Fatostatin Displays High Anti-Tumor Activity in Prostate Cancer by Blocking SREBP-Regulated Metabolic Pathways and Androgen Receptor Signaling

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Non-standard abbreviations used:

ACL, ATP citrate lyase;
AR, androgen receptor;
ER, endoplasmic reticulum;
FASN, fatty acid synthase;
HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase;
HMGCS1, 3-hydroxy-3-methyl-glutaryl-CoA synthase 1;
INSIG1, insulin-induced gene 1;
LDLR, low-density lipoprotein receptor;
MVK, mevalonate kinase;
MVD, mevalonate 5-pyrophosphate decarboxylase;
PARP, poly (ADP-ribose) polymerase
PSA, prostate-specific antigen;
SCAP, SREBP cleavage activating protein;
SCD-1, stearoyl-CoA desaturase-1;
SRE, sterol regulatory element;
SREBP, sterol regulatory element-binding protein.
Abstract

Current research links aberrant lipogenesis and cholesterogenesis with prostate cancer development and progression. Sterol regulatory element-binding proteins (SREBPs; SREBP-1 and SREBP-2) are key transcription factors controlling lipogenesis and cholesterogenesis via the regulation of genes related to fatty acid and cholesterol biosynthesis. Overexpression of SREBPs has been reported to be significantly associated with aggressive pathologic features in human prostate cancer. Our previous results showed that SREBP-1 promoted prostate cancer growth and castration resistance through induction of lipogenesis and androgen receptor (AR) activity. In the present study, we evaluated the anti-prostate tumor activity of a novel SREBP inhibitor, fatostatin. We found that fatostatin suppressed cell proliferation and anchorage-independent colony formation in both androgen-responsive LNCaP and androgen-insensitive C4-2B prostate cancer cells. Fatostatin also reduced \textit{in vitro} invasion and migration in both cell lines. Further, fatostatin caused G2/M cell cycle arrest and induced apoptosis by increasing caspase-3/7 activity and the cleavages of caspase-3 and PARP. The \textit{in vivo} animal results demonstrated that fatostatin significantly inhibited subcutaneous C4-2B tumor growth and markedly decreased serum PSA level compared to the control group. The \textit{in vitro} and \textit{in vivo} effects of fatostatin treatment were due to blockade of SREBP regulated metabolic pathways and the AR signaling network. Our findings identify SREBP inhibition as a potential new therapeutic approach for the treatment of prostate cancer.
Introduction

Prostate cancer is the most prevalent malignancy diagnosed in males and the second leading cause of cancer-related mortality in the United States and Europe (1, 2). There is currently no effective therapy for advanced cancer progression. Epidemiologic evidence is mounting for a close relationship between dietary high-fat intake and prostate carcinogenesis and lethal progression (3-5). Accumulated experimental research also suggests that activation of de novo lipogenesis (6-8) and cholesterogenesis (9-11) induces prostate cancer cell proliferation and promotes cancer development and progression. Therefore, pharmacological intervention blocking fatty acid and cholesterol anabolisms could potentially be a novel therapy for malignant prostate cancer.

Sterol regulatory element-binding proteins (SREBPs) are basic helix-loop-helix leucine (bHLH) zipper transcription factors that transcriptionally activate genes involved in fatty acid and cholesterol biosynthesis and homeostasis (12, 13). Precursor SREBPs are synthesized as endoplasmic reticulum (ER) membrane-bound forms. Through sequentially proteolytic cleavage by site-1 (S1P) and site-2 (S2P) proteases, the N-terminus of SREBPs translocate into the nucleus and trigger the expression of target genes having sterol regulatory elements (SRE), cis-acting elements in their 5′-flanking promoter regions (13, 14). Three isoforms of SREBPs have been identified including SREBP-1a, SREBP-1c and SREBP-2 (13, 15). SREBP-1a mainly regulates genes associated with fatty acid and cholesterol biosynthesis (12, 13). SREBP-2 is particularly involved in the transcriptional control of cholesterol anabolism (16). Overexpression of SREBP-1 has been found in human prostate cancer tissues and LNCaP xenograft tumor tissues during androgen refractory/castration-resistant progression (17, 18). Additionally,
our recent data showed that SREBP-1 and its nuclear form were highly elevated in clinical prostate cancer specimens with aggressive pathologic features compared to non-tumor prostate tissues (18). Furthermore, SREBP-1 promoted cell viability and castration-resistant progression via alterations of lipogenesis, oxidative stress and androgen receptor (AR) expression in prostate cancer cells (18). Previous reports revealed that SREBP-2 activity was increased in prostate cancer cells and targeting this activity altered cell membrane cholesterol content and inhibited signaling transduction mediated by cholesterol-rich lipid rafts (11, 17, 19-21). These discoveries provide a rationale for blocking SREBPs and their controlling metabolic and signaling pathways by pharmacological intervention as a potential approach for prostate cancer therapy.

