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Full Length Research Paper

Zerumbone protects HEK 293 cells from irradiation-induced DNA damage via activating Keap1/Nrf2/ARE pathway

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Zerumbone, a sesquiterpene compound occurring in tropical ginger *Zingiber zerumbet* Smith, was shown to attenuate the ^{60}Co γ-rays irradiation-induced cell damage and cell apoptosis in HEK 293 cells. The γ-H2AX focus formation and the protein expression levels, which were important markers of ionizing radiation-induced DNA double-strand breaks, were significantly inhibited too. To elucidate the mechanism via which Zerumbone exerts its cell protective activity, the effects of this compound on Keap1/Nrf2/ARE pathway, which are cellular sensors of chemical- and radiation-induced oxidative and electrophilic stress, were next assayed. The results showed that 5 to 20 μM of Zerumbone could increase Nrf2 protein levels, bring about a dose-dependent induction in ARE-dependent transcriptional activity and subsequently up-regulate the expression of phase II detoxifying enzymes of NQO1 and HO-1. When 1 μM of ATRA, an ARE response inhibitor, was co-administrated, the Zerumbone inductions in ARE-dependent transcriptional activity and HO-1 up-regulation were almost abolished. As a result, the protective effects of Zerumbone on irradiation-induced cell apoptosis and DNA damage were significantly attenuated. These results, taken together, suggested that Zerumbone could protect HEK 293 cells from irradiation-induced cell apoptosis and DNA damage via, at least partly, activating the Keap1/Nrf2/ARE pathway.

Key words: Zerumbone, DNA double-strand break, γ-H2AX, HEK 293 cell, HO-1, NQO1, antioxidant response element.

INTRODUCTION

Ionizing radiation is particles or electromagnetic waves that are energetic enough to detach electrons from atoms or molecules, therefore producing ions (Li et al., 2007). Direct ionization produces free radicals, especially reactive oxygen species (ROS), not only directly damage proteins, lipids and nucleic acids, but also elicit a series of cell signaling transduction, which may lead to cell apoptosis or cancerization (Cadet et al., 2004; Szabolcs et al., 2007; Tiwari et al., 2009). Under physiological condition, redox homeostasis is achieved by a number of antioxidant systems including the two major thiol redox systems: Glutathione and thioredoxin systems (Bjornstedt et al., 1994; Holmgren et al., 2005; Koharyova and Kolarova, 2008), which are under the regulation of Keap1-Nrf2-ARE signaling pathway. It has been approved that switching on the regulative activation of defensive genes leads to protection of cells against

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adverse effects of oxidative and electrophilic stress and promote cell survival (Niture et al., 2010). Considering that irradiated cells produce damaging ROS, compounds with ROS scavenging characteristics or with Keap1-Nrf2-ARE activating activities are expected to protect cells against irradiation-induced damage.

Zerumbone, a sesquiterpene compound occurring in tropical ginger *Zingiber zerumbet* Smith, is notable for its anti-inflammatory (Sulaiman et al., 2010; Sung et al., 2009; Yodkeeree et al., 2009) and tumor cell growth inhibition activities (Murakami and Ohigashi, 2006; Sakinah et al., 2007; Xian et al., 2007; Abdel Wahab et al., 2009; Murakami, 2009). There are also studies revealed that this compound could potentiate the gene expression of several Nrf2/ARE-dependent phase II detoxification enzymes including gamma-glutamylcysteine synthetase (GCS), glutathione peroxidase (GPx), and hemeoxygenase-1 (HO-1) (Shin et al., 2011; Nakamura et al., 2004). These enzyme systems, by performing conjugating reactions, play an important role in the metabolic inactivation of pharmacologically active substances, and subsequently function to minimize the potential damage (Jancova et al., 2010). This study focused on evaluating the protective effect of Zerumbone on irradiation-induced HEK293 cells damage and investing the possible mechanisms underlying the protective activities.

MATERIALS AND METHODS

Reagents

Zerumbone (purity > 98 %, HPLC) and tretinoin (all-trans retinoic acid, ATRA) were purchased from Merlin Standard Chemicals Pte. Ltd (Singapore). Primary antibodies to γH2AX, Nrf2, HO-1, NQO1, β-actin and secondary antibody horseradish peroxidase (HRP) conjuncted IgG were purchased from Santa Cruz Biotechnology, Inc (USA).

Cell culture and irradiation

HEK 293 cells were purchased from American Type Culture Collection (Rockville, USA) and cultured routinely in Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich Pte Ltd, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, USA), 100 µg/ml streptomycin and 100 U/ml penicillin G. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Experimental radiation was performed at room temperature with ^{60}Co γ-rays at a dose rate of 1.12 Gy/min (Gammacell 40, Canada) and cells were immediately returned to the cell incubator.

