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Rational Design, synthesis and biological evaluation of novel *triazole* derivatives as potent and selective PRMT5 inhibitors with antitumor activity

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Abstract

Protein arginine methyltransferase 5 (PRMT5) is responsible for the mono-methylation and symmetric dimethylation of arginine, and its expression level and methyl transferring activity have been demonstrated to have a close relationship with tumorigenesis, development and poor clinical outcomes of human cancers. Two PRMT5 small molecule inhibitors (**GSK3326595** and **JNJ-64619178**) have been put forward into clinical trials. Here, we describe the design, synthesis and biological evaluation of a series of novel, potent and selective PRMT5 inhibitors with antiproliferative activity against Z-138 mantle cell lymphoma cell line. Among them, compound **C_4** exhibited the highest potency with enzymatic and cellular level IC_{50} values of 0.72 and 2.6 μM, respectively, and displayed more than 270-fold selectivity toward PRMT5 over several other isoenzymes (PRMT1, PRMT4 and PRMT6). Besides, **C_4** demonstrated obvious cell apoptotic effect while reduced the cellular symmetric arginine dimethylation levels of SmD3 protein. The potency, small size, and synthetic accessibility of this compound class provide promising hit scaffold for medicinal chemists to further explore this series of PRMT5 inhibitors.

Keywords PRMT5 inhibitor · Anti-proliferative · Cellular target validation · Design and synthesis

Introduction

Protein arginine methylation catalyzed by protein arginine methyltransferases (PRMTs) is an important type of post-translational modification that affects crucial cellular processes including cell growth and proliferation, apoptosis, metastasis and angiogenesis via modulation of gene transcription and/or protein function [1–3]. The identified nine human PRMTs (PRMT1–9) are further subdivided into type I, type II, and type III enzymes according to the states of the methylated arginine. PRMT5 belongs to the predominant type II PRMTs which transfer methyl groups from S-adenosylmethionine (SAM) to a guanidine nitrogen of protein arginine residues, resulting in the reaction products ω-N^G-monomethylarginine (MMA), symmetric ω-N^G, N'^G-dimethylarginine (sDMA) and S-adenosylhomocysteine (SAH). Proteomic analysis of human tissues revealed that PRMT5, -1 and -4 exhibited more universal expression compared to PRMT2, -3, -6, -7, and -8 [4]. Through the symmetric dimethylation of diverse substrate proteins in the nucleus and cytoplasm, PRMT5 mediated various cellular processes, such as transcription [5], RNA metabolism [6], signal transduction [7], and cellular differentiation [8],

Kongkai Zhu and Jingwei Shao have contributed equally to this work.

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etc. The symmetric dimethylation of histones (H4, H3, and H2A), Sm proteins (B/B′, D1, and D3) are the hallmarks associated with PRMT5 activity [9, 10].

Interestingly, accumulating studies implicated that PRMT5 was an attractive therapeutic target in cancer. It is overexpressed or upregulated in several human cancers, including lymphomas [11–13], breast cancer [14], lung cancer [15], colorectal cancer [16], hepatocellular carcinoma [17], multiple myeloma [18], and glioblastoma [19]. It was reported that selective inhibition of PRMT5 could block initiation and maintenance of B cell transformation [20]. The importance of PRMT5 in breast cancer stem cell (BCSC) maintenance was also highlighted [21]. Besides, PRMT5 was documented to control melanoma growth through the SKI/SOX10 regulatory axis [22]. PRMT5 has been proposed as an effective target in mantle cell lymphoma (MCL) [23], glioblastoma [19], multiple myeloma [18], and mixed lineage leukemias (MLL)-rearranged acute myeloid leukemia [24].

Tremendous efforts from a number of groups have been made to discover PRMT5 inhibitors [20, 23, 25–33], and all reported PRMT5 inhibitors are summarized in Fig. 1. Among them, **LLY-283** [31], **Sinefungin** [34], and **A9145C** [10] are SAM binding site inhibitors, while **EPZ015666** [23] together with its analogue **GSK3326595** [35] are substrate binding site inhibitors. **EPZ015666** is the first PRMT5 inhibitor to be described with potent activities in both in vitro and in vivo models of Mantle cell lymphoma. Besides, the phase I of clinical trial of **GSK3326595** for the treatment of solid tumors and non-Hodgkin's lymphoma is going on. And compound **JNJ-64619178** [36] was reported to bind into the SAM binding pocket and meanwhile to reach the substrate binding pocket. Although more than 10 classes of PRMT5 inhibitors have been reported, most of them exhibit low potency or lack in vivo activity. Therefore, the search for more potent PRMT5 inhibitors remains a pressing task.

