Department of Urology[1], Shaanxi Provincal People's Hospital; Center of Maternal and Child Health[2], the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an, P. R. China

Zerumbone induces apoptosis in human renal cell carcinoma via Gli-1/Bcl-2 pathway

*Yi Sun[1,1], *Qiu Sheng[2], Yongyi Cheng[1], Yonggang Xu[1], Yili Han[1], Jianzhong Wang[1], Liang Shi[1], Huacai Zhao[1], Chun Du[1]

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Yi Sun, Department of Urology, Shaanxi Provincal People's Hospital, No. 256 Youyi West Road, Xi'an 710068, P. R. China
adoremidfall@yahoo.cn
Co-first authors

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Renal cell carcinoma (RCC) is a malignant disease insensitive to conventional treatments such as radiochemotherapy and immunotherapy. Search for new approaches to induce cancer cell apoptosis will improve the management of RCC. Here, we reported that zerumbone, a monosesquiterpine, shows anticancer effects on human RCC cells *via* induction of apoptosis *in vitro*. Human renal clear cell carcinoma 786-O and 769-P cell lines were used as the model system. Exposure of RCC cells to zerumbone resulted in cell viability inhibition, accompanied by DNA fragmentation and increased apoptotic index. Mechanically, treatment of RCC cells with zerumbone activated caspase-3 and caspase-9, and finally led to cleavage of PARP. In addition, downregulation of Gli-1 and Bcl-2, which were closely related to the chemoresistance of RCC, was observed in zerumbone-treated RCC cells. Taken together, our study provided the first evidence that zerumbone imparted strong inhibitory and apoptotic effects on human RCC cells. The zerumbone-induced apoptosis might be related to the activation of the caspase cascade and deregulation of the Gli-1/Bcl-2 pathway. Our results suggest that zerumbone merit further investigation as an apoptosis inducer as well as a novel RCC chemotherapeutic agent in the clinical setting.

1. Introduction

Renal cell carcinoma (RCC) is a common malignant disease of the human genitourinary system, accounting for approximately 210,000 new cases and 150,200 deaths each year all over the world (Parkin et al. 2005). Clear cell carcinoma is the main pathological type of RCC, originating from the renal proximal tubule (Dormoy et al. 2009). Surgical excision is the main approach for localized RCC treatment, however, metastatic RCC, which occurs frequently in RCC patients, is difficult to be removed surgically (Rajandram et al. 2012). Unlike most of other cancers, RCC is highly resistant to conventional treatments such as radiochemotherapy and immunotherapy (Berger et al. 2009). In recent years, molecular-targeted therapies have been developed in treatment of metastatic RCC, which greatly improved the outcomes of patients. However, resistance to these drugs has also been reported (Gross-Goupil et al. 2012). Thus, it is tremendously important to search for new drugs or approaches in the management of RCC.

Zingiber zerumbet, a perennial herb found in many tropical countries, was used for the treatment of various kinds of diseases such as inflammation and worm infestation (Yob et al. 2011). Zerumbone (ZER), a monosesquiterpine recognized as a main compound of *Zingiber zerumbet*, is demonstrated to have multiple biological and pharmacological activities (Kitayama 2011) (Fig. 1A). Recently, accumulating evidence indicates that ZER has chemopreventive and chemotherapeutic effects in various cancers, including cancers of liver (Sakinah et al. 2007), cervix (Abdel et al. 2009; Abdul 2009), colon (Yodkeeree et al. 2009), and leukemia (Abdelwahab et al. 2011). Mechanically, it was reported that ZER can trigger apoptotic death, which was believed to be one of the major mechanisms of action for ZER against cancer cells (Sakinah 2007; Abdel Wahab 2009; Abdelwahab et al. 2011). However, to the best of our knowledge, the pharmacological activity of ZER on RCC remains unknown.

In the present study, we firstly investigated the potential anticancer effect of ZER on RCC cells *in vitro*. We focused on the apoptotic effect of ZER on human RCC cells, and investigated its possible mechanisms.

2. Investigations and results

2.1. Inhibitory effect of ZER on cell viability

Firstly, we investigated the potential inhibitory role of ZER on cell viability of 786-O and 769-P cells. As shown in Fig. 1B and C, ZER significantly inhibited cell viability in a time- and concentration-dependent manner.

