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Title: *In Vitro* and *In Vivo* Studies of Pharmacological Effects of Zerumbone on Human Malignant Melanoma

Abstract approved: _____

Objective: This study aims to dissect the molecular mechanisms by which sesquiterpene zerumbone (ZER) exerts its inhibitory effects on human melanoma cells, and examine the potential therapeutic effect of ZER on melanoma development and metastasis in mouse xenograft model.

Methods: The inhibitory effect of ZER at physiological concentration on WM1552C cell was examined by MTT (methyl thiazolyl tetrazolium) assay. Subcutaneous inoculation and tail vein injection of melanoma cells in murine model were used for *in vivo* study of potential therapeutic effect of ZER. The roles of apoptosis, autophagy, and oxidative stress in the ZER-induced cell death of WM1552C cells were assessed using the according inhibitors. Autophagy was confirmed by MDC staining, and detecting autophagic marker LC3B. The expression of apoptosis, autophagy, and cancer- related genes were measured by real-time RT-PCR and Western Blot at mRNA and protein level. The expression profile of microRNA in ZER-treated WM1552C cells was analyzed by miRNA PCR array.

Results: ZER inhibited the proliferation of WM1552C in long term at physiological concentrations and induced apoptosis, autophagy, and oxidative stress in cells, which lead to cell death. Meanwhile, *in vivo* study showed that ZER significantly reduced the tumor

mass and lung metastasis ($p < 0.05$) in C57 BL/6 mice. In line with this finding, ZER was found to induce apoptosis and autophagy in melanoma cell lines. In addition, ZER inhibited the activation of Akt and MAPK and abolished NF-κB activation in does-dependant manners. In WM1552C cells treated with ZER, 6 human miRNAs were up-regulated and 13 miRNAs were down-regulated as detected with miRNA PCR array analysis.

Conclusion: ZER may exert potent anti-melanoma effects by inducing apoptosis, autophagy, and oxidative stress, and regulating cancer survival pathway and expression of miRNA *in vitro*. The *in vivo* study suggests that zerumbone can be a potential chemotherapeutic candidate for deterring growth and metastasis of melanoma.

In Vitro and *In Vivo* Studies of Pharmacological Effects of Zerumbone on Human Malignant Melanoma

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PREFACE

This thesis was prepared following the publication style of *Cancer Research*.

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Introduction

As one of the seven most common cancers in American men and women, cutaneous malignant melanoma, a prevalent skin cancer derived from melanocytes, is an extremely aggressive and often fatal disease. It currently becomes the leading cause of death from cutaneous malignancies and the incidence has increased steadily for several decades [1-3]. It is estimated that one out of 91 persons in United States will develop malignant melanoma in his or her life time [4-7]. From 2001 to 2012, the worldwide deaths caused by melanoma climbed by more than 20%, with a 1.9% increase each year [8, 9].

In spite of significant advances over the last three decades in my understanding of the carcinogenesis and metastasis of melanoma and the application of chemo- and immunotherapy, metastatic malignant melanoma is refractory to current therapies and has a very poor prognosis [5]. For example, chemotherapy of late-staged and metastasized melanoma with dacarbazine and other alkylating agents is often associated with severe toxicity, and does not provide significant benefits [10, 11]. Hence, there is a critical need to develop a more effective and less toxic therapeutic strategy targeting this aggressive cancer. At the same time, the therapeutic targets and pathways found in melanoma can provide a therapeutic clue that may be transformed into therapies for other types of cancers. Therefore, there is an urgent need to seek new strategies in melanoma prevention and treatment with minimal side effect.

In recent years, researchers have focused on several dietary phytochemicals that are capable to prevent carcinogenesis and inhibit the growth, invasiveness and metastasis of

melanoma cells [12-16]. Among them are sesquiterpenes, that have exhibited significant chemo-preventive, anti-oxidant, anti-inflammatory and anti-cancer properties in recent preclinical study [13, 17, 18].

Zerumbone [2,6,9,9-tetramethyl-(2E,6E,10E)-cycloundeca-2,6,10-trien-1-one] (ZER) belongs to a cyclic monosesquiterpene that was first isolated in 1956 from the essential oil of the rhizome of *Zingiber zerumbet* Smith, a subtropical wild shampoo ginger widely distributed throughout in Southeast Asia [19, 20]. In recent years, a variety of functions have been found in ZER, especially, anti-tumor activities targeting various cancer types with minimal side effect on several kinds of normal human cell [13, 21, 22]. For instance, ZER rarely influences the growth of normal human dermal (2F0-C25) and colon (CCD-18 Co) fibroblasts, however, it significantly inhibits the proliferation of various human colonic adenocarcinoma cell lines (LS174T, LS180, COLO205, and COLO320DM) in a dose-dependent manner via the induction of apoptosis [21, 23]. *In vivo,* ZER had chemopreventive and anti-cancer properties against azoxymethane-induced colon cancer in rats [24, 25], diethylnitrosamine- and 2-acetylaminofluorene-induced liver cancer in rats [26], azoxymethane-induced aberrant crypt formation in rats [27], cholecystokinin octapeptide induced acute pancreatitis in rats [28], myeloid tumors in mice [29], experimental skin cancer initiation and promotion in ICR mice [30], cervical intraepithelial neoplasiain in mice by inducing mitochondria-regulated apoptosis [31], and human breast cancer-induced bone loss in nude mice [32].

The mechanisms through which ZER may exert its anti-cancer properties fall into several categories. Firstly, ZER can influence the expression of genes, directly involved

in programmed cell death (PCD), a fundamental biological process required for removal of unnecessary, abnormal or damaged cells to maintain tissue homeostasis [33]. ZER prompted or induced apoptosis in human HCT116 colon cancer cells through up-regulation of apoptosis-inducing ligand death receptor DR4 and DR5 and down-regulation of cFLIP [34] and HepG2 liver cancer cells by up- and down-regulation of Bax/BCL-2 proteins [35]. Studies have shown that the demise of cancer cells not only can follow the well-characterized type I apoptotic pathway but also can proceed by stimulating of type II (macroautophagy-related) and type III (necrosis) programmed cell death (PCD) or combinations thereof [36-38]. During tumor development, autophagy has paradoxically been reported to have roles in promoting both cell survival and cell death [39]. Mechanistic studies have showed that ZER up-regulated expressions of pro-autophagic genes in Hepa1c1c7 cells [40].

Secondly, ZER has been shown to suppress some signaling pathways essential to the survival of cancer cells. Aberrant activations in signaling pathways emerged as promising therapeutic targets in melanoma including MAPK, Akt, NF-κB and IκBα [41-43]. Several studies already confirmed that inhibition of MAPK and PI3K/Akt pathways suppressed growth and invasion in melanoma cells [44, 45]. Weng et al. reported that ZER suppressed IKKα and Akt activation, resulting in apoptosis of GBM 8401 cells [46].

Thirdly, ZER was found to induce protective effects of oxidative damage on cancer cells. For example, ZER increased the ROS generation in a concentration-dependent manner in pancreatic carcinoma cells [47] and ZER induced TRAIL-induced apoptosis depending on reactive oxygen species [34].

MicroRNA (miRNA) are short non-coding RNAs of 18-25 nucleotides in length [48] that mediate post-transcriptional silencing either by the destabilization of the target mRNA [49-51] or by the repression of translation [52, 53]. They are involved in many biological processes, including proliferation, apoptosis, and differentiation in physical and pathogenic conditions [54-57]. In addition, they have direct influence on cancer development, progression and metastasis [58, 59]. Mueller and Caramuta et al. both reported the miRNA expression profiles associated with mutation, formation, progression and survival of malignant melanoma cell compared with normal human melanocytes [60, 61]. This study employed miRNA PCR array to analyze the expression profile of miRNA in ZER-treated melanoma cells.

As discussed previously, there have been several published research papers showing the anti-cancer activities of ZER, however, the study concerning the inhibitory effect of ZER on melanoma has not been conducted in melanoma cell culture or mouse model. Therefore, this study explored the *in vitro* anti-melanoma activities of ZER and characterized the *in vivo* therapeutic properties of ZER against malignant melanoma in a mouse model.

Materials and Methods

Mouse and cell line

This protocol of animal experiment has been approved by Institutional Animal Care and Use Committee (permit #ESU-ACUC-11-018). Fifty 4-6 week aged male C57 BL/6 mice (Harlan Laboratories, Indianapolis, Indiana) were housed in the Emporia State University (ESU) animal facility. Mice were fed with Harlan Teklad LM 485 rodent chow (Harlan Laboratories, Indianapois, Indiana) 10 grams per week and were allowed to have *ad libitum* access to tap water according to the guide for the care and use of laboratory animals [62].

WM1552C human melanoma cell line and B16-F0 mouse melanoma cell line obtained from the American Type Culture Collection (Manassas, Virginia) were cultured in complete medium (American Type Culture Collection, Manassas, Virginia) including MCDB 153 medium and Dulbecco's Modified Eagle Medium supplemented with 20 µl/ml and 100 µl/ml FBS (fetal bovine serum) (Hyclone, Logan, Utah) and 2 mM L-glutamine at 37 ℃ in 5% CO2 incubator (Thermo Fisher Scientific Inc., Waltham, Massachusetts) with humidity, according to the ATCC instruction. Powder of ZER was purchased from Kingherbs Inc. (Wuhan, China) and dissolved in dimethyl sulfoxide (DMSO) (Fisher scientific Inc., Fair Lawn, New Jersey) to make the stock concentration of 10^4 µM or 200 mg/ml and was kept at -20 ℃.

Cell viability assay

WM1552C cells (5×10^3/ well) were seeded in 96-well plates and incubated for 12 hours to allow cells to attach. To test the long time treatment effect of ZER, the experimental wells were treated with 10^4 μM ZER dissolved in DMSO at the final concentration of 1 μM, 2 μM, and 5 μM ZER diluted with medium. The control cells were treated with drug vehicle (DMSO) at the same dilution as the 5 μM ZER treatment for 96 hours.

To test the effects of caspase inhibitors on ZER-induced apoptosis of WM1552C cells, the ZER experimental and control wells were pretreated with Z-VAD-FMK (pan-caspase inhibitor) (R&D Systems Inc., Minneapolis, Minnesota) or Z-DEVD-FMK (caspse-3 inhibitor) (R&D Systems Inc., Minneapolis, Minnesota) at the concentration of 10 μM or 20 μM or the drug vehicle DMSO at the same dilution for 2 hours before ZER (25 μM) or DMSO treatment for 36 hours.

To test the effects of inhibitors on ZER-induced autophagy of WM1552C cells, the experimental and control wells were pretreated with 3-MA (3-Methyladenine) (Sigma-Aldrich Co., Louis, Missouri) at 25 μM, 40 μM, 50 μM or the same dilution of DMSO for 2 hour before the cells were treated by ZER (25 μM) or DMSO treatment of same dilution for 36 hours. The ZER experimental and control wells were treated with bafilomycin A1 (BA1) (Sigma-Aldrich Co., Louis, Missouri) in 10 nM, 50 nM or with the same dilution of PBS (phosphate buffered saline) (Sigma-Aldrich Co., Louis, Missouri) for 12 hours after ZER (25 μM) or the same dilution of DMSO treatment for 36 hours.

To test the effects of NAC (N-acetyl-L-cystein) (Sigma-Aldrich Co., Louis, Missouri) and GSH (L-glutathione) (Sigma-Aldrich Co., Louis, Missouri) on ZER-induced oxidative stress of WM1552C cells, the ZER experimental and control wells were pretreated with NAC or GSH in 1 mM or 5 mM respectively, or distilled water for 2 hours before ZER (25 μM) or the same dilution of DMSO treatment for 36 hours.

After proper treatment, TACS® MTT Cell Proliferation Assays (Trevigen Inc., Gaithersburg, Maryland) was performed to determine the survival percentage of the experimental groups relative to the control according to the instruction. The MTT assays were read at the wavelength of 570 nm on a micro-plate reader (Bio-rad Laboratory, Hercules, California) to determine the cell viability and compare to the control group or ZER alone within the treated groups.

