Oleandrin induces apoptosis via activating endoplasmic reticulum stress in breast cancer cells

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ABSTRACT

Background: Breast cancer is the most common malignant tumor in women. Due to limited treatment outcome and high rate of metastasis, the prognosis is especially poor for triple-negative breast cancer. It is urgent to discover and develop novel agents for treatment of breast cancer. Herein, we investigated the potential mechanisms of Oleandrin’s cytotoxic activity against breast cancer cells.

Methods: Cell proliferation was assessed by xCELLigence Real-Time Cell Analyzer (RTCA)-MP system. Apoptotic cells were detected by using Annexin V/PI staining and nuclear fragments observation. The effect of oleandrin on ATP1B3 expression and markers of ER stress were determined by western blot. A primary cell sensitivity assay was performed via a collagen gel droplet-embedded culture drug sensitivity method (CD-DST).

Results: Oleandrin suppressed cell proliferation and colony formation in the three breast cancer cell lines but did not affect normal mammary epithelial cells. Additionally, the expression of ATP1B3 was higher in the three breast cancer cell lines compared to MCF10A cells. Treatment with oleandrin increased the number of apoptotic cells and led to nuclear pyknosis, fragmentation, and apoptotic body formation in breast cancer cells. Furthermore, oleandrin treatment increased expression of Bax and Bim but decreased that of Bcl-2. Treatment with oleandrin also upregulated the expression of endoplasmic reticulum stress associated proteins, including eIF2α, ATF4, and CHOP, but not PERK. Oleandrin treatment also induced the phosphorylation of PERK and eIF2α. Of note, oleandrin exhibited antitumor effects on patient-derived breast cancer cells under three-dimensional culture conditions.

Conclusions: Taken together, our results suggest that oleandrin induces mitochondrial-mediated apoptosis by activating endoplasmic reticulum stress in breast cancer. Moreover, oleandrin may be an effective strategy for the treatment of breast cancer.

1. Introduction

Breast cancer is the most common malignant tumor in women with approximately 2.89 million new cases per year and 626,000 deaths worldwide in 2018 [1,2]. Currently, the treatment of breast cancer relies on surgery, chemotherapy, radiotherapy, endocrine therapy and antibody therapy [3]. The prognosis is especially poor for triple-negative breast cancer, which accounts for 15 % of breast carcinomas, due to limited treatment options and high rate of metastasis [4,5]. Although the standard treatment and novel drug delivery system has improved survival, further improvements must be made to increase survival [6–9]. Therefore, it is crucial to discover new and more effective drugs.

Cardiac glycosides (CGs), derived from Digitalis, such as digoxin, digitoxin, and ouabain, have been used to treat congestive heart failure and several types of arrhythmias [10]. In myocytes, CGs increase the Na+ influx and decrease the K+ influx through inhibition of Na+/K+-ATPase [11]. The increased intracellular Na+ concentration activates the exchange of Na+ and Ca2+, and the resulting increased intracellular Ca2+ concentration increases cardiomyocyte contractility. Recently, studies have discovered the antitumor activity of CGs [12,13]. UNBS1450, which belongs to the CG family, induces tumor apoptosis by regulating the cleavage of caspase 8, 9, and 3/7 via the NF-kB pathway.

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Incubator (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured at 37 °C in a 5 % CO2 atmosphere.

2. Materials and methods

2.1. Cell lines and cell culture

The mammary epithelial cell line MCF10A was purchased from the American Type Culture Collection (ATCC). Human breast cancer cell lines: MDA-MB-231, MCF7, and SK-BR-3 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF10A cells were cultured in MEGM (Lonza, Morristown, NJ, USA) with 100 ng/ml cholera toxin. MDA-MB-231 cells were cultured in Leibovitz L-15 (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA). MCF7 cells were cultured in minimum Eagle’s medium (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA). SK-BR-3 cells were cultured in high-glucose Dulbecco’s Modified Eagle medium (Hyclone, GE Healthcare Life Sciences, Logan, UT, USA). All medium contained 10 % fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cells were cultured at 37 °C in a 5 % CO2 incubator (Thermo Fisher Scientific, Inc.).

2.2. Patients

This study was approved by the ethics committee of the Liaoning Cancer Hospital Ethics Review Approval No. 20181235. A total of 20 female participants with pathologically diagnosed breast cancer without neoadjuvant chemotherapy was enrolled in the present study. Informed consents were provided to all the participants. The clinical features of 20 participants were shown in Table 1.

