Carcinogenesis vol.23 no.5 pp.795–802, 2002

Zerumbone, a Southeast Asian ginger sesquiterpene, markedly suppresses free radical generation, proinflammatory protein production, and cancer cell proliferation accompanied by apoptosis: the α,β-unsaturated carbonyl group is a prerequisite

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Zerumbone (ZER), a sesquiterpene from the edible plant *Zingiber zerumbet* Smith, has recently been found to suppress tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus activation in a potent manner. In the present study, we evaluated the anti-inflammatory and chemopreventive potentials of ZER in a variety of cell culture experiments. 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. 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 were also markedly diminished. These suppressive events were accompanied with a combined decrease in the medium concentrations of nitrite and prostaglandin E_2, while the expression level of COX-1 was unchanged. ZER inhibited the proliferation of human colonic adenocarcinoma cell lines (LS174T, LS180, COLO205, and COLO320DM) in a dose-dependent manner, while the growth of normal human dermal (2F0-C25) and colon (CCD-18 Co) fibroblasts was less affected. It also induced apoptosis in COLO205 cells, as detected by dysfunction of the mitochondria trans-membrane, Annexin V-detected translocation of phosphatidylserine, and chromatin condensation. Intriguingly, α-humulene, a structural analog 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). Taken together, our results indicate that ZER is a food phytochemical that has distinct potentials for use in anti-inflammation, chemoprevention, and chemotherapy strategies.

Introduction

In nature, terpenoids are biosynthesized by tandem reactions of the phosphorylated isoprene unit bearing five carbons. According to the number of combined isoprene units, they are classified into mono- (C10), sesqui- (C15), di- (C20), triterpenoids (C30), and so on. Recently, Suh *et al.* reported that novel synthetic triterpenoids have prominent potentials for suppressing inflammatory and carcinogenic processes, as demonstrated by cell-culture experiments (1,2). These are 3,12-dioxoolean-1-en-28-oic acid (TP-69) and 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), which, in a previous study, markedly suppressed the activity of a proinflammatory transcriptional factor, nuclear factor-kappaB (NF-κB), and thereby reduced the expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (1). Those experiments were followed by extensive studies on the differentiation-inducing and anti-proliferative activity of another analog, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) (2). Those results indicated that one of the notable and common structural characteristics among these three triterpenoids is the carrying of the α,β-unsaturated carbonyl group (1,2).

In our previous study, zerumbone (ZER, Figure 1), a sesquiterpene occurring in rhizomes (3) of *Zingiber zerumbet* Smith (Zingiberaceae), was identified as a distinct suppressor of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus (EBV) activation in Raji cells (3). In some Southeast Asian countries, the rhizomes of the plant are employed as a traditional medicine for anti-inflammation and the like (4), while the young shoots and inflorescence are used as condiments (5). It should be noted that the α,β-unsaturated carbonyl group is a notable moiety of ZER and, intriguingly, α-humulene (HUM, Figure 1), a ZER analog lacking this functional group, has been found to be virtually inactive to disrupt the TPA-induced biochemical pathways for EBV activation (3).

Inflammatory leukocyte-induced oxidative stress is associated with the biological processes of certain cancers (6,7). Superoxide anion (O_2^-) is a free radical generated from NADPH oxidase, predominantly present in leukocytes, and from xanthine oxidase (XO) in epithelial cells. O_2^- may be subsequently converted into more reactive intermediates such as the hydroxyl radical responsible for DNA mutations. On the other hand, there is a large body of data showing that expressions of iNOS and COX-2 are involved in the development of certain cancers (8,9). iNOS-produced nitric oxide (NO)

Abbreviations: AP, allopurinol; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; DPI, diphenyleneiodonium; EBV, Epstein–Barr virus; FBS, fetal bovine serum; FITC, fluorescence isothiocyanate; HUM, α-humulene; IFN, interferon; iNOS, inducible NO; synthase; IR, inhibitory rate; LPS, lipopolysaccharide; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-ΦB, nuclear factor-kappaB; NO, nitric oxide; NO_2^-, nitrite; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PG, prostaglandin; PI, propiodium iodide; PS, phosphatidylserine; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; XO, xanthine oxidase; ZER, zerumbone.

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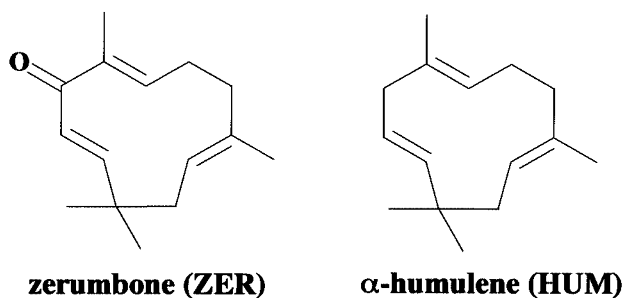


Fig. 1. Chemical structures of ZER and HUM. Their structural difference lies in the carbonyl group present in ZER, but not in HUM.

is non-enzymatically reacted with O_2^- to form peroxynitrite, a highly toxic anion causing a wide range of DNA and protein modifications (10). In contrast to COX-1, COX-2 activity is inducible and its elevation enhances the biosynthesis of prostaglandins (PGs), including PGE_2, which is one of the physiologically active and stable PGs produced in the pathways downstream of COX enzymes. PGE_2 stimulates bcl-2 activity and thereby prevents apoptosis (11). Food phytochemicals suppressing or inhibiting the above-mentioned biological activities are anticipated to be highly useful in anti-inflammatory and anti-carcinogenic strategies. These findings led us to investigate the suppressive effects of ZER in a series of *in vitro* bioassays designed to reflect inflammatory and carcinogenic processes. The activity of HUM was concurrently examined to discern if the α,β-unsaturated carbonyl group of ZER is a prerequisite component for exerting activities. The high potentials of ZER for anti-inflammation and anti-carcinogenesis as well as its responsive chemical moiety are herein demonstrated.

