Int J Clin Exp Med 2017;10(4):6640-6647
www.ijcem.com /ISSN:1940-5901/IJCEM0026922

Original Article

Zerumbone induces G1 cell cycle arrest and apoptosis in cervical carcinoma cells

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Received February 29, 2016; Accepted July 4, 2016; Epub April 15, 2017; Published April 30, 2017

Abstract: Cervical carcinoma is a cancer arising from the cervix, and it is the fourth most common cause of death in women. However, currently the candidate drugs to suppress cervical carcinoma remain lacking. This research was carried out to investigate if zerumbone, a natural cyclic sesquiterpene isolated from Zingiber zerumbet Smith, will produce the anticancer effects on cervical carcinoma cell lines. We demonstrated here for the first time that zerumbone significantly suppressed the viability of SiHa cells by inducing G1 cell cycle arrest followed by apoptosis with different concentrations (10, 20 and 30 mM). Zerumbone induced apoptosis and a production of the reactive oxygen species (ROS) and a loss of the mitochondrial membrane potential (MMP). Furthermore, zerumbone also induced an increase of Bax/Bcl-2 ratio, concomitant with the activation of caspase-3. Zerumbone also triggered a release of cytochrome c (cyt-c) into the cytoplasm. Taken together, zerumbone is an inducer of cell cycle arrest and apoptosis in cervical cancer cells.

Keywords: Cervical carcinoma, zerumbone, apoptosis, Bax/Bcl-2

Introduction

Carcinoma of the cervix is a critical public health problem that is the second most common cause of cancer death among women [1], and is the most prevalent malignancy in women in many developing countries, resulting in approximately 300,000 deaths each year [2]. However, the underlying mechanisms of cervical cancer pathogenesis are still poorly understood. Cervical carcinoma in patients with a poor prognosis is characterized by rapid cellular proliferation and strong expression of anti-apoptotic genes. Emerging evidence indicates that natural products including plants, microorganisms and halobios provide rich resources for discovery of anticancer drug [3]. Characterization of indentified molecular mechanisms should help to disclose the role of anticancer drug on cervical cancer for diagnostic, prognostic and therapeutic use.

Zerumbone is a component of *Zingiber zerumbet* which are employed as medicine in relieving stomachache and macerated in alcohol [4], and that it has been found to exert a variety of pharmacological effects, including antitumor, anti-inflammatory, antitioxidants, antibacterial and antivirus [5-7]. In addition, zerumbone has subsequently been shown to inhibit proliferation in a variety of cancer cell, but not in normal cells. It was reported that zerumbone treatment of cervical, liver and pancreatic cancer through inducing apoptosis and inhibiting invasion [4, 8, 9]. Zerumbone inhibits proliferation of HL-60 cells via G2/M cell cycle arrest [10], and induces antiproliferation and apoptosis of HCT116 cells [11]. Moreover, zerumbone causes G2/M cell cycle arrest and Bax/Bak-mediated apoptosis in MDA-MB-231 cells, and retards growth of MDA-MB-231 xenografts *in vivo* [12]. But, whether zerumbone played the inhibitory role in cervical cancer cells remains unknown.

Apoptosis is an essential process and playing an important role in the development and progression of diseases including cancer [13]. Two signaling pathways, the extrinsic and the intrinsic pathway, that are considered to be involved in apoptosis through TNF receptor superfamily activation and the mitochondria- and the Bcl-2 family-mediated signaling transduction path-

way, respectively [14]. Recently, it has been reported that zerumbone mediated its activity through the modulation of NF-κB activation, which is also correlated with the suppression of TNF-induced invasion activity [15]. Zerumbone induced apoptosis of pancreatic cancer cells through increases in Caspase-3 activity and ROS production, as well as p53 activation in PNAC-1 cells [16]. The antiproliferative effect of zerumbone on Jurkat cells is through activation of caspase-3 and caspase-9, release of cytochrome c from the mitochondria into the cytosol, and subsequent cleavage of poly (adenosine diphosphate-ribose) polymerase (PARP) [17].

