6-Shogaol from Dried Ginger Inhibits Growth of Prostate Cancer Cells Both \textit{In Vitro} and \textit{In Vivo} through Inhibition of STAT3 and NF-κB Signaling

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Abstract

Despite much recent progress, prostate cancer continues to represent a major cause of cancer-related mortality and morbidity in men. Prostate cancer is the most common non-skin neoplasm and second leading cause of death in men. 6-Shogaol (6-SHO), a potent bioactive compound in ginger (\textit{Zingiber officinale} Roscoe), has been shown to possess anti-inflammatory and anticancer activity. In the present study, the effect of 6-SHO on the growth of prostate cancer cells was investigated. 6-SHO effectively reduced survival and induced apoptosis of cultured human (LNCaP, DU145, and PC3) and mouse (HMVP2) prostate cancer cells. Mechanistic studies revealed that 6-SHO reduced constitutive and interleukin (IL)-6–induced STAT3 activation and inhibited both constitutive and TNF-α–induced NF-κB activity in these cells. In addition, 6-SHO decreased the level of several STAT3 and NF-κB–regulated target genes at the protein level, including cyclin D1, survivin, and cMyc and modulated mRNA levels of chemokine, cytokine, cell cycle, and apoptosis regulatory genes (IL-7, CCL5, BAX, BCL2, p21, and p27). 6-SHO was more effective than two other compounds found in ginger, 6-gingerol, and 6-paradol at reducing survival of prostate cancer cells and reducing STAT3 and NF-κB signaling. 6-SHO also showed significant tumor growth inhibitory activity in an allograft model using HMVP2 cells. Overall, the current results suggest that 6-SHO may have potential as a chemopreventive and/or therapeutic agent for prostate cancer and that further study of this compound is warranted. Cancer Prev Res; 7(6); 627–38. ©2014 AACR.

Introduction

Prostate cancer is the most common noncutaneous neoplasm and is the second leading cause of cancer death in American men (1). Approximately 1 in 6 men will be diagnosed with prostate cancer during their lifetime and about 1 in 36 will die of prostate cancer (2). It is estimated that approximately 238,590 new cases of prostate cancer will be diagnosed and 29,720 prostate cancer-related deaths will occur in the United States in 2013 (1, 3). During the early stages of the disease, prostate cancer cells are dependent on androgen for their growth and can be successfully treated with androgen ablation therapy. However, in a majority of cases, the disease eventually progresses to an androgen-independent state and becomes unresponsive to chemotherapy, radiotherapy, or hormonal therapy (4–6).

Moreover, the toxicity associated with chemotherapy seriously compromises the quality of life of patients with prostate cancer (7). The identification of agents that are less toxic as well as effective against both androgen-dependent and androgen-independent prostate cancer would provide better options for prostate cancer management. Furthermore, such compounds may also have value in the prevention of prostate cancer.

Ginger is a natural dietary ingredient with antioxidant, anti-inflammatory, and anticarcinogenic properties (8). Ginger contains several pungent constituents such as gingerols, shogaols, paradols, and gingerdiols (9). Gingerols were identified as the major active components in fresh ginger rhizome with 6-gingerol (6-GIN) being the most abundant constituent (10). The shogaols are the predominant pungent constituents in dried ginger (11). Recently, there has been a growing interest in ginger and its components for their potential chemopreventive effects (12). For example, an ethanol extract of ginger was shown to decrease the incidence, size, number, and multiplicity of skin papillomas in SENCAR mice (13). Lee and colleagues reported that 6-GIN inhibited cell proliferation, induced apoptosis, and G1 cell-cycle arrest in human colorectal cancer cells (14). Several \textit{in vitro} and \textit{in vivo} studies also showed that 6-Shogaol (6-SHO) has more potent anti-inflammatory activity than 6-GIN and another widely studied phytochemical, curcumin (15, 16). Nonetheless, the...
beneficial effects of 6-SHO and its mechanism of action have not been clearly evaluated in prostate cancer.