Materials and methods

Chemicals

ZER was isolated from the rhizomes of *Z.zerumbet* Smith as previously reported (purity >99%) (3). HUM was purchased from Tokyo Kasei Kogyo, (Tokyo, Japan). TPA was obtained from Research Biochemicals International (Natick, MA). Dulbecco's modified Eagle medium (DMEM), Minimum essential medium (MEM), RPMI 1640 medium, Ham's F-12 medium, and fetal bovine serum (FBS) were purchased from Gibco RBL, Rockville, NY. Lipopolysaccharide (LPS, *E.coli* serotype 0127, B8) was purchased from Difco Labs (Detroit, MI) and interferon (IFN)-γ from Genzyme (Cambridge, MA). Cytochrome *c* was obtained from Sigma, St Louis, MO. Ethidium bromide and acridine orange were obtained from Nakarai tesque (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless specified otherwise.

Cells

HL-60 (human promyelocytes), LS174T, LS180, COLO205 and COLO320DM (each human colon adenocarcinoma cells), and CCD-18 Co (human colon normal fibroblasts) were purchased from American Type Culture Collection, Rockville, MA. AS52 cells (Chinese hamster ovary cells) and RAW 264.7 cells (mouse macrophages) were kindly donated by Dr Marshall V.Williams, Department of Medical Microbiology and Immunology, Ohio State University, and Ohtsuka Pharmaceutical Co., Ohtsu, Japan, respectively. 2F0-C25 cells (human normal dermal fibroblasts) were obtained from Cell Systems, Kirkland, WA. The media used for each cell line culture were as follows: MEM (LS174T, LS180, and CCD-18 Co); RPMI 1640 (HL-60, COLO205, and COLO320DM); DMEM (RAW 264.7 and 2F0-C25); and Ham's F-12 (AS52). Ten percent FBS, 200 U/ml penicillin, and 250 μg/ml streptomycin were added to each medium, and cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

O_2^- generation in differentiated HL-60 cells and AS52 cells

An inhibitory test of TPA-induced O_2^- generation was performed as previously reported (12) with some modifications. HL-60 cells (5×10^5 cells/ml) were incubated in 1.3% dimethylsulfoxide (DMSO) in RPMI 1640 medium for 6 days. Differentiated HL-60 cells or AS52 cells (1×10^6) were incubated with the test compound dissolved in DMSO (0.5%, v/v), in 1 ml of Hank's buffer for 15 min. After washing, the cells were further incubated with 100 nM TPA and 1 mg/ml cytochrome *c* at 37°C for 60 min. The level of extracellular O_2^- was then measured using a cytochrome *c* reduction method. Cells treated only with the vehicle plus cytochrome *c* and with TPA plus cytochrome *c* were used as negative and positive controls, respectively.

NO_2^- production

A suppressive test of NO generation was done as previously reported (12). RAW 264.7 cells (8×10^5 cells/2 ml) on a 60 mm dish were treated with LPS (100 ng/ml), tetrahydrobiopterin (10 mg/ml), IFN-γ (100 U/ml), L-arginine (2 mM), and specified concentrations of the test compound dissolved in DMSO (0.5%, v/v). After 12 h, the levels of nitrite (NO_2^-) and cytotoxicity were measured using Griess and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively. Cells treated only with the vehicle and with LPS/IFN-γ were used as negative and positive controls, respectively.

PGE_2 production

RAW 264.7 cells were treated in the same manner described above. After 18 h, the concentrations of PGE_2 in the media were measured using a commercial experimental kit (Cayman, Ann Arbor, MI) according to the protocol of the manufacturer. The media was used without dilution. Cytotoxicity was measured using an MTT assay. Cells treated only with the vehicle and with LPS/IFN-γ were used as negative and positive controls, respectively.

Western blotting

Western blotting for the detection of iNOS, COX-2, and COX-1 expressions was done as previously reported (12). RAW 264.7 cells were stimulated in the same manner as described above. After the cells were washed, a boiling lysis solution was added, and then the cells were scraped from the dish, sonicated, and boiled for 10 min. Ten microgram protein samples were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, MA). After blocking, the membranes were incubated with a primary antibody (anti-mouse iNOS, 1:1000 dilution, Affinity Bioreagents, Golden, CO), and then a secondary antibody (peroxidase-conjugated swine anti-rabbit IgG, 1:1000 dilution, Dako, Glostrup, Denmark). The blots were developed using an ECL detection kit (Amersham Life Science, Buckinghamshire, UK). The antibodies were stripped and the blots were successively reprobed with each primary antibody. The first incubation was with: (i) goat anti-rat COX-2 antibody (cross-reacts with mouse counterparts, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); (ii) goat anti-rat COX-1 antibody (cross-reacts with mouse counterparts, 1:1000 dilution, Santa Cruz Biotechnology); or (iii) rabbit polyclonal anti-β-actin antibody (1:1000 dilution, Biochemical Technologies, Stoughton, MA). Each membrane was then incubated with a corresponding secondary antibody; (i) and (ii) with peroxidase-conjugated rabbit anti-goat IgG, 1:1000 dilution, Dako; and (iii) with peroxidase-conjugated swine anti-rabbit IgG (1:1000 dilution, Dako). The levels of iNOS COX-2 and COX-1 bands, which were observed in the LSP- and IFN-γ-stimulated cells, were corrected to 100% using those of β-actin as an internal standard.