Fatostatin, a non-sterol diarylthiazole derivative, was originally developed from a chemical library to inhibit insulin-induced adipogenesis (22) and decreased the amounts of fatty acid, triglyceride and low-density lipoprotein and reduce body weight by perturbing the nuclear translocation of SREBP in obese mice with low cytotoxicity (23). Fatostatin has been shown to have the potential efficacy in glioma cells (24). Here, we report that fatostatin is a novel anti-prostate tumor agent that suppresses cell proliferation, tumorigenesis and progression and induces apoptotic death in prostate cancer cells in vitro and in vivo by blocking SREBP activation and inhibiting fatty acid and cholesterol biosynthesis as well as AR signaling. This study indicates that inhibition of SREBP could be exploited as a promising therapy against malignant prostate cancer.
Materials and Methods

Cell lines and culture conditions

The human prostate cancer LNCaP cell line and C4-2B, a LNCaP lineage-derived bone metastatic subline were kindly provided by Dr. Leland W.K. Chung in December 2010 (25). Mouse embryo fibroblast NIH-3T3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) in November 2013. These cell lines have not been further authenticated. LNCaP and C4-2B cells were cultured in T-Medium (Life Technologies). NIH-3T3 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies). All cell lines were grown in medium with 10% FBS (Atlanta biologicals), 100 IU/mL of penicillin and 100 µg/mL of streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Compounds and reagents

Fatostatin (4-[4-(4-methylphenyl)-1,3-thiazol-2-yl]-2-propylypyridine hydrobromide) was purchased from Chembridge Corporation and its chemical structure was described in Supplementary Fig. S1. Ribonuclease A, propidium iodide (PI), Oil Red O and Filipin III were purchased from Sigma-Aldrich. Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit I and Matrigel Basement Membrane Matrix were purchased from BD Bioscience. The Free Fatty Acid Quantification Kit and Cholesterol/Cholesterol Ester Detection Kit were obtained from Abcam. pHMGCoASyn-Luc construct was obtained from Dr. Hitoshi Shimano of Tsukuba University. pFASN-700-Luc and pFASN-700-mutSRE-Luc constructs were obtained from Dr. Timothy F.
Osborne of Sanford-Burnham Medical Research Institute. pLDLR-Luc and pLDLR-mutSRE constructs were obtained from Addgene (Cambridge, MA).

**Cell proliferation, clonogenicity, invasion and migration assays**

Prostate cancer cells were seeded on 96-well plates in triplicate and treated with vehicle or fatostatin for 72 hours. Cell proliferation was determined by MTS assay (Promega) according to the manufacturer’s instructions. For the growth curve assay, cells were seeded on 24-well plates and treated with vehicle or fatostatin (2.5, 5 or 10 µmol/L) for 5 days. Cell numbers from triplicate wells were counted. For the clonogenic assay, cells were suspended in culture medium containing 0.3% agarose (FMC BioProducts) with vehicle or fatostatin, and placed on top of solidified 0.6% agarose in 6-well plates. The developed colonies were counted and recorded under a microscope after 3-week incubation. *In vitro* cell invasion or migration was determined in Boyden chambers pre-coated with growth factor reduced BD Matrigel matrix (invasion assay) or collagen I (migration assay) as cells as previously described (18). After incubation for 48 hours, invading or migrating cells were photographed and counted to calculate the relative cell invasion and migration.

**Quantitative real-time RT-PCR (qRT-PCR) analysis**

Total RNA from each sample was isolated by RNaseasy mini Kit (Qiagen). First-strand cDNA was synthesized from total RNA (1 µg) using SuperScript III reverse transcriptase (Life Technologies) with random hexamer primers. qPCR was performed on ABI 7500 Fast Real-Time PCR System using the SYBR Green PCR Master mix from
Applied Biosystems. The oligonucleotide primer sets, including ATP citrate lyase (ACL), fatty acid synthase (FASN), stearoyl-CoA desaturase-1 (SCD-1), 3-hydroxy-3-methyl-glutaryl-CoA synthase 1 (HMGCS1), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), mevalonate kinase (MVK), mevalonate 5-pyrophosphate decarboxylase (MVD), low-density lipoprotein receptor (LDLR), insulin-induced gene 1 (INSIG1), SREBP cleavage activating protein (SCAP) and β-actin, were listed in Supplementary Table S1.

**Western blot Analysis**

Cell lysates were prepared from prostate cancer cells as previously described (26) and Western blot was performed using Mini-PROTEAN system (Bio-Rad). Primary antibodies against SREBP-1 (sc-8984), FASN (sc-48357), HMGCR (sc-33827), PSA (sc-7638) and β-actin (sc-47778) (Santa Cruz Biotechnology), SREBP-2 (ab72856, ab112046, Abcam), AR (PG-21, Millipore) and GAPDH (2118S, Cell Signaling Technology) and horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Bio Science Corp) were used and membranes were visualized using a chemiluminescence reagent (Amersham Biosciences).

**Filipin and Oil Red O stainings**

For filipin staining, cells were fixed, quenched with glycine and stained by filipin solution (50 µg/mL in PBS) for 1 hour. The filipin staining images were visualized by fluorescence microscopy. For Oil Red O staining, cells treated with vehicle or fatostatin
were stained by Oil Red O working solution and were examined by a phase contrast microscope (18).

