A selective inhibitor of PRMT5 with in vivo and in vitro potency in MCL models

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Protein arginine methyltransferase-5 (PRMT5) is reported to have a role in diverse cellular processes, including tumorigenesis, and its overexpression is observed in cell lines and primary patient samples derived from lymphomas, particularly mantle cell lymphoma (MCL). Here we describe the identification and characterization of a potent and selective inhibitor of PRMT5 with antiproliferative effects in both in vitro and in vivo models of MCL. EPZ015666 (GSK3235025) is an orally available inhibitor of PRMT5 enzymatic activity in biochemical assays with a half-maximal inhibitory concentration (IC50) of 22 nM and broad selectivity against a panel of other histone methyltransferases. Treatment of MCL cell lines with EPZ015666 led to inhibition of SmD3 methylation and cell death, with IC50 values in the nanomolar range. Oral dosing with EPZ015666 demonstrated dose-dependent antitumor activity in multiple MCL xenograft models. EPZ015666 represents a validated chemical probe for further study of PRMT5 biology and arginine methylation in cancer and other diseases.

The importance of PRMT5 in tumorigenesis is highlighted in several reports. PRMT5 is upregulated in several human malignancies, including lymphomas1–3, lung cancer4, breast cancer5 and colorectal cancer6. In addition, PRMT5 is reported to have a role in MCL, as evidenced not only by upregulation of PRMT5 in patient samples, but also by antiproliferative effects observed after PRMT5 knockdown in MCL cell lines1,2,3. The mechanism behind the cell-transforming capabilities of PRMT5 is unclear, but the enzyme is postulated to have roles in cell death, cell-cycle progression and cell growth and proliferation4. Whether PRMT5 drives tumorigenesis by regulating gene expression (for example, through histone methylation, transcription factor binding or promoter binding), by signal transduction or by some other mechanism is still unknown.

PRMT5 catalyzes the transfer of up to two methyl groups to arginine residues, forming ω-ω-monomethyl arginine and symmetrical ω-ω-dimethyl arginine on protein substrates5. Nine mammalian PRMTs have been identified so far and are classified into three types6. Each type is defined by its ability to transfer one or two methyl groups to the nitrogen atoms of the guanidinium side chains of arginine residues using S-adenosylmethionine (SAM) as the methyl donor. PRMT5 is the predominant type II PRMT that is responsible for the symmetric dimethylation of arginine residues (the other is PRMT9)5,11. PRMT5 is reported to participate in several diverse cellular processes through the methylation of a variety of cytoplasmic and nuclear substrates6, including histones H4 residue Arg3 (H4R3) and H3 residue Arg8 (H3R8)1,2,3. PRMT5 interacts with a number of binding partners that influence its substrate specificity. A core component of these multimeric complexes is a member of the WD40 family of proteins, MEP50, a protein partner that is required for PRMT5 enzymatic activity. Pan-dimethyl arginine antibodies are used to probe the biology of methylated arginine-containing substrates and their enzymes5,9,10. Studies with pan-dimethyl arginine antibodies have shown that knockdown of PRMT5 modulates the methylation status of several proteins. More specifically, PRMT5 can methylate proteins involved in RNA splicing, one of which is Smd3, which can be used to track the cell biochemical activity of PRMT5 (refs. 14–16).

A number of groups have discussed small-molecule inhibitors of PRMT517–28. Although the majority of these reports describe compounds with biochemical half-maximal enzyme-inhibition concentrations (IC50) in the micromolar range, there are a limited set of inhibitors22,24 with nanomolar biochemical IC50 values or micromolar IC50 inhibition of a specific methyl mark in cells26,28. Despite these recent advances, none of these compounds has demonstrated the ability produce a phenotypic response that can be correlated to a reduction in levels of an enzyme-specific methyl mark. We thus set out to identify a potent and selective PRMT5 inhibitor that could be used as a powerful tool in efforts to explore and potentially further validate PRMT5 as a clinically relevant target.

Here we describe what is to our knowledge the first cell-potent and orally bioavailable inhibitor of PRMT5 with antiproliferative effects in both in vitro and in vivo models of MCL. Biochemical inhibition correlated well with inhibition of symmetric dimethylation of arginine-containing substrates and proliferation in a time- and concentration-dependent manner. Oral dosing of EPZ015666 demonstrated dose-dependent antitumor activity in several MCL xenograft models. Corresponding decreased levels of symmetrically dimethylated PRMT5 substrates in the excised
tumors strongly suggest that the antiproliferative effects were a direct consequence of PRMT5 inhibition.

**RESULTS**

**Tool compound discovery**

To identify inhibitors of PRMT5, we developed a homogeneous time-resolved fluorescence (HTRF) assay under balanced conditions to monitor the monomethylation of H4R3 on a histone H4 peptide by PRMT5:MEP50 and used it to screen a diversity library containing 370,000 small molecules (Supplementary Results and Supplementary Table 1). With a cutoff of 3 s.d. from the average percent inhibition in compound wells, the hit rate was approximately 0.7%. Roughly half of the hits were confirmed upon cherry picking and re-testing, and after the removal of compounds containing pan-assay interference structures (PAINS) and additional known frequent-hitter substructures, a subset of 800 compounds was selected for follow-up. Counterscreens for HTRF artifacts and compounds or contaminants present in compound wells were performed on a subset of hits; these screens included an orthogonal PRMT5:MEP50 activity assay that followed the transfer of a tritiated methyl group from SAM to a histone H4 peptide that was captured on a FlashPlate and a redox assay to identify reactive compounds that inhibited the enzyme in an intractable manner. These activities resulted in the identification of a prioritized chemical series whose members were further validated as inhibitors of PRMT5. Most activity was observed with crystallographic data for the ternary PRMT5:MEP50–SAM–enzyme complex (described below). PRMT5 has been reported to follow a random order of substrate binding (Fig. 1c); fitting of these data to a competitive inhibition model yielded a $K_i$ value of $5 \pm 0.3$ nM. Inhibition of PRMT5 by EPZ015666 was uncompetitive with respect to SAM, as clearly demonstrated by the descending, curvilinear dependence of the $IC_{50}$ value on the SAM concentration (Fig. 1c); fitting the data to an uncompetitive inhibition model yielded an $IC_{50}$ value for each compound shown. These results indicate that although the inhibitor had some modest affinity for the free enzyme, its affinity for the enzyme was greatly augmented by SAM binding (i.e., for all practical purposes, an uncompetitive mode of inhibition), consistent with crystallographic data for the ternary PRMT5:MEP50–SAM–EPZ015666 complex (described below).

After a number of iterative design cycles, EPZ015666 (2) was identified with a biochemical $IC_{50}$ of $22 \pm 14$ nM ($n=12$; Fig. 1b and Supplementary Note). At saturating concentrations of EPZ015666, a small amount of residual enzyme turnover was consistently observed, resulting in a maximal inhibition of $93 \pm 3$% ($n=12$) (Fig. 1b), the cause of which remains unclear. EPZ015666 showed an unambiguous pattern of competitive inhibition with respect to the peptide substrate, as demonstrated by an ascending, linear dependence of the $IC_{50}$ value on the peptide substrate concentration (Fig. 1c); fitting of these data to a competitive inhibition model yielded a $K_i$ value of $5 \pm 0.3$ nM. Inhibition of PRMT5 by EPZ015666 was uncompetitive with respect to SAM, as clearly demonstrated by the descending, curvilinear dependence of the $IC_{50}$ value on the SAM concentration (Fig. 1c); fitting the data to an uncompetitive inhibition model yielded an $IC_{50}$ constant at saturating [SAM] ($\alpha K$) of $20 \pm 0.9$ nM. These results indicate that although the inhibitor had some modest affinity for the free enzyme, its affinity for the enzyme was greatly augmented by SAM binding (i.e., for all practical purposes, an uncompetitive mode of inhibition), consistent with crystallographic data for the ternary PRMT5:MEP50–SAM–EPZ015666 complex (described below). PRMT5 has been reported to follow a random order of substrate binding (Fig. 1c). Nevertheless, the uncompetitive nature of EPZ015666 inhibition with respect to SAM might indicate a preferred binding sequence for productive ternary complex formation in which SAM binds before the methyl group–accepting substrate. EPZ015666 demonstrated exquisite selectivity against a panel of 20 other protein methyltransferases, showing no inhibition up to the maximum tested concentration of 50 μM (Fig. 1d). At the time of this writing we had not evaluated activity against PRMT9. The compound was also shown to have a favorable pharmacokinetic profile in mice, with plasma clearance of $30 \text{ ml min}^{-1} \text{ kg}^{-1}$, a volume of distribution of $1.7 \text{ kg}^{-1}$ after intravenous dosing at $2 \text{ mg kg}^{-1}$, and oral bioavailability of 69% following oral administration of 10 mg kg$^{-1}$. EPZ015666 was therefore selected as an appropriate biological tool for both in vitro and in vivo studies (Supplementary Fig. 2).

