

Zerumbone Down-regulates Chemokine Receptor CXCR4 Expression Leading to Inhibition of CXCL12-Induced Invasion of Breast and Pancreatic Tumor Cells

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Abstract

CXC chemokine receptor 4 (CXCR4), initially linked with leukocyte trafficking, is now known to be expressed in various tumors including breast, ovary, prostate, gastrointestinal, head and neck, bladder, brain, and melanoma. This receptor mediates homing of tumor cells to specific organs that express the ligand CXCL12 for this receptor. Thus, agents that can down-regulate CXCR4 expression have potential against cancer metastasis. In this study, we report the identification of zerumbone, a component of subtropical ginger (*Zingiber zerumbet*), as a regulator of CXCR4 expression. This sesquiterpene down-regulated the expression of CXCR4 on HER2-overexpressing breast cancer cells in a dose- and time-dependent manner. The decrease in CXCR4 by zerumbone was found to be not cell type specific as its expression was abrogated in leukemic, skin, kidney, lung, and pancreatic cancer cell lines. The down-regulation of CXCR4 was not due to proteolytic degradation but rather to transcriptional regulation, as indicated by down-regulation of mRNA expression, inhibition of nuclear factor- κ B activity, and suppression of chromatin immunoprecipitation activity. Suppression of CXCR4 expression by zerumbone correlated with the inhibition of CXCL12-induced invasion of both breast and pancreatic cancer cells. An analogue of zerumbone, α -humulene, which lacks the carbonyl group, was found to be inactive in inducing CXCR4 down-regulation. Overall, our results show that zerumbone is a novel inhibitor of CXCR4 expression and thus has a potential in the suppression of cancer metastasis. [Cancer Res 2008;68(21):8938–44]

Introduction

Metastasis, which occurs in as many as 90% of cancer-associated deaths, is a highly complex process that is organ selective and involves interactions between the cancer cells and the host (1). Numerous molecules have been linked with cancer metastasis including matrix metalloproteases, vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF), platelet-derived growth factor, transforming growth factor β , and TWIST (2–6). The mechanism of organ-specific tumor metastasis, however, is still

not completely understood. Evidence that chemokines can mediate tumor metastasis, first identified in breast cancer (7), is becoming increasingly evident for other cancers as well (8, 9). Chemokines constitute a superfamily of cytokine-like small chemoattractant proteins that orchestrate immunologic and inflammatory processes such as leukocyte trafficking, adhesion, hematopoiesis, and angiogenesis.

One of the most extensively studied chemokines in tumor cell migration and metastasis is stromal cell-derived factor 1 α [SDF-1 α ; also known as CXC chemokine ligand 12 (CXCL12)] and its receptor, CXCR4 (10). CXCR4 is expressed on a diverse array of cancer cell types, including those of lung, prostate, breast, kidney, and ovary (7, 11). The ligand of CXCR4, CXCL12 (SDF-1), is expressed at the site of tumor metastasis involved in homing of the tumors to different organs (12).

CXCR4 has been linked with leukocyte trafficking (13), B-cell lymphopoiesis and myelopoiesis (14), neuronal cell migration (15), and HIV invasion of host cells (16). In addition, CXCR4/SDF-1 plays an important role in the targeted metastasis of breast cancer (7). The SDF-1/CXCR4 attraction leads breast cancer cells to leave the circulation and migrate into organs that express large amounts of chemokines, where the cancer cells proliferate, induce angiogenesis, and form metastatic tumors.

The HER2 receptor tyrosine kinase has been shown to induce the up-regulation of CXCR4 that leads to breast cancer invasion (17). CXCR4 is also involved in the metastasis of prostate cancer to the bone marrow (18) and of colon cancer to the liver (19). CXCR4 expression has been correlated with poor overall survival rate in patients with breast cancer (20) and colon cancer (21). Thus, CXCR4 is viewed as a probable therapeutic target for preventing cancer metastasis.

Natural products, generally regarded as safe, have been shown to mediate anticancer activities against variety of cell types. In most cases, however, neither their active component nor their mechanism of action is well understood. Zerumbone, a sesquiterpene derived from the rhizome of *Zingiber zerumbet* (also called shampoo ginger or Narkachur), has been shown to exhibit antitumor activities as indicated by inhibition of skin tumor initiation and promotion in ICR mice (22), suppression of azoxymethane-induced aberrant crypt formation in rat (23), inhibition of dextran sodium sulfate-induced colitis (24), suppression of cholecystokinin octapeptide-induced acute pancreatitis in rats (25), and increase in survival of p388-bearing CDF-1 mice (26). Because CXCR4 is known to mediate growth and metastasis of tumor, we hypothesized that zerumbone may modulate the expression of CXCR4 and inhibit tumor cell invasion. Our results show that this sesquiterpene can down-regulate both the