2.3. Compounds and antibodies

Oleandrin was purchased from MedChemExpress (Monmouth Junction, NJ, USA) and its chemical structure was shown in Fig. 1. Oleandrin was dissolved in sterile dimethyl sulfoxide (DMSO), and the final concentration of DMSO is less than 0.1 % in all experiment. The information of primary antibodies as follow: anti-Bax #2774, anti-Bim #60434 and anti-β-actin #3700 were purchased from Cell Signaling Technology, Inc. Danvers, MA, USA. The anti-Bcl-2 (33-6100) and SuperSignal West Pico PLUS Chemiluminescent Substrate were obtained from Thermo Fisher Scientific, Inc. Anti-PERK (ab227593), anti-eIF2α (phospho S52) (ab8426), anti-ATF4 (ab182760), anti-CHOP (ab11419), goat anti-mouse IgG Horseradish peroxidase (HRP) (ab205719), goat anti-Rabbit IgG-HRP (ab6721), anti-PERK (phospho Y982) (ab192591) and anti-ATP1B3 antibody (ab137055) were purchased from Abcam (Cambridge, MA, USA).

Table 1: Clinical characteristics of the participants.

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<td>+</td>
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</table>

Abbreviation: ER, estrogen receptor. PR, progesterone receptor. C-erbB-2, erb-b2 receptor tyrosine kinase 2.

* TNM staging is carried out according to the American Joint Committee on Cancer (AJCC) eighth edition standard.

2.4. Cell viability assay

Before we performed xCELLigence Real-Time Cell Analyzer (RTCA)-MP system (ACEA Biosciences, Inc., San Diego, CA, USA) experiment, the IC50 of Oleandrin in MCF-7, SK-BR-3 and MB-MDA-231 cells was determined by MTT assay. The IC50 of Oleandrin ranged from 1 to 30 nM. Based on this result, the Real-Time Cell Analyzer experiment was designed. Cell concentration was adjusted to 1 × 104 cells/ml in complete medium. Next, 100 µl of cell suspension were seeded into E-16 plates, which contained a biocompatible microelectrode array. The E-16 plates and the device were placed in an incubator. Electrical impedance, which reflects cell viability, was monitored in every 5 min and presented as the cell index. The cell index was normalized to the time point of agent treatment.

2.5. Clonogenic assay

A total of 2 × 103 cells were seeded into a 6-well plate in 2 ml complete medium. When the cells were attached to the plate after 18 h, oleandrin was added into each well at the half-maximal inhibitory concentration (IC50) concentration. After 24 h treatment, cells were then rinsed twice with PBS and cultured in fresh medium without oleandrin for 14 days. Subsequently, the colonies were fixed with formalin and stained with crystal violet.
2.6. Apoptosis analysis

The 5 × 10^5 cells harvested in each group were stained with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 5 μl propidium iodide (PI) in 500 μl buffer. After 10 min, cells were rinsed twice with PBS. DMSO-treated cells were served as the untreated control. Apoptosis was analyzed by flow cytometry (BD Accuri™ C6, BD Biosciences, Franklin Lakes, NJ, USA).

2.7. Nuclear morphology characterization

The cells were treated with oleandrin for 24 h. Cells were then rinsed twice with PBS and stained with Hoechst 33342 Solarbio, Beijing, China at a concentration of 10 μg/ml at 37 °C. After 30 min, cells were washed with PBS 3 times and observed under a DMi8 fluorescent microscope Leica, Wetzlar, Germany.

2.8. Western blotting analysis

The treated and control cells were harvested and protein was extracted for western blotting. Primary antibodies were diluted according to the product description and incubated at 4 °C overnight. The secondary antibodies (goat anti-mouse IgG-HRP (1:10,000) or goat anti-rabbit IgG-HRP (1:15,000) against the primary antibodies were applied.