The inhibitory effect of ZER on development of tumor size

For the subcutaneous primary tumor model, male C57BL/6 mice, 4-6 weeks of age, were randomized into three groups and each group had 8 mice fed in two cages. The mice were taken care and habituated for several days. I performed subcutaneous inoculation with 1×10^6 B16-F0 mouse melanoma cells in 100 μl ice-cold HBSS (Hank's balance solution) (Sigma-Aldrich Co., Louis, Missouri) of each mouse to establish the mouse subcutaneous melanoma model according to Willem and Nicholas' work [63]. To examine the inhibitory effect of ZER against the newly implanted tumor, I injected daily each mouse in one experimental group (experimental group 1) with 50 μl of ZER solution 1 (ZER 20 μg/g in DMSO), each mouse in a second experimental group

(experimental group 2) with 50 µl of ZER solution 2 (ZER 40 µg/g in DMSO), and each mouse in a control group (Control group 3) with 50 µl of vehicle (DMSO).

Treatments were delivered intraperitoneally by bolus injection beginning from 7 days before tumor implantation. When the tumor size of mice in the control group had exceeded 4 cm^2, usage of ZER were ended. Animals were sacrificed with CO_2 gas in a cage. The tumor of each mouse was removed from the normal tissue. The growth of the tumor in each animal was determined by measuring the size of the tumor. Tumor sizes were calculated by the formula: $\pi/6\ [w_1 \times (w_2)^2]$, (where w_1 represents the largest tumor diameter and w_2 represents the smallest tumor diameter).

The inhibitory effect of ZER on pulmonary metastasis of B16-F0 mouse melanoma cells

In the pulmonary metastasis experimental model, male C57BL/6 mice, 4-6 weeks of age, were randomized into three groups and each group had 8 mice housed in two cages. I inoculated 2.5×10^5 B16-F0 mouse melanoma cells in 0.5 ml ice-cold HBSS by intravenous injection into the tail vein.

I injected each mouse daily in one experimental group (experimental group 4) with 50 µl of ZER solution 1, each mouse in a second experimental group (experimental group 5) with 50 µl of ZER solution 2, and each mouse in a control group (control group 6) with 50 µl of vehicle (DMSO). Treatments were delivered intraperitoneally by bolus injection beginning from 7 days before tumor implantation. ZER treatment ended after 19 days and animals were sacrificed with CO_2 gas in a cage. The lung of each mouse was removed from the mice. The melanoma tumor nodules on each lung of each mouse were

counted. Furthermore, all lungs in each group were fixed overnight in 10% paraformaldehyde (Fisher scientific Inc., Fair Lawn, New Jersey) immediately and the fixed tissues were sent to the Mass Histology Service (Worcester, Massachusetts) to complete the hematoxylin and eosin (HE) staining for microscopic examination with Olympus Research System microscope BX51 (Olympus America Inc., Melville, New York). Pictures were captured with a camera **(**Diagnostic Instruments Inc., Sterling Heights, Michigan) and imported into Adobe Photoshop.

WM1552C cell culture conditions

WM1552C cells were seeded at 1 × 106 cells per T-25 cell culture flask (Corning, Corning, New York) or 3 × 106 cells per T-75 cell culture flask with 4 ml or 15 ml complete medium supplemented with 20 µl/ml fetal bovine serum. After incubation for 18 hours at 37 ℃ in 5% CO2 incubator with humidity, cells can be treated in different ways according to different assay.

MDC (Monodansylcadaverine) staining

MDC staining can be used to detect the mature autophagic vacuoles under fluorescence microscope [64]. Five flasks of cells were treated with ZER at the final concentration of 25 µM in complete cell medium and three flasks of cells were treated with 0.5% DMSO in complete cell medium for 48 hours in CO2 incubator. I incubated the experimental cells and control cells with 0.05 mM MDC (Sigma-Aldrich Co., Louis, Missouri) in medium without FBS at 37 ℃ in 5% CO2 incubator for 10 minutes. After incubation, cells were washed 3 times with PBS and immediately analyzed with Olympus

Research System microscope BX51 with DAPI filter set. Picture were captured with a camera and imported into Adobe Photoshop.

RNA and MicroRNA (miRNA) isolation

Five flasks of cells were treated with ZER at the final concentration of 25 μM in complete cell medium and three flasks of cells were treated with 0.5% DMSO in complete cell medium for 48 hours in CO2 incubator. I extracted total RNAs using a PureLinkTM RNA Mini Kit (Ambion Inc., Carlsbad, California) according to manufacturer's instructions. Small RNAs ranging between 15-30 nucleotides in length were extracted using a mirVanaTM miRNA Isolation Kit (Ambion Inc., Carlsbad, California) according to manufacturer's instructions. I used a NanoDrop 2000c spectrophotometer (Thermo Scientific Inc., Wilmington, Delaware) to determine the RNA concentrations and purity according to manufacturer's instructions. The isolated RNAs were stored at -80℃ in ultra cold freezer (Thermo Electron Co., Asheville, North Carolina).

One-step quantitative RT- PCR (qRT-PCR)

The total RNAs were used in one-step qRT-PCR experiments to determine the mRNA levels of significantly altered expression of genes including BECN-1, LC3A, LC3B, Atg9B, Atg12, ULK1, PIK3C3, Caspase3, Caspase9, Caspase8, EZH2, PTEN and MACC1. Primers (Table 1) (Integrated DNA Technologies Inc., Coralville, Iowa) were retrieved from PrimerBank established and maintained by Harvard Medical School. These experiments were performed on an Eppendorf Mastercycler® RealPlex2 Thermal Cycler (Eppendorf , Hauppauge, New York) using GoTaq® 1 Step RT-qPCR System

(Promega Co., Madison, Wisconsin) according to the manufacturer's instruction [65]. All the qRT-PCR experiments were performed in triplicates. All the data were analyzed in RT^2profiler^{TM} PCR Arrays software (SABiosciences, Frederick, Maryland) to show the significantly altered expression of genes affected by ZER treatment in WM1552C melanoma cells, according to the guidelines of the software.

miRNA PCR Array

The small RNA samples from both control (DMSO) and ZER treatment group were reverse-transcribed into cDNAs using miScrip II RT Kit (Qiagen, Valencia, California). miRNA PCR arrays were performed using miScript SYBR Green PCR Kit (Qiagen, Valencia, California) and miRNome miScript miRNA PCR Array in 96-well format (Qiagen, Valencia, California) on an Eppendorf Mastercycler® RealPlex2 Thermal Cycler according to the manufacturer's instructions. All the data were analyzed in RT^2 profiler^{TM} PCR Arrays software (SABiosciences, Frederick, Maryland) to show the miRNA expression profile affected by ZER treatment in WM1552C melanoma cells, according to the guidelines of the software.

Two-step qRT-PCR for miRNA

The small RNA samples from both control (DMSO) and ZER treatment group were reverse-transcribed into cDNAs using miRCURY LNA^{TM} Universal RT microRNA PCR Universal cDNA Synthesis kit (EXIQON, Woburn, Massachusetts). miRNA two-step qRT-PCR were performed using miRCURY LNA^{TM} Universal RT microRNA PCR SYBR® Green master mix (Universal RT, 2,5ml) (EXIQON, Woburn, Massachusetts) on an Eppendorf Realplex 2 Thermal Cycler according to the manufacturer's instructions.

Data were analyzed in RT^2 profiler™ PCR Arrays software to measure the miRNA expression of hsa-miR-16 (ID 204409) and hsa-miR-26a (ID 204724) with U6snRNA (ID 203907) as an internal control. The primers were purchased from EXIQON.

Western-blot analysis

For whole cell protein extraction, three T-75 flasks of cells were treated with ZER at the final concentration of 50 μM, two flasks of cells were treated with 25 μM ZER, one flask of cells was treated with 5 μM ZER, and one flask of cells was treated with 0.5% DMSO as a control group in complete cell medium for 48 hours in CO_2 incubator. The whole protein extractions from treated cell groups were performed according to the previous study [66].

To determine the levels of NF-κB (nuclear factor-κB) expression in the nucleus, three T-75 flasks of cells were treated with ZER at the final concentration of 50 μM in complete medium (CM) (without FBS) for 12 hours and then treated with 10 ng/ml TNF-α (tumor necrosis factor alpha) (Sigma-Aldrich Co., Louis, Missouri) in CM for additional 45 minutes, three flasks of cells were treated with ZER (50 μM) in CM for 12 hours, two flasks of cells were treated with 0.5% DMSO in CM for 12 hours and then treated with 10 ng/ml TNF-α in CM for additional 45 minutes, and two flasks of cells were treated with 0.5% DMSO in CM for 12 hours. After the treatment, nuclear fractions were prepared according to the protocol involved quick fractionation of cytoplasmic and nuclear proteins from GeneTex Inc..

The concentration of each sample was determined by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware), and then I

adjusted the experimental sample concentration to be equal to the control sample. The protein samples were diluted by 2×Laemmli buffer solution (Bio-rad Laboratory, Hercules, California) and then separated by 12% Mini-PROTEAN® TGXTM Precast Gels (Bio-rad Laboratory, Hercules, California) according to the quick start guide in Mini-PROTEAN® 3 cell (Bio-rad Laboratory, Hercules, California). I applied electrical field using Fisher Scientific FB3000 electrophoresis power supply (Fisher Scientific Inc., Hayward, California).

After the electrophoresis, the proteins bands in gels were transferred to Protran BA85/3MM nitrocellulose membranes (Whatman Inc., Sanford, Maine) in PAGEgel Dual Run & Blot Vertical Mini- Gel System (PAGEgel Inc., San Diego, California) according to the instruction manual. After transfer, the membranes were agitated in TBS (Tris-buffered saline) (Bio-rad Laboratory, Hercules, California) with 0.1% Tween 20 (TBST), and then incubated in 5% fat free milk (blocking buffer) for 2 hours in room temperature. After that, the membranes were incubated with required primary rabbit anti-human antibodies (Table 2) for 18 hours in 4℃. After incubation and washing with TBST, Anti-rabbit IgG, HRP (horseradish peroxidase)-linked Antibody (Cell Signaling Technology Inc., Danvers, Massachusetts) in a 1:1000 dilution was used for 2 hours incubation in room temperature.

The protein bands were detected using Luminata^{TM} Crescendo Western HRP Substrate (Millipore Co., Billerica, Massachusetts) on a dish in Kodak GEL Logic 2200 Imaging System (Eastman Kodak Co., Rochester, New York) and the signals were captured and analyzed by Kodak software (Eastman Kodak Co., Rochester, New York) according to the manual.

Statistical analysis

Data were analyzed by the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, Illinois). A t-test at $\alpha = 0.05$ was applied to examine differences of the tumor size or metastasis tumor number between experiment groups and control groups. All values were expressed as mean ± standard deviation (SD). P-values were considered significant at ≤ 0.05. Up-regulated or down-regulated gene expression levels were calculated by RT^2profiler^{TM} PCR Arrays software. Data represented the average change (*n*-fold) ± SD determined from three independent experiments.