**Quantification of fatty acid and total cholesterol**

The amounts of fatty acid and cholesterol were measured using Free Fatty Acid Quantification Kit and Cholesterol/Cholesterol Ester Detection Kit according to the manufacturer’s instructions (Abcam) in cells treated with vehicle or fatostatin (21).

**Cell cycle and apoptosis analysis**

Cells treated with vehicle or fatostatin for 48 hours were fixed, stained with PI (25 µg/mL) and analyzed by FACScan flow cytometer on the basis of 2N and 4N DNA content. Apoptotic cell death was assessed in cells treated vehicle or fatostatin using Annexin V-FITC/PI Apoptosis Detection Kit (BD Bioscience). For caspase activity assay, cells treated with vehicle or fatostatin for 48 hours were measured for caspase 3/7 enzymatic activities using Caspase-Glo® 3/7 Assay Systems (Promega). After treatment with vehicle or fatostatin for 72 hours, anti-caspase-9 (9502), -3 (9665) and PARP (9542) primary antibodies (Cell Signaling Technology) were used for Western blot analysis.

**Luciferase activity assay**

NIH-3T3 cells were transfected with pHMGCoASyn-Luc, pFASN-700-Luc, pFASN-700-mutSRE-Luc, pLDLR-Luc and pLDLR-mutSRE constructs using Lipofectamine LTX reagent (Life Technologies), following which they were treated with vehicle or fatostatin for 20 hours. The luciferase activity was measured by the Luciferase
Assay Kit (Promega) and normalized to β-Galactosidase luciferase activity by co-transfection of β-Galactosidase control vector. pSRE-Luc, SREBP-1 (OriGene Technologies), SREBP-2, CMV500 A-SREBP1 and CMV500 A-SREBP2 (Addgene) vectors were transfected into cells as described above.

**Mouse xenograft experiments**

All animal experiments were performed in accordance with the protocol approved by the Cedars-Sinai Medical Center Institution Animal Care and Use Committee. Athymic nu/nu male mice (4-week-old, Taconic) were implanted subcutaneously with C4-2B cells (1 × 10^6). Tumor burdens were monitored by calipers and calculated using the formula: \( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \). Mice bearing C4-2B tumors with a mean volume of 100 mm^3 were randomly divided into vehicle control (sterile PBS) or fatostatin (15 mg/kg) groups with intraperitoneal (i.p.) injection for 42 days (23). Blood specimens were collected and serum PSA was determined by AIA-360 Immunoassay Analyzer (Tosoh Bioscience). At the end of the animal experiments, tumor xenografts were harvested for further analysis.

**Immunohistochemistry staining**

Immunohistochemical (IHC) staining was performed as described previously (27). The paraffin-embedded C4-2B tumor tissue slides were analyzed for Ki67 (1:200 dilution, Abcam), cleaved PARP (1:30 dilution), HMGCR (1:50 dilution), FASN (1:100 dilution, Santa Cruz Biotechnology), AR (1:100 dilution, Millipore) and PSA (1:100 dilution, Vector Lab) expression. The percentages of Ki67-positive cells (proliferation) or
cleaved PARP-positive cells (apoptosis) were calculated in 5 randomly selected microscopic fields at a 200 × magnification.

**Statistical Analysis**

All quantitative results are expressed as means ± SD. Statistically significant differences were obtained using a 2-tailed Student’s *t* test. A value of *P* < 0.05 was considered to be statistically significant in all experiments.
Results

Fatostatin inhibits cell proliferation, invasion and migration of prostate cancer cells

_in vitro_

To evaluate the effects of fatostatin on human prostate cancer cell lines with biological heterogeneity, we carried out a set of cell survival assays by treating androgen-responsive LNCaP and androgen-independent C4-2B cells with fatostatin at serial dilutions, as described in Materials and Methods. The half maximal inhibitory concentrations (IC₅₀, 72 hour treatment) of fatostatin in LNCaP and C4-2B cells were 10.4 and 9.1 μmol/L, respectively (Fig. 1A). Additionally, we determined the growth rate of each cell line by counting the total number of cells with or without fatostatin treatment. The growths of LNCaP and C4-2B cells were significantly inhibited by fatostatin in a dose- and time-dependent manner (Fig. 1B). Next we examined the effect of fatostatin on the anchorage-independent colony formation ability in prostate cancer cells. After 3-week incubation, fatostatin notably inhibited the number and size of colony formation in LNCaP and C4-2B cells in a dose-dependent manner (Fig. 1C).

