Abrogation of STAT3 Signaling Cascade by Zerumbone Inhibits Proliferation andInducesApoptosisinRenal Cell Carcinoma Xenograft Mouse Model

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Persistent activation of signal transducer and activator of transcription 3 (STAT3) is one of the characteristic features of renal cell carcinoma (RCC) and often linked to its deregulated proliferation, survival, and angiogenesis. In the present report, we investigated whether zerumbone, a sesquiterpene, exerts its anticancer effect through modulation of STAT3 activation pathway. The pharmacological effect of zerumbone on STAT3 activation, associated protein kinases and phosphatase, and apoptosis was investigated using both RCC cell lines and xenograft mouse model. We observed that zerumbone suppressed STAT3 activation in a dose- and time-dependent manner in RCC cells. The suppression was mediated through the inhibition of activation of upstream kinases c-Src, Janus-activated kinase 1, and Janus-activated kinase 2. Pervanadate treatment reversed zerumbone-induced downregulation of STAT3, suggesting the involvement of a tyrosine phosphatase. Indeed, we found that zerumbone induced the expression of tyrosine phosphatase SHP-1 that correlated with its ability to inhibit STAT3 activation. Interestingly, depletion of SHP-1 gene by siRNA abolished the ability of zerumbone to inhibit STAT3 activation. The inhibition of STAT3 activation by zerumbone also caused the suppression of the gene products involved in proliferation, survival, and angiogenesis. Finally, when administered i.p., zerumbone inhibited STAT3 activation in tumor tissues and the growth of human RCC xenograft tumors in athymic nu/nu mice without any side effects. Overall, our results suggest for the first time that zerumbone is a novel blocker of STAT3 signaling cascade and thus has an enormous potential for the treatment of RCC and other solid tumors. © 2014 Wiley Periodicals, Inc.

Key words: renal cell carcinoma; STAT3; zerumbone; proliferation; apoptosis

INTRODUCTION

Renal cell carcinoma (RCC) is the third leading cause of death among genitourinary malignancies and the tenth leading cause of overall cancer death in the United States [1]. At the time of diagnosis, approximately 30% of patients have distant metastases and 25% have locally advanced disease [2]. For patients with locally advanced disease, the 5-yr survival rate is still 61.7%. However, when RCC is present in the metastatic stage, it is associated with a 5-yr survival rate of as low as 9.5% [3]. Although immunotherapies, such as interleukin 2 and interferon-alpha (IFN-α), have displayed modest activity in RCC patients, the vast majority of individuals are either primarily resistant to these treatments or relapse after an initial response [4]. In fact, <5% of patients treated with high-dose interleukin two experience durable, complete remissions, and also metastatic RCC is quite resistant to chemotherapy, because of the significant expression of the multidrug resistance gene [5]. Two recently approved multi-targeted tyrosine kinase inhibitors, sorafenib and sunitinib, have demonstrated modest activity against RCC, but the patients suffer from various kinds of adverse effects, including hepatotoxicity [6].
Therefore, the need to develop novel therapeutic strategies for RCC is of vital importance.

Signal transducer and activator of transcription (STAT) proteins originally identified about two decades ago [7,8] consist of seven diverse members that have been found to play a critical role in both inflammation and tumorigenesis. Among STAT family proteins, STAT3 has received considerable attention in the last few years since it is a convergent point for a number of oncogenic signaling pathways and controls intracellular signal transduction pathways of several proinflammatory cytokines and growth factors implicated in tumorigenesis [8,9]. Signal transducer and activator of transcription 3 (STAT3) has been reported to regulate the expression of plethora of genes involved in anti-apoptosis, proliferation, and angiogenesis in a wide variety of solid tumors, including RCC [10–13]. Thus, small molecule inhibitors of STAT3 activation have a great potential both for the prevention and treatment of RCC.

In the present report, we describe the identification of a compound called zerumbone (2,6,9-tetramethyl-2E,6E,10E-cycloundeca-2,6,10-trien-1-one), a cyclic 11-membered sesquiterpene, isolated from the rhizomes of the tropical plant Zingiber zerumbet Smith as a novel STAT3 blocker in RCC cells. Zerumbone has been previously found to inhibit the proliferation of a wide variety of tumor cells [14], including breast [15], pancreatic [16], ovarian [17], lymphoblastic leukemia [18], cervical [17], and cholangiocarcinoma [19]. In vivo, zerumbone has been reported to prevent diethylnitrosamine-initiated and 2-acetylaminofluorene-promoted hepatocarcinogenesis [20], inhibit colon and lung carcinogenesis in mice [21], suppress human breast cancer-induced bone loss in athymic nude mice [22], and abrogate skin tumor initiation and promotion stages in ICR mice [23]. Zerumbone exhibits its anticancer effects through the modulation of various oncogenic targets including nuclear factor kappa B, PI3K/AKT, CXC chemokine receptor 4, receptor activator of nuclear factor-kappaB ligand (RANKL) signaling, and pro-apoptotic markers (Bax, Bak, p53) in diverse tumor cell lines and mouse models [14]. These reports indicate that zerumbone may be a suitable candidate agent for cancer treatment, although the detailed mechanism(s) of its action have not been completely elucidated so far.