In the present study, we evaluated the efficacy of 6-SHO for inhibition of cell survival and induction of apoptosis in both human and mouse prostate cancer cells. The effects of 6-SHO were compared with the effects of two other ginger compounds, 6-GIN and 6-paradol (6-PAR). 6-SHO was also evaluated for its efficacy at inhibiting HMVP2 cell growth, a prostate tumor cell line derived from HiMyc mice (J. Blando and colleagues; unpublished data) in vivo. We report for the first time that 6-SHO produces significant anticancer activity against human and mouse prostate cancer cells by inhibiting cell survival and inducing apoptosis through reduction of STAT3 and NF-κB activity. Collectively, the current results suggest that 6-SHO may have a role to play as a chemopreventive and/or therapeutic agent for prostate cancer.

Materials and Methods

Reagents
6-SHO was synthesized from zingerone using a modification of published procedures (refs. 17, 18; see Supplementary Information). Samples of 6-SHO, 6-GIN, and 6-PAR were also purchased from Dalton Pharma. Media (RPMI-1640), FBS was purchased from Life Technologies. Antibodies were purchased as follows: STAT3, pSTAT3Y705, pSTAT3S727, cyclin D1, survivin, cMyc, pNF-κBp65S536, NF-κBp65, pJAK2Y1007/08, JAK2, pSrcY416, PARP, and caspase-7. Cell Signaling Technology; p21, p27, and pIκBαS32/36, Santa Cruz Biotechnology; and β-actin, Sigma-Aldrich. Secondary antibodies were purchased from GE Healthcare.

Cell culture
The human prostate cancer cells LNCaP, DU145, and PC-3 were purchased from American Type Culture Collection. Cells were maintained in RPMI-1640 medium with 10% FBS. The prostate tumor cell line, HMVP2, was derived from the ventral prostate of a one-year-old HiMyc mouse (19) and cultured in RPMI-1640 medium containing 10% FBS. The prostate tumor cell line, HMVP2, was derived from the ventral prostate of 5.5-month-old FVB/N mice and cultured in RPMI-1640 medium containing 10% FBS. A secondary antibody was purchased from GE Healthcare.

Cell survival assay
Cell viability was measured by MTT assay (20). Briefly, cells (5 x 10^4/mL) in 96-well plates were treated with indicated concentrations of 6-SHO, 6-GIN, and 6-PAR. After incubation, the cells were treated with MTT solution, incubated for an additional 3 hours, and were dissolved in SDS solution, and the absorbance was measured at 570 nm using a microplate reader (Tecan Group Ltd.).

Apoptosis assay
The percentage of apoptotic cells was determined using a Guava Nexin apoptosis detection kit and Annexin V-positive cells were measured by Guava-based flow cytometry according to the manufacturer’s instructions (Millipore).

Western blotting
Expression of phosphorylated and total protein levels was measured by Western blot analysis with slight modifications as described previously (21). Protein lysates were prepared from cultured cells following treatment as indicated. Proteins were visualized using a Commercial Chemiluminescent Detection Kit (Thermo Scientific).

Quantitative real-time PCR
Cells were treated with 6-SHO for 24 hours. Total RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen). Reverse transcription was performed as previously described (21). Gene expression levels were quantitatively determined by the ddCt method using a ViiA7 Real-Time PCR System (Applied Biosystems) in conjunction with SYBR Green PCR Master Mix (Qiagen).

Immunofluorescence staining
DU145 or LNCaP cells (1 x 10^5) were plated on coverslips, exposed to vehicle or 6-SHO for 2 hours, stimulated with either 10 ng/mL interleukin (IL)-6 or 10 ng/mL IL-1β for 30 minutes, fixed with ice-cold methanol at 10 minutes at −20°C, and permeabilized with 0.05% Triton X-100 for 5 minutes. Cells were then incubated with PBS (10% goat serum, 1% bovine serum albumin) for 1 hour followed by overnight incubation with primary antibodies at 4°C, washed and treated with 2 μg/mL of Alexa Fluor 568-conjugated secondary antibody (Molecular Probes) for 1 hour at room temperature, and mounted with medium containing 4′, 6-diamidino-2-phenylindole (22). Cells were visualized using a fluorescence microscope (Olympus Optical Co. Ltd.).