In this study, we demonstrated that zerumbone exhibited antitumor effect in vitro, and further study showed that apoptosis induction of zerumbone involved in its antitumor effects. Then we found that apoptotic pathways induced by zerumbone involved the modulation of Bax/Bcl-2 ratio and a mitochondria-mediated pathway. These results provided a mechanistic framework for further exploring of zerumbone as a novel drug for human cervical cancer.

Materials and methods

Cell culture

Human cervical carcinoma lines SiHa were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone Company, USA), in which 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, Rockville, MD, USA) were supplemented. All the incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA) was set to 37°C, 100% humidity and 5% CO_2.

Cell viability analysis

The cellular viability of cervical carcinoma cells was determined by CCK-8 (Cell Counting Kit-8, Signalway Antibody LLC, College Park, MD, USA). Briefly, the cells were collected and seeded in 96-well plates to attach overnight in DMEM supplemented with 10% FBS. Then cervical carcinoma cells were incubated with zerumbone 10, 20 and 30 mM for 24, 48 and 72 h, respectively. Then, the culture media were washed out and the fresh media containing 10 mL CCK-8 were added. The cells were continuously incubated at 37°C for an additional 1 h. The optical density of each well was measured using a microplate reader at 450 nm. With each time in triplicate, experiments were performed at least three times.

Cell cycle analysis

Cells were seeded in 12-well plates at the density of 3×10^3 cells/well and then treated with 10, 20 and 30 mM of zerumbone for 48 h. Then cells were washed with PBS, trypsinized and centrifuged at 1000 × g at 4°C for 5 min. Pellets were fixed overnight in 70% cold ethanol. After fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/mL) for 10 min at room temperature. Finally, samples were stained with propidium iodide (50 mg/mL) for 10 min at 4°C. Data acquisition was done by flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA) using Cell Quest software. With each time in triplicate, experiments were performed at least three times.

Assays for apoptosis

Apoptotic cells were evaluated by both annexin V-FITC and propidium iodide double staining using a staining kit purchased from eBioscience (San Diego, CA, USA). Briefly, cells were collected after treatment with zerumbone (10, 20 and 30 mM) for 48 h. Both floating and trypsinized adherent cells were collected, resuspended in 195 mL of annexin-V FITC and 5 mL of propidium iodide, and then incubated for 10 min in the dark at room temperature before flow cytometry analysis. With each time in triplicate, experiments were performed at least three times.

Measurement of reactive oxygen species (ROS)

To quantify intracellular ROS level, we used Dihydroethidium (DHE, Shanghai Yope Biotechnology Co., Ltd, Shanghai, China). DHE is a poorly fluorescent 2-electron reduction product of ethidium that on oxidation produces DNA sensitive fluorochromes that generate a red nuclear fluorescence when excited at 510 nm. The results obtained with this probe were validated as a measure of the ability of cervical carcinoma cells to generate ROS. After treat-

