Blocking Myristoylation of Src Inhibits Its Kinase Activity and Suppresses Prostate Cancer Progression

Sungjin Kim1, Omar Awad Alsaaidan1, Octavia Goodwin1, Qianjin Li1, Essilvo Sulejmani1, Zhen Han1, Aiping Bai2, Thomas Albers3, Zanna Beharry4, Y. George Zheng1, James S. Norris5, Zdzislaw M. Szulc2, Alicja Bielawska2, Iryna Lebedyeva5, Scott D. Pegan1, and Houjian Cai1

Abstract

Protein N-myristoylation enables localization to membranes and helps maintain protein conformation and function. N-myristoyltransferases (NMT) catalyze co- or posttranslational myristoylation of Src family kinases and other oncoproteins, thereby regulating their function. In this study, we provide genetic and pharmacologic evidence that inhibiting the N-myristoyltransferase NMT1 suppresses cell-cycle progression, proliferation, and malignant growth of prostate cancer cells. Loss of myristoylation abolished the tumorigenic potential of Src and its synergy with androgen receptor in mediating tumor invasion. We identified the myristoyl-CoA analogue B13 as a small-molecule inhibitor of NMT1 enzymatic activity. B13 exposure blocked Src myristoylation and Src localization to the cytoplasmic membrane, attenuating Src-mediated oncogenic signaling. B13 exerted its anti-invasive and antitumor effects against prostate cancer cells, with minimal toxic side-effects in vivo. Structural optimization based on structure–activity relationships enabled the chemical synthesis of LCL204, with enhanced inhibitory potency against NMT1. Collectively, our results offer a preclinical proof of concept for the use of protein myristoylation inhibitors as a strategy to block prostate cancer progression. Cancer Res; 77(24); 6950–62. ©2017 AACR.

Introduction

N-myristoylation is a co- and posttranslational modification that results in the covalent attachment of the 14-carbon saturated myristic acid to the N-terminus of a target protein (1). N-myristoyltransferase (NMT) catalyzes this transfer of the myristoyl group of myristoyl-CoA to a glycine in the N-terminus. N-myristoylation is ubiquitously found in eukaryotes, and two mammalian NMT isoforms, NMT1 and NMT2, have been identified that share 77% identity (2). NMTs have been considered as promising targets for the development of antifungal, antiparasitic, and antitumor progression therapeutics (3).

One set of proteins where myristoylation has been observed to play an important role is Src family kinases (SFK). Myristoylation together with other modifications allow SFKs to attach to the cytoplasmic membrane and mediate their kinase activity and cellular functions (4, 5). SFKs are pleiotropic activators in signal transduction pathways and numerous studies have documented their role as oncogenic driver genes in a variety of cancers (6). SFKs interact with a variety of cellular receptors, and are downstream effectors of G protein–coupled receptors, integrins, and many receptor tyrosine kinases (RTK; refs. 7, 8). Activation of SFKs also activates a variety of downstream signaling to facilitate tumor growth, angiogenesis, and metastatic invasion (6, 9, 10). Particularly, aberrant expression of Src kinase facilitates the phosphorylation of androgen receptor (AR) and bypasses ligand dependent AR activation in castration-resistant prostate cancer (9, 11). Our previous study showed that co-overexpression of Src and AR promotes invasive prostate adenocarcinoma (11, 12).

In vitro studies have indicated that NMT1 regulates Src kinase myristoylation and phosphorylation or kinase activity in COS-1 cells (5) or HT-29 cells (13). Here we further study if genetic and pharmacologic inhibitions of NMT1 regulate proliferation of prostate cancer cells and growth of prostate tumor in vivo. We demonstrate that knockdown of NMT1 suppressed proliferation of prostate cancer cells by blocking cell-cycle progression, and inhibited the myristoylation and tyrosine phosphorylation of Src kinase. The inhibitory effect increased with increasing expression levels of NMT1. Myristoylation was shown to facilitate SFKs-mediated prostate tumorigenesis, and mediate the interaction of Src kinase with AR, with the synergistic effect of promoting prostate tumor progression. Screening a panel of small-molecule compounds based on the myristoyl-CoA scaffold identified a compound that blocked the enzymatic activity of NMT1 and...
myristoylation of Src kinase. The inhibitor suppressed proliferation, migration, and invasion of prostate cancer cells and tumor growth with a limited toxicity to normal cells or major organs in vivo. The inhibitory activity of this compound was optimized through structurally guided and SAR-based methods. Our study provides a novel therapeutic approach for the treatment of prostate cancer by targeting lipidaion.

Materials and Methods

Plasmid constructs

The open reading frames of the Src(WT) and its mutants [(Src(WT)/G2A), Src(Y529F/G2A), Src(Y529F/S3C/S6C), Src(Y529F/G2A/S3C/S6C), Src(Y529F/K298M)], Fyn(WT) and its mutants [(Fyn(WT)/G2A), Fyn(Y528F/G2A), Fyn(Y528F/C3S/C6S), Fyn(Y528F/G2A/C3S/C6S), Fyn(Y528F/K298M)], and AR genes were cloned into FUCRW or FUCGW lentiviral vectors as described previously (14, 15). Src(Y529F) and Src(Y529F/G2A) were also cloned into the pTK380 vector (16), designated as TRE/Src(Y529F), which expresses the reverse tetra-cycloprene-controlled transactivator (rtA), and is regulated by doxycycline (Dox). To knockdown the human Src and NMT1 genes, shRNAs targeting Src and NMT1 were generated using primers listed in Supplementary Table S1. After the primers were annealed, the inserts were cloned into pSiRNA-w[H1.4] vector at the BbsI site. The shRNA constructs with the H1 promoter were further subcloned into FUCRW or FUCGW lentiviral vectors as described previously (14, 15). Src(Y529F) and Src(Y529F/G2A) were also cloned into the pTK380 vector (16), designated as TRE/Src(Y529F), which expresses the reverse tetra-cycloprene-controlled transactivator (rtA), and is regulated by doxycycline (Dox).

