

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 lymphomas^{1–3}, 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 lines^{1–3,7}. 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 proliferation⁸. 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-NG-monomethyl arginine and symmetrical ω-NG-dimethyl arginine on protein substrates⁹. Nine mammalian PRMTs have been identified so far and are classified into three types¹⁰. 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)¹¹. PRMT5 is reported to participate in several diverse cellular processes through the methylation of a variety of cytoplasmic and nuclear substrates⁸, including histones H4 residue Arg3 (H4R3) and H3 residue Arg8 (H3R8)^{1,3,7}. 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 enzymes^{12,13}. 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 PRMTs^{17–28}. 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 inhibitors^{22,24} with nanomolar biochemical IC₅₀ values or micromolar IC₅₀ inhibition of a specific methyl mark in cells^{26,28}. 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

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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 conditions²⁹ 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)³⁰ 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 FlashPlate³¹ and a redox assay³² 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 by enzymology, biophysical methods and X-ray crystallography. EPZ007345 (**1**) (Fig. 1a) was part of a 17-member series with IC_{50} values ranging from 0.4–7 μ M with early evidence of structure-activity relationships (SAR) emerging. Analogs with structural changes distal to the tetrahydroisoquinoline (THIQ) group were well tolerated, yielding significant improvements in potency and in absorption, distribution, metabolism and excretion (ADME) properties. The ADME optimization of this series toward EPZ015666 will be the subject of a future publication. Potency SAR were tracked using a combination of three assays: (1) the previously mentioned biochemical IC_{50} assay, (2) a cell biochemical IC_{50} in-cell western assay following inhibition of symmetric dimethylation of arginine-containing substrates in Z-138 cells over 4 d, and (3) a cell-proliferation IC_{50} assay in Z-138 cells over 5 d of continuous treatment. Good correlations were observed between the biochemical and cell biochemical IC_{50} values, with an average shift between the two assays of

$\sim 10\times$ (Supplementary Fig. 1a). A similar relationship was observed between cellular methylation and high-throughput-proliferation IC_{50} values (Supplementary Fig. 1b), suggesting that the observed antiproliferative effects in Z-138 cells were a direct result of PRMT5 inhibition.

After a number of iterative design cycles, EPZ015666 (**2**) was identified with a biochemical IC_{50} of 22 ± 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 ± 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 inhibitor constant at saturating [SAM] (αK_i) of 20 ± 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 binding³³. 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 μ M (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 ml $min^{-1} kg^{-1}$, a volume of distribution of 1.7 l kg^{-1} after intravenous dosing at 2 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).