Because of the pivotal role of STAT3 in tumor cell survival, proliferation, and angiogenesis, and its aberrant expression in RCC, we analyzed whether zerumbone can mediate its anticancer effects in part through the abrogation of the STAT3 activation pathway. We observed that zerumbone can indeed suppress STAT3 activation in RCC cells, concomitant with the inhibition of multiple upstream kinases and induction of a phosphatase SHP-1. Zerumbone also inhibited the growth of human RCC cells in a xenograft mouse model and attenuated the activation of STAT3 in tumor tissues.

Molecular Carcinogenesis

MATERIALS AND METHODS

Reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-(3-(4,5-Dimethylthiazol-2-yl))-2,5-Diphenyltetrazolium Bromide), tris, glycine, NaCl, SDS, BSA, EGF, DAPI, β-actin antibody, and zerumbone were purchased from Sigma–Aldrich (St. Louis, MO). Zerumbone was dissolved in dimethylsulfoxide as a 10 mM stock solution and stored at 4°C. Further dilution was done in cell culture medium. DMEM, McCoy’s 5A, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Life Technologies (Carlsbad, CA). Rabbit polyclonal antibodies to STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr 705), Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, MMP-9, SHP-1, VEGF, pro-caspase-3, and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-specific Src (Tyr 416), Src, phospho-specific JAK1 (Tyr 1022/1023), JAK1, phospho-specific JAK2 (Tyr 1007/1008), and JAK2 were purchased from Cell Signaling Technology (Beverly, MA). CD31 antibody was purchased from Cell Signaling Technology (Danvers, MA). Ki-67 antibody was purchased from BD PharMingen, Inc. (San Diego, CA). The siRNA for STAT3 and mouse monoclonal antibodies against phospho-STAT3 (Tyr 705), Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, MMP-9, SHP-1, VEGF, pro-caspase-3, and PARP were obtained from Sigma–Aldrich. Bradford reagent was purchased from Bio-Rad. Cell Death Detection ELISA PLUS DNA fragmentation kit was purchased from Roche Applied Science (Indianapolis, IN). Nuclear extraction and STAT3 DNA binding kits were obtained from Active Motif (Carlsbad, CA).

Cell Lines

Human RCC cell line 786-O and Caki-1 were obtained from American Type Culture Collection (Manassass, VA). RCC4 cells were kindly provided by Dr. John Yuen, Department of Urology, Singapore General Hospital, Singapore. 786-O and RCC4 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 1× antibiotic-antimycotic solution with 10% FBS. Caki-1 cells were cultured in McCoy’s 5A Medium containing 1× antibiotic-antimycotic solution with 10% FBS. RCC cells were treated with zerumbone for indicated doses and time intervals and thereafter cultured in complete culture media before being harvested for various experiments as listed below.

Western Blotting

For detection of various proteins, zerumbone-treated whole-cell extracts were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mM PMSF, and 4 mM
NaVO₄). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 7.5% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with indicated antibodies (1:1000) overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by chemiluminescence (Western Bright Sirius; Advansta, Inc., CA).

**Immunocytochemistry for STAT3 Localization**

786-O cells were plated in chamber slides in DMEM containing 10% FBS and allowed to adhere for 24 h. On next day, the cells were fixed with cold acetone for 10 min, washed with PBS and blocked with 5% normal goat serum for 1 h. The cells were then incubated overnight at 4°C with anti-STAT3 (1:100; Santa Cruz Biotechnology), washed three times, and incubated with Alexa 488-labeled goat anti-rabbit IgG (1:200; Molecular Probes, Eugene, OR) for 1 h at room temperature. Next, the cells were stained with a 1 μg/mL DAPI solution and mounted on glass slides with Fluorescent Mounting Medium (GIBI Laboratories, Manchester, UK). Using an Olympus Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan), DAPI and FITC fluorescence were excited (Ex: 405 and 488 nm) and detected (Em: 461 and 519 nm) with 2.1% laser transmissivity and 5.0% laser reflectivity, respectively.

**DNA Binding Assay**

DNA binding was performed with a STAT3 DNA binding ELISA kit (Active Motif, Carlsbad, CA) as described previously [24]. The enzymatic product was measured at 450 nm with a microplate reader (Tecan Systems, San Jose, CA).

**Transfection With SHP-1/STAT3 siRNA**

786-O cells were plated in each well of six-well plates and allowed to adhere for 24 h. On day of transfection, 4 μL of lipofectamine Life Technologies was added to 50 nM SHP-1 siRNA in a final volume of 100 μL of culture medium. After 48 h of transfection, cells were treated with zerumbone, and whole-cell extracts were prepared to investigate SHP-1, phospho-STAT3, phospho-JAK2, STAT3, and JAK2 expression by Western blot analysis. For transfection experiments with STAT3 siRNA, 786-O cells were plated in 96-well plates and allowed to adhere for 24 h. On the day of transfection, 4 μL of lipofectamine was added to 50 nM scrambled or STAT3 siRNA in a final volume of 100 μL of culture medium. After 48 h, cells were treated with indicated concentrations of zerumbone and then subjected to MTT assay and Western blot analysis for PARP cleavage.