Cell culture

PNT2 cells were purchased from Sigma (Catalog No. 95012613). 293T and prostate cancer cell lines including LNCaP, 22Rv1 and DU145, and PC-3 were purchased from ATCC. The above cell lines were obtained in September 2013 and were defined as passage 1 (the first thawing) when arrived in the lab. SYF1 (Src−/−, Ysc−/−, Fyn−/−) mouse fibroblast cell line and LNCaP-abl, and 293T expressing rtTA (293T-rtTA) were gifts from Dr. Jonathan Cooper’s lab (Fred Hutchinson Cancer Research Center, Seattle, WA) in August 2008 (17). Dr. Qianwen Wang’s lab (The Ohio State University, Columbus, OH) in March 2016 (18), and Dr. Kathrin Plath’s lab (University of California, Los Angeles, Los Angeles, CA) in May 2008 (12), respectively. Cancer cell lines were cultured in ATCC-recommended medium, and LNCaP-abl was grown in 10% charcoal-stripped FCS. Cell lines from ATCC and Sigma had a certificate of mycoplasma-free and authentication when purchased. Other cell lines including SYF1 and LNCaP-abl were tested by the Universal Mycoplasma Detection Kit (ATCC catalog no. 30-1012K) and determined to be mycoplasma-free. PNT2, LNCaP, 22Rv1 DU145, and PC-3 were used within no more than 8–10 passages. LNCaP-abl cells were at 62 passages in this study. SYF1 cells and 293T-rtTA cells were used at the passage of no less than 35. Loss of Src and Fyn expression in SYF cells was not confirmed by Western blot analysis as indicated in each experiment. The doxycycline induction ability in 293T-rtTA did not show any difference in terms of cell passage number. SYF1 cells transduced with Src/Fyn(WT) or their mutants, or 293T-rtTA transduced with TRE/Src(Y529F) by lentiviral infection (12) were grown in DMEM with 10% FBS (HyClone). The expression of Src kinase was induced by 1 μg/mL doxycycline. All the cell lines were periodically examined for mycoplasma contamination.

Protein fractionation

The fractionation protocol for the cytosol and the cytoplasmic membrane were described previously with slight modifications (19). Briefly, SYF1 cells expressing Src/Fyn(WT) or its mutants were incubated in DMEM including 2% BSA for 24 hours. After lysing with TNE lysis buffer (50 mmol/L, Tris, 150 mmol/L NaCl, 2 mmol/L EDTA pH 7.4, protease inhibitor cocktail, and phosphatase inhibitor cocktails), the protein extracts were homogenized using a 25-gauge needle syringe (15 strokes) and centrifuged at 14,000 rpm for 10 minutes. The supernatant was collected as the cytosolic (Cyt.) fraction. Pellets were rinsed twice with TNE lysis buffer and resuspended in TNE lysis buffer, which contained 60 mmol/L β-octylglycoside. Samples were incubated on ice for 30 minutes and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatants were collected as the total membrane (TM) fraction.

Crystallization of NMT1 and structure determination

Purified NMT1 was dialyzed overnight against a buffer containing 20 mmol/L Tris pH 7.5, 100 mmol/L NaCl, and 1 mmol/L DTT. The enzyme was concentrated to 6.3 mg/mL and incubated at a 1:5 molar ratio with myristoyl CoA. This mixture was filtered with a 0.2-μm filter. NMT1 crystals were obtained through vapor drop diffusion using a 50-μL reservoir with a 4-μL hanging drop containing equal parts of NMT1 and reservoir solution (22.5% PEG 4000, 5 mmol/L NiCl2, 100 mmol/L sodium citrate pH 4.5, and 2.5% glycerol; ref. 20). The crystals were flash frozen in liquid nitrogen using the reservoir solution as a cryoprotectant. The data were collected at the SER-CAT 22ID beamline at 1 Å using a MAR300hs detector. HKL-2000 was used to index, integrate, and scale datasets. Phenix and coot were used for molecular replacement and refinement of the structure.

Detecting the myristoylation of Src kinase by Click chemistry

To evaluate myristoylation of proteins, PNT2, LNCaP, LNCaP-abl, 22Rv1, PC-3, and DU145 cancer cells were grown in the ATCC-recommended medium with myristic acid azide. The protein lysates were extracted using M-PER buffer, and a 40-μL protein lysate aliquot was used for the Click reaction. Myristoyl proteins were detected by immunoblotting using streptavidin-HRP.

