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Discovery of a Potent and Selective Fragment-like Inhibitor of Methyllysine Reader Protein Spindlin 1 (SPIN1)

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ABSTRACT: By screening an epigenetic compound library, we identified that UNC0638, a highly potent inhibitor of the histone methyltransferases G9a and GLP, was a weak inhibitor of SPIN1 (Spindlin 1), a methyllysine reader protein. Our optimization of this weak hit resulted in the discovery of a potent, selective and cell-active SPIN1 inhibitor, compound **3** (MS31). Compound **3** potently inhibited binding of trimethyllysine-containing peptides to SPIN1, displayed high binding affinity, was highly selective for SPIN1 over other epigenetic readers and writers, directly engaged SPIN1 in cells, and was not toxic to non-tumorigenic cells. The crystal structure of the SPIN1–compound **3** complex indicated that it selectively binds Tudor domain II of SPIN1. We also designed a structurally similar but inactive compound **4** (MS31N) as a negative control. Our results have demonstrated for the first time that potent, selective and cell-active fragment-like inhibitors can be generated by targeting a single Tudor domain.

INTRODUCTION

Epigenetic regulation plays an important role in gene expression and transcription, which are critical for a variety of cellular processes. Epigenetic modifications can be divided into two main categories: DNA methylation and histone modifications.[1] Histone post-translational modifications (PTMs)[2] require three types of proteins: the enzymes that create the modifications (the "writers"),[3-7] the enzymes that remove the modifications (the "erasers"),[7, 8] and the proteins that recognize the modifications (the "readers").[9, 10] Growing evidence suggests that reader proteins are implicated in a number of human diseases including cancer.[11] Therefore, reader proteins are increasingly being pursued as potential therapeutic targets.[12, 13] However, unlike the significant progress made in the discovery of small-molecule inhibitors of histone methyltransferases, bromodomain containing proteins (which recognize acetylated lysine residues) and histone demethylases,[5-7, 14-16] only a very limited number of small-molecule inhibitors targeting methyllysine reader proteins have been reported, including UNC1215 (a potent and selective L3MBTL3 inhibitor), UNC3866 (a potent and selective peptide-based CBX7/4 inhibitor), EML631 (a selective and cell-active SPIN1 inhibitor with 685 dalton molecule weight) and A366 (a non-selective SPIN1 inhibitor) (Figure 1).[17-24] Furthermore, it is quite challenging to achieve sufficient potency and selectivity by targeting a single methyllysine reader domain such as a Tudor domain or MBT (Malignant Brain Tumor) domain as several reported potent and selective inhibitors of methyllysine reader proteins such as UNC1215 and EML631 achieved their potency and selectivity by simultaneously targeting two methyllysine reader domains.[21, 24]

SPIN1 (Spindlin 1) is a chromatin reader protein, which recognizes trimethylated histone H3 lysine 4 (H3K4me3) through the protein-protein interaction at the defined "aromatic cage" in Tudor domain II, and the interaction between asymmetrically dimethylated histone H3 arginine 8

(H3R8me2a) and Tudor domain I further increases the affinity.[25-27] SPIN1 was found to be overexpressed in several types of malignant tumors, including ovarian cancer, certain types of liver carcinomas, non-small-cell lung cancers and liposarcoma.[28-33] Upregulation of SPIN1 has been shown to increase cellular proliferation, abnormal mitosis and chromosomal instability.[34] In addition, SPIN1 is involved in several signaling pathways, such as Wnt/TCF-4 and RET signaling pathways.[29, 35] Therefore, small molecules that selectively disrupt the protein–protein interactions between SPIN1 and its respective binding partners (such as H3K4me3) are valuable chemical tools for investigating biological functions of SPIN1 and assessing the potential of SPIN1 as a therapeutic target. Two small-molecule inhibitors of SPIN1 have been reported to date. We previously reported that A366 (Figure 1) was a potent, but not selective inhibitor of SPIN1 (Supporting Figure S1).[23] A class of bivalent compounds represented by EML631 (Figure 1) that occupy Tudor domains I and II of SPIN1 were shown to be selective and cell-active inhibitors of SPIN1 with a relatively weak binding affinity.[24] The relatively high molecule weight of EML631 (685 dalton) may also render difficulties for further optimization.

Fragment-like inhibitors may possess high intrinsic binding energy and often exhibit high ligand efficiency (LE).[36, 37] Their low molecule weights leave large room for installing additional functional groups in lead optimization, which could result in drug candidates with higher potency and improved physicochemical properties, such as aqueous solubility, membrane permeability, and oral bioavailability. Therefore, fragment-like inhibitors are valuable starting points for drug development efforts, in addition to being useful tools for chemical biology studies.[38] Here, we report our discovery of a fragment-like inhibitor, compound **3 (**MS31) with a molecule weight less than 350, which potently and selectively disrupted the protein–protein interactions between SPIN1 and H3K4me3.

