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# Zerumbone, a tropical ginger sesquiterpene, activates phase II drug metabolizing enzymes

Yoshimasa Nakamura*, Chiho Yoshida, Akira Murakami, Hajime Ohigashi, Toshihiko Osawa, Koji Uchida

*Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya 464-8601, Japan*
*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*

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**Abstract Zerumbone (ZER), a sesquiterpene compound occurring in tropical ginger *Zingiber zerumbet* Smith, has been implicated as one of the promising chemopreventive agents against colon and skin cancer. In the present study, we investigated the phase II detoxification enzymes induction of ZER using a cultured rat normal liver epithelial cell line. Exposure of RL34 cells to ZER resulted in the significant induction of glutathione *S*-transferase, while the reduced analogues of ZER (α-humulene and 8-hydroxy-α-humulene) did not show any inducing effect. Therefore, the electrophilic property, characterized by the reactivity with intracellular nucleophiles including protein sulfhydryls as well as low molecular weight thiols, at the 8-position α, β-unsaturated carbonyl group plays an important role in the induction of phase II enzymes. ZER induced nuclear localization of the transcription factor Nrf2 that binds to antioxidant response element (ARE) of the phase II enzyme genes, suggesting that ZER is a potential activator of the Nrf2/ARE-dependent detoxification pathway. This is consistent with the observation that ZER potentiated the gene expression of several Nrf2/ARE-dependent phase II enzyme genes, including γ-glutamylcysteine synthetase, glutathione peroxidase, and heme-oxygenase-1. The present study also implied the antioxidant role of this detoxification system activation by ZER in the neutralization of lipid peroxidation in hepatocytes, providing a new insight for cancer prevention.**


*Keywords:* Glutathione *S*-transferase; Zerumbone; Glutathione; Electrophile; Antioxidant; Cancer prevention; Detoxification; α, β-unsaturated carbonyl; RL34

## 1. Introduction

Zerumbone (ZER, Fig. 1A) is a sesquiterpene compound occurring in rhizomes of *Zingiber zerumbet* Smith (Zingiberaceae), previously identified as a distinct suppressor of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus (EBV) activation in Raji cells [1]. In some Southeast Asian countries, the rhizomes of the plant are employed as a traditional medicine for anti-inflammation, while the young shoots and inflorescence are used as condiments [1]. The recent study demonstrated that ZER effectively suppressed TPA-induced superoxide anion generation from both NADPH oxidase in dimethylsulfoxide-differentiated HL-60 human acute promyelocytic leukemia cells and xanthine oxidase in AS52 Chinese hamster ovary cells [2]. ZER also markedly diminished the combined lipopolysaccharide- and interferon-γ-stimulated protein expressions of inducible nitric oxide synthase and cyclooxygenase (COX)-2, together with the release of tumor necrosis factor-α, in RAW 264.7 mouse macrophages. Moreover, ZER inhibited the proliferation of human colonic adenocarcinoma cell lines in a dose-dependent manner, while the growth of normal human dermal and colon fibroblasts was less affected. Intriguingly, α-humulene (HUM, Fig. 1A), a structural analogue lacking only the carbonyl group in ZER, was virtually inactive in all experiments conducted, indicating that the α, β-unsaturated carbonyl group in ZER may play some pivotal roles in interactions with unidentified target molecule(s) [2]. Subsequently, ZER was demonstrated to inhibit both azoxymethane-induced rat aberrant crypt foci and phorbol ester-induced papilloma formation in mouse skin as a further indication for its efficacy to prevent colon and skin cancers [3,4]. Therefore, ZER is a promising dietary agent that has distinguishable potentials for use in anti-inflammation, chemoprevention, and chemotherapy strategies.

Several lines of evidence indicate that phase II xenobiotic metabolizing enzymes, such as glutathione *S*-transferase (GST) and NAD(P)H:(quinone-acceptor) oxidoreductase (NQO), play a major role in the cellular detoxification of oxidative damaging, genotoxic and carcinogenic chemicals [5]. It is widely accepted that the induction of phase II enzymes results in protection against toxicity and chemical carcinogenesis, especially during the initiation phase. GSTs are a family of soluble proteins, which conjugate xenobiotics with glutathione. Metabolites after glutathionylation are more hydrophilic and thus biologically inactive. Therefore, they are readily excreted in bile or urine as conjugates. This action is thus believed to be a major mechanism for the detoxification of reactive ultimate carcinogens. It is now generally accepted that the GSTs are encoded by at least eight different gene families (including classes alpha, mu, pi, theta, zeta, omega, sigma and kappa) of cytosolic GSTs. Class pi GST (GSTP1-1), one of the GST isozymes, can profoundly alter susceptibility to chemical carcinogenesis possibly through glutathione (GSH) conjugation of carcinogens [6–8], including widespread environmental

* Corresponding author. Fax: +81-52-789-5741.
*E-mail address:* yossan@agr.nagoya-u.ac.jp (Y. Nakamura).