To detect the effect of NMT1 on expression levels of myristoylated Src protein, PC-3 cells were transduced with shRNA-control or shNMT1. In addition, to examine the inhibition of B13 on Src myristoylation, PC-3 cells were treated with 0, 1, 15, and 30 μmol/L of B13 for 2 hours. The shNMT1 transduced or B13-treated cells were cultured in the medium with 60 μmol/L of myristic acid-azide for 24 hours. Proteins were extracted with M-PER buffer (Thermo Scientific) containing protease inhibitors and phosphatase inhibitors. Cell lysates were centrifuged at 14,000 rpm for 20 minutes. The supernatants were incubated with Src antibody for 16 hours at 4°C. Protein A agarose beads were added, and the mixtures were incubated for 1 hour at 4°C. After washing five times with IP lysis buffer, the Click chemistry reaction was accomplished by adding Click reagents according to the manufacturer's instructions (Life Technologies). In brief, 30 μL of the Click-iT reaction buffer containing 40 μmol/L alkyne-biotin, 10 μL of CuSO4, and 10 μL of additive 1 solution was mixed with the equal volume of immunoprecipitated agarose beads. Then 20 μL of additive 2 solution was added.
The myristoyl-Src was further mixed with loading buffer and boiled. The lysate was detected by immunoblotting using Streptavidin-HRP.

To screen the compounds inhibiting the Src myristoylation, SYF1 (Src(-/Fyn(-/Yes(-/))) cells were transduced with Src(WT) or Src(G2A) by lentiviral infection. The transfected cells were grown in DMEM with 2% fatty acid free BSA containing 60 μmol/L myristic acid-azide for 24 hours after pretreatment of LCL or GRU compounds for 2 hours. To examine Src myristoylation in a dose-dependent manner, SYF1 +Src(WT) cells were treated at 0, 1, 5, 15, and 30 μmol/L of B13 for 2 hours, followed by the addition of 60 μmol/L of myristic acid azide. The expression levels of myristoyl-Src (60 kDa) were detected by immunoblotting.

Screening for NMT1 inhibitors and measurement of IC50

For screening of NMT1 inhibitors or the measurement of IC50, the 1 x reaction buffer (50 mmol/L HEPES and 0.5 mmol/L EDTA), 140 nmol/L of NMT1 purified enzyme (see the NMT1 purification Section, the enzyme stock was preserved in the buffer containing 1 mmol/L EDTA, 250 mmol/L NaCl, and 20 mmol/L Tris pH 8.5), 5 μmol/L of peptide Gly-Ser-Asn-Lys-Ser-Lys-Pro-lys (derived from the N-terminus of human pp60Src tyrosine kinase), and the inhibitor at 0, 10, 20, 40, 80, 120, 160, or 200 μmol/L, respectively, were mixed in a 96-well plate. After incubation at 30°C for 10 minutes, the reaction was started by adding 1 μmol/L myristoyl coenzyme A (Avanti Polar Lipids). The total volume of the above mixture was set 80 μL/well. After incubation at 30°C for 60 minutes, the released coenzyme A was detected by adding 80 μmol/L of 30 μmol/L of 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CMU) stock solution (Sigma Aldrich) to each well and incubated in the dark for 12 minutes. The fluorescence intensity was measured by a FlexStation 3, microplate reader (excitation at 390 nm; emission at 479 nm).

Analysis of mRNA copy number in The Cancer Genome Atlas database

Expression values were extracted from the cBioPortal for Cancer Genomics (http://www.cbioportal.org/) for the following genes using the Gene Set Query functions: NMT1 and Src. The expression values were then cross-referenced with the datasets from The Cancer Genome Atlas (excitation at 390 nm; emission at 479 nm).

Prostate regeneration assay and xenograft tumors

C57BL/6J and CB.17SCID/SCID (SCID) mice were purchased from Taconic. For the prostate regeneration assay, primary prostate cells were isolated from 8- to 12-week-old male C57BL/6J mice, and infected with lentivirus expressing Src/Fyn(WT) or mutants, or co-infected with Src(WT)/Src(G2A) and AR according to the experimental setup. Infected cells (2–3 x 10^5 cells/grant) were combined with urogenital sinus mesenchyme (UGSM; 2–3 x 10^3 cells/grant) together with 25 μl of collagen type I (adjusted to pH 7.0; ref. 12). After overnight incubation, grafts were implanted under the kidney capsule in SCID mice by survival surgery. All animals were sacrificed at 8 weeks after grafts were implanted.

To examine the role of Src or NMT1 in tumor progression, the xenograft tumor model was applied. PC-3 cancer cells transduced with Src-shRNA, NMT1-shRNA, or control shRNA by lentiviral
infection and were grown in DMEM with 10% FBS. A total of 3 x 10^5 cells were mixed with 50 μL of collagen type I (pH 7.0; BD Biosciences) and inoculated subcutaneously in both lateral flank sides of SCID mice. The size of tumors was measured weekly. The host mice were sacrificed, and xenograft tumors were harvested after 2-month incubation.

For examining the inhibition of B13 on xenograft tumors, PC-3 cells were subcutaneously inoculated in the flank side of SCID mice. Mice carrying xenografts were randomly separated into two groups after 2-3 weeks. B13 was dissolved in the vehicle solution containing 30% kolliphor, 65% saline (0.9% NaCl), and 5% ethanol. Mice were administrated intravenously (i.v) with 200 μL of the drug solution at a concentration of 75 mg/kg body weight or vehicle twice a week for 4–6 weeks. Body weight and tumor size were measured (length × width) weekly. Xenograft tumors, the liver, lung, and kidney were harvested for IHC analysis. All animals were maintained according to the surgical and experimental procedures of the protocol A2013-03-008 approved by IACUC at the University of Georgia (Athens, GA).

Statistical analysis
Prism software was used to carry out statistical analyses. The data are presented as mean ± SEM and analyzed using the Student t test. All t tests were performed at the two-sided 0.05 level for significance. * P < 0.05; ** P < 0.01; N.S., not significant.

