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Cardiac glycoside induces cell death via FasL by activating calcineurin and NF-AT, but apoptosis initially proceeds through activation of caspases

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Abstract

Decrease in caspase activity is a common phenomenon in drug resistance. For effective therapeutic intervention, detection of such agents, which affects other pathway independent of caspases to promote cell death, might be important. Oleandrin, a polyphenolic glycoside induced cell death through activation of caspases in a variety of human tumour cells. In this report we provide evidence that besides caspases activation, oleandrin interacts with plasma membrane, changes fluidity of the membrane, disrupts Na⁺/K⁺-ATPase pump, enhances intracellular free Ca²⁺ and thereby activates calcineurin. Calcineurin, in turns, activates nuclear transcription factor NF-AT and its dependent genes such as FasL, which induces cell death as a late response of oleandrin. Cell death at early stages is mediated by caspases where inhibitors partially protected oleandrin-mediated cell death in vector-transfected cells, but almost completely in Bcl-xL-overexpressed cells. Overall, our data suggest that oleandrin might be important therapeutic molecule in case of tumors where cell death pathway occurs due to deregulation of caspase-mediated pathway

Keywords

NF-AT; FasL; Calcineurin; Bcl-xL; Apoptosis

Introduction

Apoptosis requires activation of a number of caspases leading to the cleavage of a variety of proteins like structural proteins, signaling proteins, regulatory proteins of transcription, replication and DNA/RNA metabolism, and fragmentation of the chromosomal DNA. Inactivation of caspase 9 confers resistance to cisplatin-mediated cell death in testicular cancer cells [1]. Human papilloma virus (HPV) positive cervical cancer cells also showed resistance to camptothecin- and cisplatin-mediated cell death through caspase 3-insensitive pathway [2]. Downregulation of these caspases in several tumor cells may lead to drug resistance in cancer therapy. Induction of the apoptosis machinery in tumor cells is the key intervention in drug resistance tumors.

Several plant products exhibit chemo preventive effects [3]. Oleandrin, a polyphenolic cardiac glycoside derived from the leaves of *Nerium oleander* was used to treat congestive heart failure

and is known to be toxic to a wide variety of tumor cells [4,5]. Oleandrin and oleandrogin inhibit export of fibroblast growth factor-2 possibly by interacting with membrane Na^+/K^+ -ATPase pump [6]. Oleandrin was found to activate NF- κ B in different cell types and induce apoptosis by caspase-dependent PARP cleavage and DNA fragmentation [7,8].

Calcineurin, a ubiquitous serine-threonine phosphatase activated by Ca^{2+} -calmodulin is involved in many signaling pathways. Calcineurin has long been a therapeutic target to prevent post-surgical rejection of transplanted organs [9,10]. The calcineurin activity was found to reduce significantly in tissues of cervical carcinoma patients [11]. A therapeutic agent that potentiates cell death without interacting calcineurin pathway would be able to regulate cancer cells where calcineurin is downregulated. Immunosuppressant drugs, cyclosporin (CsA) and FK506, block calcineurin by forming complex with the catalytic subunit. Calcineurin regulates many physiologic processes such as neuronal death, T-cell activation, and cardiac and skeletal myocytes differentiation [12]. Calcineurin is a downstream target of intracellular Ca^{2+} signaling [13]. Sustained elevation of calcium in cells keeps high calcineurin activity. Transcription factors of nuclear factor of activated T lymphocytes (NF-AT) are the substrates for calcineurin [12,14–16]. Upon stimulation of calcineurin several residues at regulatory domain of NF-AT are dephosphorylated, which leads to translocation of NF-AT to nucleus and activation of target genes of AP-1 [17], MEF2 [18,19], FasL [20], and GATA [21]. This nuclear translocation is blocked by CsA, which blocks calcineurin activity. On blocking of the Ca^{2+} signal, NF-AT is rephosphorylated by kinases, such as GSK-3 [22,23] and is exported from nucleus. Calcium-dependent calpains are involved in autocatalytic activation of caspases and a calpain inhibitor like ALLN blocks these activities [24]. Bcl-2 family of proteins, mainly Bcl-2 and Bcl-xL, bind with calcineurin and inactivate it. Thus, calcineurin-mediated downstream signaling such as dephosphorylation and translocation of NF-AT, expression of FasL, and cell death are blocked [20].