Tumor necrosis factor (TNF)-α release

The supernatants from RAW 264.7 cells, stimulated as mentioned above, were measured for TNF-α concentrations in the media using a commercial experimental kit (Endogen, Woburn, MA) according to the protocol of the manufacturer. The media was used without dilution. Cells treated only with the vehicle and with LPS/IFN-γ were used as negative and positive controls, respectively.

Proliferation of cancer and normal cell lines

Each cell line was preincubated at a density of 5×10^4 cells/ml on a 24-well plate. After a 24 h incubation followed by medium refreshment, the test compound or vehicle (0.5% v/v DMSO) was added to the cell culture, which was incubated for another 72 h. Cell proliferation was measured using a cell counting kit (Dojin Laboratories, Kumamoto, Japan) according to the protocol of the manufacturer. Cells incubated only with the vehicle were used as a control and corrected to 100% of cell growth.

Dysfunction of mitochondria transmembrane

Cells with mitochondria transmembrane dysfunction were stained using an experimental kit, DePsipher^{TM} (Trevigen, Gaithersburg, MD), according to the protocol of the manufacturer. Briefly, COLO205 cells (5×10^5/2 ml) were incubated on a 35 mm-dish for 12 h, followed by incubation with ZER (50 μM) for 0, 6, 12, 24, and 36 h. After removal of the media, 1 ml of a lipophilic cation solution, containing 5,5′,6,6'-tetrachloro-1,1',3,3′-tetraethylbenzimidazoyl carbocyanin iodide, was added to the cells, and then they were incubated in a 5% CO_2 incubator for 20 min. After washing and stabilization, the cells were observed under a fluorescein microscope. Those in a normal condition were stained with reddish orange dye (emission at

590 nm) and apoptotic cells with green (emission at 530 nm) to determine disruption of the mitochondrial transmembrane potential. At least 500 cells were counted in each experiment.

Translocation of phosphatidylserine (PS)

Apoptotic cells were detected using an Annexin V-fluorescence isothiocyanate (FITC) Apoptosis Detection kit (Medical and Biological Laboratories, Nagoya, Japan) according to the protocol of the manufacturer. In brief, COLO205 cells (5×10^5/2 ml) were incubated on a 35 mm-dish for 12 h, followed by incubation with ZER (50 μM) or the vehicle (0.5% DMSO, v/v) for 0, 12, 24, or 48 h. After harvesting, the cells were suspended in 200 μL of binding buffer, and then 1 μl of Annexin V-FITC and 1 μl of propidium iodide (PI, final concentration; 1 μg/ml) were added. Following incubation at room temperature for 5 min in the dark, Annexin V-FITC binding and PI staining were detected with a flow cytometer (CytoACE 150, JASCO, Tokyo, Japan) (Ex 488 nm; Em = 530 nm) using FITC signal detector (FL1) and PI staining with a phycoerythrin emission signal detector (FL2).

Chromatin condensation

COLO205 cells (5×10^5/2 ml) were incubated on a 35 mm-dish for 12 h, and then incubated with ZER (50 μM) (0.5% DMSO, v/v) for 0 or 24 h. After harvesting and centrifugation (500 $g \times 5$ min), the cells were suspended at a density of 5×10^6 cells/ml. One milliliter of ethidium bromide solution (100 μg/ml in PBS) and 1 μl acridine orange solution (100 μg/ml in PBS) were added to a 25 μl cell suspension. A 5 μl cell suspension was placed on a slide glass and observed under a fluorescence microscope (magnification, ×200).

Protein determination

Protein concentrations in each bioassay were determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the protocol of the manufacturer, with γ-globulin employed as the standard.

Statistical analysis and inhibitory rate (IR)

Each experiment was done four times unless specified otherwise, and the data are shown as mean ± standard deviation (mean ± SD) values. The statistical significance of differences between groups in each assay was assessed by a Student's *t*-test (two-sided) that assumed unequal variance. The IR in each assay was calculated by the following equation: IR (%) = {1– [(test sample data) – (negative control data)][(positive control data) – (negative control data)]$^{-1}$} × 100.

Quantitative analysis of ZER

Fresh rhizomes, young stems, and inflorescence of *Z.zerumbet*, purchased at a local market in Bangkok, Thailand, were cut into small pieces and extracted with acetone at room temperature for 1 week. After filtration, the extracts were concentrated using an evaporator and subjected to HPLC analysis (column, Bondasphere C_{18}, Waters, Tokyo, Japan; mobile phase, 70% methanol in water; detection, UV 254 nm; flow rate, 1.0 ml/min; retention time, 25.0 min).

Results

Suppression of O_2^- generation from differentiated HL-60 cells and AS52 cells

DMSO-differentiated HL-60 cells, which mimic polymorphonuclear leukocytes, were used as a model for measuring the suppressive effect of ZER on O_2^- generation by NADPH oxidase. TPA treatment of differentiated HL-60 cells for 1 h led to marked extracellular O_2^- generation (190 ± 13 nmol/mg protein), as has been previously reported (12). As shown in Figure 2A, ZER (3–50 μM) reduced radical generation in a dose-dependent manner (IR = 70–100%), with complete suppression at a concentration of 50 μM. HUM (50 μM) showed no suppressive effect on radical generation. An NADPH oxidase inhibitor, diphenyleneiodonium (DPI, 100 μM), diminished O_2^- production by 78%, while an XO inhibitor, allopurinol (AP, 100 μM), did not. On the other hand, TPA stimulation of AS52 Chinese hamster ovary cells produced a significant concentration of O_2^- (13.7 ± 0.6 nmol/mg protein, Figure 2B). ZER (13–50 μM), but not HUM (50 μM) or DPI (100 μM), markedly suppressed radical generation (IR = 53–56%). AP (100 μM) also reduced extracellular O_2^- concentration (4.1 ± 0.4 nmol/mg protein) by 70%. Cell viability in each experiment was consistently 90% or higher (data not shown).

