Zerumbone Enhances TRAIL-Induced Apoptosis through the Induction of Death Receptors in Human Colon Cancer Cells: Evidence for an Essential Role of Reactive Oxygen Species

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Abstract

Identification of the active component and mechanisms of action of traditional medicines is highly desirable. We investigated whether zerumbone, a sesquiterpene from tropical ginger, can enhance the anticancer effects of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). We found that zerumbone potentiated TRAIL-induced apoptosis in human HCT116 colon cancer cells and that this correlated with the up-regulation of TRAIL death receptor (DR) 4 and DR5. Induction of DRs occurred at the transcriptional level, and this induction was not cell-type specific, as its expression was also up-regulated in prostate, kidney, breast, and pancreatic cancer cell lines. Deletion of DR5 or DR4 by small interfering RNA significantly reduced the apoptosis induced by TRAIL and zerumbone. In addition to up-regulating DRs, zerumbone also significantly down-regulated the expression of cFLIP but not that of other antiapoptotic proteins. The induction of both DRs by zerumbone was abolished by glutathione and *N*-acetylcysteine (NAC), and this correlated with decreased TRAIL-induced apoptosis, suggesting a critical role of reactive oxygen species. Inhibition of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase but not of Jun NH_2-terminal kinase abolished the effect of zerumbone on DR induction. Zerumbone also induced the p53 tumor suppressor gene but was found to be optional for DR induction or for enhancement of TRAIL-induced apoptosis. Both bax and p21, however, were required for zerumbone to stimulate TRAIL-induced apoptosis. Overall, our results show that zerumbone can potentiate TRAIL-induced apoptosis through the reactive oxygen species–mediated activation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase leading to DR4 and DR5 induction and resulting in enhancement of the anticancer effects of TRAIL. [Cancer Res 2009;69(16):6581–9]

Introduction

Many traditional medicines have been used for thousands of years, and although they are safe and affordable, neither the active components nor the mechanisms of action are understood. Moreover, as many as 70% of all drugs approved by the U.S. Food and Drug Administration in the past 25 years have been based on natural products. Zerumbone, a sesquiterpene, was first isolated from the rhizome of a subtropical wild shampoo ginger (*Zingiber zerumbet*) and determined to be an anti-inflammatory agent (1). It was found to have anticancer activity against a wide variety of tumor cells, including colon cancer (1, 2), leukemia (3), myeloid cancer (4), and liver cancer (5), and to inhibit phorbol ester–induced EBV activation (6). *In vivo*, this agent was found to suppress azoxymethane-induced colonic aberrant crypt foci in rats (2, 7), dextran sodium sulfate–induced colitis in mice (8), skin tumor initiation and promotion in mice (9), myeloid tumors in mice (4), cholecystokinin-induced acute pancreatitis in rats (10), CXCR4-mediated invasion of breast and pancreatic tumor cells (11), colon and lung carcinogenesis in mice (12), and human breast cancer–induced bone loss in nude mice (13). How zerumbone mediates all these effects is not understood, but suppression of constitutive and inducible nuclear factor-κB activation and nuclear factor-κB–regulated gene products has been shown by different groups (12, 14). In contrast, zerumbone has been found to have very little or no cytotoxic effect on the normal human endothelial cells (3) and dermal fibroblasts (1). The cytotoxic effect of zerumbone on leukemia cells was found to be mediated through the induction of Fas receptors (3), leading to activation of caspase-8. Whether zerumbone has any effect on apoptosis mediated through other death receptor (DR) is not known.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily, is believed to regulate tumor cells killing through five different receptors, but only two of them, DR4 and DR5, are transmembrane receptors and mediate apoptosis. Engagement of DR4 and DR5 leads to the activation of caspase-8, which then leads to activation of caspase-9 and caspase-3 (15–17). The activation of caspase-8, also called FLICE, is inhibited by FLICE-interacting protein (cFLIP). The evidence that TRAIL may have an anticancer role also stems from the report that TRAIL deficiency accelerates the progression of hematologic malignancies (18). Because of its selectivity toward tumor cells, both TRAIL and agonistic antibodies against its receptor are currently in clinical trials for cancer treatment in combination with various chemotherapeutic agents (17).

Some tumor cells have been shown to be resistant to TRAIL, and this resistance appears to be mediated through the loss of TRAIL receptors, enhanced expression of caspases inhibitors such as cFLIP, X-linked inhibitor of apoptosis protein, cellular inhibitor of apoptosis protein, and survivin, or alternations in expression of the Bcl-2 family proteins (19). Thus, agents that can up-regulate TRAIL receptors and down-regulate antiapoptotic proteins have the potential to enhance the apoptotic effects of TRAIL. In this study, we sought to determine the effect of zerumbone on TRAIL-induced apoptosis in colon cancer cells. Our results show that zerumbone can potentiate the apoptosis induced by TRAIL

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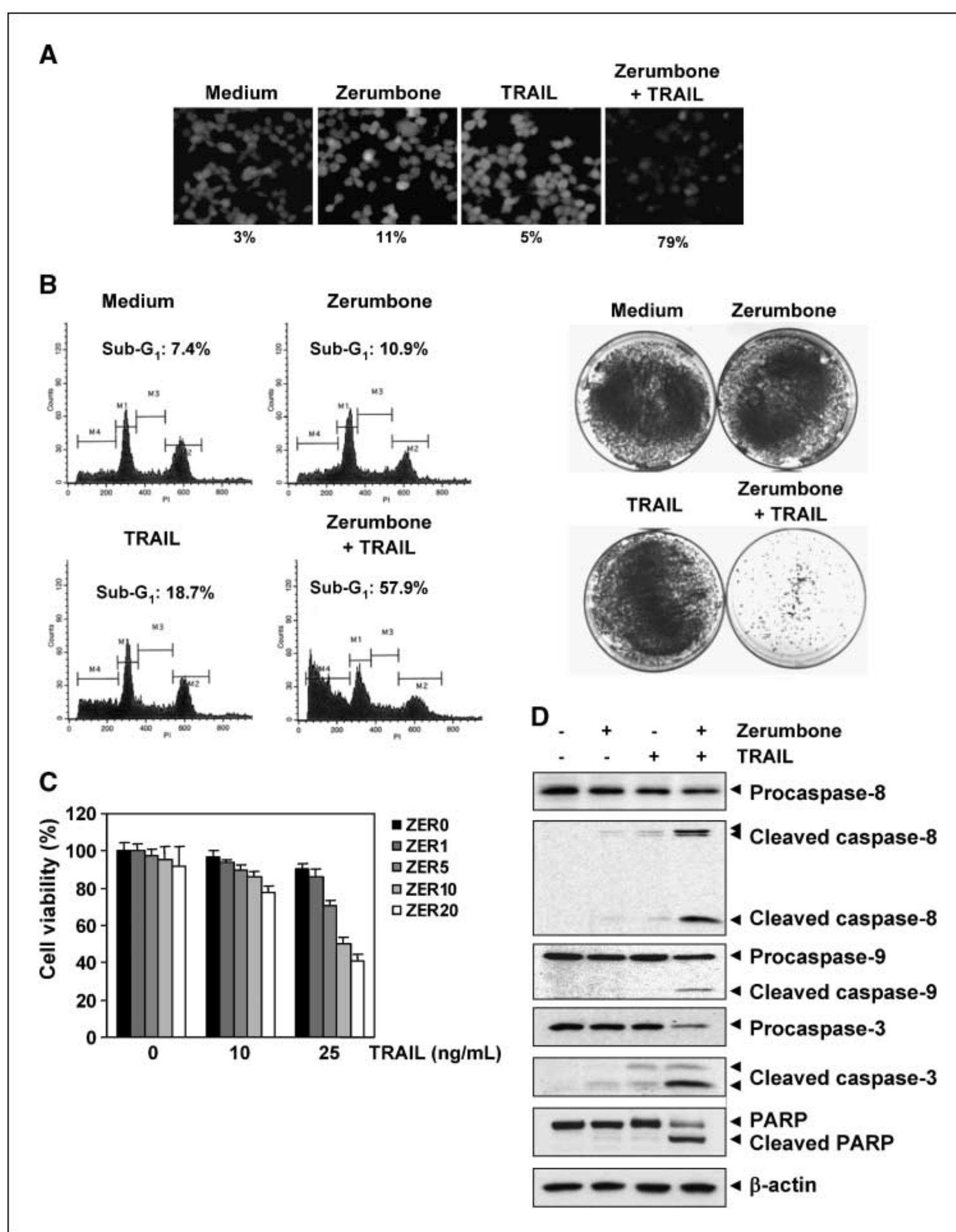