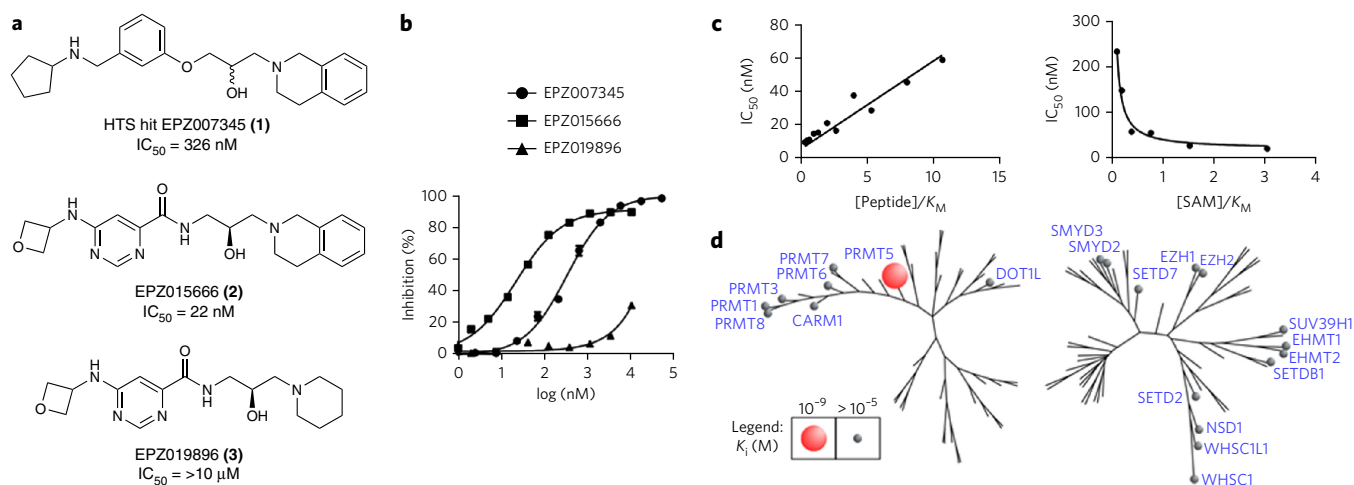


Figure 1 | Identification, characterization and optimization of diversity high-throughput screening (HTS) hit to a potent and selective inhibitor

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-uncompetitive, 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 αK_i 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.

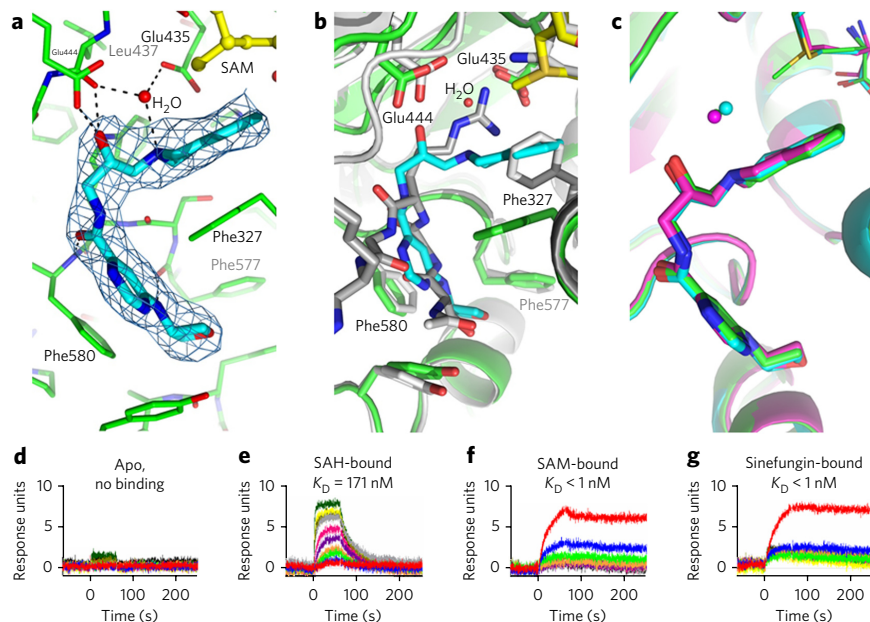


Figure 2 | Characterization of EPZ015666 binding mode using X-ray crystallography and surface plasmon resonance. (a) Crystal structure of EPZ015666 (cyan) bound to PRMT5:MEP50 complex (green) in the presence of SAM (yellow; PDB accession 4X61). The $2F_o - F_c$ electron density for the ligand is shown 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 4GQB) 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 4X60) 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.

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 in the pocket occupied by the substrate arginine side chain³⁴. 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 methylation³⁵ 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 partial 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 moment³⁶. 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 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.

To test this hypothesis further, we developed a surface plasmon resonance (SPR) assay for PRMT5:MEP50 (Supplementary Fig. 3) and used it to characterize the contribution of the cation- π interaction to binding affinity. Binding of EPZ015666 was examined against apo-PRMT5:MEP50 and SAH-, SAM- and SFG-bound PRMT5:MEP50 surfaces. Examination of the SPR sensorgrams in Figure 2d–g revealed a striking effect on EPZ015666 binding on the basis of the identity of the bound nucleoside analog. Apo-PRMT5:MEP50 showed no ability to bind EPZ015666. SAH-bound PRMT5 had a modest affinity for EPZ015666 ($K_D = 171$ nM) and dissociated rapidly, whereas SAM- and SFG-bound PRMT5:MEP50 bound EPZ015666 with very high affinity ($K_D < 1$ nM) and dissociated very slowly. Comparing affinities of EPZ015666 for SAH- versus SAM- or SFG-bound PRMT5:MEP50 indicated that the cation- π interaction contributed at least 3 kcal mol $^{-1}$ of binding energy in the reductionist SPR assay. Further SPR studies showed that the PRMT5:MEP50–SAM–EPZ015666 complex was stable upon withdrawal of SAM from the

running buffer; therefore EPZ015666 must dissociate first or simultaneously with SAM (Supplementary Fig. 4). Additionally, the dissociation rate of EPZ015666 from PRMT5:MEP50 was measured 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).

