RESEARCH ARTICLE

Curdione Inhibits Proliferation of MCF-7 Cells by Inducing Apoptosis

Juan Li[1&], Wei-He Bian[2&], Juan Wan[1&], Jing Zhou[3], Yan Lin[1], Ji-Rong Wang[1], Zhao-Xia Wang[1], Qun Shen[4], Ke-Ming Wang[1*]

Abstract

Background: Curdione, one of the major components of *Curcuma zedoaria*, has been reported to possess various biological activities. It thus might be a candidate anti-flammatory and cancer chemopreventive agent. However, the precise molecular mechanisms of action of curdione on cancer cells are still unclear. In this study, we investigated the effect of curdione on breast cancer. Materials and Methods: Xenograft nude mice were used to detect the effect of curdione on breast cancer *in vivo*; we also tested the effect of curdione on breast cancer in vitro by MTT, Flow cytometry, JC-I assay, and western blot. Results: Firstly, we found that curdione significantly suppressed tumor growth in a xenograft nude mouse breast tumor model in a dose-dependent manner. In addition, curdione treatment inhibited cell proliferation and induced cell apoptosis. Moreover, after curdione treatment, increase of impaired mitochondrial membrane potential occurred in a concentration dependent manner. Furthermore, the expression of apoptosis-related proteins including cleaved caspase-3, caspase-9 and Bax was increased in curdione treatment groups, while the expression of the anti-apoptotic Bcl-2 was decreased. Inhibitors of caspase-3 were used to confirm that curdione induced apoptosis. Conclusions: Overall, our observations first suggested that curdione inhibited the proliferation of breast cancer cells by inducing apoptosis. These results might provide some molecular basis for the anti-cancer activity of curdione.

Keywords: Curdione - MCF-7 cells - apoptosis - proliferation

Asian Pac J Cancer Prev, **15 (22)**, 9997-10001

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths (Jemal et al., 2011). In China, the incidence of breast cancer is now 20/100,000, about 12 million women suffer from breast cancer, and 50,000 people die of breast cancer every year. Chemotherapy is an effective treatment of malignant tumors, but the toxicity and side effect of chemotherapy is difficult to tolerate. Therefore, finding effective anticancer drugs with low side effect has been one of the main objectives in developing anticancer drugs. At present, looking for active antitumor drugs from natural products and traditional Chinese medicine has become a hotspot.

Nature products are the most important sources of novel cancer therapeutics (Zhou et al., 2013). Curdione was first separated from Curcuma zedoaria in 1966 by Hikino et al. (1967). Curcuma zedoaria has been shown to display anticarcinogenic properties in a wide variety of cell lines and animals. Recent studies have determined that the main bioactive constituents of Curcuma zedoaria are its essential oils, which possess anti-tumor, anti-flammatory, and neuroprotective properties (Lai et al., 2004; Makabe et al., 2006; Dohare et al., 2008; Li Y et al., 2009). Curdione is one of the main components of the essential oil (Li et al., 2009), at a relative content of 14.13%, and the molecular structure of curdione is shown in below (Yan et al., 2005).

Recently, Oh et al. (2007) indicated that curdione may be a candidate for cancer chemopreventive agents. However, the detail mechanism of curdione suppressed tu, mor growth was not studied. In this study, we found that curdione effectively suppressed tumor growth in MCF-7 xenograft nude mouse breast cancer models. Furthermore, we investigated the effect of curdione on the apoptosis of MCF-7 cells and its underlying molecular mechanisms *in vivo* and *in vitro*. Based on the experiments, we determined that curdione significantly induced the apoptosis of MCF-7 cells *in vivo* and *in vitro*.

Materials and Methods

Cell culture and reagents

Curdione (Batch No. TCM069-090910) with a purity >99%, purchased from Nanjing Tcm Institute of Chinese Materia Medical. It was dissolved in DMSO (Sigma) to make a stock solution. A stock solution of 200mg/ml was

*[1]Department of Oncology, Second Affiliated Hospital of Nanjing Medical University, [2]Department of Oncology, First Clinical Medicine School, [4]Department of Hematology, First Clinical Medicine School, Nanjing University of Chinese Medicine, Nanjing, [3]Taizhou People's Hospital, Taizhou, China [&]Equal contributors *For correspondence: tianyr1@163.com*

stored at -20°C and then diluted as needed in complete culture medium immediately before use. Human breast cancer cell line MCF-7 was provided by Dr Jianwei Zhou (Molecular Toxicology Laboratory, Nanjing Medical University). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% FBS (Invitrogen) at 37°C in incubator with 5% CO_2.