**RNA Isolation and Reverse Transcription**

Total cellular RNA was extracted from untreated and zerumbone treated RCC cells using TRIZOL reagent (Life Technologies) as described previously [24]. The expression of SHP-1 was analyzed with QIAGEN One Step RT-PCR kit (Qiagen, Hilden, Germany) with GAPDH as an internal control. The RT-PCR reaction mixture contained 10 μL of 5× QIAGEN OneStep RT-PCR buffer, 1 μg of total RNA, 0.6 μM each of forward and reverse primers, 2 μL of dNTP mix, and 2 μL of QIAGEN OneStep RT-PCR enzyme mix in a final volume of 50 μL. The reaction was performed at 95°C for 30 min, 95°C for 5 min, 95°C for 1 min, and 72°C for 1 min for 33 cycles with a final extension at 72°C for 10 min. PCR products were run on 1% agarose gel containing 1× Gel red nucleic acid gel stain from Biotium (Hayward, CA). Stained bands were visualized under UV light and photographed. The primer sequences for SHP-1 mRNA were as follows: 5′-TGCGTGCCAGGAGAACAG-3′ (forward) and, 5′-CTGCATGGTCACAGATGGGC-3′ (reverse). The primer sequences for GAPDH were 5′-CCACAGTCATGCCCATCAD-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

**Real-Time Polymerase Chain Reaction**

Real-time polymerase chain reaction for Bcl-2, cyclin D1, and Mcl-1 genes was performed as described previously [9].

**MTT Assay**

The anti-proliferative effect of zerumbone against RCC cells was determined by the MTT dye uptake method as described previously [24].

**Clonogenic Assay**

Clonogenic assay was performed as described previously [25]. Briefly, six-well dishes were seeded with RCC4/786-O cells (500 cells/well) in complete medium and allowed to grow for 24 h. The cells were then incubated in the presence or absence of zerumbone (50 μM) for indicated time periods. The zerumbone-containing medium was then removed, and the cells were washed in Dulbecco’s phosphate-buffered saline (DPBS) and incubated for an additional 9 d in complete medium. Each treatment was carried out in duplicate. The colonies obtained were stained in clonogenic reagent (50% methanol and 0.25% crystal violet) for 5 min at room temperature followed by washing with PBS twice. The colonies were counted and compared with those formed by untreated cells.

**Flow Cytometric Analysis**

To determine the effect of zerumbone on cell cycle distribution, cells were exposed to zerumbone for 48 h. Thereafter cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37°C with 0.1% RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 μg/mL propidium iodide (PI) for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako Cytomation).
DNA Fragmentation Assay

Analysis of DNA fragmentation upon zerumbone treatment in RCC cells was determined with the Cell Death Detection ELISAPLUS kit (Roche Applied Science) according to the manufacturer's protocol and as described previously [26].

RCC Xenograft Mouse Model

All procedures involving animals were reviewed and approved by NUS Institutional Animal Care and Use Committee. Six week-old athymic nu/nu female mice were implanted subcutaneously in the right flank with \(3 \times 10^6\) 786-O cells/100 \(\mu\)L saline. When tumors have reached 0.3 cm in diameter, the mice were randomized into the following treatment groups (\(n = 5\)/group): (a) untreated control (DMSO 0.1%, v/v, 100 \(\mu\)L i.p. injection); and (b) zerumbone (50 mg/kg of body weight, suspended in DMSO 0.1% v/v, i.p. injection) five times/week. Therapy was continued for 6 wks, and the animals were euthanized at the end of therapy. Tumor dimensions were measured with a digital caliper, and the tumor volume (\(V\)) calculated using the formula: \(V = 1/2 \text{length} \times (\text{width})^2\). Growth curves were plotted using average relative tumor volume within each experimental group at the set time points.

Immunohistochemical Analysis of Tumor Samples

Immunohistochemical staining of xenograft tumors was done as described previously [9]. In brief, solid tumors harvested from control and zerumbone treated mice were fixed with 10% phosphate buffered formalin, processed and embedded in paraffin. Sections were cut and deparaffinized in xylene, and dehydrated in graded alcohol and finally hydrated in water. Antigen retrieval was performed by boiling the slide in 10 mM sodium citrate (pH 6.0) for 30 min. Immunohistochemistry was performed following manufacturer instructions (DAKO LSAB kit; Dako, Carpinteria, CA). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Nonspecific binding was blocked by incubation in the blocking reagent in the LSAB kit according to the manufacturer's instructions. Sections were incubated overnight with primary antibodies as follows: anti-phospho-STAT3, anti-Ki67, anti-CD31, anti-Bcl-2, and anti-caspase-3 (each at 1:100 dilution). Slides were subsequently washed several times in Tris buffered saline with 0.1% Tween 20 and were incubated with biotinylated linker for 30 min, followed by incubation with streptavidin conjugate provided in LSAB kit (Dako) according to the manufacturer's instructions. Immunoreactive species were detected with 3,3-diaminobenzidine tetrahydrochloride (DAB) as a substrate. Sections were counterstained with Gill's hematoxylin and mounted under glass cover slips. Images were taken with an Olympus BX51 microscope (magnification, 40×). Quantitative analysis of immunohistochemistry images was done by visual scores between the control and treated images. For this quantitative analysis, each image was divided into four parts and each divided portion was individually quantified for the biomarker expression. A cell scored as positive refers simply to the presence of brown staining (peroxidase) in any part of the studied tissue. A negative cell scored refers to no staining or a relatively weaker staining.