Characterization of cell methylation and proliferation

A panel of five MCL cell lines (Z-138, Maver-1, Mino, Granta-519 and Jeko-1) was used to assess changes in cellular methylation levels and proliferation effects upon treatment with PRMT5 inhibitors. Interestingly, PRMT5 and the pan-dimethyl symmetric arginine antibody symmetric dimethyl arginine (SDMA) were not shown to be consistently upregulated in MCL cell lines compared to other cancer cell lines (Supplementary Figs. 6 and 7). Although PRMT5 overexpression has been reported in lymphoma cell lines compared to naive B cells^{1,2}, most of the published literature shows PRMT5 upregulation in primary cancer samples^{4–6}. More work is required for an understanding of how PRMT5 activity is upregulated in cell lines that are dependent on this enzyme. The effects of EPZ015666 treatment on cellular symmetric arginine dimethylation in Z-138 cells (Fig. 3a) were tested by immunoblot using SDMA. EPZ015666 treatment led to a concentration-dependent decrease in the intensity of multiple bands, including

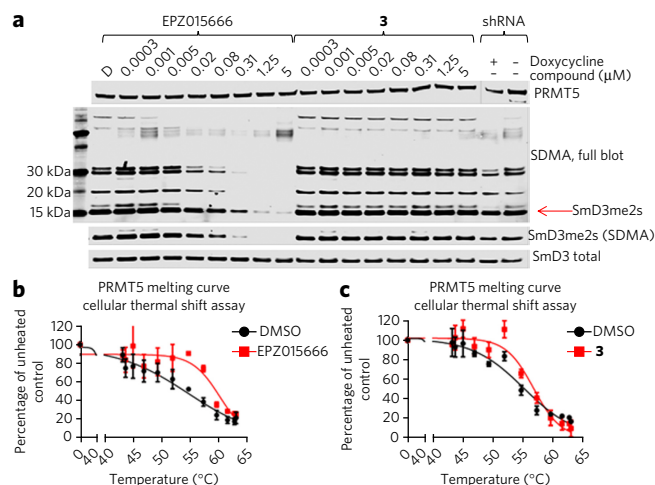


Figure 3 | Effects of EPZ015666 and control compound 3 on cellular target inhibition as determined by SMAD3 western blot and CETSA.

(a) Concentration-dependent inhibition of cellular SMAD3 substrates, including SmD3, after 4 d of treatment with EPZ015666 or 3 compared to 12-d shRNA knockdown of PRMT5 in Z-138 cells. Cells were treated with a dose titration of 0.0003–5 μM compound. The 12-d knockdown led to a 50% reduction in SmD3me2s signal when normalized to the SmD3 total. The SmD3me2s blot was cropped and the intensity decreased relative to that in the uncropped SDMA blot to more clearly distinguish changes in this specific band. (b) A375 cells treated for 18 h with 1 μM EPZ015666 showed stabilization of PRMT5 in cellular thermal shift analysis in whole-cell lysates. The melting temperature for PRMT5 was shifted by 5.9 °C. (c) No shift in melting temperatures was observed for PRMT5 in the presence of the inactive compound 3, as any changes observed were within the s.e.m. All data were analyzed using a Boltzmann sigmoidal fit. Each point plotted in b and c represents the mean of three replicates for each temperature; error bars denote $\pm$ s.e.m. Full SmD3 blots can be found in **Supplementary Figure 17**.

one that colocalized with the previously reported PRMT5 substrate SmD3. To further confirm cellular PRMT5-dependent substrates, we used short hairpin RNA (shRNA) to generate stable Z-138 PRMT5-knockdown cell lines and achieved >60% protein knockdown. The same substrate bands seen in EPZ015666-treated cells were diminished by PRMT5 knockdown (Fig. 3a), but not by treatment with 3, a structurally similar control (Fig. 3a and **Supplementary Fig. 8**).