Allograft tumor experiments
Syngeneic FVB/N male mice were obtained from an in-house breeding colony and were fed a semi-purified diet (AIN76A, 10 Kcal% fat, Research Diets) and water ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin (Austin, TX). HMVP2 cells were plated in ultra-low attaching tissue culture dishes for 3 days to generate spheroids. Spheroids were harvested, mixed with Matrigel (1:1), and injected subcutaneously into the mouse flank. Mice were randomly divided into three groups of 5 mice and treated with vehicle control or 6-SHO (50 and 100 mg/kg body weight) intraperitoneally (i.p.) every other day for 32 days. The doses of 50 and 100 mg/kg were determined on the basis of the existing literature of 6-SHO (23), where 10 mg/kg/daily doses of 6-SHO were previously used. However, we decreased the frequency of treatments to every other day to minimize the stress associated with injection and therefore a higher dose was utilized (100 mg/kg). Tumor size was measured twice a week using a digital caliper and tumor volume was calculated by the formula: 0.5236 D1(D2)^2, where D1 and D2 are the long and short diameter, respectively. Food consumption and body weight of the
mice were measured weekly. Allograft tumors were weighed at the end of the study.

**Statistical analyses**

Data are representative of at least three independent experiments unless otherwise indicated. Statistical analyses were performed using one-way ANOVA followed by Bonferroni multiple comparison test except for the single-dose studies where a two tailed Student t test was used. Significance was set at $P \leq 0.05$ in all cases.

**Results**

**Inhibition of human prostate cancer cell survival by 6-SHO**

The effect of 6-SHO on cell survival was evaluated using both androgen-dependent (LNCaP) and androgen-independent (DU145, PC-3) human prostate cancer cells. As shown in Fig. 1A, 6-SHO at concentrations of 10 to 40 μmol/L reduced the survival of LNCaP cells. Reductions in survival of LNCaP cells at a concentration of 40 μmol/L were 67%, 85%, and 96% at 24, 48, and 72 hours of treatment, respectively. 6-SHO also decreased the survival of DU145 and PC-3 cells at the same concentration by 64% and 66% after 24 hours, 80% and 78% after 48 hours, and 80% and 76% after 72 hours, respectively.

**6-SHO induces apoptosis in human prostate cancer cells**

Given the robust survival inhibition of prostate cancer cells observed following treatment with 40 μmol/L 6-SHO, we investigated whether this effect was due to the induction of apoptosis. As shown in Fig. 1B, 6-SHO (40 μmol/L) significantly increased the number of apoptotic cells in all three human prostate cancer cells as assessed by flow cytometry. Furthermore, treatment with 6-SHO led to the cleavage of both caspase-7 and PARP (Fig. 1C).

**Inhibition of constitutive and IL-6–induced STAT3 activation by 6-SHO**

To further explore the mechanism for the effects of 6-SHO on cell survival, we examined its effects on constitutive and IL-6–induced activation of STAT3 in human prostate cancer cells. Previous studies have shown in both LNCaP and DU145 cells that STAT3 is constitutively phosphorylated on Ser727, whereas constitutive phosphorylation on Tyr705 is observed only in DU145 cells (refs. 22, 24; see Fig. 2A). PC-3 cells did not express significant levels of either unphosphorylated or phosphorylated STAT3 and therefore were not used for these studies. As shown in Fig. 2B and C, 6-SHO inhibited the phosphorylation of STAT3Ser727 in both LNCaP and DU145 cells and STAT3Tyr705 in DU145 cells in both a concentration- and time-dependent manner.

