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LLY-283, a Potent and Selective Inhibitor of Arginine Methyltransferase 5, PRMT5, with Antitumor Activity

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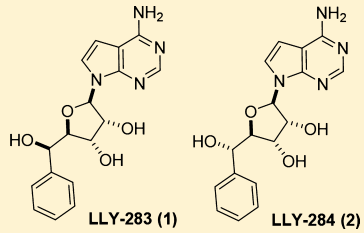
Supporting Information

ABSTRACT: Protein arginine methyltransferase 5 (PRMT5) is a type II arginine methyltransferase that catalyzes the formation of symmetric dimethylarginine in a number of nuclear and cytoplasmic proteins. Although the cellular functions of PRMT5 have not been fully unraveled, it has been implicated in a number of cellular processes like RNA processing, signal transduction, and transcriptional regulation. PRMT5 is ubiquitously expressed in most tissues and its expression has been shown to be elevated in several cancers including breast cancer, gastric cancer, glioblastoma, and lymphoma. Here, we describe the identification and characterization of a novel and selective PRMT5 inhibitor with potent *in vitro* and *in vivo* activity. Compound **1** (also called LLY-283) inhibited PRMT5 enzymatic activity *in vitro* and in cells with IC_{50} of 22 ± 3 and 25 ± 1 nM, respectively, while its diastereomer, compound **2** (also called LLY-284), was much less active. Compound **1** also showed antitumor activity in mouse xenografts when dosed orally and can serve as an excellent probe molecule for understanding the biological function of PRMT5 in normal and cancer cells.



KEYWORDS: *PRMT5, methyltransferase, inhibitor, splicing, methylation*

Arginine methylation of proteins is an important class of post-translational modification that is known to play a key functional role in crucial cellular pathways including cell growth and proliferation, apoptosis, angiogenesis, and metastasis by regulating both transcription and post-transcriptional RNA processing.[1] This modification is catalyzed by the PRMT (Protein aRginine MethylTransferase) family of arginine methyltransferases, which transfer a methyl group from S-adenosyl methionine (AdoMet) to the terminal guanidino nitrogen atoms of arginine side-chains of histones and nonhistone proteins. Nine human PRMTs have been identified to date,[2] and they are further subdivided into type I, type II, type III, and type IV enzymes based on the kind of arginine methylation catalyzed. PRMT5 is the predominant type II PRMT[3] that catalyzes the formation of ω-NG-monomethyl and ω-NG,N′G-symmetric dimethyl arginine residues and can methylate arginine residues in both a GAR and PGM motif.

PRMT5 is involved in the regulation of diverse cellular processes including transcription,[4] RNA metabolism,[5] signal transduction,[6] maintenance of Golgi apparatus architecture,[7] and cellular differentiation.[8] However, how PRMT5 impacts these biological processes is not yet clearly defined. In the nucleus, PRMT5 has been shown to be involved in transcriptional repression including that of tumor suppressor and cell cycle genes like ST7 (suppressor of tumorigenicity 7 protein),[9] cyclin E1,[10] and CDKN2A (cyclin-dependent kinase inhibitor 2A).[11] PRMT5 brings about these epigenetic modifications through the symmetric dimethylation of histone H4 on the R3 residue (H4R3me2s) and on the R8 residue of histone H3 (H3R8me2s).[12] H4R3me2s is generally associated with transcriptional repression,[13] while H3R8me2s has been seen as a mark for both transcriptional activation and repression.[14] Besides these marks, H2AR3me2s and H3R2me2s modifications have also been associated with PRMT5 activity. H3R2me2s is present in euchromatic regions and at several promoter regions like those of gluconeogenic genes; this methylation is followed by the recruitment of WDR5 and the

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subsequent expression of corresponding genes.[15] The function of the cytoplasmic H2AR3me2s modification remains unclear.