Proliferation assay

MCF-7 cells (2×10^3) were seeded into 96-well plates (Corning). After cells were treated with the indicated concentrations of curdione for 24h, 48h and 72h, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide (MTT) (Sigma) was added to the culture medium, and the cells were incubated for another 4h at 37°C. The negative control was 0.1% DMSO. After the culture medium was removed, 150ml DMSO was added, and the plates were placed on a shaking table at 150 rpm for 10 min. Optical density (OD) was measured at 490 nm. The experiment was repeated three times, and the rate of cell inhibition was calculated using the following formula: inhibition rate =[1- (ODtest/ODnegative control)]×100%. The IC_{50} was calculated using SPSS 19.0 software.

Xenograft nude mouse models

The 5-week-old to 6-week-old female BALB/c nude mice (purchased from the Shanghai Laboratory Animal Commission) weighing ~20g were divided into groups. All of the mice were injected subcutaneously with MCF-7 cells (1×10^7) in the scruff of neck. After the tumors were established (~0.5 cm^3), the mice were injected with different concentrations curdione every two days. Mouse body weights and tumor sizes were recorded every four days, and the tumor sizes were determined by vernier caliper measurements and calculated as $a\times b^2/2$ (a and b refer to the longer and shorter dimensions, respectively). All manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission.

Flow cytometry

Cells in log phase were digested and inoculated in a 6-well plate, and after adherence of cells, curdione culture medium was added for different groups, also a blank control group was set, Collect 5×10^5 cells after incubated for another 72h. The cells were then treated with 500μl binding buffer, 5μl annexin V-FITC and 5μl Propidium iodide (PI) dye (BD Biosciences). After mixing at room temperature in the dark for 5-15min, a flow cytometry analysis was performed immediately following the manufacturer's protocol. Finally, the percentages of viable cells, apoptotic cells and necrotic cells were analyzed.

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MCF-7 cells (5×10^4) were seeded in 6-well plates and incubated (37°C, 5% CO_2) for 24h. After removing the media and washing twice with PBS, 1ml complete DMEM medium containing different concentrations of curdione was added to each well. After 72h of treatment, the cells were collected, washed twice with PBS, and resuspended in 500ml JC-1 working solution (KeyGEN) for 15-20min. Cells were centrifuged at room temperature, and the supernatant was removed. The cells were then resuspended in 500ml 1× incubation buffer, and fluorescence changes were detected by flow cytometry (Ex/Em =488nm/530nm).

Western blot

After treatment with 25, 50 and 100μg/ml curdione for 72h, MCF-7 cells were collected and washed twice with cold PBS before lysis. The cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and PMSF (Roche). The protein concentration was measured using the Bio-Rad protein assay kit. Protein (70 μg) was loaded into precast 4% stacking, 10% tris-glycine gels and separated by gel electrophoresis. After electrophoresis, the proteins were transferred onto 0.22μm NC membranes (Sigma). The membranes were blocked with 5% non-fat milk for 1h followed by incubation with primary antibodies for Bcl-2 and Bax (1:400) (Bioworld), and cleaved caspase-3, and caspase-9 (1:500) (Bioworld) overnight at 4°C with shaking. After washing with TBST, membranes were then blotted with the secondary antibodies (goat anti-rabbit; 1:5000 Bioworld) for 2h at room temperature and washed again with PBST. The blots were visualized using the enhanced chemiluminescence detection system (Amersham Life Science). The gray values were determined by a gel image analysis system (Bio-Rad) normalized with β-actin. To confirm the caspase cascade activation effect caused by curdione 10μM Az-DEVD-FMK (a caspase-3 inhibitor) (KeyGEN) combined with 100μg/ml curdione was added at the same time.

Statistical analysis

Data were represented as the mean±SD. The data were analyzed with a single-factor analysis of variance by SPSS 19.0 software. $p<0.05$ was considered statistically significant.