Table 1: Primer sequences of the genes studied in the qRT-PCR assay

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
CASP3 (8G10)	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA
CASP8	TGTCCAGTTGTTCCCCAATA	GGTCACTTGAACCTTGGGAA
CASP9	CTGCATTTCCCCTCAAACTC	AGGTTCTCAGACCGGAAACA
PIK3C3	CCTGGAAGACCCAATGTTGAAG	CGGGACCATACACATCCCAT
MAP1LC3A	AACATGAGCGAGTTGGTCAAG	GCTCGTAGATGTCCGCGAT
MAP1LC3B	AAGGCGCTTACAGCTCAATG	CTGGGAGGCATAGACCATGT
ULK1	AGCACGATTTGGAGGTCGC	GCCACGATGTTTTCATGTTTCA
ATG9B	TGTGCTCACCGTCTACGAC	GGGAGGTAGTGCATGTGGG
ATG12	TAGAGCGAACACGAACCATCC	CACTGCCAAAACACTCATAGAGA
EZH2	GGACCACAGTGTTACCAGCAT	GTGGGGTCTTTATCCGCTCAG
PTEN	AGGGACGAACTGGTGTAATGA	CTGGTCCTTACTTCCCCATAGAA
MACC1	CGCTCCTGCCTTGATTTGAAT	ACCCTCCTTGATGGTTTACTTTG
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

Table 2: Dilutions of the primary antibody used in Western-blot assay

Target protein	Dilution	Source
Caspase-3 (8G10)	1:250	Cell Signaling Technology Inc., Danvers, Massachusetts
LC3B (light chain 3B)	1:500	Cell Signaling Technology Inc., Danvers, Massachusetts
PARP (poly ADP ribose polymerase)	1:100	Thermo Fisher Scientific Inc., Wilmington, Delaware
NF-κB p65	1:500	Thermo Fisher Scientific Inc., Wilmington, Delaware
Akt (pan)	1:1000	Thermo Fisher Scientific Inc., Wilmington, Delaware
Phospho-Akt (Ser473)	1:1000	Cell Signaling Technology Inc., Danvers, Massachusetts
p44/42 MAPK (Erk1/2)	1:1000	Thermo Fisher Scientific Inc., Wilmington, Delaware
Phospho- p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	1:1000	Cell Signaling Technology Inc., Danvers, Massachusetts
GAPDH	1:1000	Cell Signaling Technology Inc., Danvers, Massachusetts
β-actin	1:1000	Sigma-Aldrich Co., Louis, Missouri

Results

Effect of ZER in melanoma tumorgenesis, and melanoma metastasis in vitro and in vivo

The long term inhibitory effect of ZER at physiological concentrations was examined by MTT assay in WM1552C cells. As shown in Figure 1, exposure of WM1552C cells to ZER for 96 hours at physiological concentrations ranging from 0 to 5 μM caused significant reduction of proliferation in a dose-dependent manner.

To evaluate the inhibitory effect of ZER on melanoma tumor development, B16-F0 cells were inoculated subcutaneously into the hind flanks of male C57BL/6 mice. Either drug vehicle (DMSO; n = 8) or ZER (20 μg/g or 40 μg/g; n = 8) was injected into each mice groups via intraperitoneal injection every 2 days beginning at 7 days before tumor implantation. After treatment, the tumors were dissected from the skin of mice. As shown in Figure 2, ZER significantly inhibited melanoma growth (tumor formation) in C57BL/6 mice (Figure 2).

To further confirm the hypothesis that ZER can inhibit the lung metastasis, B16-F0 cells were injected into the tail veins of male C57BL/6 mice, and either vehicle (DMSO; n = 8) or ZER (20 μg/g or 40 μg/g; n = 8) was injected into each mice groups via intraperitoneal injections every 2 days beginning at 7 days before implantation. Metastatic nodules in each group were determined as described in Materials and Methods. As indicated in Table 3, B16-F0 cell-injected mice displayed 100% incidence of lung metastasis. ZER notably reduced the incidence of metastasis to liver and kidney

to zero at the dose of 40 μg/g. In addition, ZER significantly reduced the number of metastatic nodules growing in the lung (Figure 3A, B), suggesting that it exerted the inhibitory effect on lung metastasis of melanoma cells. HE stain of metastatic melanoma nodules in the lung confirmed the inhibitory effect of ZER on melanoma metastasis at histopathological level (Figure 3C).

Figure 1: Long term effect of ZER at low concentration on proliferation of WM1552C melanoma cells.

Cells were incubated in the presence of ZER at the physiological low concentrations for 96 hours, and the survival percentages relative to control group were measured by MTT as described in Material and Methods. The experiment data was reported as mean ± SD for all concentrations columns. Asterisks indicate a significant differences between experimental groups and control group ($p < 0.05$) (n = 9).

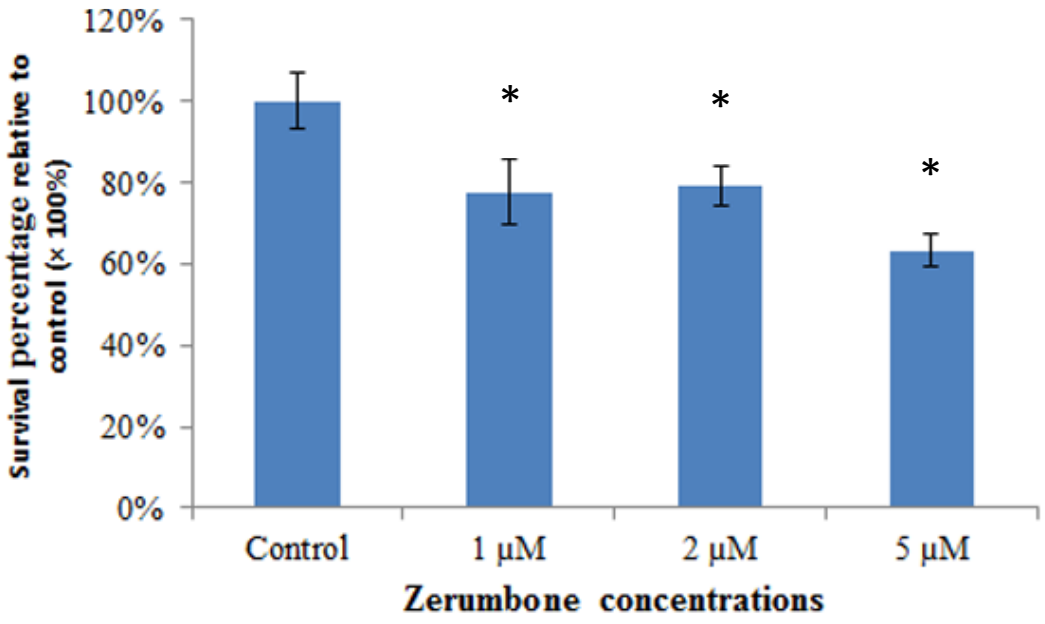

120%
100%
80%
60%
40%
20%
0%
Survival percentage relative to control (× 100%)
*
*
*
Control
1 μM
2 μM
5 μM
Zerumbone concentrations

Figure 2: ZER inhibited melanoma tumor growth in a xenograft mouse model.

C57BL/6 mice implanted with B16-F0 xenografts were administered with either vehicle (DMSO) or ZER via intraperitoneal injections every 2 days as described in Materials and Methods. Representative photography of tumor formation in each group is shown (A). Tumor sizes from animals treated with ZER and those treated with vehicle groups were compared (n = 8, mean ± SD; * $p < 0.05$) (B).

A

a. Control (B16 + DMSO) **b. B16 + 20 µg/g ZER** **c. B16 + 40 µg/g ZER**

B

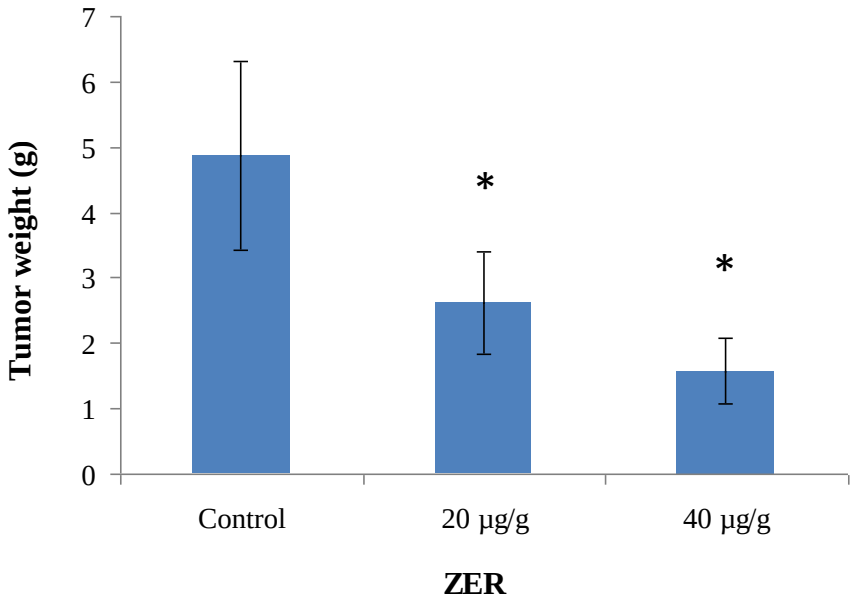
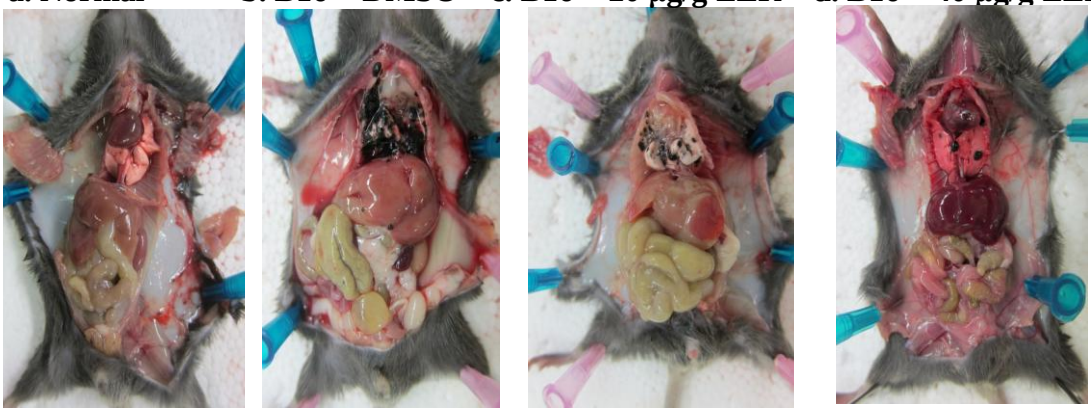


Figure 3: ZER suppressed melanoma metastasis in lung.

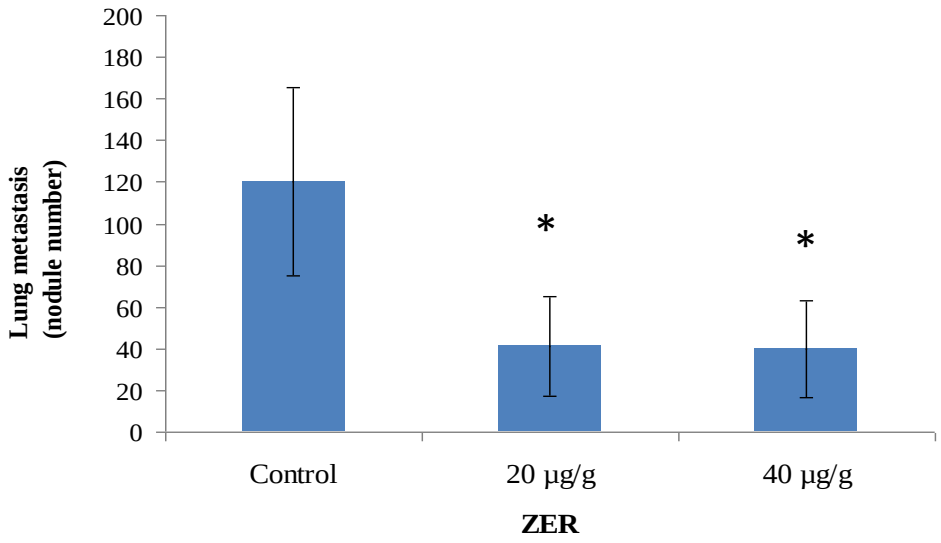
C57BL/6 mice implanted with B16-F0 cells from tail vein were administered with either vehicle (DMSO) or ZER via intraperitoneal injections every 2 days as described in materials and methods. Representative photography of lung and liver metastasis in each group is shown (A). Comparison of number of metastatic nodules from animals treated with ZER with those treated with vehicle groups (n = 8, mean ± SD; * $p < 0.05$) (B). HE staining of lung tissues from each group was visualized by photography (C). Original magnification, ×100.

