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Oxymatrine Promotes S-Phase Arrest and Inhibits Cell Proliferation of Human Breast Cancer Cells in Vitro through Mitochondria-Mediated Apoptosis

Jie Wu,* Yan Cai, Maolan Li, Yijian Zhang, HuaiFeng Li, and Zhujun Tan

*MOE Key Laboratory of Hydrodynamics and School of Naval Architecture, Ocean and Civil Engineering, Shanghai Jiao Tong University; 800 Dongchuan Road, Shanghai 200240, China; School of Biological Science and Medical Engineering, Southeast University; 2 Sipailou, Nanjing 210096, China; and Department of General Surgery, Xinhua Hospital, Affiliated to School of Medicine, Shanghai Jiao Tong University; 1665 Kongjiang Road, Shanghai 200092, China.

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Breast cancer is one of the most lethal malignancies in the world. Oxymatrine is the major effective and toxic alkaloid component which is derived from the root of Sophora flavescent Ait, a traditional Chinese medicine which is widely distributed in Asia and the Pacific Islands. In the current research study, we investigated the effects and mechanisms of action of oxymatrine on breast cancer cells. We demonstrated that the viability and single cell proliferation capability of MCF-7 and MDA-MB-231, two breast cancer cell lines which are widely used in in vitro study, were significantly suppressed in a time- and concentration-dependent manner. Furthermore, the cell cycle of breast cancer cells treated with oxymatrine was arrested at the S-phase of the cell cycle. Oxymatrine also triggered apoptosis in breast cancer cells by modulating apoptosis-related proteins, such as cleaved Caspase-3, cleaved Caspase-9 and poly(ADP-ribose)polymerase (PARP). The remarkable reduction in the ratio of Bel-2/Bax was also observed in oxymatrine treated breast cancer cells. In conclusion, our research demonstrated that oxymatrine plays a critical role in suppressing carcinogenesis of breast cancer cells through cell cycle arrest and induction of mitochondria-mediated apoptosis, which suggests a promising application of this drug in breast cancer therapy.

Key words oxymatrine; apoptosis; breast cancer; cell cycle

Breast cancer is the most highly prevalent and diagnosed carcinoma in women worldwide and is the leading cause of cancer deaths in women, with approximately 500,000 newly diagnosed cases reported in 2012. Currently, the main treatment options for breast cancer cases include surgery, radiotherapy, chemotherapy, hormone and immunological therapy. Although effectiveness could be observed in breast cancer patients receiving a variety of therapies, the high toxicity of these treatments to normal tissues is practically inevitable. Furthermore, breast cancer cells often propagate distant metastases and multi-drug resistance, which represents a main cause for treatment failure in cancer patients. Thus, the search for novel effective therapies and targeted drugs are an urgent priority with the aim of improving the efficacy of breast cancer treatment.

Traditional Chinese medicine has been shown to be a fertile source of novel drug discovery. Many active components which are extracted from traditional Chinese herbs exhibit effects in many human diseases. For example, oxymatrine is the main effective and toxic alkaloid component derived from the root of Sophora flavescent Ait, which is widely distributed in Asia and the Pacific Islands. It was previously reported that oxymatrine displays specific pharmacological effects in targeting hepatitis B infections and liver fibrosis. Furthermore, it has been demonstrated that oxymatrine exerts anti-tumor properties in several cancers via different signaling pathways, such as suppression of proliferation and promotion of apoptosis. Although previous studies showed that oxymatrine mediates Bax and Bel-2 expression in the human breast cancer MCF-7 cell-line, the pharmacological property and underlying mechanism of action of oxymatrine against breast cancer remains largely unknown.

In the present study, we demonstrated the anti-tumor effects of oxymatrine against the breast cancer cell-lines, MCF-7 and MDA-MB-231, and investigated potential molecular mechanisms which might mediate the anti-neoplastic activity of oxymatrine. Our results suggest that oxymatrine may offer a novel approach in the treatment of breast cancer patients.