Therefore, whole-cell lysates from the panel of MCL cell lines treated with a dose titration of EPZ015666 were harvested on day 4 and assessed by western blotting using the SDMA antibody. Concentration-dependent decreases in SmD3me2s were observed in all cell lines tested. Levels of SmD3me2s from the western blots were quantified by densitometry, and ratios of SmD3me2s to total SmD3 were calculated to determine the IC_{50} values (**Supplementary Figs. 9 and 10 and Supplementary Table 3**).

Further confirmation of target engagement *in vitro* was achieved with a recently described technique known as the cellular thermal shift assay (CETSA)³⁷. Assays with pretreated A375 cells (routinely used in-house because of their favorable culture characteristics) resulted in a 5.9 °C shift in the melting curve of PRMT5 in the presence of EPZ015666, but not of 3 (Fig. 3b,c and **Supplementary Fig. 11**), indicating specific binding of the active compound to PRMT5 in cells.

The effects of PRMT5 inhibition on cellular histone methylation were evaluated by western blot analysis of acid-extracted histones. In experiments using both commercial and custom-made antibodies recognizing H4R3me2s and H3R8me2s, Z-138 cells treated with EPZ015666 for 4 d did not show in any significant decreases in global histone methylation levels (**Supplementary Figs. 12 and 13**). We investigated H2AR3me2 methylation status; however, sensitive

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_{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_{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 ($mg\ kg^{-1}$). After 21 d of continuous dosing, animals were euthanized, and blood and tissues were analyzed to determine the relationship between methyl-mark 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 $mg\ kg^{-1}$ 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 $mg\ kg^{-1}$ group (**Supplementary Fig. 15**). EPZ015666 was well tolerated in all three models, with minimal bodyweight loss in the 200 $mg\ kg^{-1}$ dose group and no other clinical observations (**Supplementary Fig. 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 non-histone 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. DOT1L³⁸ 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.