A

a. Normal **b. B16 + DMSO** **c. B16 + 20 μg/g ZER** **d. B16 + 40 μg/g ZER**



B



C

a. Control (B16 + DMSO) **b. B16 + 20 μg/g ZER** **c. B16 + 40 μg/g ZER**

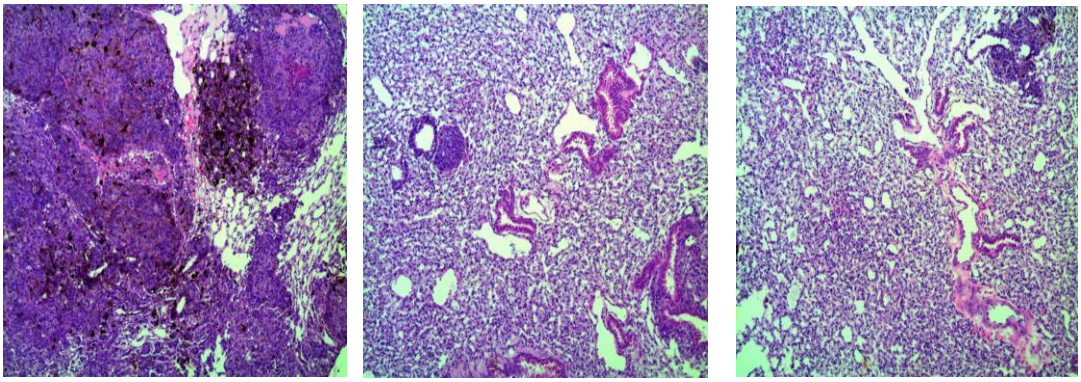


Table 3: Incidence of lung, liver, and kidney metastasis in C57BL/6 mice were inhibited by ZER administration.

	Mice No.	incidence of metastasis		
		lung	liver	kidney
Normal	8	0/8	0/8	0/8
ZER[a]	8	0/8	0/8	0/8
B16 + vehicle	10	10/10	5/10	3/10
B16 + 20μg/g ZER	10	10/10	2/10	2/10
B16 + 40 μg/gZER	9	9/9	0/9	0/9

[a] ZER, zerumbone

ZER induced apoptosis in WM1552C cells

As shown previously, ZER long term treatment exhibited an inhibitory effect on melanoma cells. Previous research in my lab showed that ZER induced condensed and fragmented nuclei under Hoechst stain, suggesting the induction of apoptosis. Thus, to further confirm an apoptotic mechanism contributed to ZER-induced death of WM1552C cells, the effects of pretreatment with the pan-caspase inhibitor (Z-VAD-FMK) or the caspase 3 inhibitor (Z-DEVD-FMK) were examined. WM1552C cells were pretreated with Z-VAD-FMK or Z-DEVD-FMK 2 hours before ZER treatment (25 μM). After 36 hours incubation, cell viabilities were determined with MTT assay. As shown in Figure 4, Z-VAD-FMK significantly inhibited the cytotoxicity induced by ZER treatment (Figure 4A), but caspase 3 inhibitor Z-DEVD-FMK did not significantly alleviated the cytotoxicity of ZER (Figure 4B).

To analyze the altered expression of apoptosis related genes in WM1552C melanoma cells exposed to ZER (25 μM), mRNAs of genes CASP3, CASP8, and CASP9, which are critical genes involved in apoptosis induction, were determined by qRT-PCR in three independent experiments. As shown in Figure 5, the expression of CASP9 were significantly up-regulated (with more than two-fold alternation), but the other two genes did not change significantly after ZER treatment.

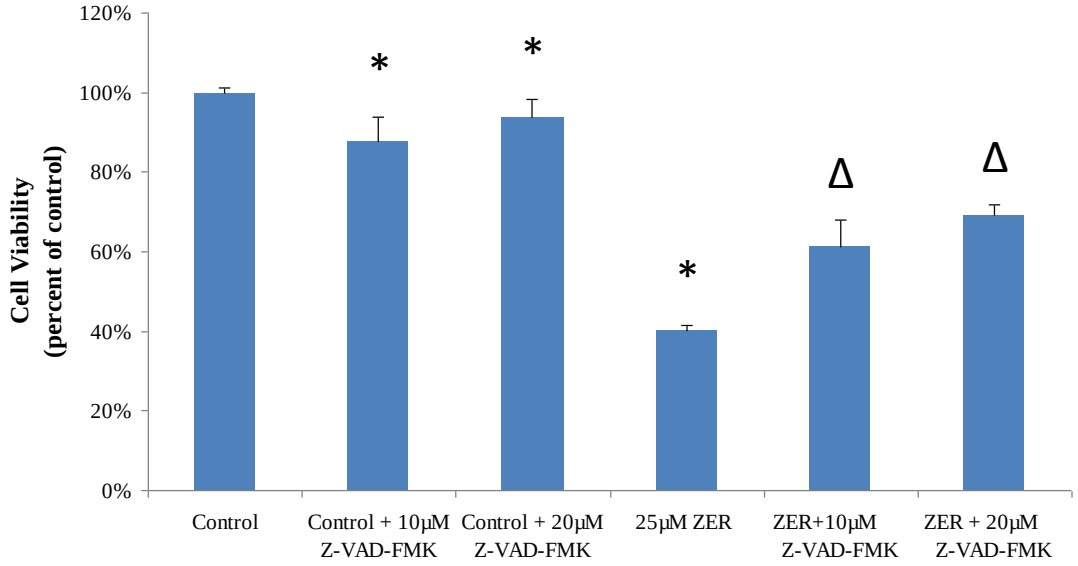
Since caspase 3 inhibitor did not rescue cells from the cytotoxicity of zerumbone, and caspase 3 plays central roles in both extrinsic (death receptor) and intrinsic (mitochondrial) pathways, the dependency of zerumbone-induced apoptosis on caspase 3 deserves more scrutiny. The proteins levels of PARP and caspase 3 in WM1552C

melanoma cells exposed to ZER (0, 5, 15, or 25 μM) for 48 hours were examined by Western-blot analysis. It revealed that the level of PARP and cleaved PARP proteins were markedly increased in ZER treatment group compared to the control group (Figure 6A), indicating the induction of apoptosis in the cells treated with ZER. However, the expression of caspase 3 at protein level did not show significant difference between experimental and control groups, and the cleaved caspase 3 was even reduced by ZER relative to the control group, as shown in Figure 6B. These results indicated that the cytotoxicity of ZER was at least partially attributed to apoptosis, and the apoptosis was induced by ZER in a caspase 3-independent pathway.

Figure 4: Effect of pan-caspase or caspase 3 inhibitor on ZER-treated WM1552C cells.

Effect of the executers of apoptosis on ZER-treated WM1552C melanoma cells was examined in a co-culture with caspase inhibitors. Cells were pretreated with Z-VAD-FMK (A) or Z-DEVD-FMK (B) (10 or 20 μM) 2 hours before 25 μM ZER treatment for additional 36 hours. Cell viabilities relative to control group were analyzed by MTT assay as described in Materials and Methods. The experiment data was reported as mean ± SD for all columns. Asterisks indicate a significant difference between experimental groups and control group ($p < 0.05$) (n = 9), Δ shows a significant difference between the ZER-treated groups and ZER alone group ($p < 0.05$) (n = 9).

A


Cell Viability
(percent of control)
120%
100%
80%
60%
40%
20%
0%
*
*
*
Δ
Δ
Control
Control + 10μM Z-VAD-FMK
Control + 20μM Z-VAD-FMK
25μM ZER
ZER+10μM Z-VAD-FMK
ZER + 20μM Z-VAD-FMK

B

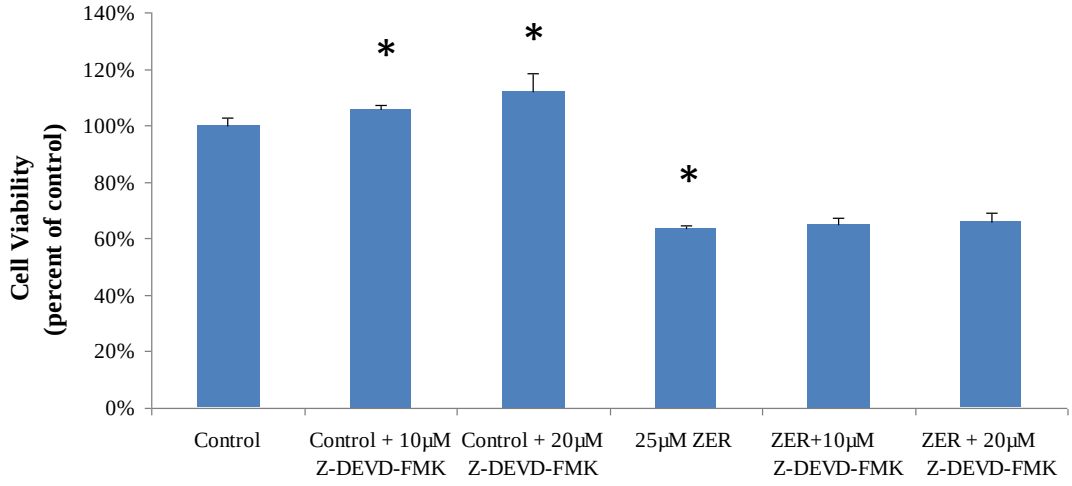

Cell Viability
(percent of control)
140%
120%
100%
80%
60%
40%
20%
0%
*
*
*
Control
Control + 10μM Z-DEVD-FMK
Control + 20μM Z-DEVD-FMK
25μM ZER
ZER+10μM Z-DEVD-FMK
ZER + 20μM Z-DEVD-FMK

Figure 5: Expression of apoptosis related genes in WM1552C cells exposed to ZER treatment using qRT-PCR.

WM1552C cells were treated with vehicle DMSO or 25 μM ZER for 36 hours. Then, the total RNAs were isolated and the qRT-PCR analysis was performed as described in Materials and Methods. The logarithm values of relative ratio of mRNA level were expressed as mean ± SD of three independent experiments relative to vehicle control. Expression levels were standardized by the HPRT1 mRNA level.

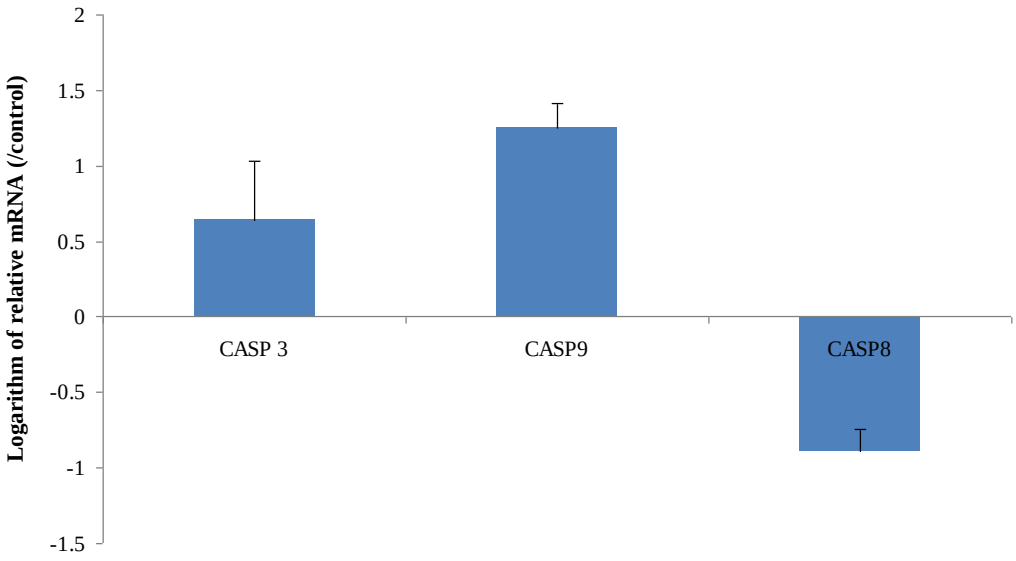
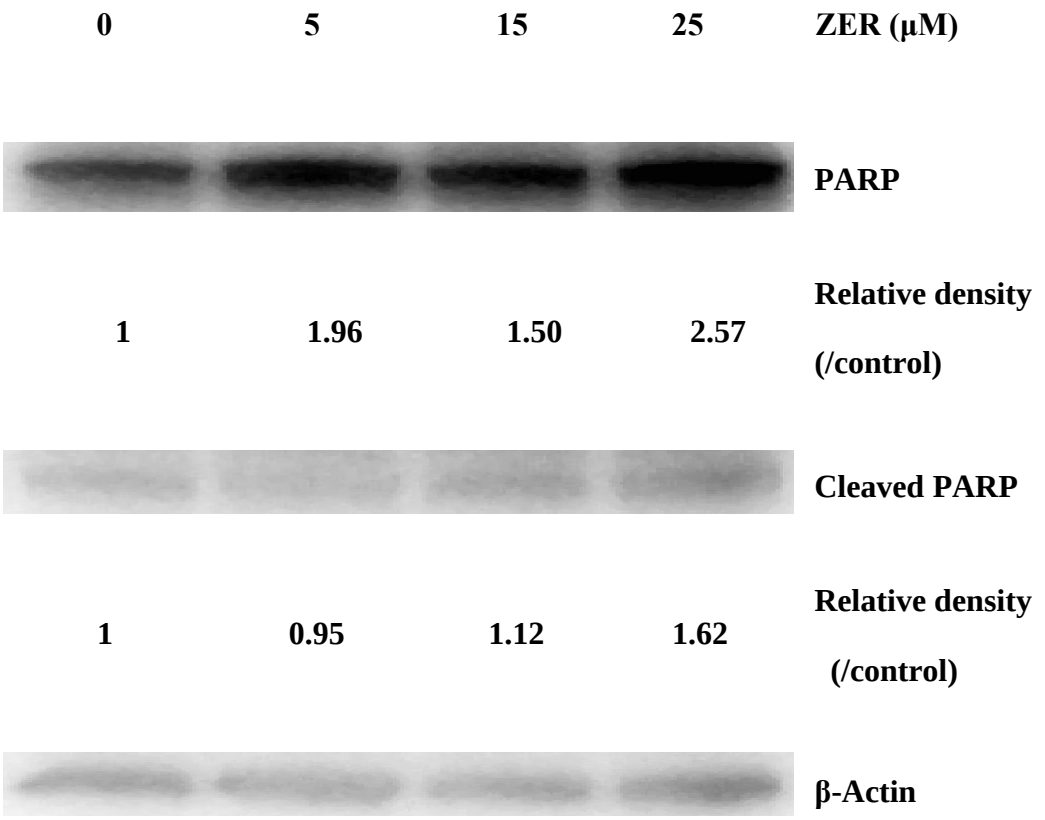