Figure 1 | Identification, characterization and optimization of diversity high-throughput screening (HTS) hit to a potent and selective inhibitor of PRMT5. (a,b) Structures (a) and representative $IC_{50}$ plot (b) of initial HTS-hit EPZ007345, tool compound EPZ015666 and inactive analog EPZ019896 ($n=2$ for each compound shown). (c) EPZ015666 is a SAM-competitive, peptide-competitive inhibitor of PRMT5:MEP50. $IC_{50}$ data were fit to competitive and uncompetitive forms of the Cheng–Prusoff equation (Online Methods), in which $K_i$ represents the binding constant of the inhibitor to free enzyme and $\alpha K$ represents the binding constant of the inhibitor to the enzyme-SAM complex (experiments performed with $n=2$; data shown for $n=1$). (d) Ligand-affinity maps of EPZ015666 across the family trees of human arginine methyltransferases and lysine methyltransferase enzymes show that EPZ015666 is a selective and potent inhibitor of PRMT5.
Characterization of unique cation-π binding mode

To understand further the mechanism of inhibition and SAR trends for this compound, we obtained a crystal structure of PRMT5:MEP50 with EPZ015666 and SAM (Fig. 2a and Supplementary Table 2). Consistent with the enzymological data, the compound was found to bind in the peptide-binding site, including the pocket occupied by the substrate arginine side chain. The compound interacted directly with many of the residues involved in peptide binding or residues that are postulated to be involved in the catalytic mechanism of methyl transfer. These included the backbone NH of Phe580 and the side chains of Glu444. Another residue thought to be important for enzyme catalysis, Glu435, was engaged in a water-mediated interaction with the tertiary nitrogen of the THIQ ring system. The THIQ group formed a π-π stacking interaction with Phe327. This residue had a role in directing symmetric arginine methylation and underwent significant conformational change at both the side chain and the backbone to accommodate the bulky THIQ moiety (Fig. 2b). The phenyl ring of the THIQ was clearly involved in a cation-π interaction with the partially positively charged methyl group of SAM, which to our knowledge is the first example of an interaction of this type. Removal of the phenyl ring of the THIQ produced a closely related but inactive analog, EPZ019896 (3). In addition, SAR around the THIQ phenyl ring of EPZ015666 were consistent with requiring a group capable of maintaining a favorable quadrupole moment. To further confirm the feasibility of this interaction, we determined co-crystal structures of PRMT5:MEP50 and EPZ015666 in the presence of sinefungin (SFG), a SAM mimic, and S-adenosylhomocysteine (SAH), without the methyl group. The three PRMT5 protein structures and EPZ015666 superimposed well (Fig. 2c). For the structures with SAM and SFG, the aryl ring of the THIQ was oriented 3.6–3.8 Å from the positively charged moiety. The absence of the positively charged group in the SAH structure did not change the overall binding mode of the compound, as EPZ015666 has all the same interactions with the protein as seen in the SAM and SFG structures. Therefore, any differences in potency seen between SAH and SAM or SFG complexes were most likely due to the cation-π interaction.

Characterization of cell methylation and proliferation

A panel of five MCL cell lines (Z-138, Mave-1, Mino, Granta-519, and Jeko-1) was used to assess changes in cellular methylation levels using the fast-on/fast-off high-throughput screening (HTS) hit compound (1) as a tracer molecule to probe the complex and SPR single-cycle kinetics. The half-life of EPZ015666 on PRMT5:MEP50 was determined to be 94 min with the tracer method and 130 min with the single-kinetics method, indicating that the two techniques were in good agreement (Supplementary Fig. 5).

Supplementary Table 2

<table>
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<th>compound</th>
<th>Kd (nM)</th>
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<tr>
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Supplementary Fig. 2 | Characterization of EPZ015666 binding mode using X-ray crystallography and surface plasmon resonance. (a) Crystal structure of EPZ015666 (cyan) bound to PRMT5:MEP50 complex (green) in the presence of SAM (yellow; PDB accession 4X61). The 2F_{o}-F_{c} electron density for the ligand is shown at 1.0σ. Select interactions are shown by dashed lines. (b) Superposition of EPZ015666-SAM (green and cyan, respectively) and H4 peptide–SFG (gray; PDB accession 4G0B) complexes reveals that EPZ015666 binds in the peptide-binding site and Phe327 changes conformation significantly to accommodate the inhibitor. (c) Crystal structures of EPZ015666 bound to PRMT5:MEP50 complex in the presence of SAM (green), SFG (magenta; PDB accession 4X6D) and SAH (cyan; PDB accession 4X63) show no change to the binding mode of the ligand with different nucleotides. (d–g) Surface plasmon resonance studies of EPZ015666 binding to (d) apo-PRMT5:MEP50 and (e) SAH-, (f) SAM- and (g) sinefungin-bound PRMT5:MEP50. EPZ015666 binding occurred only with aminonucleoside-bound PRMT5:MEP50, confirming the SAM-uncompetitive mechanism of inhibition. EPZ015666 had modest affinity for SAH-bound PRMT5:MEP50 (K_{d} = 171 nM) and high affinity for SAM- or sinefungin-bound PRMT5:MEP50 (K_{d} < 1 nM), indicating that the cation-π interaction of EPZ015666 and SAM contributed >3 kcal mol^{-1} of binding energy.

Supplementary Fig. 3 | Binding modes of EPZ015666. (a) Crystal structure of EPZ015666 (cyan) bound to PRMT5:MEP50 complex (green) in the presence of SAM (yellow; PDB accession 4X61). The 2F_{o}-F_{c} electron density for the ligand is shown at 1.0σ. Select interactions are shown by dashed lines. (b) Superposition of EPZ015666-SAM (green and cyan, respectively) and H4 peptide–SFG (gray; PDB accession 4G0B) complexes reveals that EPZ015666 binds in the peptide-binding site and Phe327 changes conformation significantly to accommodate the inhibitor. (c) Crystal structures of EPZ015666 bound to PRMT5:MEP50 complex in the presence of SAM (green), SFG (magenta; PDB accession 4X6D) and SAH (cyan; PDB accession 4X63) show no change to the binding mode of the ligand with different nucleotides. (d–g) Surface plasmon resonance studies of EPZ015666 binding to (d) apo-PRMT5:MEP50 and (e) SAH-, (f) SAM- and (g) sinefungin-bound PRMT5:MEP50. EPZ015666 binding occurred only with aminonucleoside-bound PRMT5:MEP50, confirming the SAM-uncompetitive mechanism of inhibition. EPZ015666 had modest affinity for SAH-bound PRMT5:MEP50 (K_{d} = 171 nM) and high affinity for SAM- or sinefungin-bound PRMT5:MEP50 (K_{d} < 1 nM), indicating that the cation-π interaction of EPZ015666 and SAM contributed >3 kcal mol^{-1} of binding energy.
and specific antibodies to this mark have been difficult to obtain, and technologies for assessing arginine methylation remain challenging.

The antiproliferative effects of the inhibitor manifested over several days, necessitating the development of a long-term proliferation assay allowing for the measurement of cell growth over 12 d. EPZ015666 demonstrated potent concentration-dependent antiproliferative effects, with IC\textsubscript{50} values of 96 nM and 450 nM for Z-138 and Maver-1 cells, respectively (Fig. 4a,b). Antiproliferative effects with EPZ015666 were also observed in additional MCL cell lines, with IC\textsubscript{50} values ranging from 61 to 904 nM (Supplementary Fig. 14 and Supplementary Table 3).

**Antitumor effects in MCL xenograft models**

EPZ015666 is orally bioavailable and amenable to in vivo studies. We performed 21-d efficacy studies in severe combined immunodeficiency (SCID) mice bearing subcutaneous Z-138 and Maver-1 xenografts, with twice-daily (BID) oral dosing on four dose groups: 25, 50, 100 and 200 mg per kilogram of body weight (mg kg\textsuperscript{-1}). After 21 d of continuous dosing, animals were euthanized, and blood and tissues were analyzed to determine the relationship between methylmark pharmacodynamics and tumor-growth inhibition (TGI).

EPZ015666 showed dose-dependent exposure and TGI after 21 d in both MCL models (Fig. 4c,d). Tumors in all EPZ015666 dose groups measured on day 21 showed statistically significant differences in weight, volume and tumor growth compared to vehicle-treated tumors. Dosing at 200 mg kg\textsuperscript{-1} BID induced tumor stasis in Z-138 cells, with >93% TGI after 21 d, whereas Maver-1 cells showed >70% TGI. Additionally, a third MCL xenograft was tested using the Granta-519 cell line, a fast-growing model that reached endpoint on day 18 and showed dose-dependent efficacy with 45% TGI in the 200 mg kg\textsuperscript{-1} group (Supplementary Fig. 15). EPZ015666 was well tolerated in all three models, with minimal bodyweight loss in the 200 mg kg\textsuperscript{-1} dose group and no other clinical observations (Supplementary Figs. 16).

To measure in vivo target inhibition, we developed a highly quantitative SDMA ELISA to allow for higher throughput and to complement the SDMA western blot. In the SDMA ELISA, Z-138 xenograft tumors collected on day 21 showed dose-dependent changes of >40% and >95% inhibition (>48% and >87% for Maver-1 tumors at day 21; >66% and >95% for Granta-519 tumors at day 18) of the methyl mark achieved at the lowest dose and highest dose, respectively (Fig. 4e,f and Supplementary Figs. 17–22).