Treatment with IL-6 induced phosphorylation of STAT3 at both Ser727 and Tyr705 in LNCaP cells (Fig 2D and E) and 6-SHO at concentrations of 20 and 40 μmol/L inhibited phosphorylation of STAT3 at both phosphorylation sites. The inhibition of IL-6–induced levels of pSTAT3Ser727 and pSTAT3Tyr705 by 6-SHO in both cells was further confirmed by immunocytochemical staining (see Supplementary Fig. S1A and S1B). Following phosphorylation at Tyr705, STAT3

![Figure 1. 6-SHO inhibits cell survival and induces apoptosis in human prostate cancer cells. A, LNCaP, DU145, and PC-3 cells were treated with the indicated concentrations of 6-SHO for 24, 48, and 72 hours and cell survival was measured by MTT assay. The data are presented as mean ± SEM. B, cells were treated with vehicle or 40 μmol/L 6-SHO for 48 hours and apoptosis was measured by Annexin V staining. The data are presented as mean ± SEM. C, cells were treated with vehicle or 6-SHO for 24 hours and Western blot analysis was performed for apoptosis markers. a, significantly different ($P < 0.05$) compared with the control group and b, compared with the other treated groups. Changes in relative band intensities (normalized to β-actin) for Western blot data in C are given at the top of each column and represent the average of two separate experiments.]
Figure 2. Reduction of constitutive and IL-6 induced STAT3 activation by 6-SHO in human prostate cancer cells. A–E, cell lines were cultured as indicated in Materials and Methods. Western blot analysis was performed to determine STAT3 status and level (pSTAT3Y705, pSTAT3S727, and total STAT3) in whole-cell lysates collected after the indicated treatments. A, cell lysates were prepared from untreated cultures of LNCaP, DU145, and PC-3 cells. B, LNCaP and DU145 cells were treated with 20 or 40 μmol/L 6-SHO and subjected to Western blot analysis. (Continued on the following page.)
is translocated to the nucleus where it becomes bound to specific DNA sites (25). As shown in Fig. 2F, 30 minutes after treatment of DU145 cells with IL-6, STAT3 was observed primarily in the nucleus. Pretreatment of the cells with 6-SHO prevented the nuclear translocation of STAT3. These results are consistent with the hypothesis that 6-SHO inhibits nuclear translocation of STAT3 as a result of a decrease in pSTAT3 Tyr705. We also examined the effect of 6-SHO on I Jak2 and Src activity in LNCaP and DU145 cells and found that 6-SHO decreased the levels of IL-6–induced pJak2 Tyr1007/1008 and pSrc Tyr416 (Fig. 2G).

**Inhibition of constitutive and TNF-α–induced NF-κB activation by 6-SHO**

As shown in Fig. 3A, treatment with 6-SHO (40 μmol/L) reduced the pNF-κBp65 Ser536 and its negative regulator pIkBα Ser32/36 following treatment with TNF-α (Fig. 3B) in all three human prostate cancer cells. Furthermore, we found that 6-SHO treatment decreased the level of nuclear NF-κBp65 in both LNCaP and DU145 cells following treatment with TNF-α (Fig. 3C).

**Inhibition of STAT3 and NF-κB downstream targets by 6-SHO**

Both STAT3 and NF-κB regulate the expression of various cell cycle and apoptosis genes (25–27). As shown in Fig. 4A and B, treatment with 6-SHO reduced the levels of cyclin D1, survivin, and cMyc in both LNCaP and DU145 cells. 6-SHO also inhibited the expression of cyclin D1, survivin, and cMyc in both LNCaP and DU145 cells following treatment with IL-6 (Fig. 4C). Finally, 6-SHO also decreased TNF-α–induced cMyc expression in all three human prostate cancer cells (Fig. 4D). Thus, inhibition of STAT3 and NF-κB activation by 6-SHO was also accompanied by downregulation of their target proteins cyclin D1, survivin, and cMyc.

6-SHO modulates inflammatory cytokines, chemokines, cell cycle, and apoptosis-related genes in human prostate cancer cells

Inflammation is now considered one of the major components in cancer development and progression, including in prostate cancer (28), and various inflammatory cytokines and chemokines seem to be involved in this process (29). As shown in Fig. 4E, 6-SHO decreased the mRNA expression of IL-7 and CCL5 in both DU145 and LNCaP cells. 6-SHO also increased the expression of the apoptosis regulatory gene BAX and decreased the expression of BCL2. Interestingly, 6-SHO decreased the expression of p21, p27, SOCS1, and IRF1.