Statistical Analysis

Data are expressed as the mean ± SEM. In all figures, vertical error bars denote the SEM. The significance of differences between groups was evaluated by Student's t-test and a P-value of <0.05 was considered statistically significant.

RESULTS

Zerumbone Inhibits Constitutive STAT3 Phosphorylation in 786-O Cells

We investigated the effect of zerumbone on STAT3 activation in RCC cells in vitro and in vivo xenograft mouse model. The structure of zerumbone is shown in Figure 1A. We first determined the effect of zerumbone on constitutive STAT3 activation in RCC cells. 786-O cells were incubated with different concentrations of zerumbone for 6 h, whole cell extracts were prepared and the phosphorylation of STAT3 was examined by Western blot analysis with antibodies which recognize STAT3 phosphorylation at tyrosine 705. As shown in Figure 1B, zerumbone inhibited the constitutive activation of STAT3 in 786-O cells in a dose-dependent manner, with maximum inhibition occurring at around 50 \(\mu\)M. Zerumbone had no effect on the expression of STAT3 protein (Figure 1B; lower panel). We also analyzed the incubation time needed for the suppression of STAT3 activation by zerumbone in 786-O cells. As shown in Figure 1C, the inhibition was time-dependent, with complete inhibition occurring at around 6 h, again with no substantial effect on the expression of STAT3 protein (Figure 1C; lower panel).

Zerumbone Suppresses the Nuclear Translocation of STAT3 in RCC Cells

Because the active STAT3 dimer is capable of translocating to the nucleus and inducing transcription of specific target genes [7], we next investigated whether zerumbone can suppress nuclear translocation of STAT3. Figure 1D clearly indicates that zerumbone inhibited the translocation of STAT3 to the nucleus in 786-O cells.

Zerumbone Abrogates Binding Ability of STAT3 to the DNA in RCC Cells

Because tyrosine phosphorylation can induce the dimerization of STAT3 followed by its translocation to the nucleus, where this transcription factor can bind
to the DNA and control gene transcription [8], we next analyzed whether zerumbone can modulate the DNA binding ability of STAT3. Analysis of nuclear extracts prepared from 786-O cells with ELISA based TransAM STAT3 assay kit showed that zerumbone inhibited STAT3-DNA binding activity in a dose-dependent manner (Figure 1E).

Zerumbone Suppresses Constitutive Activation of c-Src

STAT3 has also been reported to be activated by tyrosine kinases of the Src kinase families [8] and targeted inhibition of Src kinase has been found to suppress both the proliferation and migration in RCC cells [27]. Hence, we next determined whether...

Figure 1. Zerumbone inhibits constitutively active STAT3 in 786-O cells. (A) The chemical structure of zerumbone. (B) Zerumbone suppresses phospho-STAT3 levels in a dose dependent manner. 786-O cells (2 × 10^6/mL) were treated with the indicated concentrations of zerumbone for 6h, after which whole-cell extracts were prepared, and 30μg of protein was resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. (C) Zerumbone suppresses phospho-STAT3 levels in a time-dependent manner. 786-O cells (2 × 10^6/mL) were treated with the 50μM zerumbone for the indicated times, after which Western blotting was performed as described for panel B. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. (D) Zerumbone causes inhibition of translocation of STAT3 to the nucleus. 786-O cells (1 × 10^5/mL) were incubated with or without 50μM zerumbone for 6h and then analyzed for the intracellular distribution of STAT3 by immunocytochemistry. STAT3 (green) was immunostained with rabbit anti-STAT3 followed by FITC-conjugated secondary antibodies and the nucleus (blue) was stained with DAPI. The third panel shows the merged images of the first and second panels. (E) Zerumbone suppresses STAT3 DNA binding ability in 786-O cells. 786-O cells were treated with indicated concentrations of zerumbone for 6h; nuclear extracts were prepared, and 50μg of the nuclear extract protein was used for ELISA based STAT3 DNA binding assay as described in Material and Methods section. The results shown are representative of two independent experiments *P < 0.05.
zerumbone can modulate the constitutive activation of Src kinase in 786-O cells. We found that zerumbone suppressed the constitutive phosphorylation of c-Src kinase in a time-dependent manner (Figure 2A). The levels of nonphosphorylated Src kinase remained unaffected under the same conditions.

Zerumbone Modulates Constitutive Activation of JAK1 and JAK2

Because STAT3 is also activated by tyrosine kinases of the Janus family (JAKs) [7], and the defective JAK/STAT activation in RCC has been previously linked to IFN-α resistance [28], we also analyzed whether zerumbone can suppress the activation of JAK1 in 786-O cells. We observed that zerumbone suppressed the constitutive phosphorylation of JAK1 in a time-dependent manner (Figure 2B). The levels of nonphosphorylated JAK1 remained unchanged under the same conditions (Figure 2B, bottom panel). To analyze the potential effect of zerumbone on JAK2 activation, 786-O cells were treated for indicated time intervals with zerumbone and the phosphorylation levels of JAK2 were determined (Figure 2C). We observed that zerumbone suppressed the constitutive phosphorylation of JAK2 in a time-dependent manner (Figure 2C). The levels of nonphosphorylated JAK2 remained unaffected under the same conditions (Figure 2C, bottom panel).

