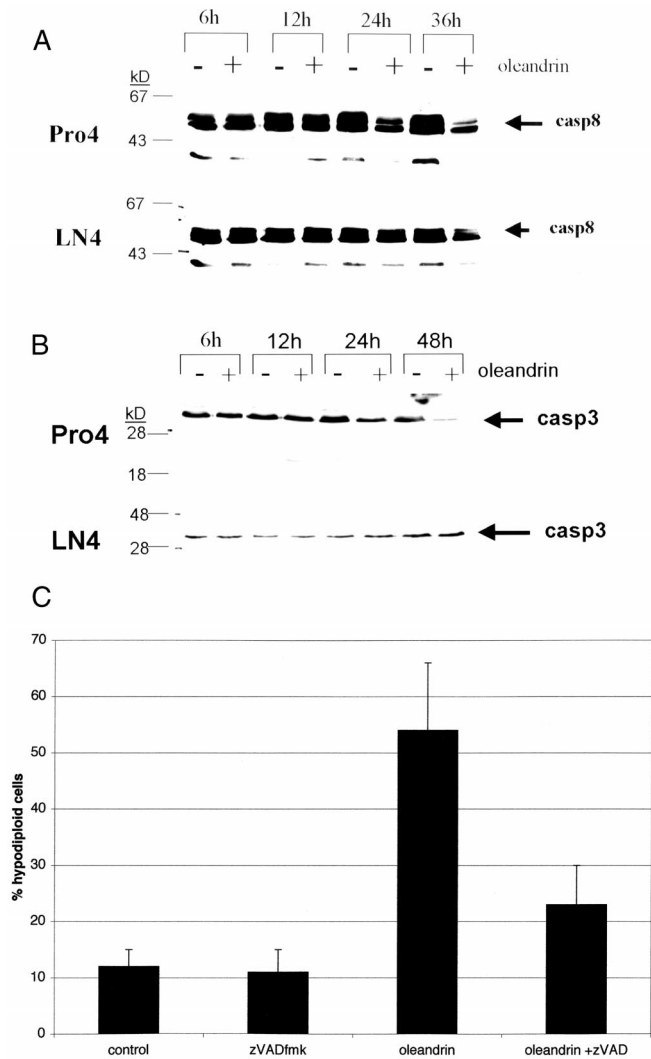


Fig. 6. Oleandrin-induced caspase activation. *A* and *B*, procaspase processing. Cells were treated with 100 nM oleandrin for the times indicated, and procaspase-3 and procaspase-8 were detected by immunoblotting. Reduction in band intensity corresponds to procaspase processing and activation. *A*, procaspase-8; *B*, procaspase-3. *C*, effects of zVADfmk on DNA fragmentation. Cells were pretreated with 100 nM oleandrin in the absence or presence of 50 μ M zVADfmk (a pan-caspase inhibitor), and DNA fragmentation was measured by propidium iodide/FACS. Data are means; bars, SD; *n* = 3. DNA fragmentation was significantly lower in the oleandrin plus zVADfmk group compared with the group treated with oleandrin alone; *P* < 0.01 by paired Student's *t* test.

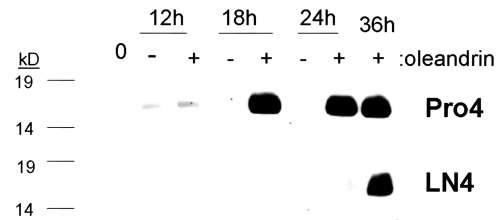


Fig. 7. Oleandrin-induced cytochrome *c* release. Cells were treated with 100 nM oleandrin for the times indicated. Cytosolic extracts were prepared, and the cytochrome *c* content was determined by immunoblotting. (Extracts from equivalent cell numbers were loaded in each lane.)

caspase-3 and caspase-8 (compare Figs. 6 and 7). Importantly, oleandrin-induced cytochrome *c* release in the LN4 cells was only obvious after 36 h (Fig. 7). Therefore, the slower kinetics of apoptosis in the LN4 cells treated with cardiac glycosides are probably attributable to impaired cytochrome *c* release.