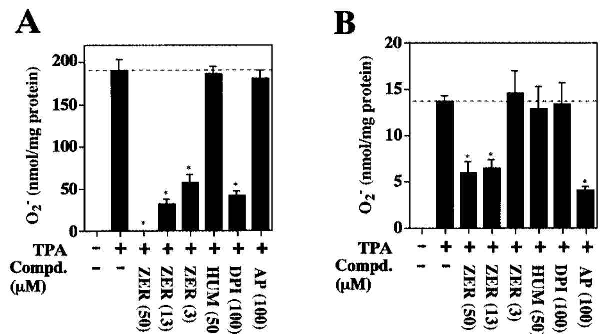


Fig. 2. Suppressive effects of ZER and HUM on TPA-induced O_2^- generation in DMSO-differentiated HL-60 cells (**A**) and AS52 cells (**B**). HL-60 cells (5×10^5 cells/ml) were differentiated by incubation in 1.3% DMSO in RPMI 1640 medium for 6 days. The differentiated HL-60 cells or AS52 cells (1×10^6) were incubated with the test compound, dissolved in DMSO (0.5%, v/v), in 1 ml of Hank's buffer for 30 min. After washing, the cells were further incubated with 100 nM TPA and 1 mg/ml cytochrome *c* at 37°C for 30 min. The level of extracellular O_2^- was then measured using a cytochrome *c* reduction method. *$P < 0.001$ versus positive control in Student's *t*-test.

Suppression of iNOS and COX-2 expression in RAW 264.7 cells

As shown in Figure 3A, the combined LPS- and IFN-γ-stimulated RAW 264.7 cells, from a mouse macrophage line, expressed a high expression level of iNOS protein after 12 h of incubation, in contrast to the vehicle control. ZER (6–50 μM) dramatically suppressed iNOS induction by 66–94%, which caused a decrease in NO_2^- concentrations that reflected NO production in the media (IR = 48–85%). Interestingly, HUM (50 μM) was virtually inactive to suppress both iNOS induction and NO_2^- formation. Along a similar line, ZER (13–50 μM) effectively suppressed both COX-2 protein expression (IR = 66–95%) and media concentration of PGE_2 (IR = 21–97%), whereas HUM failed to attenuate these LPS/IFN-γ-enhanced biological parameters. The expression levels of COX-1 in each experiment were steadily constant. Cell viability in each experiment was consistently 90% or higher (data not shown).

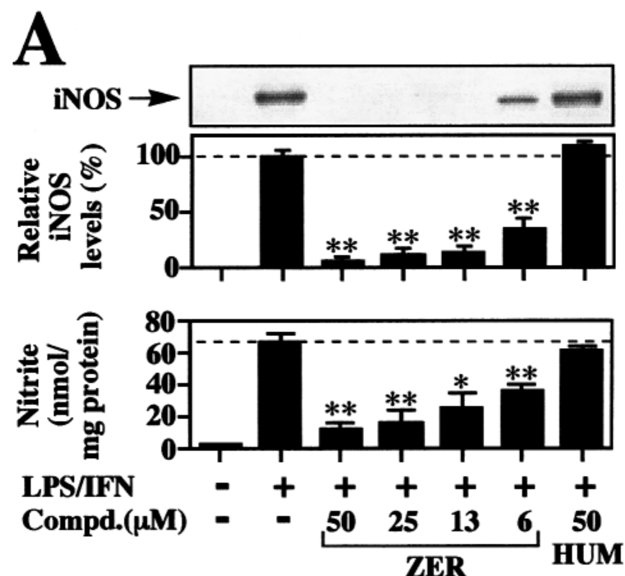
Suppression of TNF-α release from RAW 264.7 cells

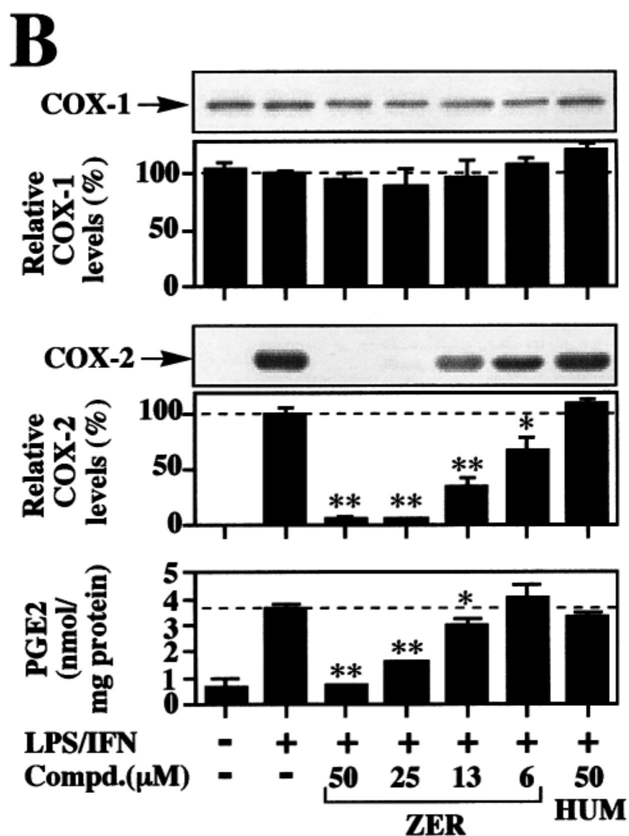
Besides iNOS/COX-2 expression, endotoxin and/or cytokine-stimulated RAW 264.7 cells have been reported to release a wide range of proinflammatory cytokines. In the present study, we examined the suppressive effect of ZER on LPS/IFN-γ-induced release of TNF-α, as well as its multiple roles in inflammation and tumor promotion. As shown in Figure 4, while TNF-α was undetectable in the media of non-stimulated RAW 264.7 cells, a significant amount (6.0 ± 0.8 ng/mg protein) of TNF-α was released into the media after 12 h of stimulation, whereas ZER (13–50 μM) counteracted it by 75–100%. HUM (50 μM) did not reduce the TNF-α concentration. Cell viability in each experiment was consistently 90% or higher (data not shown).