Logarithm of relative mRNA (/control)
2
1.5
1
0.5
0
-0.5
-1
-1.5
CASP 3
CASP9
CASP8

Figure 6: Expression of apoptosis related proteins in WM1552C cells exposed to ZER treatment using Western-blot.

Western-blot analysis of PARP (A) and caspase 3 (B) expression in WM1552C cells treated with ZER (0, 5, 15, or 25 μM) for 48 hours were performed as described in Materials and Methods for three independent times, and the three independent experiments results showed a similar tendency. The data were quantified by densitometric analysis and the representative data is shown. Expression levels were standardized by the β-actin as loading control.

A


0
5
15
25
ZER (μM)
PARP
1
1.96
1.50
2.57
Relative density (/control)
Cleaved PARP
1
0.95
1.12
1.62
Relative density (/control)
β-Actin

B

0	5	15	25	ZER (μM)
				**Caspase 3**
1	**0.95**	**0.89**	**0.91**	**Relative density (/control)**
				**Cleaved caspase 3**
1	**--**	**0.33**	**0.21**	**Relative density (/control)**
				**β-Actin**

ZER induced autophagy in WM1552C cells

As ZER triggered apoptosis in WM1552C cells, I examined if autophagy, another type of programmed cell death, was also induced by ZER. As shown in Figure 7, ZER increased the number of MDC-labeled vesicles and overall MDC fluorescence intensity in WM1552C cells in a dose-dependent manner.

To examine the effect of pharmacological inhibition at different steps on ZER-induced cytotoxicity, two autophagy inhibitors 3-MA or BA1 were used to inhibit the autophagy. WM1552C cells were pretreated with 3-MA (25, 40, or 50 μM) for 2 hours before the cells were treated with 25 μM ZER for 36 hours, or treated with BA1 (10 or 50 nM) for additional 12 hours after ZER treatment. Cell viabilities were determined with MTT assay. As shown in Figure 8, 3-MA significantly inhibited the cytotoxicity induced by ZER treatment (Figure 8A), but BA1 did not significantly affected the cytotoxicity of ZER (Figure 8B).

To confirm the induction of autophagy, the mRNA level of related genes at different steps of autophagy in WM1552C melanoma cells exposed to ZER (25 μM) were analyzed by qRT-PCR. The expression of LC3A, LC3B, ATG9B, ATG12, ULK1 and PIK3C3 genes were all up-regulated by ZER, especially for LC3B and ULK1, each with more than two-fold alternation (Figure 9).

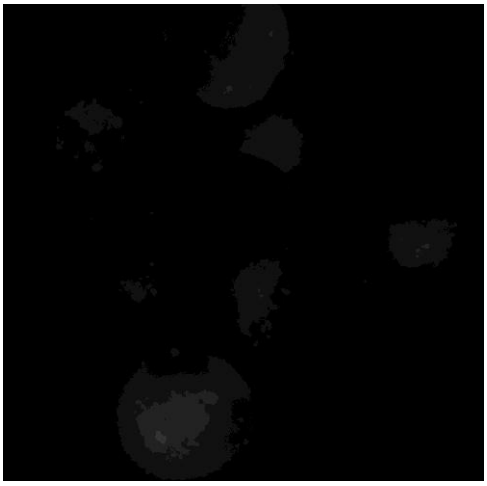
To validate the up-regulation of LC3B at protein level in ZER-treated WM1552C melanoma cells (0, 5, 15, or 25 μM for 48 hours), the effect of ZER on LC3B level was assessed by Western-blot analysis. It evidently showed that LC3B protein level was significantly up-regulated by ZER (Figure 10). These results indicated that ZER induced

autophagy, which may contribute to the cytotoxicity of zerumbone against melanoma cell.

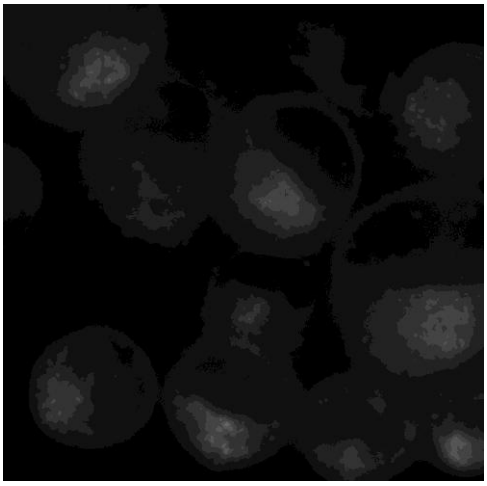
Figure 7: MDC-labeled autophagic vacuoles were induced after ZER treatment.

WM1552C cells were treated with drug vehicle alone (A) or 30 μM (B) or 50 μM (C) of ZER for 48 hours, and then were stained with MDC (50 μM) for 10 minutes. The MDC-labeled vacuoles were observed under an inverted fluorescent microscope (400 × magnification). The figure shows representative images from three independent experiments.

A Control



B 30 µM ZER



C 50 µM ZER

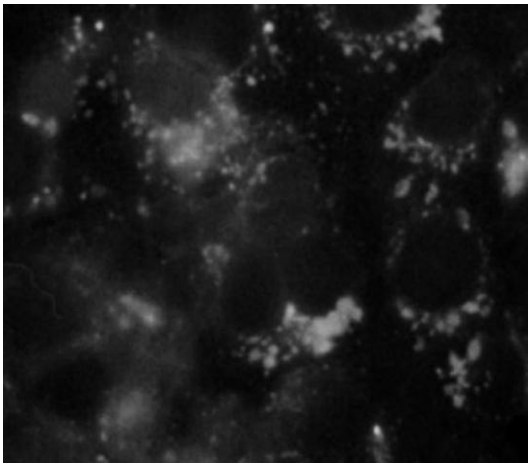
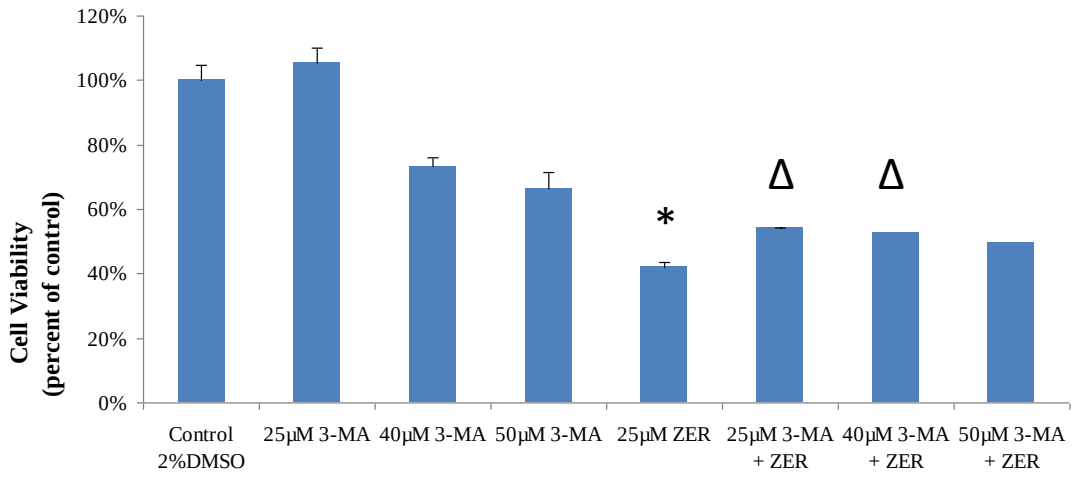


Figure 8: Effects of inhibitors of autophagy at different steps on ZER-induced cytotoxicity of WM1552C cells.

WM1552C cells were pretreated with various doses of 3-MA for 2 hours before ZER (25 μM) treatment for 36 hours (A) or treated with 10 nM or 50 nM BA1 for additional 12 hours after ZER treatment for 36 hours (B). Cell viabilities relative to control group were analyzed by MTT assay as described in Materials and Methods. The experiment data were reported as mean ± SD for all columns. Asterisks indicate a significant difference between experimental groups and control group ($p < 0.05$) (n = 9), Δ shows a significant difference between the ZER-treated groups and ZER alone group ($p < 0.05$) (n = 9).

A


Cell Viability (percent of control)
120%
100%
80%
60%
40%
20%
0%
*
Δ
Δ
Control 2%DMSO
25μM 3-MA
40μM 3-MA
50μM 3-MA
25μM ZER
25μM 3-MA + ZER
40μM 3-MA + ZER
50μM 3-MA + ZER

B

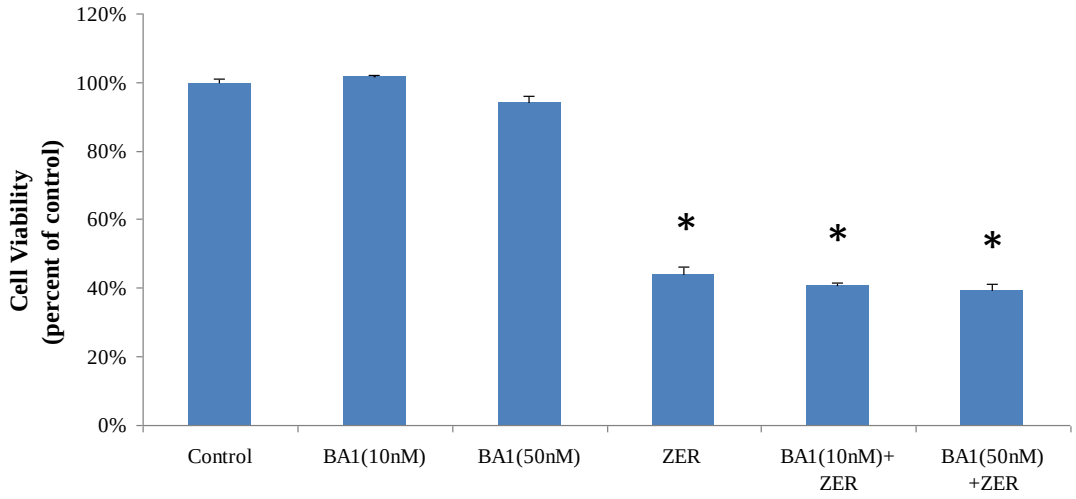

Cell Viability (percent of control)
120%
100%
80%
60%
40%
20%
0%
*
*
*
Control
BA1(10nM)
BA1(50nM)
ZER
BA1(10nM)+ ZER
BA1(50nM) +ZER

Figure 9: Autophagy related gene expression at mRNA level in WM1552C cells exposed to ZER treatment using qRT-PCR.