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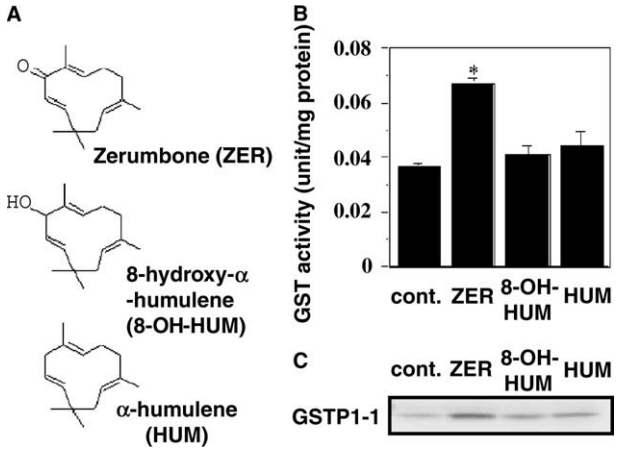


Fig. 1. Electrophilic ZER, but 8-OH-HUM, and HUM does not, induces GST in RL34 cells. (A) Chemical structures of zerumbone (ZER), 8-hydroxy-α-humulene (8-OH-HUM), and α-humulene (HUM). (B) Effect of ZER, 8-OH-HUM, and HUM on total cellular GST activity. Cells' post-confluency was exposed to the test compounds (25 μM) in the medium containing 5% fetal bovine serum for 24 h. GST activity was measured using CDNB as a substrate. Statistical significance was determined by the Student's $t$ test and is expressed as: *, versus control treated only with DMSO, $P < 0.05$. (C) Induction-of pi-Class GST protein (GSTP1) by ZER, 8-OH-HUM, and HUM. Cells' post-confluency were exposed to the test compounds (25 μM) in the medium containing 5% fetal bovine serum for 24 h. The GSTP1 level was evaluated by an immunoblot analysis.

pollutants. An antioxidant/electrophile response element (ARE/EpRE; consensus sequence TGACNNNGC) or the related element, regulating both its basal and inducible expression, was mostly found in the 5′-flanking region of genes of the phase II enzymes and may be recognized by a similar series of transcriptional factors [5]. The induction of phase II xenobiotic metabolizing enzymes has been reported to be evoked by a variety of chemical agents, including Michael reaction acceptors, quinines and others, most of which are electrophiles [9]. Among them, Isothiocyanate (ITC) compounds, including sulforaphane and benzyl ITC, are well known to induce the phase II enzyme in rat liver and cancer chemopreventive effects in several rodent models [10]. Epidemiological studies also demonstrated that dietary consumption of the ITC compounds or urinary ITC level was associated with significantly reduced risk of several cancers [11,12]. These results implied that the enhanced detoxification of carcinogens by the ITC compounds might be involved in cancer prevention in humans. Therefore, food chemicals inducing phase II enzyme activities are anticipated to be highly useful in anti-carcinogenic strategies.

In the present study, we evaluated ZER as an inducer of GSH-related phase II enzymes including GST. We also examined the structure–activity relationship study of the α,β-unsaturated carbonyl moiety and discussed, based on the structural factor, the chemical reactivity required for enzyme induction. In addition, the $Fe^{2+}$-induced lipid peroxidation in hepatocyte can be affected by the detoxification system activation by ZER.