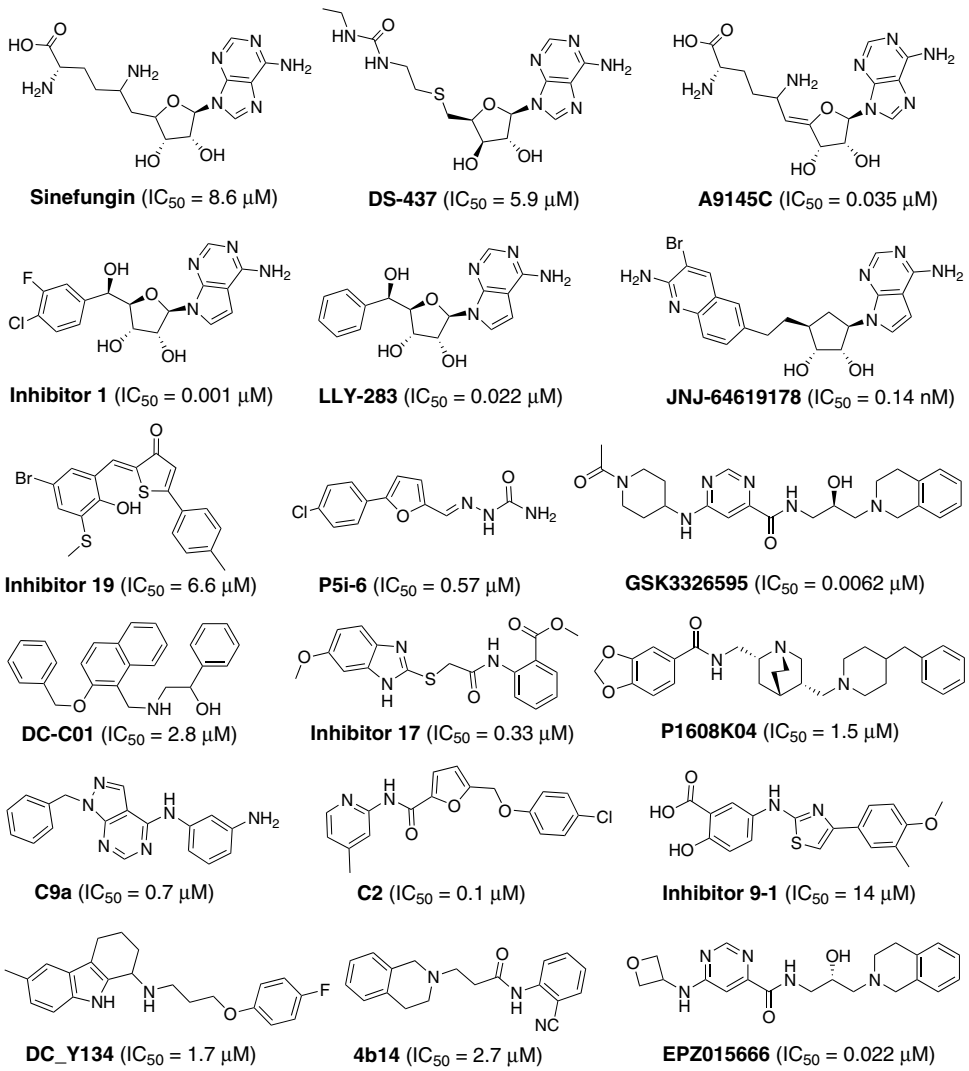


Fig. 1 Previously reported PRMT5 inhibitors

In the present study, we report the identification of a series of PRMT5 inhibitors with potent antiproliferative activity against Z-138 mantle cell lymphoma cell line. The most potent one (**C_4**) was employed to characterize its cell apoptotic effect and cellular target validation.

Materials and methods

PRMT5, PRMT1, PRMT4 and PRMT6 enzymatic activity inhibition assays

Using a radioactivity-based assay to monitor the transfer of the methyl group from ^{3}H-SAM to peptide substrate, we tested the inhibitory activity of our compounds against PRMT5:MEP50. The detailed methods to test the inhibition activity of compound **C_4** against PRMT5, PRMT1, PRMT4 and PRMT6 were as we have previously described [29].

Molecular docking

Molecular docking was performed using Glide program inserted in Maestro 7.5. The coordinates of PRMT5:**EPZ015666** (PDB code: 4X61) were used to construct docking model. First, the protein part was minimized by Protein Preparation Wizard Workflow. After that, docking grids were created by defining residues within 15 Å around **EPZ015666**. Then the prepared (using LigPrep panel) **GSK3326595** was docked to the pre-defined docking grids with standard precision mode.

In vitro anti-proliferation assay

Long-term proliferation assays were performed on Z-138 cells using the method we previously reported. In brief, exponentially growing cells were seeded in 24-well cell plates in growth medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) at a density of 1×10^5 cells per well. Cells were incubated in the presence of increasing concentrations of the tested compounds up to 200 μM. Viable cell number was determined every 4 days for up to 8 days using the Cell Titer-Glo Luminescent Cell Viability kit (Promega). On days of cell counting, growth media and compounds were replaced and cells split back to a density of 1×10^5 cells per well. IC_{50} values were determined from at least three independent assays and calculated from the concentration-dependence curves.