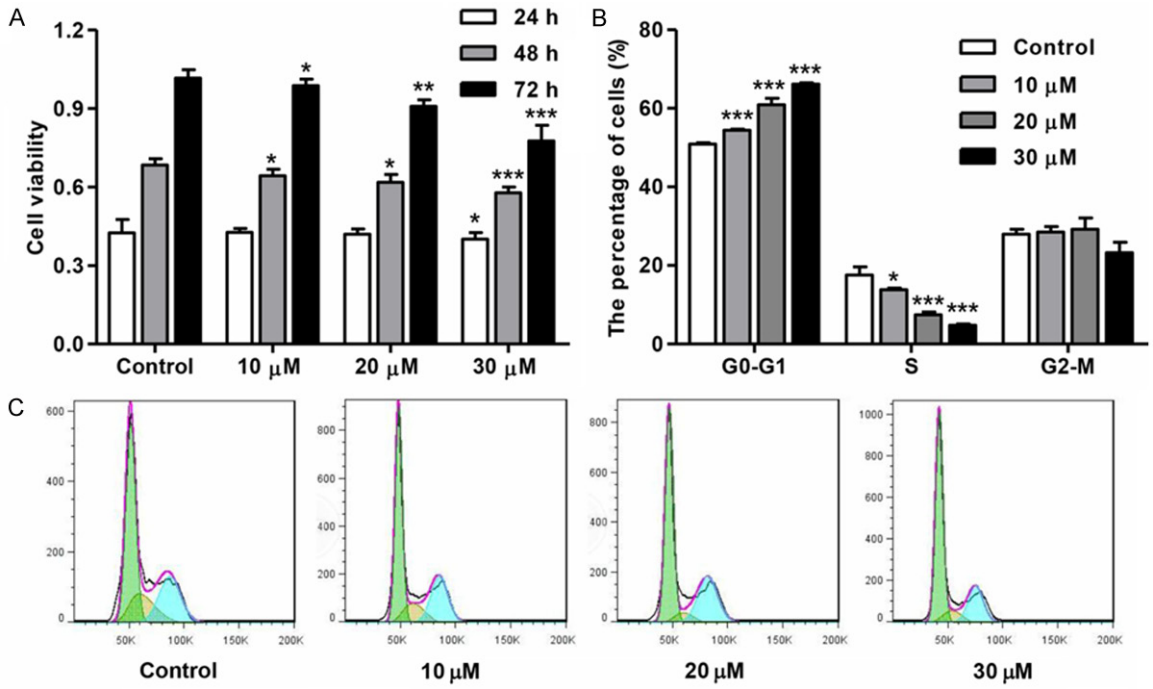

Figure 1. Effects of zerumbone on the cellular viability and cell cycle of SiHa cells. A. The cellular viability of SiHa was significantly reduced by zerumbone 10, 20 and 30 μM after 24, 48 and 72 h incubation. B, C. The cell cycle of SiHa cells was significantly blocked at G1 phase by zerumbone 10, 20 and 30 μM after 48 h incubation. **P*<0.05, ***P*<0.01 and ****P*<0.001 versus Control.

ment with zerumbone at doses of 10, 20 and 30 mM for 48 h, cells were resuspended with 50 μmol/L DHE, and fluorescence intensity was measured using flow cytometry. With each time in triplicate, experiments were performed at least three times.

Measurement of mitochondrial membrane potential (MMP)

Cells (1×10^6) were collected after treatment with zerumbone (10, 20 and 30 mM) for 48 h and then were resuspended with 100 nM Tetramethyl rhodamine methyl ester (TMRM, Amyjet Scientific Inc, Wuhan, China), and fluorescence intensity was measured using flow cytometry. With each time in triplicate, experiments were performed at least three times. With each time in triplicate, experiments were performed at least three times.

Western blot analysis

Cells were harvested, aliquoted and lysed in lysis-buffer. Protein samples (50 ug) from both zerumbone-treated and untreated cells were separated on 15% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polyvinyl-difluoride (PVDF) membranes (PolyScreen, NEN Life Sciences, USA). The membranes were dried, preblocked with 5% non-fat milk in PBS-Tween (0.1%), then incubated with the primary antibodies (Bax, Bcl-2, cyt-c and caspase-3) diluted in 1:1000. The secondary antibody used was horseradish peroxidase labeled to rabbit or mouse IgG. Antibody binding was detected with an enhanced chemiluminescence kit and ECL film (Amersham, Buckinghamshire, UK). The membranes were reprobed with GAPDH antibodies as an internal control and to confirm equal loading.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The unpaired Student's *t*-test was used to compare quantitative data populations with normal distribution and equal variance. All statistical analysis was performed using the SPSS 13.0 software for Windows. A value of $P<0.05$ was considered statistically significant or otherwise specified.