Results

Inhibitory effect of curdione on MCF-7 cells

The inhibitory effect of curdione on MCF-7 cells proliferation was measured by the MTT assay (Figure 1). Curdione significantly inhibited the proliferation of

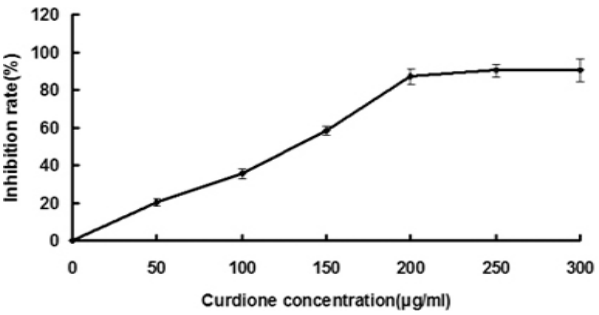

Figure 1. The Inhibitory Effect of Curdione on the Proliferation of Breast Cancer Cells. Cells in 96-well plates were treated with various concentrations of curdione for 72h. MCF-7 cells viability was assessed by the MTT assay. The values are the mean±SD of at least three independent experiments

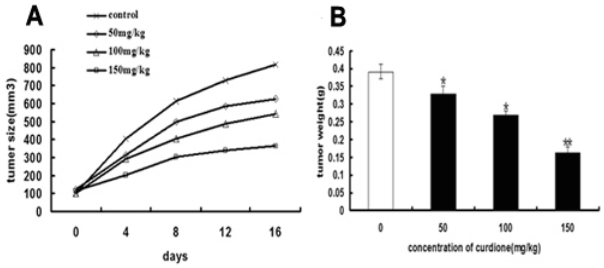

Figure 2. Curdione Inhibits Tumor Growth *in vivo*. MCF-7 nude mice were injected with different concentrations of curdione. **A)** Tumor size was measured using a sliding caliper every four days; **B)** Tumors were isolated from different groups with or without curdione treatment. The tumors in the curdione -treated groups were significantly smaller than those of the control group. (*p<0.05; **p<0.01)

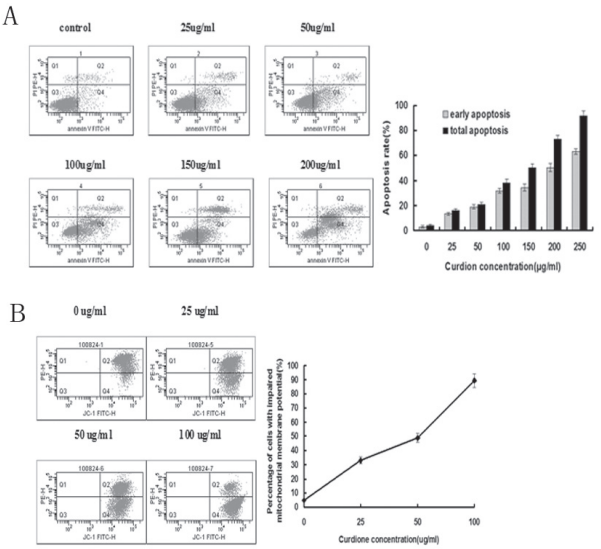

Figure 3. The Effect of Curdione on MCF-7 Cells Apoptosis. A) Curdione induces the apoptosis of MCF-7 cells. Curdione was used to treat MCF-7 cells at concentrations of 0, 25, 50, 100, 150, 200μg/ml. The cells were collected after 72h, and the apoptosis rates were detected by flow cytometry; **B)** MCF-7 cells were treated with different concentrations of curdione for 72h and the proportion of cells with impaired mitochondrial membrane potential was calculated by flow cytometry

MCF-7 cells at 50-200μg/ml in a dose-dependent manner. The IC_{50} was 125.632μg/ml.

Curdione inhibits tumor growth in vivo

To determine the effect of curdione on tumor growth at different doses, we used xenograft nude mouse breast tumor models. The tumor sizes were recorded every four days. As shown in Figure 2A, at day 16 after curdione injection, the average tumor size in the control group was 744±17mm^3, whereas those of the curdione-treated (50, 100, 150mg/kg) groups were 613±22mm^3, 517±28mm^3, and 374±13mm^3.The average tumor weight of the control group was 386±34mg, whereas those of the curdione-treated groups were 332±12mg, 279±24mg and 154±10mg (Figure 2B).