WM1552C cells were treated with vehicle DMSO or 25 μM ZER for 36 hours. Then, the total RNAs were isolated and the qRT-PCR analysis was performed as described in Materials and Methods in three independent experiments. The logarithm values of relative ratio of mRNA level were expressed as mean ± SD of three independent experiments relative to vehicle control. Expression levels were standardized by the HPRT1 mRNA level.

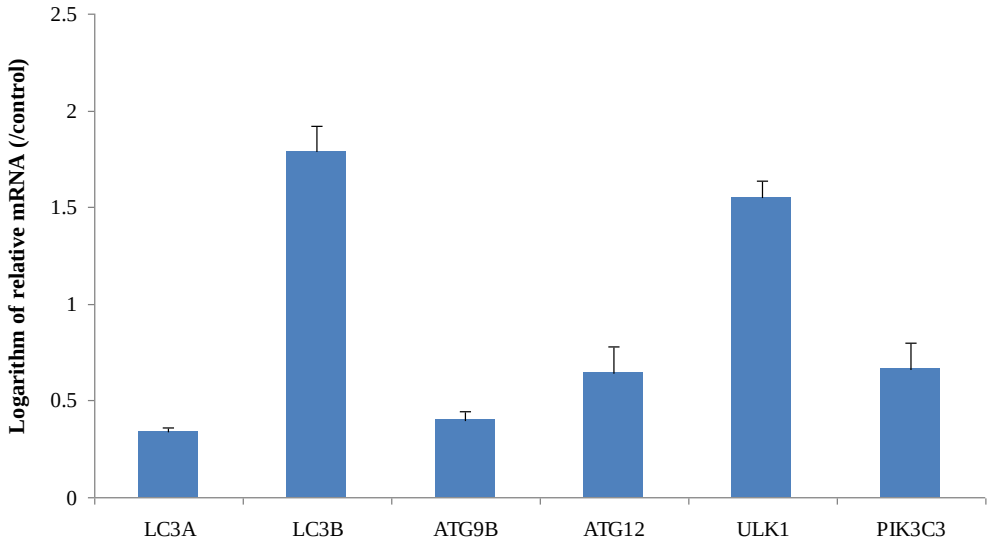
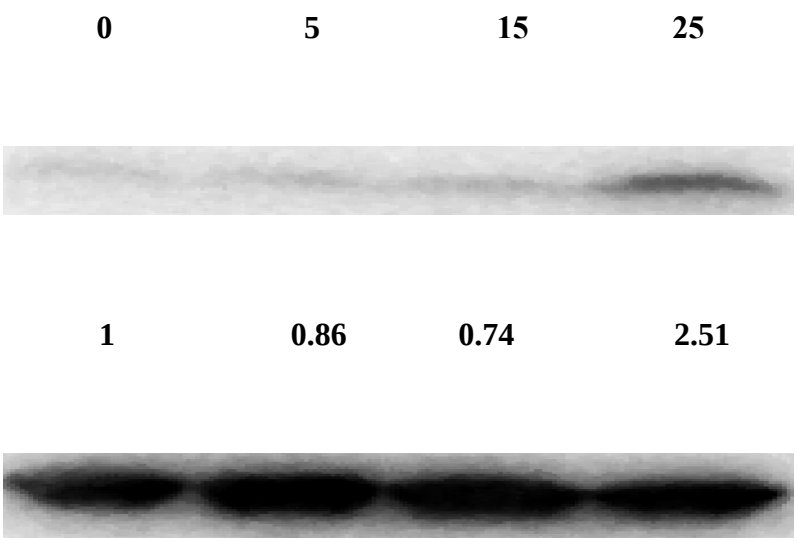

Logarithm of relative mRNA (/control)
2.5
2
1.5
1
0.5
0
LC3A
LC3B
ATG9B
ATG12
ULK1
PIK3C3

Figure 10: Zerumbone up-regulated the expression of LC3B at protein level in WM1552C cells exposed to ZER treatment using Western-blot.

Western-blot analysis of LC3B expression in WM1552C cells treated with ZER (0, 5, 15, or 25 μM) for 48 hours were performed as described in Materials and Methods. The data were quantified by densitometric analysis. The representative picture is shown here. Expression levels were normalized by the GAPDH protein level.


0
5
15
25
ZER (μM)
LC3B
1
0.86
0.74
2.51
Relative density
(/control)
GAPDH

Molecular mechanisms involved in ZER induced cell death

To further address the molecular mechanisms of cytotoxicity of ZER exhibited on melanoma *in vitro* and *in vivo*, I investigated the effects of ZER treatment on several survival-signaling pathways in melanoma that were aberrantly activated and caused uncontrolled proliferation and resistance to apoptosis.

To investigate the effect of ZER on PI3K/Akt signaling and MAPK/Erk signaling in WM1552C cells, WM1552C melanoma cells exposed to ZER (0, 5, 25, or50 μM) for 48 hours were prepared for Western-blot analysis to examine the effect of ZER on the activation of Akt and MAPK. The phosphorylated form of Akt was significantly reduced by ZER treatment (Figure 11A), suggesting that ZER deactivated Akt signaling pathway. Similarly, ZER remarkably decreased the phospho-MAPK in a dose-dependent manner, indicating the deactivation of MAPK/Erk pathway signaling exerted by ZER (Figure 11B).

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) has been well recognized as a primary transcriptional factor that regulates the expression of genes involved in cell proliferation and survival, thus it has been the subject of much research that targets it for anti-cancer therapy. NF-κB promptly responds to harmful cellular stimuli including tumor necrosis factor-α (TNF-α), reactive oxygen species (ROS), interleukin 1-β, bacterial lipopolysaccharide (LPS) and ionizing radiation. Upon activation, NF-κB is trans-located to the nucleus. To determine whether ZER inhibits TNF-induced NF-κB activation in W1552C cells, the nuclear extracts were isolated and

analyzed for the level of NF-κB protein. As shown in Figure 12, ZER evidently inhibited TNF-α induced NF-κB activation in WM1552C cells.

MACC1 (metastasis-associated in colon cancer-1), PTEN (Phosphatase and tensin homolog), and EZH2 (Enhancer of zeste homolog 2) genes are important genes that involved in tumor progression and metastasis. The effect of ZER on the expression of these genes at mRNA level in WM1552C melanoma cells was investigated by qRT-PCR. MACC1 was significantly down-regulated (with more than two-fold alternation) by ZER; however, the other two genes did not show significant change in gene expression by ZER treatment (Figure 13).

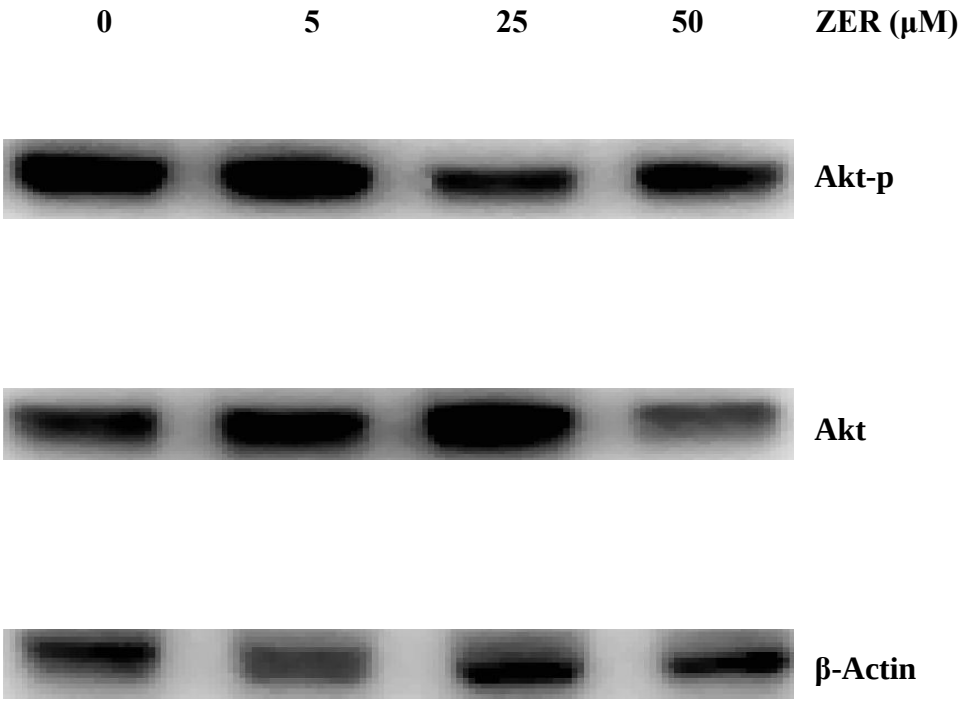
ZER may exert cytotoxicity by inducing oxidative stress. To test this hypothesis, I pretreated the WM1552C cells with NAC (1 mM) and GSH (5 mM), two exogenous antioxidants, before the cells were treated with ZER (25 μM) for 36 hours. NAC (Figure 14A) and GSH (Figure 14B) were each found to significantly inhibit the cytotoxicity induced by ZER treatment, suggesting that ZER induced oxidative stress cell death in WM1552C cells.

Figure 11: ZER inactivated Akt and MAPK signaling pathways in WM1552C cells.

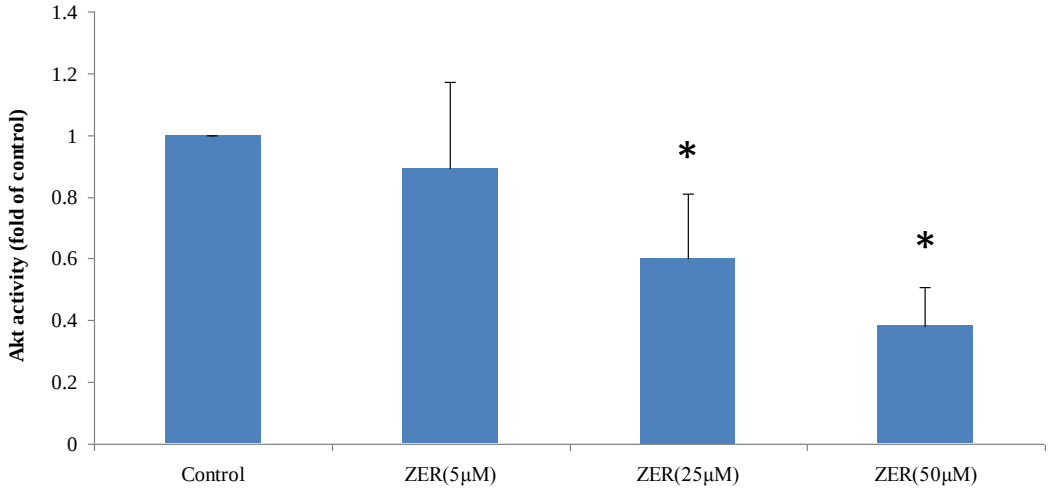
Western-blot analysis of Akt (A) and MAPK (B) activity in WM1552C cells treated with ZER (0, 5, 25, or 50 μM) for 48 hours were performed as described in Materials and Methods. The data were quantified by densitometric analysis. Three independent experiments have been performed and the representative data is shown. Expression levels of Akt and PAPK were normalized by the β-actin and GAPDH, respectively.