An important facet of PRMT5 biology involves the regulation of RNA splicing and snRNP biogenesis in the cytoplasm where PRMT5 mainly exists in a 20S methylosome complex together with MEP50 and pICln.[16,17] In this complex, PRMT5 catalyzes the symmetric dimethylation of the Sm proteins B/B′, D1, and D3 of U1, U2, U4, U5 snRNP and U6 snRNA-associated LSm4[18] thus enabling their binding to Survival Motor Neuron (SMN)[19] and subsequent deposition onto U-snRNAs thereby forming U-snRNPs.[20] It is believed that Sm protein methylation increases their binding affinity for SMN though the effect was apparent only *in vitro*.[21] However, the spliceosomal role of PRMT5 appears to be conserved across species, and mutations in PRMT5 in *Arabidopsis thaliana*[22] and *Drosophila melanogaster* (Dart5)[23] are believed to affect alternative splicing of critical genes involved in germ cell speciation in *Drosophila* and circardian rhythms in *Arabidopsis*. Similarly, PRMT5-null knockouts are embryonic lethal in mice showing that PRMT5 is essential for mammalian development.[24] Remarkably, deletion of PRMT5 in neural stem/progenitor cells (NPCs) resulted in widespread effects on alternative splicing events including that of Mdm4[5] producing, in the latter case, a truncated splice variant lacking exon 6 that is thus targeted for degradation by nonsense-mediated decay. This causes a decrease in full-length MDM4 leading to induction of p53 pathway and, subsequently, cell cycle arrest and apoptosis. However, p53 activity is not solely responsible for the phenotype arising from the loss of PRMT5.[5]

The important role of PRMT5 in cancer including its interaction with pathways deregulated in cancer implicates it as an attractive therapeutic target in cancer.[4,25] Several small molecule inhibitors of PRMTs including PRMT5 have been identified.[26−28] However, most of these are low potency inhibitors or lack cellular and/or *in vivo* activity. Recently, a substrate competitive and cell active PRMT5 inhibitor, EPZ015666, was demonstrated to have antiproliferative effects in both *in vitro* and *in vivo* models of Mantle cell lymphoma (MCL).[29,30] We had earlier reported the crystal structure of human PRMT5:MEP50 complex,[31] which aided the synthesis of novel inhibitors of PRMT5. Here, we describe the identification and characterization of a potent and selective inhibitor of PRMT5, compound **1** (Figure 1), that specifically binds in the SAM pocket of PRMT5 in contrast to EPZ015666, which is histone peptide (substrate) competitive. The unique characteristics of compound **1** enable it to be highly potent and cell permeable with well-defined PK characteristics.

Deazapurine analogue **3** was prepared according to literature procedures.[32,33] Hypervalent iodine oxidation of primary alcohol **3** gave acid **4**, which was converted to morpholinoamide **5** without evidence of epimerization of the C4 center. Grignard addition proceeded smoothly at low temperatures to yield acetophenone **6**. Chemical probe **1** and diastereomer **2** are readily prepared from this common intermediate. Asymmetric transfer hydrogenation of the ketone with (*R,R*)-Ts-DENEB[34] delivered the hydride from the *re*-face, producing **7** with high diastereoselectivity. The relative configuration of the 5′-hydroxyl was confirmed from single crystal X-ray structure of the *para*-bromo benzoate (Supporting Information). The chloride was displaced by ammonia in MeOH, and the acetal **8** was hydrolyzed to produce **1**. To obtain the 5′-OH diastereomer, L-selectride was used to enforce delivery of the hydride from the *si*-face of acetophenone **6**, yielding **9** with high

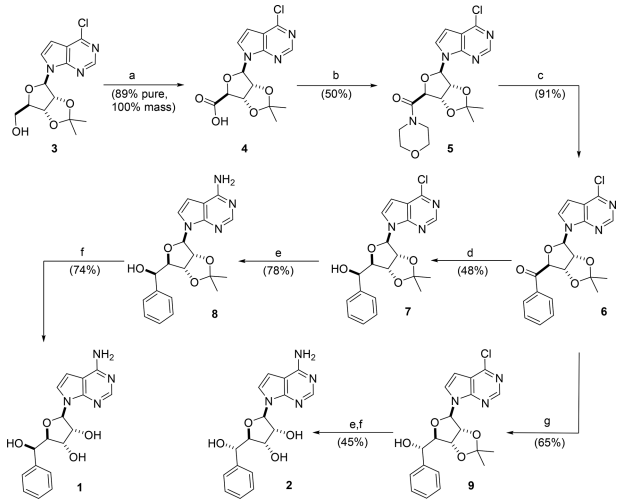

Figure 1. Synthesis of PRMT5 chemical probe **1** and negative control compound **2**.

facial selectivity. Finally, treatment with ammonia and mild acid hydrolysis of the acetal delivered compound **2**.