Results
Ablation of NMT1 inhibits proliferation of prostate cancer cells
Protein lipidation including myristoylation is essential for regulation of the structure and function in numerous disease-related proteins (3). NMT catalyzes protein myristoylation, and has been considered a major target to block cancer progression (13, 21). NMT1 was expressed in normal or prostate cancer cells, and mRNA and protein expression levels of NMT1 were significantly elevated in DU145 and PC3 cancer cells (Fig. 1A and B). Increased expression of NMT1 correlated with elevated protein myristoylation at approximately 60 kDa (Fig. 1C). To examine if NMT1 regulates the growth of prostate cancer cells, shRNA targeting NMT1 (shNMT1) was generated (Supplementary Fig. S1A and S1B). Knockdown efficiency was validated in SYF1 (Src−/−Yea−/−Fyn−/−) cells expressing Src(WT) by demonstrating a reduction in myristoylated Src kinase using Click chemistry (Supplementary Fig. S1C). Although knockdown of NMT1 showed no inhibition on the growth of PNT2 cells (normal cells), it inhibited the proliferation of LNCaP, 22Rv1, DU145, and PC-3 prostate cancer cells (Fig. 1D, Supplementary Fig. S1D). In particular, the inhibitory effect on cell proliferation correlated with the expression levels of NMT1 (Fig. 1E) and the lack of inhibition of normal cells (PNT2 cells) suggests that NMT1 might serve as a potential inhibition target in prostate tumors without causing major toxicity.

PC-3 cells were selected for xenograft studies because NMT1 expression was highest in this cell line. Knockdown of NMT1 significantly inhibited the growth of PC-3 xenograft tumors, leading to a decrease in the size and weight of tumors (Fig. 1F and G). Suppression of NMT1 led to cell-cycle arrest at the S-phase with a decrease of the cell population at the G1–G2/M phase (Fig. 1H), but had no significant effect on cell apoptosis (Fig. 1I).

NMT1–Src axis mediates proliferation of prostate cancer cells
Myristoylation of Src kinase regulates its kinase activity (5). SFKs are important oncogenic driver genes in a variety of cancers including advanced stages of prostate cancer (9, 22). Expression levels of NMT1 and Src kinase were significantly correlated in human tumors (Fig. 2A) and expression levels of NMT1 correlated with the active Src kinase [detected by p5Src(Y416) antibody] in prostate cancer cells (Fig. 1B). Similar to the effect of shRNA-NMT1 on PC-3 xenografts, the growth of PC-3 cancer cells (Supplementary Fig. S2A and S2B) and xenografts (Fig. 2B) were dependent on expression of Src kinase. Knockdown of another SFK member Fyn kinase led to only mild inhibition of PC-3 and LNCaP cell proliferation (Supplementary Fig. S2C and S2D). Downregulation of NMT1 appears to inhibit Src myristoylation (Fig. 2C), tyrosine phosphorylation (pSrcY416), and the level of FAK phosphorylation, a downstream target of Src kinase (Fig. 2D), highlighting a potential NMT1-Src axis to inhibit tumor growth, particularly in Src-driven tumors.

Single and double knockdown of NMT1 and Src were performed to evaluate the effect on cell proliferation in LNCaP, 22Rv1, and PC-3 cell lines. Single knockdown of Src or NMT1 inhibited proliferation of the three cell lines (Fig. 2E) but double knockdown (shSrc and shNMT1) showed no additive effect in LNCaP and 22Rv1 cells. However, double knockdown in PC-3 cells (showing the highest expression of NMT1 and pSrc) showed greater inhibition of proliferation compared with either single knockdown (Fig. 2E). These results demonstrate an NMT1–Src axis that plays a role in prostate cancer cell growth.

Loss of N-myristoylation inhibits SFK-induced oncogenic signaling in vitro and prostate tumorigenesis in vivo
The tumorigenic potential of the following Src and Fyn kinase mutants were examined in vitro and in vivo: constitutively active that recapitulates activated Src kinase in tumors [Src(Y529F) or Fyn(Y528F)], loss of the myristoylation site [Src(Y529F/G2A) or Fyn(Y528F/G2A)], gain [Src(Y529F/S3C/S6C)] or loss [Fyn(Y528F/C3S/C6S)] of two palmitoylation sites, and loss of both myristoylation and palmitoylation sites [Fyn(Y528F/C3S/C6S/G2A)] (Fig. 3A and B). Src(Y529F/G2A), Fyn(Y528F/G2A), and Fyn(Y528F/C3S/C6S/G2A) inhibited phospho-Erk, pFAK expression (Supplementary Fig. S3A and S3B), and colony formation (Supplementary Fig. S3C and S3D), suggesting that specifically myristoylation and not palmitoylation is essential for SFKs-mediated oncogenic signaling and transformation in vivo.

As previously reported (17), although regenerated tissue derived from Src(Y529F) or Fyn(Y528F/C3S/C6S) infected epithelial cells formed a solid tumor (Fig. 3C and D), tissue from Src(Y529F/S3C/S6C) showed normal tubule structure (Fig. 3C and E). Src(Y529F)-induced tumors were composed of sheets of poorly differentiated carcinoma cells without glandular structures and with focal sarcomatoid areas (Fig. 3C). In contrast, the regenerated tissue derived from Src(Y529F/G2A) showed normal tubule structure (Fig. 3E). In addition, regenerated prostate tissue derived from Fyn(Y528F) and Fyn(Y528F/C3S/C6S) exhibited high-grade adenocarcinoma and invasive tumor, respectively (17). The tissues from Fyn(Y528F/C3S/C6S) showed solid tumors with un-differentiated tumorigenic cells. In contrast, tissues from Fyn(Y528F/G2A) or Fyn(Y528F/C3S/C6S/G2A) showed normal glandular tubules (Fig. 3D and F). Collectively, these results indicate that myristoylation is essential for SFKs-induced
tumorigenesis and loss of myristoylation abolishes tumorigenic potential, suggesting that myristoylation is an important oncogenic target.