Western blot analysis

Exponentially growing Z-138 cells were incubated with compound **C_4** with different concentrations for 4 days. Then cells were harvested and lysed in 100 μL of total lysis buffer, and total cell lysates were separated on 4–12% SDS-PAGE and transferred to PVDF membrane (Millipore). Afterward, blocking buffer (5% nonfat milk in 0.1% Tween 20 PBS buffer) was used to block the Blots. Subsequently, primary antibodies [SDMA (CST, 13222s), GAPDH (CST, 5174) and SmD3 (Sigma, HPA001170-100UL), all prepared in a 1:3 dilution of blocking buffer] were added to Blots and incubated overnight at 4 °C. The next day, after being washed three times with PBST, the Blots were incubated with 1:10,000 dilution of donkey anti-rabbit secondary antibody (HRP conjugated) for 2 h. Finally, the signal was detected using ChemiScope3400 imaging system (Clinx Science Instruments).

Flow cytometric analysis

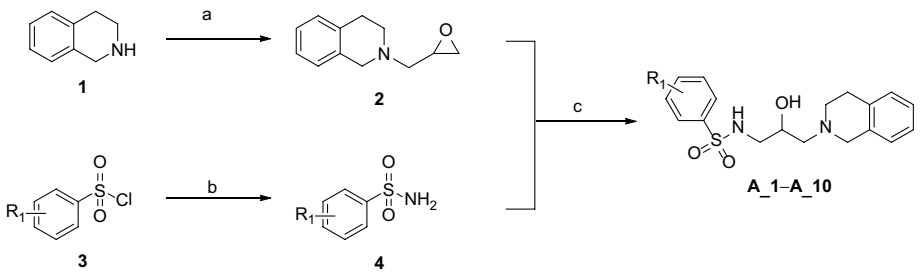
Exponentially growing Z-138 cells were plated in 6-well plates at a density of 5×10^5 and treated with compound **C_4**, **EPZ015666** (positive control) or DMSO (control). Cells were harvested at 48 h and were then measured using AnnexinV-FITC Apoptosis Detection Kit (Vazyme Biotech). FlowJo V7.6.1 was used to detect samples and to analyze data.

Chemistry

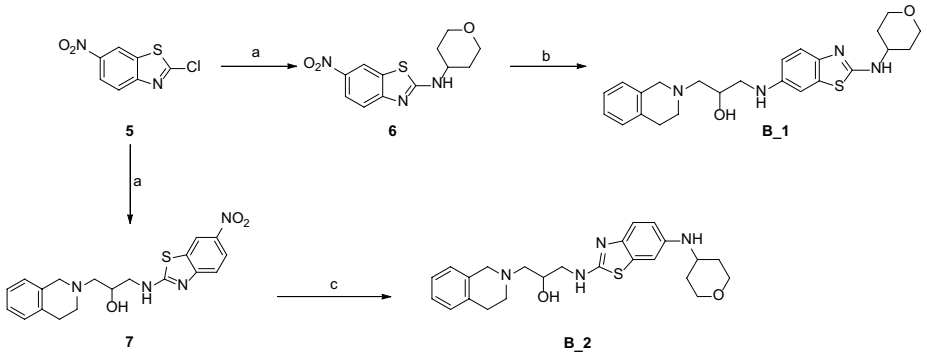
All purchased chemicals and solvents were used without further purification unless otherwise noted. Flash chromatography was performed using silica gel (300–400 mesh). All reactions were monitored by thin-layer chromatography (TLC), using silica gel plates with fluorescence F254 and visualized under UV light. ^{1}H NMR data were recorded on Varian Mercury 300 or 400 NMR spectrometers. Chemical shifts (δ) were reported in parts per million (ppm), coupling constant (J) values were in hertz, and the splitting patterns were abbreviated as follows: s for singlet; d for doublet; t for triplet; q for quartet and m for multiplet. High resolution ESI–MS spectra were recorded on a Finnigan/MAT95 spectrometer. All the final compounds are racemates. Compound **5** was synthesized according to the reported procedures [37].

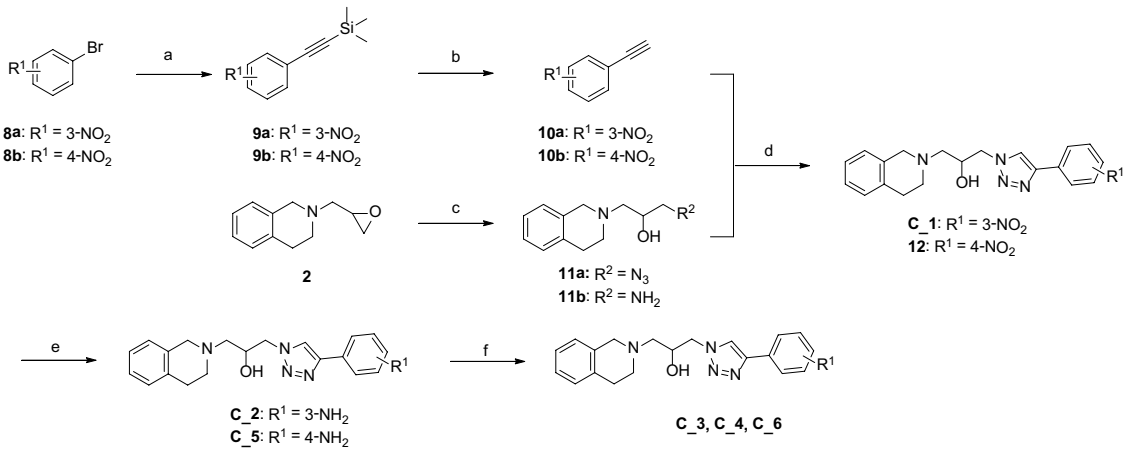
All the compounds were synthesized as illustrated in Schemes 1, 2, and 3. Compound **1** was reacted with 2-(chloromethyl)oxirane in the presence of base to afford the intermediate epoxide **2**. Benzenesulfonyl chloride derivatives were treated with ammonium hydroxide to afford benzenesulfonamides. Compounds **A_1**–**A_10** were prepared through ring-opening reaction of epoxide (compound **2**) under solid–liquid phase-transfer catalytic conditions.