As noted above, the mechanisms of action of cardiac glycosides in cardiac myocytes involve sustained Ca^{2+} increases. We and others have demonstrated that sustained Ca^{2+} increases can also trigger apoptosis in prostate cancer cells and certain other cell types (6). To verify that this mechanism was involved in the proapoptotic effects of cardiac glycosides, we measured intracellular Ca^{2+} in single, fura-2-loaded Pro4 and LN4 cells by dual-wavelength fluorescence microscopy. Treatment with oleandrin or ouabain led to rapid, sustained Ca^{2+} increases in all of the cells analyzed (Fig. 8). Importantly, drug-induced Ca^{2+} increases were comparable in the Pro4 and LN4 cells, indicating that the block in the apoptotic pathway in the LN4 cells is downstream of Ca^{2+} .

DISCUSSION

Very few effective therapies exist for androgen-independent, metastatic prostate cancer. Part of the problem may be attributable to the fact that most conventional chemotherapeutic agents target proliferating cells, whereas the growth fraction in prostate tumors is considered to be very low. However, previous work has shown that thapsigargin and other Ca^{2+} active agents exert potent cytotoxic effects in both normal prostate epithelial cells and cell lines derived from advanced cancer (11). Notably, Ca^{2+} -induced apoptosis is not dependent on cell cycle progression (22), suggesting that targeting this pathway could circumvent the problems encountered using current therapies in this disease.

Here we provide the first evidence that cardiac glycosides are potent inducers of apoptosis in cells derived from the PC-3 human prostate adenocarcinoma. Because these cells lack functional androgen receptor and p53, apoptosis is by definition independent of these pathways. The data indicate that oleandrin-induced apoptosis involves an early, sustained Ca^{2+} increase that precedes release of cytochrome *c* from mitochondria and caspase activation, and the involvement of Ca^{2+} in the pathway is consistent with the known mechanism of action of cardiac glycosides in the heart. Importantly, we have confirmed that prostate cancer cells express the Na^+/K^+ -ATPase that serves as the molecular target for the drug and that cardiac glycosides inhibit its activity.⁴ The relative potency of oleandrin compared with the Anvirzel crude oleander extract (~50-fold higher) strongly suggests that much of the proapoptotic activity of Anvirzel is directly attributable to oleandrin, which makes up ~ 0.25% of the extract. Their high activities and water solubility make Anvirzel and possibly oleandrin attractive choices for future prostate cancer therapy. In

⁴ Y. Lin, unpublished observations.