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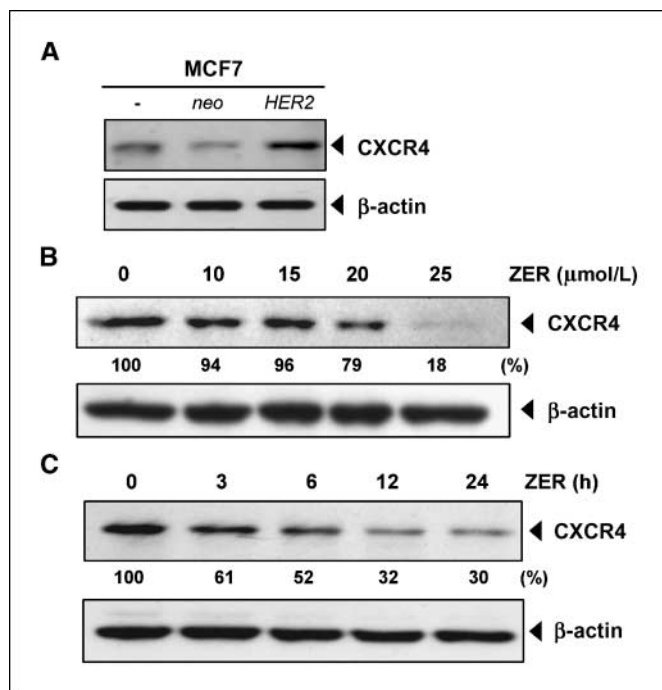


Figure 1. Zerumbone suppresses CXCR4 in MCF7/HER2 cells. **A**, Western blot analysis of CXCR4 expression. Whole-cell extracts of MCF7, MCF7/neo, and MCF7/HER2 (30 μg) were resolved on SDS-PAGE gel and probed with anti-CXCR4 antibody. As a loading control, stripped membrane was probed with β-actin antibodies. **B**, zerumbone suppresses CXCR4 levels in a dose-dependent manner. MCF7/HER2 cells (1×10^6) were treated with the indicated concentrations of zerumbone (ZER) for 24 h. Whole-cell extracts were then prepared, and 30 μg of protein were resolved on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for CXCR4. **C**, zerumbone suppresses CXCR4 levels in a time-dependent manner. MCF7/HER2 cells (1×10^6) were treated with 25 μmol/L zerumbone for the indicated times, after which Western blotting was done as described in above. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. Representative results of three independent experiments.

constitutive and HER2-induced expression of CXCR4. Zerumbone was also found to inhibit CXCL12-induced invasion of breast and pancreatic tumor cells.

Materials and Methods

Reagents. Zerumbone, kindly supplied by Dr. Akira Murakami (Kyoto University, Kyoto, Japan), was dissolved in DMSO as a 25 mmol/L stock solution and stored at 4°C. Further dilution was done in cell culture medium. RPMI 1640, DMEM/F12, Iscove's modified Dulbecco's medium (IMDM), DMEM, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and an antibiotic-antimycotic mixture were obtained from Life Technologies. Rabbit polyclonal antibody to CXCR4 was obtained from Abcam. Lactacystin was obtained from Calbiochem.

Cell lines and cell culture. Breast cancer cell lines that express different levels of HER2, including stably transfected MCF7/HER2 and their vector control, were kindly provided by Dr. D. Yu of our Institute (27). The KBM-5 (human chronic myeloid leukemia) cell line was provided by Dr. Nicholas J. Donato (University of Michigan Comprehensive Cancer Center, Ann Arbor, MI). U266 (multiple myeloma), H1299 (lung adenocarcinoma), SCC4 (squamous cell carcinoma), PANC-1 (pancreatic duct cell carcinoma), PANC-28 (pancreatic carcinoma), MIA PaCa-2 (pancreatic carcinoma), AsPC-1 (pancreatic adenocarcinoma), and A293 (embryonic kidney carcinoma) were obtained from American Type Culture Collection. MCF7/HER2 and its control cells were cultured in DMEM/F12 supplemented with 10% FBS. H1299, PANC-1, PANC-28, MIA PaCa-2, AsPC-1, and U266 cells were cultured in RPMI 1640 with 10% FBS. KBM-5 cells were

cultured in IMDM with 15% FBS. A293 cells were cultured in DMEM with 10% FBS. SCC-4 cells were cultured in DMEM containing 10% FBS, 100 μmol/L nonessential amino acids, 1 mmol/L pyruvate, 6 mmol/L L-glutamine, and $1 \times$ vitamins. Culture media were also supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin except media for MCF7/HER2 and its control. Cells were maintained at 37°C in an atmosphere of 5% CO₂-95% air.

Western blotting. For detection of CXCR4, zerumbone-treated whole-cell extracts were lysed in lysis buffer (20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, and 4 mmol/L NaVO₃). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 10% SDS gel. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-CXCR4 antibodies (1:3,000) overnight at 4°C. The blot was washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 2 h, and finally examined by chemiluminescence (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay. To evaluate the activation of nuclear factor κB (NF-κB) following radiation therapy, electrophoretic mobility shift assay was done as described previously (28).