Cell viability

Cell viability was determined by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA) according to the manufacturer's instructions. In brief, HEK 293 cells were plated in 96-well plates (5000 cells per well) and incubated for approximately 24 h to allow cell attachment, after which, the cells were pre-treated with different concentrations of Zerumbone for 2 h and then irradiated with 4 Gy of γ-ray. MTS assays were performed on these cells 24 h after the irradiation. The absorbance of the dissolved formazan was read at 490 nm on an Infinite® M200 PRO (Tecan Group Ltd, Switzerland) plate reader and data was automatically transferred to a computer, where the absorbance readings (corrected to background absorbance in blank wells) were displayed. The intensity of absorbance was directly proportional to cell viability.

Cell apoptosis

The treated with or without Zerumbone or irradiation HEK 293 cells were washed twice with cold phosphate-buffered saline (PBS) and then suspended in 200 µl ice-cold 1 X binding buffer. The cells were fixed with 1 ml of 70% ice-cold ethanol for minimum of 4 h at 4°C. The fixed cells were washed with ice cold PBS, and resuspended in 100 µl of PBS containing 100 µg/ml ribonuclease A (Sigma-Aldrich Pte Ltd, USA). Finally, 400 µl of propidium iodide (PI, 50 µg/ml, purchased from Sigma-Aldrich Pte Ltd, USA) was added and the cell samples were left in darkness at room temperature for 30 min. The tubes were shaken occasionally to maintain the cells in suspension. Propidium iodide (PI)-stained cells were then analyzed by a BD FACSCalibur™ flow cytometer (BD Biosciences, USA). Cell death was measured as the percentage of cell population with a sub-G_0/G_1 DNA content in the PI intensity-area histogram plot.

Confocal microscopy

The treated with or without Zerumbone or irradiation HEK 293 cells were washed twice with pre-warmed PBS (37°C) and fixed in 3.7% paraformaldehyde for 10 min at room temperature. In order to permeabilize cell membrane, the cells were incubated in 0.3% Triton X-100 for 3 to 5 min. After blocked with 5% bovine serum albumin (BSA), the cells were stained with primary antibody γH2AX in 1:100 dilution and Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, USA) in 1:1,000 dilution for 1 h each, and then mounted in Fluorsave (Merck, Germany) mounting medium. DRAQ5 (Biostatus, UK) was diluted 1:1,000 in mounting medium for nuclear staining. The stained cells were examined by Zeiss LSM510 confocal microscopy.

Nrf2-dependent luciferase reporter assay

HEK 293 cells were seeded in 12-well culture plates and incubated for approximately 24 h to allow cell attachment, after which, the cells were cotransfected with 1.0 µg pGL3-ARE plasmid and 0.05 µg pRL-CMV plasmid (Promega, USA) per well for 24 h. After which the cells were treated with different concentrations of Zerumbone, as well as 10 µM sulforaphane (used as a positive control) for 10 h. Firefly and renilla luciferase activities were assayed using the Dual Luciferase® Assay System (Promega, USA) and results were expressed as firefly luciferase activity normalized to renilla luciferase activity.

GSH measurement

HEK 293 cells were seeded onto 60 mm diameter plates at a density of 5×10^4 cells/plate and allowed to grow to 60 to 70% confluency before the addition of Zerumbone. After 24 h of treatment, cells were pooled, washed once in ice-cold PBS and pelleted by centrifugation (1,200 rpm, 4°C, 5 min). To each pellet, 1 ml of 3% w/v 5-sulfosalicylic acid was added to extract glutathione (GSH). Total GSH content was determined by the enzymatic

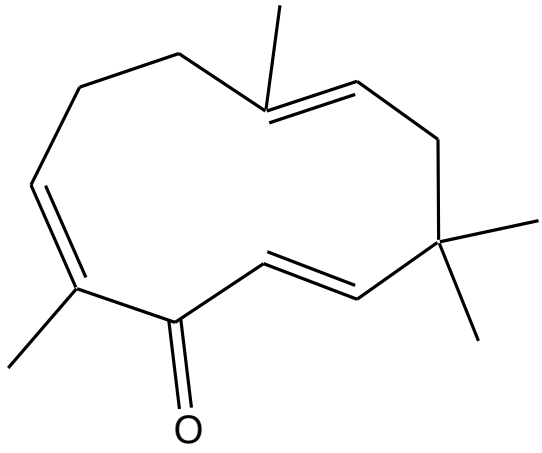


Figure 1. Chemical structure of Zerumbone.