Figure 1. Zerumbone enhances TRAIL-induced HCT116 cell death. *A,* cells were treated with 20 μmol/L zerumbone for 12 h and washed with PBS. Cells were treated with 25 ng/mL TRAIL for 24 h. Cell death was determined using the Live/Dead Assay. *B, left,* cells were treated with zerumbone for 12 h and washed with PBS. Cells were treated with 25 ng/mL TRAIL for 24 h. Cells were stained with propidium iodide, and the sub-G_1 fraction was analyzed using flow cytometry. *Right,* cells were treated with zerumbone for 12 h, washed, and treated with TRAIL (25 ng/mL) for 12 h. Cells were then reseeded in 100 mm dishes and incubated. After 14 d, cells were stained with crystal violet. *C,* cells were pretreated with various concentrations of zerumbone for 12 h, medium was removed, and cells were exposed to TRAIL for 24 h. Cell viability was then analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide method. *D,* cells were pretreated with zerumbone for 12 h and washed out. Cells were treated with TRAIL for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against caspase-3, caspase-8, caspase-9, and PARP.

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through the up-regulation of DR4 and DR5 expression and the down-regulation of cFLIP.

Materials and Methods

Materials. Zerumbone was kindly supplied by Dr. Akira Murakami (Kyoto University). Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech. Penicillin, streptomycin, DMEM, RPMI 1640, and fetal bovine serum were obtained from Invitrogen. All antibodies were obtained from Santa Cruz Biotechnology, BD Biosciences, or Cell Signaling. Small interfering RNA (siRNA) for DR5, DR4, and extracellular signal-regulated kinase (ERK) 1 were synthesized or purchased from Qiagen.

Cell lines. Human cell lines HCT116 (colon adenocarcinoma), HT29 (colon adenocarcinoma), H1299 (lung adenocarcinoma), A293 (embryonic kidney carcinoma), PC3 (prostate adenocarcinoma) and DU145 (prostate adenocarcinoma), MDA-MB-231 (breast adenocarcinoma) and MCF-7 (breast adenocarcinoma), and AsPC-1 (pancreatic adenocarcinoma) were obtained from the American Type Culture Collection. HCT116 variants with deletions in p53, p21, and bax were kindly supplied by Dr. Bert Vogelstein (John Hopkins University). The colon cancer cells (HCT116 and HT29) were cultured in McCoy's 5A medium with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). A293, MDA-MB-231, and MCF-7 were cultured in DMEM, and other cells lines were cultured in RPMI 1640 with 10% fetal bovine serum and penicillin/streptomycin.

Live/Dead Assay. This assay was done as described previously (14).

Cytotoxic assay. The effects of zerumbone on the cytotoxic effects of TRAIL agents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake method as described (20).

Clonogenic assay. Treated and untreated cells were seeded in 100 mm Petri dishes, allowed to form colonies for 14 days, and then stained as described (21).

Analysis of cell surface expression of DR4 and DR5. Treated and untreated cells were stained with phycoerythrin-conjugated mouse monoclonal anti-human DR5 or DR4 (R&D Systems) for 45 min at 4°C according to the manufacturer's instructions, resuspended and analyzed

by flow cytometry with phycoerythrin-conjugated mouse IgG2B as an isotype control.

Propidium iodide staining for DNA fragmentation. Cells were pretreated with zerumbone for 12 h and then exposed to TRAIL for 24 h. Propidium iodide staining for DNA content analysis was done as described elsewhere (22).

RNA analysis and reverse transcription-PCR. Total RNA was extracted from the treated cells according to the manufacturer's instructions (Invitrogen) and RT-PCR was done as described (11).

Transfection with siRNA. HCT116 cells were plated in each well of 6-well plates and allowed to adhere for 24 h. On the day of transfection, 12 μL Hiperfect transfection reagent (Qiagen) was added to 50 nmol/L siRNA in a final volume of 100 μL culture medium. After 48 h of transfection, cells were treated with zerumbone for 12 h and then exposed TRAIL for 24 h.

Western blot analysis. To determine the levels of protein expression, we prepared whole-cell extracts and analyzed by Western blot as described previously (14). To measure activation of mitogen-activated protein kinase (MAPK), whole-cell extracts from treated cells were subjected to Western blotting for phosphorylated ERK1/2, p38, and Jun NH_2-terminal kinase (JNK). The same blots were stripped and reprobed with ERK1/2, p38, and JNK to ensure equal loading.

Results

The present studies were designed to investigate the effect of zerumbone on TRAIL-induced apoptosis. HCT116 colon cancer cells were used for most studies, but other cell types were also used to determine the specificity of this effect.

Zerumbone sensitizes colon cancer cells to TRAIL-mediated apoptosis. We determined whether zerumbone enhances TRAIL-induced apoptosis in HCT116 colon cancer cells. For this, cells were pretreated with zerumbone (20 μmol/L) for 12 h, washed with PBS to remove zerumbone, and then exposed to TRAIL (25 ng/mL) for 24 h. The results indicated that zerumbone and TRAIL treatment alone induced 11% and 5% apoptosis, respectively. Combination treatment with both zerumbone and TRAIL enhanced apoptosis to 79% (Fig. 1*A*). When apoptosis was examined using propidium iodide staining, we found that apoptosis was induced at 10.9% by zerumbone, 18.7% by TRAIL, and 57.9% by the combination of the two (Fig. 1*B*, *left*).

Whether zerumbone enhances the effect of TRAIL in long-term colony formation assay was also examined. We found that, under the conditions when zerumbone or TRAIL alone had minimal effect on colony formation of HCT116 cells, the combination treatment completely suppressed the colony-forming ability of these tumor cells (Fig. 1*B*, *right*).