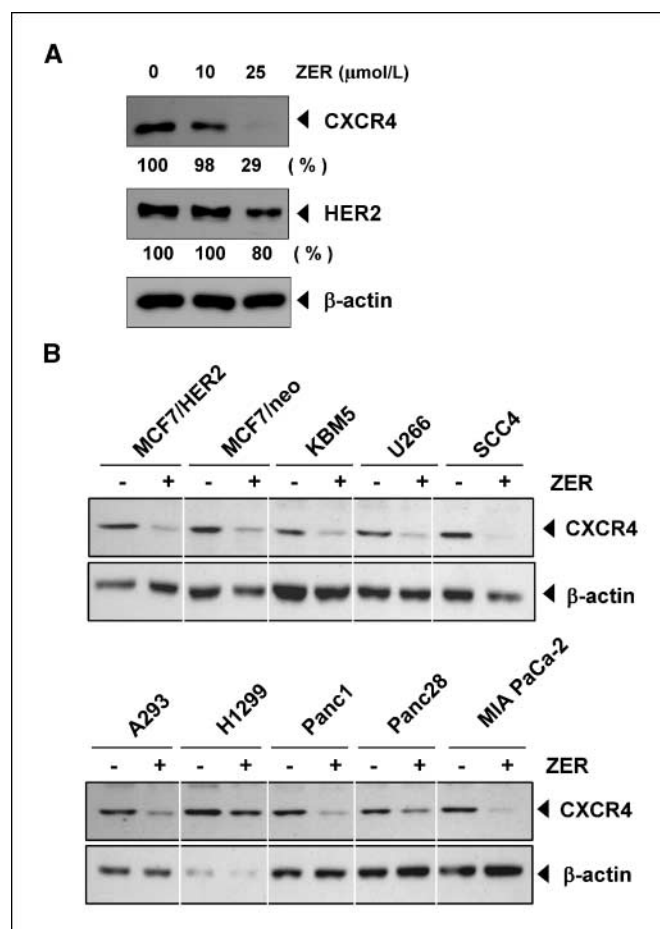


Figure 2. Zerumbone down-regulates CXCR4 in different cell types. **A**, zerumbone inhibits CXCR4 and HER2 expression. MCF7/HER2 cells were incubated with the indicated concentrations of zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against HER2 and CXCR4. **B**, cells were incubated with 25 μmol/L zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against CXCR4. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. Representative results of three independent experiments.