We have investigated the effect of oleandrin on the induction of apoptosis. Initially oleandrin induces cell death mediated by several caspases. It goes through FasL-mediated apoptosis as late response. Induction of FasL is mediated sequentially by alteration of membrane fluidity, impairment of Na^+/K^+ -ATPase activity, release of intracellular Ca^{2+} , activation of Ca^{2+} -dependent calcineurin, translocation of NF-AT to nucleus and increase in NF-AT binding to DNA and expression of NF-AT-dependent gene products such as FasL. For the first time, we provide evidence in this report that oleandrin induces cell death in two ways—early activation of caspases and late expression of FasL. This double-edged activity of oleandrin indicates beneficial effects in its use as therapeutic agent. Oleandrin-mediated cell death via caspases activation might be beneficial to regulate the cervical cancer, where calcineurin activity decreased.

Materials and methods

Materials

Glycine, MTT, oleandrin, diphenylhexatriene (DPH), cholesterol, sphingosine, cephalin, chondroitin sulphate, lecithine, ALLN, actinomycin D, cyclosporin A, BAPTA-AM, Fura-2AM, propidium iodide, DAPI, and anti-tubulin antibody were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Penicillin, streptomycin, neomycin, RPMI-1640 medium, and fetal bovine serum (FBS) were obtained from Life Technologies. Antibodies against NF-AT, CRM1, FasL, Bcl-xL, cytochrome *c*, proteinase 3, caspase 3, and caspase 8 and double stranded NF-AT oligonucleotide were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Plasmids for NF-AT-luciferase and FasL-luciferase were gifted by Dr. LeiChen (Crabtree laboratory, Beckman Center, Stanford University).

Cell lines

The Jurkat (T-cell) and U-937 (histiocytic lymphoma) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Human promyelocytic lymphoma HL-60 cells stably transfected with vector and Bcl-xL constructs [designated as HL-60 (neo) and HL-60 (Bcl-xL)] were obtained from Prof. Bharat B. Aggarwal, MD Anderson Cancer Center, Houston, USA. Cells were culture RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). All cells were free from mycoplasma, as detected by Gen-Probe mycoplasma detection kit (Fisher Scientific, PA, USA).

Calcineurin activity assay

After different treatments, cells (2×10^6) were extracted and extracts were passed through sephadex G-25 column to eliminate the free phosphate and proteins fractions were pulled together and used for calcineurin activity assay [11,25]. The extract (10 µg protein) was incubated with 25 µl of 2x assay buffer (200 mM NaCl, 100 mM Tris [pH 7.5], 12 mM MgCl₂, 1 mM CaCl₂). The mixture was incubated without or with RII phosphopeptide (5 µM) for 10 min at 30°C. Reaction was terminated by addition of 100 µl of Malachite green mix (3 volume of 0.045% Malachite green and 1 volume of 4.2% ammonium molybdate in 4N HCl) and allowed for 30 min incubation at 30°C. The absorbance was read at 660 nm. The calcineurin activity (fold) was calculated in terms of inorganic phosphate release from the total protein and unstimulated cells value was considered as one fold.

Determination of caspases activity

The activities of different caspases were determined using colorimetric (paranitroaniline conjugated) caspase substrates as per manufacturer's protocol Calbiochem (San Diego, CA) as described previously.

Cytotoxicity assay

The cytotoxicity was assayed by the MTT dye [26]. Briefly, cells (10^4 cells/well of 96-well plate) were incubated with test sample for 72 h at 37°C. Thereafter, 100 µg MTT dye was added to each well. After 2 h of incubation, cells were solubilized and the absorbance was read at 570 nm using multiscanner auto reader (Biorad), with the extraction buffer as a blank.

Annexin staining

The apoptotic cells were detected by Annexin V-PE apoptosis detection kit (BD Pharmingen, San Diego). After different treatments, cells were washed, suspended in Binding buffer, and incubated with Annexin V-PE and 7 AAD at 37°C for 15 min. Then cells were analyzed in FACS Scan [27].

Determination of nuclear fragmentation

U-937 cells were treated with oleandrin for different times. Cells were then harvested and fixed in 80% methanol, stained with propidium iodide (PI), and viewed under fluorescence microscope [28].