One of hallmarks of progressive and metastatic cancer cells is their ability to invade surrounding tissues and migrate efficiently. The effect of fatostatin on the invasion and migration potentials of LNCaP and C4-2B cells was determined. After 48h treatment, fatostatin significantly decreased the invasive (Fig. 1D) and migratory (Fig. 1E) capabilities of LNCaP and C4-2B cells compared with the untreated group. Take together; these data indicate that fatostatin inhibits proliferation, anchorage-independent colony formation, invasion and migration in both androgen-responsive and -independent prostate cancer cells.
Fatostatin causes G2/M cell cycle arrest and induces caspase-medicated apoptosis in prostate cancer cells

The effects of fatostatin on cell cycle distribution and apoptosis in prostate cancer cells were determined. LNCaP and C4-2B cells were exposed to various concentrations of fatostatin for 48 hours. Subsequently, the percentage of cell cycle distribution was measured by flow cytometry-based PI staining. As shown in Fig. 2A, fatostatin induced a significant accumulation in the G2/M phase in LNCaP and C4-2B cells. Next, the level of apoptosis induced by fatostatin was examined using simultaneous staining with FITC-coupled Annexin V and PI. The population of LNCaP and C4-2B cells in the early (lower right quadrant) or late apoptotic (upper right quadrant) stage was increased by fatostatin treatment (Fig. 2B). Only 1.84% or 0.57% of the late stage of apoptotic cells was detected in LNCaP or C4-2B without fatostatin treatment. However, the percentages of late apoptotic cells were significantly increased in both LNCaP (52.88%) and C4-2B (30.01%) cells after fatostatin (20 μmol/L) treatment.

To further define the mechanism underlying fatostatin-induced apoptotic cell death, we first examined a number of well-defined apoptotic markers involving caspase-3/7 enzymatic activity in prostate cancer cells by luminescence cleavage of specific substrates (Promega). A marked increase of caspase-3/7 enzymatic activities was found in LNCaP and C4-2B cells exposed to fatostatin with a dose-dependent manner (Fig. 2C). Next, we determined the expression patterns of caspases by Western blot analysis. Fatstatin decreased the expression of full-length caspases and increased the expression of cleaved caspase-9, -3 and PARP, a downstream factor of caspases, in LNCaP and C4-2B cells (Fig. 2D). This indicates that fatostatin activates the apoptotic cascade pathway in...
prostate cancer cells. Collectively, these results suggest that fatostatin causes G2/M cell cycle arrest and induces caspase-dependent programmed cell death in LNCaP and C4-2B cells.

**Fatostatin inhibits SREBP processing and transcriptional activity, and key enzymes for lipogenesis and cholesterogenesis, to reduce fatty acid and cholesterol levels in prostate cancer cells**

To reveal the molecular mechanism of fatostatin action in prostate cancer, we first investigated the nuclear translocation and transcriptional activity of SREBPs and their regulated anabolic pathways affected by fatostatin. As shown in Fig. 3A, expression of both nuclear SREBP-1 and SREBP-2 were decreased by fatostatin in a dose-dependent manner in LNCaP and C4-2B cells. In addition, immunofluorescence staining showed that fatostatin led to a reduction of SREBP-1 and SREBP-2 in the nuclear compartmentalization (Supplementary Fig. S2).

Next, we determined transcriptional expression of SREBP-controlled anabolic genes affected by fatostatin, including ACL, FASN and SCD-1 for lipogenesis, HMGCS1, HMGCR, MVK, MVD and LDLR for cholesterogenesis, two chaperones, INSIG1 and SCAP. As shown in Fig. 3B, fatostatin significantly down-regulated mRNA expression of these genes in LNCaP and C4-2B cells. Fitting with the effects of fatostatin on SREBP target mRNA expression, FASN and HMGCR proteins were also suppressed by fatostatin in a dose dependent pattern in prostate cancer cells (Fig. 3A).

To confirm that fatostatin acts on the SREBP pathways, the transcriptional ability of SREBP was determined by the promoter-luciferase (Luc) and mutant constructs assays
for several SREBP-regulated genes. As shown in Fig. 3C, fatostatin significantly decreased the activities of the pHMGCoASyn-Luc, pFASN-700-Luc and pLDLR-Luc reporters, but not that of the mutant pFASN-700-mutSRE and pLDLR-mutSRE reporters. Moreover, the dominant negative SREBP forms (A-SREBP1 and A-SREBP2) were utilized to prove the specific effect of fatostatin on SREBP activation. Fatostatin significantly decreased the SRE-Luc activity in the presence of SREBP-1 or -2 expression vector but didn’t affect the SRE-Luc activity in the presence of dominant negative forms (Supplementary Fig. S3). The results indicated that fatostatin specifically blocked SREBP activation and processing.

Since fatostatin inhibited key genes related to lipogenesis and cholesterogenesis through SREBPs, we subsequently performed staining and quantification assays to determine the changes of intracellular fatty acid and cholesterol levels caused by fatostatin treatment. The Oil Red O staining results showed that lipid droplet accumulation in fatostatin-treated LNCaP and C4-2B cells was decreased in comparison with vehicle-treated cells (Fig. 3D). Additionally, fatostatin significantly decreased the levels of intracellular fatty acid compared to vehicle-treated cells (Fig. 3E). The filipin fluorescent staining demonstrated that cholesterol accumulation was decreased in fatostatin-treated cells compared to vehicle-treated prostate cancer cells (Fig. 3F). To further validate the results of the filipin fluorescent staining, we assayed the intracellular cholesterol levels in both cells. When LNCaP and C4-2B cells were exposed to fatostatin, total cholesterol amounts were significantly lower than vehicle-treated cells (Fig. 3G). These data suggest that inhibition of SREBPs by fatostatin caused a decrease in the levels of fatty acid and cholesterol in prostate cancer cells.
Fatostain exhibits anti-tumor efficacy in a subcutaneous C4-2B xenograft mouse model