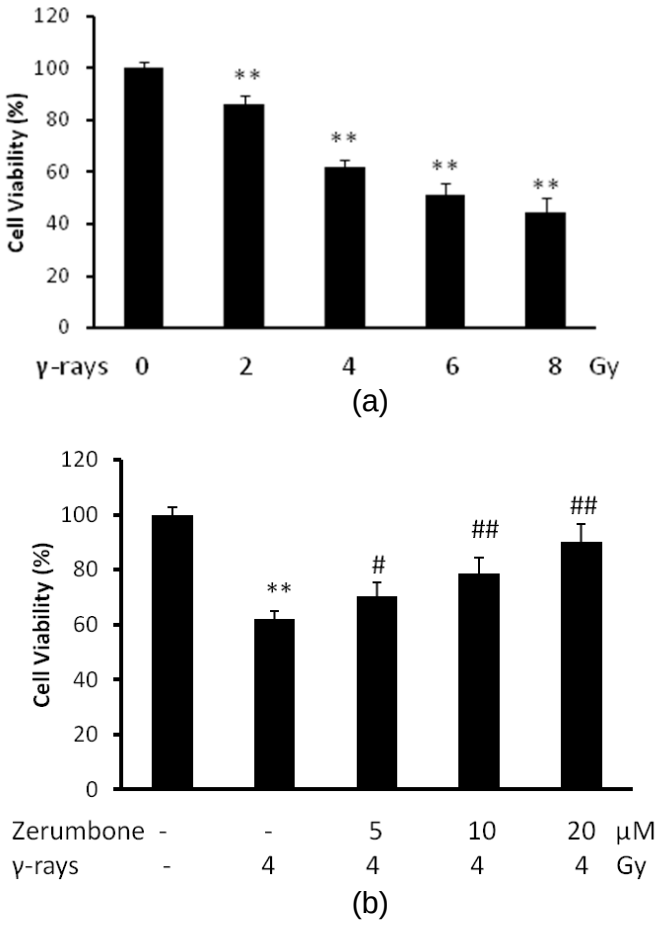


Figure 2. Zerumbone protected HEK 293 cells from ^{60}Co γ- rays irradiation-induced cell death. (a) 0 to 8 Gy of irradiation reduced cell viability in a dose-dependent manner, with cell viability being reduced to around 60% after exposure to 4 Gy-irradiation. (b) Pretreatment of the cells with 5 to 20 μM of Zerumbone significantly improved the reduced viability. **P < 0.01 versus DMSO control; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 versus only γ-rays irradiated group.

method of Tietze (Tietze, 1969).

Western blot analysis

The treated with or without Zerumbone or irradiation HEK 293 cells were washed with ice-cold PBS and lysed with lysis buffer (Cell Signaling Technology, USA) containing freshly added protease inhibitor cocktail (Roche Diagnostics, Germany). Proteins in whole cell lysates were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat milk for 1 h at room temperature. After immunoblotting with the first specific antibodies, membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Membranes were then visualized by enhanced chemiluminescence [SuperSignal West Femto (Pierce, USA)]. To make sure equal amounts of sample protein were applied for electrophoresis and immunoblotting, β-actin was used as an internal control.

Statistics

All experiments were conducted at least three times. Statistical calculations were done using SPSS 13.0 software with one way variance analysis. Results were expressed as means ± S.D. P < 0.05 was considered as significant difference.

RESULTS

Zerumbone protected HEK 293 cells from ^{60}Co γ-rays irradiation-induced cell death

The protective effect of Zerumbone (Figure 1) on HEK293 cells against ^{60}Co γ-rays irradiation induced cell death were carried out by MIT assays. As shown in Figure 2a, 0 to 8 Gy of irradiation reduced cell viability in a dose-dependent manner, with cell viability being reduced to around 60% after exposure to 4 Gy-irradiation, while Zerumbone alone did not show any cytotoxicity at concentration of 1 to 25 μM (P > 0.05, data not shown), pretreatment cells with 5 to 20 μM of Zerumbone significantly improved the reduced viability (Figure 2b). Furthermore, flow cytometry analysis of HEK 293 cells treated or untreated with Zerumbone and ^{60}Co γ-rays irradiation revealed that pretreatment of the cells with Zerumbone (5 to 20 μM) attenuated the irradiation-induced cell apoptosis (Figure 3).