Blockade of myristoylation inhibited synergy of Src and AR in prostate tumorigenesis

Coexpression of c-Src and AR induces activation of Src kinase and leads to invasive prostate tumorigenesis in vivo (12). The role of myristoylation in the synergy of Src-AR induced tumorigenesis was also examined. Prostate primary cells were transduced with AR, Src(WT), Src(G2A), AR+Src(WT), or AR+Src(G2A) by lentiviral infection (Fig. 4A). Their expression was visualized in the regenerated tissues by fluorescence imaging of the GFP/RFP markers (Fig. 4B). Although the size of regenerated tissue showed no visible difference, the weight of regenerated tissue derived from Src(WT)+AR increased significantly in comparison with Src(WT), Src(G2A), AR, or Src(G2A)+AR (Fig. 4B). As reported previously (12), overexpression of AR or Src(WT) alone did not induce prostate tumorigenesis, and regenerated tissues contained histologically normal prostate tubules (Fig. 4C). Regenerated tissues derived from overexpression of Src(G2A) alone or Src(G2A)+AR showed normal tubule structure (Fig. 4C), suggesting that loss of Src kinase myristoylation blocks the synergy of Src(WT) and AR induced tumorigenesis in vivo. Because
myristoylation was important for Fyn transformation, the synergy of AR and Fyn was examined. However, the results showed no synergistic effect in the regenerated tissues (Supplementary Fig. S4A and S4B), suggesting differential functions of Src family kinases (17), likely dictated by differential intracellular trafficking pathways (23).

Loss of Src myristoylation interfered with the protein interaction of exogenously expressed Src and endogenous AR in LNCaP (Supplementary Fig. S5A–S5C) and 22Rv1 prostate cancer cells (Supplementary Fig. S5D–S5F) in the presence or absence of AR agonist (R1881), and inhibited AR-regulated expression of the PSA, KLK2, and TMPRSS2 genes (Supplementary Fig. S5G). These results further suggest that myristoylation is a potential therapeutic target for the inhibition of Src kinase function and its mediated tumorigenesis.

Small-molecule targeting of NMT1 enzymatic activity

To identify small-molecule compounds that target NMT activity, recombinant NMT1 protein (with an exclusion of the 108 amino acids DNA sequence in the N-terminus) was used (Supplementary Fig. S6A and S6B). The protein was purified by affinity chromatography (Supplementary Fig. S6C; ref. 20). A fluorescence-based in vitro assay was developed (Supplementary Fig. S7A; ref. 24) and the myristoylation process was found to occur by a ‘Ping-Pong’ mechanism (Supplementary Fig. S7B). The detection of Src myristoylation using click chemistry was developed to examine the inhibition of compounds at the cellular level (Supplementary Fig. S8A and S8B). The assays were used to screen a selected panel of LCL compounds of previously synthesized myristoyl-CoA analogues (Supplementary Figs. S8C and S9). D-NMAPPD, N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-tetradecanamide, also named B13 (or LCL4), was the top hit that inhibited NMT1 activity and Src kinase myristoylation (Fig. 5). The IC50 of B13 (77.6 μmol/L) was not improved with analogues with longer or shorter N-acyl carbon chains such as LCL7 or LCL35 likely due to steric clashes of the longer tails or loss of hydrophobic interactions with shorter tails with the NMT1 protein (Fig. 5). In addition, when the nitro (R2 group) was removed from the p-position of the aromatic ring of LCL4 along with the addition of hydroxymethyl (R4 group) such as the compound LCL1, it showed a significant increase in IC50 value. However, N,N′-disubstituted urea as a linker between aromatic ring and aliphatic tail such as LCL17 led to a decrease of the IC50 value, which could be due to the planar and non-flexible nature of the –NH-CO-NH- linker (Fig. 5).

B13 and its derivative LCL204 compete with the myristoyl-CoA binding site of NMT1

B13 is structurally similar to myristoyl-CoA as both contain a 14-carbon alkyl tail (Fig. 6A). The crystal structure of NMT1 was solved to understand how B13 interacts with NMT1
Similar to the reported structure (20), the myristoyl-CoA binding site of NMT1 was identified in our structure and placement of B13 within this site was performed initially by overlaying B13’s alkyl tail with that of myristoyl-CoA (Fig. 6B). In addition, orientation of B13 within the active site was driven by the SAR data (Fig. 6B and Supplementary Fig. S9). Several favorable interactions between B13 and the NMT1 the binding pocket, including the interaction of (i) the aromatic ring with a hydrophobic patch comprised of Tyr180 and Val181; (ii) the aliphatic tail with Asn246; (iii) potential hydrogen bonds between R2 nitro group and Arg255, between the amide group and Thr282 and the backbone amide of Leu248, and between the R4 hydroxymethyl group and the hydroxyl group of Tyr180 (Fig. 6B).