To understand the binding mode of compound **1** and to characterize its molecular interactions with PRMT5, we determined the crystal structure of the PRMT5:MEP50 complex bound to compound **1** (Figure 2). The crystal

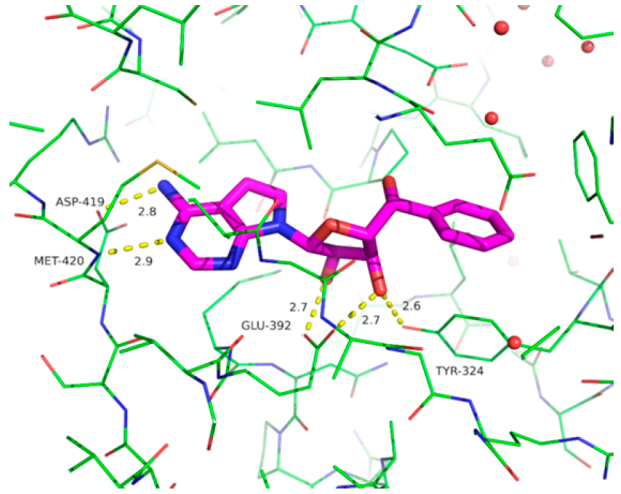

Figure 2. Crystal structure of the PRMT5:MEP50 complex (green) bound to compound **1** (purple). Key hydrogen bonds are indicated in dotted yellow lines as the ligand binds in the AdoMet pocket. Select residues are labeled.

structure was determined to 2.9 Å resolution using published methods.[31] Coordinates and structure factors have been deposited with the Protein Data Bank under the accession code 6CKC. Data quality and refinement statistics are presented in Table S1 (Supporting Information). In contrast to the previously published inhibitors of PRMT5 that bind in the peptide substrate pocket,[29] compound **1** is the first potent inhibitor that binds in the AdoMet pocket, with the adenine and ribose moieties adopting very similar conformations and making similar interactions observed in other PRMT5:MEP50 structures with AdoMet analogs.[31] The adenine makes hydrogen bonds to the side chain carboxylate of Asp419 and the main chain amino of Met420, while the ribose makes a pair

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of hydrogen bonds to the glutamate side chain of Glu392, as well as a hydrogen bond to the side chain of Tyr324. Comparison to the PRMT5:MEP50 complex with a substrate peptide in addition to an AdoMet analog reveals that the phenyl group of compound **1** displaces the side chain of Phe327, which adopts an alternate rotamer with minimal movement of the main chain.

Compounds **1** and **2** were screened for their *in vitro* inhibitory activity toward PRMT5:MEP50 complex (Figure 3).