Zerumbone protected HEK 293 cells from ^{60}Co γ- rays irradiation-induced DNA damage

The predominant mechanism of cell killing by ionizing radiation is DNA damage, most significantly double-strand breaks (DSBs) (Bernier et al., 2004). Within minutes of the induction of DNA double-strand breaks, histone H2AX becomes phosphorylated in the serine 139 residue at the damage site. The phosphorylated H2AX, designated as

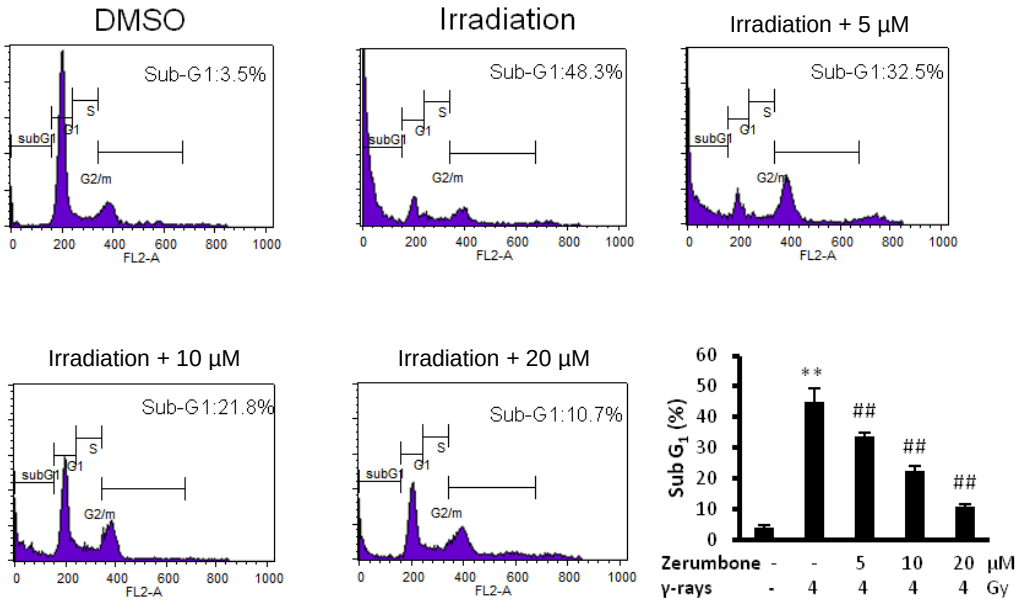


Figure 3. Zerumbone protected HEK 293 cells from ^{60}Co γ- rays irradiation-induced cell apoptosis. Flow cytometry analysis of HEK293 cells treated or untreated with Zerumbone and ^{60}Co γ-rays irradiation. Cell apoptosis was measured as the percentage of cell population with a sub-G_0/G_1 DNA content in the PI intensity-area histogram plot. **P < 0.01 versus DMSO control; $^{\#\#}$P < 0.01 versus only γ-rays irradiated group.

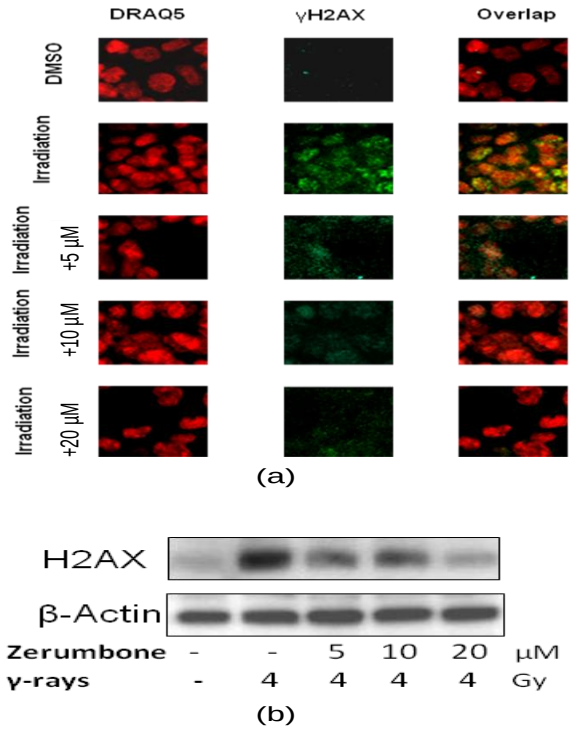


Figure 4. Zerumbone protected HEK 293 cells from ^{60}Co γ-rays irradiation-induced DNA damage. γ-H2AX focus formation was seen at 30 min after exposure to 4 Gy of irradiation. Pretreatment of the cells with Zerumbone 4 h before the irradiation could reduce the foci of γ-H2AX (a) and inhibit the γ-H2AX expression (b).