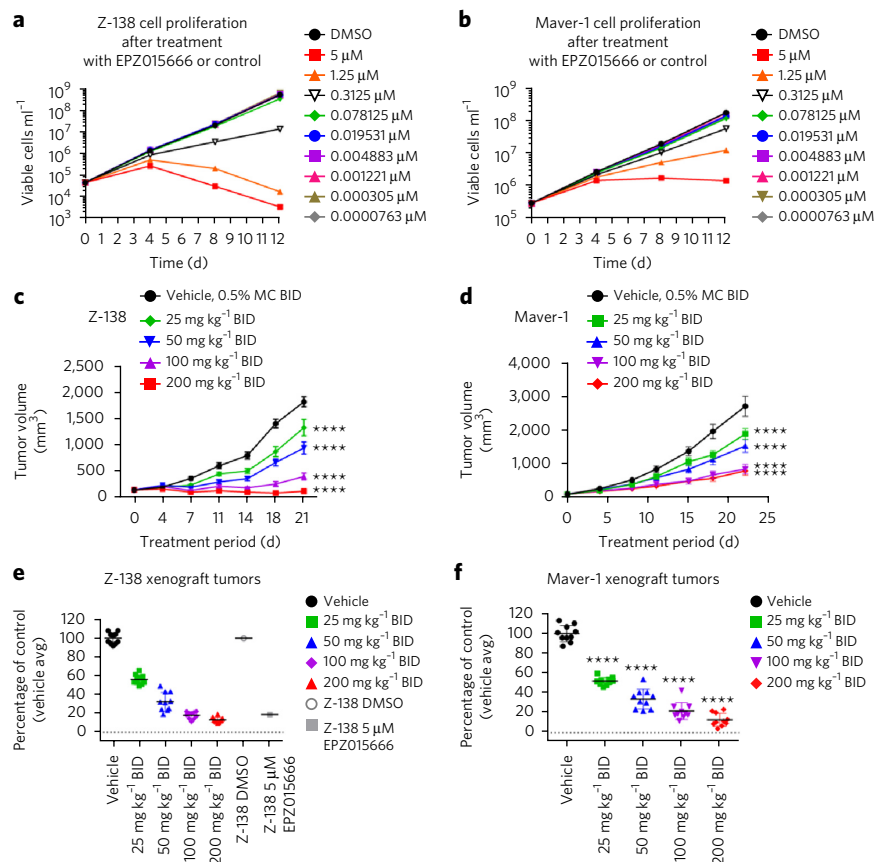


Figure 4 | EPZ015666 effects on *in vitro* cellular proliferation and *in vivo* antitumor activity in MCL cell lines and xenografts in SCID mice. (a,b) Inhibition of proliferation of (a) Z-138 and (b) Maver-1 cells by EPZ015666 *in vitro* (measured by cell viability as determined by Guava ViaCount Reagent Assay) over 12 d in culture. Cells were treated with a dose titration of 0.0007–5 μ M compound (see key). 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. (c,d) Antitumor activity induced by BID administration of EPZ015666 or vehicle (0.5% methyl-cellulose (MC) in water) for 21 d at the indicated doses for (c) Z-138 and (d) Maver-1 xenografts. Compound administration was stopped on day 21, and tumors were harvested for pharmacodynamic analysis (data shown as mean $\pm$ s.e.m. for ten animals per group). (e,f) PRMT5 target inhibition in (e) Z-138 and (f) Maver-1 xenograft tumor tissue collected from mice euthanized on day 21. Each point represents the ratio of SDMA to total SmD3 normalized to the vehicle control, as measured by ELISA. The horizontal lines represent group mean values $\pm$ s.d. for two replicates per sample (ten mice per group). Z-138 ELISA included samples from the Z-138 cell line treated *in vitro* with EPZ015666 for 4 d as a positive control. **** P < 0.0001 versus vehicle, one-way analysis of variance with a Tukey test.

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}, breast⁵, lung⁴ and colorectal cancers⁶. 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 *in vitro* and *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 H2AR3, 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 H2AR3me2s 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^{1,3,7,41}. 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 known⁴²; 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.

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Author contributions

K.W.D., M.J.M. and R.C. designed compounds. L.J. and P.A.B.-S. performed X-ray crystallography and produced X-ray protein. M.P., T.F.H., K.N., K.P.O. and K.T.G. made the initial protein for biochemistry. E.C.-P., K.G.K., T.L. and L.D.J. performed *in vitro* methyl mark and proliferation studies. N.R. and N.J.W. performed ADME pharmacokinetics studies. E.C.-P., K.G.K., R.M.P. and K.A.W. ran *in vivo* studies. C.R.M. and T.J.W. ran biochemical and SPR studies. K.W.D., E.C.-P., K.G.K., S.L.J., M.P.S., M.P.M., R.A.C., O.B., R.K., N.J.W., N.R., K.S., J.J.S., R.C., A.R. and S.A.R. designed studies and interpreted results. K.W.D., E.C.-P., K.G.K., T.J.W. and P.A.B.-S. wrote the paper.

Competing financial interests

The authors declare competing financial interests: details are available in the [online version of the paper](#).

Additional information

Supplementary information, chemical compound information and chemical probe information is available in the [online version of the paper](#). Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. Correspondence and requests for materials should be addressed to K.W.D.

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 (MHHHHHH) fused directly to Arg2 of MEP50. The amplified genes were subcloned into pENTR/D/TEV (Life Technologies) and subsequently transferred by Gateway *attL* × *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 1.2×10^6 cells/ml with a 5,000-fold dilution of BIIC stock or through infection of Sf9 cells at 1.2×10^6 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, 25 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 87%.

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, 1 mM TCEP, pH 8.0.

Crystallography. SAM, SAH or SFG, and EPZ015666, 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. Trays were incubated at 18 °C. Crystals appeared within 1 d and grew to full size after 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 1.000 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 dictionaries were generated using ProDr⁴⁷ within the CCP4 software package⁴⁸, and ligand fitting was performed manually. Structure refinement was performed using iterative cycles of refinement and model building using REFMAC5 (ref. 49) and COOT⁵⁰, 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, 4X61; SFG, 4X60; and SAH, 4X63.

Surface plasmon resonance studies. SPR immobilization was performed at 25 °C. Biotinylated PRMT5:MEP50 complex was diluted to 1.15 µM in 20 mM Bicine (pH 7.6) and injected into flow cell 1 (FC1) of a Series S Sensor Chip SA (GE Healthcare) in a Biacore T200 (GE Healthcare) that was equilibrated with 20 mM Bicine (pH 7.6) using a flow rate of 1 µL/min. Approximately 2,500 response units of the biotinylated PRMT5:MEP50 were captured on the streptavidin-coated biosensor chip. We created a reference surface in flow cell 2 (FC2) by injecting 20 mM Bicine (pH 7.6). FC1 and FC2 were blocked by an injection of 1 mM PEG-biotin (Sigma-Aldrich). The activity of the biotinylated PRMT5:MEP50 surface was validated by the injection of a dose-response curve of SAM at the start and end of each experiment.