We also examined the effect of zerumbone on TRAIL-induced cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, which detects the mitochondrial activity. For this experiment, cells were pretreated with various

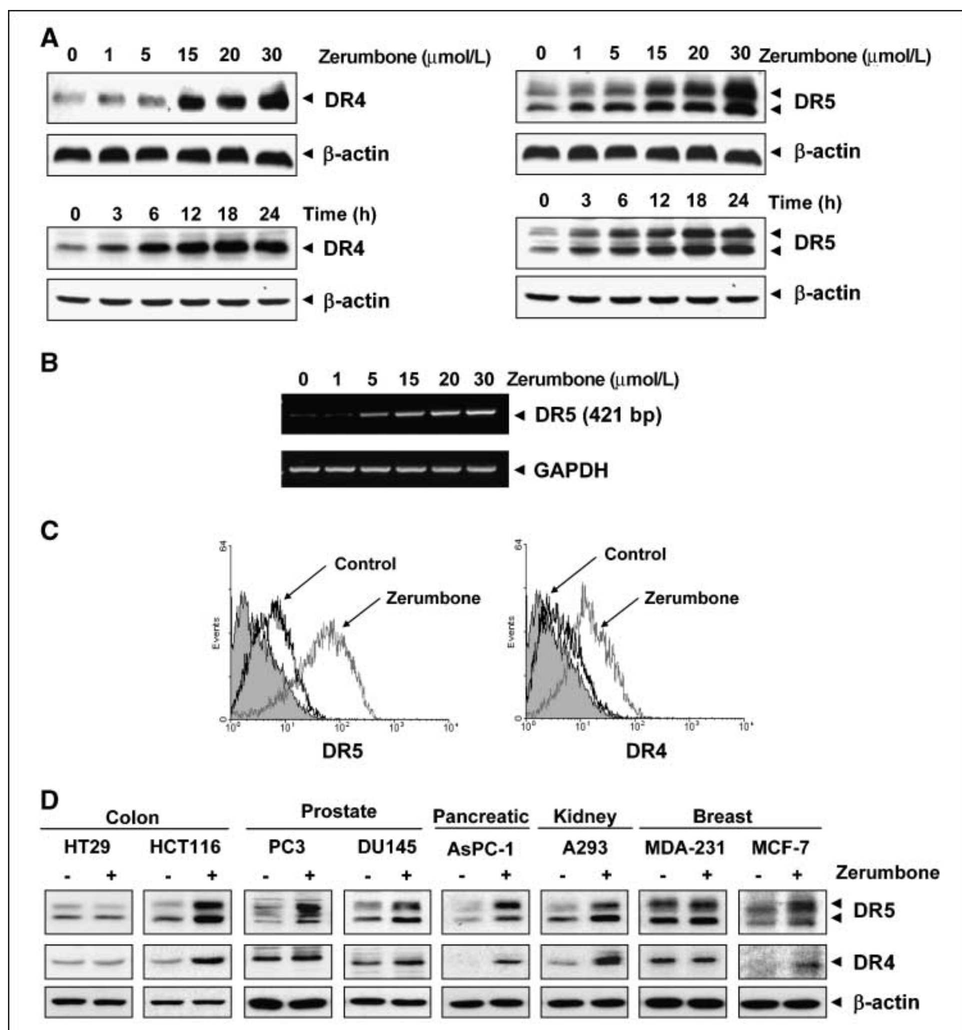

Figure 2. Zerumbone-induced DR5 and DR4 expression. *A,* HCT116 cells were treated with indicated doses of zerumbone at the indicated times. Whole-cell extracts were prepared and analyzed for DR4 and DR5 expression by Western blotting. *B,* zerumbone-induced DR5 gene expression. HCT116 cells were treated with zerumbone for 24 h, and total RNA was extracted and examined for expression of DR5 by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control to show equal RNA loading. *C,* HCT116 cells were treated with 20 μmol/L zerumbone for 24 h and then harvested for analysis of cell surface DR4 and DR5 by immunofluorescent staining and subsequent flow cytometry. *Filled gray peaks,* cells stained with a matched control phycoerythrin-conjugated IgG isotype antibody. *D,* cells were treated with 20 μmol/L zerumbone for 24 h, and whole-cell extracts were prepared and analyzed by Western blotting using. Equal protein loading was evaluated by β-actin.

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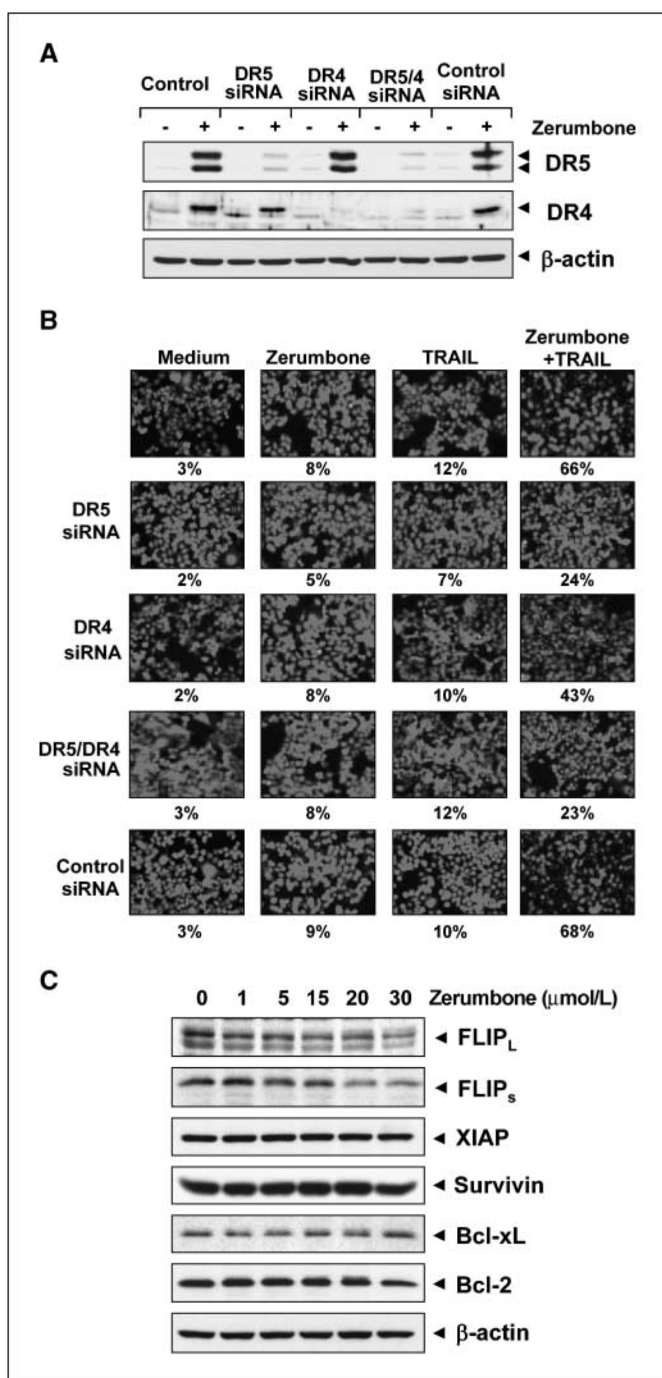

Figure 3. Effects of knockdown of DRs on zerumbone-induced sensitization of TRAIL. *A,* HCT116 cells were transfected with DR5 siRNA, DR4 siRNA, and control siRNA alone or combined. After 48 h, cells were treated with 20 μmol/L zerumbone for 24 h, and whole-cell extracts were subjected to Western blotting for DR5 and DR4. *B,* cells were seeded in a chamber slide and transfected with siRNAs. After 48 h, cells were pretreated with 20 μmol/L zerumbone for 12 h and then incubated with 25 ng/mL TRAIL for 24 h. Cell death was determined by the Live/Dead Assay. *C,* effects of zerumbone on antiapoptotic protein expression. HCT116 cells were pretreated with the indicated dose of zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using relevant antibodies.

concentrations of zerumbone for 12 h and then exposed to TRAIL for 24 h. HCT116 cells were moderately sensitive to either zerumbone or TRAIL alone. However, pretreatment with zerumbone enhanced TRAIL-induced cytotoxicity (Fig. 1*C*).

Next, we examined the effect of zerumbone, TRAIL, and the combination on the activation of caspase-8, caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP) cleavage. We found that although zerumbone and TRAIL had little effect on the caspases and PARP, the two together were highly effective (Fig. 1*D*). Together, our results indicate that zerumbone can enhance TRAIL-induced apoptosis.