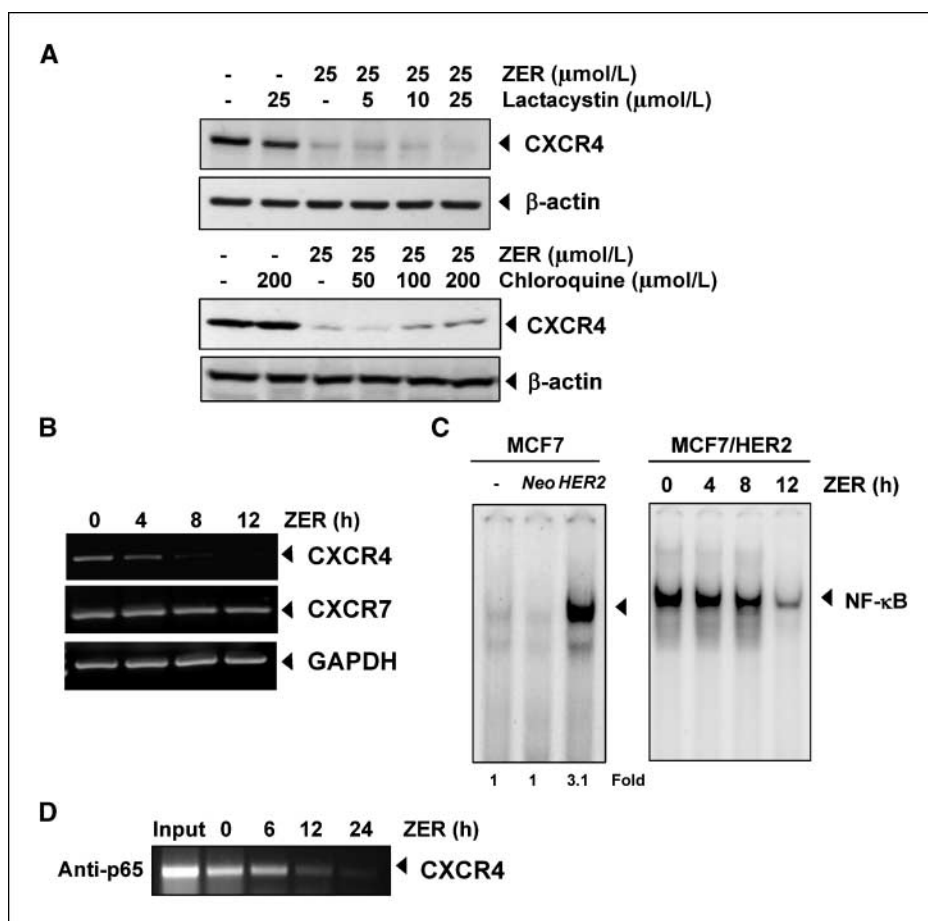


Figure 3. Zerumbone suppresses CXCR4 through mRNA level. **A**, zerumbone does not suppress CXCR4 through lysosomal and proteosomal degradation. Cells were treated with the indicated concentration of lactacystin or chloroquine for 1 h at 37°C, followed by treatment with 25 μmol/L zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against CXCR4. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. **B**, zerumbone suppresses expression of CXCR4 mRNA. Cells were treated with 25 μmol/L zerumbone for the indicated times. Total RNA was isolated and analyzed by RT-PCR assay as described in Materials and Methods. GAPDH was used to show equal loading of total RNA. **C**, HER2 overexpression leads to NF-κB activation (*left*) and zerumbone inhibits HER2 induced NF-κB activation (*right*) in breast cancer cells. MCF7/HER2 cells were incubated with zerumbone for the indicated times. The nuclear extracts were assayed for NF-κB activation by electrophoretic mobility shift assay. **D**, zerumbone inhibits binding of NF-κB to the CXCR4 promoter. MCF7/HER2 cells were pretreated with 25 μmol/L zerumbone for 6, 12, and 24 h, and the proteins were cross-linked with DNA with formaldehyde and then subjected to chromatin immunoprecipitation assay with an anti-p65 antibody with the CXCR4 primer. Reaction products were resolved by electrophoresis.

RNA analysis and reverse transcription-PCR. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies). One microgram of total RNA was converted to cDNA by Superscript reverse transcriptase and then amplified by Platinum Taq polymerase using Superscript One-Step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The relative expression of CXCR4 and CXCR7 was analyzed by quantitative RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The following pairs of forward and reverse primer sets were used: CXCR4, 5'-GAAGCTGTTGGCTGAAAAGG-3' and 5'-GAGTCGATGCTGATCCCAAT-3' (PCR product size, 345 bp; GenBank accession no. NM_003467); and CXCR7, 5'-CTCACGTGCAAAGTCACACA-3' and 5'-CGATAATGGAGAAGGGAACG-3' (PCR product size, 343 bp; GenBank accession no. NM_001047841). The RT-PCR reaction mixture contained 12.5 μL of 2× reaction buffer, 10 μL each of cDNA, 0.5 μL each of forward and reverse primers, and 1 μL of RT-Platinum Taq in a final volume of 50 μL. The reaction was done at 50°C for 30 min, 94°C for 2 min, 94°C for 30 cycles of 15 s each, 54°C for 30 s, and 72°C for 1 min with extension at 72°C for 10 min. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was done as previously described with some modifications (29). MCF7/HER2 cells (2×10^7) were incubated with or without 25 μmol/L zerumbone for the indicated times. PCR analyses were carried out for 39 cycles with primers 5'-CTTAGGAAATGCCTCTGGGAGGTCC-3' (forward) and 5'-GCAGACGCGAGGAAGGAG-3' (backward) for CXCR4 [the amplified DNA fragment (-187 to -50) contains the NF-κB binding site of -109GGGCTCCC₋₁₀₁; ref. 30].

Invasion assay. *In vitro* invasion assay was done using BD Bio-Coat Matrigel invasion assay system (BD Biosciences) according to the manufacturer's instructions. Cancer cells (2×10^5) were suspended in

medium (2% FBS-RPMI 1640 for AsPC-1 and 10% FBS-DMEM/F12 for MCF7/HER2) and seeded into the Matrigel-precoated transwell chambers with polycarbonate membranes of 8-μm pore size. After preincubation with or without zerumbone (25 μmol/L) for 6 h, transwell chambers were then placed into 24-well plates, in which was added the basal medium only or basal medium containing 100 ng/mL CXCL12. After incubation (24 h for AsPC-1 and 60 h for MCF7/HER2), the upper surface of transwell chambers was wiped off with a cotton swab and invading cells were fixed and stained with a Diff-Quick stain. The invading cell numbers were counted in five randomly selected microscope fields ($\times 200$).

Statistical analysis. The experiments have been done in triplicate and repeated twice. The *P* value was obtained after ANOVA and Student-Newman-Keul tests.

Results

The present study was designed to investigate the effect in tumor cells of zerumbone on both the constitutive and inducible expression CXCR4, the chemokine receptor that plays a critical role in tumor cell invasion and metastasis. We also investigated the effect of zerumbone on CXCR4 in various tumor cell types and invasive activity.