Scheme 1 Synthesis of compounds **A_1**–**A_10**[a]. [a]Reagents and conditions: **a** 2-(chloromethyl)oxirane, K_2CO_3, MeOH, rt; **b** NH_2OH, MeCN, 0 °C; **c** K_2CO_3, TEBAC, 1,4-dioxane, 90 °C



Scheme 2 Synthesis of compounds **B_1** and **B_2**[a]. [a]Reagents and conditions: **a** tetrahydro-2*H*-pyran-4-amine or **11b**, K_2CO_3, DMF, 115 °C; **b** i Pd/C, H_2, MeOH; ii **2**, EtOH, 120 °C, sealed; **c** i Pd/C, H_2, MeOH; ii $NaBH_3CN$, dihydro-2*H*-pyran-4(3*H*)-one, MeOH





Scheme 3 Synthesis of compounds **C_1**–**C_6**[a]. [a]Reagents and conditions: **a** trimethylsilylacetylene, CuI, $Pd(PPh_3)_2Cl_2$, Et_3N, DMF, microwave irradiation, 120 °C; **b** K_2CO_3, MeOH, rt; **c** for **11a**: NaN_3, EtOH, reflux; for **11b**: NH_3 in MeOH, 80 °C in sealed tube; **d** $CuSO_4(H_2O)_5$, sodium ascorbate, THF/H_2O; **e** Pd/C, H_2, MeOH; **f** $NaBH_3CN$, ketone, MeOH

The synthesis of compounds **B_1** and **B_2** was shown in Scheme 2. Compound **5** was treated with tetrahydro-2*H*-pyran-4-amine or **11b**, affording **6** or **7**. Reduction of **6** yielded the amine intermediate which was converted to compound **B_1** by reacting with **2**. Compound **B_2** was obtained by a two-step procedure: reduction of **7** and reductive amination reaction.

The synthesis of compounds **C_1**–**C_6** was started from 3- or 4-bromonitrobenzene, which was first subjected to a Sonogashira cross-coupling with TMS-acetylene and then deprotected with K_2CO_3 to yield **10**. Compound **2** was treated with NaN_3 or NH_3 in MeOH to furnish **11**. Compounds **C_1** and **12** were prepared via click chemistry from intermediates **10** and **11a**. Reduction of **C_1** and **12** with hydrogen afforded **C_2** or **C_5** that were reacted with ketones to afford the target compounds.

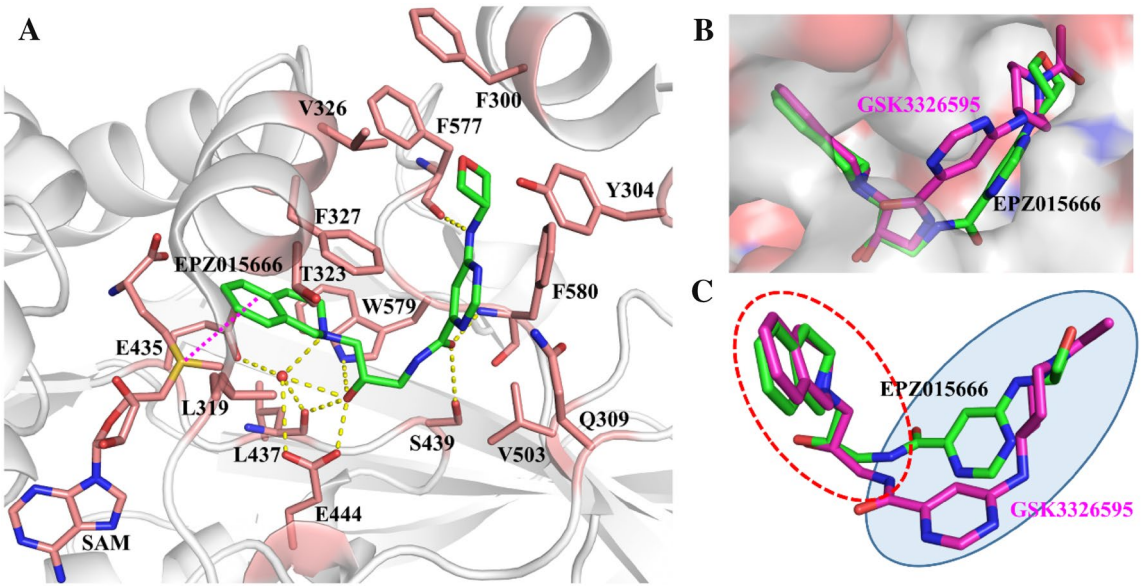


Fig. 2 Binding mode analysis. **a** The detailed interactions of **EPZ015666** with PRMT5. Residues involved in H-bond and hydrophobic interactions with **EPZ015666** and SAM which established cation–π interaction with **EPZ015666** were shown as sticks. Yellow and pink dashed lines indicate H-bond and cation–π interaction, respectively. **b** The comparison of binding modes of **GSK3326595** and **EPZ015666**. The protein and the ligands were shown as surface and sticks, respectively. **c** Only the ligands were shown for the purpose of clarity