Zerumbone induces the expression of TRAIL receptors DR4 and DR5 in cancer cell lines. To determine how zerumbone potentiates TRAIL-induced apoptosis, we investigated its effect on TRAIL receptors (DR4 and DR5). For this, HCT116 cells were treated with different concentrations of zerumbone for 24 h, and whole-cell extracts were prepared and examined for expression of DR4 and DR5 proteins. Zerumbone induced both DR4 (Fig. 2*A, top left*) and DR5 (Fig. 2*A, top right*) in a dose-dependent manner, with optimum induction occurring at around 20 to 30 μmol/L. Whether this induction of the DRs was dependent on time was also examined. Zerumbone induced both DR4 (Fig. 2*A, bottom left*) and DR5 (Fig. 2*A, bottom right*) in a time-dependent manner.

To determine whether induction of TRAIL receptors by zerumbone occurs at the transcriptional level, we examined mRNA for DR5 expression after cells had been treated with various concentrations of zerumbone. As shown in Fig. 2*B*, zerumbone induced the transcript for DR5 in a dose-dependent manner, thus suggesting that zerumbone acts at the transcriptional level.

Whether zerumbone enhances the expression of DRs on cell surface was also examined. For this, we analyzed cell surface expression of DR5 and DR4 in cells exposed to zerumbone. We found that zerumbone increased cell surface levels of DR5 and DR4 (Fig. 2*C*). The level of DR4 cell surface expression induced by zerumbone was lower than that of DR5. Collectively, these results indicate that zerumbone up-regulated the expression of both DRs on the cell surface.

We also investigated whether up-regulation of DR5 and DR4 by zerumbone was specific to HCT116 or also occurs in other cell types. For this, colon cancer cells (HT29 and HCT116), prostate cancer cells (PC3 and DU145), pancreatic cancer cells (AsPC-1), embryonic kidney cells (A293), and breast cancer cells (MDA-MB-231 and MCF-7) were exposed to zerumbone (20 μmol/L) for 24 h and then examined for DR5 and DR4 protein expression. Zerumbone induced the expression of DR5 (Fig. 2*D, top*) in HCT116, PC3, DU145, AsPC-1, A293, and MCF-7 cells. The sesquiterpene induced the expression of DR4 (Fig. 2*D, middle*) in HCT116, AsPC-1, A293, and MCF-7 cells. No significant induction of DR4 was noted in prostate cancer cell lines PC3 and DU145 (Fig. 2*D*). Induction of either DR4 or DR5 was minimal in breast cancer MDA-MB-231 and colon cancer HT29 cells after exposure to zerumbone. Together, these findings suggest that the up-regulation of DR5 and DR4 by zerumbone is not cell type specific.

DR induction by zerumbone is needed for TRAIL-induced apoptosis. To determine the role of DR5 and DR4 in TRAIL-induced apoptosis, we used siRNA specific to DR5 and DR4 to down-regulate the expression of these receptors. Transfection of cells with siRNA for DR5 but not with the control siRNA reduced zerumbone-induced DR5 expression (Fig. 3*A*). Similarly, transfection of cells with siRNA for DR4 reduced the zerumbone-induced DR4 expression (Fig. 3*A*). However, DR4 siRNA had minimal effect on the zerumbone-induced up-regulation of DR5.

We next examined whether the suppression of DR5 or DR4 by siRNA could abrogate the sensitizing effects of zerumbone on TRAIL-induced apoptosis using an esterase staining assay (the

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Live/Dead Assay). The results reveal that the effect of zerumbone on TRAIL-induced apoptosis was effectively abolished in cells transfected with either DR5 or DR4 siRNA (Fig. 3*B*), whereas treatment with control siRNA had no effect (Fig. 3*B*). Silencing of DR5 had more dramatic effect on TRAIL-induced apoptosis than that of DR4. The silencing of both receptors abolished the apoptosis as much as silencing of DR5 alone, thus suggesting that DR5 is a major player in TRAIL-induced apoptosis.

Zerumbone down-regulates the expression of cFLIP but has no effect on X-linked inhibitor of apoptosis protein, survivin, and Bcl-2 family proteins. We examined whether zerumbone has any effect on the expression of any of the antiapoptotic proteins. Cells were exposed to different concentrations of zerumbone for 24 h and then examined for expression of cFLIP, X-linked inhibitor of apoptosis protein, survivin, Bcl-xL, and Bcl-2 expression. Zerumbone down-regulated the expression of both forms of cFLIP but had little effect on the other antiapoptotic proteins (Fig. 3*C*). Thus, it is possible that zerumbone affects TRAIL-induced apoptosis not only through induction of DR4 and DR5 but also through the down-regulation of cFLIP, an inhibitor of caspase-8.

Zerumbone-induced up-regulation of TRAIL receptors is dependent on reactive oxygen species. There has been a report that TRAIL-induced apoptosis is regulated by reactive oxygen species (ROS; ref. 23). Another study suggested that induction of DR5 by agents such as curcumin requires ROS and exposure of renal cancer cells to peroxide induces DR5 (24). We sought to determine whether zerumbone-induced TRAIL receptors are also regulated by ROS. As shown in Fig. 4*A*, pretreating HCT116 cells with the ROS scavenger *N*-acetylcysteine (NAC) reduced the zerumbone-induced up-regulation of both DR5 and DR4 expression in a dose-dependent manner. We also examined the effect of glutathione on the zerumbone-induced expression of TRAIL receptors and found that glutathione pretreatment abolished the induction of DR4 and DR5 by zerumbone in a dose-dependent manner (Fig. 4*B*). This suggests the critical role of ROS in the induction of TRAIL receptors by zerumbone.

We next examined whether scavenging of ROS could attenuate the TRAIL-induced cell death enhanced by zerumbone. As shown in Fig. 4*C*, zerumbone significantly enhanced TRAIL-induced apoptosis in colon cancer cells, and pretreatment of cells with

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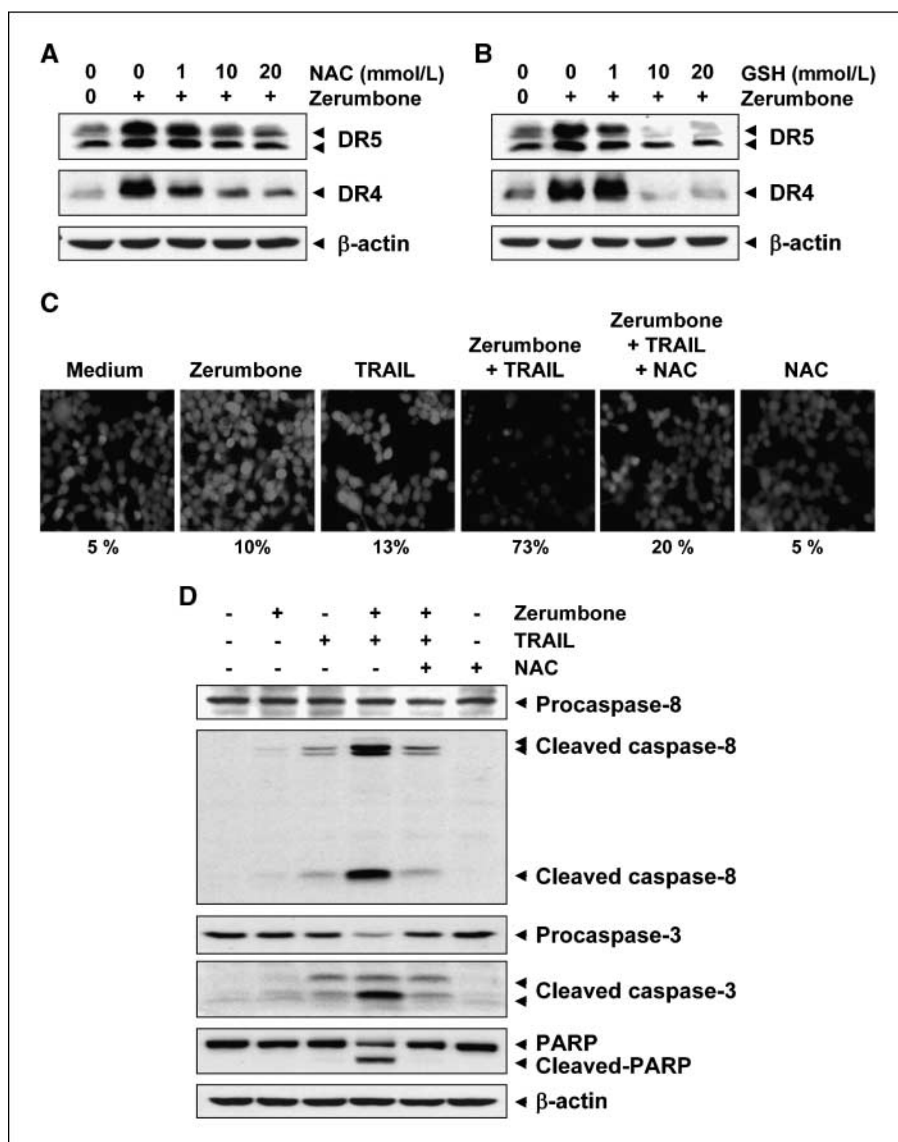