**DISCUSSION**

Protein lysine and arginine methylation on both histone and nonhistone substrates is increasingly recognized to have a significant biological role. In particular, the misregulation of arginine methylation is gaining importance as a potential driver of human cancers. Although lysine methyltransferases are widely studied and have been shown to regulate gene expression patterns that are critical for cancer development, less is known about arginine methyltransferases and their roles in oncogenesis. High-quality molecular probes are necessary not only to investigate the biological effects of protein methyltransferase inhibition, but also to assist in defining strategies that will benefit patients in the clinic. DOT1L\textsuperscript{18} and EZH2 (refs. 11,39,40) are recent examples from the lysine methyltransferase enzyme family for which the identification of potent and selective tool compounds has been successful in demonstrating that these methyltransferases are drivers of multiple cancers. A cell-potent and orally bioavailable inhibitor of PRMT5, EPZ015666, has now been identified. To our knowledge, EPZ015666 is also the first described inhibitor of an arginine methyltransferase enzyme that has demonstrated both in vivo target engagement and efficacy in xenograft tumor models. The identification of this compound could represent a watershed in the chemical biological evaluation of biological mechanisms driven by PRMT5-mediated arginine methylation in cancer and other indications.
PRMT5 is the major symmetric arginine methyltransferase in mammals, with one of the largest collections of substrates among the family of PRMTs. The variety of reported PRMT5 substrates that exist in both the cytoplasm and the nucleus has provided an understanding of potential physiological roles of PRMT5 in both normal and neoplastic cells. PRMT5 has been shown to be upregulated in a number of different cancers, including lymphoma\(^1\)–\(^3\), breast\(^4\), lung\(^5\) and colorectal cancers\(^6\). Although many groups have reported various mechanisms for PRMT5 in driving oncogenesis, including cell death, cell-cycle effects and RNA processing, this is still an area of active investigation.

The multitude of reports implicating PRMT5 in cancer reflects the need for potent and selective inhibitors of PRMT5 so that its pathobiology can be further explored and validated. Previous studies used RNA interference technologies to investigate the role of PRMT5 in \textit{in vitro} and \textit{in vivo} models of cancer. This type of system has proven difficult to use because of the low-throughput method of investigating multiple tumor cell lines, as well as off-target effects and the often incomplete loss of protein in cells. We have shown in this study that even a small amount of remaining enzyme can lead to residual methylation on physiologically relevant substrates, resulting in partial or no detectable phenotypes and thus confounding interpretation.

PRMT5 is reported to modulate transcription through the methylation of H2A3, H3R8 and H4R3. The effects of PRMT5 inhibition were tested using both small molecules and shRNA knockdown of histone methylation by western blotting analysis of acid-extracted histones, with both commercial and custom-made histone antibodies. Early on in our studies there were no commercially available H3R8me2s antibodies, and those that were available for H4R3me2s were identified as nonspecific. As a result, we generated our own custom antibodies that were highly specific and sensitive to SDMA through rigorous testing against two of the most contaminating methyl marks, monomethyl arginine and asymmetric dimethyl arginine (Supplementary Fig. 23). Sensitive and specific H2A3me2s antibodies have been difficult to obtain, and technical challenges to the assessment of methylation at this site remain. The use of stable knockdown cell lines and cells treated with EPZ015666 did not result in any significant decreases in global H4R3me2s or H3R8me2s levels as detected by western blotting with highly specific antibody reagents (commercial or custom). This result was observed despite complete reduction of the methyl mark on multiple dimethylated arginine substrates (measured by SDMA antibody). Also contributing to the lack of detectable global histone arginine methylation in cells is the low abundance of arginine methylation on histones compared to lysine methylation. Therefore, detection of the loss of one or two methyl groups on arginine in a population that is already in low abundance might not be possible even with highly specific antibody reagents. Our results do not rule out the potential modulation of histone methylation at specific gene promoters that cannot be detected on a global level, as suggested by a few reports\(^1\)–\(^3\),\(^7\)–\(^14\). The existence of cell-potent PRMT5 inhibitors will allow for further investigation into the transcriptional regulatory function of PRMT5 through histone methylation.

In summary, the action of EPZ015666, to our knowledge the first cell-potent and orally bioavailable inhibitor of PRMT5, has been exemplified and characterized. X-ray crystallography revealed a unique binding mode within the substrate channel of PRMT5 that has not been observed previously in any protein methyltransferase enzyme. The potential for a cation–π interaction involving SAM is known\(^{16}\); however, the key cation–π interaction between EPZ015666 and cofactor SAM is, to our knowledge, the first example of an interaction of this type within a SAM-dependent enzyme active site. We believe that this interaction may contribute to the selectivity of EPZ015666 against all other protein methyltransferase enzymes tested so far. Biochemical inhibition correlated well with inhibition of symmetric dimethylation of arginine-containing substrates and proliferation in a time- and concentration-dependent manner. Further evidence for cellular target engagement of EPZ015666 with PRMT5 was shown by CETSA. Oral dosing of EPZ015666 demonstrated dose-dependent antitumor activity in multiple MCL xenograft models, with near 95% TGI observed after 21 d of dosing.
Corresponding decreased levels of symmetrically dimethylated PRMT5 substrates in the excised tumors strongly suggested that the antiproliferative effects were a direct consequence of PRMT5 inhibition. We believe EPZ015666 is a powerful probe compound that could be used to understand more about the biological roles of PRMT5 and potentially assist in defining a therapeutic strategy across multiple cancer indications.

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METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank (PDB): Structures have been deposited under accession codes 4X61 for SAM, 4X60 for SFG and 4X63 for SAH.

References

**ONLINE METHODS**

**PRMT5:MEP50 molecular biology.** Full-length human PRMT5 (NM_006109.3) transcript variant 1 clone was amplified from a fetal brain complementary DNA (cDNA) library, incorporating a flanking 5' sequence encoding a FLAG tag (MDYKDDDDK) fused directly to Ala2 of PRMT5. Full-length human MEP50 (NM_024102) clone was amplified from a human testis cDNA library incorporating a 5' sequence encoding a six-histidine tag (MHHHHHHH) fused directly to Arg2 of MEP50. The amplified genes were subcloned into pENTR/D/TEV (Life Technologies) and subsequently transferred by Gateway attL x attR recombination to the pDEST8 baculovirus expression vector (Life Technologies). Additional constructs of FLAG-PRMT5 and His-MEP50 were made using the pFastbac1 vector system, and FLAG-Avi-PRMT5 was made in the pDEST vector.

**PRMT5:MEP50 protein expression.** Recombinant baculovirus and baculovirus-infected insect cells (BIICs) were generated according to the Bac-to-Bac Kit instructions (Life Technologies) and ref. 43, respectively. Protein overexpression was accomplished by infecting exponentially growing *Spodoptera frugiperda* (Sf9) cell culture at 1.2 x 10⁶ cells/ml with a 5,000-fold dilution of BIIC stock or through infection of Sf9 cells at 1.2 x 10⁶ cells/ml with FLAG-PRMT5 or FLAG-Avi-PRMT5 and His-MEP50 P2 viruses at infection ratios of 1:200 for each virus. Infections were carried out at 27 °C for either 48 or 72 h, and cells were harvested by centrifugation and stored at −80 °C for purification.

**PRMT5:MEP50 purification (assay).** Expressed full-length human FLAG-PRMT5–6His-MEP50 protein complex was purified from cell paste by nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography after a 5-h equilibration of the resin with buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) at 4 °C, to minimize the adsorption of tubulin impurity to the resin. FLAG-PRMT5–6His-MEP50 was eluted with 300 mM imidazole in the same buffer. The purity of recovered protein was 67%.

**PRMT5:MEP50 purification (crystallography).** Cells were resuspended in a buffer containing 50 mM Tris-HCl, 500 mM NaCl, 100 mM Glu, 100 mM Arg, 1 mM TCEP, pH 8.0, with protease inhibitors (Roche), and cell lysis was performed by sonication. After centrifugation, the clarified supernatant was purified by Ni-NTA (GE Healthcare) chromatography using buffers containing 50 mM Tris-HCl, 250 mM NaCl, 1 mM TCEP, pH 8.0, 0–250 mM imidazole. Protein was then dialyzed into a buffer containing 20 mM Tris, 150 mM NaCl, 5% glycerol, pH 8.0, and loaded onto a column containing FLAG affinity resin (Sigma). Protein was eluted with buffer containing 200 μg/ml peptide, 20 mM Tris, 150 mM NaCl, 5% glycerol, pH 7.8, before final exchange into a buffer containing 50 mM Tris-HCl, 250 mM NaCl, 1 mM TCEP, pH 8.0. This protein was concentrated to 32–38 mg/ml and flash-frozen for storage at −80 °C.

**Biotin-PRMT5:MEP50: purification (SPR).** FLAG-Avi-PRMT5:MEP50 was purified in a similar manner as the protein for crystallography. After enrichment by nickel affinity, the complex was dialyzed into biotinylation buffer (50 mM Tris, 7.5 mM MgCl₂, pH 7.8). The Avi tag was enzymatically biotinylated by the addition of 2 mg/ml BirA, 5 mM biotin and 100 mM ATP and incubation for 4 h at 18 °C. The biotinylated PRMT5:MEP50 complex was further purified by FLAG chromatography and stored in 50 mM Tris-HCl, 250 mM NaCl, pH 8.0.