Results

Rational design, synthesis, and structure–activity relationship analysis

As the best two previously reported substrate binding site inhibitors are **GSK3326595** and **EPZ015666**, we thus choose them as template compounds for further structure optimization. Fortunately, the complex crystal structure of PRMT5 with **EPZ015666** was previously determined, which provided many clues for structure-based molecular design. Therefore, the detailed interaction between **EPZ015666** and PRMT5 was analyzed and shown in Fig. 2a. As can be seen from Fig. 2a, the *N*-(3-(3,4-dihydroisoquinolin-2(1*H*)-yl)-2-hydroxypropyl) amino fragment of **EPZ015666** forms crucial interaction with many residues (L319, T323, F327, E435, L437, E444 and W579) contributing to its high inhibitory potency against PRMT5. It is worth to note that the cation-π interaction between tetrahydroisoquinoline ring and the cofactor SAM was first proposed and believed to contribute to the good selectivity of **EPZ015666**. Most importantly, the hydroxyl group in **EPZ015666** forms stable H-bond interactions with L437, E444 and W579, which indicates that it is the key pharmacophore. So we retained this hydroxyl group in the subsequent molecular design. Giving that the chirality of the hydroxyl-bonding carbon was different in **EPZ015666** and **GSK3326595,**

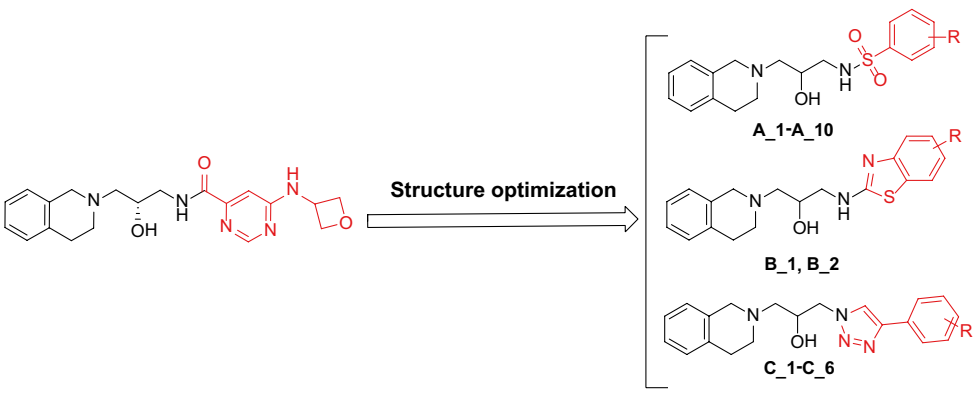


Fig. 3 Design of new PRMT5 inhibitors

Table 1 PRMT5 inhibitory activity of compounds

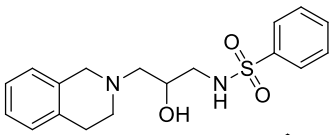
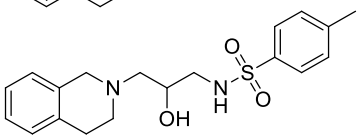
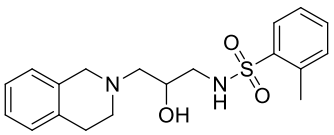
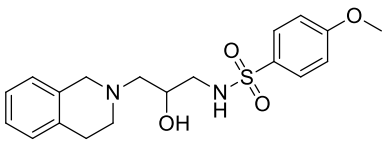
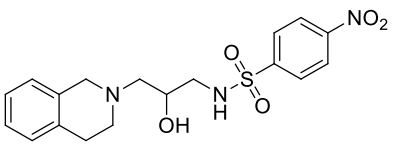
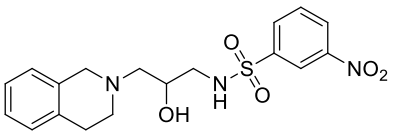
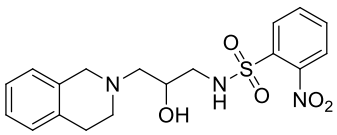
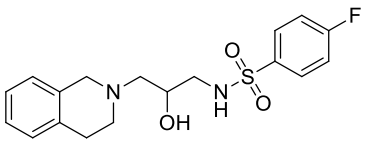
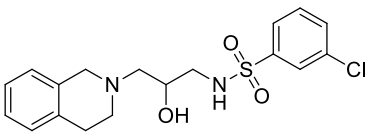
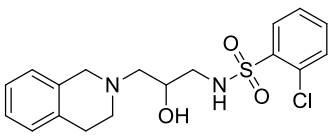
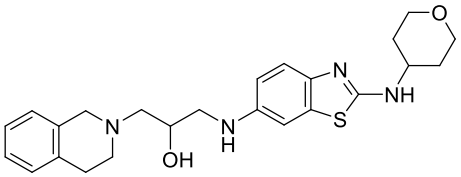
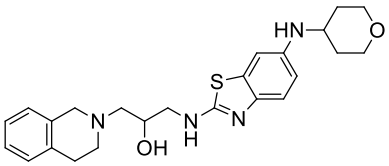
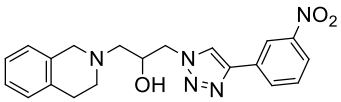
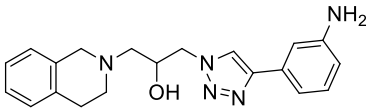
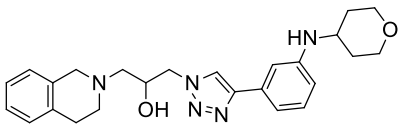
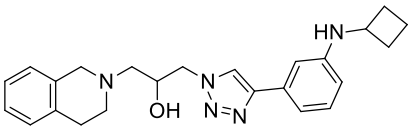
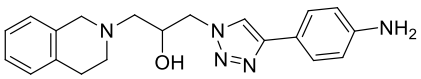
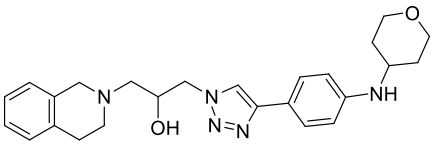