Figure 4. Up-regulation of DR4 and DR5 by zerumbone was mediated by ROS. HCT116 cells were pretreated with various concentrations of NAC (*A*) or glutathione (*GSH*; *B*) for 1 h. Cells were treated with 20 μmol/L zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using DR5 and DR4 antibodies. *C,* NAC reversed cell death induced by the combination of zerumbone and TRAIL. HCT116 cells were pretreated with NAC for 1 h and then treated with zerumbone for 12 h. Next, cells were washed with PBS and treated with TRAIL for 24 h. Cell death was determined by the Live/Dead Assay. *D,* NAC suppressed caspase activation and PARP cleavage induced by combination of zerumbone and TRAIL. HCT116 cells were pretreated with NAC for 1 h and then treated with zerumbone for 12 h. Cells were then washed with PBS and treated with TRAIL for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using relevant antibodies. β-Actin was used as a loading control.

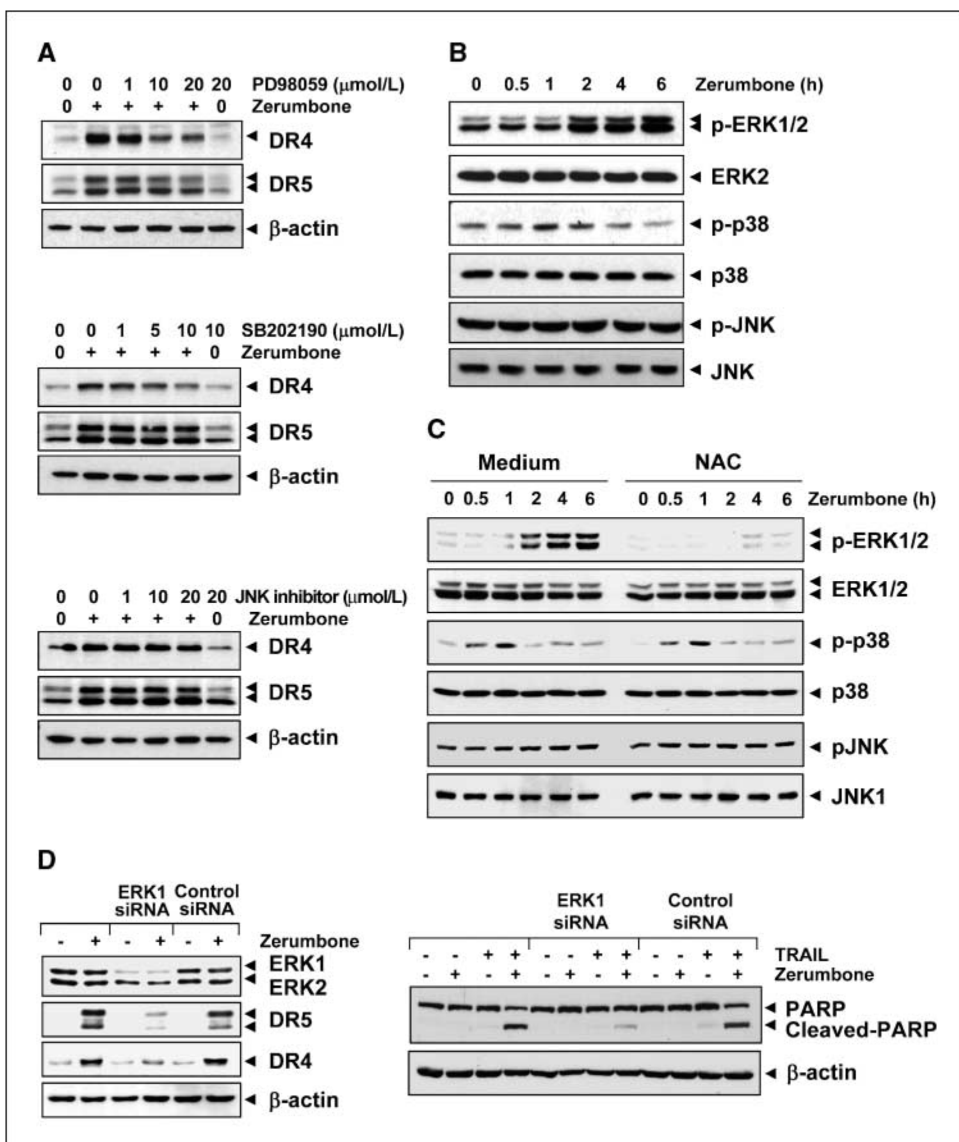

Figure 5. *A,* up-regulation of DR4 and DR5 by zerumbone was mediated through the activation of MAPK pathway. Cells were pretreated with various concentrations of PD98059, SB202190, and JNK inhibitor for 1 h and then treated with 20 μmol/L zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using DR4 and DR5 antibodies. *B,* zerumbone-activated ERK. Cells were treated with 20 μmol/L zerumbone and whole-cell extracts were subjected to Western blotting for phosphorylated ERK1/2, p38, and JNK. The same blots were stripped and reprobed with ERK1/2, p38, and JNK to ensure equal loading. *C,* zerumbone-induced ERK activation is dependent on ROS. Cells were pretreated with NAC (20 mmol/L) for 1 h and then exposed to 20 μmol/L zerumbone for the indicated times. Whole-cell extracts were prepared and subjected to Western blotting using relevant antibodies. *D,* blockade of ERK reversed effect of zerumbone on TRAIL-mediated cell death. Cells were transfected with either ERK1 siRNA or control siRNA. After 48 h, cells were treated with 20 μmol/L zerumbone for 24 h, and whole-cell extracts were subjected to Western blotting (*left*). *Right,* cells and siRNA-transfected cells were pretreated with 20 μmol/L zerumbone for 12 h and then incubated with 25 ng/mL TRAIL for further 24 h. Cell death was determined by the PAPR cleavage. Equal protein loading was evaluated by β-actin.

NAC markedly reduced this zerumbone-induced enhancement from 73% to 20%.

Then we determined whether NAC pretreatment also attenuates the zerumbone-induced increase in activation of caspase-8, caspase-3, and PARP cleavage (Fig. 4*D*). We found that zerumbone enhanced TRAIL-induced cleavage of procaspase-8, procaspase-3, and PARP and that NAC abolished this increase, again suggesting the critical role of ROS.