Results

Zerumbone reduced cellular viability and blocked cell cycle of SiHa cells

The effects of zerumbone on the viability of SiHa cells were measured by the CCK-8 assay. As displayed in **Figure 1A**, the exposure of SiHa cells to zerumbone 10, 20 and 30 μM for indicated times resulted in a significant reduction of cellular viability, compared with control cells. Zerumbone 10, 20 and 30 μM decreased the viability of SiHa cells from 0.653±0.002 to 0.643±0.002, 0.618±0.003, and 0.578±0.002 at 48 h, whereas from 1.016±0.003 to 0.987±0.002, 0.908±0.002, and 0.776±0.006 at 72 h, respectively. The results suggest that zerumbone reduces the cell viability of SiHa in a concentration- and time-dependent manner. SiHa cells were incubated with zerumbone (10, 20 and 30 μM) for 48 h and then the cells were analyzed in terms of cell cycle distribution by means of flow cytometry. The cell cycle analysis demonstrated that zerumbone induced an accumulation of cells in the G1 phase and a depletion of cells in the S phase (**Figure 1B** and **1C**), suggesting that zerumbone induced blockade of G1 cell cycle in SiHa cells.

Apoptosis of SiHa cells was induced by exposure to zerumbone

To further define the mechanism of antiproliferative effect of zerumbone, SiHa cells were treated with zerumbone at 10, 20 and 30 μM to determine whether this bioactive compound induced SiHa cells death via apoptosis or necrosis. As shown in **Figure 2**, increased apoptotic rate could be detected more significantly in SiHa cells treated with zerumbone than in control SiHa cells. Zerumbone 10, 20 and 30 μM significantly increased the apoptotic rate of SiHa cells from 0.067±0.058% to 3.667±0.351%, 6.567±0.0751%, 12.57±0.874% after 48 h incubation.

Zerumbone increased ROS and decreased MMP level in SiHa cells

The effect of zerumbone on the ROS production and MMP level in SiHa cells was further investigated. As illuminated in **Figure 3A**, the exposure of SiHa cells to zerumbone 10, 20 and 30 μM markedly increased the production of ROS by approximately 195.803%, 357.407% and 5679.433%, respectively. These results further confirmed that zerumbone induced apoptosis of SiHa cells. Then, we explored the influences of zerumbone on the MMP level. SiHa cells were exposed to zerumbone 10, 20 and 30 μM for 48 h and analyzed for the level of MMP by flow cytometry using TMRM a lipophilic fluorochrome taken up by the mitochondria in proportion to the MMP. **Figure 3B** demonstrated the fluorescence image of MMP in the absence and presence of zerumbone in SiHa cells. The level of MMP was decreased by zerumbone in a concentration-dependent manner. The exposure of SiHa cells to zerumbone 10, 20 and 30 μM markedly decreased the level of MMP by approximately 33.949%, 32.756% and 57.030%, respectively.

Effects of zerumbone on the expression of Bax/Bcl-2, cyt-c and caspase-3

We next examined the expression of apoptosis-associated proteins during treatment with zerumbone. Zerumbone induced the cleavage of the pro-apoptotic Bax protein and degradation of Bcl-2 was observed at 48 h after treatment with zerumbone. The effect of zerumbone on the caspase-3 activity and cyt-c release in SiHa cells was further investigated. The exposure of SiHa cells to zerumbone 10, 20 and 30 μM markedly increased the activity of caspase-3 and induced release of cyt-c from the mitochondria into the cytosol (**Figure 4**).

Discussion

Previous studies have shown that zerumbone could effectively induce programmed cell death and suggested that it could be a promising antitumor drug [18]. In the present study, we observed that zerumbone exhibited anti-tumor activity against SiHa human cervical cancer cells. These findings indicated that zerumbone exerted a strong anti-cervical cancer activity in vitro and might be with low toxicity. We also found that zerumbone induced the apoptosis of SiHa cells in vitro by flow cytometry assay. All these data suggested that zerumbone should be further explored as a possible therapeutic agent for the treatment for cervical cancer.