Compound	Structure	IC_{50} [a] ± SD [b] (μM)
A_1		3.40 ± 0.62
A_2		6.09 ± 0.73
A_3		4.27 ± 0.45
A_4		5.72 ± 0.56
A_5		14.80 ± 2.38
A_6		24.74 ± 2.95
A_7		59.88 ± 6.34
A_8		42.66 ± 5.63
A_9		21.69 ± 4.14
A_10		21.72 ± 3.67

Table 1 (continued)

Compound	Structure	IC_{50} [a] ± SD [b] (μM)
B_1		4.1 ± 0.35
B_2		6.6 ± 0.64
C_1		5.5 ± 0.64
C_2		34 ± 3.54
C_3		2.4 ± 0.85
C_4		0.72 ± 0.20
C_5		31 ± 3.54
C_6		5.1 ± 1.27
EPZ015666		0.047 ± 0.015

IC_{50} half maximal inhibitory concentration, *SD* standard deviation

and the co-crystal structure of PRMT5 complexed with **GSK3326595** was not determined, molecular docking was thus used to probe the binding mode of **GSK3326595**. As can be seen from Fig. 2b, c, the *N*-(3-(3,4-dihydroisoquinolin-yl)-2-hydroxypropyl) group in **GSK3326595** could be well overlapped with that in **EPZ015666**, which suggested that it was the privileged fragment and the chirality of the hydroxyl-bonding carbon had little effect on the PRMT5 inhibitory activity. As shown in Fig. 2a, apart from the *N*-(3-(3,4-dihydroisoquinolin-2(1*H*)-yl)-2-hydroxypropyl) amino fragment, the remaining fragment (left part) of **EPZ015666** established well-defined hydrophobic

Table 2 Inhibitory activity of **C_4** and **EPZ015666** against PRMT1, -4 and -6 (IC_{50} in μM) at enzymatic level

Compd No.	PRMT1	PRMT4	PRMT6
C_4	> 200	> 200	> 200
EPZ015666	> 200	> 200	> 200

Table 3 The in vitro effects of **B** and **C** series of compounds on cell proliferation

Compound	IC_{50} (μM)
B_1	6.25 ± 1.07
B_2	12.85 ± 2.35
C_1	17.35 ± 1.65
C_2	40.5 ± 3.26
C_3	3.23 ± 0.36
C_4	2.60 ± 0.81
C_5	57.57 ± 5.21
C_6	7.81 ± 2.24
EPZ015666	0.11 ± 0.02

interactions with PRMT5, which indicated that the relative pocket was a hydrophobic pocket. Thus, the design strategy was to keep the *N*-(3-(3,4-dihydroisoquinolinyl)-2-hydroxypropyl) amino moiety and to replace the left side part of **EPZ015666** with groups that could form hydrophobic interactions with PRMT5. A total of 18 compounds (Fig. 3) were synthesized according to our design strategy by utilizing the traditional structure optimization methods, such as scaffold hopping, bioisosteric replacement and aromatic ring replacement (see Supplementary Material for synthetic details).