Up-regulation of TRAIL receptors by zerumbone is reversed by inhibitors of MAPK. MAPKs, including ERK1/2, p38 MAPK, and JNK, have been shown to mediate the up-regulation of DR5 by bisindolylmaleimide and LY303511 (25, 26). We investigated whether these MAPKs have any role in zerumbone-induced TRAIL receptors. As shown in Fig. 5*A*, pretreatment of cells with an ERK1/2 inhibitor (PD98059) suppressed the zerumbone-induced up-regulation of DR4 and DR5 in a dose-dependent manner.

Pretreatment of cells with a p38 inhibitor (SB202190) also reduced zerumbone-induced DR4 up-regulation but to a lesser extent (Fig. 5*A, middle*). No effect of the p38 inhibitor was observed on zerumbone-induced DR4 and DR5 expression. Compared with inhibitors of ERK and p38 MAPK, JNK inhibitor had no effect on zerumbone-induced DR4 or DR5 expression (Fig. 5*A, bottom*).

We also examined whether zerumbone can activate ERK, p38 MAPK, and JNK. We found that zerumbone activated ERK in a time-dependent manner (Fig. 5*B, top*). Zerumbone activated p38 MAPK transitorily between 1 and 2 h and declined thereafter (Fig. 5*B, middle*). No effect of zerumbone was observed on the activation of JNK. Thus, the activation of these various MAPKs is consistent with the results obtained with the effect of their inhibitors on the zerumbone-induced expression of TRAIL receptors.

Zerumbone-induced activation of MAPK is dependent on ROS. Thus far, our data indicated that ERK and ROS are involved in expression of DR by zerumbone. Whether zerumbone-induced activation of MAPK is also dependent on ROS production was examined. To do this, cells were exposed to antioxidant NAC (20 mmol/L) for 1 h and then treated with zerumbone (30 μmol/L) for indicated times and examined for MAPK activation. We found that zerumbone activated ERK and the presence of NAC inhibited the zerumbone-induced phosphorylation of ERK but had no effect on activation of p38 MAPK and JNK (Fig. 5*C*). These results

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indicate that zerumbone-induced activation of MAPK is dependent on ROS.

Silencing of ERK blocks DR up-regulation and TRAIL sensitization. We next determined whether silencing of ERK blocks DR up-regulation and inhibits TRAIL sensitization. To examine this, HCT116 cells were transfected with control and specific siRNA against ERK1. After 48 h, cells were then exposed to zerumbone for 24 h and harvested, and lysates were subjected to Western blotting. The result indicates that the expression of ERK1 was significantly reduced in cells treated specific siRNA when compared with cells left untreated or treated with control siRNA (Fig. 5*A, left*). The reduction in expression of ERK1 by the siRNA correlated with suppression of zerumbone-induced up-regulation of DR5 and DR4 (Fig. 5*A, left*).

Furthermore, siERK1 significantly inhibited the enhancement of TRAIL-induced PARP cleavage by zerumbone. However, control siRNA had no effect (Fig. 5*D, right*).

Zerumbone can induce p53 but is not required for expression of TRAIL receptors. Numerous reports have suggested that p53 can induce DRs (27, 28). We examined whether zerumbone can induce p53 and, if so, whether this action mediates the induction of TRAIL receptors. As shown in Fig. 6*A*, zerumbone induced p53 expression in a dose- and time-dependent manner.

To determine whether p53 is needed for zerumbone-induced DRs induction, we used H1299 lung cancer cells that are deficient in p53 protein expression. As shown in Fig. 6*B* (*left*), zerumbone induced DR5 but not DR4 in p53-deficient H1299 cells, although these cells do not express p53 protein. To clarify the difference between results using colon and lung cancer cell lines, we also used a HCT116 variant in which p53 is deleted. Again, we found that zerumbone induced DR5 as well as DR4 in both wild-type and p53-deleted cells, thus indicating that p53 has no role in the induction of either DR by zerumbone (Fig. 6*B, right*).

To determine whether deletion of p53 modulates the apoptotic effect of zerumbone on TRAIL-induced apoptosis, we examined PARP cleavage. Enhanced apoptosis was observed when TRAIL was combined with zerumbone in wild-type cells, but this was unaffected in p53-deleted cells, suggesting that p53 induction plays little role in TRAIL-induced apoptosis (Fig. 6*C, left*). The enhancement of apoptosis by zerumbone, however, was abolished in cells in which either p21 (Fig. 6*C, middle*) or bax was deleted (Fig. 6*C, right*), suggesting the critical role of these two proteins.

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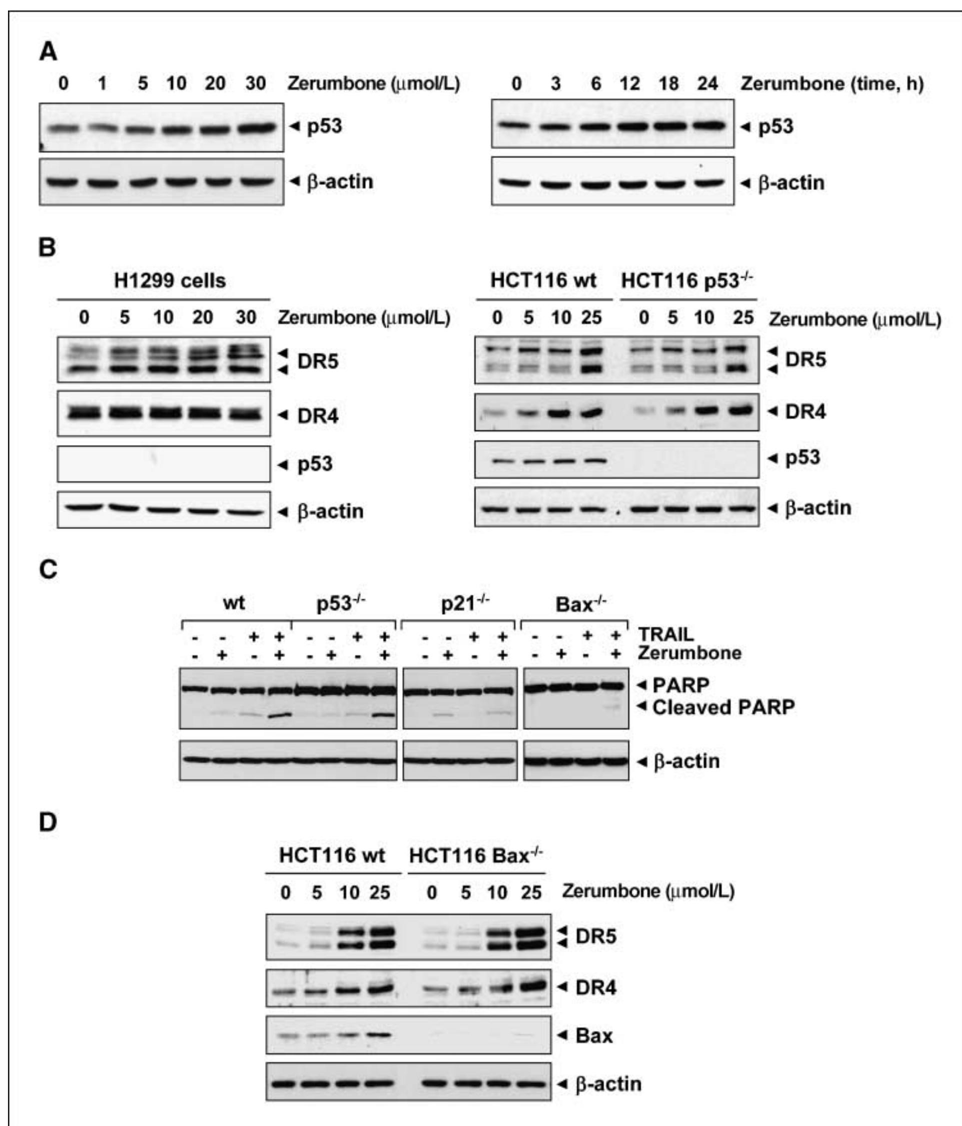