Inhibition of colon adenocarcinoma cell proliferation

The inhibitory effects of ZER on the proliferation of four cell lines derived from human colon adenocarcinomas (LS174T, LS180, COLO205, and COLO320DM) were examined and the activity was compared with that of a known anti-proliferative agent, *n*-butyric acid (13). In addition, normal human dermal (2F0-C25) and colon (CCD-18 Co) fibroblast lines were used to assess the ability of ZER for selectively inhibiting the growth of cancer cell lines. As summarized in

Table I, ZER at a concentration of 50 μM showed a wide spectrum of potent inhibition towards cancer cell proliferation (IR = 85–97%), as well as at 13 μM (IR = 51–90%). The anti-proliferating potency of ZER (13–50 μM) was comparable to or higher than that of *n*-BA (3 mM, IR = 58–91%). Interestingly, while ZER at 50 μM significantly inhibited the proliferation of two normal fibroblast lines (IR = 35 and 32%, each $P < 0.01$), a concentration of 13 μM did not have any effect on the growth of these cells. HUM (50 μM) did not affect normal or transformed cell proliferation.





Apoptosis induction in COLO205 cells

COLO205 cells were selected for the studies of apoptosis because they were most sensitive to ZER treatment (Table I). ZER (50 μM) time-dependently caused a dysfunction of the mitochondria transmembrane in COLO205 cells, as shown in Figure 5A where viable and mitochondria-dysfunctioned cells can be seen stained reddish orange and green, respectively. The dysfunction of the mitochondria transmembrane was initiated in a small fraction (10.4%) of the cells 6 h after treatment, and grew to become predominant after 12 (52%), 24 (8.2%), and 36 h (100%). Next, translocation of PS was detected on a flow cytometer using Annexin V-FITC as a fluorescence probe. PI was used to distinguish viable and dead

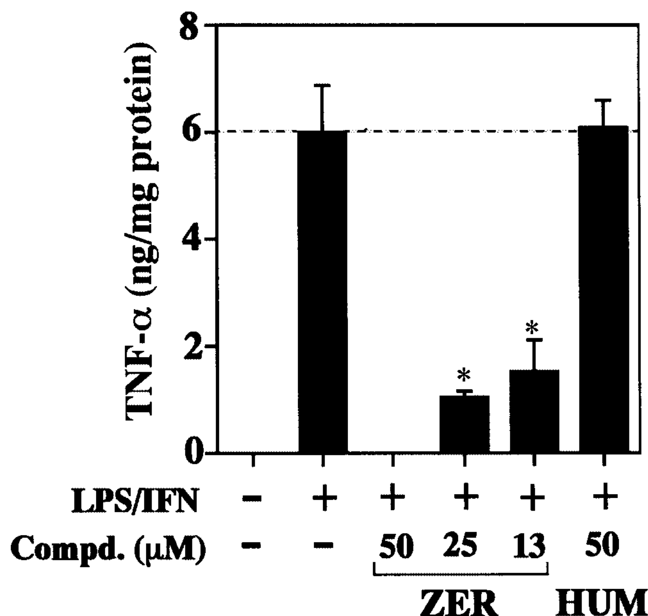


Fig. 4. Suppressive effects of ZER and HUM on LPS- and IFN-γ-induced TNF-α release from RAW264.7 cells. The supernatants from RAW 264.7 cells stimulated with LPS and IFN-γ for 12 h were measured for TNF-α concentrations in the media using a commercial experimental kit. The media was used without dilution. $^*P < 0.001$ versus LPS/IFN-γ control in Student's *t*-test.

Fig. 3. Suppressive effects of ZER and HUM on LPS- and IFN-γ-induced iNOS and COX-1 and COX-2 expressions in RAW264.7 cells. RAW 264.7 cells (8×10^5 cells/2 ml) in a 60 mm dish were treated with LPS (100 ng/ml), tetrahydrobiopterin (10 mg/ml), IFN-γ (100 U/ml), L-arginine (2 mM) and specified concentrations of the test compound dissolved in DMSO (0.5%, v/v). After 12 or 18 h of incubation, the levels of NO_2^- (12 h), PGE_2 (18 h) and cytotoxicity (12 and 18 h) were measured as described in the 'Materials and methods' section. Western blotting for detecting iNOS, COX-2 and COX-1 expressions was performed using cells stimulated in the same manner as described above. Ten microgram protein samples were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with appropriate antibodies as described in the 'Materials and methods' section. The blots were developed using an ECL detection kit. The levels of iNOS, COX-2 and COX-1 bands, observed in the LSP- and IFN-γ-stimulated cells, were corrected to 100% using those of β-actin as an internal standard. (**A**) iNOS protein expression and NO_2^- concentrations, $^*P < 0.01$, $^{**}P < 0.001$ versus LPS/IFN-γ control in Student's *t*-test. (**B**) COX-1 and COX-2 protein expressions, $^*P < 0.05$, $^{**}P < 0.01$ versus LPS/IFN-γ control in Student's *t*-test.