RESULTS AND DISCUSSION

Discovery of a Fragment-like Hit (Compound 2). Using AlphaLISA and fluorescence polarization (FP)-based biochemical assays, we screened the epigenetic compound library generated by our lab, which includes hundreds of small-molecule modulators of epigenetic writers, readers and erasers, and identified UNC0638 (compound **1**, Figure 2), a highly potent inhibitor of the histone methyltransferases G9a and GLP,[39, 40] as a weak SPIN1 inhibitor (IC_{50} = 3.2 ± 0.3 μM (AlphaLISA) and 7.4 ± 0.4 μM (FP)) (Supporting Figure S1). To improve potency and selectivity of UNC0638 for SPIN1 over G9a and GLP, we analyzed the co-crystal structure of compound **1** in complex with G9a (PDB: 3RJW)[41] and the co-crystal structure of SPIN1 in complex with an H3 peptide (PDB: 4H75),[26] and generated a docking model of compound **1** in complex with SPIN1 (Supporting Figure S2). The docking model suggests that the 3-(pyrrolidin-1-yl)propoxyl group of compound **1** likely mimics H3K4me3, interacting with the aromatic cage of the SPIN1 Tudor domain II, while the 4-amino piperidine group on the quinazoline ring extends out of the SPIN1 Tudor domain II and the 2-cyclohexyl group on the quinazoline ring does not appear to make any interactions. Based on these observations, we simplified the structure of compound **1** by replacing the 2,4-disubstituted quinazoline with a disubstituted phenyl ring while keeping the 3-(pyrrolidin-1-yl)propoxyl and methoxy groups. As a result, we discovered a much simpler compound with a molecular weight less than 300, **2** (Figure 2), as a SPIN1 inhibitor. Importantly, compared with compound **1**, compound **2** showed approximately 10-fold improvement in potency for SPIN1 (IC_{50} = 338 ± 30 nM (AlphaLISA) and 741 ± 46 nM (FP)) (Figures 3A and 3B). We determined that compound **2** bound SPIN1 with a K_D value of 390 ± 54 nM using isothermal titration calorimetry (ITC) (Figure 3C). To our delight, **2** did not bind either G9a or GLP using the same ITC experiments (Figure 3D and Supporting Figure S3).

Discovery of Compound 3. To improve potency of compound **2**, we replaced the pyrrolidinyl group with an isoindolinyl group, trying to achieve some π-π stacking interactions in the aromatic cage of the SPIN1 Tudor domain II. As what we expected, compared with **2**, the resulting compound **3** (Figure 2), showed > 4-fold higher potency for SPIN1 (IC_{50} = 77 ± 3 nM (AlphaLISA) and 243 ± 10 nM (FP)) (Figure 4A), while maintaining a low molecular weight of 341. The potency of compound **3** is comparable to that of A366 (IC_{50} = 72 ± 2 nM (AlphaLISA) and 207 ± 10 nM (FP)) (Supporting Figure S1), a G9a/GLP inhibitor[42] whose SPIN1 activity was previously reported by us.[23] We next assessed binding affinity of compound **3** to SPIN1 using ITC and found that it bound SPIN1 with a K_D value of 91 ± 4 nM (Figure 4B left panel), which is consistent with its potency in biochemical assays. The molar ratio of 1 was observed, suggesting that compound **3** likely bound only one out of three Tudor domains of SPIN1. Importantly, similar to compound **2**, compound **3** did not bind either G9a or GLP in ITC experiments (Figure 4C). In addition, compound **3** showed moderate binding affinities (ranging from 170 nM to 1.7 μM) to other closely related SPIN subfamily members (SPIN2B, SPIN3 and SPIN4) in ITC experiments (Supporting Figure S4), suggesting similar domain architectures and binding modes amongst the SPIN subfamily members.[43-45] To further assess its selectivity, we tested compound **3** against 33 methyltransferases (including G9a and GLP) and 5 acetyltransferases and found that it did not significantly inhibit these methyltransferases and acetyltransferases at up to 50 μM (Figure 5A and Supporting Table S1). In addition, we assessed selectivity of compound **3** against 20 methyllysine, methylarginine and acetyllysine reader proteins using a thermal shift assay and found that compound **3** did not induce a significant thermal shift for any of the 20 reader proteins at 20 or 200 μM (Figure 5B and Supporting Table S2). Thus, compound **3** is selective over a broad range of epigenetic proteins.

Determination of Cocrystal Structure of Compound 3 in Complex with SPIN1. We next solved the X-ray crystal structure of SPIN1 in complex with compound **3** at 1.6 Å resolution (PDB code: 6QPL, Figure 6, and Supporting Table S3). As illustrated in Figure 6A, compound **3** only occupied the SPIN1 Tudor domain II, which recognizes H3K4me3. As expected, the isoindolinyl group occupied the aromatic cage (Figure 6B and 6C). The protonated amino group in the isoindolinyl group not only formed a hydrogen bond (H-bond) with Y179, but also interacted with Y170, W151 and F141 in the aromatic cage through cation-π interactions (Figure 6C). The phenyl ring of the isoindoline group interacted with W151 through π-π stacking, which may explain the increased potency of compound **3** over compound **2** (Figure 6C). Besides these important interactions formed by the isoindolinyl group, two aminomethylene side chains formed a few critical H-bonds. The amino group of the 3-aminomethylene side chain formed a H-bond with D95 on the loop of the Tudor domain I of SPIN1 (Figure 6C). The amino group of the 5-aminomethylene side chain formed a direct H-bond with D184 on the α-helix of Tudor domain II, and two water-mediated H-bonds with M140 and D189. Disrupting these H-bond interactions by switching the 5-aminomethylene group of compound **3** to an amido group resulted in a structurally similar but inactive compound, **4** (Figure 2), which was completely inactive in the SPIN1 AlphaLISA and FP assays (Figure 4A). In addition, compound **4** did not show detectable binding affinity to SPIN1 in ITC experiments (Figure 4B right panel). Thus, compound **4** could be used as a negative control in chemical biology studies.

Evaluation of compound 3 in Cellular Assays. Using a NanoBRET target engagement assay,[46] we next demonstrated that compound **3** can engage SPIN1 in cells. As illustrated in Figures 7A and 7B, compound **3**, but not compound **4**, reduced the interaction between SPIN1 and histone H3 in U2OS cells in a concentration dependent manner with an IC_{50} value of 3.2 ± 0.7 μM.