In view of the potent *in vitro* effect of fatostatin against prostate cancer cells (Fig. 1 and 2), we next examined the efficacy of fatostatin on human prostate cancer growth in immunodeficient mice. The volumes of subcutaneous C4-2 tumors treated with fatostatin were significantly smaller than those in the vehicle-treated control group over 42 days of observation (Fig. 4A). Moreover, at the end of the animal experiment, the average weight of the excised tumors collected from the fatostatin group was greatly decreased to 18% of the control group (Fig. 4B). Serum PSA levels of C4-2B tumor-bearing mice with fatostatin treatment were lower compared to control mice (Fig. 4C). Additionally, the toxicity profile of fatostatin seemed quite favorable as body weight was not significantly affected by treatment with fatostatin compared to controls (Fig. 4D).

Next, we examined the proliferation (Ki67) and apoptosis (cleaved PARP) in C4-2B tumor tissues by IHC staining. Consistent with the potent anti-tumor effects, the results showed that the fatostatin-treated tumors exhibited a significant decrease in a proliferative index (Ki67 status) compared to the vehicle-treated group (Fig. 4E). A significant increase in cleaved PARP staining was observed in the fatostatin-treated tumors compared to the control tumors (Fig. 4E). The *in vivo* data suggest that the predominant effect of fatostatin on subcutaneous C4-2B tumors is inhibition of proliferation and induction of apoptosis.

Fatostatin exhibits significant *in vivo* anti-tumor activity by blocking SREBP-regulated metabolic pathways
Fatostatin has been demonstrated to inhibit fat biosynthesis and reduced body fat through SREBPs in obese mice (23). We subsequently determined expression of SREBP downstream target genes involved in lipogenesis and cholesterogenesis in the vehicle- and fatostatin-treated subcutaneous C4-2B tumors. The results of qRT-PCR showed that fatostatin significantly reduced mRNA levels of ACL, FASN, SCD-1, HMGCS1, HMGCR, MVK, MVD, INSIG1 and SCAP (Fig. 5A). In addition, expression of FASN and HMGCR proteins in tumors harvested from control and fatostatin-treated groups were examined by Western blot and immunohistochemical staining analysis. As shown in Fig. 5B and 5C, the expressions of HMGCR and FASN were lower in fatostatin-treated tumors than in vehicle-treated tumors. This suggests that fatostatin exerted its anti-tumor effects by decreasing the expression of SREBP target genes associated with biosynthesis of fatty acid and cholesterol in a xenograft mouse model, in accord with the observation that lipogenesis and cholesterogenesis have been shown to be linked with prostate cancer malignancy.

**Fatostatin decreases expression of AR and its target gene PSA in prostate cancer in vitro and in vivo**

AR activity is essential for prostate cancer development, growth and castration-resistant progression. We previously showed that SREBP-1 transcriptionally induced AR expression and activity in prostate cancer cells (18). Here, we tested the idea that blocking SREBP-1 transcriptional activity by fatostatin inhibits the AR signaling in AR-positive LNCaP and C4-2B cells. As we expected, fatostatin affected the expression of AR and its downstream target gene PSA via dose-dependent inhibition in LNCaP and
C4-2B cells (Fig. 6A). We also observed that fatostatin blocked AR nuclear translocation in both cell lines (Supplementary Fig. S4). Furthermore, the results of Western blot analysis at different time points showed that fatostatin firstly inhibited the nuclear translocation of SREBP-1 and -2 (at 6 hours) and subsequently decreased AR expression (at 12 hours) in LNCaP and C4-2B cells (Fig. 6B). It indicates that SREBPs are main targets of fatostatin but not AR. Consistent with the in vitro results, fatostatin inhibited the expression of both AR and PSA compared to the vehicle group as determined by Western blot (Fig. 6C) and IHC staining (Fig. 6D) in a C4-2B tumor xenograft mouse model. Additionally, the serum PSA levels of the fatostatin-treated mice were significantly reduced compared to the vehicle control group (Fig. 4C). These in vitro and in vivo results suggest that this specific SREBP inhibitor inhibited AR expression and function in prostate cancer.
Discussion

Cancer cells reprogram the metabolic pathways to meet their abnormal demands for uncontrolled proliferation and survival. Accumulated research suggests that up-regulation of de novo lipid synthesis is associated with prostate cancer malignancy (28, 29). The altered fatty acid and cholesterol biosynthesis in cancer cells regulated by oncogenic signaling pathways, tumor microenvironment and metabolism-related transcription regulators are believed to be important for the initiation and progression of tumors (30, 31). Understanding the molecular mechanism for reprogramming lipogenesis and cholesterogenesis is the key to developing a successful therapy for eliminating prostate cancer.