Zerumbone suppresses the expression of CXCR4 protein in HER2-overexpressing breast cancer cells. HER2 overexpression has been linked with metastasis of breast cancer. Furthermore, HER2 has been shown to induce the expression of CXCR4 on breast cancer cells (17). As shown in Fig. 1A, expression of HER2 in MCF7 cells indeed induced the expression of CXCR4. Whether zerumbone can modulate the expression of CXCR4 in MCF7/HER2 cells was

investigated. When MCF7/HER2 cells were incubated either with different concentrations of zerumbone for 24 hours or with 25 $\mu\text{mol/L}$ zerumbone for different times, zerumbone suppressed the expression of CXCR4 in a dose- and time-dependent manner (Fig. 1B and C). Zerumbone-induced suppression could be observed as early as 3 hours and with a concentration as low as 20 $\mu\text{mol/L}$. The exposure of cells to 25 $\mu\text{mol/L}$ zerumbone for 24 hours significantly inhibited CXCR4 expression. This down-regulation was not due to decrease in cell viability because >90% cells were viable under these conditions (data not shown).

Down-regulation of CXCR4 by zerumbone accompanies down-regulation of HER2 in breast cancer cells. Because HER2 enhances the expression of CXCR4 by stimulating CXCR4 translation and attenuating CXCR4 degradation (31), whether zerumbone down-regulates CXCR4 expression through regulation of HER2 expression was examined. For this, HER2-overexpressing MCF7 cells were incubated with 10 and 25 $\mu\text{mol/L}$ zerumbone for 24 hours and then examined for both CXCR4 and HER2 expression by Western blot analysis with specific antibodies. We found that zerumbone completely suppressed CXCR4 expression at 25 $\mu\text{mol/L}$, but HER2 expression was only partially affected (Fig. 2A), thus suggesting that

down-regulation of CXCR4 expression by zerumbone is not entirely due to modulation of HER2 expression.

Zerumbone down-modulates CXCR4 in different cell types. Up to this point, our all studies were carried out with HER2-overexpressing breast cancer cells. CXCR4, however, is overexpressed on wide variety of tumor cells. Thus, we investigated whether zerumbone down-regulates expression of CXCR4 in leukemia (KBM-5), myeloma (U266), head and neck squamous cell carcinoma (SCC4), kidney (A293), lung adenocarcinoma (H1299), and pancreatic (PANC-1, PANC-28, and MIA PaCa-2) cancer cell lines. Cells were treated with 25 $\mu\text{mol/L}$ zerumbone for 24 hours and then examined CXCR4 expression. Figure 2B shows that zerumbone down-regulated CXCR4 on all these cell lines but most dramatically in head and neck (SCC4) and pancreatic cancer cells (MIA PaCa-2; Fig. 2B, bottom). Thus, CXCR4 down-regulation by zerumbone is not cell type specific.

Down-regulation of CXCR4 by zerumbone is not mediated through its degradation. Because zerumbone could down-regulate CXCR4 expression by enhancing its degradation, and CXCR4 has been shown to undergo ubiquitination at its lysine residue followed by degradation (32, 33), we first explored the possibility that zerumbone enhances the rate of degradation through the activation of proteasomes. To determine this, we examined the ability of lactacystin, a proteasome inhibitor, to block zerumbone-induced degradation of CXCR4 in MCF7/HER2 cells. Cells were pretreated with lactacystin for 1 hour before being exposed to zerumbone. As shown in Fig. 3A, lactacystin had no effect on zerumbone-induced degradation of CXCR4, suggesting that this is an unlikely mechanism for zerumbone effects on CXCR4 regulation.

Because CXCR4 can undergo ligand-dependent lysosomal degradation (33), we examined the ability of chloroquine, a lysosomal inhibitor, to block zerumbone-induced degradation of CXCR4. To examine this, cells were pretreated with chloroquine for 1 hour before exposure to zerumbone. Chloroquine at 200 $\mu\text{mol/L}$ only slightly prevented the degradation of CXCR4 (Fig. 3A, bottom), indicating that this was not the primary pathway for suppression of expression of CXCR4.

Down-regulation of CXCR4 by zerumbone occurs at the transcriptional level. Because zerumbone did not down-regulate CXCR4 expression by enhancing its degradation, we investigated whether suppression occurs at the transcriptional level using RT-PCR. Cells were treated with zerumbone for different times and then examined for mRNA. As shown in Fig. 3B, zerumbone induced the down-regulation of CXCR4 mRNA. Interestingly, zerumbone had no effect on the mRNA of another chemokine receptor, CXCR7, thus indicating that the effects are specific for CXCR4.

Zerumbone suppresses constitutive activation of NF- κ B in MCF7/HER2 cells. Previously, Biswas and Iglehart (34) reported that HER2 expression and NF- κ B activation were related in breast cancer. We used a DNA-binding assay to explore whether overexpression of HER2 affects NF- κ B activation in MCF7 breast cancer cells. HER2-overexpressing MCF7 cells showed constitutive NF- κ B activation, although nontransfected and vector-transfected (neo) cells did not show any NF- κ B activation (Fig. 3C, left). Treatment of HER2-overexpressing cells with zerumbone down-regulated NF- κ B activation in a time-dependent manner (Fig. 3C, right). Thus, these results suggest that zerumbone may down-regulate CXCR4 expression through down-regulation of NF- κ B activation.