These results indicate that 6-SHO modulates expression of a number of genes that may play an important role in its ability to alter the growth and survival of prostate cancer cells.

**Effects of 6-SHO on HMVP2 cells**

Recently, we developed a prostate tumor cell line from the ventral prostate of one-year-old HiMyc transgenic mice. These cells (called HMVP2) display characteristics of cancer stem cells in that they produce spheroids under appropriate culture conditions and express stem cell markers (LINneg/Sca1high/CD49f high). These cells form well-differentiated adenocarcinomas upon subcutaneous injection into syngeneic mice (I. Blando and colleagues; unpublished data; see also Supplementary Fig. S5). Similar to the results observed in the human prostate cancer cells, 6-SHO decreased the survival of cultured HMVP2 cells, at concentrations 10 to 40 μmol/L (Fig. 5A). 6-SHO (40 μmol/L) also induced apoptosis in HMVP2 cells (Fig. 5B) and reduced phosphorylation of STAT3 as shown in Fig. 5C. Notably, 6-SHO inhibited both constitutive and IL-6–induced phosphorylation of STAT3 Tyr705 in HMVP2 cells. In addition, 6-SHO also decreased both constitutive and TNF-α–induced NF-κB activation (Fig. 5D). Furthermore, 6-SHO decreased both constitutive and IL-6–induced expression of cyclin D1 and TNF-α–induced cMyc in HMVP2 cells similar to that seen in human prostate cancer cells (see Fig. 5E and F). Thus, 6-SHO was capable of reducing cell survival while inducing apoptosis in HMVP2 cells in a manner similar to that seen for human prostate cancer cells; again, these effects correlated with reduced activation of both NF-κB and STAT3 signaling.

As shown in Supplementary Fig. S2, 6-SHO, when used at the same concentration range as above (i.e., 10–40 μmol/L), did not have a significant inhibitory effect on the survival of a nontumorigenic mouse prostate cell line, NMVP.

**Comparison of the effects of 6-GIN and 6-PAR with 6-SHO in human and mouse prostate cancer cells**

Because ginger contains several biologically active components, we compared the effects of 6-SHO with those of 6-GIN and 6-PAR in both human and mouse prostate cancer cell lines. As shown in Fig. 5G, both 6-GIN and 6-PAR reduced the survival of human and mouse prostate cancer cells. However, each of these compounds was less active on a molar basis than 6-SHO. For example, approximately 4-fold higher concentrations of both 6-GIN and 6-PAR were required to produce equivalent reductions in cell survival as compared with what was seen for 40 μmol/L 6-SHO in both the human and mouse prostate cancer cell lines. Western
blot analyses with 6-GIN and 6-PAR provided confirmation that pSTAT3 Tyr705 was reduced by both compounds at a concentration of 100 μmol/L (Supplementary Fig. S3A) in both LNCaP and DU145 cells. Both 6-GIN and 6-PAR decreased the level of survivin and increased the expression of cyclin-dependent kinase inhibitors p21 and p27 in all three human prostate cancer cells, at the 100 μmol/L concentration (Supplementary Fig. S3B). TNF-α-induced phosphorylation of NF-κBp65 and IkBα was also reduced by 6-GIN and 6-PAR in both DU145 and PC-3 cells at the 100 μmol/L concentration (see Supplementary Fig. S3B). 6-PAR also decreased the phosphorylation of STAT3, NF-κBp65, and the level of survivin in HMVP2 cells (Supplementary Fig. S3B). Collectively, these results demonstrate that 6-GIN and 6-PAR have the ability to block growth and reduce survival of both human and mouse prostate cancer cells but that they are both less potent than 6-SHO.