Recently, researchers reported that zerumbone exerts antiproliferative effects on various human colon cancer cells, including COLO205, COLO320DM and LS180 cells, but had only a

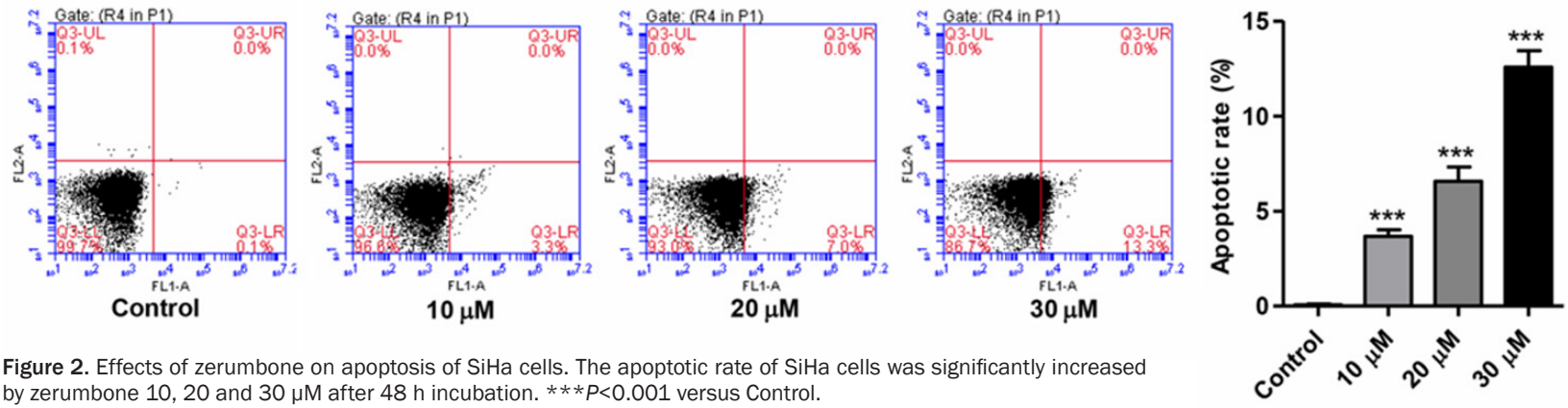

Figure 2. Effects of zerumbone on apoptosis of SiHa cells. The apoptotic rate of SiHa cells was significantly increased by zerumbone 10, 20 and 30 μM after 48 h incubation. ***P<0.001 versus Control.