2.2. Induction of apoptosis by ZER in vitro

Since we have observed the significant inhibitory effect of ZER on RCC cells, we next examined the effects of ZER on renal cancer cell apoptosis by DNA ladder assay and annexin V/PI double staining. DNA fragmentation was analyzed to detect the

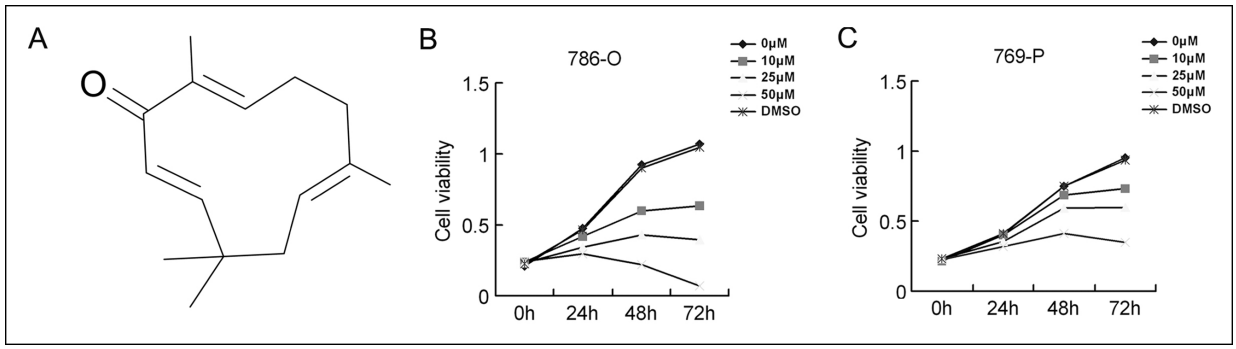

Fig. 1: Inhibitory effect of ZER on cell viability of RCC cells. (A) chemical structure of ZER. (B, C) cells were treated with various concentration of ZER. Cell viabilities were detected at different time points by MTT assay

characteristic breakdown of DNA. The results showed typical DNA ladder in agarose gel electrophoresis after treatment with ZER (Fig. 2A). In addition, when the treated cells were subjected to annexin V and PI staining, we found that ZER increased apoptotic index in a concentration-dependent manner (Fig. 2B and C). These results clearly revealed that ZER induced apoptosis in 786-O and 769-P cells.

2.3. Activation of caspase activity by ZER

It is well known that the final pathway leading to the execution of apoptosis is the activation of a series of proteases termed caspases, which were downsteam effectors. To determine whether ZER induces apoptosis *via* caspases, we analyzed activities of caspase-3 and caspase-9 in cells treated with ZER. Data from our analysis showed that ZER activated caspase-3 and caspase-9 in a concentration-dependent manner, as observed by its increased cysteine protease activity for caspase-3 and caspase-9 substrate (Fig. 3A and B). This was consistent with the followed experiment by western blot, which showed an increased cleavage of PARP, a substrate of caspases (Fig. 3C and D). All of these results confirmed the activation of caspase cascade by ZER in RCC cells.

2.4. Targeting Gli-1/Bcl-2 pathway by ZER

The transcription factor Gli-1, an executor of hedgehog signal pathway, was reported to play important roles for cancer cell survival and invading of apoptosis, partly *via* its downstream protein Bcl-2 (Chen et al. 2008). To determine whether ZER induces apoptosis *via* Gli-1/Bcl-2 pathway, we detected levels of these two proteins by western blot. Our data showed that after treatment for 48 h, ZER significantly suppressed the expression of Gli-1 and Bcl-2 (Fig. 4).

3. Discussion

Apoptosis, a programmed cellular process that occurs in physiological and pathological conditions, is closely correlated with the development and progression of most cancers (Wong 2011). It is reported that many kinds of drugs, including conventional chemotherapeutic drugs and plant extracts, exert anticancer activities through inducing apoptosis (Rebillard et al. 2010; Zeng et al. 2011). However, cancer is considered as the result of a succession of genetic changes during cell transformation, and the reduced apoptosis apoptotic its resistance plays a vital role in carcinogenesis and tumor progression (Wong 2011). Some types of human cancers, such as RCC, are insensitive to anticancer drugs (Rajandram et al. 2012). Search for new drugs which can effectively induce apoptosis may provide promising approaches to improve the management of RCC.