γ-H2AX, is emerging as an important marker of ionizing radiation-induced DSBs. As shown in Figure 4a, γ-H2AX focus formation was seen at 30 min after exposure to 4 Gy of irradiation. Both foci per cell and percent of γ-H2AX-positive cells were significantly increased. When observed by Western blot analysis, the total γ-H2AX protein level was also up-regulated (Figure 4b). Pretreatment of the cells with Zerumbone 4 h before the irradiation could reduce the foci of γ-H2AX and inhibit the γ-H2AX expression (Figure 4a and b), suggesting a protective effect of this compound on ^{60}Co γ-rays irradiation-induced DNA damage.

Zerumbone up-regulated Nrf2 protein levels and ARE-dependent transcriptional activity on HEK 293 cells

To elucidate the mechanism via which Zerumbone exerts its cell protective activity, the effects of this compound on Keap1/Nrf2/ARE pathway, which are cellular sensors of chemical- and radiation-induced oxidative and electrophilic stress, were next assayed. As shown in Figure 5a, 5 to 20 μM of Zerumbone brought about a dose-dependent induction in ARE-dependent transcriptional activity. In particular, 20 μM of Zerumbone showed an induction fold that was comparable to positive control sulforaphane. In view that the up-regulation of ARE-dependent transcriptional activity could be due to an enhancement in the activity of the transcription factor Nrf2, the effects of

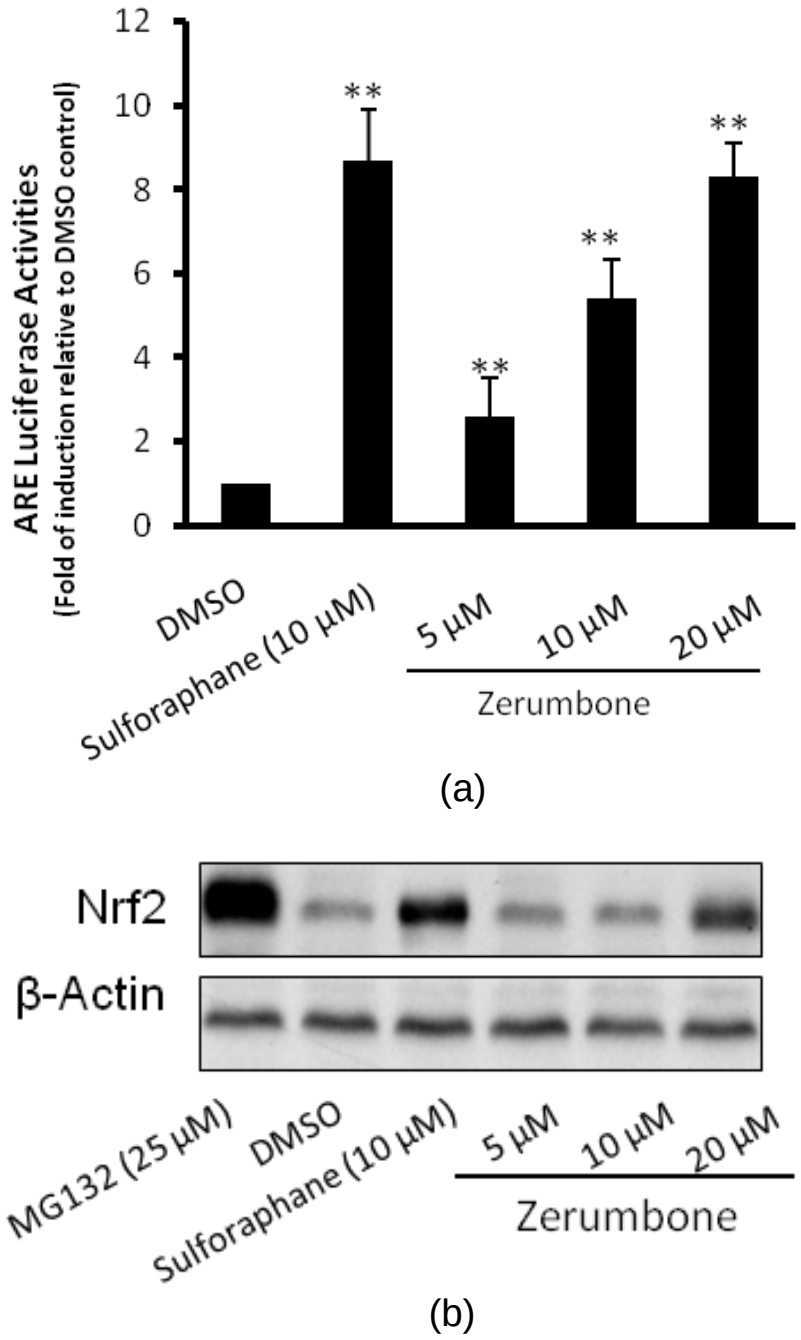
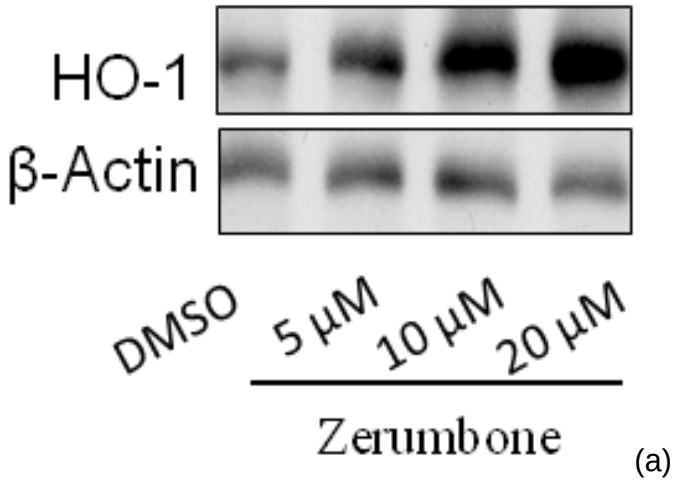