MATERIALS AND METHODS

Oxymatrine, Cell-Lines, Culture Conditions and Antibodies Oxymatrine was obtained from Sigma-Aldrich (U.S.A.). The MCF-7 and MDA-MB-231 of breast cancer cells were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were cultured in Rosewell Park Memorial Institute (commonly referred to as RPMI) 1640 culture medium (Gibco, U.S.A.) and MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) culture medium. Both MCF-7 and MDA-MB-231 cells were cultured as monolayers. The medium used in this research was supplemented with 10% FBS (fetal bovine serum; Gibco) and PS antibiotics (penicillin–streptomycin antibiotics; HyClone, U.S.A.) and maintained at 37°C with 5% CO2. Primary antibodies, including cleaved Caspase-9, cleaved Caspase-3, poly(ADP-ribose)polymerase (PARP), Bel-2, Bax and the internal control β-actin were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Secondary antibodies were purchased from Abcam (Cambridge, U.K.).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoli-
Bromide (MTT) Assay Drug sensitivity and cell proliferation of MCF-7 and MDA-MB-231 cells was measured by using MTT cell viability assay. In brief, cells were trypsinized and plated into 96 well plates. After the cells were cultured overnight, fresh medium containing different concentrations (0, 2, 4, 6 and 8 mg/mL) of oxymatrine were replenished for three different time points, 24, 48 and 72h, respectively. Then, MTT solution was added to each well and the well-plates were cultured at 37°C for 3h. After addition of acidic isopropanol, each well was measured on a microplate reader (Molecular Devices, U.S.A.) at a wavelength of 590 nm.

**Colony Formation Assay** Breast cancer cells, MCF-7 and MDA-MB-231 cells, were resuspended as single cell suspension and plated into 6-well plates with a density of 400 cells/well. After 48h of incubation, cells were treated with oxymatrine at different concentrations (0, 4, 8 mg/mL) at 37°C with 5% CO₂ in air. The cells were cultured for additional two weeks for colony formation. At last, breast cancer cells were stained with 0.1% crystal violet (Sigma-Aldrich) after fixation with 4% paraformaldehyde (PFA) and analyzed by a light microscopy (Leica, Germany). Only the colonies containing more than 50 cells were counted.

**Flow Cytometry** Breast cancer cells, MCF-7 and MDA-MB-231, were seeded in six-well plates and treated with different concentrations of oxymatrine (0, 4, 6 and 8 mg/mL) for 24, 48 and 72h. Breast cancer cells, MCF-7 and MDA-MB-231, were harvested with trypsinization after incubation for 48h and then washed in pre-chilled phosphate buffered saline (PBS) and fixed with ethanol. After fixation, breast cancer cells were incubated with ribonuclease (RNase) (10 mg/mL) and then stained with propidium iodide (PI; 1 mg/mL) for 30 min at 37°C in a dark condition. The DNA content was accessed through flow cytometry (BD, U.S.A.). Cell Quest acquisition software (BD) was employed to analyze the percentage of cells in different phases (G0/G1, S, and G2/M). To evaluate apoptosis, the breast cancer cells were stained with Annexin V-fluorescein isothiocyanate (FITC) (100 µg/mL) and propidium iodide (100 µg/mL) for 15 min in a dark condition at room temperature. After centrifugation for 3 min, cells were resuspended in PBS. Samples were subjected to flow cytometry for further analysis.

**Western Blotting** Breast cancer cells, MCF-7 and MDA-MB-231, were cultured in a six-well plate. After incubation for 24h, the cells were treated with different concentrations of oxymatrine (0, 4, 6 and 8 mg/mL) for 48h. Breast cancer cells were collected and lysed with lysis buffer (Beyotime, China) containing a protease inhibitor (Complete Mini Protease Inhibitor Cocktail, Roche, Switzerland). The protein concentrations of different conditions were assessed via bicinchoninic acid assay system (Beyotime). Subsequently, equivalent amounts of proteins were loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel for further separation and then proteins were transferred to polyvinylidene difluoride membranes after electrophoresis. Then Tris-buffered saline with tween-20 (TBST) with 1% bovine serum albumin (BSA) was used to block the membranes for 1h. The polyvinylidene difluoride membranes were then incubated with primary antibodies at 4°C overnight. The primary antibodies used in this research and the corresponding working concentrations are as follows: cleaved Caspase-9 (1:1000 dilution), cleaved Caspase-3 (1:1000 dilution), PARP (1:1000 dilution), Bax (1:1000 dilution), Bel-2 (1:1000 dilution) and the internal control β-actin (1:1000 dilution). After washing with TBST buffer, the polyvinylidene difluoride membranes were washed and incubated with secondary antibodies for 2h, respectively. The secondary antibodies used in this research and the corresponding working concentrations are as follows: horseradish peroxidase conjugated goat-anti-mouse (1:5000 dilution) and goat-anti-rabbit (1:5000 dilution). The membranes were then washed and visualized by chemiluminescent ECL reagent (Pierce, Rockford, IL, U.S.A.). Photographs were scanned and used to analyse the optical densities for protein quantification.