Immunodetection was performed using a Bio-Rad GelDoc XR + system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.9. Primary breast cancer cell sensitivity assay

A primary cell sensitivity assay was performed with collagen gel droplet-embedded culture drug sensitivity method (CD-DST; Kurabo, Osaka, Japan) as previously described [22]. Briefly, breast cancer specimens were obtained from patients undergoing surgical resection. Tissues were isolated and digested with EZ enzyme (Nitta Gelatin Inc., Osaka, Japan). Disassociated primary breast cancer cells were cultured in a collagen gel-coated flask (Nitta Gelatin Inc.) for 24 h. Adherent cells were then harvested and seeded in three-dimensional (3D) collagen droplets, and then treatment with 20 nM or 40 nM oleandrin. After 24 h of treatment, the medium was removed and replaced with prepared culture media-2 (PCM-2; Kurabo) without FBS for 7 days. Droplets were stained with neutral red and fixed with formalin. Cell viability relative to optical density was analyzed using the Primag System (Solution Systems, Tokyo, Japan).

2.10. Statistical analysis

The results were analyzed via one-way analysis of variance by using the SPSS software (version 21, IBM Corp., Armonk, NY, USA). Multiple comparisons between the control and treatment groups were evaluated using Dunnett’s Least Significant Difference test. Data is presented as mean ± SD. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Oleandrin suppresses the proliferation and colony formation of breast cancer cells, but not mammary epithelial cells

To investigate the inhibitory effects of oleandrin on breast cancer cell viability, MCF7 (luminal A subtype), SK-BR-3 (HER-2 + subtype), and MDA-MB-231 (triple negative breast cancer, TNBC) cells were treated with oleandrin, then cell proliferation was monitored using the RTCA-MP system every 5 min for 72 h. The control group was treated with DMSO. As shown in Fig. 2A, oleandrin significantly inhibits breast cancer cell growth (MCF7, SK-BR-3 and MDA-MB-231) in a dose- and time-dependent manner. Of note, oleandrin did not affect MCF10A proliferation, even at higher concentrations (Fig. 2A). The IC50 of oleandrin at 24 h and 48 h for MCF7 was 14.5 and 6.07 nM, respectively; for SK-BR-3 cells, 6.13 and 1.42 nM, respectively; and for MDA-MB-231 cells, 24.62 and 11.47 nM, respectively. Proliferation of each of the three molecular subtypes of breast cancer cells was inhibited by oleandrin at nanomolar level. To further demonstrate this result, breast cancer cells were treated with oleandrin and colony formation was analyzed (Fig. 2B). Oleandrin significantly suppressed colony formation of the three breast cancer cell lines. Previous studies have suggested that the Na+/K+-ATPase α3 subunit (ATP1B3) may be the target of oleandrin [23]. Herein, the protein expression level of ATP1B3 in 3 breast cancer cells (MCF7, SK-BR-3, MDA-MB-231) and MCF10A were determined by western-blot analysis. As result shown in Fig. 2C, the expression level of ATP1B3 was lower in MCF10A than MCF7, SK-BR-3, and MDA-MB-231 cells. This result suggests that the cytotoxic activity of oleandrin against breast cancer cells may be associated with the expression level of ATP1B3.

3.2. Effect of oleandrin on inducing apoptosis of breast cancer cells

Given that oleandrin exhibited potent cytotoxic effect in breast cancer cells, we further investigate the effects of oleandrin on apoptosis of breast cancer cells, including non-triple negative breast cancer cell line MCF7 and TNBC cell line MDA-MB-231. After treated with oleandrin at IC50 concentration for 24 h, apoptotic cells were analyzed by staining with Annexin V-FITC and PI. DMSO-treated cells served as the control. Apoptotic cells were defined as either Annexin V-FITC+ PI− (early apoptosis) or Annexin V-FITC+ PI+ (late apoptosis). Compared to the DMSO-treated control group, the number of apoptotic cells increased in the breast cancer cells after oleandrin treatment (Fig. 3A). Additionally, oleandrin-induced apoptosis was further assessed with nuclear staining and observation by fluorescence microscopy. As seen in Fig. 3B, nuclear pyknosis, fragmentation, and apoptotic bodies were observed in oleandrin-treated breast cancer cells, whereas the control cells displayed normal round nuclei. These results suggest that oleandrin induces breast cancer cell apoptosis.