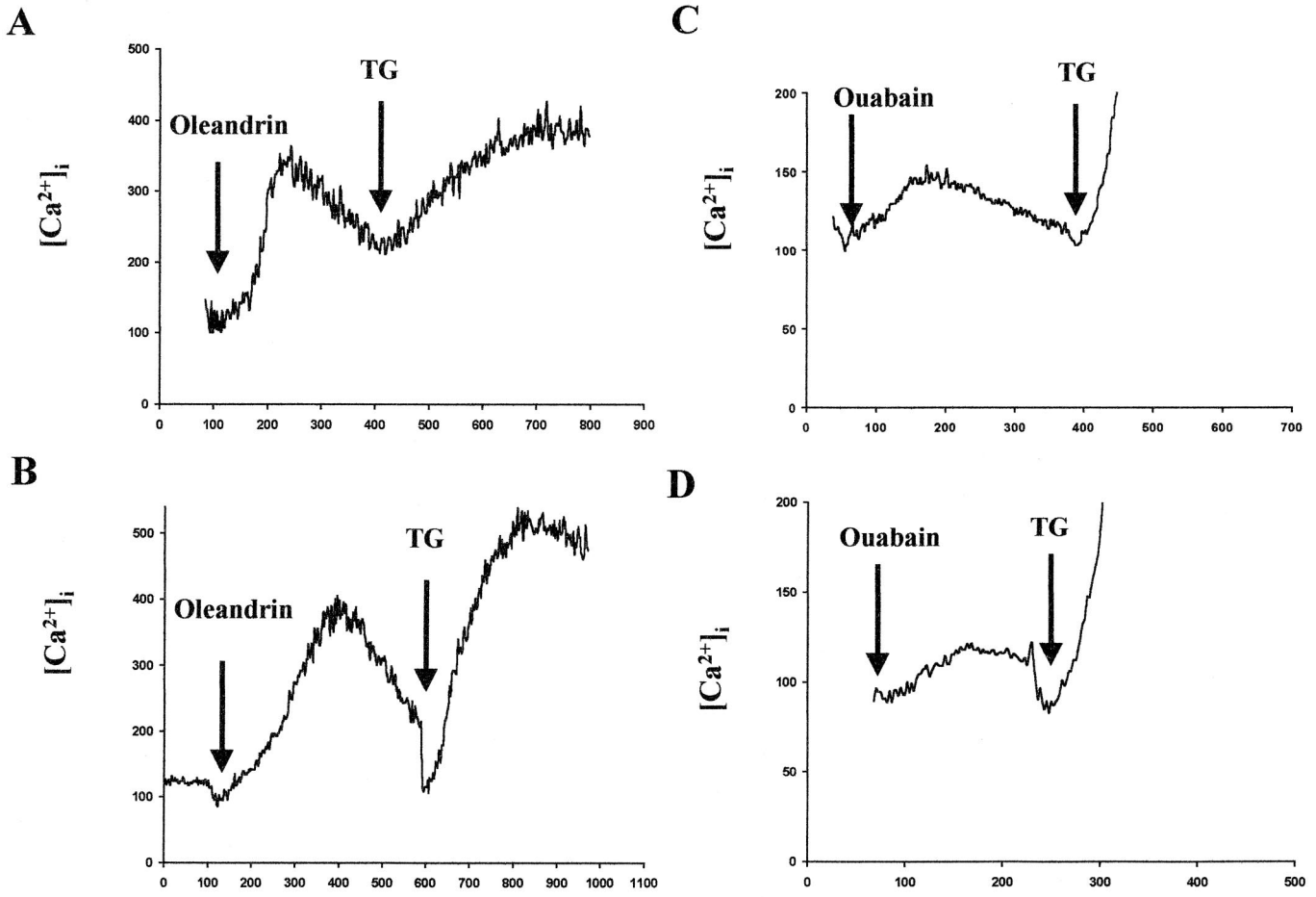


Fig. 8. Cardiac glycosides stimulate cytosolic Ca^{2+} increases. PC3 M-Pro4 and PC3 M-LN4 cells were loaded with fura-2 acetoxymethyl ester as outlined in "Materials and Methods." Cells suspended in HBSS containing 1 mM Ca^{2+} were stimulated with ouabain (1 mM), oleandrin (10 μM), or thapsigargin (TG; 5 μM , positive control). Traces represent results from 10 to 24 individual cells. A, Pro4 cells stimulated with oleandrin; B, LN4 cells stimulated with oleandrin; C, Pro4 cells stimulated with ouabain; D, LN4 cells stimulated with ouabain.

ongoing studies, we are directly investigating the effects of Anvirzel and the cardiac glycosides on orthotopic PC-3 tumors in nude mice (13). Importantly, serum oleandrin concentrations reached therapeutic levels in a cancer patient administered 15 mg of Anvirzel via i.m. injection [14 nM (8 ng/ml) at 3 h, with sustained levels above 10 nM (5 ng/ml) for an additional 3 h, as quantified using a validated LC/MS analytical assay⁵].

Previous work has established the importance of intracellular ion fluxes in the regulation of apoptosis. For example, many investigators have shown that intracellular Ca^{2+} alterations often precede cell death, and that the antiapoptotic BCL-2 protein acts (at least in part) to prevent these changes (6). More recent work has shown that caspase activation and DNA fragmentation are preceded by a drop in intracellular K^+ levels (23, 24), and that inhibition of this drop blocks caspase activation (20, 24) and cell death (24). Importantly, cardiac glycosides induce both an increase in Ca^{2+} and a decrease in K^+ . In addition, parallel studies have shown that oleandrin suppresses nuclear factor- κB activation (25), which could also contribute to cell death induction (26). However, the cell death-promoting activity of cardiac glycosides appears cell type specific, because other work has shown that they inhibit multiple pathways of apoptosis in vascular smooth muscle cells (27). Whether this antiapoptotic activity will be

observed in all excitable tissues (including the heart) remains to be determined.