SREBPs are transcription factors that predominantly activate expression of genes related to biosynthesis of long-chain fatty acid, triglyceride and cholesterol and cholesterol uptake by binding to the SRE located in the 5’-flanking promoter region of these genes in the nucleus (32-34). Nuclear SREBP-1 proteolytic activation is under the control of hormones or signal transduction systems, and SREBP-2 nuclear translocation is regulated by the ER cholesterol content, reflecting its deep involvement in cholesterol homeostasis (35). SREBPs have been shown to promote prostate cancer growth, development and progression. Importantly, clinical evidence revealed that overexpression of SREBP-1 and its nuclear form is associated with prostate cancer development and lethal castration resistance (17, 18). These experimental and clinical results offer a rationale for targeting SREBP transcriptional activity and regulated downstream metabolic pathways as a potential therapeutic approach for prostate cancer.
Transcription factors may be difficult to directly target by small molecules. However, the cytoplasmic maturation and nuclear translocation of SREBPs provides a feasible targeting approach. In the present study, a new SREBP blocker, fatostatin, was tested for anti-prostate cancer efficacy \textit{in vitro} and \textit{in vivo} by perturbing SREBP maturation and nuclear translocation. Fatostatin significantly inhibited cell proliferation and induced G2/M cell cycle arrest and caspase-dependent apoptosis in AR- and PSA-positive LNCaP and C4-2B prostate cancer cells. Fatostatin also suppressed subcutaneous C4-2B tumor growth and decreased serum PSA levels in a xenograft mouse model. Furthermore, through down-regulation of SREBPs-controlled metabolism-related enzymes including FASN and HMGCR, fatostatin inhibited the levels of fatty acid and cholesterol in prostate cancer cells. Our data reveal an important and significant insight, that blocking the maturation and translocation of SREBPs provides a promising approach for perturbing the cancer metabolic program and attenuating prostate tumor growth.

Fatty acid is an essential constituent of all biological membrane lipids and an important substrate for energy storage and metabolism. ACL, FASN and SCD-1 are three primary enzymes at rate-limiting steps for biosynthesis of long-chain fatty acid. Markedly increased ACL expression and activity have been reported in cancer cells (36). FASN is highly expressed and associated with poor prognosis in human carcinomas including prostate cancer and has been considered as a metabolic oncogene (37-39). SCD-1 plays a critical role in the regulation of carcinogenesis, cancer cell proliferation and programmed cell death (40). In this study, we demonstrated that fatostatin inhibited expression of ACL, FASN and SCD-1 and the levels of intracellular fatty acid in prostate cancer cells and a xenograft tumor model. The inhibitory mechanism of fatostatin could be due to
decreasing SREBP-1 transcriptional activity, since SREBP-1 has been demonstrated to transcriptionally mediate expression of these three lipogenic genes (12). The data suggest that fatostatin decreased expression of cancer-associated lipogenic genes and further reduced intracellular fatty acid, to inhibit prostate cancer growth \textit{in vitro} and \textit{in vivo}.

Cholesterol is one of major components of lipid rafts, specialized microdomains of the plasma membrane that serve as organizing centers for the assembly of signaling molecules (41). Therefore, rapidly proliferating cancer cells with highly activated signal transduction networks, such as prostate cancer cells, are likely to have an enhanced requirement for cholesterol (42). Besides, cholesterol is a metabolic precursor for biosynthesis of steroid hormones, including androgens. SREBP-2 is a master transcription regulator that up-regulates several key cholesterogenic genes, such as HMGCS1, HMGCR and LDLR. HMGCR activity was increased in \textit{ex vivo} LNCaP tumors during progression to a castration-resistant state in xenograft mouse models (43, 44). Expression of LDLR has been demonstrated to be elevated in prostate cancer cells compared to normal cells, which could be due to the lack of sterol feedback regulation controlled by SREBP-2 in prostate cancer cells (45). Blocking the intracellular cholesterol supply through inhibition of the LDLR pathway induced tumor cell death \textit{in vivo} (46). Our results showed that inhibition of SREBP-2 activity by fatostatin suppressed expression of these cholesterogenic genes and significantly reduced the levels of intracellular cholesterol in prostate cancer \textit{in vitro} and \textit{in vivo}, which is an essential component and precursor for the lipid raft-mediated survival signaling pathways and intracrine androgen biosynthesis. These results suggest that interrupting the SREBP-2 regulated cholesterogenic pathway may offer an attractive anti-prostate cancer approach.
Androgens exert their effects by interacting with AR to release heat shock proteins and allowing nuclear localization and DNA interaction. AR, a critical transcription, growth and survival factor, plays a key role in regulation of androgen-responsive organs during normal development and maintenance as well as prostate cancer growth and lethal hormone refractory/castration-resistant progression (47, 48). The AR signaling axis provides a promising targeted therapy for prostate cancer malignancy. Different strategies have been successfully attempted to target AR directly through gene transcription or translation or blocking the interaction between AR and its co-factors and their downstream functions in prostate cancer cells (26, 49). We previously reported that SREBP-1 mediated AR transcriptional expression and activity by a mechanism of binding the AR promoter region in prostate cancer cells (18). Additionally, knocking-down SREBP-1 led to decreases in AR mRNA and protein expression and inhibited prostate cancer cell viability (18, 27). Furthermore, unpublished results showed that silencing SREBP-2 by specific shRNA decreased expression of AR and its downstream target gene PSA in prostate cancer cells. These findings suggest that SREBPs could have an essential role as an important regulator for AR signaling. In this study, we demonstrated that interrupting SREBP activity by fatostatin suppressed expression of AR and PSA in both androgen-responsive LNCaP and androgen-independent C4-2B cells. In a C4-2B xenograft mouse model, fatostatin also inhibited AR and PSA expression and decreased serum PSA levels. Taken together, the in vitro and in vivo results revealed an important mechanism by which fatostatin inhibited AR expression and activity via blockade and inhibition of the nuclear translocation and transcriptional activity of SREBP in prostate cancer cells. This could be exploited as a novel and improved therapeutic
application by co-targeting the dysregulated metabolic pathways and the AR signaling axis using a SREBP inhibitor, such as fatostatin, to treat prostate cancer and hormone refractory/castration-resistant progression.