Figure 2. (A) Zerumbone suppresses phospho-Src levels in a time-dependent manner. 786-O cells (2 × 10^6/mL) were treated with 50 μM zerumbone, after which whole-cell extracts were prepared and 30 μg aliquots of those extracts were resolved on 10% SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed for phospho-src antibody. The same blots were stripped and reprobed with Src antibody to verify equal protein loading. (B) Zerumbone suppresses phospho-JAK1 levels in a time-dependent manner. 786-O cells (2 × 10^6/mL) were treated with 50 μM zerumbone for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK1 antibody. The same blots were stripped and reprobed with JAK1 antibody to verify equal protein loading. (C) Zerumbone suppresses phospho-JAK2 levels in a time-dependent manner. 786-O cells (2 × 10^6/mL) were treated with 50 μM zerumbone for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS–PAGE, electrotransferred onto nitrocellulose membranes, and probed against phospho-JAK2 antibody. The same blots were stripped and reprobed with JAK2 antibody to verify equal protein loading. (D) Caki-1 cells (2 × 10^6/mL) were treated with 50 μM zerumbone for the indicated times and then stimulated with EGF (100 ng/mL) for 2 h. Whole-cell extracts were then prepared and analyzed for phospho-STAT3/STAT3 by Western blotting. (E) Caki-1 cells (2 × 10^6/mL) were treated with 50 μM zerumbone for the indicated times and then stimulated with EGF (100 ng/mL) for 2 h. Whole-cell extracts were then prepared and analyzed for phospho-JAK1/JAK1 by Western blotting. (F) Caki-1 cells (2 × 10^6/mL) were treated with 50 μM zerumbone for the indicated times and then stimulated with EGF (100 ng/mL) for 2 h. Whole-cell extracts were then prepared and analyzed for phospho-JAK2/JAK2 proteins. The results shown are representative of two independent experiments.
of JAK2 was analyzed by Western blot analysis. As shown in Figure 2C, JAK2 was constitutively active in 786-O cells and pretreatment with zerumbone suppressed this phosphorylation in a time-dependent manner.

Zerumbone Inhibits Inducible STAT3 and JAK1/2 Phosphorylation in Caki-1 Cells

Because EGF induces STAT3 phosphorylation [7], we next determined whether zerumbone could also inhibit EGF-induced STAT3 phosphorylation in Caki-1 cells that display relatively low levels of constitutively active STAT3. Interestingly, we observed that EGF-induced STAT3 as well as JAK1 phosphorylation was suppressed by zerumbone in a time-dependent manner. Exposure of cells to zerumbone for 4 h was sufficient to substantially suppress EGF-induced STAT3 and JAK1 phosphorylation in Caki-1 cells (Figure 2D and E). Also, we found that zerumbone can abrogate EGF-induced JAK2 phosphorylation in Caki-1 cells (Figure 2F). These results clearly suggest that zerumbone can also downregulate inducible STAT3 activation in RCC cells.

Tyrosine Phosphatases Are Involved in Zerumbone-Induced Inhibition of STAT3 Activation

Because protein tyrosine phosphatases (PTPs) have also been reported to play a critical role in STAT3 activation [8], we determined whether zerumbone-induced inhibition of STAT3 tyrosine phosphorylation could be because of activation of a PTP. Treatment of 786-O cells with the broad-acting tyrosine phosphatase inhibitor sodium pervanadate reversed zerumbone-induced suppression of STAT3 activation in a dose-dependent manner (Figure 3A). This result indicates that tyrosine phosphatases may play an important part in zerumbone-induced inhibition of STAT3 activation in RCC cells.

Zerumbone Induces the Expression of SHP-1 in RCC Cells

SHP-1 is a SH-2 containing tyrosine phosphatase involved in the suppression of a variety of cytokine signals, including those mediating STAT3 activation [29]. We therefore examined whether zerumbone can induce the expression of SHP-1 in 786-O cells. Cells were incubated with 50 μM of zerumbone for 4 h, whole cell extracts were prepared and examined for SHP-1 protein expression by Western blot analysis. As shown in Figure 3B, zerumbone induced the expression of SHP-1 protein in 786-O cells in a time-dependent manner, with maximum expression observed at 4 h. This stimulation of SHP-1 expression by zerumbone correlated with down-regulation of constitutive STAT3 activation in 786-O cells (Figure 1B). Whether modulation of SHP-1 by zerumbone is also regulated at the transcriptional level was investigated as well. We observed that treatment of zerumbone also induced the expression of SHP-1 mRNA in a dose-dependent manner (Figure 3C). Thus, these finding suggest that the induction of SHP-1 expression by zerumbone may mediate the downregulation of constitutive STAT3 activation in RCC cells.

Transfection With siRNA Downregulated the Expression of SHP-1 Induced by Zerumbone in RCC Cells

Whether the suppression of SHP-1 expression by siRNA abrogates the zerumbone-induced SHP-1 expression in RCC cells, was also determined. As observed by Western blot analysis, zerumbone-induced SHP-1 expression was effectively inhibited in the cells transfected with SHP-1 siRNA but not in those treated with the scrambled siRNA (Figure 3D).