Several studies revealed that ZER has chemopreventive and chemotherapeutic activities against cancers with different origins (Tanaka et al. 2001; Yodkeeree et al. 2009). It is believed that ZER mediated therapy involves inhibition of cell proliferation and induction of cell apoptosis (Abdelwahab et al. 2012). In the present study, we observed that ZER significantly inhibited cell viability of renal clear cell carcinoma 786-O and 769-P cells *in vitro*. To confirm that ZER-induced cell inhibition was associated with induction of apoptosis, DNA fragmentation was analyzed to detect the characteristic breakdown of DNA, which is a hallmark of apoptosis (Wong 2011). The results showed typical DNA ladder in agarose gel electrophoresis after treatment with ZER, indicating that ZER did induce apoptosis in 786-O and 769-P cells. This was confirmed by a followed apoptotic index assessment, which showed that ZER increased apoptotic index in a concentration-dependent manner.

Activation of caspases is one of the main biochemical changes central to the mechanisms of apoptosis. As both the initiators and

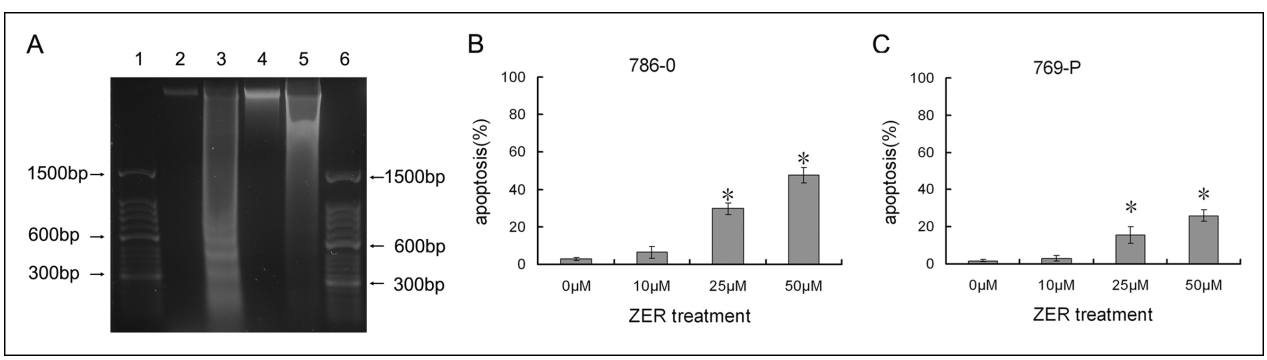

Fig. 2: ZER induced apoptosis in RCC cells. (A) DNA fragmentation was analyzed for cells treated with (lane 3 for 786-O and lane 5 for 769-P cells) or without (lane 2 for 786-O and lane 4 for 769-P cells) 25 μM ZER for 48 hours. 50 bp DNA ladder marker (lane 1 and lane 6) was used for electrophoresis. (B, C) Cells were treated with various concentration of ZER for 48 hours; the apoptotic index was calculated by flow cytometry. Percentage of apoptotic cells was presented as mean ± SE of triplicate samples and are representative of 3 independent experiments. Error bars represent SEs. *, $P < 0.05$ *vs* 0 μM group