## 2. Materials and methods

### 2.1. Materials

ZER was isolated from the rhizomes of *Z. zerumbet* Smith as previously reported (purity >99%) [1]. HUM was purchased from Tokyo Kasei Kogyo, Tokyo, Japan. 8-Hydroxy-α-humulene (8-OH-HUM) was synthesized as previously reported [1]. TPA was obtained from Research Biochemicals International, Natick, MA. Cycloheximide (CHX) was obtained from Sigma, St. Louis, MO. MG132 was purchased from Calbiochem, Darmstadt, Germany. Anti-rat GSTP1-1 antiserum was obtained from Biotrin International, Dublin, Ireland. Anti-Nrf2 polyclonal antibody was purchased from Santa Cruz, Santa Cruz, CA. Anti-cytochrome P450 (CYP) 1A1/2 antiserum was obtained from Daiichi Nippon Pharmacology Co., Tokyo, Japan. Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin was purchased from Dako, Glostrup, Denmark. The protein concentration was measured using the BCA protein assay reagent from Pierce, Rockford, IL.

### 2.2. Cell cultures

RL34 cells were obtained from the Health Science Research Resources Bank, Osaka, Japan [13]. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml) and 0.37% $NaHCO_3$ at 37 °C in an atmosphere of 95% air and 5% $CO_2$.

### 2.3. Enzyme assay

GST activity was measured using l-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) as substrates according to the method of Habig and Jakoby [14].

### 2.4. Western blot analysis

The ZER-treated and untreated cells were rinsed twice with PBS (pH 7.0) and lysed by incubation at 37 °C for 10 min with a solution containing 0.8% digitonin and 2 mM EDTA (pH 7.8). Each whole cell lysate was then treated with Laemmli sample buffer for 3 min at 100 °C [15]. The samples (20 μg) were run on 12.5% SDS–PAGE slab gel. One gel was used for staining with Coomassie brilliant blue and the other was transblotted on a nitrocellulose membrane with a semi-dry blotting cell (Trans-Blot SD, Bio-Rad), incubated with Block Ace (40 mg/ml) for blocking, washed, and treated with the antibody.

### 2.5. Immunochemical detection of Nrf2 nuclear translocation

The cells treated with ZER were fixed overnight in PBS containing 2% paraformaldehyde and 0.2% picric acid at 4 °C. The membranes were permeabilized by exposing the fixed cells to PBS containing 0.3% Triton X-100. The cells were then sequentially incubated in PBS solutions containing blocking serum (5% normal rabbit serum) and immunostained with the anti-Nrf2 polyclonal antibody. The cells were then incubated for 1 h in the presence of fluorescein isothiocyanate-labeled rabbit anti-goat (DAKO A/S, Glostrup, Denmark), rinsed with PBS containing 0.3% Triton X-100, and covered with anti-fade solution. Images of the cellular immunofluorescence were acquired using a confocal laser microscope (Bio-Rad, Hercules, CA) with a 40× objective (488-nm excitation and 518-nm emission).

### 2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The RT reaction was performed with 5 μg of total RNA and an oligo(dT) primer using the First-Strand cDNA synthesis kit (Life Technology Inc., Rockville, MD). The reaction mixture was then subjected to brief incubation at 65 °C in order to inactivate the enzyme. PCRs were carried out as previously reported [16]. The following primers were used: GSTP1, (F) 5′-GCAGATATCCAATGCCGCCGTACACCATT-3′ and (R) 5′-GGACTCGAGCTACTGTTTACCATTGCCGT-3′ (25 cycles, product size 630 bp); glutathione reductase (GR), (F) 5′-GATAACCAGTGATGGGTTCTTT-3′ and (R) 5′-TTGGAGCAAAGTAGAGTGGTGA-3′ (25 cycles, product size 472 bp); NQO1, (F) 5′-GGCTGGTTTGAGAGAGTG-3′ and (R) 5′-GTCGGCTGGAATGGACTTG-3′ (35 cycles, product size 459 bp); hemeoxygenase-1 (HO-1), (F) 5′-CAGCACTACGTAAAGCGTCTCC-3′ and (R) 5′-AGTGCTGATCTGGGATTTTCCT-3′ (25 cycles, product size 452 bp); γ-glutamylcysteine synthetase light chain (γGCSl), (F) 5′-CTTGAATGAATGGAGTTCCCA-3′ and (R) 5′-TACCTGT GCCCACTGATACAGC-3′ (27 cycles, product size 400 bp); γ-GCS heavy chain (γGCSh), (F) 5′-CCAGTTCCTGCACATCTACCACGC-3′ and (R) 5′-GCAGAAATCACTCCCCAGCGAC-3′ (27 cycles, product size 800 bp); GAPDH, (F) 5′-CGAGATCCCTCCAAAATCAA-3′ and (R) 5′-AGGTCCACCACTGACACGTT-3′ (25 cycles, product size 345 bp).