Zerumbone Downregulates the Expression of Cyclin D1, Bcl-2, Bcl-xL, Mcl-1, Survivin, MMP-9, and VEGF in RCC Cells

STAT3 activation has been shown to regulate the expression of various gene products involved in proliferation, anti-apoptosis, invasion, angiogenesis, and chemoresistance [8]. We found that the expression of cell cycle regulator protein (cyclin D1), the antiapoptotic proteins (Bcl-2, Bcl-xL, survivin, Mcl-1), (MMP-9) a protein that plays an important role in cellular invasion, and the angiogenic gene product (VEGF) was inhibited upon zerumbone treatment. The expression of these oncogenic proteins decreased in a time-dependent manner, with maximum suppression observed at around 24 h (Figure 4A). We also found that mRNA expression of cyclin D1, Bcl-2, and Mcl-1 was down-modulated by zerumbone treatment in a time-dependent manner with maximum reduction observed at 24 h after treatment (Figure 4B).

Zerumbone Inhibits the Proliferation of RCC Cells in a Dose and Time Dependent Manner

Because zerumbone downregulated the expression of cyclin D1, a gene involved in cell cycle progression, we investigated whether zerumbone can also suppress the proliferation of RCC cells by using the MTT assay. We indeed observed that zerumbone inhibited proliferation of 786-O, and RCC4 cells significantly in a dose and time dependent manner (Figure 5A).
Zerumbone Inhibits Colony Forming Ability of Human RCC Cells in Long-Term Clonogenic Assay

Tumors in vivo do not grow as monolayers but as colonies. Hence we further assessed the effects of zerumbone treatment on cell survival by using a clonogenic assay, which measures the long-term effects of drugs on permanent cell growth arrest and apoptosis.

The exposure of both RCC4 and 786-O cells to zerumbone resulted in time dependent reduction in colony formation compared with that of untreated control (Figure 5B and C).

Zerumbone Causes the Accumulation of the Cells in the sub-G1 Phase of the Cell Cycle

Because D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase [8] and suppression in the levels of cyclin D1 was noticed in zerumbone treated cells, we next analyzed the effect of zerumbone on cell cycle distribution in 786-O cells. We found that zerumbone can cause substantial increased accumulation of cell population in sub-G1 phase, which is indicative of apoptosis (Figure 5D).

**Figure 3.** Zerumbone induces the expression of SHP-1 protein in 786-O cells. (A) Pervanadate reverses the phospho-STAT3 inhibitory effect of zerumbone. 786-O cells (2 × 10^7/mL) were co-incubated with the indicated concentrations of pervanadate and 50 μM zerumbone for 6 h, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 7.5% SDS–PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3 and STAT3. (B) 786-O cells were treated with 50 μM zerumbone for the indicated times, after which Western blotting was performed. (C) Effect of zerumbone on SHP-1 mRNA expression in 786-O cells. 786-O cells were treated with indicated concentrations of zerumbone for 6 h, after which RNA samples were subjected to RT-PCR with SHP-1 and GAPDH specific primers. PCR products were run on 1% agarose gel containing Gel Red. Stained bands were visualized under UV light and photographed. (D) Effect of SHP-1 knockdown on zerumbone-induced expression of SHP-1. 786-O cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 48 h, cells were treated with 50 μM zerumbone for 6 h and whole-cell extracts were subjected to Western blot analysis. (E) 786-O cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 48 h, cells were treated with 50 μM zerumbone for 6 h and whole-cell extracts were subjected to Western blot analysis for phosphorylated STAT3 and total STAT3. (F) 786-O cells were transfected with either SHP-1 siRNA or scrambled siRNA (50 nM). After 24 h, cells were treated with 50 μM zerumbone for 6 h and whole-cell extracts were subjected to Western blot analysis for phosphorylated JAK2 and total JAK2. The results shown are representative of two independent experiments.
Zerumbone Activates Caspase-3 and Causes PARP Cleavage

Whether suppression of constitutively active STAT3 in 786-O cells by Zerumbone leads to apoptosis was also investigated. In 786-O cells treated with zerumbone there was a time-dependent increase in the percentage of DNA fragmentation as determined by Cell Death Detection ELISA PLUS kit (Figure 6A). Also, in cells treated with zerumbone there was a time-dependent activation of pro-caspase-3 which led to the cleavage of a 116 kDa PARP protein into an 85 kDa fragment (Figure 6B). These results clearly suggest that zerumbone can induce significant apoptosis in 786-O cells.