6-SHO inhibits growth of HMVP2 cells in an allograft model

The finding that 6-SHO inhibits growth and survival of both human and mouse prostate cancer cell lines prompted us to look at the efficacy of this compound in vivo. Toward this end, HMVP2 cells, grown as spheroids, were used to initiate tumors in FVB/N male mice following subcutaneous injection as described in the Materials and Methods section. Cells were allowed to grow for approximately 2 weeks, at which time small tumors at the injection site were palpable. As shown in Fig. 6A, treatment with 6-SHO produced statistically significant decreases in tumor volume at both the 50 and 100 mg/kg doses (62% and 73%, respectively; \( P < 0.05 \)) compared with vehicle control treated mice at the end of the study. In addition, tumor weights were also reduced at both doses of 6-SHO (48% and 65% reduction, respectively; Fig. 6B). The reduction in tumor...
weight at the 100 mg/kg dose of 6-SHO was statistically significant \((P < 0.05)\) compared with the vehicle-treated control group. There were neither appreciable changes in body weight or the daily food consumption among the 6-SHO-treated and control mice (Fig. 6C and D) nor did gross observations at necropsy reveal tissue abnormalities. As shown in Fig. 6, Western blot analyses of protein lysates from tumor tissue showed a decrease in pSTAT3\(^{Y705}\) (Fig. 6E) and both cyclin D1 and survivin levels (Fig. 6F) in 6-SHO–treated groups compared with the vehicle-treated control group. Taken together, these data demonstrate that 6-SHO has potent in vivo antitumor activity at the doses tested and is free of apparent adverse effects.

Discussion

In the work presented here, we report for the first time that 6-SHO inhibits the survival of both human and mouse prostate cancer cells in culture, a reduction that is accompanied by the induction of apoptosis. Previously published studies have shown the potential antiproliferative and apoptosis inducing activity of 6-SHO in lung, colorectal, liver and hematologic cancer cells (15, 30–32). In the present study, 6-SHO was also shown to be effective at inhibiting the growth of HMVP2 cells in vitro. The effect of 6-SHO on survival of both human and mouse prostate cancer cells in vitro was compared with two additional ginger constituents, 6-GIN and 6-PAR. The results support the conclusion that...
Figure 5. Effect of 6-SHO on mouse prostate cancer HMVP2 cells. A, HMVP2 cells were treated with the indicated concentrations of 6-SHO for 24, 48, and 72 hours and cell survival was measured by MTT assay. The data are presented as mean ± SEM. B, HMVP2 cells were treated with vehicle or 40 μmol/L 6-SHO for 48 hours and apoptosis was measured by Annexin V staining. The data are presented as mean ± SEM. C, HMVP2 cells were treated with 40 μmol/L 6-SHO for 30 and 60 minutes and Western blot analysis was performed for pSTAT3 Tyr705, STAT3, and β-actin (top two). D, HMVP2 cells were treated with vehicle or 40 μmol/L 6-SHO followed by TNF-α treatment for 30 minutes and Western blot analysis was performed for pNF-κBp65, NF-κBp65, and β-actin (bottom two). E, HMVP2 cells were treated with vehicle or 40 μmol/L 6-SHO for 2 hours followed by TNF-α and the level of cyclin D1 was measured by Western blot analysis. F, HMVP2 cells were treated with vehicle or 40 μmol/L 6-SHO for 2 hours followed by TNF-α and the level of cMyc was measured by Western blot analysis. Changes in relative band intensities (normalized to β-actin and total protein) for Western blot data are given at the top of each column. The results are significant (P < 0.05) where a decrease in phosphorylation or protein level is ≥40%. G, LNCaP, DU145, PC-3, and HMVP2 cells were treated with the indicated concentrations of 6-PAR, 6-GIN, or 6-SHO for 48 hours and cell survival was measured by MTT assay. The data are presented as mean ± SEM. a, significantly different (P < 0.05) compared with the control group and b, compared with the other treated groups of the same compound.
Figure 6. 6-SHO reduces HMVP2 allograft tumor growth in vivo. Spheroids from HMVP2 cells were injected subcutaneously into the flank of male FVB mice. Two weeks after injection, mice were treated intraperitoneally with 6-SHO (50 or 100 mg/kg body weight, BW) every other day for the duration of the experiment. A, tumor volume is expressed as average tumor volume/mouse (mm³). B, tumor weight is presented as average tumor weight (mg)/mouse. C, average mouse BW (g) for each group. D, average feed consumption/mouse/day. Data are mean ± SEM; n = 5 mice per group; * P < 0.05 compared with the control group. Lysates of allograft tumor tissues were subjected to Western blot analysis for pSTAT3 Tyr705 and STAT3 (E) and cyclinD1 and survivin (F) and β-actin was used as control for both sets of blots. Changes in relative band intensities (normalized to β-actin and total protein) for Western blot data are given at the top of each column. The results are significant (P < 0.05) where a decrease in phosphorylation or protein level is >40%.
among the three compounds tested, 6-SHO was the most potent for inhibiting growth of the prostate cancer cells in vitro. More broadly, 6-SHO seems to possess characteristics that make it worthy of further study as an agent for the prevention and/or treatment of prostate cancer.