Cytochrome c release assay

Cells (5×10^7) were treated with oleandrin for different times. Cytoplasm and mitochondria were prepared by differential centrifugation, as described previously [29]. Cells were pelleted, washed once with phosphate-buffered saline, resuspended in 1 ml of ice-cold Buffer A (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 170 µg/ml phenyl-methylsulfonyl fluoride, 16 µg/ml aprotinin, and 4 µg/ml leupeptin), and incubated for 10 min on ice. Cells were lysed in a Down's homogenizer.

Unlysed cells and nuclei were pelleted at $120 \times g$ for 10 min at 4°C . The supernatant was spun at $10,000 \times g$ for 25 min at 4°C . The supernatant was collected as cytosol and the pellet (mitochondrial fraction) was re-suspended in same extraction buffer (Buffer A) to get mitochondrial proteins. Protein concentrations were determined and $150 \mu\text{g}$ of the mitochondrial fraction and an equal volume of the cytosolic fraction were loaded on a 12% SDS-polyacrylamide gel and subjected to Western blotting for cytochrome *c*.

Intracellular Ca^{2+} release assay

Intracellular Ca^{2+} mobilization was measured using the Fura-2AM method as described previously [30,31]. Briefly, taking U937 (2×10^6 cells/ml) were suspended in low Calcium Buffer A (136 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2 , 5 mM HEPES, 5.6 mM glucose, 0.42 mM NaH_2PO_4 and 0.5 mM EGTA buffer, pH 6.6). Cells were centrifuged for 6 min at $900 \times g$, then resuspended in low Calcium Buffer B (same composition as buffer A, except CaCl_2 0.16 mM and without EGTA, pH 7.4) and incubated with $2.5 \mu\text{M}$ Fura-2AM at 37°C for 30 min. Then the cells were washed and suspended in Buffer B and fluorescence intensity was monitored at a fixed excitation wavelength at 330 nm and an emission wavelength at 510 nm in a Perkin Elmer spectrofluorometer.

Measurement of membrane fluorescence

Diphenylhexatriene (DPH), a widely used membrane-interacting fluorescent probe was used to detect the alteration of membrane fluidity. DPH is soluble in tetrahydro-furan (THF). For the preparation of aqueous solution, $5 \mu\text{l}$ DPH solution (2 mM in THF) was taken in 10 ml of rapidly stirred PBS for 4 h in a dark place. For interaction with U-937 cells, either untreated or treated with oleandrin were incubated with DPH solution (final concentration, $1 \mu\text{M}$) at 37°C for 2 h. After incubation, the cells were washed, suspended in PBS, and the emission spectra of DPH bound to cells were recorded at 430 nm when the fluorescent probe was excited at 365 nm in a Perkin Elmer spectrofluorometer [32,33].

Immunocytochemistry

The level of NF-AT was examined by the immunocytochemical method as described [34] with slight modifications. Briefly, cells were plated on a poly-L-lysine-coated glass slide air-dried, fixed with 3% formaldehyde, and permeabilized with 0.1% of Triton X-100. Slides were incubated with anti-p65 Ab for 6 h followed by incubation with anti-rabbit IgG-Alexa Fluor or -FITC for 1 h. Slides were mounted with mounting medium with DAPI and analyzed under a fluorescence microscope.

NF-AT DNA binding activity assay

To determine NF-AT activation, EMSA were conducted essentially as described [11,35]. Briefly, cells, after different treatments were used to prepared cytoplasmic and nuclear extracts. Nuclear extract proteins ($8 \mu\text{g}$) were incubated with ^{32}P end-labeled double-stranded NF-AT oligonucleotide of, 5'-CGCCCAAAGAGGAAAATTTGTTTCATA-3' for 30 min at 37°C , and the DNA-protein complex was separated from free oligonucleotide on 6.6% native PAGE. Visualization of radioactive bands was done in a PhosphorImager (Fuji, Japan).

FasL- or NF-AT-dependent luciferase gene transcription assay

The level of FasL- or NF-AT-dependent luciferase reporter gene expression was carried out as described previously [36,37]. Cells were transiently transfected with SuperFect transfection reagent for 3 h containing $0.5 \mu\text{g}$ of each reporter plasmid containing NF-AT binding site cloned upstream of luciferase (NF-AT-Luciferase) or NF-AT binding site cloned upstream of FasL-luciferase (FasL-Luciferase) and GFP constructs. Cells were cultured for 12 h followed by treatment with oleandrin. GFP positive cells were counted. The cell pellets were extracted with

lysis buffer (part of Luciferase assay kit) and incubated with the firefly luciferin substrate. Light emission was monitored with a Luminometer and values were calculated as fold of activation over vector-transfected value.