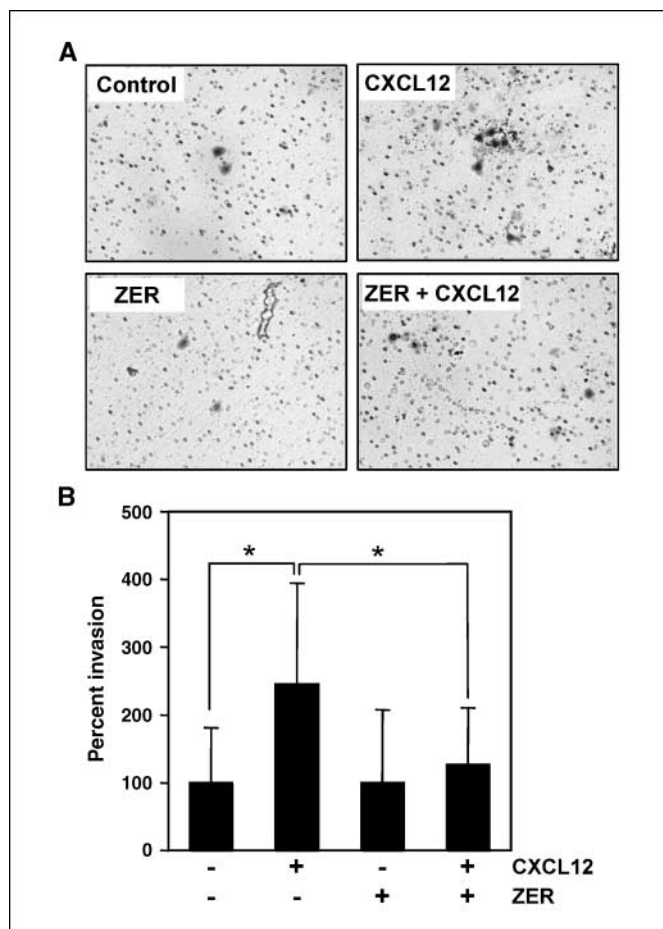


Figure 4. Zerumbone suppresses invasion in breast cancer cells. A and B, MCF7/HER2 cells (2×10^5 ; 10% FBS-DMEM/F12) were seeded in the top chamber of Matrigel. After preincubation with or without zerumbone (25 $\mu\text{mol/L}$) for 6 h, transwell chambers were then placed into 24-well plates, in which was added the basal medium only or basal medium containing 100 ng/mL CXCL12. After incubation, invasion assay was done as described in Materials and Methods. B, columns, mean number of invaded cells; bars, SE. *, $P < 0.05$.

Zerumbone inhibits binding of NF- κ B to the CXCR4 promoter. Whether the down-regulation of CXCR4 by zerumbone in MCF7/HER2 cells was due to suppression of NF- κ B activation *in vivo* was examined by chromatin immunoprecipitation assay targeting NF- κ B binding in the CXCR4 promoter. We found that zerumbone suppressed the NF- κ B binding to CXCR4 promoter (Fig. 3D). Overall, these results suggest that zerumbone inhibits CXCR4 expression by suppressing NF- κ B binding to the CXCR4 promoter.

Zerumbone suppresses CXCL12-induced breast cancer cell invasion. Several lines of evidence implicate the role of CXCR4 in breast cancer metastasis (7, 35). Müller and colleagues (7) found that the motility and migration of breast cancer cells can be induced when they are exposed to their ligand, CXCL12. In addition, breast cancer metastasis can be inhibited by silencing CXCR4 (35). Whether down-regulation of CXCR4 by zerumbone correlates with breast cancer cell migration was examined using an *in vitro* invasion assay. We found that CXCL12 induced the invasion of breast cancer cells and that zerumbone effectively abrogated the invasion (Fig. 4A and B). Because MCF7 cells are not as invasive as AsPC-1, only a small number of MCF7 cells invade through the Matrigel. This may explain why the SE is high for MCF7 cells.

Zerumbone inhibits CXCL12-induced pancreatic cancer cell invasion. Whether suppression of invasion by zerumbone is tumor cell specific was also investigated. CXCL12/CXCR4 signaling has been shown to play a critical role in pancreatic cancer metastasis (36). To elucidate whether zerumbone had an effect on pancreatic cancer cell metastasis, we examined the effect of zerumbone on CXCL12-induced cell invasion. As shown in Fig. 5A and B, treatment of zerumbone suppressed CXCL12-induced invasion of pancreatic cancer AsPC-1 cells.

Whether zerumbone suppressed invasion through the down-regulation of CXCR4 in AsPC-1 cells was also examined. We found that zerumbone down-regulated the expression of both mRNA (Fig. 5C) and protein (Fig. 5D) for CXCR4.