*2.7. Glutathione titration*

The quantitation of GSH was fluorometrically performed according to the method of Hissin and Hilf [17]. Cells were lysed and extracted with 5% metaphosphoric acid solution containing 5 mM EDTA. After centtrifugation (10 000 ×*g*, 20 min), a 1.8 ml aliquot of 0.1 M phosphate solution (pH 8.0) containing 5 mM EDTA and 100 μl of the *o*-phthalaldehyde solution (1 mg/ml) were then added to the supernatant (100 μl). The fluorescence intensity at 420 nm was next determined with activation at 350 nm.

*2.8. Lipid peroxidation test and TEARS quantification*

Briefly, to determine the inhibitory effect of ZER on lipid peroxidation in RL34 cells, ZER (25 μM) dissolved in 5 μl of DMSO was added to the cell suspension and incubated at 37 °C for the different periods as indicated. After rinsing twice with PBS, the cells were treated with $H_2O_2$ (100 μM) and $FeSO_4$ solutions (50 μM), which were incubated for another 1 h. The reaction was terminated by the addition of 0.4 mM *t*-butymydroxytoluene solution. Thiobarbituric acid-reacting substances (TEARS) level of the collected cells was determined by our previously reported method [18]. The final results are expressed as equivalents of nmols of malondialdehyde per mg protein, on the basis of a standard line of TBARS formation using authentic malondialdehyde.

*2.9. Statistics*

Data were the means of at least three independent experiments. Specific differences among treatments were examined using the Student's *t* test (two sided) that assumed unequal variance.

## 3. Results

*3.1. ZER is a potential inducer of pi class GST isozyme*

As shown in Fig. 1B, the treatment of rat hepatocyte RL34 cells with ZER resulted in a significant induction of total GST activity evaluated using a representative substrate, CDNB. The GST activity was induced approximately 1.5-fold when cells were exposed to 25 μM ZER for 24 h. To classify the structural requirement of ZER for GST activity potentiation, the cells were exposed to a variety of ZER-related compounds at a concentration of 25 μM for 24 h. Not only the total GST activity but also the GSTP1-1 protein were not significantly elevated in the cells with HUM or 8-OH-HUM (Fig. 1B and C), suggesting that an α, β-unsaturated carbonyl group as a Michael reaction acceptor is an essential structural factor for GST induction by ZER. To examine the GST isozyme responsible for the increase in the GST activity of the ZER-treated RL34 cells, an enzyme assay and immunoblot analysis were carried out using the GST class-specific antibodies, to confirm the apparent induction of the GST proteins. The immunoblot analysis demonstrated a significant increase in the level of the GSTP1-1 protein level by treatment with ZER [$3.2 \pm 0.3$-fold of the DMSO control ($n = 3$), as determined by densitometry analysis]. By contrast, untreated RL34 cells express an undetectable or trace level of the Class alpha and Class mu proteins whose genes also contain ARE/EpRE sequences and these were nearly unchanged by ZER treatment (data not shown). Insensitivity of their basal and inducible expression in RL 34 cells may be due to cell specific effect. The increase in the GSTP1-1 protein, therefore, might coincide with a substantial rise in the GST activity. In addition, we examined the modifying effects of ZER on the GST activities toward EA, a specific substrate of Class pi GSTs compared with the total GST activity using CDNB. The mean ZER-enhanced specific activities of the cytosolic GSTs in RL34 cells were $67.8 \pm 2.1$ (CDNB) and $35.3 \pm 3.2$ (EA) ($\times 10^{-3}$) units per mg protein, respectively. This also supported that half of the GST induction by ZER was accounted for by the induction of Class pi GST isozymes. As shown in Fig. 2A and B, the GST-inducing activity of ZER was confirmed to be in dose- and time-dependent manners. In addition, ZER did not affect the CYP1A1 protein level (Fig. 2B), suggesting that ZER might not activate the pathway dependent on xenobiotic response element, which is contained in and required for carcinogen-induced expression of some cytochrome P450 isozymes.