Detection of FasL by semi quantitative RT-PCR

The expression of Fas was detected by RT-PCR followed by PCR with FasL specific primers from extracted RNA as reported [38], keeping expression of housekeeping actin gene as control.

Membrane preparation and Na⁺/K⁺-ATPase activity assay

U-937 cells membrane was prepared by sucrose gradient centrifugation as described previously [32]. The membrane pellet was extracted in the TES buffer (1% Triton X-100) and equal amount of membrane proteins were incubated with 100 μ l of ATP assay buffer (0.6 mM EGTa, 156 mM NaCl, 24 mM KCl, 3.6 mM MgCl₂, 60 mM imadizole, and 3.6 mM ATP), pH 7.2 and 200 μ l of Malachite Green Mix in triplicates for 30 min at 37°C [39,40]. The absorbance was taken at 655 nm and the activity. The ATPase activity (%) was calculated in terms of inorganic phosphate release from the total membrane protein and unstimulated cells value was considered as cent percent.

Results

Oleandrin increases calcineurin activity in U-937 cells

Cells were treated with oleandrin (100 ng/ml) for different times and then activity of calcineurin was measured in whole cell extracts. Calcineurin activity increased in a time-dependent manner by oleandrin treatment. Specificity of the assay of calcineurin activity was established by its inhibition by cyclosporin A in the extract from 36 h of oleandrin treatment (Fig. 1). These results suggest that oleandrin treatment induces calcineurin activity.

Oleandrin induces nuclear translocation of NF-AT and DNA binding

Cells were treated with oleandrin (100 ng/ml) for different times and cytoplasmic and nuclear extracts were assayed for NF-AT levels. The concentration of NF-AT decreased with increase in time in the cytoplasmic extracts and increased in the nuclear extracts (Fig. 2(A)). High concentration of NF-AT was found by immunofluorescence technique in the cytoplasm of unstimulated cells and nucleus of oleandrin-treated cells (Fig. 2(B)). By gel shift assay the NF-AT DNA binding was enhanced with increasing time of oleandrin treatment in U-937 and Jurkat cells (Fig. 2(C)). Oleandrin increased NF-AT-dependent reporter gene luciferase activity in a time-dependent manner in U-937 cells transfected with NF-AT-luciferase construct (Fig. 2(D)). These data suggested that oleandrin increases NF-AT nuclear translocation and DNA binding activity.

Oleandrin increases FasL expression

U-937 cells, transfected with FasL-luciferase construct were treated with oleandrin for different times and luciferase activity was assayed in cell extracts. Oleandrin increased luciferase activity in a time-dependent manner (Fig. 2(E1)).

The amount of FasL mRNA, measured by RT-PCR followed by PCR increased with increase in oleandrin treatment (Fig. 2(E2)). The amount of FasL in U-937 cells extract increased at 24 and 36 h of oleandrin treatment (Fig. 2(E3), upper panel). The amount of FasL was also increased in the culture supernatant collected from oleandrin-treated cells at 24 and 36 h (Fig. 2(E3), lower panel). These data suggest that oleandrin induces expression and release of FasL in U-937 cells.

Inhibition of calcineurin or Ca^{2+} level partially protects oleandrin-induced cell death

U-937 cells were pretreated with cyclosporin A or BAPTA-AM ($2.5 \mu\text{M}$ each) for 4 h and then treated with oleandrin for 24 h. Oleandrin induced 48% cell death at 36 h of oleandrin treatment and almost 50% protection of oleandrin-mediated cell death was observed either cyclosporin A or BAPTA-AM pretreated cells (Fig. 3(A)). Oleandrin increased intracellular Ca^{2+} level as detected by Fura-2AM. BAPTA-AM almost completely blocked intracellular free Ca^{2+} level induced by oleandrin (Fig. 3(B)). The calcineurin activity increased almost 60 fold. Oleandrin-mediated calcineurin activity completely inhibited by pretreatment of cyclosporin A or BAPTA-AM (Fig. 3(C)). All these results suggested that oleandrin-mediated cell death was partially blocked by inhibiting calcineurin activity or intracellular Ca^{2+} level at 24 h of oleandrin treatment. Role of Ca^{2+} or calcineurin in oleandrin-mediated cell death is indicated.