**Crystallography.** SAM, SAH or SFG, and EPZ01566 were each solubilized at 100 mM in DMSO, were added to PRMT5:MEP50 complex to a final concentration of 1.3 mM each, after which samples were incubated on ice for 1 h. Vapor-diffusion methods using hanging drop trays with a 0.5-ml reservoir were used for crystallization. Typically, 1 μl protein was added to 1 μl well solution containing 0.2 M Na acetate, 0.1 M Na citrate, pH 6.1, 10% wt/vol PEG400; microseeds of previously grown PRMT5:MEP50 complex were added to the drops to facilitate crystal growth. Crystals were incubated at 18 °C. Crystals appeared within 1 d and grew to full size within 1 week. Crystals were cryoprotected in a stepwise fashion to a final solution containing 80% mother liquor and 20% glycerol before being frozen in liquid nitrogen. All data sets were collected at synchrotron sources (Advanced Photon Source beamlines 22-ID (SAM, SAH) or 21-ID-G (SFG)) at 1.0 Å at −180 °C. Data reduction and scaling were performed using either XDS⁴⁴ and AIMLESS⁴⁵ or HKL2000 (ref. 46).

Structure determination was performed using previously solved structures of PRMT5:MEP50 and visual inspection of difference density maps. Ligand-dictionary methods were generated using ProsD⁴⁷ within the CCP4 software package⁴⁸, and ligand fitting was performed manually. Structure refinement was performed using iterative cycles of refinement and model building using REFMACS (ref. 49) and COOT⁴⁹, respectively. Analysis of the structures showed that more than 99% of all residues were in preferred or allowed regions of the Ramachandran plot. Data collection and refinement statistics are shown in Supplementary Table 2. Structures have been deposited in the Protein Data Bank with the following accession codes: SAM, 4X6L; SFG, 4X60; and SAH, 4X63.

**Surface plasmon resonance half-life determination studies.** The dissociation of the PRMT5:MEP50 complex on the same biotinylated PRMT5:MEP50 complex was performed using a fast-on/fast-off compound, EPZ007345 (Kₐ = 70 nM), from the same chemical series (the ‘tracer’). PRMT5:MEP50 was immobilized as described above, and we characterized the binding of the tracer molecule by acquiring a full dose-response curve in the presence of 20 μM SAM to validate that the protein was properly folded and active on the SPR chip surface, after which the surface was extensively washed to remove all tracer and SAM. Then, 20 μM SAM and 20 μM EPZ015666 were coinjected in apo running buffer until saturation of the PRMT5:MEP50 was achieved, creating a long-lived complex of PRMT5:MEP50-SAM-EPZ015666. Because the tracer and EPZ015666 compete for the same binding site in PRMT5, we measured the dissociation of EPZ015666 from PRMT5:MEP50 by assessing the ability of a saturating amount of the tracer (2 μM) and SAM (20 μM) to regain the ability to bind over a long period of time. The tracer and SAM were injected repeatedly during a 12-h period, and the percentage of blocked PRMT5:MEP50 was determined through comparison to the response-unit signal measured for the tracer or SAM at the start of the run and fit to a one-site exponential decay model in GraphPad Prism 6.0. In the second method we used the technique of single-cycle kinetics in the Biacore T200 (ref. 51).

For a simple 1:1 interaction under steady state conditions, the equilibrium dissociation constant Kₐ is calculated using the following formula:

\[
K_a = \frac{R_a \cdot [A]}{R_m \cdot [A]} = \frac{[A]}{K_D \cdot [A]}
\]

\(R_a\) is the response at time zero, and \(R_m\) is the maximum response.
Where:

\[ R_{\text{max}} = \text{response observed when all binding sites on the immobilized protein are occupied}. \]

\[ [A] = \text{concentration of injected analyte A}. \]

\[ R = \text{response units elicited by analyte A}. \]

The kinetic association rate constant \( k_1 \) and the dissociation rate constant \( k_2 \) are calculated by fitting to a 1:1 interaction model.

\[
\frac{dR}{dt} = k_1[A]R_{\text{max}} - (k_2[A] + k_2)R
\]

Where:

\[ dR = \text{change in response unit}, \]

\[ dt = \text{change in time}, \]

\[ [A] = \text{concentration of injected analyte A}. \]

\[ R_{\text{max}} = \text{response observed when all binding sites on the immobilized protein are occupied}, \]

\[ R = \text{response units elicited by analyte A}. \]

**Biochemical methods.** Biotinylated histone peptides were synthesized by 21st Century Biochemicals and HPLC-purified to >95% purity. 384-well FlashPlates were purchased from PerkinElmer, and 3H-labeled S-adenosylmethionine (3H-SAM) was obtained from American Radiolabeled Chemicals with a specific activity of 80 Ci/mmol. Unlabeled SAM and SAH were obtained from Sigma-Aldrich. FlashPlates were washed in Biotek ELx-406 with 0.1% Tween. 384-well FlashPlates were read on a TopCount microplate reader (PerkinElmer). Compound dilutions were performed on a Freedom EVO (Tecan) and spotted into assay plates using a Thermo Scientific Matrix PlateMate (Thermo Scientific). Reagent cocktails were added by Multidrop Combi (Thermo Scientific). Streptavidin-D2 and anti-rabbit immunoglobulin G (IgG)-cryptate were obtained from Cisbio, and histone H4 monomethyl R3 antibody was obtained from Abcam. HTS plates were read on a PerkinElmer EnVision.

**Determination of enzyme-inhibition IC\(_{50}\) values in radioactive 384-well FlashPlate format.** Ten-point curves of EPZ015666 were made using serial threefold dilutions in DMSO, beginning at 0.5 μM (the final top concentration of compound was 10 μM, and the fraction of DMSO was 2%). A 1-μL aliquot of the inhibitor dilution series was spotted in a 384-well microtiter plate. The 100% inhibition control consisted of a 100 μM final concentration of the product inhibitor SAH. Compound was incubated for 30 min with 40 μL per well of 4 nM PRMT5-MEP50 (final assay concentration in 50 μL) and 40 nM peptide representing human histone H4 residues 1–15 (final assay concentration in 50 μL) in 1× assay buffer (20 mM Bicine, pH 7.6, 0.002% Tween-20, 0.005% bovine skin gelatin, and 1 mM TCEP). A total of 10 μL per well of substrate mix comprising assay buffer with 75 nM 3H-SAM (final assay concentration in 50 μL) was added to initiate the reaction. Reactions were incubated for 30 min at room temperature and quenched with 1 μL per well of 3 nM Streptavidin-D2, 3 nM anti-rabbit IgG-cryptate, 1× anti-histone H4 monomethyl R3, and 200 mM potassium fluoride (all final concentrations in 4 μL of assay buffer). After an incubation time of 30 min, the plate was read on an EnVision reader.

**Methyltransferase cross-screening panel.** Cross-screening against the protein methyltransferase enzymes listed in Figure 1c was done according to general procedures as previously described.

**Tissue culture and cell lines.** Cell lines used in these experiments were obtained from various sources and were cultured according to the conditions specified by the respective cell banks. The following cell lines were purchased from ATCC: Z-138 (CRL-3001), Mauer-1 (CRL-3008), Mino (CRL-3000), Jeko-1 (CRL-3006), JVM-2 (CRL-3002), MP-2 (CRL-1420), Panc-1 (CRL-1469), MDAMB-453 (HTB-131), MDAMB-468 (HTB-132), MDA-MB-231 (HTB-22), and A549 (CCL-185). Granta-519 (ACC 342) cells were purchased from DSMZ.

**In vitro compound treatment.** Cultured cells in linear/log-phase growth were split to a seeding density of 2 × 10\(^4\) cells/mL in 2–20 mL of media, depending on the yield required at the end of the growth period. Compound was diluted in DMSO and added to each culture vessel with a final DMSO concentration of 0.2%. Cells were allowed to grow for 96 h undisturbed. At the conclusion of each treatment period, cells were harvested by centrifugation (5 min, 1,200 rpm), and cell pellets were rinsed once with PBS before being frozen on dry ice pending further processing.

**In vitro proliferation assay.** Long-term proliferation assays were performed on all MCL lines using the method previously described, with slight adjustments to initial seeding densities, depending on growth characteristics for each cell line. All assays were carried out for 12 d.

**Whole-cell lysate protocol.** Cells or powdered tumor tissue were lysed in 1× RIPA buffer (Millipore, 20-188) with 0.1% SDS and Protease Inhibitor Cocktail tablet (Roche, 04693124001) and sonicated on ice before being spun at 4 °C. Clarified supernatant was assayed for protein concentration by BCA (Pierce, 23225).