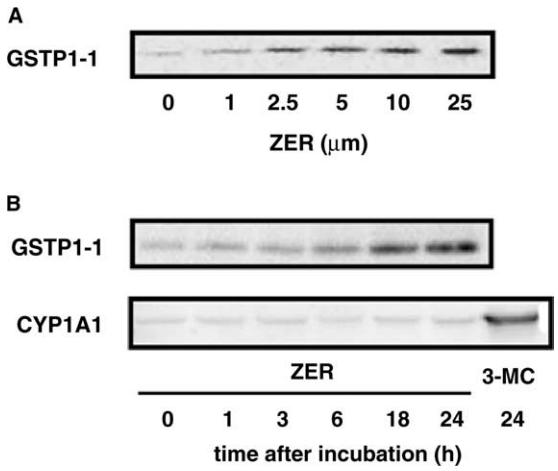


Fig. 2. Induction of pi-Class GST protein (GSTP1) by ZER in RL34 cells. (A) Dose-dependent effect of ZER (1–25 μM) on GSTP1 protein level. Cells' post-confluency was exposed to the test compounds for 24 h. (B) Time-dependent effect of ZER on GSTP1 and CYP1A1 protein levels. Cells' post-confluency was exposed to the test compound (25 μM) for the different periods as indicated. GSTP1 and CYP1A1 levels were evaluated by an immunoblot analysis. 3MC; 3-methylcholanthrene (1 μM) as a positive control for the CYP1A1 induction.

*3.2. ZER activates the Nrf2-dependent pathway*

Several lines of evidence indicate that a member of the basic leucine zipper transcription factor family, Nrf2 (NF-E2-related factor 2), is involved in the activation of gene expression of phase II enzymes including GSTP1-1 [19]. To determine whether this transcription factor contributes to the ZER-stimulated GST induction, we examined the nuclear localization of Nrf2 in the ZER-treated RL34 cells. As shown in Fig. 3A, only cytoplasmic labeling of Nrf2 with no nuclear staining was observed in the non-stimulated cells (0 h), whereas an intense nuclear labeling was observed in the ZER-stimulated cells (6 h). Next, we sought to provide further information about how the function of Nrf2 is controlled by ZER. To investigate this point, the fact that MG132-mediated inhibition of the proteasome is reversible was exploited to generate a significant pool of Nrf2 in RL34 cells [20]. RL34 cells were pretreated with MG132 for 2 h before the proteasome inhibitor was removed and replaced with media containing CHX and either ZER or vehicle. When cotreated with CHX and vehicle, a notable reduction in the expression of Nrf2 was observed after a chase period of 60 min (Fig. 3B), indicative of proteasomal-mediated degradation [$24.5 \pm 4.5\%$ of the 0-h control ($n = 3$) as determined by densitometry analysis]. Most importantly, this degradation was clearly inhibited by cotreatment with ZER [25 μM; $62.5 \pm 6.5\%$ of the 0-h control ($n = 3$)]. Overall, these data suggest that Nrf2 is subject to proteasome-dependent degradation under homeostatic condition but that the rate of degradation is reduced significantly during the ZER treatment. As shown in Fig. 3C,

treatment of the cells with ZER (25 μM) indeed enhanced the GSTP1, GR, NQO1 and HO-1 mRNA expressions, all of which are reported to be regulated by the Nrf2-dependent pathway [21].

### 3.3. ZER is a biologically active antioxidant

In order to protect against excessive reactive oxygen species (ROS), aerobic organisms have developed a number of cellular defenses composed of non-enzymatic and enzymatic components. Some GST isozymes, being able to utilize the major products of lipid peroxidation including fatty acid hydroperoxide and 4-hydroxy-2-nonenal as substrates, play a physiological role in the protection against oxidative stress [22,23]. We preliminarily confirmed that ZER did not show any