Thus, compound **3** is cell permeable and can effectively engage SPIN1 in cells. Finally, we assessed off-target toxicity of compound **3** in two transformed cell lines, C2C12 and 293T, and one normal human primary fibroblasts cell line, HFF-1. We found that compound **3** was not toxic to these cells at up to 30 μM (Figure 7C). Thus, compound **3** is a useful tool compound for cellular studies.

Chemical Synthesis. Compounds **2**, **3** and **4** were synthesized using the synthetic routes shown in Schemes 1, 2 and 3.

The preparation of compound **2** was started from 3,5-dibromo-2-methoxyphenol (**5**).[47] Protection of the phenol group using benzyl bromide under basic conditions provided benzyl ether intermediate **6**. Next, the dibromo groups on the phenyl ring were converted to dicyano groups under Rosenmund-von Braun reaction conditions, resulting in intermediate **7**. Benzyl ether deprotection under palladium catalyzed hydrogenation conditions provided phenol **8**, which was subsequently converted to 3-chloropropyl ether **9**. Substitution of the chloro group with pyrrolidine in the presence of potassium iodide yielded intermediate **10**. Lastly, reduction of the dicyano groups under Raney nickel mediated hydrogenation provided compound **2** (Scheme 1).

Compound **3** was synthesized following similar procedures for preparation of compound **2**. Briefly, isoindoline substitution of the chloro group of intermediate **9** yielded compound **11**. The dicyano groups were subsequently reduced to diaminomethylene groups to afford compound **3** (Scheme 2).

Compound **4** was prepared from commercially available compound **12**. The carboxylic acid and phenolic hydroxyl groups of **12** were simultaneously protected as benzyl ester and benzyl ether groups, respectively, to provide intermediate **13**. The bromo group of **13** was substituted with

cyano group using the Rosenmund-von Braun reaction to yield intermediate **14**. After the hydrolysis of the benzyl ester under basic conditions, the resulting carboxylic acid was activated as acid chloride, which was subsequently converted to amide **16** in the presence of ammonium hydroxide aqueous solution. The benzyl ether was deprotected to provide intermediate **17** with a free phenol group, which was converted to the 3-isoindolinylpropyl ether product **4** in three steps (Scheme 3) using the similar reaction sequences for the preparation of compounds **2** and **3**.

CONCLUSION

In summary, we optimized compound **1**, a weak SPIN1 but highly potent G9a/GLP inhibitor, into compound **3**, a potent and selective fragment-like inhibitor of SPIN1. Compound **3** displayed high potency in SPIN1 biochemical assays (IC_{50} = 77 nM (AlphaLISA) and 243 nM (FP)) and high binding affinity to SPIN1 (K_D = 91 nM) by ITC. Compound **3** was completely inactive against G9a and GLP and selective for SPIN1 over a broad range of epigenetic proteins. We also obtained an X-ray crystal structure of SPIN1 in complex with compound **3**, which confirmed that compound **3** occupied Tudor domain II of SPIN1. Based on the cocrystal structure, we designed compound **4**, a close analog of compound **3** as a negative control, which was indeed inactive in SPIN1 biochemical and biophysical assays. We demonstrated that compound **3**, but not the negative control compound **4**, engaged SPIN1 in cells using a NanoBRET assay and compound **3** was not toxic in the non-tumorigenic cells evaluated. Thus, compound **3** and compound **4** are a pair of useful tool compounds for investigating biological functions and disease associations of SPIN1. Remarkably, compound **3** achieved high potency and selectivity for SPIN1 by targeting a single methyllysine reader domain. These results have demonstrated that it is feasible to generate potent, selective and cell-active inhibitors by targeting a single Tudor domain and paved the way for discovering improved inhibitors of methyllysine reader proteins.

EXPERIMENTAL SECTION

Chemistry General Procedures. HPLC spectra for compounds were acquired using an Agilent 1200 Series system with DAD detector. Chromatography was performed on a 2.1×150 mm Zorbax 300SB-C_{18} 5 μm column with water containing 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B at a flow rate of 0.4 mL/min. The gradient program was as follows: 1% B (0−1 min), 1−99% B (1−4 min), and 99% B (4−8 min). UPLC spectra for compounds were acquired using a Waters Acquity I-Class UPLC system with PDA detector. Chromatography was performed on a 2.1×30 mm ACQUITY UPLC BEH C_{18} 1.7 μm column with water containing 3% acetonitrile, 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B at a flow rate of 0.8 mL/min. The gradient program was as follows: 1−99% B (1−1.5 min), and 99− 1% B (1.5−2.5 min). High-resolution mass spectra (HRMS) data were acquired in positive ion mode using an Agilent G1969A API-TOF with an electrospray ionization (ESI) source. Nuclear Magnetic Resonance (NMR) spectra were acquired on either a Bruker DRX-600 spectrometer (600 MHz ^{1}H) or a Bruker DXI 800 MHz spectrometer (800 MHz ^{1}H, 200 MHz ^{13}C). Chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to 254 nm. Samples were injected into a Phenomenex Luna 75 x 30 mm, 5 μm, C_{18} column at room temperature. The flow rate was 40 mL/min. A linear gradient was used with 10% (or 50%) of MeOH (A) in H_2O (with 0.1 % TFA) (B) to 100% of MeOH (A). HPLC and UPLC were used to establish the purity of target compounds. All final compounds had > 95% purity using the HPLC and UPLC methods described above. Compounds **2** and **3** were tested in biological assays in their HCl salt forms and compound **4** in its CF_3CO_2H salt form.