**Western blot analysis.** 4–12% Bis-Tris gels (Invitrogen, WG1402BOX) were run with 10–15 μg protein per lane and transferred to nitrocellulose via iBlot (Invitrogen). Blots were blocked (Lico, 927-40010) at room temperature and then incubated with primary antibody (SDMA, CST, 13222s, SmD3, Sigma, HPA001170-100μL; or β-actin, CST, 3700S, all prepared in a 1:3 dilution of blocking buffer and water with 0.1% Tween-20) at 4 °C overnight and secondary antibody (goat anti-rabbit IR700, Invitrogen, A21076, and donkey anti-mouse
IR800, Licor, 92632212; all prepared in a 1:3 dilution of blocking buffer and water) at room temperature for 45 min. Imaging was performed using a Licor Odyssey, and methylation changes in the SmD3 band were quantified by densitometry. Ratios between methylated and total protein were calculated for each sample, and compound-treated samples were normalized to controls (DMSO or vehicle). IC<sub>50</sub> values were calculated using GraphPad Prism.

**ELISA protocol.** Whole-cell lysates were diluted in 1× PBS carbonate-bicarbonate buffer (Sigma, C3041), and 62.5, 125, or 250 ng was added to each well of a 96-well titer plate (ThermoFisher, 3855) in duplicate, depending on the sample and antibody to be tested (SDMA, CST, 13222S; SmD3, Abgent, AP12451a). Plates were incubated at room temperature for a minimum of 2 h. After washing with PBS–TWEEN 20 (Biotek plate washer), wells were blocked with 5% BSA in PBS at room temperature for 2 h. A second round of washes with PBS/TBST was followed by incubation with primary antibody (SDMA or SmD in diluted 1× PBS) at 4 °C overnight. On the second day, plates were washed with PBS/TBST before secondary antibody was added (anti-rabbit IgG horseradish peroxidase conjugate, CST, 70745, diluted 1:500000) and were incubated in the dark for 60 min at room temperature. Once again, plates were washed with PBS/TBST, and TMB solution (SurModics, TMBS-1000-01) was added to each well and allowed to develop for up to 8 min in the dark at room temperature. Reactions were stopped with 1N H<sub>2</sub>SO<sub>4</sub>; before plates were analyzed using an EnVision reader (PerkinElmer) scanning at 450 nM. Ratios were calculated for methylation decreases in the SDMA plates compared to total protein in SmD3 plates. We calculated percentages of control values by comparing individual sample ratios from each group to the average ratio of the vehicle-treated group. Individual sample data were then plotted in GraphPad Prism.

**Histone extraction analysis.** Histones were extracted using a previously described method.<sup>25</sup>

**Histone western blot analysis.** 4–12% Bis-Tris gels (Invitrogen, WG1402BOX) were run with 1 μg histone per lane and transferred to nitrocellulose via iBlot (Invitrogen). Blots were blocked (Licor, 927-40010) at room temperature and incubated at 4 °C overnight with primary antibodies (Total H3, Abcam, ab10799; Total H4, Abcam, ab31830; H4R3me2s, CST, custom; H4R4me2s, 21st Century Biochemicals, custom) prepared in a 1:3 dilution of blocking buffer and water with 0.1% Tween-20 and then for 45 min at room temperature with secondary antibodies (goat anti-rabbit IR700, Invitrogen, A21076; donkey anti-mouse IR800, Licor, 92632212) prepared in a 1:3 dilution of blocking buffer and water. Imaging was performed using a Licor Odyssey, and methylation changes in the SmD3 band were quantified by densitometry. Ratios between methylated and total protein were calculated for each sample, and compound-treated samples were normalized to controls (DMSO or vehicle). IC<sub>50</sub> values were calculated using GraphPad Prism.

**Cellular thermal shift assay.** Cellular thermal shift assays were performed on A375 cells using the method previously described.<sup>26</sup> Cells were pretreated with 1 μM compound for 18 h before the assay on whole-cell lysates. Western blotting was performed as described above, probing with antibodies to either PRMT5 (Abcam, ab12151) or β-actin (CST, 3700) and loading 20 μL of reaction mix per lane. Bands were quantified by densitometry. The percentage of control was calculated on the basis of the unheated control samples for each condition. Melt curves were plotted in GraphPad Prism using a Boltzmann sigmoidal fit.

**In-cell western assay in Z-138 cells.** Materials. IMDM/Glutamax Medium, penicillin-streptomycin, and heat-inactivated fetal bovine serum were purchased from Life Technologies (Grand Island, NY, USA). RPMI 384-well plates were purchased from Greiner Bio-One (Monroe, NC, USA). Cell-culture 384-well white opaque plates were purchased from PerkinElmer (Waltham, MA, USA). SpectraMax M5 plate reader was purchased from Molecular Devices (Sunnyvale, CA, USA).

Z-138 suspension cells were maintained in growth medium (RPMI 1640 supplemented with 10% vol/vol heat-inactivated FBS) and cultured at 37 °C under 5% CO<sub>2</sub>. Under assay conditions, cells were incubated in assay medium (RPMI 1640 supplemented with 10% vol/vol heat-inactivated FBS and 100 units/mL penicillin-streptomycin) at 37 °C under 5% CO<sub>2</sub>.

**Methods.** For the assessment of the effect of compounds on the proliferation of the Z-138 cell line, exponentially growing cells were plated in 384-well white opaque plates at a density of 10,000 cells/well in a final volume of 50 μL of assay medium. To prepare the compound source plate, we performed triplicate nine-point threefold serial dilutions in DMSO, beginning at 10 mM (the final concentration of compound in the assay was 20 μM, and the fraction of DMSO was 0.2%). A 100-nL aliquot from the compound stock plate was added to its respective well in the cell plate. The 100%-inhibition control consisted of cells treated with a 200 nM final concentration of staurosporine, and the 0%-inhibition control consisted of DMSO-treated cells. After addition of the compounds, assay plates were incubated for 5 d at 37 °C, 5% CO<sub>2</sub>, relative humidity > 90%.

Each plate included 14 control wells of DMSO-only treatment (minimum inhibition), as well as 14 control wells for maximum inhibition treated with 3 μM of the positive control compound EPZ012737 (background wells). The positive control was a compound that gave maximum inhibition of the SYM11 signal in the in-cell western assay without antiproliferative effects.

The average of the ratio values for each control type was calculated and used to calculate the percent inhibition of each test well in the plate. The positive control was serially diluted threefold in DMSO for a total of nine test concentrations, beginning at 3 μM. The percent inhibition was determined and IC<sub>50</sub> curves were generated using triplicate wells per concentration of compound.

Percent inhibition =

\[
100 - \left( \frac{\text{Individual test sample ratio} - \langle \text{Background average ratio} \rangle}{\langle \text{Minimum inhibition ratio} \rangle - \langle \text{Background average ratio} \rangle} \cdot 100 \right)
\]

**High-throughput proliferation assay.** Materials. RPMI/Glutamax Medium, penicillin-streptomycin, and heat-inactivated fetal bovine serum were purchased from Life Technologies (Grand Island, NY, USA). V-bottom polypropylene 384-well plates were purchased from Greiner Bio-One (Monroe, NC, USA). Cell-culture 384-well white opaque plates were purchased from PerkinElmer (Waltham, MA, USA). Cytocell Glo was purchased from Promega (Madison, WI, USA). SpectraMax M5 plate reader was purchased from Molecular Devices (Sunnyvale, CA, USA).

Z-138 suspension cells were maintained in growth medium (RPMI 1640 supplemented with 10% vol/vol heat-inactivated FBS) and cultured at 37 °C under 5% CO<sub>2</sub>. Under assay conditions, cells were incubated in assay medium (RPMI 1640 supplemented with 10% vol/vol heat-inactivated FBS and 100 units/mL penicillin-streptomycin) at 37 °C under 5% CO<sub>2</sub>.

Methods. For the assessment of the effect of compounds on the proliferation of the Z-138 cell line, exponentially growing cells were plated in 384-well white opaque plates at a density of 10,000 cells/well in a final volume of 50 μL of assay medium. To prepare the compound source plate, we performed triplicate nine-point threefold serial dilutions in DMSO, beginning at 10 mM (the final concentration of compound in the assay was 20 μM, and the fraction of DMSO was 0.2%). A 100-nL aliquot from the compound stock plate was added to its respective well in the cell plate. The 100%-inhibition control consisted of cells treated with a 200 nM final concentration of staurosporine, and the 0%-inhibition control consisted of DMSO-treated cells. After addition of the compounds, assay plates were incubated for 5 d at 37 °C, 5% CO<sub>2</sub>, relative humidity > 90%.

\[
\text{Symmetric dimethyl arginine 800nm value} = \frac{\text{DRAQ5}700\text{nm value}}{\text{DRAQ5}800\text{nm value}}
\]
Cell viability was measured by quantitation of ATP present in the cell cultures, with the addition of 35 μL of Cell Titer Glo reagent to the cell plates. Luminescence was read in the SpectraMax M5 microplate reader. The concentration of compound inhibiting cell viability by 50% was determined using a four-parametric fit of the normalized dose-response curves.

**Generation of stable inducible knockdown cell line.** Z-138 cells were infected with shRNA (TRIPZ Inducible Lentivirus, Open Biosystems) targeting PRMT5 (sequence in supplemental) using 8 μg/mL polybrene in culture media. After 18 h, virus was removed and the media was replaced with fresh media. Cells were allowed to grow for 96 h before selection was begun with puromycin (1 μg/mL in media). Pools were allowed to propagate for 1–2 weeks before knockdown was induced with doxycycline (1 μg/mL in media) for 12 d and cells were harvested for western blotting.