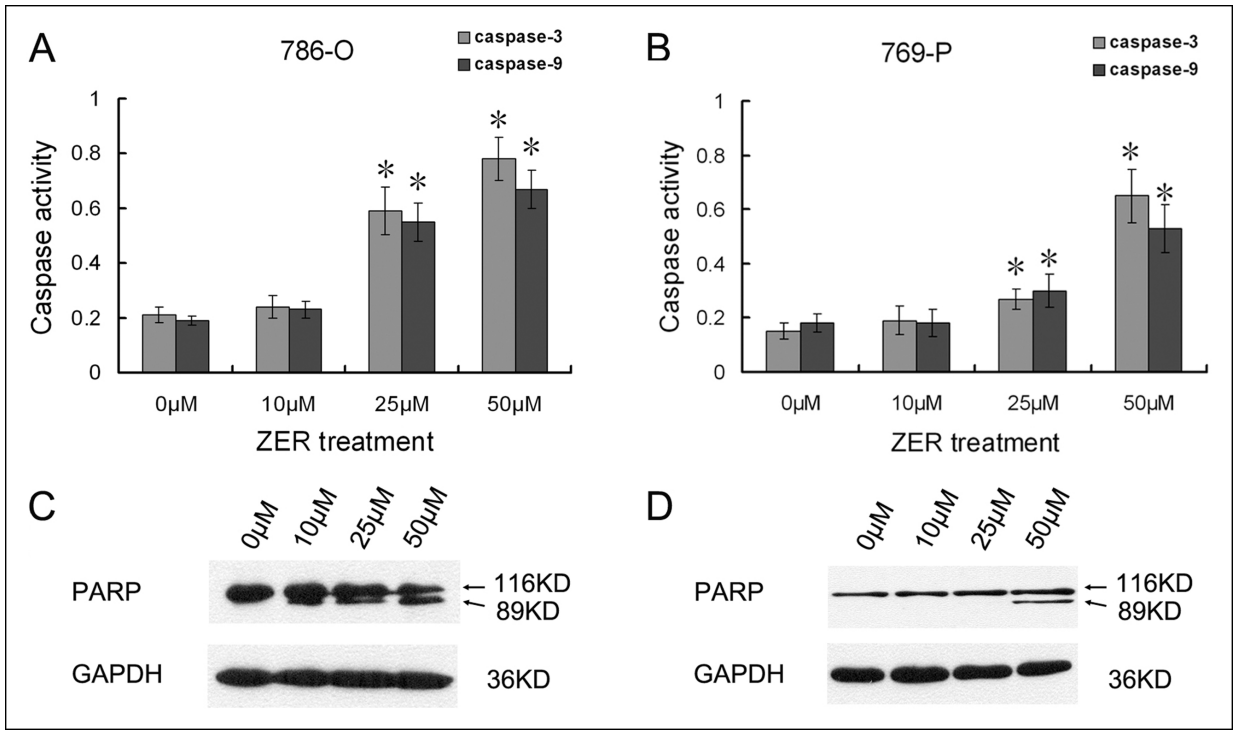

Fig. 3: ZER treatment activated caspases in RCC cells. (A, B) after treatment with various concentration of ZER for 48 h, activities of caspase-3 and caspase-9 were assessed by a colorimetric system. Data are presented as mean ± SE of triplicate samples and are representative of 3 independent experiments. Error bars represent SEs. *, $P < 0.05$ *vs* 0 μM group. (C,D) in similar treatment, total lysates of 786-O (C) and 769-P (D) cells were analyzed for PARP cleavage by western blot