(4-Methoxy-5-(3-(pyrrolidin-1-yl)propoxy)-1,3-phenylene)dimethanamine (2). Compound **2** was synthesized from the intermediate **6** following the procedures described below. To a solution of **10** (127 mg, 0.32 mmol) in MeOH (2 mL) was added Raney Ni (50 mg), followed by aqueous ammonia solution (0.1 mL). The mixture was stirred under hydrogen atmosphere (1 atm) for 12 h, before the insoluble solid was filtered. The filtrate was collected and concentrated. The resulting residue was purified by prep-HPLC to yield the title compound as brown oil (90 mg, 70% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.38 (s, 1H), 7.16 (s, 1H), 4.28 (t, J = 5.5 Hz, 2H), 4.16 (s, 2H), 4.14 (s, 2H), 4.01 (s, 3H), 3.82 – 3.69 (m, 2H), 3.52 – 3.44 (m, 2H), 3.23 – 3.13 (m, 2H), 2.41 – 2.32 (m, 2H), 2.26 – 2.16 (m, 2H), 2.14 – 2.04 (m, 2H). HPLC >95%, t_R = 1.24 min. HRMS (ESI-TOF) m/z: $[M+H]^+$ calcd for $C_{16}H_{28}N_3O_2$ 294.2176, found 294.2150.

(5-(3-(Isoindolin-2-yl)propoxy)-4-methoxy-1,3-phenylene)dimethanamine (3). Compound **3** was synthesized from the intermediate of **11** according to the procedures for the preparation of **2** as brown oil (65% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.49 – 7.42 (m, 4H), 7.40 (s, 1H), 7.15 (s, 1H), 5.02 (d, J = 13.8 Hz, 2H), 4.68 (d, J = 13.9 Hz, 2H), 4.33 (t, J = 5.8 Hz, 2H), 4.16 (s, 2H), 4.14 (s, 2H), 4.01 (s, 3H), 3.74 (t, J = 7.8 Hz, 2H), 2.50 – 2.38 (m, 2H). ^{13}C NMR (200 MHz, MeOD) δ 151.60, 148.24, 133.57, 129.30, 128.79, 126.98, 122.93, 122.60, 115.77, 65.82, 60.35, 58.51, 52.32, 42.47, 38.30, 25.46. HPLC >95%, t_R = 1.31 min. HRMS (ESI-TOF) m/z: $[M+H]^+$ calcd for $C_{20}H_{28}N_3O_2$ 342.2176, found 342.2145.

3-(Aminomethyl)-5-(3-(isoindolin-2-yl)propoxy)-4-methoxybenzamide (4). Compound **4** was synthesized from intermediate **19** according to the procedures for the preparation of compound **2** as brown oil (72% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.67 (s, 1H), 7.59 (s, 1H), 7.48 – 7.44 (m, 4H), 5.08 – 4.94 (m, 2H), 4.77 – 4.58 (m, 2H), 4.32 (t, J = 5.7 Hz, 2H), 4.22 (s, 2H), 3.99 (s, 3H), 3.78 (d, J = 7.8 Hz, 2H), 2.42 – 2.32 (m, 2H). ^{13}C NMR (200 MHz, MeOD) δ 169.50, 152.05,

148.83, 133.55, 129.93, 128.83, 126.40, 122.60, 121.41, 112.85, 70.05, 58.60, 55.26, 52.59, 38.20, 26.39. UPLC >95%, t_R = 0.66 min. HRMS (ESI-TOF) m/z: $[M+H]^+$ calcd for $C_{20}H_{26}N_3O_3$ 356.1969, found 356.1994.

1-(Benzyloxy)-3,5-dibromo-2-methoxybenzene (6). To a solution of **5**[47] (1.71 g, 6.1 mmol) in DMF (10 mL) was added K_2CO_3 (1.60 g, 11.6 mmol), followed by benzyl bromide (0.89 mL, 7.5 mmol). The resulting suspension was stirred at rt for 12 h, before water (10 mL) was added to quench the reaction. The mixture was extracted with ether (3 X 20 mL). The combined organic phase was dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting residue was purified by flash chromatography to yield the title compound as white solid (2.05 g, 90% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.49 – 7.36 (m, 5H), 7.33 (s, 1H), 7.08 (s, 1H), 5.12 (s, 2H), 3.89 (s, 3H).

5-(Benzyloxy)-4-methoxyisophthalonitrile (7). To a solution of **6** (2.05 g, 5.5 mmol) in NMP (10 mL) was added CuCN (1.08 g, 12.1 mmol). The mixture was heated in a sealed tube for 18 h. After cooling to room temperature, aqueous ammonia solution (2 mL) was added. The reaction was extracted with ether (3 X 20 mL). The combined organic phase was dried with anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography to yield the title compound as colorless oil (580 mg, 40% yield) ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.49 (s, 1H), 7.48 – 7.40 (m, 5H), 7.37 (s, 1H), 5.18 (s, 2H), 4.18 (s, 3H). UPLC >95%, t_R = 1.62 min.

5-(3-Chloropropoxy)-4-methoxyisophthalonitrile (9). To a solution of **7** (580 mg, 2.2 mmol) in ethyl acetate (10 mL) was added 10% palladium on carbon (50 mg). The mixture was stirred in hydrogen atmosphere (1 atm) for 5 h, before being filtered. The filtrate was concentrated. The resulting residue was dissolved in DMF (5 mL). To the resulting solution was added K_2CO_3 (455 mg, 3.3 mmol), followed by 1-bromo-3-chloropropane (0.43 mL, 4.4 mmol). The mixture was

stirred at room temperature for 12 h, before water was added to quench the reaction. The mixture was extracted with ether (3 X 20 mL). The combined organic phase was combined, dried over anhydrous Na_2SO_4, and concentrated. The resulting residue was purified by flash chromatography to yield the title compound as colorless oil (335 mg, 60% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.50 (s, 1H), 7.37 (s, 1H), 4.24 (t, J = 5.8 Hz, 2H), 4.16 (s, 3H), 3.78 (t, J = 6.1 Hz, 2H), 2.39 – 2.32 (m, 2H). UPLC >95%, t_R = 1.47 min.