**shRNA construct.** The sequence of the TRIPZ inducible lentiviral shRNA construct (Open Biosystems) used to knock down PRMT5 in these experiments was TCGAGAGGTATATTGCTGTGTGACAGTGAGGAAAGAGATCTCTATGATTGCAACTAGTGAAGCCACAGTGAGTGTCAATCATAGGATCCTTGCTACTGCTCGG.

**Pharmacokinetic study in mice.** Male CD-1 mice (25–40 g; Vital River) were fasted overnight and weighed before protein precipitation and LC-MS/MS analysis. We constructed standard curves, with the addition of 35 μg/mL labetalol as an internal standard and 1.0–3,000 ng/mL EPZ015666. We determined the concentration of compound (3.0–2,400 ng/mL EPZ015666). We constructed standard intervals (seven time points). For the last time point (24 h), samples were collected via cardiac puncture while the animals were under anesthesia (70% N2O:30% O2). Blood samples were transferred into K2-EDTA tubes and placed on wet ice before centrifugation at 4 °C (3,000 g, 15 min) to obtain plasma within 15 min after sample collection. Plasma samples were stored at −70 ± 10 °C before protein precipitation and LC-MS/MS analysis. We constructed standard calibration curves by analyzing a series of control plasma aliquots containing 100 ng/mL labelatol as an internal standard and 1.0–3,000 ng/mL EPZ015666. Four levels of quality control were also included in the analysis (3.0–2,400 ng/mL EPZ015666). We determined the concentration of compound in each unknown sample by solving the linear calibration-curve equation for each corresponding drug or internal-standard ratio. Data were analyzed using Phoenix WinNonlin 6.2.1.

**21-d efficacy xenograft studies.** All of the procedures related to animal handling, care and treatment in this study were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Chempartner following the guidance of the AAALAC. All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the IACUC at GlaxoSmithKline or according to the ethical review process at the institution where the work was performed. For in vivo efficacy studies, each mouse was inoculated subcutaneously at the right flank with Z-138, Maver-1 or Granta-519 tumor cells (5 × 105 cells/mouse, 50% Matrigel) in 0.2 mL of a mixture of base media and Matrigel (IMDM:Matrigel or RPMI:Matrigel, 1:1) for tumor development. The treatments were started when the mean tumor size reached 141.98 mm3 for the Z-138 efficacy study (12 d after inoculation), 120.02 mm3 for the Maver-1 efficacy study (13 d after inoculation), or 155.2 mm3 for the Granta-519 efficacy study (10 d after inoculation). Mice were assigned into groups using a randomized block design. EPZ015666 or vehicle (0.5% methylcellulose in water) was administered orally BID at a dose volume of 10 mL/kg for 21 d (Granta-519 was dosed for 18 d). Body weights were measured every day for the first week, then twice weekly for the remainder of the study. Tumor size was measured twice weekly in two dimensions using a caliper, and the volume was expressed in cubic millimeters. Animals were euthanized 3 h after the final dose on day 21, 22 or 18 for Z-138, Maver-1, or Granta-519, respectively, at which time blood and tissues were collected for analysis.

Supplementary Information

A Selective Inhibitor of PRMT5 with In Vivo and In Vitro Potency in MCL Models

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Supplementary Results

Supplementary Figures

Supplementary Figure 1. Correlation plots of high throughput assay data from lead series. (a) Correlation of biochemical IC$_{50}$ from PRMT5:MEP50 radioactive Flashplate assay against cell biochemical IC$_{50}$ with a high throughput (HT) In-Cell Western format (ICW) assay following symmetric methylation of SmD3 with SMY11 antibody in Z-138 cells over 4 days. (b) Correlation of HT ICW assay IC$_{50}$ against HT cell proliferation IC$_{50}$ using a 96 well format in Z-138 cells over a 5 day continuous treatment. All data points were determined using a minimum 8 point dose response curve with $n \geq 2$. 
Supplementary Figure 2. Preclinical pharmacokinetics in CD-1 mouse. (a) Table of EPZ015666 pharmacokinetic (PK) parameters as determined in mouse. All values
reported as mean ± standard deviation (SD), n=3. PK data analysis was performed using noncompartmental analysis and WinNonlin Phoenix 6.2.1 software. Calculated PK parameters show low clearance (CL) and high oral bioavailability (%F) for EPZ015666. Volume of distribution at steady state (VD_{ss}) was greater than total body water of 0.7 L/kg. Mean terminal half-life (t_{1/2}) and residence time (MRT) ranged from 0.9 to 1.6 hours. C_{max}, the maximum plasma concentration, was determined at time t_{max}. AUC_{0-t} and AUC_{0-inf} are the areas under the curve to the last measurable data point and extrapolated to infinity, respectively. (b) EPZ015666 data are shown graphically as concentration vs. time profile of mean ± SD (n=3) plasma concentrations following i.v. bolus (2 mg/kg) or oral gavage (po; 10 mg/kg) administration to CD-1 mouse (formulated in 20% N-N-dimethylacetamide in water).
Supplementary Figure 3. Assessment of SAM, SAH and Sinefungin binding using a PRMT5:MEP50 SPR assay. Biotinylated PRMT5:MEP50 complex was immobilized in a Biacore T200 and assay buffer, flow-rate and temperature were optimized to detect binding of small molecules to PRMT5. SAM, SAH and Sinefungin (SFG) binding was then measured.
Supplementary Figure 4. Complex of PRMT5:MEP50-SAM-EPZ015666 is stable upon withdrawal of SAM from SPR running buffer indicating that EPZ015666 must dissociate first or simultaneously with SAM. Injections of a saturating amount of SAM (20 µM) (blue) or co-injections of saturating SAM (20 µM) + saturating EPZ015666 (0.5 µM) (green) were performed against apo PRMT5:MEP50 for 60s then dissociation rates were monitored. SAM underwent rapid and complete dissociation from PRMT5:MEP50, while the co-injected sample of SAM and EPZ015666 demonstrated very slow dissociation from PRMT5:MEP50.
Supplementary Figure 5. Surface plasmon resonance analysis of half-life of EPZ015666 for PRMT5:MEP50 complex measured using a fast-on/fast-off tracer molecule by SPR and by single-cycle kinetics. The tracer EPZ007345 (2 µM) was injected with SAM (20 µM) to measure the dissociation of EPZ015666 and SAM. (a) The tracer method gave a $t_{1/2}$ of 94 min, while (b) the single-cycle kinetic method gave a $t_{1/2}$ of 130 min.
Supplementary figure 6. PRMT5 levels in cancer cell lines. Western blot analysis of whole cell lysates from MCL cell lines and cancer cell lines from other indications. PRMT5, SDMA and alpha-tubulin (loading control) levels were assessed as shown. Cell lines “453” and “468” refer to MDA-MB-453 and MDA-MB-468 respectively. Uncut western blots for PRMT5 and Tubulin can be seen in Supplementary Figure 7.
Supplementary Figure 7. Uncut western blots from Supplementary Figure 6.
Supplementary Figure 8. Uncut western blots from Figure 3a.
Supplementary Figure 9. Effects of EPZ015666 on cellular symmetric dimethyl arginine substrate SmD3 in MCL cell lines. Concentration-dependent inhibition of SmD3me2s after four days of treatment with a dose-titration from 0.0003 to 5 µM of EPZ015666 in MCL cell lines Granta-519, Maver-1, Mino, and Jeko-1. SmD3me2s panel is cropped and the intensity decreased from the uncropped SDMA panel above to more clearly distinguish changes in this specific band. Uncut western blots for SmD3 can be seen in Supplementary Figure 10.
Supplementary Figure 10. Uncut western blots from Supplementary Figure 9.
Supplementary Figure 11. Effects of EPZ015666 and control compound 3 on cellular control protein using CETSA. A375 cells treated for 18 hours with either (a) 1uM EPZ015666 or (b) the inactive compound 3 in cellular thermal shift analysis of whole cell lysates. No shift in melting temperatures was observed for β-Actin in the presence of either compound as any changes observed are within SEM. All data analyzed using a Boltzmann Sigmoidal fit. Each point represents the mean of three replicates for each temperature ± SEM.
Supplementary Figure 12. Lack of global histone methylation changes upon compound treatment and shRNA KD in Z-138 cells. Acid-extracted histones from 4-day compound treatment of Z-138 cells with EPZ015666 or the paired inactive compound 3 compared to 12 day shRNA KD of PRMT5. Cells were treated with a dose-titration of 0.0003 to 5µM EPZ015666 or compound 3. No observed global histone methylation changes with multiple antibodies to several histone marks. Uncut western blots for all marks shown can be seen in Supplementary Figure 13.
Supplementary Figure 13. Uncut western blots from Supplementary Figure 12.
Supplementary Figure 14. Effects of EPZ015666 on cell proliferation. Inhibition of proliferation of Jeko-1, Granta-519, and Mino cells by EPZ015666 in vitro (measured by Guava Viacount Reagent assay) over 12 days in culture. Cells were counted and replated at the original seeding densities on days 4 and 8. Each point represents the mean for three replicates at each concentration.
**Supplementary Figure 15. Anti-tumor activity of EPZ015666 in Granta-519 xenografts in SCID mice.** (a) Anti-tumor activity induced by twice daily (BID) administration of EPZ015666 for 18 days at the indicated doses. Compound administration was stopped on day 18, and tumors were harvested for PD analysis (data shown as mean values ± SEM, n=10 mice per group). (b) PRMT5 target inhibition in Granta-519 xenograft tumor tissue collected from mice euthanized on day 18. Each point represents the ratio of SDMA to total SmD3 normalized to the vehicle control, measured by ELISA. The horizontal lines represent group mean values ± SD for 2 replicates per sample (10 mice per group). **** $P < 0.0001$, versus vehicle, 1-way ANOVA with a Tukey Test.
Supplementary Figure 16. Bodyweight change (%) of mice in EPZ015666-Treated (a) Z-138 Xenograft Mice and (b) Maver-1 xenograft mice in 21-Day and (c) Granta-519 xenograft mice in 18-day Efficacy Studies. Mean body weight changes induced by twice daily (BID) administration of EPZ015666 for 21 or 18 days at the indicated doses. Compound administration was stopped on day 21 (or 18), and tumors were harvested for PD analysis (data shown as mean values ± SEM, n=10 mice per group). All doses were well tolerated, with no group experiencing greater than 8.3% body weight loss over the course of these studies.
Supplementary Figure 17. EPZ015666 decreases cellular methylation of SmD3 in tumors of Z-138 xenograft SCID mice collected on day 21. (a) Western blots performed on whole cell lysates of tumors from 6 representative animals for each group. SmD3me2s panel is cropped and the intensity decreased from the uncropped SDMA panel above to more clearly distinguish changes in this specific band. (b) Quantification performed by comparing SmD3me2s to total SmD3 signal for each individual tumor and normalizing to vehicle average. The horizontal lines represent group mean values ± SD for 6 mice per group. Uncut western blots for SmD3 can be seen in Supplementary Figure 18.
Supplementary Figure 18. Uncut western blots from Supplementary Figure 17a.
Supplementary Figure 19. EPZ015666 decreases cellular methylation of SmD3 in tumors of Maver-1 xenograft SCID mice collected on day 22. (a) Western blots
performed on whole cell lysates of tumors from 6 representative animals for each group. SmD3me2s panel is cropped and the intensity decreased from the uncropped SDMA panel above to more clearly distinguish changes in this specific band. (b) Quantification performed by comparing SmD3me2s to total SmD3 signal for each individual tumor and normalizing to vehicle average. The horizontal lines represent group mean values ± SD for 6 mice per group. Uncut western blots for SmD3 can be seen in Supplementary Figure 20.
Supplementary Figure 20. Uncut western blots from Supplementary Figure 19a.
Supplementary Figure 21. EPZ015666 decreases cellular methylation of SmD3 in tumors of Granta-519 xenograft SCID mice collected on day 18. (a) Western blots
performed on whole cell lysates of tumors from 6 representative animals for each group. SmD3me2s panel is cropped and the intensity decreased from the uncropped SDMA panel above to more clearly distinguish changes in this specific band. 