3.3. Oleandrin initiates mitochondrial apoptosis signaling pathway

Endogenous activation of the mitochondrial-signaling pathway is involved in apoptosis regulation. The Bcl-2 family of proteins, including the anti-apoptotic protein Bcl-2 and pro-apoptotic proteins Bax and Bim, serve important roles in the mitochondrial apoptotic pathway [24]. In the present study, Bcl-2, Bax, and Bim were detected by western blot. As presented in Fig. 3C, compared to the control group, the expression levels of Bax and Bim were upregulated and Bcl-2 was downregulated following treatment with oleandrin. Overall, these results demonstrate that oleandrin-induced apoptosis is associated with the mitochondrial apoptosis signaling pathway.

3.4. Oleandrin activates ER stress in breast cancer cells

ER stress plays an important role in the regulation of the mitochondrial apoptosis pathway [25]. In the present study, the key ER stress proteins were examined after 12 h of oleandrin treatment. Expression of PERK, a protein located on the ER that detects ER stress, did not significantly differ between the control and oleandrin-treated groups. However, phospho-PERK increased after treatment with oleandrin in both MCF7 and MDA-MB-231 cells. Phospho-PERK phosphorylates eIF2α, which further upregulates CHOP [26]. As shown in Fig. 4, increased expression and phosphorylation of eIF2α correlates with increased expression of ATF4 and CHOP. These results suggest that the ER stress pathway is activated in oleandrin-treated MCF7 and MDA-MB-231 cells.
3.5. Effect of oleandrin on the proliferation of primary breast cancer cells

To investigate whether oleandrin may be a potential therapeutic drug for breast cancer, the primary breast cancer cells were treated with oleandrin via a CD-DST assay. DMSO treated cells served as the control. Cells with high viability were prominently stained with neutral red (Fig. 5). Compared with the control group, the cell viabilities were reduced by oleandrin in Lumina A subtype, Lumina B subtype, HER-2+ subtype and TNBC.
Fig. 3. Oleandrin induces apoptosis in human breast cancer.

(A) A total of $1 \times 10^5$ MCF7 and MDA-MB-231 cells were treated with oleandrin or DMSO for 24 h, and then stained with Annexin V-FITC and PI. Cell apoptosis was detected using flow cytometry. The Annexin V-FITC$^+$/PI$^-$ cells were compared to control groups. **$P < 0.01$ vs. control. B Control and oleandrin-treated cells were stained with Hoechst 33342, and observed by fluorescence microscopy. C MCF7 and MDA-MB-231 cells were treated with oleandrin or DMSO for 24 h. The expression of Bcl-2, Bim, and Bax was detected by western blot using $\beta$-actin as a loading control.
4. Discussion

Oleandrin, isolated from the leaves of *Nerium oleander*, is cytotoxic to a variety of tumors. Compared to other CGs (including gitoxigenin, convallatoxin, strophanthidin, and nerifolin), oleandrin selectively and more strongly suppresses colon cancer cell growth without reducing the viability of normal human colonic epithelial cells. Na+/K+-ATPase α3 subunit maybe the target of oleandrin, and this may explain the selectivity [23,27]. In the present study, the expression of the Na+/K+-ATPase α3 subunit in mammary epithelial cell line MCF10A and breast cancer cell lines MDA-MB-231, MCF7, and SK-BR-3 were compared using western blot. The result showed that protein expression of the Na+/K+-ATPase α3 subunit was lower in MCF10A compared to the breast cancer cell lines. We observed that oleandrin significantly inhibited proliferation in MDA-MB-231, MCF7, and SK-BR-3 cells, but not in MCF10A cells. In addition, the genotype background of breast cancer cells as follows: MCF-7 (Luminal A), SK-BR-3 (HER2+), MDA-MB-231 (TNBC). The IC50 of Oleandrin in MCF7, SK-BR-3 and MDA-MB-231 is 14.5, 6.12 and 24.62 nM, respectively. SK-BR-3 exhibits more sensitivity to Oleandrin than MCF7 and MDA-MB-231 cells, this result may be associated with high-level expression of ATP1B3 in SK-BR-3 cells. In the future, we will provide the evidences that Oleandrin could directly target ATP13B in vitro and in vivo breast cancer model [28,29].