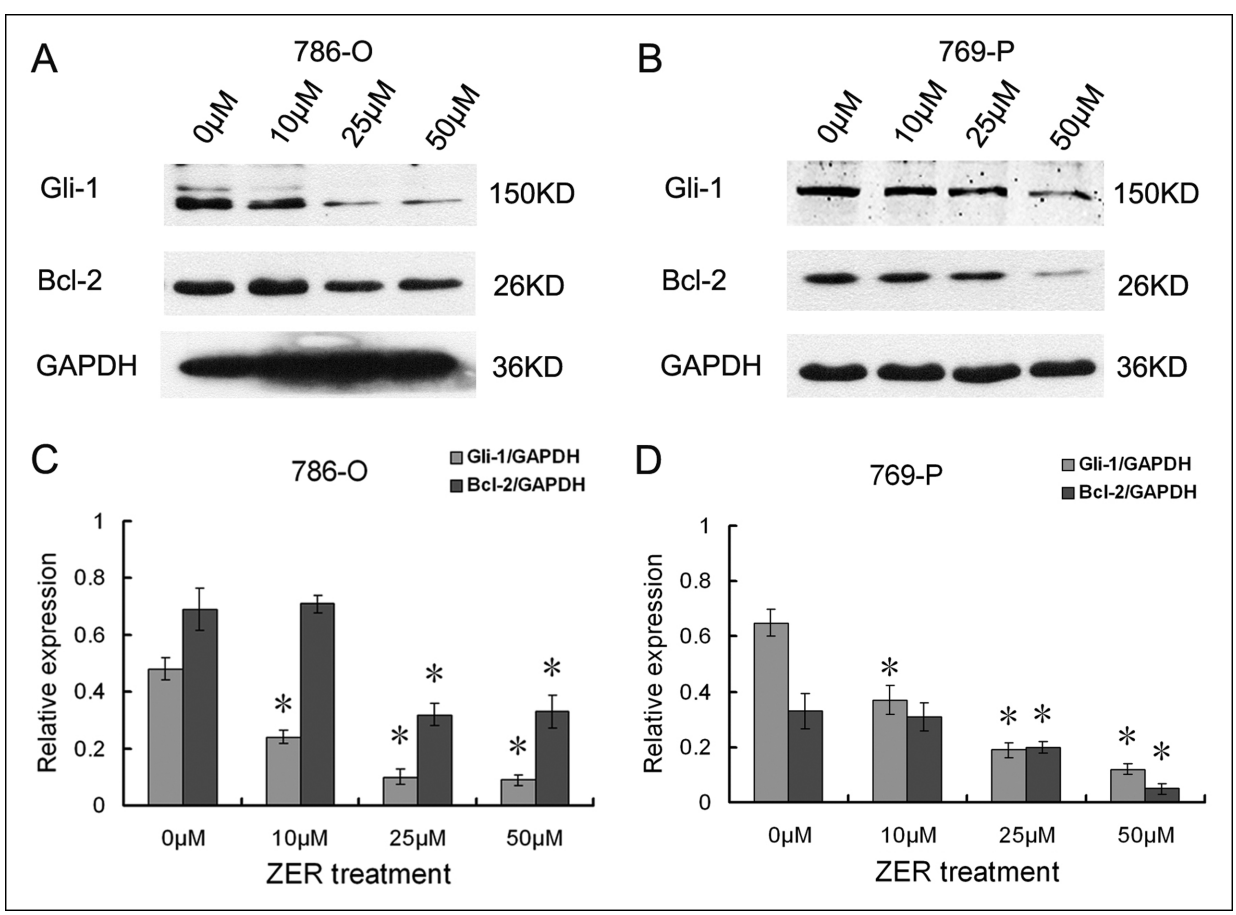

Fig. 4: Targeting Gli-1/Bcl-2 pathway by ZER. After treatment with various concentration of ZER for 48 h, cell lysates of 786-O and 769-P cells were analyzed for Gli-1 and Bcl-2 expression by western blot. GAPDH was detected as an internal control. (A,B) Representative western blot results. (C,D) Quantitative analyses on relative expression as compared with GAPDH. All data are presented as mean±SD of three independent experiments. Error bars represent SDs.*, $P < 0.05$ vs 0 μM group

executors of apoptosis, caspases can be activated by mitochondrial pathway or death receptor pathway (Wong 2011). However, in some conditions, apoptosis could be caspase-independent (Misirlic et al. 2012). To determine whether caspases were involved in ZER-induced apoptosis in RCC cells, we detected activities of caspase-3 and caspase-9 in cell lysate treated with ZER. The data revealed that ZER treatment increased caspase activity. In addition, we also observed increased cleavage of PARP, which could be cleaved by activated caspases (Soldani and Scovassi 2002). We concluded that ZER induced apoptosis in 786-O and 769-P cells was caspase-dependent, at least, partly dependent on caspases.

The Bcl-2 family of proteins, which is comprised of pro-apoptotic and anti-apoptotic proteins, plays a pivotal role in the regulation of mitochondrion-mediated apoptosis (Soldani and Scovassi 2002). The balance of anti-apoptotic and pro-apoptotic members regulates mitochondrial release of cytochrome-c and determines the initiation of apoptosis (Soldani and Scovassi 2002). During carcinogenesis and tumor progression, the upregulated anti-apoptotic members, or downregulated pro-apoptotic members, might lead to insensitivity to apoptotic stimuli, and therefore, resistance to therapy, recurrence and poor prognosis (Michaud et al. 2009). Recent evidence indicated that ZER induced cancer cell apoptosis *via* modulation of the Bcl-2/Bax ratio, indicating that it is a potential agent to reverse resistance to apoptosis contributed by disbalance of Bcl-2 family (Sakinah et al. 2007).

The hedgehog (Hh) signal pathways are critical for embryonic and postnatal organ development (Watson et al. 2010). Recent evidence also indicates that the Hh signals are reactivated in some cancers, including melanoma, pancreatic cancer and RCC, contributing to the growth and maintenance of various cancers (Dormoy et al. 2009; Watson et al. 2010). The zinc finger transcription factor Gli-1 is regarded as a nuclear executor of Hh pathway and as a marker of Hh signal activation (Mahindroo et al. 2009). Over expression of Gli-1 in cancers results in malignant phenotypes such as decreased cell apoptosis and accelerated metastasis through regulating a series of target genes (Mori 2006; Chen 2008). For example, Gli-1 transcriptionaly upregulates the anti-apoptotic protein Bcl-2, which is important for cell survival (Bigelow et al. 2004). In the present study, we found that ZER treatment significantly downregulated the expression of Gli-1 and its downstream protein Bcl-2. Thus, we concluded that ZER may initiate apoptosis through downregulating Gli-1-mediated Bcl-2 expression. Further studies are needed to investigate its chemotherapeutic effects *in vivo*. Also, the detailed mechanism of ZER-induced apoptosis in RCC remains to be investigated. For example, the exact role of Gli-1 in ZER-induced apoptosis and its importance should be explored. Taken together, our study provided the first evidence that ZER imparted strong inhibitory and apoptotic effects on human RCC cells. ZER-induced apoptosis might be related to the activation of the caspase cascade and deregulation of the Gli-1/Bcl-2 pathway. Our results suggest that ZER merits further investigation as an apoptosis inducer as well as a novel RCC chemotherapeutic agent in the clinical setting.