(b) Quantification performed by comparing SmD3me2s to total SmD3 signal for each individual tumor and normalizing to vehicle average. The horizontal lines represent group mean values ± SD for 6 mice per group. Uncut western blots for SmD3 can seen in Supplementary Figure 22.
Supplementary Figure 22. Uncut western blots from Supplementary Figure 21a.
Supplementary Figure 23. Specificity of custom H3R8me2s antibody against full-length synthetic histones with alternative modifications. Western blot of full-length single-modification synthetic histones (1µg per lane loaded) probed with H3R8me2s affinity-purified antibody (1:5000 dilution).
## Supplementary Table 1: Summary of high throughput screening data

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<th>Parameter</th>
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<td>Primary measurement</td>
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<td>Key reagents</td>
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<td>Assay protocol</td>
<td>Compound was incubated for 30 min with 2 µL per well of 8nM PRMT5/MEP50 (final assay concentration in 4 µL) and 50nM of peptide representing human histone H4 residues 1-15 (final assay concentration in 4 µL) in 1X assay buffer (20 mM Bicine [pH 7.6], 0.010% Tween-20, 0.005% Bovine Skin Gelatin, and 2 mM DTT, 25mM NaCl). A total of 1 µL per well of substrate mix comprised of assay buffer with 200nM SAM (final assay concentration in 4 µL) was added to initiate the reaction. Reactions were incubated for 90 min at room temperature and quenched with 1 µL per well of 3nM Streptavidin-D2, 3nM Anti-Rabbit IgG-Cryptate, 1x Anti-Histone H4 mono-methyl R3 antibody, and 200 mM potassium flouride (all final concentrations in 4ul of assay buffer). After an incubation time of 30 minutes, the plate was read on an Envision.</td>
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<tr>
<td></td>
<td>Reagent/ compound dispensing system</td>
<td>Multifo peristaltic pump</td>
</tr>
<tr>
<td></td>
<td>Detection instrument and software</td>
<td>PerkinElmer, Envision</td>
</tr>
<tr>
<td></td>
<td>Assay validation/QC</td>
<td>50% Inhibition between 30%-70%, Z' &gt; 0.5</td>
</tr>
<tr>
<td></td>
<td>Correction factors</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Normalization</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Additional comments</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Post-HTS analysis</strong></td>
<td>Hit criteria</td>
<td>30% Inhibition and above</td>
</tr>
<tr>
<td></td>
<td>Hit rate</td>
<td>0.7%</td>
</tr>
<tr>
<td></td>
<td>Additional assay(s)</td>
<td>After removal of compounds containing pan-assay interference structures (PAINS) and additional known frequent hitter substructures, a subset of 800 compounds were selected for follow-up activity confirmation in enzyme based flashplate assay and a redox assay to identify reactive compounds that inhibit the enzyme in an intractable manner. Compounds were repurchased, re-synthesized and verified by LC/MS, and potency was confirmed at every step.</td>
</tr>
<tr>
<td></td>
<td>Confirmation of hit purity and structure</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Additional comments</td>
<td>N/A</td>
</tr>
</tbody>
</table>
**Supplementary Table 2: Data collection and refinement statistics for EPZ015666 complexes in PRMT5-MEP50**

<table>
<thead>
<tr>
<th>Data collection</th>
<th>SAM</th>
<th>SFG</th>
<th>SAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>I222</td>
<td>I222</td>
<td>I222</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$a$, $b$, $c$ (Å)</td>
<td>102.3, 138.2, 178.0</td>
<td>103.7, 138.0, 178.0</td>
<td>102.4, 138.1, 178.3</td>
</tr>
<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
<td>90.0, 90.0, 90.0</td>
<td>90.0, 90.0, 90.0</td>
<td>90.0, 90.0, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50.00-2.85</td>
<td>44.75-2.35</td>
<td>50.00-3.05</td>
</tr>
<tr>
<td>$R_{\text{sym}}$ or $R_{\text{merge}}$</td>
<td>0.117 (0.576)</td>
<td>0.045 (0.646)</td>
<td>0.148 (0.653)</td>
</tr>
<tr>
<td>$I_{\text{obs}}/\sigma(I)$</td>
<td>13.8 (2.2)</td>
<td>21.0 (2.5)</td>
<td>11.3 (2.2)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.5 (78.0)</td>
<td>99.6 (99.4)</td>
<td>95.1 (75.8)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>5.9 (4.9)</td>
<td>5.0 (5.0)</td>
<td>5.8 (5.3)</td>
</tr>
</tbody>
</table>

| Refinement |     |     |     |
| Resolution (Å) | 50.00-2.85 | 44.75-2.35 | 50.00-3.05 |
| No. reflections | 21760 | 48418 | 21785 |
| $R_{\text{work}}$ / $R_{\text{free}}$ | 0.206/0.260 | 0.202/0.254 | 0.214/0.274 |
| No. atoms |     |     |     |
| Protein | 7358 | 7412 | 7402 |
| EPZ015666/Cofactor/Glycerol | 28/27/6 | 28/27/18 | 28/26/0 |
| Water | 61 | 140 | 1 |
| $B$-factors |     |     |     |
| Protein | 65.8 | 63.3 | 83.7 |
| EPZ015666/Cofactor/Glycerol | 49.3/50.8/82.8 | 46.2/42.6/76.3 | 87.0/66.8/- |
| Water | 45.0 | 51.0 | 36.2 |
| R.m.s. deviations |     |     |     |
| Bond lengths (Å) | 0.005 | 0.008 | 0.004 |
| Bond angles (°) | 0.972 | 1.242 | 0.927 |

1 crystal was used for each structure. *Values in parentheses are for highest-resolution shell.
Supplementary Table 3. Long term proliferation IC₅₀/IC₉₀ values and SmD3me2s (SDMA) western IC₅₀ values in MCL cell lines dosed with EPZ015666. A panel of 5 MCL cell lines were treated with EPZ015666 for 12 days in the long-term proliferation assay. IC₅₀s and IC₉₀s were calculated in GraphPad Prism (non-linear regression analysis, top and bottom of the curves were fixed to 100 and 0%, respectively) for each line using split-adjusted cell counts at day 12. IC₅₀s and IC₉₀s ranged from 4-347nM across all types of MCL cell lines tested (n=1 for all cell lines). SDMA western blot IC₅₀s for 4-day EPZ015666 treatment are shown for the MCL cell line panel. Western blot images for this data are available in Supplementary fig. 7.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>LTP IC₅₀ (nM)</th>
<th>LTP IC₉₀ (nM)</th>
<th>SDMA Western Blot IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-138</td>
<td>96</td>
<td>208</td>
<td>44</td>
</tr>
<tr>
<td>Granta-519</td>
<td>61</td>
<td>243</td>
<td>4</td>
</tr>
<tr>
<td>Maver-1</td>
<td>450</td>
<td>3060</td>
<td>42</td>
</tr>
<tr>
<td>Mino</td>
<td>103</td>
<td>2080</td>
<td>78</td>
</tr>
<tr>
<td>Jeko-1</td>
<td>904</td>
<td>3430</td>
<td>347</td>
</tr>
</tbody>
</table>
Supplementary Note

Many of the intermediates have previously been described in WO2014100719.