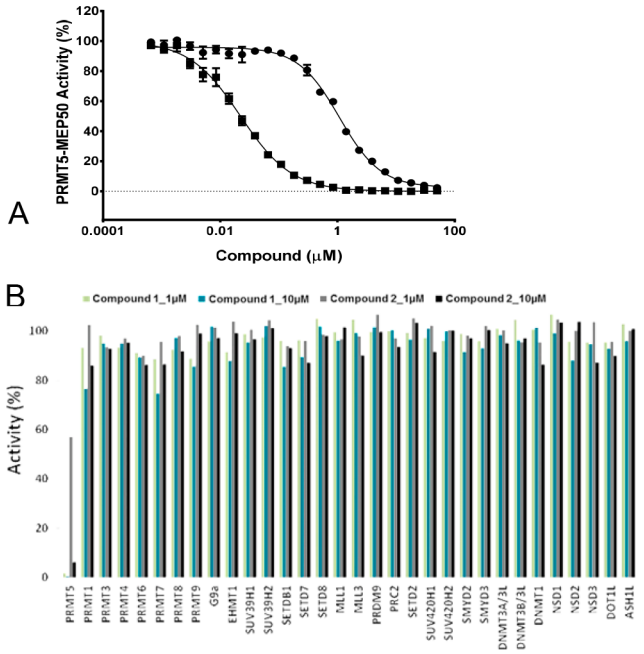

Figure 3. (A) Enzyme inhibition assay for compound **1** and its negative control (compound **2**). The IC_{50} values of 22 ± 3 nM (Hill Slope of 1) and 1074 ± 53 nM (Hill Slope of 1.2) were determined for compound **1** (■) and compound **2** (●), respectively. The experiments were performed in triplicate. (B) Selectivity of compounds **1** and **2** at 1 and 10 μM (as indicated on the plot) were assessed against a collection of 32 methyltransferases.

Using a radioactivity-based assay monitoring the transfer of the methyl group from ^{3}H-SAM to peptide substrate, we confirmed that compound **1**, potently inhibited PRMT5:MEP50 activity *in vitro* with an IC_{50} of 22 ± 3 nM, while its 5′-stereoisomer, compound **2**, was almost 50-fold less active (IC_{50} of 1074 ± 53 nM). Binding of compound **1** to PRMT5:MEP50 complex was confirmed by surface plasmon resonance (SPR; Supporting Information) with a K_D (equilibrium dissociation constant) value of 6 ± 2 nM (n = 3) and dissociation- (k_{on}) and association- (k_{off}) rate constants of 3.9 ± 0.4 × 10^5 M^{-1} s^{-1} and 2.2 ± 0.8 × 10^{-3} s^{-1}, respectively. The mechanism of action (MOA) of compound **1** was assessed by determining the IC_{50} values in the presence of various concentrations of peptide and SAM. No dramatic change was observed in IC_{50} values upon increasing the concentration of each substrate to as high as 25× of the respective K_m (Michaelis–Menten constant) values. Even though binding of compound **1** in the SAM pocket was clearly seen in cocrystal structures, the SAM competition assay did not reveal competitive inhibition with respect to SAM; rather the MOA data supports apparent noncompetitive patterns with respect to both substrates. The reason(s) for noncompetiveness is (are) unknown at this time. We have seen this earlier in other cases, e.g., PRMT4 and PRMT6 for MS049[35] (a dual PRMT4 and 6 inhibitor) and PRMT6 for MS023[36] (a pan-Type 1 PRMT inhibitor). However, in both these cases the ligand is binding in the peptide pocket.

Compounds **1** and **2** were also assessed for their activity against a panel of 32 methyltransferases using radioactive assays as described in Supporting Information. In spite of being a close analogue of SAM, compound **1** showed remarkable specificity for PRMT5 with the activity dropping significantly even against other arginine methyltransferases including the closely related arginine methyltransferase PRMT7 and the Type I PRMTs such as PRMT4 and 6.

The cellular activity of the compounds was evaluated by monitoring SmBB′ symmetric dimethylation in MCF7 cells in Western blot (Figure 4) and alternative splicing of MDM4 in

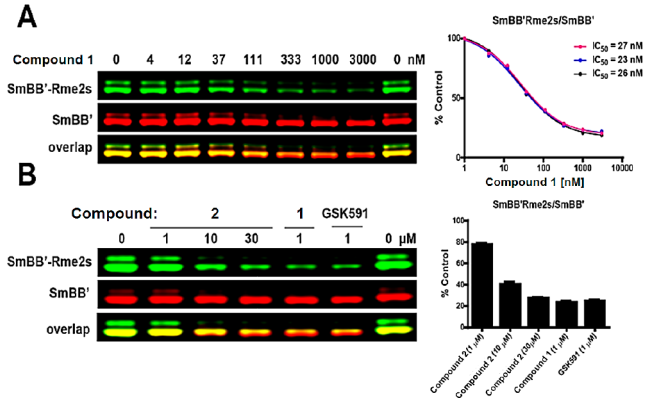

Figure 4. Compound **1** inhibits PRMT5 activity in MCF7 cells. MCF7 cells were treated with compounds at indicated concentrations for 48 h and SmBB′-Rme2s levels were determined by Western blot. (A) SmBB′-Rme2s dose response to compound **1**. The graph represents nonlinear fits of SmBB′-Rme2s signal intensities normalized to total SmBB′. (B) Effect of compound **1**, GSK3203591, and compound **2** on SmBB′-Rme2s levels. The graph represents SmBB′-Rme2s signal intensities normalized to total SmBB′ represented as % DMSO control. The results are mean ± SEM of three replicates.