Figure 4. Zerumbone suppresses STAT3 regulated gene products involved in proliferation, survival and angiogenesis. (A) 786-O cells (2 × 10^6/mL) were treated with 50 μM zerumbone for indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS–PAGE, membrane sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-xL, survivin, Mcl-1, MMP-9, and VEGF antibodies. (B) 786-O cells (3 × 10^5/mL) were treated with 50 μM zerumbone for the indicated time intervals, after which cells were harvested after treatment and RNA samples, were extracted. One microgram portions of the respective RNA extracts then proceed for reverse transcription to generate corresponding cDNA. Real-time PCR was performed to measure the relative quantities of mRNA. Each RT product was targeted against cyclin D1, Bcl-2, and Mcl-1 TaqMan probes, with 18S as endogenous control for measurement of equal loading of RNA samples. Results were analyzed with Sequence Detection Software version 1.3 provided by Applied Biosystems (Foster City, CA). Relative gene expression was obtained after normalization with endogenous 18S and determination of the difference in threshold cycle (ΔΔCt) between treated and untreated cells using 2^(-ΔΔCt) method. The results shown are representative of two independent experiments.
Figure 5. Zerumbone suppresses proliferation, colony forming ability and causes accumulation of RCC cells in sub-G1 phase of the cell cycle. (A) 786-O, and RCC4 cells (5 x 10^5/mL) were plated in triplicate, treated with indicated concentrations of zerumbone, and then subjected to MTT assay after 24, 48, and 72 h to analyze proliferation of cells. Standard deviations between the triplicates are indicated. (B and C) RCC4 and 786-O cells were incubated with zerumbone for 12 or 24 h and subsequently allowed to grow into colonies. After 9 d incubation, cells were stained with clonogenic reagent, photographed and colonies were counted and represented graphically. Results are representative of two independent experiments. (D) 786-O cells (1 x 10^6/mL) were treated with 25 μM zerumbone for 48 h, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometric analysis. The results shown are representative of two independent experiments.
Transfection With STAT3 siRNA Abrogates Anti-Proliferative/Pro-Apoptotic Effects of Zerumbone in RCC Cells

We determined whether the suppression of STAT3 expression by siRNA could abrogate the anti-proliferative/pro-apoptotic effect of zerumbone on RCC cells. Results shown in Figure 6C and D clearly indicate that the observed anti-proliferative and pro-apoptotic effects of zerumbone were substantially abolished in the cells transfected with STAT3 siRNA, whereas treatment with scrambled siRNA had minimal effect.

These results suggest that inhibition of proliferation and induction of apoptosis induced by zerumbone is mediated at least in part through the suppression of STAT3 signaling axis in RCC cells.

Zerumbone Suppresses the Growth of Human RCC In Vivo and Suppresses STAT3 Activation in Tumor Tissues

We also analyzed the antitumor potential of zerumbone in vivo via intra-peritoneal administration in a subcutaneous model of human RCC using 786-O cells. Zerumbone at dose of 50 mg/kg caused...
significant inhibition of tumor growth compared with the DMSO-treated controls (Figure 7A and B) without any observed adverse effects. The tumor inhibition rate was around 15% on week 5 and 45% on week 6 (Figure 7B). The data on body weight also clearly showed that zerumbone treatment did not cause any significant weight loss in treated mice (Figure 7C). We further evaluated the effect of zerumbone on constitutive p-STAT3 levels in RCC tumor tissues by immuno-histochemical analysis and found that zerumbone can significantly inhibit the constitutive STAT3 activation in drug treated group as compared with the control group (Figure 7D). The effect of zerumbone was also analyzed on the expression of Ki-67 (marker of proliferation), CD31 (marker of angiogenesis), Bcl-2 (marker of survival), and caspase-3 (marker of apoptosis). As shown in Figure 7D, expression of various biomarkers including Ki-67, CD31, and Bcl-2 was downregulated and that of caspase-3 was significantly increased in zerumbone treated group as compared with control group. We also found that zerumbone inhibited STAT3 activation in tumor tissues from mice treated with the agent in vivo. Interestingly, zerumbone was also found to increase SHP-1 expression in zerumbone treated tumor tissues obtained from the mice. Overall, these observations further confirmed that down-modulation of STAT3 activation by zerumbone may be through up-regulation of SHP-1 (Figure 8).

**DISCUSSION**

The goal of this study was to determine whether zerumbone exerts its anticancer effects through the abrogation of the STAT3 signaling pathway in RCC cells and xenograft mouse model. We found that zerumbone suppressed STAT3 activation in human RCC cells concomitant with the inhibition of upstream kinases such as (c-Src, JAK1, and JAK2 activation) and the induction of the phosphatase (SHP-1). Zerumbone also downregulated the expression of STAT3 regulated oncogenic gene products in RCC cells. It also caused the inhibition of proliferation, increased accumulation of cells in sub-G1 phase, and induced significant apoptosis in RCC cells. We also analyzed the therapeutic efficacy of zerumbone to suppress tumor growth in RCC xenograft mouse model. Intra-peritoneal administration of zerumbone into nude mice bearing subcutaneous 786-O xenografts resulted in significant suppression of tumor growth and the inhibition of STAT3 activation in the tumor tissues.

Whether examined by STAT3 phosphorylation at tyrosine 705 using Western blot analysis, by nuclear translocation, or by DNA binding assay, we found that zerumbone significantly suppressed STAT3 activation in RCC cells. STAT3 phosphorylation clearly plays a pivotal role in the proliferation and survival of a wide variety of tumor cells [30]. Dysregulated STAT3 activation is an important hallmark of RCC and a recent study clearly indicates that STAT3 polymorphism can even predict the response to IFN-α therapy in patients with metastatic RCC [31]. Moreover, it has been observed that the repression of suppressor of cytokine signaling (SOCS) proteins, which are negative regulators of STAT3 activation, can overcome the resistance of RCC to IFN-α [32]. Additionally, the multi-targeted tyrosine kinase inhibitor sunitinib approved by FDA for the treatment of RCC also exerts its anticancer effects through the inhibition of STAT3 activation and the induction of apoptosis [33]. Thus, the identification of pharmacological STAT3 inhibitors such as zerumbone as described here can form the basis of a novel therapy for RCC patients.