Accumulating evidence shows that STAT3 is constitutively activated in human prostate cancer and that STAT3 is involved in cell proliferation, metastasis, angiogenesis, and resistance to apoptosis (33–35). Several studies have also shown that inhibition of STAT3 activation results in growth inhibition and induction of apoptosis in cultured prostate cancer cells (36, 37). Mechanistic studies performed as part of the current study revealed that 6-SHO treatment of prostate cancer cells inhibits both constitutive (DU145 and HMVP2) and IL-6–induced (LNCaP) phosphorylation of STAT3 Tyr705. 6-SHO treatment also decreases phosphorylation of STAT3 Ser727 as assessed in LNCaP and DU145 cells. The activation of STAT3 is regulated by upstream kinases, including receptor tyrosine kinases (e.g., EGF receptor) and non-receptor tyrosine kinases such as Jak2 and Src (25). As shown in Fig. 2G, 6-SHO inhibited activation of both Jak2 and Src, suggesting that inhibition of both Jak2 and Src kinases likely plays a role in mediating the effects of 6-SHO on downstream signaling, including STAT3 activation.

Previous studies have shown that PC-3 cells do not express significant amounts of STAT3 or pSTAT3 even after prolonged exposure to IL-6 (22). This was also the case in the current experiments where PC3 cells were devoid of measurable STAT3 (Fig. 2A). However, because 6-SHO produced significant growth inhibition and induced apoptosis in PC-3 cells (Fig 1A and B), other mechanisms associated with 6-SHO action were investigated. In this regard, 6-SHO has been shown to inhibit activation of NF-kB in several cell types and in mouse skin in vivo after topical treatment (16, 38). NF-kB is constitutively active in androgen-independent prostate cancer cells such as PC-3 cells (39–41). As shown in Figs. 3 and 4, NF-κB was found to be constitutively active in PC-3, DU145, and HMVP2 cells. Treatment with 6-SHO inhibited both constitutive and TNF-α–induced phosphorylation of NF-κBp65 in all three human prostate cancer cells. Immunocytochemical data also demonstrated that NF-κBp65 was located primarily in the nucleus after treatment with TNF-α and that 6-SHO blocked this nuclear localization (Fig. 3C). Inhibitors of IkB kinase (IKK) are the major upstream regulators of NF-κB and are activated by a wide variety of stimuli including proinflammatory cytokines (e.g., TNF-α and IL-1; ref. 40). Once IKKs are activated and phosphorylated, they in turn cause phosphorylation and proteasomal degradation of IkBα, thus leaving the free active form of NF-κB for nuclear localization (26, 39–42). As shown in Fig. 3B, 6-SHO treatment also decreased the TNF-α–induced phosphorylation of IκBα, as would be expected for an inhibitory effect for NF-κB in prostate cancer cells that is mediated through the classical IκK/IκB complex (41).