Table I. Inhibitory activities of ZER on proliferation of human colon adenocarcinoma and normal cell lines

	Cell growth (%)					
	LS174T	LS180	COLO205	COLO320DM	2Fo-C25	CCD-18Co
Blank	100 ± 11	100 ± 12	100 ± 11	100 ± 17	100 ± 6.8	100 ± 13
n-BA (3 mM)	9.2 ± 2.1[a]	30.8 ± 7.5[a]	24.0 ± 9.2[a]	42.1 ± 2.6[a]	24.0 ± 6.3[a]	61.0 ± 6.9[b]
ZER (50 μM)	2.7 ± 1.0[a]	15.6 ± 3.0[a]	4.9 ± 1.9[a]	6.3 ± 0.1[a]	65.3 ± 7.8[b]	67.8 ± 6.8[b]
ZER (13 μM)	44.1 ± 5.4[a]	49.4 ± 7.1[a]	10.0 ± 1.1[a]	47.1 ± 3.5[a]	102 ± 9.8	100 ± 11
HUM (50 μM)	102 ± 8.0	93.5 ± 8.4	90.8 ± 10	89.5 ± 13	90.0 ± 11	101 ± 5.8

LS174T, LS180, COLO205 and COLO320DM are human colon adenocarcinoma cells. 2F0-C25 and CCD-18 Co are human dermal and colon normal fibroblasts, respectively. Each cell line was preincubated at a density of 5×10^4 cells/ml on a 24-well plate. After a 24 h incubation followed by medium refreshment, the test compound or vehicle (0.5% v/v DMSO) was added to the cell culture, which was incubated for another 72 h. Cell proliferation was measured using a cell counting kit according to the protocol of the manufacturer. Cells incubated only with vehicle were used as the control.
[a]$P < 0.001$ vs. Blank in Student's *t*-test. [b]$P < 0.01$ vs. Blank in Student's *t*-test.