The inhibitory activities against PRMT5 at enzymatic level (Table 1) of the 18 compounds and **EPZ015666** were evaluated using the method we previously reported [29, 38]. From Table 1, it could be seen that the activity of **EPZ015666** (47 nM) remained the same level as reported (22 nM) in the literature, demonstrating the reliability of our assay, whereas replacing the amide moiety with the sulfonamide group (**A_1**–**A_10**), benzo[d]thiazole ring (**B_1** and **B_2**) or triazole-derived fragment (**C_1**–**C_6**) caused a distinct loss of activity. Compounds **A_2**–**A_4** with an electron-donating substituent on the benzene ring showed much better inhibitory activity than compounds (**A_5**–**A_10**) with corresponding electron-withdrawing groups, which was different from the C series of compounds. Compound **C_1** with an electron-withdrawing nitro substituent on the benzene ring showed much better inhibitory activity than **C_2** with a corresponding electron-giving amino group, while the substituting position of the amino group did not seem to impact the result (**C_2** vs. **C_5**). Further structural modification of the free amino group with extra rings significantly improved the activity by 14- to 17-fold (**C_3**, **C_4** vs. **C_2**) or sixfold (**C_6** vs. **C_5**), and the best inhibition against PRMT5 was found for compound **C_4** with a cyclobutyl substitution on the amino moiety, showing a IC_{50} value of 0.72 ± 0.20 μM.

Selectivity testing indicated that C_4 is a selective PRMT5 inhibitor

As **C_4** displayed the most potency, it was selected to evaluate the selectivity over other PRMTs (PRMT1, PRMT4, and PRMT6). As shown in Table 2, the IC_{50} values of compound **C_4** against PRMT1, PRMT4, and PRMT6 were all above 200 μM, which indicated that it was a selective PRMT5 inhibitor.

Anti-proliferative activity on Z-138 cells

Z-138 cells were used to assess the anti-proliferative effects upon treatment with our synthesized compounds. A long-term proliferation assay was developed to measure the cell growth over 8 days. **B** and **C** series of compounds demonstrated potent concentration-dependent anti-proliferative effects, with IC_{50} values ranging from 2.60 to 57.57 μM (Table 3). As indicated by the assay results shown in Table 3, the cell level activities of these compounds were consistent with those of the enzyme level, and compound **C_4** also proved to be the most potent one with an IC_{50} value of 2.60 ± 0.81 μM. The dose response curve for **C_4** against Z-138 cell lines was shown in Fig. 4a.

Cellular symmetric dimethylation effects and inducement of cell apoptosis of C_4

The effect of **C_4** on cellular symmetric arginine dimethylation in Z-138 cells was tested by immunoblot using the pan-dimethyl symmetric arginine antibody symmetric dimethyl arginine (SDMA). As shown in Fig. 4b, concentration-dependent decrease in the intensity of SmD3me2s was observed. SmD3 is one of the PRMT5 substrate proteins and can be used to track the cellular biochemical activity of PRMT5, so from the western blot result, we can conclude that compound **C_4** could target PRMT5 in cellular context.

Since PRMT5 inhibitors usually induce cancer cell apoptosis [19, 33], the effect of **C_4** on cell apoptosis was studied in Z-138 cells for 48 h by flow cytometry. As shown in Fig. 5, concentration-dependent increase of apoptosis upon treatment with compound **C_4** was observed. This result further confirmed the cellular target engagement of **C_4** on PRMT5.

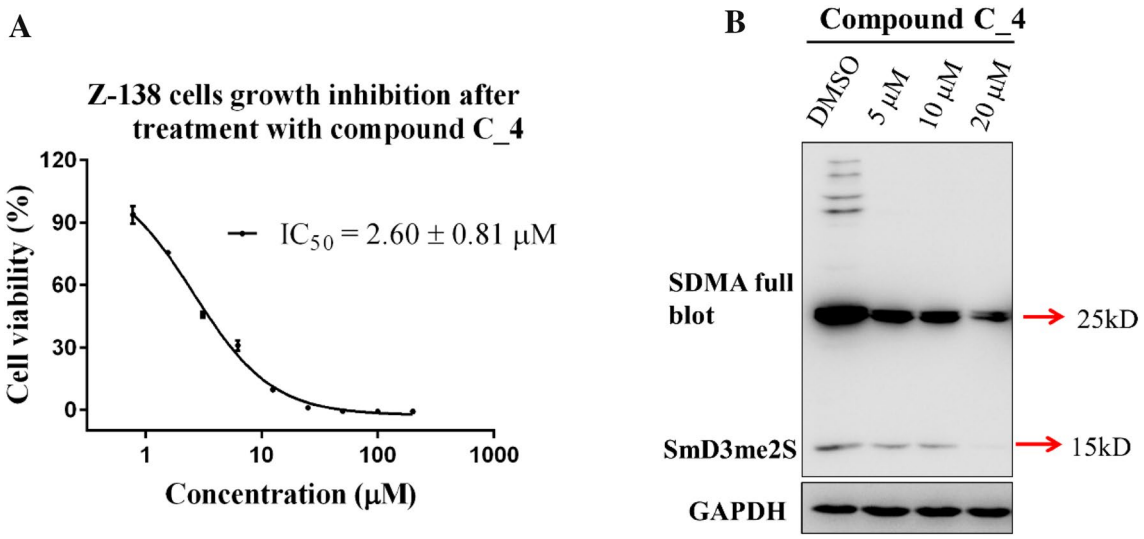


Fig. 4 Cellular anti-proliferative activity and target validation of compound **C_4**. **a** Dose response curve for compound **C_4** against Z-138 cell line. **b** Effect of **C_4** on cellular symmetric dimethyl arginine substrate SmD3 in Z-138 cells. Concentration-dependent inhibition of SmD3me2S after 4 days of treatment with a dose-titration from 5 to 20 μM of compound **C_4** in Z-138 cells