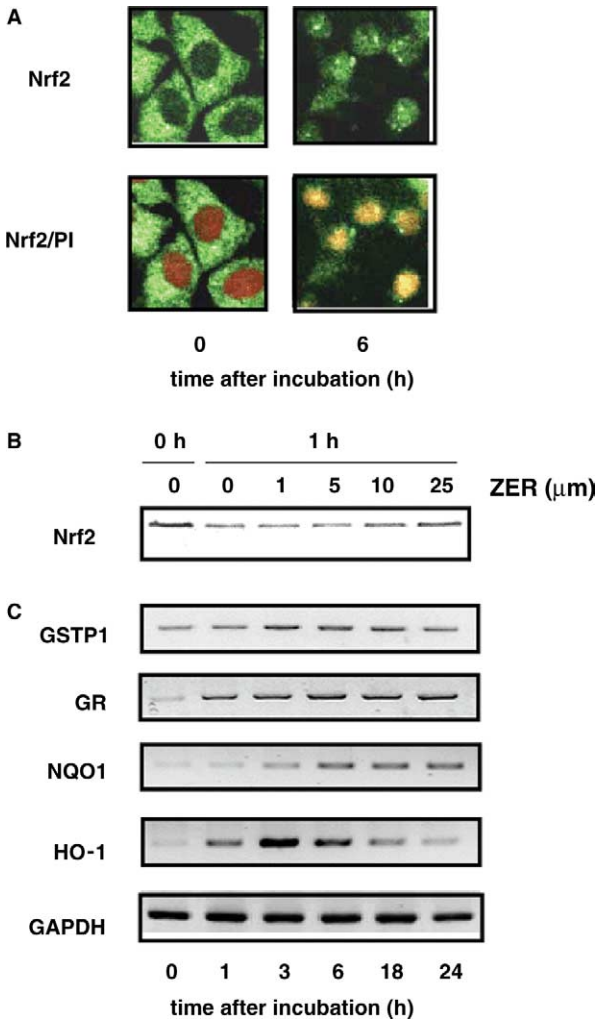


Fig. 3. ZER activates the Nrf2-dependent pathway. (A) Induction of nuclear translocation of Nrf2 by ZER. The RL34 cells treated with ZER for 0 h or 6 h were fixed in 2% paraformaldehyde and 0.2% picric acid and immunostained with the anti-Nrf2 antibody. Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope. (B) ZER stabilizes the Nrf2 protein by antagonizing its proteasome-dependent degradation. RL34 cells were cultured with 2.5 μM MG132 for 2 h before CHX was added to a final concentration of 10 μM. Whole-cell lysates were prepared 0 and 60 min after the addition of CHX. As for the ZER-treated group, RL34 cells were cultured with 2.5 μM MG132 for 2 h. Medium was removed and all monolayer cells were washed with one volume of fresh medium. Fresh medium containing 10 μM CHX and either ZER (1 ~ 25 μM) or vehicle (0 μM) was added to each dish. Whole-cell lysates were prepared and immunoblotted with the anti-Nrf2 antibody. (C) Effect of ZER on mRNA expression of the Nrf2-dependent enzyme genes (GSTP1, OR, NQO1, and HO-1). Total RNA was extracted from RL34 cells untreated (0 h) or treated with 25 μM ZER for the indicated time periods, and then a RT-PCR analysis was carried out.