The activation of both STAT3 and NF-κB signaling leads to alterations in the expression of multiple target genes involved in proliferation, survival, and apoptosis, such as cyclin D1, survivin, c-myc, and Bcl2 (22, 24, 40). Treatment of cultured human and mouse prostate cancer cells with 6-SHO reduced the levels of cyclin D1, survivin, and c-myc protein and altered mRNA expression of selected chemokine, cytokine, cell cycle, and apoptosis regulatory genes (Fig. 4). Thus, inhibition of STAT3 and NF-κB activity by 6-SHO was associated with reduced expression of their target gene products. Given that 6-SHO effectively inhibited the growth of prostate cancer cells regardless of STAT3 expression or activity, multiple mechanisms are likely involved in its effects on prostate cancer cell survival, including effects on STAT3, NF-κB, and possibly other signaling pathways. In this regard, it has been reported that 6-SHO induces apoptosis by generation of reactive oxygen species in human colorectal cancer cells, by directly regulating Akt1/2 in non–small cell lung carcinoma cells and by modulation of estrogen receptor stress in hepatocellular carcinoma cells (23, 32, 43). In addition, as shown in Fig. 2G, Src activation was inhibited following treatment with 6-SHO. Thus, 6-SHO affected multiple signaling pathways that likely contributed to its ability to inhibit cell growth and induce apoptosis in all of the prostate cancer cell lines examined in the current study.

As part of the current study, the activity of two other compounds found in ginger (6-GIN and 6-PAR) were compared with 6-SHO. The potential chemopreventive effects of 6-GIN and 6-PAR have been previously reported. For example, Jeong and colleagues reported that 6-GIN suppresses colon cancer cell growth by inhibiting leukotriene A4 (44). 6-GIN was also found to inhibit COX-2 expression by blocking mitogen-activated protein kinase and NF-κB in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin (45). In another report, both 6-GIN and 6-PAR were shown to induce apoptosis in HL-60 leukemia cells (46). 6-PAR treatment also induced apoptosis and caspase-3 activation in an oral squamous carcinoma cell line and demonstrated chemopreventive and antioxidant activity in the 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis assay (47, 48). As shown in Fig. 5G and Supplementary Fig. S3, both 6-GIN and 6-PAR inhibited survival of human and mouse prostate cancer cells and reduced activation of both STAT3 and NF-κB. However, higher concentrations (~2–3 times) were required to achieve effects comparable to 6-SHO. These results are consistent with previously published reports that 6-SHO is more potent than 6-GIN (15, 16).

On the basis of this precedent and the cell culture data showing that 6-SHO was the most effective of the three ginger-derived compounds that were considered in the present study, its effects on tumor growth in an in vivo model system were evaluated (HMVP2 cells grown in syngeneic FVB/N mice). As shown in Fig. 6, 6-SHO at two different doses (50 and 100 mg/kg, i.p. every other day) produced statistically significant reductions in the growth of HMVP2 cells at both doses (tumor volume) and at the higher dose (tumor weight) without any apparent toxic
effects. Analysis of protein lysates from tumor tissue confirmed a reduction in STAT3 activation and altered expression of several target proteins in the 6-SHO–treated groups.

In conclusion, the results of the current study demonstrate that treatment of androgen-dependent and -independent human prostate cancer cells in culture with 6-SHO inhibits survival and induces apoptosis. 6-SHO also inhibits survival and induces apoptosis in cultured mouse prostate cancer cells derived from HiMyc mice. Importantly, 6-SHO was highly effective at inhibiting the growth of HMVP2 cells in an allograft tumor model. These effects of 6-SHO were associated with inhibition of both STAT3 and NF-kB signaling and possibly other signaling pathways (e.g., Src). On the basis of our findings, we suggest that 6-SHO has a combination of activity, low toxicity, and biochemical properties that makes it of potential utility as a naturally occurring chemopreventive and/or therapeutic agent in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Authors’ Contributions

Conception and design: A. Saha, J.L. Sessler, J. DiGiovanni
Development of methodology: A. Saha, J. DiGiovanni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Saha, J. Blando, E. Silver, J. DiGiovanni
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Saha, J. Blando, J.L. Sessler, J. DiGiovanni
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Saha, J.L. Sessler
Study supervision: J. DiGiovanni

Grant Support

A. Saha and E. Silver were supported by Cancer Prevention Research Institute of Texas postdoctoral and predoctoral trainee awards under grant RP1015G01 from the State of Texas.

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Received December 5, 2013; revised February 24, 2014; accepted March 26, 2014; published OnlineFirst April 1, 2014.


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*Cancer Prev Res* 2014;7:627-638. Published OnlineFirst April 1, 2014.

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