α -Humulene, a zerumbone analogue, does not suppress CXCR4. Furthermore, the zerumbone analogue, α -humulene, which lacks a carbonyl group at the 8-position in zerumbone (Fig. 6A), has been shown to be inactive in suppressing free radical generation, cell proliferation, and apoptosis induction (37). Whether α -humulene affects CXCR4 expression was examined. As shown in Fig. 6B, α -humulene had no effect on CXCR4 expression, whereas zerumbone down-modulated CXCR4 expression at the same concentration.

Discussion

The goal of the present study was to determine whether zerumbone, a component of shampoo ginger that has been linked with anticancer activities, can suppress the expression of CXCR4, a chemokine receptor that has been closely linked with cancer cell growth, invasion, angiogenesis, and metastasis. Our results showed for the first time that zerumbone abolished the expression of CXCR4. A wide variety of tumor cells expressed CXCR4, and zerumbone down-regulated expression irrespective of the cell type. Besides constitutive CXCR4 expression, zerumbone also suppressed HER2-induced CXCR4 expression in breast cancer cells. Our results showed that down-regulation of CXCR4 did not occur through proteolytic degradation of the receptor but rather through down-regulation of the transcript. Furthermore, suppression of receptor expression led to down-regulation of invasion whether induced by tumor cells or the ligand CXCL12.

The CXCR4 chemokine receptor till now has been reported to be overexpressed in more than 20 different tumors, including breast

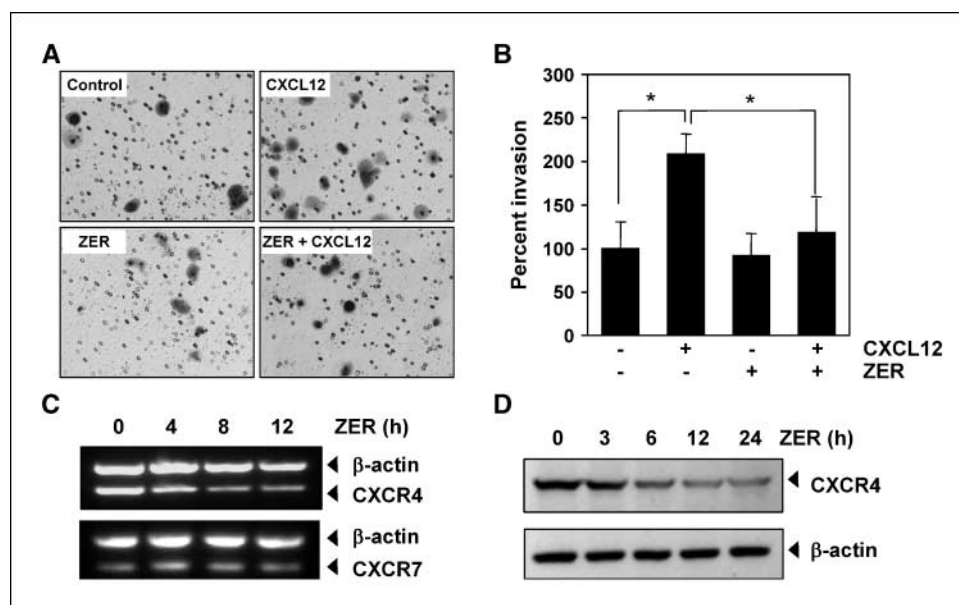


Figure 5. Zerumbone suppresses CXCR4 and invasion in pancreatic cancer cells. *A* and *B*, AsPC-1 cells (2×10^5 ; 2% FBS-DMEM) were seeded in the top chamber of Matrigel. After preincubation with or without zerumbone (25 μ mol/L) for 6 h, transwell chambers were then placed into 24-well plates, in which we added the basal medium only or basal medium containing 100 ng/mL CXCL12. After incubation, invasion assay was done as described in Materials and Methods. *B*, columns, mean number of invaded cells; bars, SE. *, $P < 0.01$. *C*, zerumbone suppresses expression of CXCR4 mRNA. AsPC-1 cells were treated with 25 μ mol/L zerumbone for the indicated times. Total RNA was isolated and analyzed by RT-PCR assay as described in Materials and Methods. GAPDH was used to show equal loading of total RNA. *D*, cells were incubated with 25 μ mol/L zerumbone for 24 h. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against CXCR4. The same blots were stripped and reprobed with β -actin antibody to show equal protein loading. Representative results of three independent experiments.