4-Methoxy-5-(3-(pyrrolidin-1-yl)propoxy)isophthalonitrile (10). To a solution of **9** (100 mg, 0.4 mmol) in DMF (2 mL) was added pyrrolidine (84 μL, 1 mmol), K_2CO_3 (140 mg, 1 mmol), and KI (15 mg, 0.1 mmol). The resulting suspension was heated at 60 °C for 12 h. After cooling to room temperature, the reaction was quenched with water (5 mL) and extracted with CH_2Cl_2 (3 X 5 mL). The combined organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated. The resulting residue was purified by pre-HPLC to yield the title compound as brown oil (127 mg, 80% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.74 (s, 1H), 7.71 (s, 1H), 4.26 (t, J = 5.7 Hz, 2H), 4.16 (s, 3H), 3.82 – 3.69 (m, 2H), 3.49 – 3.41 (m, 2H), 3.22 – 3.11 (m, 2H), 2.40 – 2.29 (m, 2H), 2.28 – 2.17 (m, 2H), 2.14 – 2.00 (m, 2H). UPLC >95%, t_R = 0.95 min. **5-(3-(Isoindolin-2-yl)propoxy)-4-methoxyisophthalonitrile (11).** Compound **11** was synthesized from intermediate **9** according to the procedures for the preparation of **10** as brown oil (60% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.72 (dd, J = 3.8, 1.7 Hz, 2H), 7.47 – 7.44 (m, 4H), 5.00 (br, 2H), 4.66 (br, 2H), 4.31 (t, J = 5.8 Hz, 2H), 4.14 (s, 3H), 3.72 – 3.69 (m, 2H), 2.49 – 2.37 (m, 2H). HPLC >95%, t_R = 1.24 min.

Benzyl 3-(benzyloxy)-5-bromo-4-methoxybenzoate (13). To a solution of 3-bromo-5-hydroxy-4-methoxybenzoic acid (1.1 g, 4.4 mmol) in DMF (12 mL) was added benzyl bromide (1.1 mL, 9.2 mmol) and K_2CO_3 (1.6 g, 11.3 mmol). The mixture was stirred at room temperature for 12 h,

before being quenched with water. The mixture was extracted with ether (3 X 30 mL). The combined organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated. The resulting residue was purified by flash chromatography to yield the title compound as white solid (1.7 g, 91% yield).

Benzyl 3-(benzyloxy)-5-cyano-4-methoxybenzoate (14). Compound **14** was synthesized from intermediate **13** according to the procedures for the preparation of **7** as brown solid (54% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.92 (d, J = 1.8 Hz, 1H), 7.60 (d, J = 1.8 Hz, 1H), 7.57 – 7.54 (m, 2H), 7.50 – 7.46 (m, 2H), 7.46 – 7.34 (m, 6H), 5.39 (s, 2H), 5.15 (s, 2H), 3.94 (s, 3H). UPLC >95%, t_R = 1.74 min.

3-(Benzyloxy)-5-cyano-4-methoxybenzamide (16). To a solution of **14** (460 mg, 1.2 mmol) in MeOH (5 mL) was added aqueous NaOH (1.5 mL, 1 N, 1.5 mmol) solution. The solution was stirred at room temperature for 2 h, before being acidified with aqueous HCl (2 mL, 1 N, 2 mmol). The resulting suspension was filtered. The collected solid was dried and used for the next step without further purification. The crude acid (370 mg, 1.3 mmol) was dissolved in CH_2Cl_2 (5 mL). To the resulting solution was added $(COCl)_2$ (0.22 mL, 2.6 mmol) and DMF (several drops). The solution was stirred at room temperature for 1 h, before being concentrated. The resulting residue was dissolved in CH_2Cl_2 (5 mL). To the solution was added aqueous ammonia solution (1 mL, 28%, 14.8 mmol) at 0 ^{o}C. The mixture was stirred for 1 h at 0 ^{o}C before being poured into separatory funnel. The organic phase was collected. The aqueous phase was extracted with CH_2Cl_2 (2 X 5 mL). The organic phase was combined, dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by flash chromatography to yield the title compound as brown solid (140 mg, 41% in 2 steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.74 (d, J = 1.9 Hz, 1H), 7.67 (d, J = 1.9

Hz, 1H), 7.45 – 7.41 (m, 2H), 7.35 – 7.25 (m, 3H), 5.30 (s, 2H), 4.69 (s, 2H), 3.96 (s, 3H). UPLC >95%, t_R = 1.29 min.

3-(3-Chloropropoxy)-5-cyano-4-methoxybenzamide (18). Compound **18** was synthesized from intermediate **16** according to the procedures for the preparation of **9** as off-white solid (55% yield in 2 steps). ^{1}H NMR (600 MHz, CD_3OD) δ 7.74 (s, 1H), 7.69 (s, 1H), 4.41 (t, J = 5.6 Hz, 2H), 4.26 (s, 2H), 3.94 (s, 3H), 3.83 (t, J = 6.2 Hz, 2H), 2.26 – 2.17 (m, 2H). UPLC >95%, t_R = 1.20 min.