Unless otherwise noted, intermediates were characterized by LC-MS to confirm the mass matched the structure and carried on to the next step without further purification.

Compound 1, EPZ007345, 1-(3-((cyclopentylamino)methyl)phenoxy)-3-(3,4-dihydroisoquinolin-2(1H)-yl)propan-2-ol was purchased from ChemBridge, Catalog #39852262, 98% HPLC purity.

Compound 2, EPZ015666, (S)-N-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-2-hydroxypropyl)-6-(oxetan-3-ylamino)pyrimidine-4-carboxamide.

Step 1: (R)-2-(oxiran-2-ylmethyl)-1,2,3,4-tetrahydroisoquinoline

To a solution of 1,2,3,4-tetrahydroisoquinoline (400g, 6mol) in tetrahydrofuran (THF) (4000mL) was added potassium fluoride (KF) (880g, 9mol) and (S)-oxiran-2-ylmethyl 3-nitrobenzenesulfonylate (856g, 6.8mol) at 0°C. After the addition was complete, the resulting mixture was stirred at 20°C for 16 h then filtered. The filtrate was concentrated in vacuum to give the desired product (400g, crude) which was used for next step without the further purification. LCMS (m/z): 190.1 (M+1).

Step 2: (S)-1-amino-3-(3,4-dihydroisoquinolin-2(1H)-yl)propan-2-ol
A solution of (R)-2-(oxiran-2-ylmethyl)-1,2,3,4-tetrahydroisoquinoline (80g, 0.42mol) in NH₃/EtOH (10L) was sealed and stirred at 80°C for 3 h. After completion, the reaction mixture was concentrated under vacuum. The residue was purified by column chromatography separation to afford desired product (48g, Yield 55%) which was used for the next step without further purification. LCMS (m/z): 207.1 (M+1).

Step 3: (S)-6-chloro-N-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-2-hydroxypropyl)pyrimidine-4-carboxamide

![Chemical Structure](image1)

To a stirred and cooled (0 °C) solution of (S)-1-amino-3-(3,4-dihydroisoquinolin-2(1H)-yl) propan-2-ol (7.15 g, 34.7 mmol) and triethylamine (Et₃N) (14.0 g, 138.8 mmol) in dichloromethane (DCM) (100 mL) was added 6-chloropyrimidine-4-carbonyl chloride (5.5 g, 34.7 mmol). After addition, the resulting mixture was stirred at 25 °C for 16 h, at which time LCMS showed the completion of the reaction. The solvent was evaporated and the residue purified by flash chromatography to give the (S)-6-chloro-N-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-2-hydroxypropyl)pyrimidine-4-carboxamide (7.2 g, yield: 60 %). LCMS (m/z): 347.0 [M+H]+

Step 4: (S)-N-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-2-hydroxypropyl)-6-(oxetan-3-ylamino)pyrimidine-4-carboxamide

![Chemical Structure](image2)
To a solution of (S)-6-chloro-N-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-2-hydroxy propyl)pyrimidine-4-carboxamide (347 mg, 1 mmol) in iso-propylamine (i-PrOH) (5 mL) was added oxetan-3-amine (73.1 mg, 1 mmol) and diisopropyl ethylamine (DIPEA) (129 mg, 1 mmol). The resulting mixture was stirred at 110 ºC for 16 hours, at which time LCMS showed the completion of the reaction. After evaporation of the solvent, the residue was purified by preparative HPLC to give the target compound (S)-N-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-2-hydroxypropyl)-6-(oxetan-3-ylamino)pyrimidine-4-carboxamide (62.5 mg, yield: 16.3 %). 1H NMR (400MHz, MeOD-d4) δ 8.24 (s, 1H), 7.15 - 7.05 (m, 4H), 7.02 - 6.98 (m, 1H), 5.09 (s, 1H), 4.95 (t, J=6.8 Hz, 2H), 4.59 (t, J=6.3 Hz, 2H), 4.10 - 4.03 (m, 1H), 3.72 (s, 2H), 3.56 - 3.46 (m, 2H), 2.96 - 2.91 (m, 2H), 2.65 (d, J=6.3 Hz, 2H); 13C NMR (101 MHz, DMSO-d6) δ ppm 29.7, 45.7, 47.4, 52.8, 57.6, 63.7, 68.1, 79.4, 104.9, 126.8, 127.4, 127.6, 129.6, 135.2, 135.5, 155.8, 158.8, 164.1, 165.9 HRMS; HRMS (m/z): [M+H]+ Calcd. for C20H26N5O3. 384.2036; found 384.2027.

Compound 3, EPZ019896, (S)-N-(2-hydroxy-3-(piperidin-1-yl)propyl)-6-(oxetan-3-ylamino)pyrimidine-4-carboxamide

Step 1: (R)-1-(oxiran-2-ylmethyl)piperidine

A solution of (S)-oxiran-2-ylmethyl 3-nitrobenzenesulfonate (18.3g, 70.6mmol), piperidine (5g, 58.8mmol) and potassium fluoride (6.7g, 117.6mmol) was combined in THF (50mL) and stirred at 20ºC. After 24 hrs, the solid was filtered off and filtrate
concentrated to provide the crude desired product (7g, 104% yield) which was used in
the next step without further purification.

Step 2: (S)-1-amino-3-(piperidin-1-yl)propan-2-ol

A solution of (R)-1-(oxiran-2-ylmethyl)piperidine (7g, 49.6mmol) in NH₃/EtOH (200mL)
was heated to 80°C in a sealed tube for 4 hrs. After cooling to 25°C, the solvent was
 evaporated to yield the desired crude product (8g, 102% yield) which was used in the
next step without further purification.

Step 3: (S)-6-chloro-N-(2-hydroxy-3-(piperidin-1-yl)propyl)pyrimidine-4-carboxamide

To a solution of (S)-1-amino-3-(piperidin-1-yl)propan-2-ol (0.93g, 5.8mmol) and Et₃N
(11.8g, 11.7mmol) in DCM (100mL) at 0°C, 6-chloropyrimidine-4-carbonyl chloride
(1.03g, 5.8mmol) was slowly added. Once the addition was complete, the mixture was
warmed to 20°C and stirred for a further 4hrs. The mixture was washed with aqueous
NaHCO₃ (2x50mL) then dried over anhydrous sodium sulfate and the solvent removed
under reduced pressure. The crude product was purified by column chromatography to
give the desired product (800mg, 47% yield). LCMS (m/z): 299.2 [M+H]⁺

Step 4: (S)-N-(2-hydroxy-3-(piperidin-1-yl)propyl)-6-(oxetan-3-ylamino)pyrimidine-4-
carboxamide
To a solution of (S)-6-chloro-N-(2-hydroxy-3-(piperidin-1-yl)propyl)pyrimidine-4-carboxamide (300mg, 1.004mmol) in isopropanol (15mL) was added oxetan-3-amine (73.4mg, 1.004mmol) and DIPEA (254mg, 2.008mmol) and the reaction mixture was stirred and heated at 80°C for 18 hrs. The reaction mixture was concentrated under reduced pressure and purified by preparative HPLC to yield the desired compound (10mg, 0.03 mmol, 3% yield). 

$^1$H NMR (400MHz, MeOD-d$_4$) $\delta$ 8.45 (s, 1H), 7.14 (s, 1H), 5.11 (m, 1H), 4.96 (t, 2H, J=6.4 Hz), 4.61 (t, 2H, J=6.4Hz), 3.94 (m, 1H), 3.47 (dd, 1H, J=5.2, 5.2Hz), 3.41 (dd, 1H, J=6.8, 6.8Hz), 2.47 (m , 4H), 2.40 (d, 2H, J=6.4Hz), 1.59 (m, 4H), 1.47 (m, 2H); 

$^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ ppm:25.3, 26.8, 45.6, 47.4, 56.3, 64.7, 68.0, 79.4, 104.9, 155.8, 158.9, 164.2, 165.9; HRMS (m/z): [M+H]$^+$ calcd. for C16H26N5O3, 336.2036; found 336.2040.