Figure 6. *A,* zerumbone increased p53 expression. HCT116 cells were treated with various concentrations and times. Whole-cell extracts were prepared and analyzed by Western blotting using p53 antibody. *B,* effect of p53 on zerumbone-induced DR expression. H1299 cells (p53-null cells; *left*) and HCT116 and p53 variant cells (*right*) were treated with zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using relevant antibodies. *C,* cells were pretreated with zerumbone for 12 h and exposed to TRAIL for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using indicated antibodies. *D,* HCT116 and bax variant cells were treated with zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using relevant antibodies. Equal protein loading was evaluated by β-actin. *wt,* wild-type.

Deletion of bax has no effect on up-regulation of DR by zerumbone. Whether the deletion of bax modulates the expression of DR by zerumbone in HCT116 cells was examined. The effect of bax on the expression of DR5 and DR4 induced by zerumbone was investigated using bax-deleted cells. Results show that zerumbone increased the expression of bax, DR5, and DR4 in wild-type cells, but deletion of bax had no effect on the induction of these receptors by zerumbone (Fig. 6*D*). These results suggest that bax is not linked to the up-regulation of DR expression by zerumbone.

Discussion

In the present report, we show that zerumbone, a component of wild ginger, can enhance the apoptotic effects of TRAIL against colon cancer cells. The mechanism by which zerumbone mediates its effects on TRAIL-induced apoptosis appears to involve the induction of TRAIL receptors and down-regulation of cFLIP, an inhibitor of caspase-8. We found that the induction of DRs by zerumbone was not cell type specific but was observed in a wide variety of cell types, including colon, breast, prostate, kidney, and pancreatic cancer cells. Induction of TRAIL receptors in some cells, however, was much more pronounced than that in other cell types. We found that zerumbone had no effect on human breast cancer MDA-MB-231 and colon cancer HT29 cells. Both of these cell lines are known to express mutant p53 (29, 30). Our results, however, show that the lack of induction of receptors by zerumbone is not due to mutation in p53. The mechanism by which this sesquiterpene induces DR5 appears to occur at the transcriptional level. We also found that zerumbone induces TRAIL receptors through the generation of ROS as shown by our results that NAC and glutathione abolished the induction of DRs. The results regarding the induction of DRs by zerumbone through ROS are consistent with those in previous reports regarding curcumin (24), inactive phosphatidylinositol 3-kinase inhibitor LY303511 (26), proteasome inhibitors (28), H_2O_2 (31), baicalein (32), and 15-deoxy-Δ12,14-prostaglandin J_2 (33). We found that an increase in TRAIL-induced apoptosis by zerumbone was also reversed by NAC and glutathione. Apoptosis induced by TRAIL alone is also known to be regulated in colon cancer cells through the generation of ROS (23).

Besides the induction of DRs, the down-regulation of cFLIP by zerumbone may also lead to the enhancement of TRAIL-induced apoptosis. Zerumbone was found to have little effect on other antiapoptotic proteins, including X-linked inhibitor of apoptosis protein, survivin, Bcl-2, and Bcl-xL. Interestingly, like zerumbone, a sesquiterpene, the synthetic triterpenoids 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid and its methyl ester analogue have also been shown to down-regulate the expression of cFLIP and to enhance TRAIL-induced apoptosis (34, 35). In addition to terpenoids, celecoxib and honokiol enhance TRAIL-induced apoptosis through the down-regulation of cFLIP (36, 37).

We found that the induction of DR4 and DR5 by zerumbone required the activation of ERK1/2 and MAPK as shown by our results that zerumbone activated these kinases and that their inhibitors abolished zerumbone-induced expression of TRAIL receptors. Given that ROS induces MAPK (26, 38, 39), it is possible that zerumbone induces TRAIL receptors through the sequential induction of ROS and MAPK. In contrast to ERK, p38 MAPK was found to be involved in induction of DR4 but not that of DR5. JNK was found to have no role in the induction of TRAIL receptors by zerumbone. That activation of ERK1/2 by Ras can lead to the up-regulation of DR4 and DR5 has been shown previously (40). In addition, quercetin has been shown to augment TRAIL-induced apoptosis through the ERK-mediated down-regulation of the survivin signal transduction pathway (41). In our studies, however, we found no effect of zerumbone on the expression of survivin.

The tumor suppressor gene p53 is a key regulator of apoptosis in cancer cells. We found that zerumbone induced p53 protein expression. Although p53 expression has been implicated in the expression of DR5 (28, 42), we found that zerumbone-induced DR5 expression was mediated through a p53-independent mechanism. No induction of DR4, however, was observed in p53-null cells. That p53 plays a role in the up-regulation of DR4 has already been shown (43). Thus, it is possible that the role of p53 in the induction of DR4 and DR5 depends on the nature of the stimulus and the cell type. We found that the ability of zerumbone to enhance TRAIL-induced apoptosis was unaffected by a lack of p53 expression in cells.

Overall, we conclude that zerumbone can enhance TRAIL-induced apoptosis through the up-regulation of DRs and the down-regulation of cFLIP. Considering that zerumbone when used alone is highly safe and exhibits anticancer activities *in vitro* (1–5) and *in vivo* (1, 2, 7, 9, 12) against a wide variety of tumors, we suggest that it can be also used in combination with TRAIL that is in clinical trials currently. Thus, these studies have an implication in treatment of cancer by TRAIL, especially tumors that develop resistance to TRAIL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Murakami A, Takahashi D, Kinoshita T, et al. 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. Carcinogenesis 2002;23:795–802.
2. Kirana C, McIntosh GH, Record IR, Jones GP. Antitumor activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid zerumbone. Nutr Cancer 2003;45:218–25.
3. Xian M, Ito K, Nakazato T, et al. Zerumbone, a bioactive sesquiterpene, induces G_2/M cell cycle arrest and apoptosis in leukemia cells via a Fas- and mitochondria-mediated pathway. Cancer Sci 2007;98:118–26.
4. Huang GC, Chien TY, Chen LG, Wang CC. Antitumor effects of zerumbone from *Zingiber zerumbet* in P-388D1 cells *in vitro* and *in vivo*. Planta Med 2005;71:219–24.
5. Sakinah SA, Handayani ST, Hawariah LP. Zerumbone induced apoptosis in liver cancer cells via modulation of Bax/Bcl-2 ratio. Cancer Cell Int 2007;7:4.
6. Murakami A, Takahashi M, Jiwajinda S, Koshimizu K, Ohigashi H. Identification of zerumbone in *Zingiber zerumbet* Smith as a potent inhibitor of

12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation. Biosci Biotechnol Biochem 1999;63:1811–2.