3-Cyano-5-(3-(isoindolin-2-yl)propoxy)-4-methoxybenzamide (19). Compound **19** was synthesized from intermediate **18** according to the procedures for the preparation of **11** as brown oil (61% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.86 (d, J = 2.0 Hz, 1H), 7.79 (d, J = 1.9 Hz, 1H), 7.56 – 7.35 (m, 4H), 5.06 – 4.96 (m, 2H), 4.72 – 4.65 (m, 2H), 4.46 (t, J = 5.6 Hz, 2H), 4.00 (s, 3H), 3.89 – 3.79 (m, 2H), 2.43 – 2.27 (m, 2H). UPLC >95%, t_R = 0.90 min.

Crystallization and X-ray cocrystal structure determination. $SPIN1_{Pro49\text{-}Ser262}$ was crystallized with compound **3** at 4 °C using the sitting drop vapor diffusion method. SPIN1 was incubated with 1.5 mM of compound **3** using 39 mg/mL protein, and crystals appeared in drops consisting of 75 nl protein compound mixture and 75 nl precipitant consisting of 60 % MPD and 0.1 M SPG (Succinate, Phosphate and Glycine) buffer pH 6.0. A 1.6 Å resolution dataset was collected on beamline I04 at Diamond Light source UK. The dataset was processed, scaled and merged at the Diamond Light source using Xia2.[48-52]

The structure was solved by molecular replacement using PHASER and SPIN1 PDB ID 6I8L as a search model. The structure was refined in PHENIX[53] with electron density map inspections and model improvement in COOT[54] and terminated when there were no significant changes in the

R_{work} and R_{free} values and inspection of the electron density map suggested that no further corrections or additions were justified. Statistics for data collection and refinement are shown in Supplementary Table 3. Structural analyses were performed with PyMOL (http://www.pymol.org) and the coordinates deposited in PDB with the ID 6QPL.

SPIN1 Fluorescence Polarization (FP) assay. FP experiments were performed as previously described.[23] The compounds were measured as a 12-point serial dilution with concentrations ranging from 24 nM to 50 µM, resulting in a final DMSO concentration of 0.5 %. Blank values were measured in six wells on the first and last row of the assay plate. Each concentration as well as positive and negative controls were tested in triplicates. The incubation time was modified to 30 min.

SPIN1 AlphaLISA assay. AlphaLISA experiments were performed as previously described.[23] The compounds were measured as a 14-point serial dilution with concentrations ranging from 6.1 nM to 50 µM, resulting in a final DMSO concentration of 0.5 %. Blank values were measured in seven wells on the first and last row of the assay plate. Each concentration as well as positive and negative controls were done in triplicates. The incubation time after addition of the beads was modified to 90 min.

IC_{50} values were calculated using the combined inhibition values of two independent experiments and applying a sigmoidal dose-response fit (variable slope) using Graphpad Prism (version 7).

G9a and GLP ITC experiments. ITC measurements were made at 25 °C on a MicroCal ITC200 Instrument (Malvern Instruments). Co-concentrated G9a−SAM and GLP-SAM (protein/SAM molar ratio of 1:5) were diluted at 35 µM in ITC buffer [50 mM Tris (pH 8.0), 150 mM NaCl] supplemented with 1% DMSO. Small molecule compounds were dissolved in DMSO

at 50 mM and diluted to 0.5 mM in ITC buffer with a final DMSO concentration of 1%. Binding constants were calculated by fitting the data using the ITC data analysis module in Origin 7.0 (OriginLab Corp.).

SPIN1 ITC experiments. ITC experiments were performed at 20 °C with a MicroCal VP-ITC microcalorimeter (GE Healthcare) by injection of 8 µL of compound solution (100-300 µM) during 16 seconds into the sample cell containing 10 µM of His-SPIN1(49-262) in ITC assay buffer. DMSO was adapted in syringe and sample cell to 0.1-0.3%. A total of 33 injections were performed with a spacing of 240 s and a reference power of 10 µcal s^{-1}.

Automated baseline assignment and peak integration were performed with NITPIC version 1.0.1.[55] Isotherms were plotted by global analysis of multiple titrations, with the Simplex algorithm, with SEDPHAT version 10.58d.[56] Isotherms were subsequently validated by two-dimensional error surface projections at P levels of 0.68 and 0.95 in SEDFIT.[57]

Other SPIN subfamily ITC experiments. Purified protein was diluted to 10 - 30 µM in ITC buffer (100 mM NaCl, 20 mM Tris, pH 8.0) and then dialyzed against a 1000 times excess of ITC buffer at 4 °C overnight using D-Tube Dialyzer tubes (Merck/Sigma Aldrich) with a 3.5 kDa molecular weight cut off. The dialyzed sample was then centrifuged at 14.000 rpm for 10 min in a standard chilled benchtop centrifuge at 4 °C. Protein concentration was then verified via absorption at 280 nm using a NanoDrop ND1000 Spectrophotometer. Compounds were diluted to 200 µM in ITC buffer. Experiments were performed on a NanoITC Standard Volume instrument (960 µL cell volume, 250 µL syringe colume; TA Instruments) using direct titration (protein in cell, compound in syringe) at 20 °C, a stir rate of 350 rpm and an injection rate of 50 ms/step (~1.4 µL/s). An initial injection of 3.7 µL followed after 200 s by 30 injections of 7.96 µL spaced at 300 s. Data

were analyzed using an independent fit model with the NanoAnalyse software (TA Instruments, version 3.8.0).