Figure 5. Zerumbone up-regulated Nrf2 protein levels and ARE-dependent transcriptional activity on HEK 293 cells. (a) 5 to 20 μM of Zerumbone brought about a dose-dependent induction in ARE-dependent transcriptional activity and (b) increased Nrf2 protein levels at the time point of 24 h. ** $P < 0.01$ versus DMSO control.

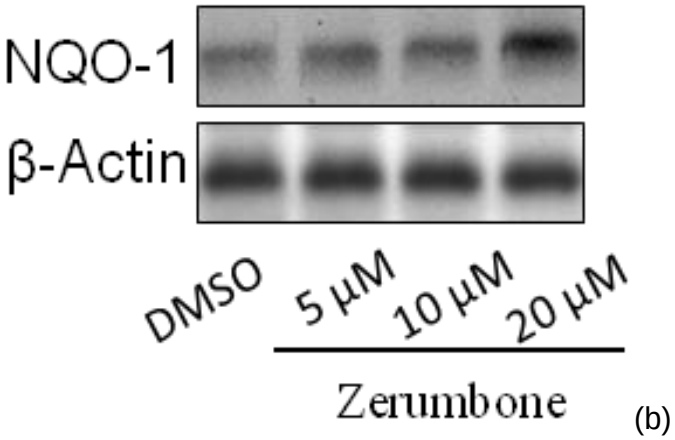
Zerumbone on the cellular levels of Nrf2 protein were next determined. As depicted in the representative Western blot in Figure 5b, Zerumbone at all concentrations were found to increase Nrf2 protein levels at the time point of 24 h.

Zerumbone increased the expression levels of phase II detoxifying enzymes HO-1 and NQO1

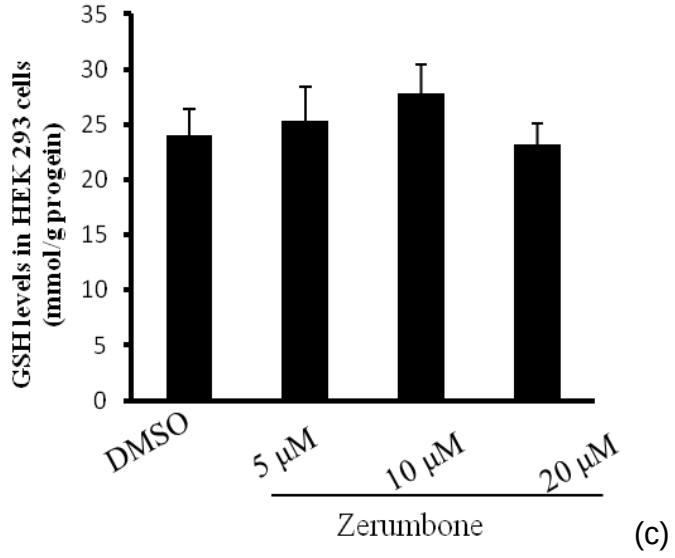
Upon translocation into the nucleus, Nrf2 binds to the ARE, the promoter region of a battery of genes encoding



(a)



(b)



(c)

Figure 6. Zerumbone increased the expression levels of phase II enzymes HO-1 and NQO1. (a) 5 to 20 μM of Zerumbone significantly increased the expression levels of HO-1. (b) The NQO1 protein levels were also slightly elevated, with 20 μM of Zerumbone showing significant difference. (c) The cellular total content of the antioxidant molecular GSH, however, was marginally changed.

phase II enzymes, leading to induction on the expression of these cytoprotective enzymes. In our experiments, treatment of HEK293 cells with 5 to 20 μM of Zerumbone, significantly increased the expression levels of HO-1 (Figure 6a). The NQO1 protein levels were also slightly elevated, with 20 μM of Zerumbone showing significant difference (Figure 6b). The cellular total content of the antioxidant molecular GSH, however, was marginally changed (Figure 6c).