Effect of curdione on MCF-7 cells apoptosis

Flow cytometry with annexin V/PI double staining was used to analyze whether curdione induced MCF-7

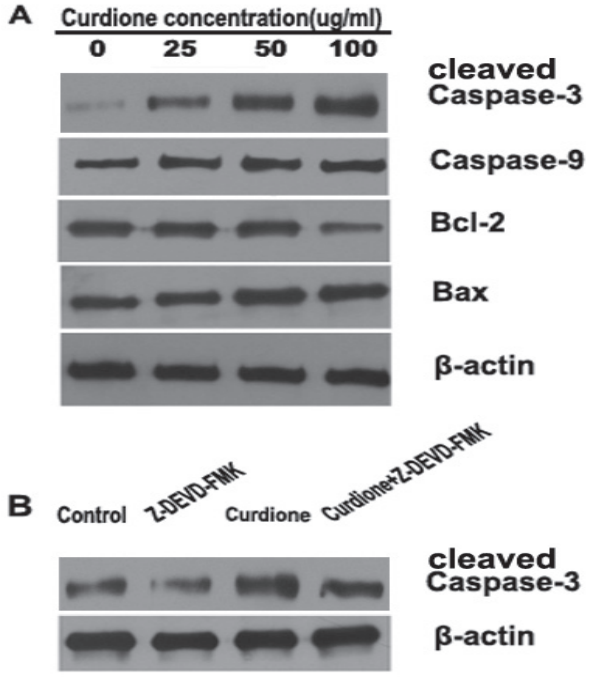

Figure 4. The Expression of Apoptosis-related Proteins was Analyzed by Western Blot. A) MCF-7 cells were treated with different concentrations of curdione for 72h, and the expression levels of cleaved caspase-3, caspase-9, Bcl-2 and Bax were detected by western blot. **B)** After treatment with 10μM Az-DEVD-FMK (a caspase-3 inhibitor) combined with curdione for 72h. The cleaved caspase-3 protein expression levels were analyzed by western blot. (*p<0.05; **p<0.01)

cells apoptosis. The rates of apoptosis after treatment with 0, 25, 50, 100, 150, 200μg/ml curdione had apparently increased. Therefore, curdione significantly induced breast cancer MCF-7 cells apoptosis in a dose-dependent manner (Figure 3A).

Effect of curdione of different concentration on MCF-7 cell mitochondrial membrane potential

In order to test the impaired mitochondrial membrane potential after curdione treatment, MCF-7 cells were treated with different concentration of curdione for 72h. The results showed that the increased of impaired mitochondrial membrane potential occurred in a concentration dependent manner. After treating the MCF-7 cells with 100μg/ml curdione, the impaired mitochondrial membrane potential has increased more than 80% (Figure 3B).

Effect of curdione of different concentration on caspase-3, caspase-9, bax and bcl2 protein expression level of MCF-7 cells

After treatment with different concentrations of curdione for 72h, the expression of apoptosis-related proteins was detected by western blot. The results showed that the expressions of Bax, cleaved caspase-3 and caspase-9 were increased by curdione treatment in a dose-dependent manner. However, Bcl-2 expression was decreased (Figure 4A). After treatment with Az-DEVD-FMK 10μM (a caspase-3 inhibitor), the expression of cleaved caspase-3 in MCF-7 cells was significantly decreased (Figure 4B).

Discussion

Curcuma zedoaria is a Zingiberaceae perennial rhizomatous herbs that belongs to the Curcuma genus. Modern research has determined that the essential oil isolated from curcuma zedoaria, exhibit multiple bioactive properties, including anti-tumor activities. At present, many volatile oil components from Curcuma, such as β-elemene, curcumol, curdione, neocurdione, germacron, etc, have been isolated and identified (Shiobara et al., 1985; Zwaving and Bos, 1991; Xu et al., 2007). β-elemene has been relatively well studied as an anti-tumor substance both *in vitro* and *in vivo*, and recent studies on germacron have demonstrated that it can inhibit the proliferation of breast cancer cell lines (Zhong et al., 2011). The bioactivities of other components in essential oil have not been clearly described. To the best of our knowledge, this is the first study on the anti-tumor potential of curdione *in vitro* and *in vivo*.