Oleandrin increases caspases activity and cell death

U-937 cells treated with oleandrin for different times and levels of procaspase 8 or 3 and active caspase 3 or 8 were measured from cell extracts. As shown in (Fig. 4(A)), the procaspase 3 or 8 level decreased at 12 and 24 h of oleandrin treatment. The level of caspase 3 or 8 increased at 12 and 24 h of oleandrin treatment. The activities of caspase 3, 8, or 9 increased with oleandrin treatment till 12 h. After this no significant increase in caspases activity was observed (Fig. 4(B1)). The amount of cytochrome *c* released from mitochondria with increasing time of oleandrin treatment, corresponded with its release into cytoplasm (Fig. 4(B2)). All these results suggest that activity of caspases increases at early time of oleandrin treatment. Oleandrin induced nuclear fragmentation in a time-dependent manner (Fig. 4(C)).

Oleandrin does not induce necrosis

The concentrations and duration of exposure of different agents used for these studies had no effect on cytolysis as detected by lactate dehydrogenase (LDH) assay from culture supernatant. Culture supernatant from oleandrin-treated U-937 cells for 0, 12, 24, and 36 h when incubated with substrate solution (0.23 M sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5) showed 1.122 ± 0.092 , 1.102 ± 0.104 , 1.092 ± 0.104 , and 1.066 ± 0.094 absorbencies, respectively at 340 nm.

Actinomycin D, z-VAD, cyclosporin A and anti-FasL antibody protect oleandrin-mediated cell death

Cell death, measured by annexin-PE, by z-VAD-fmk was completely inhibited at 12 h and partially at 24 and 36 h of oleandrin treatment (Fig. 5). Transcription blocker actinomycin D inhibited cell death partially at 36 h of oleandrin treatment. Cyclosporin A was unable to inhibit oleandrin-mediated cell death at 12 h but partially inhibited at 24 and 36 h. Calpain inhibitor, ALLN, inhibited cell death at 12 and 24 h and partially inhibited at 36 h of oleandrin treatment. Cyclosporin A and z-VAD-fmk in combination completely inhibited oleandrin-mediated cell death at any time of oleandrin treatment. Anti-FasL antibody was unable to block cell death at 12 and 24 h, partially inhibited at 36 h of oleandrin treatment. These results suggested that caspases inhibitor block oleandrin-mediated cell death at early time but cyclosporin A blocks at later time of oleandrin treatment.

Oleandrin inhibits Na^+/K^+ -ATPase activity and alters membrane fluidity

Considering the role of Na^+/K^+ -ATPase in regulation of intracellular Ca^{2+} level and oleandrin's ability to increase intracellular free Ca^{2+} level, the effect of oleandrin in Na^+/K^+ -ATPase activity was tested. U-937 cells were treated with oleandrin and ouabain and Na^+/K^+ -ATPase activity was assayed from extracts. These treatments decreased Na^+/K^+ -ATPase activity by almost 50% with oleandrin and 65% with ouabain (Fig. 6(A)). To understand the mechanism of oleandrin-mediated inhibition of Na^+/K^+ -ATPase, a membrane protein, the

membrane fluidity was measured by assaying the binding of diphenylhexatriene (DPH) with membrane lipids (Fig. 6(B)). DPH binding, as detected by its fluorescence spectrum, decreased in oleandrin-treated cells suggesting that oleandrin alters membrane fluidity.

Lipid molecules protect oleandrin-mediated cell death

As oleandrin alters DPH binding by changing lipid environment of membrane, oleandrin was first incubated with different lipid molecules and the cells were treated with this mixture. Different lipid molecules did not induce cell death by themselves. Oleandrin induced 57% cell death. Oleandrin-mediated cell death was protected by almost 60% by sphingosine, and 30–40% by cephalin or lecithin when mixed with oleandrin (Fig. 6(C)). These results suggest that oleandrin interacts with lipid molecules.