Based on the expression level of human epidermal growth factor receptor 2 (HER2), hormone receptor (HR) including estrogen (ER) and progesterone (PR), breast cancers can be classified into 5 subtypes: Luminal A (HR+/HER2-), Luminal B (HR+/HER2+), HER2-enriched (HR-/HER2+), basal-like (about 75 % of this type of breast cancers belongs to triple-negative) and normal breast-like tumors [4]. HR- or HER2-targeted therapy have successfully used for the treatment of Luminal A, Luminal B and HER2-enriched breast cancers[]. Due to loss of molecular targets, no therapeutic responses were observed in TNBC, and cytotoxic chemotherapy is the only available treatment option for TNBCs [30,31]. Though cardiac glycosides exhibit potent cytotoxic activity in breast cancer at low-nM level [27,32,33]. Of note, more evidences demonstrated that cardiac glycosides bind with ER and can increase risk of breast and uterus cancer incidences (risk ratios:1.3-1.5) [34]. Thus, we will consider the side effect and therapeutic efficacy of oleandrin in breast cancers in the future, especially in ER+ breast cancer.

Apoptosis plays a key role in cancer cell death after therapy [35]. Previous studies have shown that the NF-κB pathway, the mitochondrial apoptosis pathway, and DR4 and DR5 are involved in CG-induced apoptosis. However, in the present study, we showed that ER stress was involved in oleandrin-induced apoptosis in breast cancer cells. The ER is important for the regulation of intracellular Ca2+ homeostasis, protein folding, and protein maturation [36,37]. Several studies have found that ER stress is associated with apoptosis [38]. Hypoxia, Ca2+ concentration, and oxidative injury affect ER homeostasis and hinder protein folding [39-41]. The result of unfolded protein accumulation in the ER is ER stress. ER stress triggers a stress response via the unfolded protein response (UPR) pathway. The three major sensors on the ER that regulate ER stress are inositol-requiring protein 1 alpha (IRE1-α), activating transcription factor 6 (ATF6), and protein kinase RNA-like endoplasmic reticulum kinase (PERK) [42]. Activated PERK increases phosphorylation of Ser at position 51 in eukaryotic initiation factor 2
alpha (eIF2α) which inhibits global protein synthesis. The phosphorylated eIF2α promotes expression of activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP). CHOP is a critical pro-apoptosis transcription factor that regulates the expression of Bcl-2 family proteins [43,44]. In the present study, we observed that oleandrin induced MDA-MB-231 and MCF7 cell apoptosis after 24 h, which was represented by Annexin V-FITC+ PI−/+ staining and Hoechst 33342 staining. Furthermore, oleandrin treatment increased the expression of Bim and Bax and decreased the expression of pro-survival protein Bcl-2. The Bcl-2 family of proteins regulates the mitochondrial apoptosis pathway. Our results are consistent with a previous study showing that oleandrin induces apoptosis in human colon cancer cells via the mitochondrial pathway. To further investigate the possible mechanism involved in oleandrin-induced mitochondrial apoptosis, the expression of UPR downstream signaling proteins such as PERK were analyzed. After 6 h of oleandrin treatment, PERK, p-PERK and p-eIF2α were upregulated in MDA-MB-231 and MCF7 cells, but eIF2α was not affected. Moreover, ATF4 and CHOP, downstream targets of the PERK pathway, were upregulated. The activation of PERK signaling pathway precedes apoptosis.

5. Conclusions

Taken together, our results suggest that oleandrin induces mitochondrial-mediated apoptosis by activating endoplasmic reticulum stress in breast cancer. Moreover, oleandrin may be an effective strategy for the treatment of breast cancer.

Funding

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Fig. 5. Oleandrin decreases the viability of primary breast cancer cells. Primary breast cancer cells were obtained from lumina A, lumina B, HER-2+ and TNBC patients. Oleandrin sensitivity was evaluated by a collagen gel droplet-embedded culture drug sensitivity assay. Cell viabilities were quantified by staining with neutral red and detected by image analysis. The results presented were representative of each subtype patients. The cell viability of control group was set as 100 %. Data are presented as the mean ± SD from three droplets. **P < 0.01 vs. control.
Authors' contributions

X Li and D Wang mainly performed the experiments and drafted the manuscript. X Li, D Wang, C Sui, F Meng, S Sun, J Zheng and Y Jiang played an important role in coordinating the study and performing the analysis with constructive discussions. X Li, D Wang, C Sui, F Meng prepared the samples and performed the experiments; X Li and Y Jiang conceived the study and revised the manuscript. All authors discussed the results and contributed to the writing and editing of the manuscript.

Declaration of Competing Interest

The authors have no conflict of interest.

Acknowledgement

Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.109852.

References