A375 cells by qPCR (Figure 5). It has been reported previously that the various SMN complex proteins, including SmBB′ are symmetrically dimethylated by PRMT5.[17] We found that knocking down of PRMT5, but not PRMT1, 3, 4, 6, 7, and 9 resulted in decreased basal SmBB′ symmetric dimethylation levels in MCF7 cells (Supporting Information). Similar to PRMT5 knockdown, two day treatment of MCF7 cells with compound **1** decreased SmBB′-Rme2s levels in a dose-dependent manner (IC_{50} = 25 ± 1 nM) (Figure 4). At 1 μM, compound **1** decreased the levels of SmBB′-Rme2s to the same extent as previously published peptide-competitive PRMT5 inhibitor, GSK591.[29] At the same concentration, negative control compound, compound **2**, caused only 20% decrease in SmBB′-Rme2s levels.

Reduced methylation of Sm proteins caused by the depletion of PRMT5 has been shown to lead to aberrant alternative splicing of specific mRNAs with weak 5′ donor sites.[5] Mdm4 was seen to be one of these key mRNAs that is sensitive to the defects in the spliceosomal machinery. Similarly, compound **1** also inhibited the PRMT5 mediated regulation of the splicing of MDM4, as indicated by the ratio of MDM4 mRNA bearing exons 5 and 6 to that bearing solely exon 5 (Figure 5). Treatment of A375 melanoma cells with compound **1** for 72 h

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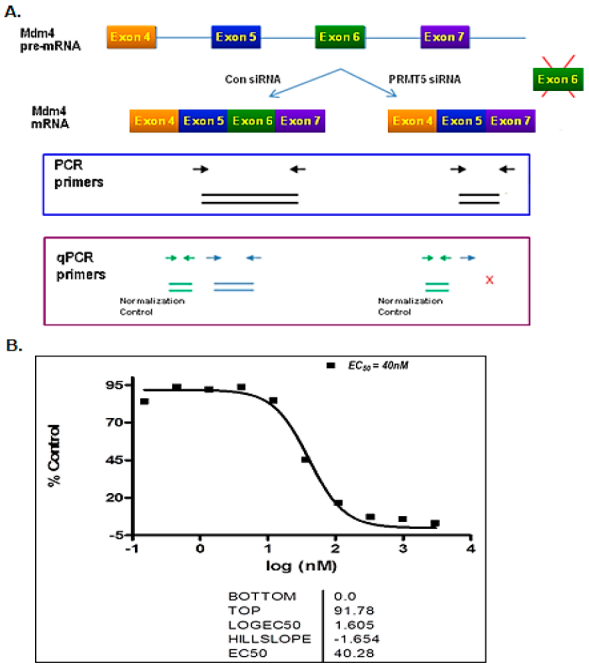

Figure 5. Compound **1** inhibits PRMT5 activity in A375 cells. (A) Scheme depicting the qPCR assay strategy. (B) Mdm4 exon 6 splicing (normalized to exon 5) dose response to compound **1** (run in duplicate; Supporting Information). Subsequent repeats of the assay (n > 3) gave an EC_{50} of 37 ± 3 nM (mean ± SEM).

caused skipping of the exon 6 (cassette exon) of Mdm4 with an EC_{50} of 40 nM when normalized to the levels of exon 5.