Oleandrin is unable to induce nuclear translocation and DNA binding activity of NF-AT in Bcl-xL-overexpressed cells

Oleandrin-mediated NF-AT nuclear translocation and DNA binding has been detected in Bcl-xL-overexpressed cells. The level of Bcl-xL increased in Bcl-xL stable transfected HL-60 cells and oleandrin did not decrease this level at any time of treatment (Fig. 7(A)). Oleandrin induced NF-AT nuclear translocation in vector transfected HL-60 (HL-60-neo) cells, but this translocation was completely protected in Bcl-xL-overexpressed cells as indicated by immunofluorescence data (Fig. 7(B)). The NF-AT DNA binding activity increased with oleandrin treatment in a time-dependent manner in HL-60 (neo) cells as determined from gel shift assay from nuclear extracts, but no activity was observed in Bcl-xL-overexpressed cells at any time of oleandrin treatment (Fig. 7(C)). These results suggest that oleandrin is unable to induce NF-AT activity in Bcl-xL-overexpressed cells.

Oleandrin induces cell death partially in Bcl-xL over expressed cells and caspases inhibitor blocks it completely

The role of Bcl-xL in oleandrin-mediated cell death and NF-AT activity was then investigated. HL-60 cells, stable transfected with vector of Bcl-xL, were treated with oleandrin for different times and cell viability measured by MTT assay. Oleandrin-mediated cell death was reduced partially in Bcl-xL overexpressed cells. z-VAD-fmk pre-treated cells almost completely blocked oleandrin-mediated cell death in Bcl-xL-overexpressed HL-60 cells (Fig. 7(D)). These results suggest oleandrin mediates cell death by caspases activation in Bcl-xL-overexpressed cells. Oleandrin induced the release of intracellular Ca^{2+} in HL-60 neo and Bcl-xL over-expressed cells equally as detected by Fura-2AM fluorescent probe (data not shown).

Discussion

Oleandrin induces apoptosis by activating caspases leading to cleavage of several cellular proteins followed by nuclear fragmentation [23]. Activation of caspases occurs in the early phase of oleandrin treatment. Inhibition of caspases partially protected oleandrin-mediated cell death confirming their role in cell death. These results prompted us to determine if any other pathways were involved in oleandrin-mediated cell death. Understanding the mechanism of action of any drug would be important to detect any alternate pathway beside caspases activation for cell death. Previously, we reported that oleandrin interacts with lipid molecules, alters the fluidity of membrane, and down regulates interleukin-8 receptors [32]. In this report we provided data that oleandrin treatment of cells decreases activity of Na^+/K^+ -ATPase, possibly by altering membrane fluidity. This enzyme keeps the cellular Ca^{2+} homeostasis [41]. Decrease in the Na^+/K^+ -ATPase activity leads to increase in the intracellular free Ca^{2+} level. Increased Ca^{2+} induces calcineurin activity. Calcineurin, a serine/threonine phosphatase dephosphorylates its substrate protein NF-AT in the cytoplasm. In unstimulated cells, NF-AT remains in the cytoplasm as phosphorylated form and thereby unable to translocate to nucleus.

As oleandrin induces calcineurin through elevation of Ca^{2+} level, the NF-AT translocates to nucleus due to its dephosphorylation. Several reports support that FasL expression is NF-AT-dependent and cyclosporin A or FK506 blocks NF-AT-dependent FasL expression [12,42–44]. As oleandrin induces nuclear translocation of NF-AT, the NF-AT-dependent gene activation is also seen by FasL expression. Increased level of FasL leads to apoptosis since 24 h of oleandrin treatment. Oleandrin mediates cell death through apoptosis, but not by necrosis as oleandrin treatment till 36 h did not show any cytoplasmic leakage as detected by LDH release assay.

Oleandrin-mediated cell death is partially inhibited either by blocking calcineurin-NF-AT pathway or caspases activation. Oleandrin induces caspases activation since 6 h of treatment and induces cell death after 12 h, whereas FasL-mediated cell death occurs at late time of oleandrin treatment (after 24 h). z-VAD-fmk, a pan caspases inhibitor blocks oleandrin-mediated cell death completely at 12 h of oleandrin treatment and partially at later time point; cyclosporin, a calcineurin inhibitor does not block at 12 h of oleandrin treatment and partially inhibits at later time of ole-andrin treatment; whereas z-VAD-fmk and cyclosporin A, in combination, block almost 80% cell death mediated by oleandrin. All these results suggest that oleandrin mediates cell death by caspases at early time of oleandrin treatment, whereas calcineurin-mediated cell death via FasL expression at later time of oleandrin treatment. Anti-FasL antibody protected oleandrin-mediated cell death at later time of incubation, which supports the involvement of FasL. Oleandrin mediates the release of FasL into supernatant, which might interact with its cell surface receptor to induce cell death. Enhancement of intracellular Ca^{2+} also induces several cal-pains, which in turn activates caspases. Calpain inhibitor ALLN inhibits cell death at the early time of oleandrin treatment. Release of cytochrome *c* from mitochondria supports the mitochondrial potential change and early activation of caspase -3 and -9 than -8 (Fig. 4(B1)).