In summary, we uncovered the underlying molecular mechanisms by which fatostatin suppresses prostate cancer growth and tumorigenicity (Fig. 7). Fatostatin was shown to inhibit cell proliferation, anchorage-independent colony formation and progression, and induce the apoptotic pathway through interruption of SREBP nuclear translocation and transcriptional inhibition of SREBP-regulated metabolic gene expression and AR activity in both androgen-responsive and -independent prostate cancer cells. These biological effects of fatostatin provide a novel strategy for prostate cancer therapy. Future studies will determine the efficacy of fatostatin in advanced prostate cancer as well as various cancer types.
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Reference


Figure Legends

Figure 1. Fatostatin inhibits cell proliferation, colony formation, invasion and migration of prostate cancer cells in vitro.

A, fatostatin suppressed the proliferation of LNCaP and C4-2 B prostate cancer cells. The values are represented as percent of viable cells where the vehicle-treated cells were regarded as 100%. The value of IC50 for 72h treatment was calculated and showed in the histogram. B, fatostatin inhibited the growth of LNCaP and C4-2B cells in a dose- (0-10 µmol/L) and time- (0-5 days) dependent manner. Cell growth was determined by counting cell numbers daily using a hemocytometer. C, fatostatin suppressed the anchorage-independent growth of LNCaP and C4-2B cells in a dose-dependent manner (0-10 µmol/L) for 3-week treatment by the soft agar colony formation method. The number of colonies was counted and pictures of colony development taken. Each bar shows the mean ± SD of triplicate experiments. D-E, after 48h treatment, fatostatin significantly decreased the invasive and migratory capabilities of LNCaP and C4-2B cells in a dose-dependent manner (0-20 µmol/L). The graphs show images of invading and migrating cells on the lower side of the inserts. * P < 0.05, ** P < 0.01 and *** P < 0.001.

Figure 2. Fatostatin causes G2/M cell cycle arrest and induces apoptosis through caspase-mediated cleavages in prostate cancer cells.

A, after 48h treatment, fatostatin induced G2/M phase arrest in both LNCaP and C4-2B cells. The effect of fatostatin on cell cycle distribution was determined by flow cytometry. Data represent the mean ± SD from triplicate experiments. B, after 48h treatment of fatostatin, LNCaP and C4-2B cells were stained with Annexin V-FITC and
PI staining. C, fatostatin (0-20 µmol/L) induced caspase 3/7 activities in LNCaP and C4-2B cells. Fluorescent detection (RFU) of caspase 3/7 activities was measured using an enzymatic activity assay. Results represent the mean ± SD of triplicate experiments. *** P < 0.001. D, fatostatin decreased full-length (F) caspase-9, -3 and PARP, and increased cleaved (C) caspase-9, -3 and PARP expression in LNCaP and C4-2B cells as assayed by Western blot. β-actin was used as a loading control.

Figure 3. Fatostatin inhibits SREBP activation and regulated gene expression, and further reduces the levels of fatty acid and cholesterol in prostate cancer cells

A, Precursor (P) and nuclear (N) forms of SREBP-1 and SREBP-2 were measured by Western blot in LNCaP and C4-2B cells treated with vehicle or fatostatin for 24 hours. Nuclear SREBPs and expression of downstream proteins, FASN and HMGCR were decreased by fatostatin in a dose-dependent pattern. β-actin was used as a loading control. B, fatostatin decreased mRNA levels of ACL, FASN, SCD-1, HMGCS1, HMGCR, MVK, MVD and LDLR and two chaperones, INSIG-1 and SCAP in LNCaP and C4-2B cells examined by qRT-PCR. Data were normalized to β-actin and represent the mean ± SD of three independent duplicate experiments. *, P < 0.05, **, P < 0.01 and ***, P < 0.001 significant differences from the absence of fatostatin. C, fatostatin decreased the activity of the HMGCoA Syn, FASN-700 and LDLR promoter-Luc reporter constructs, but did not affect the activity of the FASN-700-mutSRE and LDLR-mutSRE reporters. The relative luciferase activity was normalized by the β-galactosidase activity and assigned as 100% in the absence of fatostatin. ***, P < 0.001 significant differences from the absence of fatostatin. D-E, after treatment with vehicle or fatostatin on June 25, 2017. © 2014 American Association for Cancer Research.
for 48 h, the levels of fatty acid in LNCaP and C4-2B cell were determined by Oil Red O staining and fatty acid quantification kit. *, $P < 0.05$, significant differences from vehicle treatment, scale bar = 100 µm. F-G, after 48 h incubation, fatostatin decreased the levels of cholesterols in LNCaP and C4-2B cells determined by filipin staining and cholesterol quantitation kit. *, $P < 0.05$ and **, $P < 0.01$ significant differences from vehicle group, scale bar = 100 µm.