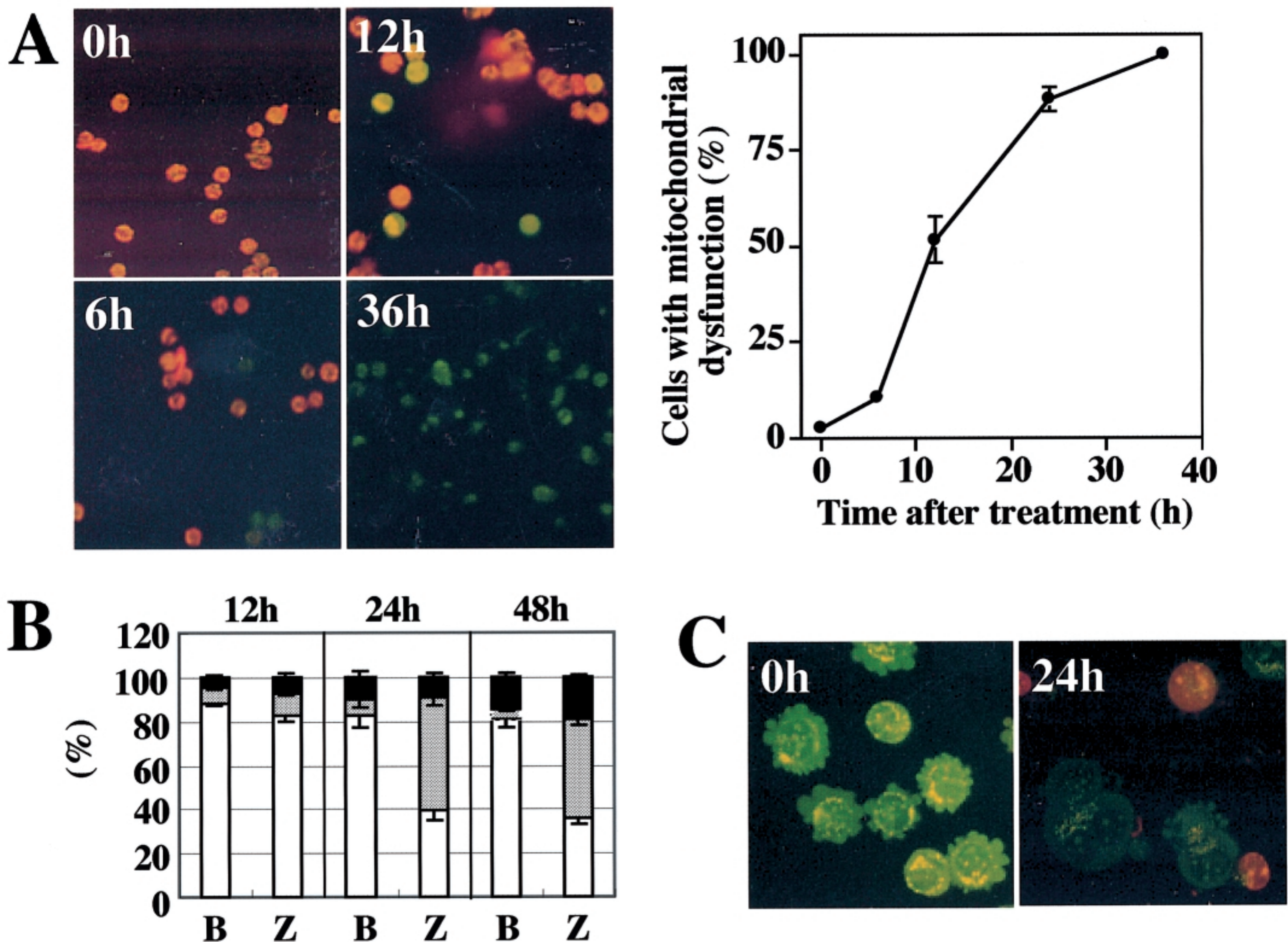


Fig. 5. Apoptosis-inducing activity of ZER in COLO205 cells. COLO205 cells (5×10^5/2 ml) were incubated in a 35 mm dish for 12 h. The cells were then incubated with ZER (50 μM) for the times indicated in the panel. (**A**) rate of cells with mitochondrial transmembrane dysfunction. Cells in normal condition were stained reddish orange (emission at 590 nm) and apoptotic cells green (at 530 nm), due to disruption of the mitochondrial transmembrane potential. Cells with transient color were recognized as green. Original magnification, ×100. (**B**) The proportions of viable, apoptotic and dead COLO205 cells treated with vehicle or ZER (50 μM). Open bars, viable cells (Annexin V-FITC-negative and PI-negative). Dotted bars, apoptotic cells (Annexin V-FITC-positive and PI-negative). Closed bars, dead cells (PI-positive). B, vehicle control. Z, ZER. (**C**) ZER-induced chromatin condensation. Left, vehicle control. Right, ZER (50 μM) after 24 h. The chromatin DNA of live cells was stained with bright green fluorescence, while in the early phase apoptotic cells chromatin was condensed and stained with green fluorescence and in the late phase apoptotic cells chromatin was condensed and stained with orange fluorescence. In necrotic cells, chromatin was normal and stained with orange fluorescence. Original magnification, ×200.

Table II. Distribution of ZER throughout *Zingiber zerumbet*

Part	Purpose for use	Content (mg/kg fresh weight)
Rhizomes	Medicine	2650 ± 294
Young stems	Condiment	38.1 ± 12.6
Inflorescences	Condiment	27.2 ± 6.70

(both apoptotic and necrotic) cells. As shown in Figure 5B, a large fraction of COLO205 cells treated with ZER (50 μM) was found to be apoptotic (both FITC-positive and PI-negative) after 24 and 36 h of incubation (51.1% and 44.4%, respectively). In addition, chromatin condensation was observed in those cells treated with ZER (50 μM) for 24 h (Figure 5C, right), which was in contrast to the vehicle control (Figure 5C, left).

Distribution of ZER throughout Z.zerumbet

We next examined the occurrence of ZER in three parts of *Z.zerumbet* (rhizomes, young stems, and inflorescence). As summarized in Table II, a very large amount of ZER (2650 p.p.m.) was detected in the rhizomes, which are used locally as an anti-inflammatory medicine (4). It is notable that ZER was also present in some of the edible parts, including young stems and inflorescence, which are used in traditional cooking (5).

Discussion

ZER has been reported to be cytotoxic (14) and to dramatically suppress EBV activation (3). In those two studies, one of the common and notable findings was that HUM, lacking only the α,β-unsaturated carbonyl group present in ZER, was virtually and consistently inactive (3,14). Compounds carrying this reactive group have been shown to exhibit versatile biological activities, e.g., inhibition of tumor cell growth (14), induction of differentiation (15), apoptosis (16), insulin-like growth factor-I and Waf-1 (17), heat shock protein (18), glutathione *S*-transferase activity (19), and cytoprotective activity (20). Modifications of cysteine residues (21) and consumption of intracellular glutathione (16) have been implicated as the essential action mechanisms of α,β-unsaturated carbonyl agents. Interestingly, Suh *et al.* demonstrated that, while natural oleanolic and ursolic acids are poor suppressors of the *de novo* synthesis of iNOS/COX-2, analogous compounds, provided with this reactive group, have a much higher activity (1,2). We assume that these synthetic analogs, but not oleanolic or ursolic acid, are highly effective, at least in part, on account of bearing the α,β-unsaturated carbonyl group.

Rossi *et al.* recently discovered that anti-inflammatory cyclopentenone PGs directly inhibit IκB kinase by modifying the cysteine residue (21) (Figure 6A), thereby attenuating IκB phosphorylation to abolish NF-κB activity. It is well known that NF-κB is the key transcriptional factor for synthesis of proinflammatory mediators, including iNOS, COX-2, and TNF-α. By structural analogy, ZER (Figure 6B), but not HUM (Figure 6C), can be recognized to have a chemical property resembling that of cyclopentenone PGs, suggesting that ZER is able to suppress IκB kinase activity. Therefore, the effect of ZER on IκB kinase activity with the use of HUM remains to be examined. If our premise is correct, the action mechanisms by which ZER attenuates iNOS/COX-2 induction and TNF-α release may be associated with a possible abrogation of

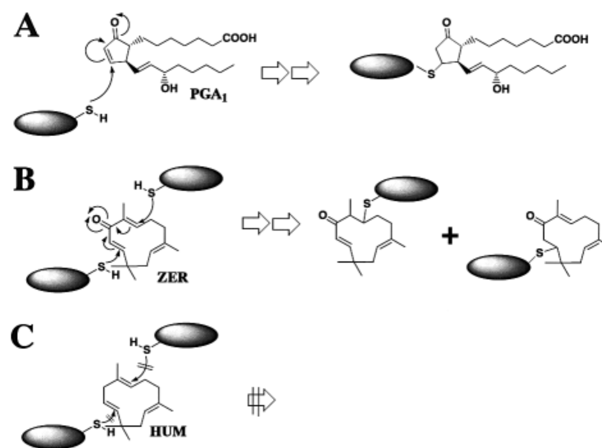


Fig. 6. Chemical property of ZER, but not HUM, to react with biological nucleophiles. PGA_1 has been reported to conjugate with a cysteine residue of IKK for inactivation (**A**) (ref. 21). ZER has a similar chemical property to mimic this reaction (**B**) to yield two possible adducts of ZER-target molecules, while HUM does not (**C**), because it lacks the carbonyl group present in ZER. Hatched object represents macromolecule(s) in biological systems that have reactive nucleophilic group(s), which is IKK in the case of PGA_1.