Alteration of membrane fluidity leads to sequential inactivation of Na^+/K^+ -ATPase, increase in intracellular Ca^{2+} level and calcineurin activity, translocation of NF-AT to nucleus, increase in NF-AT DNA binding activity, FasL expression, and induction of apoptosis. Cells, treated with incubation mixture of lipid molecules and oleandrin show partial protection of oleandrin-mediated cell death. Oleandrin interacts with lipid molecules and inhibits Na^+/K^+ -ATPase, which leads to the increase in Ca^{2+} level followed by downstream signaling to show apoptosis via FasL. Calcineurin interacts with Bcl-2 and Bcl-xL and fails to dephosphorylate NF-AT and thereby downstream signaling [20]. In Bcl-xL stably transfected HL-60 cells, oleandrin mediates cell death partially after 12 h of treatment. At 12 h of oleandrin treatment, the cell death in both neo and Bcl-xL-overexpressed cells show almost similar. As oleandrin induces caspases activation initially, the cell death has not been protected in Bcl-xL-overexpressed cells at 12 h time of oleandrin treatment. Oleandrin does not induce nuclear translocation of NF-AT and FasL expression in Bcl-xL-overexpressed cells. Though oleandrin-mediated cell death was partially protected in Bcl-xL-overexpressed cells, but the release of intracellular Ca^{2+} was similar in neo and Bcl-xL-overexpressed cells. This result indicates that the role Bcl-xL to protect oleandrin-mediated cell death lies much downstream, which may be on the nuclear translocation of NF-AT level.

We report here for the first time that oleandrin-mediated cell death is proceeding through caspases activation and FasL expression. Mutation of caspases is common in some cancers. This makes it difficult to keep cells sensitive to different therapeutic drugs that act on caspases. Detection and understanding the mechanism of action of therapeutic agent(s) are always helpful to effective chemotherapy. Induction of FasL is an important advantage conferred by oleandrin in mediating cell death in addition to apoptosis mediated by the caspases. Most of the chemotherapeutic drugs induce cell death by activation of different caspases, DNA intercalating, or mutation in tumor suppressors. Oleandrin may serve as a potential

chemotherapeutic agent especially in caspases inactive tumor cells, and induce cell death via caspase activation in calcineurin-deregulated tumor cells such as cervical carcinoma. Further this study may find application in the design of pharmaceutical interventions for cancer.

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Abbreviations

ALLN	N-acetyl-leucyl-leucyl-norleucinal
BAPTA-AM	1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetra acetic acid - acetoxymethyl ester
CsA	cyclosporin A
DAPI	4', 6-diamidino-2-phenylindole
DPH	diphenylhexatriene
GFP	green fluorescent peptide
FITC	fluorescein isothiocyanate
MTT	-(4,5-dimethyl-2-thiozoly)- 2,5-diphenyl-2H-tetrazolium bromide
NF-AT	nuclear factor of activated T lymphocytes
PE	phycoerythrin
PI	propidium iodide

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Fig. 1.

Effect of oleandrin on calcineurin activity. U-937 cells were treated with 100-ng/ml oleandrin for different times and calcineurin activity assayed from cell extracts. Cells extract from oleandrin-treated (36 h) cells was incubated with cyclosporin A (2.5 μ M) and calcineurin activity assayed

Fig. 2.