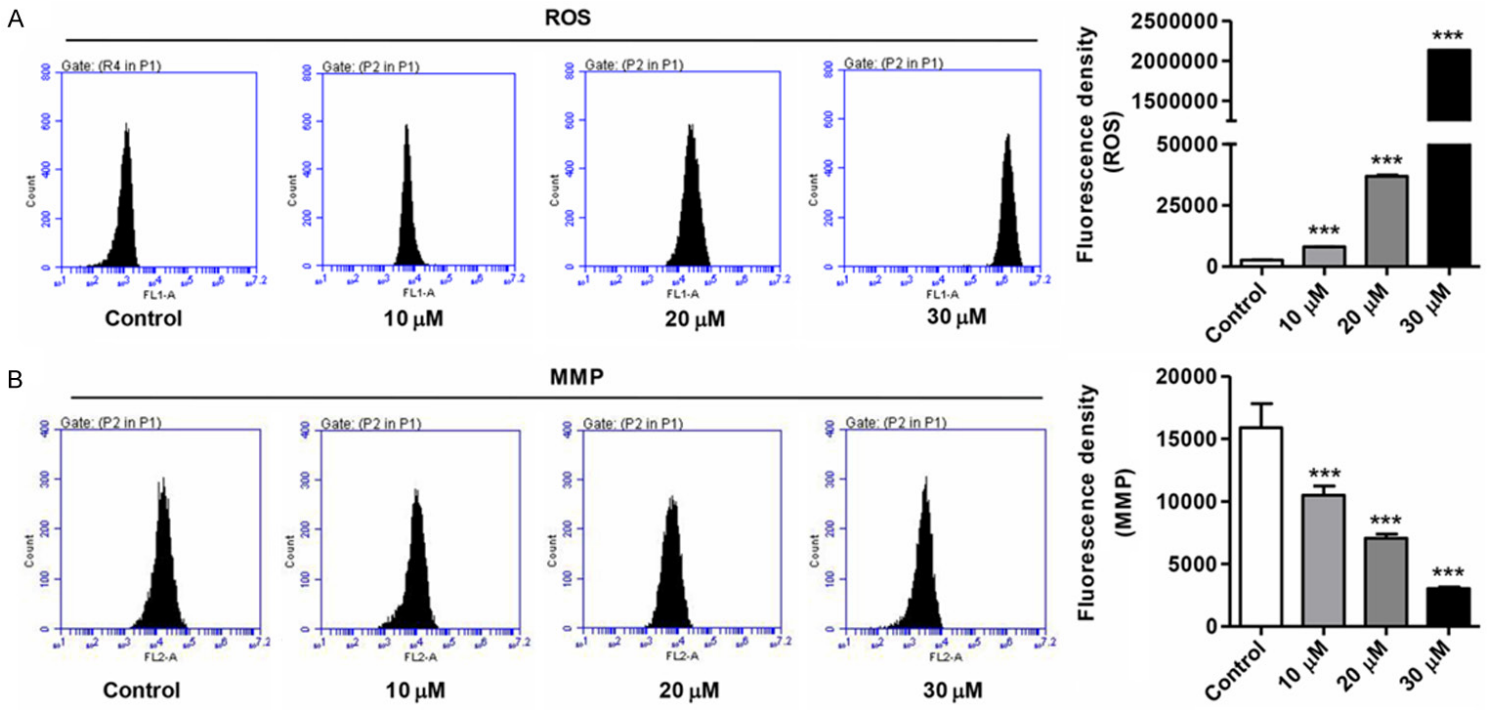

Figure 3. Effects of zerumbone on ROS production and MMP level of SiHa cells. A. The ROS production of SiHa cells was significantly increased by zerumbone 10, 20 and 30 μM after 48 h incubation. B. The MMP level of SiHa cells was significantly decreased by zerumbone 10, 20 and 30 μM after 48 h incubation. ***P<0.001 versus Control.

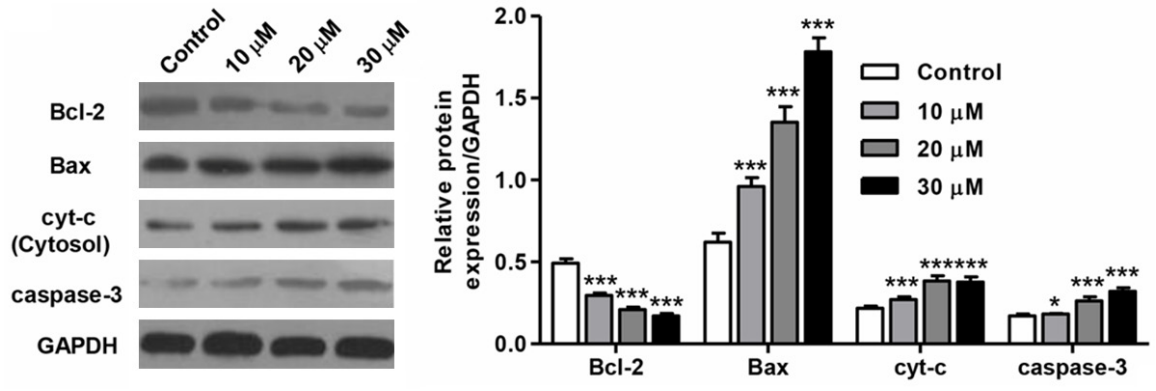

Figure 4. Zerumbone-induced apoptosis in SiHa cells is mediated through Bax/Bcl-2 and mitochondrial pathway. Effect of zerumbone on the expression of Bax, Bcl-2, cyt-c and caspase-3 proteins in SiHa cells was detected by Western blotting. GAPDH was used to confirm that equal amounts of protein were in each lane. **P*<0.05 and ****P*<0.001 versus Control.