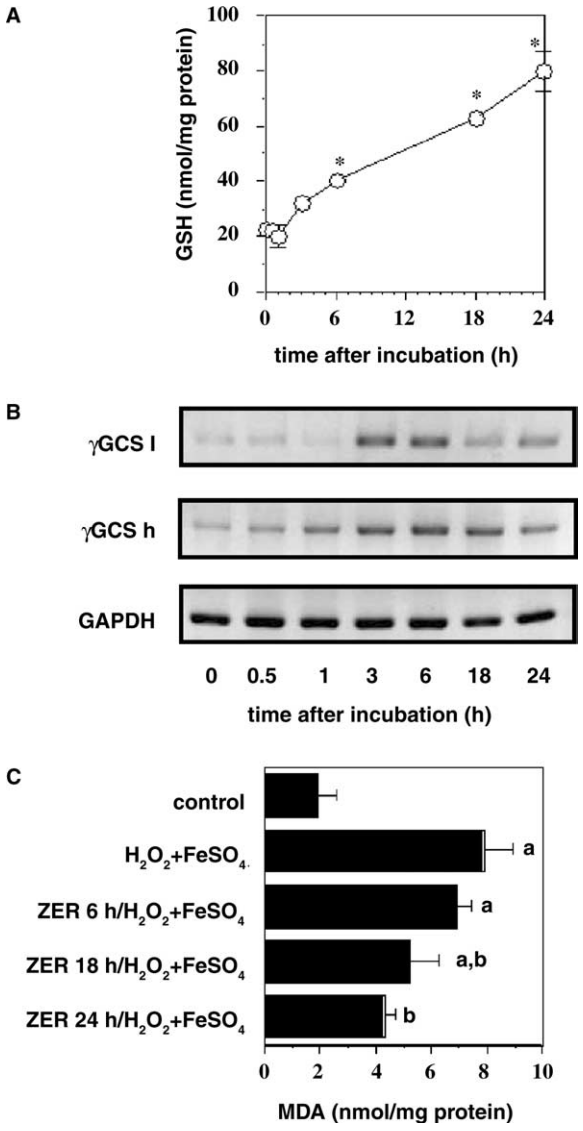


Fig. 4. ZER acts as a biologically active antioxidant. (A) Enhancement of intracellular GSH level by ZER. The cells were treated with 25 μM ZER for different time intervals. Intracellular GSH levels were colorimetrically measured as described in the Materials and Methods section. Statistical significance was determined by the Student's $t$ test and is expressed as: *, versus 0 h, $P < 0.05$ ($n = 3$). (B) Effect of ZER on mRNA expression of the GSH biosynthesis-related genes (γGCS1 and -γGCSh). Total RNA was extracted from RL34 cells untreated (0 h) or treated with 25 μM ZER for the indicated time periods, and then a RT-PCR analysis was carried out. (C) Inhibition of lipid peroxidation by ZER in RL34 cells. ZER (25 μM) was added to the cell suspension and incubated at 37 °C for the different periods as indicated. After rinsing twice with PBS, the cells were treated with $H_2O_2$ (100 μM) and $FeSO_4$ solutions (50 μM), which were incubated for another 1 h. Then, TBARS levels of the collected cells were determined. Data are means of three independent experiments. Statistical significance was determined by the Student's $t$ test and is expressed as: a, versus control, $P < 0.05$; b, versus positive control ($H_2O_2$+$FeSO_4$), $P < 0.05$.

scavenging effect against the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (data not shown). Conversely, treatment with 25 μM ZER led to an initial lowering of cellular GSH (87% of control at 1 h), which was followed by recovery and then a dramatic increase over the control (286% of controls at 24 h). The maximum effect on γGCS subunit mRNA levels was observed after 3 h of the ZER treatment (Fig. 4B), suggesting that ZER might enhance GSH biosynthesis via the gene expression of the rate-limiting enzyme. In addtion, the GPx activity towards *t*-butylhydroperoxide was significantly increased about 2.5-fold by the ZER treatment at 25 μM for 24 h (data not shown), consistent with the result of the GPx gene expression (Fig. 3C). These results prompted us to examine whether ZER inhibits lipid peroxidation in RL34 cells. The $H_2O_2/Fe^{2+}$-induced oxidative damage was evaluated by the TBARS level, a well-known biomarker of overall oxidative damage to cellular constituents such as membrane lipids. The quantitative data for the levels of TBARS formation in the $H_2O_2/Fe^{2+}$-treated cells are shown in Fig. 4C. The increased level of TBARS caused by the $H_2O_2/Fe^{2+}$ treatment was significantly higher than that of the control ($1.9 \pm 0.7$ versus $7.9 \pm 1.1$ nmol/mg protein, $P < 0.05$), Pretreatment with ZER (25 μM) before the $H_2O_2/Fe^{2+}$ treatment time-dependentry inhibited the increase in the TBARS level. Particularly, pretreatment for 24 h completely blocked the lipid peroxidation ($4.3 \pm 0.4$ nmol/mg protein, $P < 0.05$ versus $H_2O_2/Fe^{2+}$). Thus, treatment with ZER at the dose required for the induction of GSH and the GSH metabolizing enzymes actually exhibited an antioxidant effect in RL34 cells.

## 4. Discussion

Terpenoids, including mono-, sesqui-, di-, and triterpenoids, are biosynthesized by tandem reactions of the phosphorylated isoprene unit consisting of five carbons and are ubiquitously found in the plant kingdom. Some of these dietary terpenoids have anticarcinogenic activities in a variety of rodent experiments [24]. For example, *d*-limonene from the peel oil of orange or lemon has a chemopreventive activity against mammary, skin, liver, lung and forestomach carcinogenesis. Clinical trial results also demonstrated that some terpenoids have the potential of treating cancers without major toxicity [24]. The further understanding of their biological and physiological mechanisms may lead to the identification of more effective compounds in this category for the prevention and treatment of targeted cancer types. In the present study, we demonstrated that ZER, a major sesquiterpene compound of a tropical ginger *Zingiber zerumbet* Smith, induces phase II drug metabolizing enzyme activity potentially via the Nrf2-dependent pathway.