SPR compound screening was performed at 25 °C in apo running buffer (20 mM Bicine, pH 7.6, 100 mM NaCl, 0.05% Tween-20, 1 mM TCEP, 2% DMSO), SAM-bound running buffer (20 mM Bicine, pH 7.6, 100 mM NaCl, 0.05% Tween-20, 1 mM TCEP, 2% DMSO, 50 µM SAM), SFG-bound running buffer (20 mM Bicine, pH 7.6, 100 mM NaCl, 0.05% Tween-20, 1 mM TCEP, 2% DMSO, 50 µM SFG) or SAH-bound running buffer (20 mM Bicine, pH 7.6, 100 mM NaCl, 0.05% Tween-20, 1 mM TCEP, 2% DMSO, 50 µM SAH) using the following parameters:

Flow rate = 80 µL/min

Association time = 60 s

Disassociation time ≥ 120 s

Surface plasmon resonance half-life determination studies. We determined the order of dissociation of the PRMT5:MEP50 complex on the same biotinylated PRMT5:MEP50 complex by either injecting a saturating amount of SAM (20 µM) or coinjecting saturating amounts of both SAM (20 µM) and EPZ015666 (0.5 µM) for 60 s against apo-PRMT5:MEP50 and then following the dissociation for 3 min.

The half-life of EPZ015666 was measured in two ways. The first method involved probing a pre-established complex of PRMT5:MEP50–SAM–EPZ015666 with a fast-on/fast-off compound, EPZ007345 ($K_d = 70$ 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 K_D is calculated using the following formula:

$$R = \frac{R_{\max}[A]}{K_D + [A]}$$



Where:

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

$[A]$ = concentration of injected analyte A, and

R = response units elicited by analyte A.

The kinetic association rate constant k_a and the dissociation rate constant k_d are calculated by fitting to a 1:1 interaction model.

$$\frac{dR}{dt} = k_a[A]R_{\max} - (k_a[A] + k_d)R$$

Where:

dR = change in response unit,

dt = change in time,

$[A]$ = concentration of injected analyte A,

$R_{\max}$ = response observed when all binding sites on the immobilized protein are occupied, and

R = 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 serial 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 mM (the final top concentration of compound was 10 μ M, and the fraction of DMSO was 2%). A 1- μ L aliquot of the inhibitor dilution series was spotted in a 384-well microtiter plate. The 100% inhibition control consisted of a 100 μ M final concentration of the product inhibitor SAH. Compound was incubated for 30 min with 40 μ L per well of 4 nM PRMT5:MEP50 (final assay concentration in 50 μ L) and 40 nM peptide representing human histone H4 residues 1–15 (final assay concentration in 50 μ L) in 1 $\times$ 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 μ L per well of substrate mix comprising assay buffer with 75 nM 3H-SAM (final assay concentration in 50 μ L) was added to initiate the reaction. Reactions were incubated for 120 min at room temperature and quenched with 10 μ L per well of unlabeled 600 μ M SAM in assay buffer (final assay concentration in 60 μ L). A 50- μ L volume of the reaction mix was transferred to a streptavidin-coated 384-well FlashPlate (PerkinElmer). After an incubation time of 1 h, the plate was washed with 0.1% Tween-20 and then read on a TopCount (PerkinElmer) to measure the amount of tritium incorporated into the peptide substrate, reported in counts per minute.

Mechanism-of-inhibition studies in radioactive 384-well FlashPlate format. Experimental conditions for the SAM competitions in a 50- μ L total volume in 384-well format were similar to those for the IC_{50} experiments, with the following exceptions.

EPZ015666 was serially diluted threefold from 1,000 to 0.051 nM and spotted into a 384-well polypropylene V-bottom microplate. 3H-SAM was serially diluted twofold in assay buffer for a seven-point dilution series with a top concentration of 700 nM (final assay concentration). Reactions were initiated by the addition of 4 nM enzyme and 40 nM peptide (final assay concentrations for both). Reactions were incubated for 60 min and quenched by the addition of 10 μ L per well of 600 μ M unlabeled SAM in assay buffer (final assay concentration).