minimal effect on the proliferation of normal colon fibroblasts [19]. In this study, we observed that zerumbone affect the proliferation of SiHa cells at concentration of 30 mM for 24 h, while previous findings showed that 20 mM zerumbone inhibited proliferation of HUVECs and NB4 cells [10], suggesting that SiHa cells were less sensitive to zerumbone. Furthermore, we also found zerumbone induced G1 cell cycle arrest in SiHa cells in a concentration-dependent manner, from 10 mM to 30 mM, which was similar to the findings reporting that zerumbone induced G0/G1 cell cycle arrest of HT-29 cells at concentrations of 10-25 μM [5]. However, the cell cycle arrests in ovarian and cervical cancer cell lines (Caov-3 and HeLa, respectively) were observed at G2/M [20].

Upon our previous research, the inhibition of tumor proliferation by zerumbone may be through apoptosis. Then we observed the apoptosis-induction activity of zerumbone in SiHa cells. Notably, apoptosis is more important in understanding cancer because cells have developed a way avoid apoptosis. Thus, cancer is often characterized by too little apoptosis and too much proliferation of cells. The programmed cell apoptosis compared to necrosis is a desire somatic defense mechanism against cancer cells [21]. Moreover, apoptosis is reported earlier to be triggered by natural products [22, 23]. Our results showed that zerumbone induced apoptosis of SiHa cells in a concentration-dependent manner, which is agreement with previous studies in terms of the potential anticancer of this compound and the typical features of apoptosis of liver cancer, leukemia cell lines and HaLa human cervical cancer cells [20, 24, 25].

Reactive oxygen species (ROS) are a variety of molecules and free radicals derived from molecular oxygen, which was constantly generated and eliminated in the biological system, and have important role in cell signaling and homeostasis [26]. Increasing evidence supported that the excessive amounts of ROS contributes to oxidative damage to protein and DNA leading to tumorgenesis or cell apoptosis [27]. We found that zerumbone exerted a facilitated role in the production of ROS and exerted an inhibitory effect on the MMP level in a concentration-dependent manner, which is at least in part responsible for its pharmacological actions on SiHa cells.

In further analysis, we demonstrated that zerumbone markedly induced apoptosis as evidenced by increases in Bax/Bcl-2 ratio, cyt-c release and activity of caspase-3. The susceptibility of tumor cells to the induction of apoptosis by chemotherapeutic agents is controlled by the ratio of Bax/Bcl-2 proteins in the mitochondria [28]. When cells in culture received death signals, Bax moves to mitochondria and triggers release of cyt-c to the surrounding cytosol, loss of transmembrane potential and induction of mitochondrial permeability transition events that result in apoptotic cells [29]. In agreement with the previous study reporting that mitochondrial permeabilisation and cyt-c dependent caspase activation dominate in zerumbone induced cell death of MCF-7 and SiHa cells [30].

In conclusion, we demonstrated here that zerumbone inhibits the proliferation of SiHa cells via induction of G1 cell cycle arrest followed by apoptosis, accompanied with increasing production of ROS and decreased MMP level. Taken together, the present results suggest that zerumbone may possess potential as a novel therapeutic agent in the treatment of human cervical cancer.

Acknowledgements

This work was supported by the Science and Technology Commission of Shanghai Municipality Fund (14DZ1941804).

Disclosure of conflict of interest

None.

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