In this study, we firstly constructed MCF-7 xenograft nude mouse breast tumor models and found that curdione could inhibit breast tumor growth in a dose-dependent manner. Subsequently, we investigated the effect of curdione on cell proliferation *in vitro*.The results showed that curdione significantly suppressed MCF-7 cell proliferation depending on concentration, with IC_{50} at 125.632μg/ml. Taken together, curdione could inhibit breast cancer growth *in vivo* and *in vitro*.

Normally, apoptosis, a strictly regulated form of programmed cell death, is the most common form of cell death in various cell types (Kerr et al., 1972; Jing et al., 2012). The nature product of Gambogenic acid has been reported to suppress breast cancer MDA-MB-231 cell growth by mediating apoptosis (Zhou et al., 2013). In order to analyze whether curdione induced MCF-7 cells by apoptosis, Flow cytometry with annexin V/PI double staining was used. The results showed that the rates of apoptosis after treatment with 0, 25, 50, 100, 150, 200μg/ml curdione had apparently increased. Then we tested the effect of curdione on MCF-7 cells mitochondria by JC-1. The JC-1 is a very useful reagent for investigating mitochondrial function. Mitochondria play an important role in cell apoptosis. A variety of apoptotic proteins were transported to the mitochondria, which led to a decreased permeability and reduced membrane integrity resulting in a disappearance of hydrogen ions and mitochondrial membrane potential. After the drop of mitochondrial membrane potential, a variety of apoptosis-inducing factors and pro-apoptotic proteins, such as cytochrome C and caspases-3/-8/-9, were released from mitochondrial and caused typical apoptosis (Desagher and Martinou, 2000). Ours results showed that mitochondrial membrane potential damage accumulated with an increased curdione concentration. All the results suggested that curdione might induce apoptosis in MCF-7 cells.

To explore the potential mechanisms of curdione-induced cell apoptosis, cell apoptosis-related protein expressions were examined. The key biochemical event involved in the apoptotic process is the up-regulation of pro-apoptotic proteins and/or the down-regulation of anti-apoptotic protein molecules. The Bcl-2 family of proteins, including Bcl-2, Bcl-xL, Bim, and Bax proteins, serve as critical regulators of the mitochondrial pathway involved in apoptosis, acting to either inhibit or promote cell death (Reed, 1998). The Bcl-2 protein has been identified as anti-apoptotic proteins, which bind to the outer membrane of the mitochondria and prevent the release of cytochrome c. Bax is thought to be pro-apoptotic effect proteins and are responsible for permeabilizing the membrane due to damaging cellular stress (Ewings et al., 2007; Green and Chipuk, 2008). Dohare et al. (2008) reported that the volatile oil of Curcuma suppressed the elevated protein level of Bax and aided mitochondrial translocation and activation of Bcl-2 by altering $\Delta\Psi m$. Fortunately, curdione showed significant effects on Bcl-2 and Bax expressions in MCF-7 cells in this study, suggesting that modulation of apoptosis by curdione was mediated by an increase in the level of Bax and/or a decrease in Bcl-2. Further studies are needed to elucidate this pathway.

Caspases are a family of proteases involved as the central component of a proteolytic system in the apoptotic process. These enzymes take part in a cascade that results in cell disassembly due to a trigger in response to pro-apoptotic signals. This culminates in the cleavage of a set of proteins (Thornberry and Lazebnik, 1998). Caspases are classified into two groups according to their function and structure: the initiator caspases (caspase-2, 8, 9, 10) and the executioner caspases (caspase-3, 6, 7) (Kuribayashi et al., 2006). Caspase-3 reported as a final apoptotic effect molecule, activated by caspases-4/-8/-9, could hydrolyze downstream-specific substrates poly (ADP-ribose) polymerase and induced cell apoptosis (Hishikawa et al., 2000; Kaufmann et al., 2008; Shu et al., 2009). In the present study, we found that the expression of cleaved caspase-3 was increased after treatment with curdione, and this increase was blocked by caspase inhibitor, Az-DEVD-FMK. While, the expressions of caspase-9 were also increased. These results suggested that curdione induced apoptosis might be through mitochondria mediated pathway.

In conclusion, our studies showed that a natural product curdione suppressed breast cancer growth via inducing apoptosi*s in vivo* and *in vitro*, and demonstrated that curdione significantly inhibited human breast cancer cell proliferation *in vitro* possibly trough inducing apoptosis.

Acknowledgements

This work was supported by Natural Science Foundation of Jiangsu province ordinary university (10KJB320002).

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