A

(a)

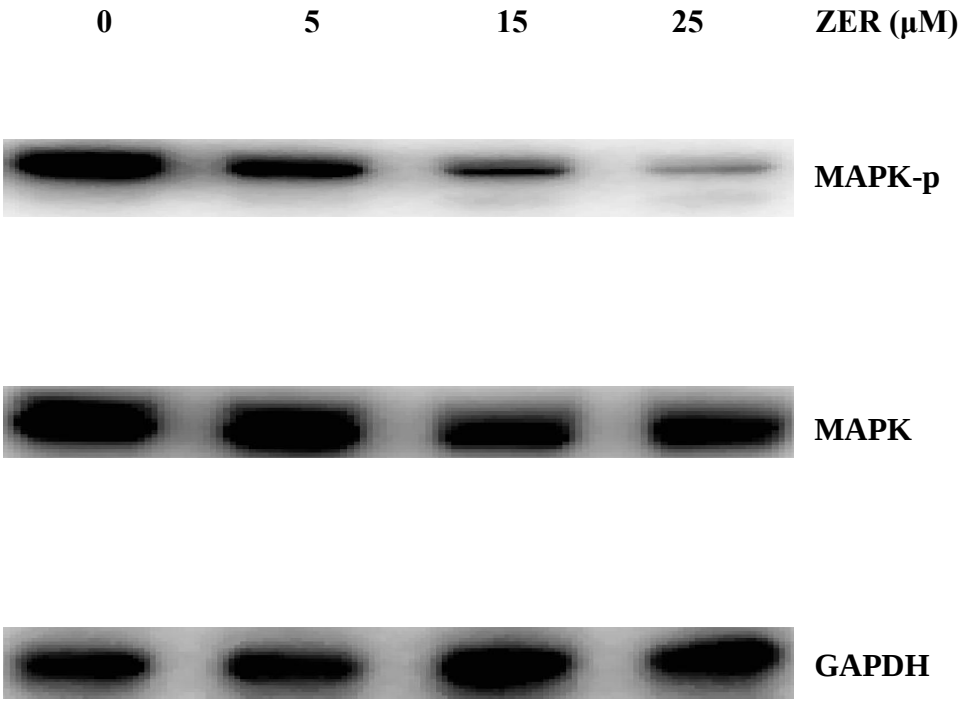

0
5
25
50
ZER (μM)
Akt-p
Akt
β-Actin

(b)


Akt activity (fold of control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
ZER(5μM)
ZER(25μM)
ZER(50μM)

B

(a)


0
5
15
25
ZER (μM)
MAPK-p
MAPK
GAPDH

(b)

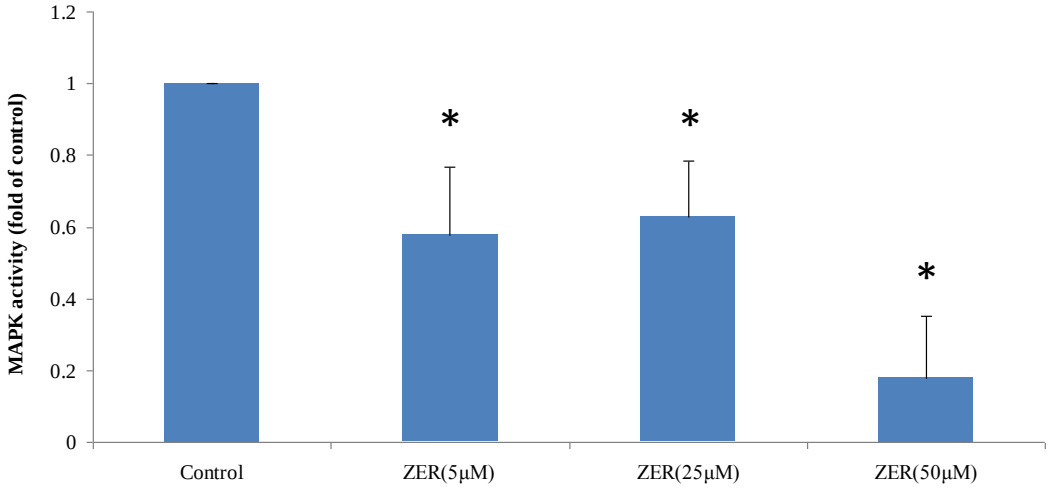
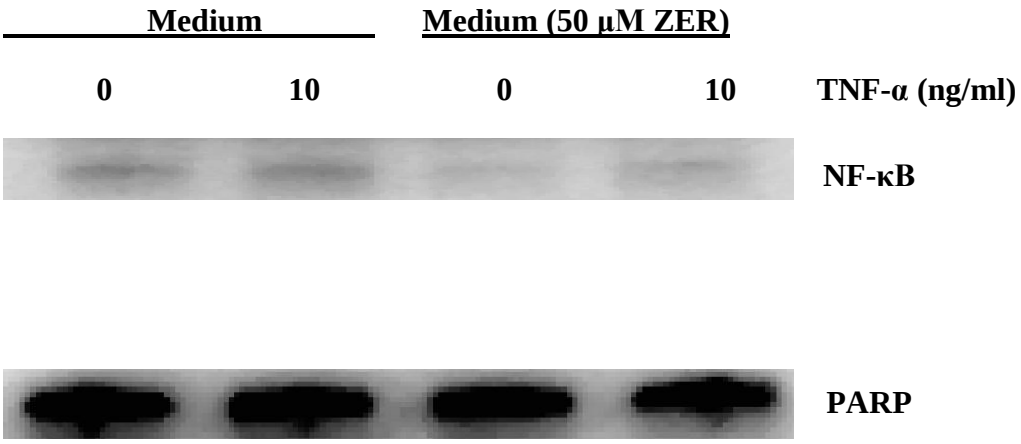

MAPK activity (fold of control)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Control
ZER(5μM)
ZER(25μM)
ZER(50μM)

Figure 12: ZER suppressed TNF-dependent NF-κB activation in WM1552C cells.

WM1552C melanoma cells were pre-incubated with 50 μM ZER in complete medium without FBS for 12 hours and then treated with 10 ng/ml TNF-α for 45 minutes. The nuclear extracts were prepared and assayed for NF-κB activation by Western-blot assay according to Materials and Methods. Densitometric data from three independent experiments were reported as mean ± SD for all columns (B), and the representative data is shown. Expression levels were normalized using PARP protein as loading control.

A


Medium
Medium (50 μM ZER)
0
10
0
10
TNF-α (ng/ml)
NF-κB
PARP

B

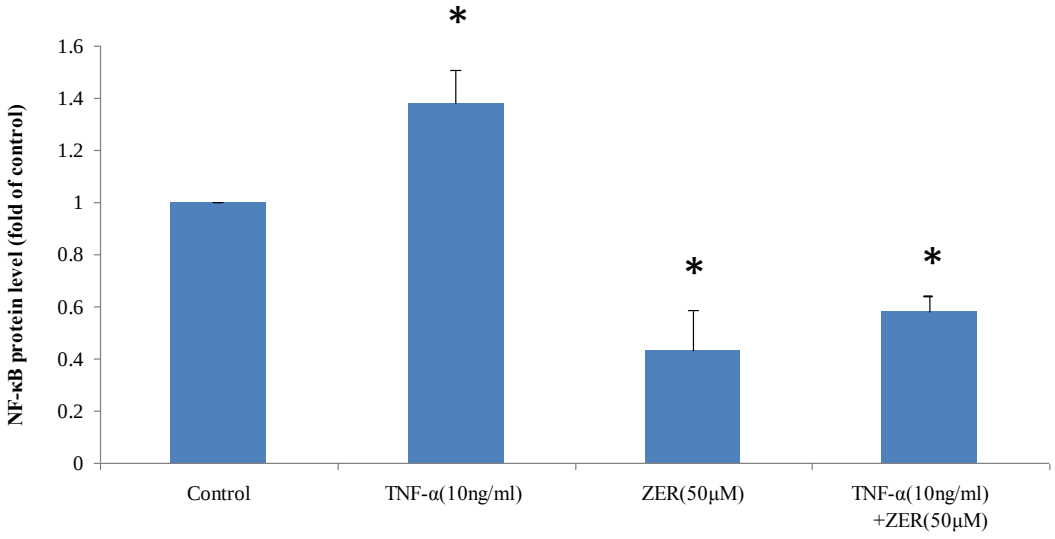

NF-κB protein level (fold of control)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Control
TNF-α(10ng/ml)
ZER(50μM)
TNF-α(10ng/ml)
+ZER(50μM)

Figure 13: Effect of ZER on expression of PTEN, EZH2, and MACC1 in WM1552C cells at mRNA level.

WM1552C cells were treated with vehicle DMSO or 25 μM ZER for 36 hours. Then, the total RNAs were isolated and the qRT-PCR analysis was performed as described in Materials and Methods. The logarithm values of relative ratio of mRNA level were expressed as mean ± SD of three independent experiments relative to vehicle control. Expression levels were standardized by the HPRT1 mRNA level.

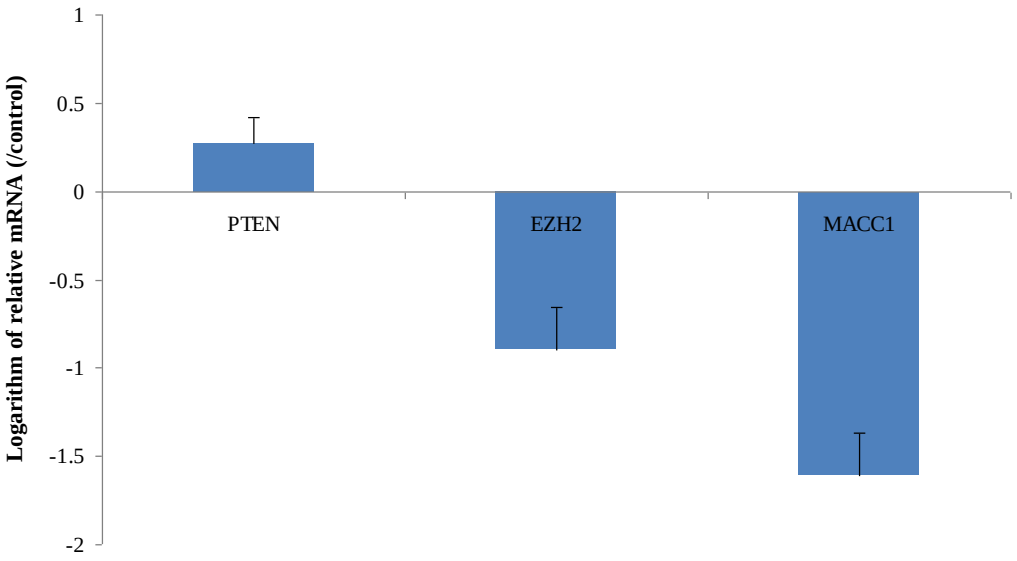
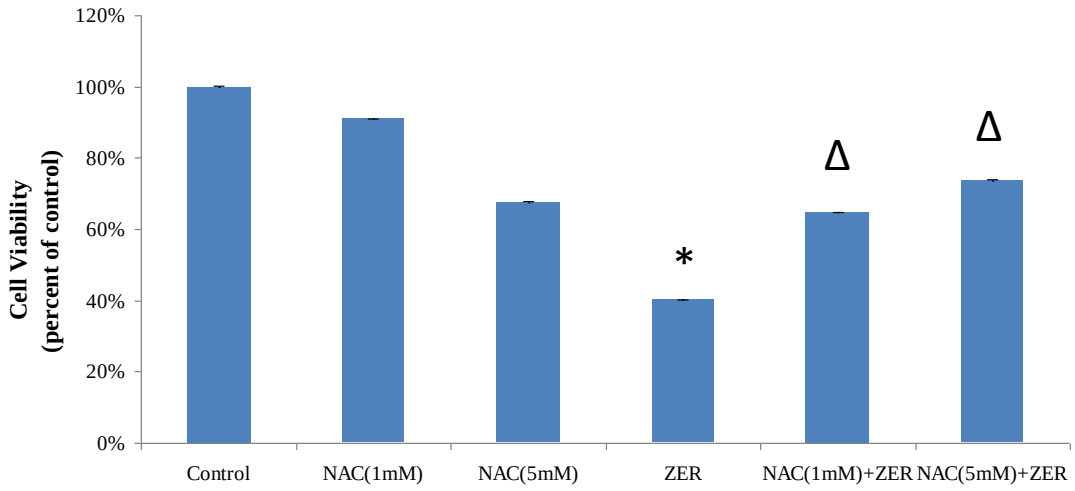