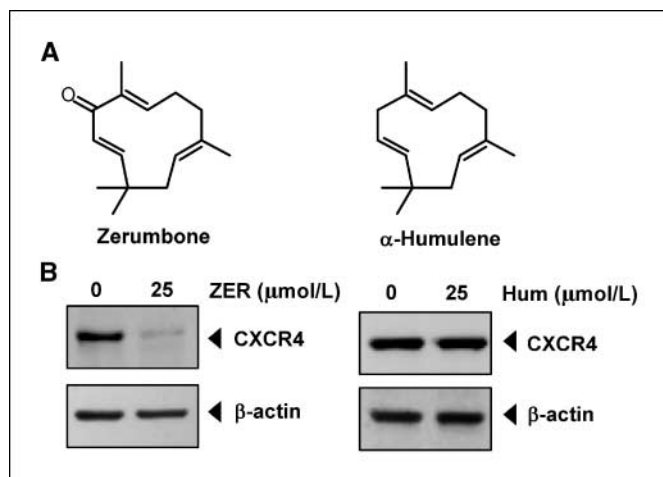


Figure 6. The zerumbone analogue α -humulene does not suppress CXCR4. **A**, structures of zerumbone and α -humulene. **B**, MCF7/HER2 cells were incubated with either 25 $\mu\text{mol/L}$ zerumbone (*left*) or humulene (*right*) for 24 h. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against CXCR4. The same blots were stripped and reprobed with β -actin antibody to show equal protein loading. Representative results of three independent experiments.

cancer, ovarian cancer, glioma, pancreatic cancer, prostate cancer, acute myelogenous leukemia, B-cell chronic lymphocytic leukemia, melanoma, cervical cancer, colon carcinoma, rhabdomyosarcoma, astrocytoma, small-cell lung carcinoma, chronic lymphocytic leukemia, renal cancer, and non-Hodgkin's lymphoma (31, 38–40). What leads to overexpression of this receptor in tumor cells is not fully understood, but the role of genetic alterations in protein degradation pathways (32, 33) and of tumor hypoxia (41) has been reported. Inflammatory cytokines such as TNF (42) and VEGF (43) can induce the expression of CXCR4. More recently, the growth factor receptor HER2 has also been linked with overexpression of the chemokine receptor (44).

The ligand-dependent down-regulation of the CXCR4 receptor by lysosomal degradation is well documented (32). Recent evidence suggests that degradation involves atrophin-interacting protein-4-mediated ubiquitination and degradation (33). Our results, however, suggest that zerumbone does not down-regulate CXCR4 through this mechanism. We found that down-regulation occurs at the transcription level. The transcription factors HIF-1 α (41, 45), PPAR γ (46), and NF- κ B (47) have been implicated in the regulation of CXCR4. von Hippel-Lindau tumor suppressor pVHL was found to down-regulate CXCR4 expression under hypoxic conditions (45). Zerumbone has been shown to down-regulate NF- κ B activation. Therefore, it is possible that down-regulation of CXCR4 occurs through down-regulation of NF- κ B activation. We found that, indeed, down-regulation of NF- κ B by zerumbone mediates down-regulation of CXCR4. Our results are in agreement with those of Li

and colleagues (17) who showed that HER2-induced CXCR4 expression requires NF- κ B activation. Other mechanisms than suppression of NF- κ B activation that are involved in down-regulation of CXCR4 by zerumbone cannot be ruled out.

Besides CXCR4, the activation of NF- κ B induces expression of various adhesion molecules including intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and endothelial-leukocyte adhesion molecule 1, which are also linked with cancer cell metastasis to other organs. Because zerumbone can inhibit both inducible and constitutively activated NF- κ B activation (28), it is possible that zerumbone can suppress the expression of these adhesion molecules as well. Our data showed that zerumbone suppressed both constitutive and inducible CXCR4 expression on various types of cancer cells. Thus, the effect of zerumbone on CXCR4 is not limited to a single cell type.

We also found that zerumbone suppressed the ligand-induced invasion of both breast and pancreatic cancers. This shows the critical role of the CXCR4 receptor in invasion of the tumor and the potential of zerumbone to down-regulate the expression or the activity of CXCR4. Elevated levels of CXCR4 have been reported with nodal metastasis of human breast cancer (48).

We found that the carbonyl group of zerumbone is critical for its activity, whereas α -humulene, which lacks this group, had no activity. How the α -carbonyl of zerumbone mediates this role is unclear. It is possible that Michael acceptor in zerumbone is needed for its activity. This is in agreement with 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂, which has a Michael acceptor and down-regulates CXCR4 through down-regulation of NF- κ B (49). Rationally designed CXCR4 antagonists such as AMD3100 and AMD3465 have Michael acceptor in their structures (50).

Zerumbone has been shown to inhibit tumor initiation and promotion (22), azoxymethane-induced aberrant crypt formation (23), dextran sodium sulfate-induced colitis (24), cholecystokinin octapeptide-induced pancreatitis (25), and antitumor effects against p388 (26). It is possible that some of these antitumor effects of zerumbone are also mediated through CXCR4 regulation. Taken together, our data suggest that zerumbone can down-regulate the expression of CXCR4, a key receptor involved in the cross talk between tumor cells and its microenvironment, which contributes to its anti-invasive activity. Further *in vivo* studies are needed to show the relevance of these observations to cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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