NF-κB activity. Conversely, we recently observed that ZER suppressed the combined LPS- and IFN-γ-induced degradation of IκB protein (Murakami *et al.*, unpublished observation), suggesting the efficacy of ZER for inhibiting IκB kinase activity. In any case, the chemical property of ZER to conjugate biological nucleophiles may be essential for exerting its activity.

In the present study, we showed for the first time that TPA triggers O_2^- generation in AS52 cells. XO, at least in part, is an enzyme responsible for O_2^- generation, because an XO inhibitor, AP, markedly inhibited radical generation, whereas an NADPH oxidase inhibitor DPI did not. Having neither O_2^- scavenging nor XO inhibiting activity, ZER may cause a dysfunction of the XO activation processes in AS52 cells. Although the O_2^- generating capacity of XO is much less than that of NADPH oxidase (~1/14-fold), XO has been reported to be involved in certain cancers (22–24). Along a similar line, ZER is recognized as a chemical agent that disrupts the assembly and/or activity of NADPH oxidase in DMSO-differentiated HL-60 cells. It is interesting to note that protein kinase C may be associated with the activation processes of NADPH oxidase (25), and tyrosine kinase with those of XO (26). Recent reports provide some interesting findings that O_2^- is not only an oxidant in biological systems, but also mediates the downstream effects of Ras and Rac in non-phagocytic cells (27), and yet is responsible for cellular transformation (28). The biological ability of ZER to suppress O_2^- generation *in vitro* led us to anticipate its marked chemopreventive effects in certain organs, where oxidative stress becomes dominant, by mitigating oxidative damage and/or disrupting signal transduction pathways for carcinogenesis. Accordingly, we have so far reported the notable chemopreventive activity of 1'-acetoxychaviaol acetate (29), auraptene (30), AL-1 (31), and nobiletin (12), all of which are able to suppress O_2^- generating biochemical processes (32).

iNOS-produced excess amounts of NO are involved in carcinogenic processes in the esophagus (33), stomach (34), colon (35), liver (9), and pancreas (36). There are ample reports indicating that chronic inflammation is closely associated with certain cancers, such as in the stomach and colon. Besides

oxidative stress derived from O_2^- and NO generation, activated leukocytes play some critical roles in carcinogenesis processes, e.g. production of bioactive PGs through COX-2 induction and release of proinflammatory cytokines to stimulate cancer cell growth, as well as to trigger, enhance, and maintain leukocyte activation via autocrine and paracrine loops. A proinflammatory cytokine, TNF-α (37), is an important agent in some processes of tumor development through its induction of the biosynthesis of matrix metalloproteinase and plasminogen activator (38), by expressing COX-2 (39) and iNOS (40), along with an association with tumor promotion (41). In fact, accumulating evidence from rodent and human studies demonstrates that COX-2 expression and TNF-α-release are the key steps in multi-step carcinogenic processes in a wide range of target organs (42–50). Further, it should be pointed out that the biological efficacy of ZER to suppress free radical generation, iNOS/COX-2 induction, and TNF-α-release is conspicuous among the food phytochemicals we have so far reported, including 1'-acetoxychaviaol acetate (29), auraptene (30), and AL-1 (31), as well as resveratrol and nobiletin (12).

The anti-proliferative effect of ZER toward cancer cell lines (Table I) can be attributable, at least in part, to its apoptosis-inducing activity (Figure 5). Although the precise molecular mechanisms for inducing apoptosis are still unknown, dysfunction of the mitochondria transmembrane is involved, since mitochondria are a common target for endogenous and exogenous apoptotic stimuli. In fact, several agents, including capsaicin (51), staurosporine (52), rotenone (53), and curcumin (54), have been previously shown to disturb mitochondrial functions in apoptotic pathways. It is known that the concentrations of intracellular calcium and cAMP are important determinants of *caspase-3* expression (55), which leads, in turn, to DNA fragmentation, and yet the possibilities of release of reactive oxygen species and cytochrome *c* from mitochondria (56–58) should be explored in the future. Interestingly, some recent reports indicate that inhibition and/or suppression of COX-2 is a determinant of apoptosis induction in the lung (59), colon (60, 61), liver (62), stomach (63), and prostate (64), suggesting the activity of ZER in these organs.

Overall, our present data and previous findings implicate that ZER has great potential to suppress carcinogenesis in multiple experimental models. In fact, we recently observed that it prevented dimethylbenz[*a*]anthracene-initiated and TPA-promoted papilloma formation in mouse skin by topical application (Murakami *et al.*, in preparation), and oral feeding of ZER inhibited azoxymethane-induced colonic aberrant crypt foci formation in rat colons, which was accompanied with a reduction of COX-2 protein expression in colonic mucosa (65). ZER is readily available from the rhizomes of *Z.zerumbet*, thus further chemopreventive studies can be attempted in multiple target organs.

In conclusion, ZER showed a wide range of suppressive activities in cell culture systems relating to inflammatory and carcinogenic processes, while HUM was found to be a useful analog to explore the role of the α,β-unsaturated carbonyl group in the action mechanisms of this intriguing sesquiterpenoid.

Acknowledgements

This work was supported by the Grant-in-Aid from the Urakami Foundation and from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Dr Marshall V.Williams, Department of Medical Microbiology and Immunology, Ohio State University, for generously donating us with AS52 cells and K.Yamamoto and A.Sasaki for their technical assistance.

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Received December 17, 2001; revised January 28, 2002; accepted January 29, 2002