4. Experimental

4.1. Chemicals and reagents

ZER and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle Media/F12 (DMEM/F12) and fetal bovine serum (FBS) were obtained from GIBCO Co. (Gibco, NY, USA). Primary antibodies against Bcl-2, Gli-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Primary antibody against poly (ADP-ribose) polymerase (PARP) and secondary antibodies conjugated with horseradish peroxidase were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Annexin V-FITC Apoptosis Detection Kit was purchased from BD Bioscience Inc (BD, San Jose, CA).

4.2. Cell culture and treatment

Human renal clear cell carcinoma 786-O and 769-P cell lines (ATCC, USA) were propagated in DMEM/F12 medium containing 10% FBS and maintained at 37 °C in a humidified incubator with 5% CO2 in air. ZER was dissolved in DMSO at a stock concentration of 20 μM. Cells were seeded in plates or dishes and incubated for 24 h. Then the supernatant was removed and fresh medium containing different concentration of ZER was added. Medium containing 0.3% DMSO was used as a control.

4.3. Cell viability assay

Cells were plated in 96-well culture plates in 0.2 ml of medium supplement containing 10% FBS. After culture for 24 h, the medium was changed by fresh medium containing various concentration of ZER (0, 10, 25, and 50 μM) and the cells were incubated for various times (0, 24, 48 and 72 h), followed by supplement of 20 μl MTT (5 mg/ml) for each well and incubation for 4 h. Viable cell-mediated reaction products were recorded by using a 96-well microplate reader (Bio-Rad, Hercules, CA) at a wavelength of 490 nm.

4.4. Flow cytometry for apoptotic index

Cells were exposed to various doses of ZER (0, 10, 25, and 50 μM) for 48 hours. The cells were collected and subjected to annexin V and propidium iodide (PI) staining using an Annexin V-FITC Apoptosis Detection Kit. Apoptotic index were then analyzed by flow cytometry.

4.5. Assay for caspase activity

Caspase activity was assessed by a colorimetric system (Zeng et al. 2011). Briefly, after treatment with 25 μM ZER for 48 h, cells were lysed in a lysis buffer by freeze/thaw cycles. Lysates were clarified by centrifugation at 10,000 g (4 °C) for 10 min. Protein (100 μg) was incubated with 30 mL of caspase assay buffer and 2 mL of caspase-3 (DEVD-pNA) or caspase-9 (LEHD-pNA) colorimetric substrate at 37 °C for 4 h. The optical density of the reaction mixture was determined spectrophotometrically at a wavelength of 405 nm using a 96-well microplate reader.

4.6. Detection for DNA fragmentation

Cells were plated in 6-cm dishes and incubated for 24 h. Then the supernatant was replaced by fresh medium with or without 25 μM ZER, followed by incubation for 48 h. DNA of treated cell was extracted as previously described (Song et al. 2005), and then electrophoresed in 1.5% agarose gels containing 0.5 μg/mL EB.

4.7. Western blot

Total proteins were extracted from cells with RIPA lysis buffer containing protease inhibitor. Protein concentration of lysates was analyzed by the Bradford method. Western blot was performed as previously described but with some modifications (Ling et al. 2005). Briefly, 30 μg protein was separated by electrophoresing on a 12% SDS–polyacrylamide gel and blotted onto nitrocellulose filter membranes. The membranes were blocked with 5% no-fat-milk in Tris-Buffered Saline and Tween 20 (TBS-T) for 1 h at room temperature. The blots were then incubated with primary antibodies overnight at 4 °C, and horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Immunoreactive signals were detected using the ECL detection system followed by exposure to X-ray film. The relative intensity of each band was determined by using Quantity One software (Bio-Rad, USA). Immunoblotting against GAPDH was performed as an internal control.

4.8. Statistical analyses

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, USA). Quantitative data were presented as mean ± SD. Student's t test was used when the comparison the difference between 2 groups. Significance was assumed for P values < 0.05.

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