There is substantial and mounting evidence that phase II drug metabolizing enzymes, e.g., GST, NQO1, epoxide hydrolase, hemeoxygenase, and UDP-glucuronosyl-transferase, play important roles in the detoxification of electrophilic toxicants and their induction protects against carcinogenesis and mutagenesis [25], Recently, two transgenic rodent studies showed that deficiency in the GSTP1 expression caused particular sensitivity towards chemical carcinogenesis in mouse skin [6] and rat liver [7]. The Class pi rat and human GST isozymes are highly efficient in the GSH conjugation of carcinogenic benz[a]pyrene derivatives [8], widespread environmental pollutants in cigarette smoke and automobile exhaust. In addition, GSTP1 isozyme is more effective for the detoxification of electrophilic α, β-unsaturated carbonyl compounds produced by radical reactions, lipid peroxidation, ionizing radiation, and the metabolism of drugs than other GSTs [26]. Overexpression of human GSTA2 in human erythroleukemia K562 cells attenuates lipid peroxidation and confers resistance to these cells from $H_2O_2$ cytotoxicity. Indeed, some GST-inducing agents have been confirmed to be biologically active antioxidants in vivo [27,28]. Also, topical application of ZER resulted in a significant decrease in the $H_2O_2$ level in inflamed mouse skin [4]. Thus, the inducing ability of GST, being able to catalyze GSH-dependent reduction of hydroperoxide, as well as the induction of GSH and the GSH metabolizing enzymes may mainly participate in its antioxidant effect in RL34 cells.

The topic of the present study is that ZER, having an electrophilic property, is effective for GST induction, while the non-electrophilic HUM and 8-OH-HUM are not. This structure–activity relationship has also been found in some physiological activities, including suppression of Epstein–Barr virus activation [1] and inhibition of $O_2^-$ generation in differentiated HL-60 cells [2]. A potential candidate of the *trans*-acting factor(s) for the gene expression of GST and other Phase II enzymes has been recently identified to be the transcription factor Nrf2 [21]. It has also been shown by gene-targeted disruption in mice that Nrf2 is a general regulator of the Phase II enzyme genes in response to electrophiles and ROS [19,29]. The general regulatory mechanism underlying the electrophile counter attack response has been demonstrated [30], in which electrophilic agents alter the interaction of Nrf2 with its represser protein (Keapl; Kelch-like ECH-associated protein 1), thereby liberating the Nrf2 activity from repression by Keapl, culminating in the induction of the Phase II enzyme genes and antioxidative stress protein genes via ARE/EpRE [21]. Keap 1 contains 25 cysteine residues, 4 of which are expected to have highly reactive sulfhydryl groups with the inducers [31,32]. As shown in the present study, the enzyme inducing ability of electrophilic ZER is more significant than those of the other related compounds. Dinkova-Kostova et al. clearly demonstrated that the enhancement of phase II enzyme-inducer potency correlates with rapid reactivity of the agents with model sulfhydryl compounds [33]. Moreover, the nuclear localization of Nrf2 was observed in the ZER-treated RL34 cells (Fig. 3A). Therefore, the Keapl-Nrf2 complex is one of the most plausible candidates for the cytoplasmic sensor system that recognizes inducers including ZER.

Recently, one plausible mechanism that Keapl negatively regulates Nrf2 function by controlling its subcellular localization and accumulation has been proposed. Two research groups demonstrated that the accumulation of Nrf2 results from inhibition of its degradation by the 26 S proteasome [34,35], a feature common to many transcription factors including p53, c-Mye, c-Jun, and β-catenin [36]. McMahon et al. also confirmed that endogenous Nrf2 is subject to 26 S proteasome-dependent degradation and that its rate of degradation depends upon the redox environment of the cell [19]. Thus, under homeostatic conditions, Keapl interacts with Nrf2 and increases its rate of proteasome-mediated degradation, leading to a reduction in the cellular level. We demonstrated here that ZER, possibly by antagonizing the interaction

between Nrf2 and Keapl, stabilized Nrf2, leading to its rapid accumulation within the cell (Fig. 3B).

In conclusion, the results in the present study provide biological evidence that ZER has a significant ability to suppress oxidative stress possibly through induction of the endogenous antioxidants such as the phase II xenobiotic metabolizing enzymes as well as GSH. Considering the importance of oxidative damage in carcinogenesis, the antioxidant effect of ZER can be explored as a cancer chemopreventive agent targeted towards inflammation-related carcinogensis such as skin cancer and colon cancer.

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