Thermal shift (T_m) assay. Compounds were dispensed on white PCR plates using an Echo 550 acoustic liquid dispenser to a final concentration of 20 or 200 μM with two technical replicates per concentration per protein. Protein in T_m shift buffer (20 mM HEPES, 500 mM NaCl, pH 7.5, SYPRO orange dye at 1:1000 dilution from purchased stock) was added at 2 μM final concentration. The lower concentrations were back-filled with DMSO to the same amount dispensed as for the highest concentration and DMSO-only controls were also included (n = 4 per protein). Experiments were performed on Agilent Mx3005P qPCR machines (reaction volume 20 μL) or Roche Lightcycler480 (reaction volume 5 μL). The temperature gradient was run from 25 °C to 95 °C over 25 minutes. Melting temperatures T_m were estimated as the inflection point of a Boltzmann equation fitted to the fluorescence intensity $I(T)$ from the onset (I_{onset}) to the peak (I_{peak}) of intensity where S is the slope of the curve:

$$I(T) = I_{onset} + (I_{peak} - I_{onset})/1+e^{((T_m-T)/S)}$$

Shifts in unfolding (ΔT_m) were then calculated as the difference between the individual T_m from the mean of the DMSO controls.

Methyltransferase and acetyltransferase selectivity assays. The effect of compound **3** on activities of 33 methyltransferases (MTs) and 5 acetyltransferases was assessed using activity assays as previously described.[58]

NanoBRET assay. U20S cell (2.8 x 10^5) were plated in each well of a 6-well plate. After 6 h cells were co-transfected with C-terminal HaloTag-Histone 3.3 (NM_002107) and an N-terminal NanoLuciferase fusion of full length SPIN1 at a 1:500 (NanoLuc® to HaloTag®) ratio

respectively with FuGENE HD transfection regent. Sixteen hours post-transfection, cells were collected, washed with PBS, and exchanged into media containing phenol red-free DMEM and 4% FBS in the absence (control sample) or the presence (experimental sample) of 100 nM NanoBRET 618 fluorescent ligand (Promega). Cells were then re-plated in a 384-well assay white plate (Greiner #3570) at $2.7x10^3$ cells per well. Compound **3** and Compound **4** were then added directly to media at final concentrations 0 – 30 μM or an equivalent amount of DMSO as a vehicle control, and the plates were incubated for 24 h at 37 °C in the presence of 5% CO2. NanoBRET Nano-Glo substrate (Promega) was added to both control and experimental samples at a final concentration of 10 μM. Readings were performed within 10 minutes using a ClarioSTAR (BMG labtech). A corrected BRET ratio was calculated and is defined as the ratio of the emission at 610 nm/460 nm for experimental samples minus the emission at 610 nm/460 nm for control samples (without NanoBRET fluorescent ligand). BRET ratios are expressed as milliBRET units (mBU), where 1 mBU corresponds to the corrected BRET ratio multiplied by 1000. The assay was further validated by domain specific site directed mutagenesis (Y170A) ablating peptide and ligand binding.

Cell viability assay. 293T (gift from Dr. Benchimol), C2C12 (gift from Dr. McPherson), HFF1 (ATTC) cells were cultured following standard protocols in DMEM (Gibco) 10% FBS (Wisent) and penicillin-streptomycin (Gibco). Cells were seeded in 96-well plates (seeding density: 2000 for HFF-1, 1000 for 293T and 500 for C2C12), recovered for 8 hours, and treated with several different concentrations of compound **3** for 6 days. Following cell treatment, cell viability was measured by adding resazurin (Sigma) to the media at 0.01 mg/mL, incubating plates for 2 – 4 h in a 37 °C CO_2 incubator, and measuring fluorescence at 590 nm on a CLARIOstar microplate reader (BMG Labtech).

Construct design, cloning, expression and purification of human SPINDLIN proteins. A plasmid encoding full-length human SPIN1 was obtained from Source Bioscience (IOH9972-pDEST26) and used as a template to clone SPIN1$_{M26–Ser262}$ and SPIN1$_{P49-S262}$ into the pNIC-CTHF vector with a TEV (tobacco etch virus) cleavable C-terminal His$_6$ tag. SPIN1$_{G21-S262}$ was cloned into the pNIC-Bio2 vector with a TEV cleavable N-terminal His$_{10}$ tag, and a C-terminal biotinylation sequence. SPIN2BP45-S258, SPIN3M27-S258 and SPIN4T36-P249 were cloned into the pNIC vector with a TEV cleavable N-terminal His6 tag using templates obtained from the Mammalian Gene Collection and Source Bioscience (SPIN2B: cDNA clone IMAGE id: 6729986, SPIN3 IMAGENE: IRCBp5005F0211Q and SPIN4 IMAGE id 40032302). The recombinant proteins were expressed in a phage-resistant derivative of *Escherichia coli* strain BL21(DE3) carrying the pRARE2 plasmid for rare codon expression. Cells were grown at 37 °C in Terrific Broth supplemented with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol, until the culture reached an OD_{600} of 2.0. The temperature was decreased to 18 °C and protein expression induced with 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside) overnight. Cells were collected by centrifugation and frozen at -80 °C. For purification, cells were resuspended in 50 mM Hepes pH 7.5, 500 mM NaCl, 10 mM Imidazole, 5 % glycerol, 0,5 mM TCEP and a protease inhibitor cocktail (Sigma), and lysed by sonication. The cell lysate was clarified by centrifugation and the proteins purified by nickel-affinity chromatography (GE Healthcare) using a stepwise gradient of imidazole. This was followed by size exclusion chromatography (Superdex 75 or Superdex 200, GE Healthcare) as previously described.[23] For ITC assay, Aliquots of purified His-SPIN1(49-262) protein were stored at -80 °C in ITC assay buffer containing 20 mM Tris/HCl (pH 8.0), 200 mM NaCl. For crystallisation studies the histidine tag was removed by incubation with TEV protease at 4 °C overnight, and the TEV protease and the uncleaved proteins were then removed by nickel-

affinity chromatography. Proteins were concentrated using an Amicon centrifugal filtration unit and the mass was verified by electrospray ionization time of flight mass spectrometry (ESI-TOF-TOF: Agilent LC/MSD).