**Figure 4. Fatostatin significantly suppresses subcutaneous C4-2B tumor growths in a xenograft tumor model.**

A-B, fatostatin significantly inhibited the growth and weight of subcutaneous C4-2B tumors compared with control vehicle-treated tumors during 6-week treatment (N=10 for each group). Photograph of tumors in each group was shown. C, fatostatin reduced serum PSA levels compared with the control group. D, the body weights of mice displayed no differences between fatostatin and the control group. E, IHC staining results showed that decrease of Ki67 (cell proliferation) and increase of cleaved PARP (apoptosis) were observed in fatostatin-treated C4-2B tumors compared with control tumors, scale bar = 100 µm. Quantifications of Ki67 and cleaved PARP were analyzed by counting positive stained cells in an average of five random fields. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ significant differences from vehicle group.
Figure 5. Fatostatin inhibits the mRNA and protein levels of SREBP downstream genes in C4-2B xenograft tumors.

A, fatostatin inhibited mRNA expression of SREBP downstream target genes, including ACL, FASN, SCD-1, HMGCS1, HMGCR, MVK and MVD and two chaperones, INSIG-1 and SCAP compared to the control group. Total RNA was extracted from C4-2B xenograft tumors in nude mice treated with vehicle or fatostatin (4 tumors for each group). *, P < 0.05, **, P < 0.01 and ***, P < 0.001 significant differences from vehicle-treated control tumors. B, Western blot analysis showed that fatostatin decreased expression of FASN and HMGCR in C4-2B xenograft tumors compared to the vehicle group. GAPDH was used as a loading control. C, the results of IHC staining showed that the expression of FASN and HMGCR were decreased in the fatostatin-treated C4-2B xenograft tumors compared to the vehicle group. Scale bar = 100 μm.

Figure 6. Fatostatin decreases expression of AR and its target gene PSA in prostate cancer cells and in a xenograft mouse model

A, after 24 h treatment, fatostatin inhibited the expression of AR and PSA in LNCaP and C4-2B cells in a dose dependent manner. B, fatostatin (10 μmol/L) blocked activation of SREBP-1 and SREBP-2 (at 6 hours) and subsequently inhibited AR and PSA protein expression (at 12 hours) in LNCaP and C4-2B cells. P, precursor form; N, nuclear form. C, fatostatin also suppressed the expression of AR and PSA in C4-2B xenograft tumors compared to the vehicle group, GAPDH was used as a loading control. D, IHC staining showed that the expression of AR and PSA were reduced by fatostatin in C4-2B xenograft tumors. Scale bar = 100 μm.
Figure 7. A proposed mechanism by which fatostatin inhibits prostate cancer growth and induces apoptosis by blocking activation of SREBPs, biosynthesis of fatty acid and cholesterol, and AR signaling.

By inhibiting the nuclear translocation and transcriptional activity of SREBPs, fatostatin decreases the expression of SREBP downstream target genes, including FASN, SCD-1, HMGCS1, HMGCR, MVK, MVD and LDLR, and further reduces the levels of intracellular fatty acid and cholesterol. Besides, fatostatin suppresses expression of AR and its target gene PSA. Through the concerted inhibition of lipogenesis, cholesterogenesis and the AR signaling pathway, fatostatin inhibits cell proliferation and induces apoptosis in prostate cancer cells. Reg, regulatory domain; bHLH, basic helix-loop-helix zipper domain; ER, endoplasmic reticulum; SRE, sterol regulatory element.
Figure 1

(A) Cell viability rate (%) of LNCaP and C4-2B cells treated with different concentrations of Fatostatin. The IC_{50} values are 10.4 μmol/L for LNCaP and 9.1 μmol/L for C4-2B.

(B) Changes in cell number over time (days) for LNCaP and C4-2B cells treated with different concentrations of Fatostatin. The graph shows significant differences (* * * p < 0.001) between the groups.

(C) Colony number for LNCaP and C4-2B cells treated with different concentrations of Fatostatin. The following concentrations were used: 0.0, 2.5, 5.0, and 10.0 μmol/L. The graph shows a significant decrease in colony number with increasing Fatostatin concentration (* p < 0.05, ** p < 0.01).

(D) Representative images of LNCaP and C4-2B cells treated with different concentrations of Fatostatin. The graph shows relative cell invasion (%) with increasing Fatostatin concentration (* * * p < 0.001).

(E) Representative images of LNCaP and C4-2B cells treated with different concentrations of Fatostatin. The graph shows relative cell migration (%) with increasing Fatostatin concentration (* * * p < 0.001).
Figure 6

A

B

C

D

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