For the peptide competition, EPZ015666 was serially diluted threefold from 1,000 to 0.051 nM and spotted into a 384-well polypropylene V-bottom microplate. Peptide was serially diluted twofold in assay buffer for a seven-point

dilution series with a top concentration of 480 nM (final assay concentration). Reactions were initiated by the addition of 4 nM enzyme and 75 nM 3H-SAM (final assay concentrations for both). Reactions were incubated for 60 min, and the reactions were quenched by the addition of 10 μ L per well of 600 μ M unlabeled SAM in assay buffer (final assay concentration).

K_i and αK_i were calculated using the following formulas:

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_M} \right)$$

$$IC_{50} = \alpha K_i \left(1 + \frac{K_M}{[S]} \right)$$

Where:

$[S]$ = substrate concentration,

K_M = Michaelis constant,

K_i = inhibitor constant for binding to enzyme, and

αK_i = inhibitor constant for binding to enzyme-substrate complex.

Determination of percent inhibition for HTS in 1,536-well-format HTRE. Compounds were incubated for 30 min with 2 μ L per well of 8 nM PRMT5:MEP50 (final assay concentration in 4 μ L) and 50 nM peptide representing human histone H4 residues 1–15 (final assay concentration in 4 μ L) in 1 $\times$ assay buffer (20 mM Bicine, pH 7.6, 0.010% Tween-20, 0.005% bovine skin gelatin, 2 mM DTT, and 25 mM NaCl). A total of 1 μ L per well of substrate mix comprising assay buffer with 200 nM SAM (final assay concentration in 4 μ L) was added to initiate the reaction. Reactions were incubated for 90 min at room temperature and quenched with 1 μ L per well of 3 nM Streptavidin-D2, 3 nM anti-rabbit IgG-cryptate, 1 $\times$ anti-histone H4 monomethyl R3, and 200 mM potassium fluoride (all final concentrations in 4 μ L 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 described⁴⁰.

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), Maver-1 (CRL-3008), Mino (CRL-3000), Jeko-1 (CRL-3006), JVM-2 (CRL-3002), MP-2 (CRL-1420), Panc-1 (CRL-1469), MDA-MB-453 (HTB-131), MDA-MB-468 (HTB-132), MCF-7 (HTP-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 2×10^5 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 described³⁸, 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 $\times$ 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 $^{\circ}$ 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 μ g protein per lane and transferred to nitrocellulose via iBlot (Invitrogen). Blots were blocked (Licor, 927-40010) at room temperature and then incubated with primary antibody (SDMA, CST, 13222s; SmD3, Sigma, HPA001170-100UL; or β -actin, CST, 3700S; all prepared in a 1:3 dilution of blocking buffer and water with 0.1% Tween-20) at 4 $^{\circ}$ 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). IC₅₀ 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 PBST was followed by incubation with primary antibody (SDMA or SmD3 diluted in 1× PBST) at 4 °C overnight. On the second day, plates were washed with PBST before secondary antibody was added (anti-rabbit IgG horseradish peroxidase conjugate, CST, 7074S, diluted in 1× PBST) and were incubated in the dark for 60 min at room temperature. Once again, plates were washed with PBST, 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 H₂SO₄ 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 method³⁸.

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; H3R8me2s, 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). IC₅₀ values were calculated using GraphPad Prism.

Cellular thermal shift assay. Cellular thermal shift assays were performed on A375 cells using the method previously described²⁹. 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, heat-inactivated horse serum, and D-PBS were purchased from Life Technologies (Grand Island, NY, USA). Odyssey blocking buffer, 800CW goat anti-rabbit IgG (H+L), and a Licor Odyssey Infrared Scanner were purchased from Licor Biosciences (Lincoln, NE, USA). Symmetric dimethyl arginine antibody (SYM11) was purchased from EMD Millipore (Billerica, MA, USA). 16% paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA, USA).

Z-138 suspension cells were maintained in growth medium (IMDM supplemented with 10% vol/vol heat-inactivated horse serum and 100 units/mL penicillin-streptomycin) and cultured at 37 °C under 5% CO₂.