7. Tanaka T, Shimizu M, Kohno H, et al. Chemoprevention of azoxymethane-induced rat aberrant crypt foci by dietary zerumbone isolated from *Zingiber zerumbet*. Life Sci 2001;69:1935–45.
8. Murakami A, Hayashi R, Tanaka T, Kwon KH, Ohigashi H, Safitri R. Suppression of dextran sodium sulfate-induced colitis in mice by zerumbone, a subtropical ginger sesquiterpene, and nimesulide: separately and in combination. Biochem Pharmacol 2003;66:1253–61.
9. Murakami A, Tanaka T, Lee JY, et al. Zerumbone, a sesquiterpene in subtropical ginger, suppresses skin tumor initiation and promotion stages in ICR mice. Int J Cancer 2004;110:481–90.
10. Szabolcs A, Tiszlavicz L, Kaszaki J, et al. Zerumbone exerts a beneficial effect on inflammatory parameters of cholecystokinin octapeptide-induced experimental pancreatitis but fails to improve histology. Pancreas 2007;35:249–55.
11. Sung B, Jhurani S, Ahn KS, et al. Zerumbone down-regulates chemokine receptor CXCR4 expression leading to inhibition of CXCL12-induced invasion of breast and pancreatic tumor cells. Cancer Res 2008;68:8938–44.
12. Kim M, Miyamoto S, Yasui Y, Oyama T, Murakami A, Tanaka T. Zerumbone, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice. Int J Cancer 2009;124:264–71.
13. Sung B, Murakami A, Oyajobi BO, Aggarwal BB. Zerumbone abolishes RANKL-induced NF-κB activation, inhibits osteoclastogenesis, and suppresses human breast cancer-induced bone loss in athymic nude mice. Cancer Res 2009;69:1477–84.
14. Takada Y, Murakami A, Aggarwal BB. Zerumbone abolishes NF-κB and IκBα kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis, and downregulation of invasion. Oncogene 2005;24:6957–69.
15. Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J, Hood L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-κB pathway. Immunity 1997;7:821–30.
16. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003;3:745–56.
17. Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol 2008;26:3621–30.
18. Zerafa N, Westwood JA, Cretney E, et al. Cutting edge: TRAIL deficiency accelerates hematological malignancies. J Immunol 2005;175:5586–90.
19. Krueger A, Baumann S, Krammer PH, Kirchhoff S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol 2001;21:8247–54.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
21. Takada Y, Sethi G, Sung B, Aggarwal BB. Flavopiridol suppresses tumor necrosis factor-induced activation of activator protein-1, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase (MAPK), p44/p42 MAPK, and Akt, inhibits expression of antiapoptotic gene products, and enhances apoptosis through cytochrome c release and caspase activation in human myeloid cells. Mol Pharmacol 2008;73:1549–57.
22. Pathak AK, Bhutani M, Nair AS, et al. Ursolic acid inhibits STAT3 activation pathway leading to suppression of proliferation and chemosensitization of human multiple myeloma cells. Mol Cancer Res 2007;5:943–55.
23. Izeradjene K, Douglas L, Tillman DM, Delaney AB, Houghton JA. Reactive oxygen species regulate caspase activation in tumor necrosis factor-related apoptosis-inducing ligand-resistant human colon carcinoma cell lines. Cancer Res 2005;65:7436–45.
24. Jung EM, Lim JH, Lee TJ, Park JW, Choi KS, Kwon TK. Curcumin sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through reactive oxygen species-mediated upregulation of death receptor 5 (DR5). Carcinogenesis 2005;26:1905–13.
25. Ohtsuka T, Zhou T. Bisindolylmaleimide VIII enhances DR5-mediated apoptosis through the MKK4/JNK/p38 kinase and the mitochondrial pathways. J Biol Chem 2002;277:29294–303.
26. Shenoy K, Wu Y, Pervaiz S. LY303511 enhances TRAIL sensitivity of SHEP-1 neuroblastoma cells via hydrogen peroxide-mediated mitogen-activated protein kinase activation and up-regulation of death receptors. Cancer Res 2009;69:1941–50.
27. Tomasetti M, Andera L, Alleva R, Borghi B, Neuzil J, Procopio A. α-Tocopheryl succinate induces DR4 and DR5 expression by a p53-dependent route: implication for sensitisation of resistant cancer cells to TRAIL apoptosis. FEBS Lett 2006;580:1925–31.
28. Chen JJ, Chou CW, Chang YF, Chen CC. Proteasome inhibitors enhance TRAIL-induced apoptosis through the intronic regulation of DR5: involvement of NF-κB and reactive oxygen species-mediated p53 activation. J Immunol 2008;180:8030–9.
29. Watson NC, Di YM, Orr MS, et al. Influence of ionizing radiation on proliferation, c-myc expression and the induction of apoptotic cell death in two breast tumour cell lines differing in p53 status. Int J Radiat Biol 1997;72:547–59.
30. Hiro J, Inoue Y, Toiyama Y, Miki C, Kusunoki M. Mechanism of resistance to chemoradiation in p53 mutant human colon cancer. Int J Oncol 2008;32:1305–10.
31. Kwon D, Choi K, Choi C, Benveniste EN. Hydrogen peroxide enhances TRAIL-induced cell death through up-regulation of DR5 in human astrocytic cells. Biochem Biophys Res Commun 2008;372:870–4.
32. Taniguchi H, Yoshida T, Horinaka M, et al. Baicalein overcomes tumor necrosis factor-related apoptosis-inducing ligand resistance via two different cell-specific pathways in cancer cells but not in normal cells. Cancer Res 2008;68:8918–27.
33. Su RY, Chi KH, Huang DY, Tai MH, Lin WW. 15-Deoxy-Δ12,14-prostaglandin J_2 up-regulates death receptor 5 gene expression in HCT116 cells: involvement of reactive oxygen species and C/EBP homologous transcription factor gene transcription. Mol Cancer Ther 2008;7:3429–40.
34. Hyer ML, Croxton R, Krajewska M, et al. Synthetic triterpenoids cooperate with tumor necrosis factor-related apoptosis-inducing ligand to induce apoptosis of breast cancer cells. Cancer Res 2005;65:4799–808.
35. Suh WS, Kim YS, Schimmer AD, et al. Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL. Leukemia 2003;17:2122–9.
36. Chen S, Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. CCAAT/enhancer binding protein homologous protein-dependent death receptor 5 induction and ubiquitin/proteasome-mediated cellular FLICE-inhibitory protein down-regulation contribute to enhancement of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by dimethyl-celecoxib in human non small-cell lung cancer cells. Mol Pharmacol 2007;72:1269–79.
37. Raja SM, Chen S, Yue P, et al. The natural product honokiol preferentially inhibits cellular FLICE-inhibitory protein and augments death receptor-induced apoptosis. Mol Cancer Ther 2008;7:2212–23.
38. Lee MW, Park SC, Yang YG, et al. The involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein (MAP) kinase in TRAIL/Apo2L-induced apoptosis. FEBS Lett 2002;512:313–8.
39. Huang J, Wu L, Tashiro S, Onodera S, Ikejima T. Reactive oxygen species mediate oridonin-induced HepG2 apoptosis through p53, MAPK, and mitochondrial signaling pathways. J Pharmacol Sci 2008;107:370–9.
40. Drosopoulos KG, Roberts ML, Cermak L, et al. Transformation by oncogenic RAS sensitizes human colon cells to TRAIL-induced apoptosis by up-regulating death receptor 4 and death receptor 5 through a MEK-dependent pathway. J Biol Chem 2005;280:22856–67.
41. Kim JY, Kim EH, Park SS, Lim JH, Kwon TK, Choi KS. Quercetin sensitizes human hepatoma cells to TRAIL-induced apoptosis via Sp1-mediated DR5 up-regulation and proteasome-mediated c-FLIPS down-regulation. J Cell Biochem 2008;105:1386–98.
42. Wu GS, Burns TF, McDonald ER III, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997;17:141–3.
43. Liu X, Yue P, Khuri FR, Sun SY. p53 upregulates death receptor 4 expression through an intronic p53 binding site. Cancer Res 2004;64:5078–83.

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