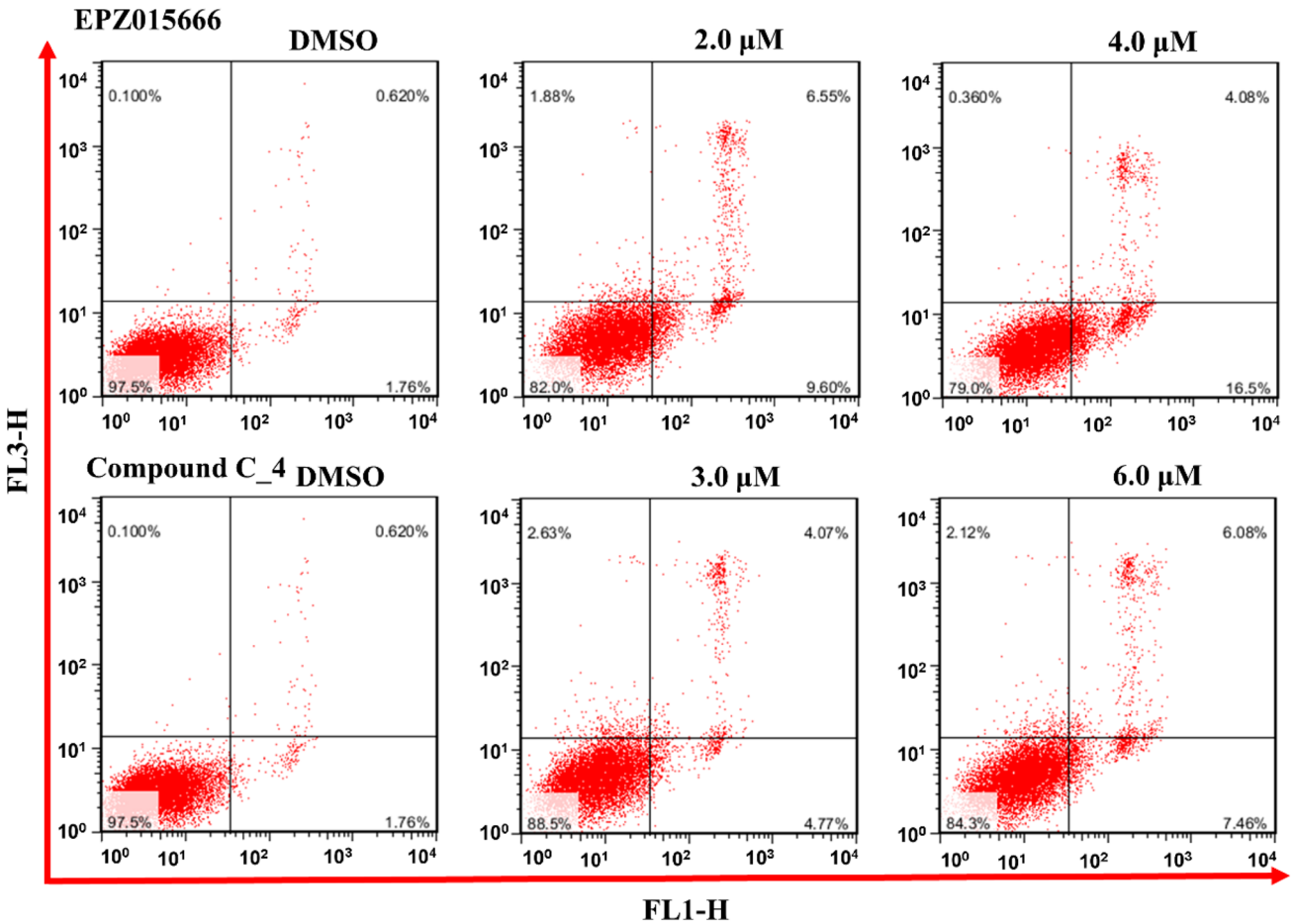


Fig. 5 Cell apoptosis induction after treatment with **C_4**. Treatment with **C_4** induced apoptosis of Z-138 cells measured after 48 h

Discussion

In the present study, new PRMT5 inhibitors were successfully discovered by combination of structure-based drug design, synthesis and biological evaluation. Among our synthesized 18 compounds, compound **C_4** showed the best PRMT5 inhibitory activity and profound cellular anti-proliferative activity with IC_{50} values of 0.72 ± 0.21 and 2.60 ± 0.81 μM, respectively. Besides, flow cytometric analysis demonstrated that **C_4** exhibited an obvious apoptotic effect. Compound **C_4** also reduced the cellular symmetric arginine dimethylation level of SmD3, a protein that can be used to track the biochemical activity of PRMT5 in cells. The compounds identified in this work provide a new scaffold for future PRMT5 inhibitor development.

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