The effects of PRMT5 target engagement manifested as an antiproliferative phenotype in many cancer cell lines when tested in a 7-day proliferation assay (Cell titer Glo assay; Supporting Information). Though hematological tumors were especially sensitive, potent antiproliferative effects were observed in many solid tumor lines including those from breast, lung, skin ovarian, and gastric cancer (representative examples from some of the common cancer types are shown in Table 1). In the cell lines where proliferation inhibition assay was repeated, reproducible IC_{50} values were obtained, for example, A375 (n = 3) gave an average IC_{50} of 46 ± 5 nM (mean ± SEM).

Further *in vitro* and *in vivo* characterization of compound **1** revealed that it possesses desirable physical and ADME properties that will enable broad exploration of the PRMT5 mechanism in animals. In brief, the compound has excellent physicochemical properties, high unbound levels in plasma, moderate to slow plasma clearance, low potential for competitive CYP inhibition, and dynamic range of oral bioavailability (Supporting Information) that result in high exposure in rodents when given once daily. As seen in Figure 6, unbound C_{max} (@10 mg/kg PO dose) exceeds the cellular MDM4 IC_{50} value for compound **1**.

Since compound **1** is amenable to *in vivo* studies, we tested its efficacy in A375 derived xenografts grown subcutaneously in severe combined immunodeficiency (SCID) mice in a 28-day study, with an oral once a day (QD) dose of compound **1** at 20 mg per kilogram of body weight (mg/kg).

After 28 days of dosing, compound **1** (20 mg/kg; QD) induced statistically significant tumor growth inhibition (TGI) compared to vehicle-treated tumors (Figure 7). A more pronounced *in vivo* response to compound **1** is predicted in

Table 1. Cancer Cell Line Response to Compound 1 in 7-Day Proliferation Assay (Supporting Information)

cell line	histology	IC_{50} (μM)	max inhibition (%)
HCC1937	breast	0.030	83
T-42D	breast	0.003	84.0
UACC-812	breast	>2.5	21.0
MX-1 cells	breast	>2.5	36.9
NUGC-3	gastric	0.017	92
KE-97	gastric	0.024	95.7
MV4−11	hematological	0.016	93
Daudi	hematological	0.017	99.3
Karpas-422	hematological	0.085	99.9
HEL 92.1.7	hematological	0.003	91.2
ARH-77	hematological	0.010	97.2
REC-1	hematological	0.022	99.7
IM-9	hematological	0.046	93
KMS-12-BM	hematological	0.006	99
RPMI-8226	hematological	0.009	88.5
OPM-2	hematological	>2.5	22.5
NCI-H2171	lung	0 010	97.7
H-2122	lung	0.027	90
NCI-H1930	lung	>2.5	49.6
NCI-H1651	lung	>2.5	32
A375	skin	0.037	91.1
A2058	skin	0.013	96
GAK	skin	0.016	99.9
Colo829	skin	0.013	51
OVCAR-3	ovarian	0.032	95

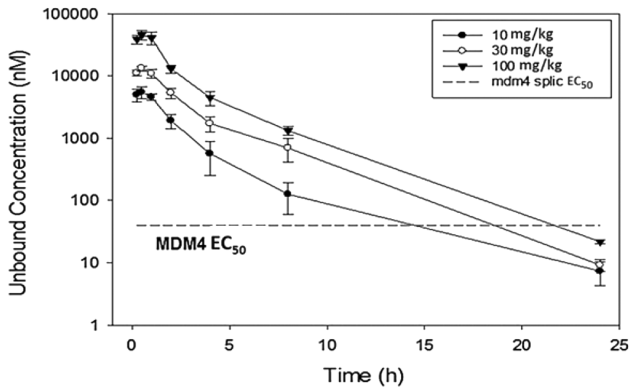

Figure 6. Compound **1** mouse PO PK: Oral dose response.

cell lines from hematological cancers like DLBCL, MCL, ALL, etc., since many such lines are highly sensitive to compound **1** *in vitro*. Further, at the doses given, compound **1** was well tolerated with moderate body weight loss (<10%) and no apparent toxicological observations.