Effect of oleandrin on NF-AT nuclear translocation, DNA binding, NF-AT-dependent luciferase activity, and FasL expression. U-937 cells were treated with 100-ng/ml oleandrin for different times. NF-AT was detected from cytoplasmic and nuclear extracts by Western blot and blots were re-probed for tubulin and CRM1 respectively (A). U-937 cells, treated with oleandrin for 12 h were incubated with anti-NF-AT antibody conjugated with FITC and DAPI and detected in Fluorescence microscope (B). U-937 and Jurkat cells were treated with oleandrin for different times and nuclear extracts were assayed for NF-AT DNA binding by EMSA (C). U-937 cells were transfected with Qiagen SuperFect reagent for 3 h with plasmids for NF-AT or FasL promoter DNA that had been linked to luciferase (NF-AT-Luciferase or

FasL-Luciferase) and GFP. After washing, cells were cultured for 12 h. The GFP positive cells were counted and transfection efficiency was calculated. Cells, treated with oleandrin (100 ng/ml) for different times were extracted and assayed for luciferase as per Promega protocol (D & E1). FasL level was detected by RT-PCR followed by PCR using FasL and actin specific primers (E2) and Western blot (E3) from oleandrin-treated cells extract and culture supernatant. The culture supernatant blot was re-probed with anti-proteinase antibody

Fig. 3.

Effect of cyclosporin A and BAPTA-AM on oleandrin-mediated cell viability, Ca^{2+} release, and calcineurin activity. U-937 cells, incubated with cyclosporin A or BAPTA-AM ($2.5 \mu\text{M}$ each) for 4 h and then treated with oleandrin (100 ng/ml) for 24 h in triplicate. Cell death detected by annexin V-PE and analyzed in FACS (A). Intracellular free Ca^{2+} detected using Fura-2AM as fluorescent probe in Fluorimeter (B). Calcineurin activity assayed from whole cell extracts (C)

Fig. 4.

Effect of oleandrin on caspases activity and cell death. U-937 cells were treated with 100-ng/ml oleandrin for different times and procaspase 3, caspase 3, procaspase 8, and caspase 8 were detected by Western blot using specific antibodies (A). Caspase 3, 8, and 9 were detected from whole cell extracts using colorimetric substrate. The absorbance was taken at 405 nm and results represented in fold activation of caspases (B1). The amount of cytochrome *c* was detected from mitochondrial extract and cytoplasm from 100 ng/ml oleandrin-treated cells for different times by Western blot (B2). Nuclear fragmentation was detected from oleandrin (100 ng/ml for different times) treated U-937 cells by propidium iodide staining (C)

Fig. 5.

Effect of Cyclosporin A, actinomycin D, z-VAD-fmk, ALLN, or anti-FasL antibody on oleandrin-mediated cell death. U-937 cells were treated with cyclosporin A (2.5 μ M), actinomycin D (30 nM), z-VAD-fmk (3 μ M), ALLN (10 μ M), anti-FasL antibody (1 μ g/ml) for 6 h and treated with 100 ng/ml oleandrin for different times. Cell death was assayed by Annexin V-PE and detected in FACS and represented in percentage. The figure represented one of the two independent experiments.

Fig. 6.

Effect of oleandrin on Na⁺/K⁺-ATPase activity and microviscosity. U-937 cells were treated with oleandrin and ouabain (100 ng/ml each) for 12 h; Na⁺/K⁺-ATPase activity was assayed from whole cell extract (A). U-937 cells, treated with oleandrin for 12 h were incubated with DPH for 2 h with moderate shaking. DPH binding was detected from cells in spectrofluorimeter (B). U-937 cells were treated with different lipid molecules (100 ng/ml) or oleandrin and lipid molecules mixture (100 ng oleandrin and 100 ng lipid molecule incubated for 4 h) for 72 h. Cell viability was detected by MTT assay and results represented as inhibition of cell viability in percentage (C)

Fig. 7. Effect of oleandrin on cell death, translocation of NF-AT to nucleus, and NF-AT DNA binding activity in Bcl-xL overexpressed cells. HL-60 (neo) and HL-60 (Bcl-xL) cells were treated with oleandrin (100 ng/ml) for different times. The level of Bcl-xL was detected by Western blot (A). Same blot was re-probed with anti-tubulin antibody. Cells were treated with oleandrin for 12 h and NF-AT was detected by immunofluorescence assay (B). Cells were treated with oleandrin for different times and nuclear extracts were assayed for NF-AT DNA binding by gel shift assay (C). Cell viability was assayed by MTT uptake and indicated as inhibition of cell death in percentage (D)