**Statistical Analysis** Statistical analysis was carried out with SPSS 19.0 software. Differences between paired groups were analyzed by the Student’s t test. A p-value of less than 0.05 was considered as statistically significant. All values are expressed as mean±standard deviation (S.D.) about the mean.

**RESULTS**

**Proliferation and Viability of Breast Cancer Cells Were Suppressed by Oxymatrine** To evaluate whether oxymatrine influences the growth and viability of breast cancer cells, various concentrations (i.e., 0, 2, 4, 6 and 8 mg/mL) of oxymatrine were employed to treat two breast cancer cell-lines, MCF-7 and MDA-MB-231, for 24, 48 and 72h, respectively. The results showed that oxymatrine exhibited a dramatic cytotoxic effect on MCF-7 and MDA-MB-231 cells. Both MCF-7 and MDA-MB-231 cells showed a time- and dose-dependent reduction of cell viability after the incubation with oxymatrine, as shown in Figs. 1A and B. Approximately 15% MDA-MB-231 cells and 20% MCF-7 cells survived after treatment with 8 mg/mL oxymatrine for 72h. Furthermore, we performed the plate-well colony formation assay to investigate the anti-proliferation effects of oxymatrine on breast cancer cell-lines. Figures 1C–F showed that the colony numbers of MCF-7 and MDA-MB-231 cells treated with oxymatrine were significantly inhibited in a dose-dependent manner as compared with the untreated breast cancer cells through the crystal violet staining. All of the above results demonstrated that cell viability and single cell proliferation capacity of breast cancer cells was remarkably suppressed in response to oxymatrine treatment.

**S-Phase Arrest Was Induced in Breast Cancer Cells by Treatment with Oxymatrine** Cell cycle progression plays an important role in proliferation of cancer cells. Thus, we investigated cell phases of the both breast cancer cell-lines in order to determine whether the inhibition of oxymatrine on breast cancer cell proliferation was mediated by dysregulation of cell cycle. Figure 2 showed the changes in the cell cycle of MDA-MB-231 cells and MCF-7 cells induced by oxymatrine. After incubation with oxymatrine for 48h, the proportion of breast cancer cells in the G0/G1-phase of the cell cycle decreased significantly in a dose-dependent manner (from 64.34 to 29.25% in MCF-7 cells and from 85.16 to 57.12% in MDA-MB-231 cells) as compared with the untreated cells. Moreover, the proportion of cancer cells in S-phase increased dramatically also in a dose-dependent manner (from 25.58 to 64.01% in MCF-7 cells and from 11.65 to 41.69% in MDA-
MB-231 cells). Our data demonstrated that S-phase arrest occurred in the cell cycle of both breast cancer cell-lines in a concentration-dependent manner, which might account for an oxymatrine-dependent suppression of cell proliferation and colony formation capacity.

**Oxymatrine Induced Apoptosis in Human Breast Can-**

Fig. 1. Oxymatrine Inhibits Cell Viability of MCF-7 and MDA-MB-231

(A, B) The viability of MCF-7 (A) and MDA-MB-231 cells (B) were assessed by MTT assays at 24, 48 and 72 h after treatment with different concentrations of oxymatrine (0, 2, 4, 6 and 8 mg/mL); (C, D) Representative images of crystal violet staining assays of MCF-7 cells (C) and MDA-MB-231 cells (D) incubated with different concentrations of oxymatrine (0, 4 and 8 mg/mL); (E, F) Statistical analysis of colony numbers of MCF-7 (E) and MDA-MB-231 cells incubated with different concentrations of oxymatrine (0, 4 and 8 mg/mL) (F). *p<0.05, **p<0.01. Three replicates were performed for each experiment.
Fig. 2. Oxymatrine Promotes Cell Cycle Arrest at S-Phase in MCF-7 and MDA-MB-231 Cells