In summary, we have discovered and characterized a potent and selective PRMT5 inhibitor that can be used as an *in vivo* tool compound to evaluate the pharmacology of PRMT5 inhibition. Unlike earlier PRMT inhibitors such as EPZ015666, which is mainly substrate competitive, compound **1** directly binds to and occupies the SAM pocket and is, to our knowledge, the first such PRMT5 inhibitor that has been reported. We have shown that compound **1** is a low nanomolar enzymatic inhibitor of the PRMT5:MEP50 complex in biochemical assays and is selective for PRMT5 over other methyltransferases including related family members. The binding mode in the SAM pocket of PRMT5 was confirmed

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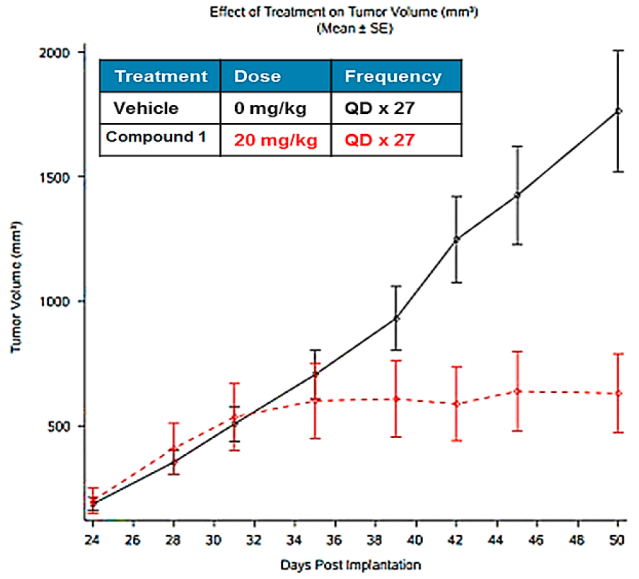

Figure 7. Compound 1 inhibits tumor growth in mouse xenograft (A375).

from a 2.9 Å resolution crystal structure. In addition, compound **1** has submicromolar cell-based potency as revealed by its ability to inhibit the arginine methylation of spliceosomal protein SmB/B′ and, consequently, affects the alternative splicing of Mdm4. We have shown that compound **1** has broad *in vitro* antiproliferative activity across several tumor types. Further, well-behaved and predictable pharmacokinetic characteristics of compound **1** enable investigation of its antitumor effects *in vivo* as seen in A375 mouse xenografts. Although PRMT5 is also known as an epigenetic modulator through its regulation of histone arginine methylation especially that of histones H3 (H3R8) and H4 (H4R3), we did not observe much evidence of such effects in our cell-based assays with compound **1** using corresponding commercially available and proprietary methylated histone antibodies. However, at this stage we cannot rule out the epigenetic role of PRMT5. Further characterization of the mechanism of antitumor effects of PRMT5 inhibition will be critical in defining the therapeutic potential of PRMT5 across different tumor types.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00014.

In vitro and *in vivo* biological methods describing biochemical activity assays, surface plasmon resonance (SPR), cellular PRMT5 assay, Western blot, MDM4 Exon5/6 PCR assay in A375 tumor cells, 7-day proliferation assay, A375 xenograft tumor model, and a table listing crystal structure data quality and refinement statistics; experimental and characterization data for **1** and **2**, and copies of ^{1}H NMR spectra; SPR and MOA data; PRMT knockdown data; summary table for ADME and PK properties (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SAM, *S*-adenosylmethionine; PRMT, protein arginine methyl transferase; SmBB′me2s, small ribonucleoprotein particle protein BB′ symmetric dimethylation; PK, pharmacokinetic; MEP50, methylosome protein 50; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl; (*R*,*R*)-Ts-DENEB, chloro{*N*-[(1*R*,2*R*)-1,2-diphenyl-2-(2-(4-methylbenzyloxy)ethylamino)-ethyl]-4-methylbenzenesulfonamide(chloro)ruthenium(II) (*R*,*R*); CYP, cytochrome P450 enzyme; DLCBL, diffused large B-cell lymphoma; MCL, mantle cell lymphoma; ALL, acute lymphoblastic leukemia; L-selectride, lithium tri-*sec*-butyl borohydride; GAR, glycine and arginine-rich motif; PGM, proline, glycine and methionine-rich motif

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