Inhibition the binding of Nrf2 to the ARE enhancer abolished the protective effect of Zerumbone

To further validate the contributing effect of Keap1/Nrf2/ARE pathway on the Zerumbone-produced cyto-protective activity, tretinoin (all-trans retinoic acid, ATRA) was adopted to reduce the binding of Nrf2 to the ARE enhancer. As shown in Figure 7a, while 1 μM of ATRA did not show any cytotoxicity ($P > 0.05$, data not shown), co-administration of ATRA with Zerumbone caused an almost complete repression of inducible luciferase activity. The expression of phase II enzyme HO-1 was also reduced to the basal level (Figure 7b). Consequently, the protective effects of Zerumbone on irradiation-induced DNA damage and cell viability reduction were partly abolished (Figure 7c and d). The results revealed that Keap1/Nrf2/ARE pathway plays a pivotal role in the cyto-protective activity of Zerumbone.

DISCUSSION

Ionizing radiation can induce DNA damage and cell death by generating ROS, including hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), which consist of the major attackers of cellular damage. DNA damage, especially double-strand breaks (DSBs), is the main event in irradiated cells (Valko et al., 2004). After induction of DNA damage by ionizing radiation, ataxia telangiectasia mutated protein (ATM) and related kinases phosphorylate Ser-139 on H2AX to form foci of phosphorylated H2AX (γ-H2AX) at DNA DSB sites. The phosphorylation of H2AX at DSBs timely recruits and/or retains DNA repair and checkpoint proteins such as BRCA1, MRN complex, MDC1 and 53bp1 to sites of DNA damage, activating downstream signal pathways that ultimately resulting in DNA damage repair (homologous recombination or non-homologous end joining), cell cycle arrest or apoptosis (Celeste et al., 2003; Fernandez-Capetillo et al., 2003; Furuta et al., 2003; Johnny et al., 2010). The phosphorylated H2AX is emerging as an important marker of ionizing radiation-induced DSBs. Our results showed that 4 Gy of ^{60}Co irradiation reduced cell viability, induced cell apoptosis and increased DNA DSBs.