Our results demonstrate that the apoptotic response of the metastatic PC-3 M-LN4 cells is attenuated compared with the response observed in the PC-3 M-Pro4 cells. This apoptosis resistance is manifest as a delay in cytochrome *c* release and caspase activation and is not attributable to defect(s) in early Ca^{2+} signaling. The uncoupling of early signaling from cytochrome *c* release is consistent with a role for BCL-2 family member(s) in apoptosis resistance, because these polypeptides are thought to act at the level of the mitochondrion to inhibit caspase activation (18). Importantly, the LN4 cells are cross-resistant to several other triggers of apoptosis (staurosporine, doxorubicin, and proteasome inhibitors⁶), indicating that the mechanism(s) of apoptosis resistance is not directly linked to the biochemical resistance mechanism of oleandrin action.

Although apoptosis was a prominent factor in oleandrin-induced growth suppression, the drug is also capable of promoting arrest at the G₂-M phase of the cell cycle (Fig. 2A). Interestingly, the effects of oleandrin on cell cycle arrest appear to be much more pronounced than those of thapsigargin,⁷ suggesting that the oleandrin-induced intracellular Ca^{2+} elevation is not principally responsible for the

⁵ R. A. Newman, unpublished observations.

⁶ L. K. Nutt, unpublished observations.

⁷ Y. Lin, unpublished observations.

effect and that alterations in K^+ and/or Na^+ may also be involved. Inhibition of proliferation by Anvirzel may contribute substantially to the drug's antitumor effects *in vivo*.

Recent work indicates that caspase activation in apoptosis occurs via two general pathways (28). In the first, so-called "death receptors," such as Fas and the type I receptor for tumor necrosis factor, can directly interact with caspase 8 and caspase 10, and ligand-induced multimerization leads directly to caspase activation. In the second, apoptotic stimuli disrupt mitochondrial homeostasis, resulting in release of cytochrome *c* (29) and assembly of a molecular complex known as the "apoptosome" (30). Formation of the apoptosome catalyzes the proteolytic activation of procaspase-9, which in turn activates downstream ("effector") caspases such as caspase-3 and caspase-7. Importantly, caspase-8 activation can also lead to mitochondrial cytochrome *c* release via cleavage of the BCL-2 family polypeptide, BID (31). Our observation that caspase-8 activation occurs coincidentally with cytochrome *c* release and precedes caspase-3 activation argues that a death receptor pathway may be involved. Alternatively, oleandrin may directly promote caspase-8 activation via a novel mechanism. The availability of bid^{-/-} mice (32) should help define the role of BID in the mitochondrial alterations observed in this and a variety of other apoptotic model systems.