(A, B) Representative plots of the cell cycle phases following different concentrations of oxymatrine (0, 4, 6 and 8 mg/mL) treated MCF-7 (A) and MDA-MB-231 cells (B) by flow cytometry. (C) Statistical analysis of cell numbers at different cell cycle phases (G0/G1, S and G2/M) following oxymatrine treatment of MCF-7 (left) and MDA-MB-231 cells (right). *p<0.05, **p<0.01. Three replicates were performed for each experiment.
cer Cells It has been reported that apoptosis could be another important mechanism by which, oxymatrine might suppress the cell proliferation and colony formation capacity in breast cancer cells. To determine whether oxymatrine induced apoptosis in MCF-7 and MDA-MB-231 cells, the cell-lines were incubated dose-dependently with oxymatrine for 48 h and then the frequency of apoptotic cells was analyzed by Annexin V/PI double-staining and flow cytometry. In live cells, phosphatidylserine (PS) was located on the cytoplasmic surface of the membranes. Externalization of PS from the inner leaflet to the outer leaflet of the plasma membrane occurred during the early phase of apoptosis and was regarded as a distinctive phenomenon for this specific biological process. Thus, early apoptotic cells could be easily recognized by the binding between Annexin V and PS. By contrast, PI expression indicates late apoptotic and necrotic cells due to its ability to permeate damaged cell membranes. As shown in Fig. 3, Q3 quadrant (Annexin V−/PI−), Q4 quadrant (Annexin V+/PI−) and Q1 quadrant (Annexin V+/PI+) represented living cells, early apoptotic cells with PS exposure/intact cell membrane and late apoptotic cells with permeable cell membrane, respectively. Our results showed that oxymatrine dose-dependently inhibited the proportion of living cells and increased the number of

**Fig. 3. Oxymatrine Induces Apoptosis of MCF-7 and MDA-MB-231 Cells**

(A, B) Representative plots showing the apoptosis patterns of oxymatrine treated MCF-7 (A) and MDA-MB-231 cells (B). MCF-7 cells and MDA-MB-231 cells treated with different concentrations of oxymatrine (0, 4, 6 and 8 mg/mL) were stained with Annexin V/PI and evaluated with flow cytometry; (C, D) Statistical analysis of cell numbers of different cell states (survival, early apoptosis and late apoptosis) in oxymatrine treated MCF-7 (C) and MDA-MB-231 cells (D). Annexin V−/PI−, survival cells; Annexin V+/PI−, early apoptotic cells with PS exposure/intact cell membrane; Annexin V+/PI+, late apoptotic cells with permeable cell membrane. *p<0.05, **p<0.01. Three replicates were performed for each experiment.
Apoptosis Induced by Oxymatrine Was Regulated by Bcl-2 Family Members and Caspases in Breast Cancer Cells

A cascade of proteolytic activities is involved in the biological process of apoptosis; much of which was exerted by caspase and Bcl-2 family member proteins. To explore the apoptotic pathway which was activated by oxymatrine treatment, we determined the expression levels of apoptosis-related proteins by Western blot assay following dose-dependent treatment with oxymatrine. As shown in Fig. 4A, incubation with oxymatrine resulted augmented functional expression of cleaved Caspase-9, cleaved Caspase-3, PARP, Bax and Bcl-2, which suggested activation of caspase-mediated apoptotic signaling. In addition, the expression levels of PARP, a well-known substrate of activated caspases, also increased remarkably, supporting the notion that treatment with oxymatrine induced apoptosis by modulating the caspase-mediated signaling pathway. Furthermore, Bcl-2 expression levels decreased significantly, which resulted in a dramatically reduced ratio of Bcl-2 (anti-apoptotic) to Bax (proapoptotic) by a dose-dependent mechanism (Fig. 4B). All these data demonstrated that induction of apoptosis by oxymatrine was mediated by regulating functional expression of caspase and Bcl-2 family members in MCF-7 and MDA-MB-231 breast cancer cells.

DISCUSSION

Breast cancer is one of the most lethal diseases in the world and a leading cause of carcinoma-related mortality in women. Although surgery, radiotherapy, chemotherapy, hormone and immunological therapy are effective in the majority of patients that present with early stage of breast cancer, many patients with advanced stages of this disease show unacceptable mortality because of disease recurrence and distant metastases. Thus, it is an urgent priority to search for new and highly effective methods and drugs for the treatment of breast cancer.