Logarithm of relative mRNA (/control)
1
0.5
0
-0.5
-1
-1.5
-2
PTEN
EZH2
MACC1

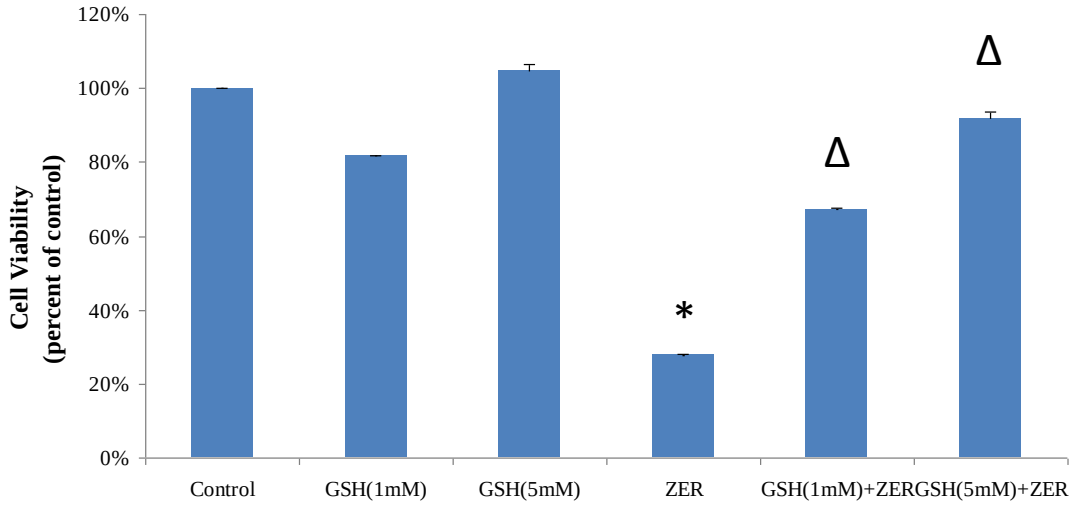
Figure 14: Effect of exogenous antioxidants on ZER-treated WM1552C cells.

The induction of oxidative stress by ZER in WM1552C melanoma cells was examined in a co-culture with NAC or GSH. Cells were pretreated with NAC (A) or GSH (B) (1 mM or 5 mM) 2 hours before ZER treatment (25 μM) for 36 hours. Cell viabilities relative to control group were analyzed by MTT assay as described in Materials and Methods. The experiment data were reported as mean ± SD for all columns. Asterisks indicate a significant difference between experimental groups and control group ($p < 0.05$) (n = 9), Δ shows a significant difference between the ZER-treated groups and ZER alone group ($p < 0.05$) (n = 9).

A


Cell Viability
(percent of control)
120%
100%
80%
60%
40%
20%
0%
*
Δ
Δ
Control
NAC(1mM)
NAC(5mM)
ZER
NAC(1mM)+ZER
NAC(5mM)+ZER

B


Cell Viability
(percent of control)
120%
100%
80%
60%
40%
20%
0%
*
Δ
Δ
Control
GSH(1mM)
GSH(5mM)
ZER
GSH(1mM)+ZER
GSH(5mM)+ZER

Preliminary investigation on the effect of ZER on the miRNA expression profile using miRNA PCR Array analysis

MicroRNAs are short non-coding RNAs that post-transcriptionally regulated the expression of genes involved in cellular processes including proliferation, apoptosis, differentiation and tumor metastasis. To further explore the molecular mechanisms by which ZER exerts its anti-melanoma effect, the miRNA expression profiles of WM1552C melanoma cells in response to ZER treatment (25 μM) were assessed using miRNA PCR Array. The expressions of 19 miRNAs showed more than 1.3-fold change in response to ZER treatment. Among these miRNAs, 6 miRNAs were up-regulated (Table 4A) and 13 miRNAs were down-regulated (Table 4B). The putative target genes of regulated miRNAs were identified from the miRBase, Pictar and Target Scan computational algorithm and the tumor suppressor genes or oncogenes were selected from those target genes by meshing in Cancer Genes database.

Table 4A: The miRNAs that were significantly up-regulated by ZER in WM1552C cells and their putative target oncogenes.

miRNA	Fold Changes	Putative target oncogenes (target score > 70%)
hsa-miR-26b-5p	1.6358	TET2, STRADB, NAA15
hsa-miR-141-3p	1.5157	ABL2, DEK, ZEB2, IBA57
hsa-miR-122-5p	1.5157	TMX3
hsa-miR-30b-5p	1.3851	RUNX2
hsa-miR-96-5p	1.366	SH3KBP1
hsa-miR-210	1.366	METTL13

Table 4B: The miRNAs that were significantly down-regulated by ZER in WM1552C cells and their putative target tumor suppressor genes.

miRNA	Fold Changes	Putative target tumor suppressor genes (target score > 90%)
hsa-miR-27b-3p	-1.7777	FBXW7, GXYLT1
hsa-let-7d-5p	-1.4142	LRIG3
hsa-miR-16-5p	-1.5476	BTRC
hsa-miR-32-5p	-1.9453	FBXW7, DCAF6
hsa-let-7g-5p	-1.454	LRIG3
hsa-miR-142-3p	-1.6818	WASL
hsa-miR-146a-5p	-2.0562	SRSF6, ZNF90
hsa-miR-15b-5p	-1.8921	BTRC
hsa-miR-374a-5p	-1.7777	MIPOL1, RORA, UBE3A
hsa-miR-25-3p	-1.7291	DCAF6, G3BP2
hsa-miR-424-5p	-1.4241	KIF1B
hsa-miR-20a-5p	-1.434	ITPRIPL2
hsa-miR-7-5p	-2.7702	CHAMP1(ZNF828)

Discussion

Due to dramatically increasing incidence and death rate of malignant melanoma, its poor prognosis, and the lack of conventional chemotherapeutic therapies, there is an urgent need to develop effective therapies that can improve melanoma prevention and patient survival time. ZER, a member of the sesquiterpenes, had a chemo-preventive property and an anti-cancer effect with relative low toxicity compared to other chemotherapy in a pre-clinical experiment [13, 21, 22]. ZER has been documented to have inhibitory effect on proliferation of a series of cancer cell lines including colon, leukemia, breast, liver, skin and cervical cancer *in vitro* and *in vivo* [21, 67]. However, the effect of ZER on human melanoma cells has not been reported yet. This study aims to preliminarily investigate the anti-melanoma effect of ZER *in vitro* and *in vivo* and explore the underlying mechanism at molecular levels.

In the presented study, ZER exhibited significant cytotoxicity against WM1552C melanoma cells at physiological concentrations (1-5 μM). According to the American National Cancer Institute guidelines, a significant activity of a new drug shows 50% inhibition of growth at no higher than 30 μM. *In vivo*, ZER retarded melanoma tumor growth and inhibited metastasis of melanoma, however, it did not cause significant difference in food intake, body weight, and survival rate in primary melanoma mouse xenograft and metastasis model. The *vitro* and *vivo* data all suggested that ZER can be a potent therapeutic candidate against melanoma with relatively low side effect.

I presented multiple evidences to show that ZER induced apoptosis, which contributes to the cytotoxicity of ZER. Interestingly, the apoptosis induced by ZER is pan-caspase dependent but caspase 3-independent. This result is very intriguing because caspase 3 is an executor caspase that plays a key role in both intrinsic and extrinsic pathways of apoptosis, and caspase 3 did not seem to be required for ZER-induced apoptosis. The pathway of apoptosis induced by ZER deserves further study.

Recent works demonstrate that radiation or chemotherapeutic agents induce autophagy instead of apoptosis in several cancer cells [68-71]. Whether autophagy promotes cell death or cell survival is circumstantial. My study is the first work to show that ZER induces autophagy in tumor cells. MDC-labeled autophagic vacuoles were induced by ZER treatment in WM1552C melanoma cells. At mRNA level, several ATGs (Autophagy-Related Genes) essential for autophagosome formation including ULK1, ATG9, PIK3C3, ATG12, and LC3 [39] showed increased expression in response to ZER treatment. The expression of LC3B, a key autophagy marker protein that is specifically recruited to autophagosome membranes [76], was evidently increased at protein level. These are all strong evidences suggesting that ZER induced apoptosis in melanoma cells.

3-MA, an inhibitor of autophagy by inhibiting phosphatidylinositol-3 kinase (PI3K)-dependent autophagic sequestration [72, 73], abolished the cytotoxicity of ZER, suggesting that autophagy induced by ZER leads to cell death instead of cell survival. ZER could inhibit the proliferation of melanoma cells by regulating autophagy.

BA1, which is known to inhibit autophagy at a later step, failed to suppress ZER-induced cytotoxicity. BA1 is a vacuolar H^+-ATPase that inhibits the fusion between

autophagosome and lysosome [72, 74, 75]. The work of Kanzava, *et al.* has shown that BA1 did not rescue tumor cells from autophagic cytotoxicity of temozolomide because the BA-1-induced accumulation of autophagosome led to induction of apoptosis. Failing to suppress the ZER-induced cytotoxicity by autophagy inhibitor BA1 could be due to the similar mechanism.

Besides the apoptosis and autophagy, some other mechanistic studies in this work have showed that ZER inhibits the proliferation of cancer cell lines by inducing oxidative stress, regulating cancer cell survival cell signaling pathways and TNF-induced NF-κB activation. Akt, also known protein Kinase B, is a serine/threonine specific protein kinase that plays critical roles in multiple cellular processes including proliferation, apoptosis and cell migration. MAPK (mitogen activated protein kinase) pathway relays the extracellular signal to DNA in nucleus and is a kinase cascade pathway playing essential roles in cell proliferation through regulating transcription and translation. This work showed that ZER suppressed both Akt and MAPK activation in WM1552C cells in dose dependent manners, suggesting that ZER inhibited the cancer cell survival pathway in melanoma cells to lead to cell death. NF-κB is a fast-acting primary transcriptional factor that responds to harmful cellular stimuli. NF- κB have been known to regulate several critical genes involved in cell proliferation and apoptosis [77-83]. This work showed that ZER significantly reduced the translocation of NF- κB to the nucleus, suggesting that zerumbone inhibited TNF-induced NF-κB activation in WM1552C cells. ZER may affect the proliferation of melanoma cells by regulation NF-κB activation.

Anti-oxidants GSH and NAC each can alleviate the cytotoxicity of ZER, suggesting that ZER may induce oxidative stress to cause cytotoxicity in WM1552C human melanoma cells.

MicroRNA profiling can be successfully employed to detect miRNAs regulated in cancer formation, progression and treatment [60, 87]. This study used microRNA profiling to show that ZER altered miRNA expression in human WM1552C melanoma cells, including 6 up-regulated and 13 down-regulated miRNAs. Since tumor suppressor and oncogenes are commonly regulated by miRNAs in the development of cancer [88, 89], the target tumor suppressor and oncogenes for these deregulated miRNAs were identified. ZER may affect the growth and other behaviors of melanoma cells by regulating the expression of miRNAs.

Taken together, my study indicated that ZER is potentially a novel chemotherapeutic agent against melanoma. The anti-melanoma mechanisms of zerumbone include type I and II programmed death, regulation of cancer cell survival signaling pathway, inducing oxidative stress, NF-κB activation inhibition and regulation of miRNA expression. These mechanistic studies should be beneficial in the development of novel cancer therapeutic strategy. Cancer researchers can use my work as a reference for further research related to the development of dietary prevention and treatment regimens of human melanoma.

Future studies aim to determine the expression level of tumor suppressor and oncogenes selected from miRNA PCR array. siRNA- and miRNA- transfection technology will be employed to evaluate the functions of those genes in ZER-treated malignant melanoma cells to see if the knock-out of the genes will influence the

anti-proliferative and anti-metastatic effect of ZER. In addition, the molecular mechanisms need to be confirmed in tissues from *in vivo* study at mRNA and protein levels. And, the relationship among apoptosis, autophagy, cancer cell survival signaling pathway, oxidative stress, TNF-induced NF-κB activation and regulation of miRNA in ZER-treatment melanoma cell need to be further investigated.

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