REFERENCES

- Hauptman, P. J., Garg, R., and Kelly, R. A. Cardiac glycosides in the next millennium. *Prog. Cardiovasc. Dis.*, *41*: 247–254, 1999.
- Rose, A. M., and Valdes, R., Jr. Understanding the sodium pump and its relevance to disease. *Clin. Chem.*, *40*: 1674–1685, 1994.
- Kasner, S. E., and Ganz, M. B. Regulation of intracellular potassium in mesangial cells: a fluorescence analysis using the dye, PBFI. *Am. J. Physiol.*, *262*: F462–F467, 1992.
- Hung, K. C. *The Pharmacology of Chinese Herbs*, Vol. 1, p. 53. Boca Raton, FL: CRC Press, Inc., 1999.
- Stenkvist, B. Is digitalis a therapy for breast carcinoma? *Oncol. Rep.*, *6*: 493–496, 1999.
- McConkey, D. J., and Orrenius, S. The role of calcium in the regulation of apoptosis. *Biochem. Biophys. Res. Commun.*, *239*: 357–366, 1997.
- Kerr, J. F. R., and Searle, J. Deletion of cells by apoptosis during castration-induced involution of the rat prostate. *Virchows Arch. Abt. B Zellpathol.*, *13*: 87–102, 1973.
- Martikainen, P., and Isaacs, J. Role of calcium in the programmed cell death of rat ventral prostatic glandular cells. *Prostate*, *17*: 175–187, 1990.
- Kyprianou, N., English, H. F., and Isaacs, J. T. Activation of a Ca^{2+} - Mg^{2+} -dependent endonuclease as an early event in castration-induced prostatic cell death. *Prostate*, *13*: 103–117, 1988.
- Kyprianou, N., and Isaacs, J. T. Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology*, *122*: 552–562, 1988.
- Furuya, Y., Lundmo, P., Short, A. D., Gill, D. L., and Isaacs, J. T. The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. *Cancer Res.*, *54*: 6167–6175, 1994.
- Koslowski, J. M., Fidler, I. J., Campbell, D., Xu, Z., Kaighn, M. E., and Hart, I. R. Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res.*, *44*: 3522–3529, 1984.
- Pettaway, C. A., Pathak, S., Greene, G., Ramirez, E., Wilson, M. R., Killion, J. J., and Fidler, I. J. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin. Cancer Res.*, *2*: 1627–1636, 1996.
- Dong, Z., Ward, N. E., Fan, D., Gupta, K. P., and O'Brian, C. A. *In vitro* model for intrinsic drug resistance: effects of protein kinase C activators on the chemosensitivity of cultured human colon cancer cells. *Mol. Pharmacol.*, *39*: 563–569, 1991.
- Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, *139*: 271–279, 1991.
- Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.*, *53*: 3976–3985, 1993.
- Gurtu, V., Kain, S. R., and Zhang, G. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. *Anal. Biochem.*, *251*: 98–102, 1997.
- Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. Prevention of apoptosis by bcl-2: release of cytochrome *c* from mitochondria blocked. *Science (Washington DC)*, *275*: 1129–1132, 1997.
- Mesner, P. W., Jr., Bible, K. C., Martins, L. M., Kottke, T. J., Srinivasula, S. M., Svingen, P. A., Chilcote, T. J., Basi, G. S., Tung, J. S., Krajewski, S., Reed, J. C., Alnemri, E. S., Earnshaw, W. C., and Kaufmann, S. H. Characterization of caspase processing and activation in HL-60 cell cytosol under cell-free conditions. Nucleotide requirement and inhibitor profile. *J. Biol. Chem.*, *274*: 22635–22645, 1999.
- Chandra, J., Gilbreath, J., Freireich, E. J., Kliche, K. O., Andreeff, M., Keating, M., and McConkey, D. J. Protease activation is required for glucocorticoid-induced apoptosis in chronic lymphocytic leukemic lymphocytes. *Blood*, *90*: 3673–3681, 1997.
- Liu, X., Zou, H., Slaughter, C., and Wang, X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, *89*: 175–184, 1997.
- Isaacs, J. T. The biology of hormone refractory prostate cancer. Why does it develop? *Urol. Clin. N. Am.*, *26*: 263–273, 1999.
- Bortner, C. D., Hughes, F. M., Jr., and Cidlowski, J. A. A primary role for K^+ and Na^+ efflux in the activation of apoptosis. *J. Biol. Chem.*, *272*: 32436–32442, 1997.
- Hughes, F. M., Jr., Bortner, C. D., Purdy, G. D., and Cidlowski, J. A. Intracellular K^+ suppresses the activation of apoptosis in lymphocytes. *J. Biol. Chem.*, *272*: 30567–30576, 1997.
- Manna, S. K., Sah, N. K., Newman, R. A., Cisneros, A., and Aggarwal, B. B. Oleandrin suppresses activation of nuclear transcription factor κ B, activator protein-1 and c-Jun NH₂-terminal kinase. *Cancer Res.*, in press, 2000.
- Wang, C. Y., Mayo, M. W., and Baldwin, A. S. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF κ B. *Nature (Lond.)*, *274*: 784–787, 1996.
- Orlov, S. N., Thorin-Trescases, N., Kotelevtsev, S. V., Tremblay, J., and Hamet, P. Inversion of the intracellular Na^+/K^+ ratio blocks apoptosis in vascular smooth muscle at a site upstream of caspase-3. *J. Biol. Chem.*, *274*: 16545–16552, 1999.
- Thornberry, N. A., and Lazebnik, Y. Caspases: enemies within. *Science (Washington DC)*, *281*: 1312–1316, 1998.
- Liu, X., Kim, C. N., Yang, J., Jemerson, R., and Wang, X. Induction of the apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell*, *86*: 147–157, 1996.
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, *91*: 479–489, 1997.
- Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell*, *94*: 481–490, 1998.
- Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature (Lond.)*, *400*: 886–891, 1999.