Traditional Chinese medicine is shown to be a fertile source for new drug discovery. Oxymatrine is extracted from the root of *Sophora flavescens* Ait which is widely distributed in Asia and the Pacific Islands. It has been demonstrated that oxymatrine is the main effective and toxic alkaloid component. Indeed, prior studies have reported that oxymatrine possesses a variety of biological functions. It has been demonstrated that oxymatrine promotes anti-tumor properties in the setting of many carcinomas, including colon carcinoma, hepatocellular carcinoma and lung cancer.\(^{19-22}\) However, the signaling pathways which mediate its anti-tumor properties are divergent. In colorectal cancer, epithelial–mesenchymal transition (EMT), which is involved in the initiation of metastasis for malignant cancer progression was inhibited by oxymatrine through regulation of the nuclear factor-kappaB (NF-κB) signaling pathway. Furthermore, in the lung cancer A549 cell line, treatment with oxymatrine suppressed the cancer cell proliferation through the induction of apoptosis via regulating Bcl-2 and

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**Fig. 4. Expression of Apoptosis-Related Proteins Were Induced by Treatment with Oxymatrine in MCF-7 and MDA-MB-231 Cells**

Both breast cancer cell lines were incubated with different concentrations of oxymatrine (0, 4, 6 and 8 mg/mL). (A) The apoptosis-related protein expression levels of cleaved Caspase-9, cleaved Caspase-3, PARP, Bax and Bcl-2 were evaluated by Western blot in oxymatrine treated MCF-7 cells (left) and MDA-MB-231 cells (right). β-Actin was used as the internal control protein; (B). The statistical analysis of the ratio of Bcl-2 to Bax was determined by band density, and data was shown as the mean±S.D. as compared with the control (designated as 1.00). Key: *p<0.05; and **p<0.01.
Bax expression. These studies demonstrated that oxymatrine exerts its anti-tumor effects in different cancer cell types via distinctive signaling pathways.

In our current research, oxymatrine was shown to suppress MCF-7 and MDA-MB-231 breast cancer cells. Cell viability of both breast cancer cells decreased significantly after incubation with oxymatrine in a time- and dose-dependent manner, which was accompanied by an impaired capacity for single cell proliferation, as determined by colony-formation assay. Further study showed that S-phase cell cycle arrest was seen following treatment with oxymatrine, which resulted in apoptosis of MCF-7 and MDA-MB-231 cells via apoptotic-related caspase and Bcl-2 family members. Lin et al. found that oxymatrine promoted apoptosis in breast cancer MCF-7 cells by regulating the expression levels of Bax and Bcl-2, which was concordant to our results. \(^\text{18}\)

Apoptosis is a process of programmed cell death, which could be triggered by many chemical factors and drugs. It has been reported that induction of apoptosis is regarded as the best strategy for targeted anti-tumor therapy.\(^\text{23,24}\) Cysteine-containing aspartate-specific protease family (i.e., caspases), including apoptotic-related proteins, such as Caspase-3 and Caspase-9, are key players in this biological process. When cells were activated by external apoptotic signals, Caspase-3 and Caspase-9, two key components of cell apoptotic signaling, are the most important executors of apoptotic signaling transduction.

In the current study, we showed that oxymatrine triggered apoptosis by augmenting cleaved Caspase-3 and PARP, a well-known substrate of activated caspases. By contrast, the expression levels of the anti-apoptotic protein Bcl-2 and the ratio of Bcl-2/Bax were significantly suppressed in human breast cancer MCF-7 and MDA-MB-231 cells. It would be of great interest to determine the precise variety of gene expression profiles that might be induced in the oxymatrine-triggered apoptosis of human breast cancer cells.

Finally, it has also been implied that oxymatrine might diminish the side population (SP cells) of breast cancer cells, which represents a population of cancer stem-like cells.\(^\text{25}\) Moreover, oxymatrine caused a dose-dependent reduction in the number of SP cells via Wnt/\beta\)-catenin signaling pathway. Thus, oxymatrine has the potential to suppress breast cancer progression by inhibiting cancer stem-like cells in addition to its inhibitory effects on cancer cell proliferation and single cell proliferation capacity.

In conclusion, it would be beneficial to comprehend in greater detail the mechanisms responsible for oxymatrine-assisted suppression of cancer progression. These studies are clearly warranted to assist in the rational design of targeted breast cancer therapeutics.

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**Conflict of Interest** The authors declare no conflict of interest.

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