The interactions of B13 and NMT1 described above were used to identify an analogue with improved efficacy. Based on the B13-NMT1 model in Fig. 6B, the hydroxymethyl group and carbonyl oxygen are competing for hydrogen bonding interactions with NMT1. Therefore, the removal of the carbonyl could potentially enhance binding and thus increase inhibition. Molecular modeling by using Autodock Vina further suggested that the NO₂ moiety (Fig. 6B) and could extend the half-life due to introduction of the stable alkane group. To probe the predictive accuracy of this structural placement and molecular modeling, LCL204 was synthesized (Fig. 6A and B) and found to exhibit an order of magnitude enhancement in potency towards NMT1 (IC₅₀ = 8.7 μmol/L; Fig. 6C). These results suggest a likely path forward for further structure optimization for greater potency in targeting NMT1 activity.

B13 inhibits Src myristoylation, localization at the cytoplasmic membrane, and its mediated oncogenic signaling and transformation

Src myristoylation is a downstream target of NMT1. B13 inhibited Src myristoylation of ectopically expressed Src kinase or endogenous Src kinase (Fig. 6D and E). Myristoylation is essential for the attachment of Src family kinases at the cytoplasmic membrane. The majority of Src/Fyn proteins were found to localize in the cytosol in the Src(G2A), Src(Y529F/G2A), Fyn(G2A), and Fyn(Y528F/G2A) mutants (Supplementary Fig. S10A–S10D). Loss of myristoylation inhibited the association of Src kinase with the cellular membrane (Supplementary Fig. S10E). B13 inhibited Src kinase tyrosine...
phosphorylation [detected by pSrc(Y416)] in association with downregulation of pFAK and pAkt (Fig. 6F), or suppression of de novo synthesized Src-induced signaling (Fig. 6G). Similarly, the amount of nonphosphorylated Src kinase in the open conformation [detected by non-pSrc(Y527)] at the cytoplasmic membrane, representing the doxycycline induced de novo synthesized Src kinase, was reduced after treatment with B13 (Fig. 6H). Expression levels of nonphosphorylated Src in the cytoplasmic fraction did not change with B13 treatment most likely because only a small portion of the total cytosol lysate was analyzed due to the limited loading volume available in the gel. In addition, although Src(Y529F) significantly increased colony formation, the transformation was inhibited by B13 (Fig. 6I).

Figure 4. Loss of myristoylation in Src kinase inhibits the synergy of Src(WT) with AR in prostate tumorigenesis. A, Schematic for examining the synergy of Src and AR in prostate tumorigenesis. Primary prostate epithelial cells were transduced with AR (GFP marker), Src(WT) (RFP marker), Src(G2A) (RFP marker), or cotransduced with Src(WT)/Src(G2A) and AR and the infected cells were combined with UGSM, and implanted under the renal capsule of SCID mice. Regenerated prostate tissue was isolated after 8 weeks. B, Representative images of regenerated prostate tissue and RFP/GFP detection (scale bar, 2 mm). The weight of prostate tissues was compared in the bar graph. *p, an unpaired, two-tailed t test. C, Hematoxylin and eosin (H&E), RFP/GFP, and IHC staining of AR and total Src, and co-staining of CK8, CK5, and DAPI in regenerated tissue. Scale bar, 100 μm.

B13 inhibits proliferation, migration, and invasion of prostate cancer cells and growth of xenograft tumors

The ability of B13 to inhibit transformation and proliferation of prostate cancer cells was examined. B13 significantly inhibited proliferation of 22Rv1, PC-3, and DU145 prostate cancer cells, but this effect was less sensitive in LNCaP cells (Fig. 7A), which is correlated with the lower expression of NMT1 (Fig. 1A and B). Cell-cycle progression was significantly inhibited in the tested prostate cancer cells (Fig. 7B). The cell-cycle inhibition was further confirmed by decreased expression of CDK2 and cyclin D1, increased expression of p27 in PC-3 and DU145 cells, and decreased expression of CDK6 in DU145 cells (Fig. 7C). B13 also suppressed invasion of 22Rv1 and migration of PC-3 cancer cells.
However, B13 had a limited effect on PNT2 normal cell proliferation and did not affect the cell cycle in PNT2 cells (Supplementary Fig. S11A–S11C) or proliferation of 293T cells (Supplementary Fig. S11D).

To examine the specificity of B13 for targeting the NMT1-Src axis, 22Rv1 or PC-3 cells transduced with shRNA-control or shRNA-Src were subjected to the B13 treatment. Single B13 treatment or knockdown of Src showed significant inhibition on proliferation of 22Rv1 and PC-3 cells (Supplementary Fig. S12A–S12B). PC-3 cells with Src knockdown and B13 treatment showed a combined effect on reducing proliferation (shRNA-Src+B13 vs. B13; Supplementary Fig. S12A). This effect on proliferation was not observed in 22Rv1 cells (shRNA-Src+B13 vs. shRNA-Src; Supplementary Fig. S12B). As PC-3 cells showed the highest and 22Rv1 the lowest expression of NMT1 and pSrc, this data suggest that the NMT1-Src axis sensitizes cells to targeting myristoylation to reduce proliferation.

The effect of B13 in host mice carrying PC-3 xenograft tumors was further examined. B13 significantly inhibited the size and weight of PC-3 xenograft tumors (Fig. 7E) with no observed pathologic toxicity to the major organs of host SCID mice including the liver, kidney, and lung (Fig. 7F), and no significant changes in body (Supplementary Fig. S13A) or organs’ weight (Supplementary Fig. S13B). Collectively, B13 exhibited inhibition on the growth of prostate cancer cells, suggesting its potential as an effective agent for the treatment of prostate tumors.