The inhibitory effect of zerumbone on STAT3 phosphorylation correlated with the suppression of upstream protein tyrosine kinases c-Src, JAK1, and JAK2 in RCC cells. Previous studies have indicated that Src family members are overexpressed and also associated with decreased survival in RCC [34]. Moreover it has been reported that Connexin 32 acts as a tumor suppressor gene in metastatic RCC because of the inactivation of Src and the concomitant inhibition of Src by pharmacological blocker (PP1) is an effective procedure to induce cytotoxic effects in RCC cells [35,36]. Our results also are in part agreement with a recent study, in which AZD1480 a potent, competitive small-molecule inhibitor of JAK1/2 kinase was found to significantly inhibit the tumor angiogenesis and metastasis mediated by STAT3 in RCC cells and mouse model [37].

We also found evidence that the zerumbone-induced inhibition of STAT3 activation involves the induction of a PTP. Numerous PTPs have been implicated in STAT3 signaling including SHP-1, SH-PTP2, TC-PTP, PTEN, PTP-1D, CD45, PTP-epsilon, low molecular weight (LMW), and PTP [38]. However, we found in this study that zerumbone induces the expression of SHP-1 protein and mRNA in RCC cells, which correlated, with its ability to negatively regulate constitutive STAT3 phosphorylation. Transfection with SHP-1 siRNA reversed the STAT3/JAK2 inhibitory effects of this sesquiterpene, thereby clearly implicating an important role of this phosphatase in zerumbone-induced downregulation of STAT3/JAK2 activation.

We further observed that zerumbone can inhibit the expression of several STAT3-regulated genes; including proliferative (cyclin D1) and antiapoptotic gene products (Bcl-2, Bcl-xl, survivin, and Mcl-1) and angiogenic gene product (VEGF). The downregulation of cyclin D1 expression correlated with suppression in proliferation as observed in RCC cell lines and corroborate well with other studies on the inhibitory effect of zerumbone on tumor cell proliferation and cell cycle progression [39]. The downregulation of the expression of Bcl-2, Bcl-xl, survivin, and Mcl-1 may be linked with the zerumbone’s ability to induce
Figure 7. Zerumbone inhibits the growth of human RCC in vivo. (A) Schematic diagram of tumors harvested from control and zerumbone treated mice. (B) Athymic mice bearing subcutaneous 786-O tumors were treated for five times a week for 6 consecutive weeks with 50 mg/kg zerumbone (each group, n = 5). *P < 0.05. (C) Zerumbone treatment did not exhibit any substantial toxic effects as no significant changes in body weight of treated mice as compared to control group was observed. (D) Immunohistochemical analysis of various biomarkers showed the inhibition in expression of p-STAT3, Ki-67, CD31, Bcl-2, and increased levels of caspase-3 expression in zerumbone treated samples as compared with control group. Percentage indicates positive staining for the given biomarker. The photographs were taken at the magnification of 40×. (E) Tumor tissues from mice treated with vehicle and zerumbone (50 mg/kg body weight) for 6 wks were homogenized and analyzed by Western blot for phospho-STAT3 and SHP-1 expression levels as indicated under Materials and Methods section. The blots were stripped and reprobed with β-actin antibody to verify equal protein loading.
apoptosis in RCC cells and may also contribute to its previously reported chemosensitizing effects [40]. Also, our findings are in part agreement with a recent study in which zerumbone was found to induce apoptosis in human RCC via Gli-1/Bcl-2 pathway [41], although its detailed mechanism of action was not investigated in this report. We further noticed that zerumbone treatment can induce significant apoptosis as evident by DNA fragmentation assay in RCC cells which correlates with its previously reported effects on apoptosis in tumor cells [14]. Additionally, we also found that knocking down the expression of STAT3 with siRNA significantly reduced the anti-proliferative and apoptotic effects of zerumbone in RCC cells, thereby supporting the hypothesis that anti-proliferative/pro-apoptotic effects of zerumbone were mediated at least in part through the abrogation of the STAT3 signaling pathway.

Whether these in vitro observations with zerumbone has any relevance to that in vivo was also investigated. Our results also show that zerumbone significantly suppressed RCC growth in xenograft mouse model, downregulated the expression of phospho-STAT3, Ki-67, CD31, Bcl-2, and increased the levels of caspase-3 in treated group as compared to the control. The Western blot analysis of tumor tissues further indicated that tissues from DMSO-treated mice express STAT3 phosphorylation, whereas that from zerumbone-treated animals did not. Additionally, zerumbone was observed to induce SHP-1 expression in tumor tissues, which further suggest that this sesquiterpene may modulate STAT3 activation in RCC through the upregulation of SHP-1. Interestingly, the doses required to inhibit STAT3 activation (50 mg/kg) was quite comparable to rationally designed JAK2 inhibitor AZ1489 that can also target STAT3 phosphorylation [37]. Overall, our results clearly indicate that zerumbone can inhibit STAT3 activation cascade, which may provide new mechanistic insights into the anticancer actions of this sesquiterpene.

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