Methods. For detection of symmetric dimethyl arginine and DNA content, Z-138 cells were seeded in assay medium at a concentration of 50,000 cells/mL

in a 384-well cell-culture plate with 50 µL per well. Compound (100 nL) from 384-well source plates was added directly to the 384-well cell plate. Plates were incubated at 37 °C, 5% CO₂ for 96 h. After 4 d of incubation, 40 µL of cells from incubated plates was added to Poly-D-Lysine-coated 384-well culture plates (BD Biosciences, 356697). Plates were incubated at room temperature for 30 min and then at 37 °C, 5% CO₂ for 5 h. After the incubation, 40 µL per well of 8% paraformaldehyde in PBS (16% paraformaldehyde was diluted to 8% in PBS) was added to each plate and incubated for 30 min. Plates were transferred to a Biotek 405 plate washer and washed five times with 100 µL per well of wash buffer (1× PBS with 0.1% Triton X-100 (vol/vol)). Next 30 µL per well of Odyssey blocking buffer was added to each plate and incubated for 1 h at room temperature. Blocking buffer was removed, 20 µL per well of primary antibody was added (SYM11 diluted 1:100 in Odyssey buffer with 0.1% Tween 20 (vol/vol)) and plates were incubated overnight (16 h) at 4 °C. Plates were washed five times with 100 µL per well of wash buffer. Next 20 µL per well of secondary antibody was added (1:200 800CW goat anti-rabbit IgG (H+L), 1:1,000 DRAQ5 (Biosstatus limited) in Odyssey buffer with 0.1% Tween 20 (vol/vol)) and incubated for 1 h at room temperature. The plates were washed five times with 100 µL wash buffer per well and then once with 100 µL water per well. Plates were allowed to dry at room temperature and then were imaged on the Licor Odyssey machine, which measures integrated intensity at 700-nm and 800-nm wavelengths. The 700-nm and 800-nm channels were both scanned.

Calculations. First, the ratio for each well was determined as follows:

$$\left(\frac{\text{Symmetric dimethyl arginine 800nm value}}{\text{DRAQ5 700-nm value}} \right)$$

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 EPZ011273 (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 determine the percent inhibition for 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 IC₅₀ curves were generated using triplicate wells per concentration of compound.

Percent inhibition =

$$100 - \left(\frac{((\text{Individual test sample ratio} - (\text{Background average ratio}))}{((\text{Minimum inhibition ratio}) - (\text{Background average ratio}))} \right) \times 100$$

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). Cell-Titer Glo was purchased from Promega (Madison, WI, USA). A 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% CO₂. 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% CO₂.

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/mL 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 top 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% CO₂, relative humidity > 90%.

Cell viability was measured by quantitation of ATP present in the cell cultures, with the addition of 35 μ L 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 μ g/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 μ g/mL in media). Pools were allowed to propagate for 1–2 weeks before knockdown was induced with doxycycline (1 μ g/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 TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAAGAGATCCTATGATTGACAACACTAGTGAAGCCACAGATGTAGTTGTCAATCATAGGATCTCTGTGCCTACTGCCTCGG.

Pharmacokinetic study in mice. Male CD-1 mice (25–40 g; Vital River Laboratory Animal Technology; $n = 6$, with 3 per time point) were treated with a single dose of EPZ015666 at 2 mg/kg by intravenous tail-vein injection and 10 mg/kg by oral gavage administration, with both doses formulated in 20% *N-N*-dimethylacetamide in water. Animals were fasted overnight and weighed before dose administration on the day of dosing. The study was performed in accordance with the guidelines and standards of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) and the National Institutes of Health. Approximately 30 μ L of blood were taken from animals by submandibular or retro-orbital bleeding at pre-specified time intervals (seven time points). For the last time point (24 h), samples were collected via cardiac puncture while the animals were under anesthesia (70% CO₂:30% O₂). Blood samples were transferred into K₂-EDTA tubes and placed on wet ice before centrifugation at 4 °C (3,000g, 15 min) to obtain plasma within 30 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 labetalol 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 the *in vivo* efficacy studies, each mouse was inoculated subcutaneously at the right flank with Z-138, Maver-1 or Granta-519 tumor cells (5×10^6 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.

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