**Discussion**

Our study demonstrates that the inhibition of NMT1 genetically or pharmacologically suppresses proliferation of prostate cancer cells and growth of xenograft tumors. The magnitude of the inhibitory effect is positively correlated with the expression levels of NMT1 and pSrc(Y416) in cancer cells. Inhibition of NMT1 suppresses myristoylation and tyrosine phosphorylation of Src kinase in vitro and in vivo. Targeting myristoylation exhibits dual effects in inhibiting Src-mediated oncogenic activity because both the catalytic domain and

![Figure 5](https://example.com/figure5.png)

**Figure 5.**

Chemical structures and IC\textsubscript{50} values of LCL compounds targeting NMT1 enzymatic activity. The IC\textsubscript{50} of LCL compounds on NMT1 enzymatic activity was measured. LCL7 and LCL35 represent a compound with a longer and shorter acyl-chain (R1 group in A) than LCL4/B13, respectively. LCL17 represents a compound with NH group in the acyl-chain, which enhances inhibitory effect. LCL86 represents a compound with longer acyl-chain and without nitro group (R2 group). LCL1 represents a compound without both nitro and hydroxyl group (R4 group). The bold highlights the chemical groups different from LCL4/B13 compound.
scaffold function are essential for SFKs (10). The myristoyl group may participate in protein folding and promote Src kinase to switch to its active conformation, leading to phosphorylation at Tyr416 (25). Loss of myristoylation suppresses downstream signaling including FAK as well as the MAPK signaling pathway (13). In addition, inhibition of myristoylation blocks the scaffold function of Src kinase, and prevents the protein–protein interaction with AR thus inhibiting androgen-independent AR activation. Several studies have reported that high levels of Src kinase dead mutants are still able to enhance FAK catalytic activity (26) and decrease osteoporosis in the Src−/− animal model (27), and promote AR activity in part (12). Inhibition of Src kinase anchoring to the intracellular membrane could change the protein intracellular trafficking path (23). Inhibition of NMT provides an additional pathway to inhibit SFK-mediated oncogenic signaling in comparison with numerous Src kinase inhibitors such as dasatinib that only target the ATP binding site (28).

We show that expression of NMT1 is correlated with expression levels of Src kinase in human tumors. Given the fact that the elevation of Src expression and activity has been well documented in advanced prostate cancer (9), targeting the
NM1-T-Src axis provides a novel approach for inhibiting tumor progression, particularly in Src-driven tumors. Although numerous NMT inhibitors have been developed as antiviral, antifungal, or antiparasitic agents (29), only a limited number of inhibitors including COPP24 and ‘Compound 1’ have been reported as anticancer agents (30, 31). NMT activity occurs via the formation of a ternary complex with myristoyl-CoA and glycine at the N-terminus of the target proteins (32).
Our study identifies B13 as a novel NMT inhibitor that can be modeled into the myristoyl-CoA binding site in NMT. B13 shows limited effect on normal cells or organs, which may be due to elevated fatty acid metabolism and lipogenesis in cancer cells in comparison with normal cells (33). Elevated expression of fatty acid synthase (FASN) has been well documented in numerous cancers, and targeting FASN shows benefit in inhibiting cancer progression (34). Biosynthesis of acyl-CoAs including myristoyl-CoA is a required step for phospholipid synthesis (35). In concert with aberrant expression of NMT in a variety of cancer cells (3, 21, 36), an increase of myristoyl-CoA production either from exogenous fatty acids or through de novo synthesis could further promote protein myristoylation to facilitate the growth of cancer cells (35). In particular, Src expression and/or activity are highly elevated in advanced prostate cancer (9). Further studies are required to determine whether the amount of myristoylated Src, which is essential for its kinase activity, is also elevated in advanced prostate cancer. Nevertheless, the differential activity of fatty acid metabolism coupled with an elevation of NMT enzymatic activity might provide a molecular basis to differentiate cancer cells with normal cells. Our study emphasizes the NMT1-Src axis in mediation of the growth of prostate cancer cells. It should be noted that targeting NMT may also lead to inhibition of other NMT downstream proteins in which myristoylation is essential for their function. For example, myristoylation of AMPK is required for its recruitment to the mitochondria for the induction of mitophagy and cell viability (37). The inhibition of B13 on NMT activity could potentially block the AMPK recruitment process to inhibit the proliferation of prostate cancer cells. Regardless, our study has demonstrated that targeting NMT activity provides a promising therapeutic approach for the treatment of prostate cancer progression.

It should be noted that B13 could have other inhibitory targets in addition to NMT1. For example, it has been reported that B13 can inhibit acid ceramidase (38, 39). The inhibition suppresses the conversion of ceramide to sphingosine, which leads to an increase of ceramide levels including C14-, C16-, C18-, C24-, C24:1-ceramides and a decrease of sphingosine (24, 38, 39). The alteration of ceramides and sphingosine triggers the cell apoptosis pathway (40). It remains to be studied if inhibition of NMT activity could also contribute to the alteration of ceramide levels. The biosynthesis of ceramides requires fatty acyl-CoA as substrates, and the accumulation of myristoylated Src, which is essential for its kinase activity, is also elevated in advanced prostate cancer. Furthermore, the differential activity of fatty acid metabolism coupled with an elevation of NMT enzymatic activity might provide a molecular basis to differentiate cancer cells with normal cells. Our study emphasizes the NMT1-Src axis in mediation of the growth of prostate cancer cells. It should be noted that targeting NMT may also lead to inhibition of other NMT downstream proteins in which myristoylation is essential for their function. For example, myristoylation of AMPK is required for its recruitment to the mitochondria for the induction of mitophagy and cell viability (37). The inhibition of B13 on NMT activity could potentially block the AMPK recruitment process to inhibit the proliferation of prostate cancer cells. Regardless, our study has demonstrated that targeting NMT activity provides a promising therapeutic approach for the treatment of prostate cancer progression.