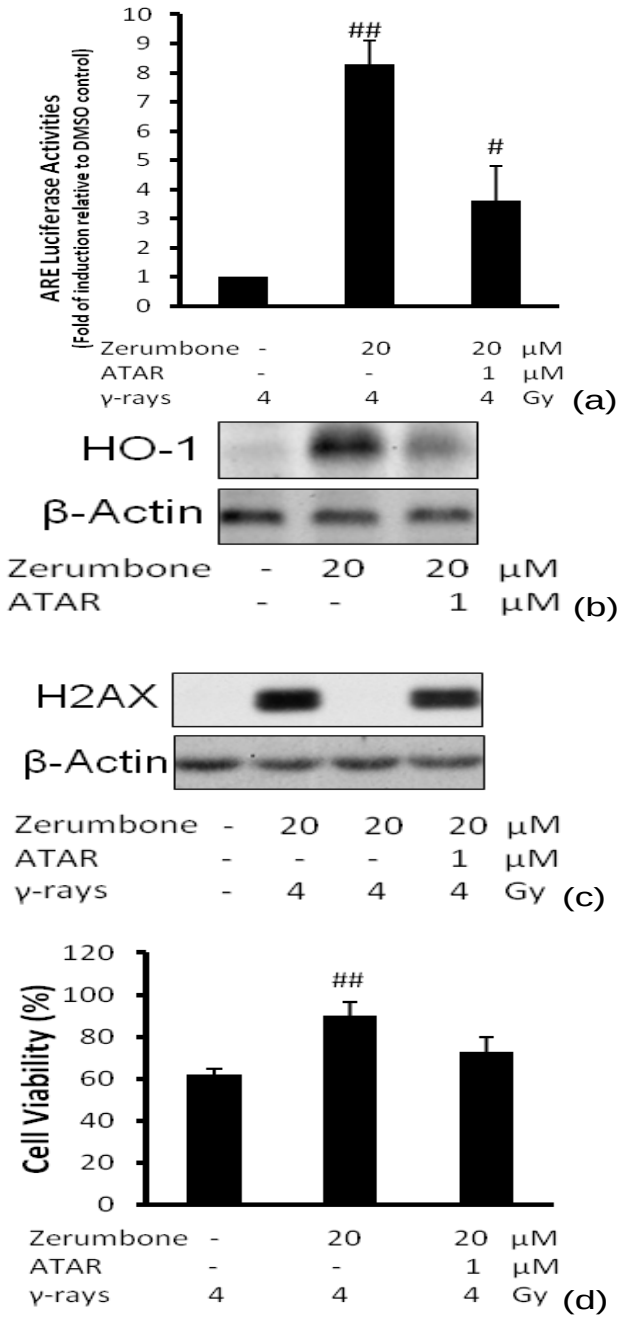


Figure 7. Inhibition the binding of Nrf2 to the ARE enhancer abolished the protective effect of Zerumbone. (a) Co-administration of ATRA with Zerumbone caused an almost complete repression of inducible luciferase activity. (b) The expression of phase II enzyme HO-1 was also reduced to the basal level. (c) Consequently, the protective effects of Zerumbone on irradiation-induced DNA damage and (d) cell viability reduction, were partly abolished. $^{\#}P < 0.05$. $^{\#\#}P < 0.01$ versus only γ-rays irradiated group.

Pretreatment of the cells with 5 to 20 μM of Zerumbone could inhibit the irradiation-induced cell damage and apoptosis, reduce the formation of γ-H2AX foci, suggesting a protective effect of this compound against γ-ray irradiation induced cellular damage.

Since irradiation-produced damaging ROS are the major determinants of cellular damage, compounds that possess ROS-scavenging activities might act as radio-protectors. Previous investigations revealed that Zerumbone could suppress free radical generation and scavenge free radical directly (Murakami et al., 2002; Eguchi et al., 2007). In addition to the direct free radical scavenging pathway, to stimulate the production of cellular antioxidant enzymes and molecules is another strategy against oxidative stress. These cellular antioxidant enzymes and molecules include superoxide dismutase (SOD), GPx, NQO1, HO-1 and GSH, which work together to maintain the redox homeostasis. Particularly, GSH contains triple functions, acting not only as a free radical scavenger, but also as a regulator to regenerate other scavengers and as a substrate in the GPx reaction (Lou, 2003). In our experiments, while the cellular total GSH content was marginally changed, Zerumbone significantly increased the expression of HO-1 and slightly elevated the NQO1 protein levels.

Among all the regulating pathways, the Keap1-Nrf2-ARE is presumably the most important modulator to coordinate the oxidative stress response. Under basal conditions, Keap1 (actin-associated and cysteine-rich Kelch-like ECH-associated protein 1) serves as an adaptor to link Nrf2 to the ubiquitin E3 ligase Cul3-Rbx1 complex that ubiquitinates Nrf2 and leads to proteasomal degradation of Nrf2. When oxidative/electrophilic stress is introduced, Keap1 lose the ability to negatively regulated Nrf2 and lead to the accumulation of Nrf2. Upon translocation into the nucleus, Nrf2 binding to the antioxidant response element (ARE), leads to expression induction of these cytoprotective antioxidant enzymes (Chew et al., 2010; Li and Kong, 2009). Results from our experiments showed that Zerumbone not only increased the Nrf2 protein levels, but also brought about a dose-dependent induction in ARE-dependent transcriptional activity. In particular, 20 μM of Zerumbone showed an induction fold that was comparable to positive control sulforaphane. When 1 μM of ATRA, an ARE response inhibitor (Wang et al., 2007), was co-administrated, the Zerumbone-induced inductions in ARE-dependent transcriptional activity was almost abolished. The expression levels of HO-1 were also reduced to the control level. Consequently, the protective effects of Zerumbone on irradiation-induced cell apoptosis and DNA damage were attenuated significantly (Figure 7).

In conclusion, Zerumbone could protect HEK 293 cells from irradiation-induced cell apoptosis and DNA damage via, at least partly, activating the Keap1-Nrf2-ARE pathway.

Abbreviations: ARE, Antioxidant response elements; **DSBs**, double-strand breaks; **HO-1**, Heme oxygenase 1; **Keap**1, Kelch-like ECH-associated protein 1; **NQO1**,

NAD(P)H dehydrogenase (quinone 1); **Nrf2**, NF-E2-related factor 2; **ROS**, reactive oxygen species.

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