It should be noted that B13 could have other inhibitory targets in addition to NMT1. For example, it has been reported that B13 can inhibit acid ceramidase (38, 39). The inhibition suppresses the conversion of ceramide to sphingosine, which leads to an increase of ceramide levels including C14-, C16-, C18-, C24-, C24:1-ceramides and a decrease of sphingosine (24, 38, 39). The alteration of ceramides and sphingosine triggers the cell apoptosis pathway (40). It remains to be studied if inhibition of NMT activity could also contribute to the alteration of ceramide levels. The biosynthesis of ceramides requires fatty acyl-CoA as substrates, and the accumulation of myristoylated Src, which is essential for its kinase activity, is also elevated in advanced prostate cancer.

Recipients of B13 as a novel NMT inhibitor that can be modeled into the myristoyl-CoA binding site in NMT. B13 shows limited effect on normal cells or organs, which may be due to elevated fatty acid metabolism and lipogenesis in cancer cells in comparison with normal cells (33). Elevated expression of fatty acid synthase (FASN) has been well documented in numerous cancers, and targeting FASN shows benefit in inhibiting cancer progression (34). Biosynthesis of acyl-CoAs including myristoyl-CoA is a required step for phospholipid synthesis (35). In concert with aberrant expression of NMT in a variety of cancer cells (3, 21, 36), an increase of myristoyl-CoA production either from exogenous fatty acids or through de novo synthesis could further promote protein myristoylation to facilitate the growth of cancer cells (35). In particular, Src expression and/or activity are highly elevated in advanced prostate cancer (9). Further studies are required to determine whether the amount of myristoylated Src, which is essential for its kinase activity, is also elevated in advanced prostate cancer. Nevertheless, the differential activity of fatty acid metabolism coupled with an elevation of NMT enzymatic activity might provide a molecular basis to differentiate cancer cells with normal cells. Our study emphasizes the NMT1-Src axis in mediation of the growth of prostate cancer cells. It should be noted that targeting NMT may also lead to inhibition of other NMT downstream proteins in which myristoylation is essential for their function. For example, myristoylation of AMPK is required for its recruitment to the mitochondria for the induction of mitophagy and cell viability (37). The inhibition of B13 on NMT activity could potentially block the AMPK recruitment process to inhibit the proliferation of prostate cancer cells. Regardless, our study has demonstrated that targeting NMT activity provides a promising therapeutic approach for the treatment of prostate cancer progression. It should be noted that B13 could have other inhibitory targets in addition to NMT1. For example, it has been reported that B13 can inhibit acid ceramidase (38, 39). The inhibition suppresses the conversion of ceramide to sphingosine, which leads to an increase of ceramide levels including C14-, C16-, C18-, C24-, C24:1-ceramides and a decrease of sphingosine (24, 38, 39). The alteration of ceramides and sphingosine triggers the cell apoptosis pathway (40). It remains to be studied if inhibition of NMT activity could also contribute to the alteration of ceramide levels. The biosynthesis of ceramides requires fatty acyl-CoA as substrates, and the accumulation of myristoylated Src, which is essential for its kinase activity, is also elevated in advanced prostate cancer.

Authors’ Contributions

Conception and design: S. Kim, O.A. Alsaidan, Z. Han, A. Bielawska, S.D. Pegan, H. Cai

Development of methodology: S. Kim, O.A. Alsaidan, Q. Li, Z. Han, Y.G. Zheng, Z.M. Szule, H. Cai

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kim, O.A. Alsaidan, O. Goodwin, E. Sulejmani, T. Albers, Z. Beharry, I. Lebedyeva, S.D. Pegan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kim, O.A. Alsaidan, O. Goodwin, T. Albers, S.D. Pegan, H. Cai

Writing, review, and/or revision of the manuscript: S. Kim, O.A. Alsaidan, O. Goodwin, Z. Beharry, Y.G. Zheng, J.S. Norris, Z.M. Szule, A. Bielawska, S.D. Pegan, H. Cai

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kim, O.A. Alsaidan, H. Cai

Other (myristoyl-CoA analogues’ synthesis): A. Bai

Other (synthesis of compounds studied in this project): I. Lebedyeva

Grant Support

This work was supported by the NIH (R01CA172495) and DOD (W81XWH–15–1–0507 to H. Cai), and P30 CA138313 (to A. Bielawska). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 7, 2017; revised August 24, 2017; accepted October 9, 2017; published OnlineFirst October 16, 2017.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Kim et al.


Downloaded from cancerres.aacrjournals.org on December 4, 2021. © 2017 American Association for Cancer Research.
Blocking Myristoylation of Src Inhibits Its Kinase Activity and Suppresses Prostate Cancer Progression

Sungjin Kim, Omar Awad Alsaidan, Octavia Goodwin, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-17-0981

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2017/10/14/0008-5472.CAN-17-0981.DC1

Cited articles
This article cites 41 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/77/24/6950.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/77/24/6950.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/77/24/6950.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.