Construct design, cloning, expression and purification of G9a and GLP proteins. Human G9a and GLP catalytic domains were cloned, expressed, and purified as previously described.[59]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge via the internet at http://pubs.acs.org.

Concentration response curves of A366 and compound **1** in the SPIN1 AlphaLISA and FP biochemical assays; Docking model of compound **1** in complex with SPIN1; Binding of compound **2** to GLP by ITC; Binding of compound **3** to SPIN2B, SPIN3 and SPIN4 by ITC; ^{1}HNMR and ^{13}CNMR spectra of compounds **3** and **4**; Raw data of the enzymatic inhibition assay against 33 methyltransferases and 5 acetyltransferases; Raw data results of the T_m shift assay against 20 methyllysine, methylarginine and acetyllysine reader proteins; Crystallography data and refinement statistics; Description of cell lines for cytotoxicity assays; Molecular formula strings (CSV)

Accession Codes

The structure of SPIN1 in complex with compound **3** has been deposited under PDB ID 6QPL. Authors will release the atomic coordinates and experimental data upon article publication.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS USED

SPIN1, spindlin 1; GLP, G9a-like protein 1; L3MBTL3, lethal(3) malignant brain tumor-like protein 3; CBX, chromobox; MBT, malignant brain tumor; H3K4me3, trimethylation of lysine 4 on histone H3; H3R8me2, dimethylation of Arginine 9 on histone H3; PTMs, post-translational modifications; Wnt, wingless/integrated; TCF, T-cell factor/lymphoid enhancer-binding factor; LE, ligand efficiency; SAR, structure–activity relationship; Alpha, Amplified luminescent proximity homogeneous assay; FP, fluorescence polarization; ITC, isothermal titration calorimetry; BRET, bioluminescence resonance energy transfer.

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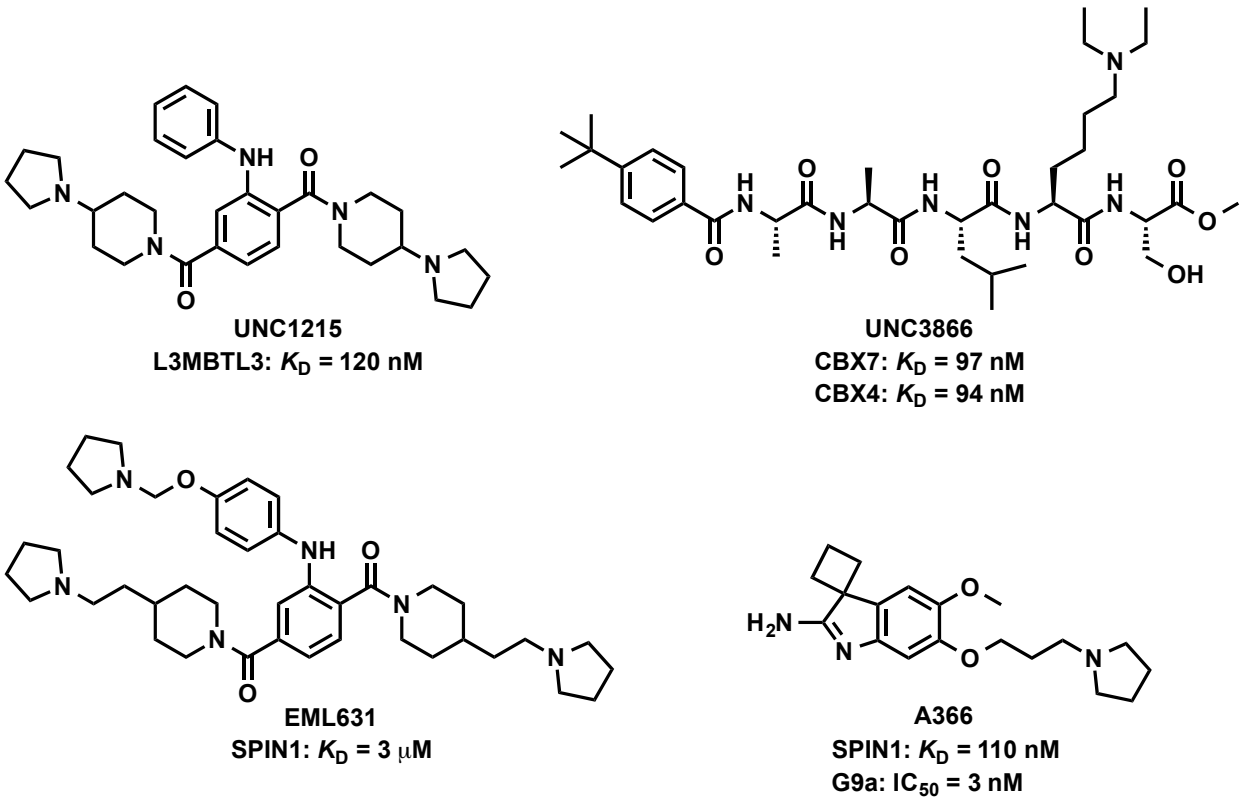


Figure 1. Representative inhibitors of methyllysine reader proteins.

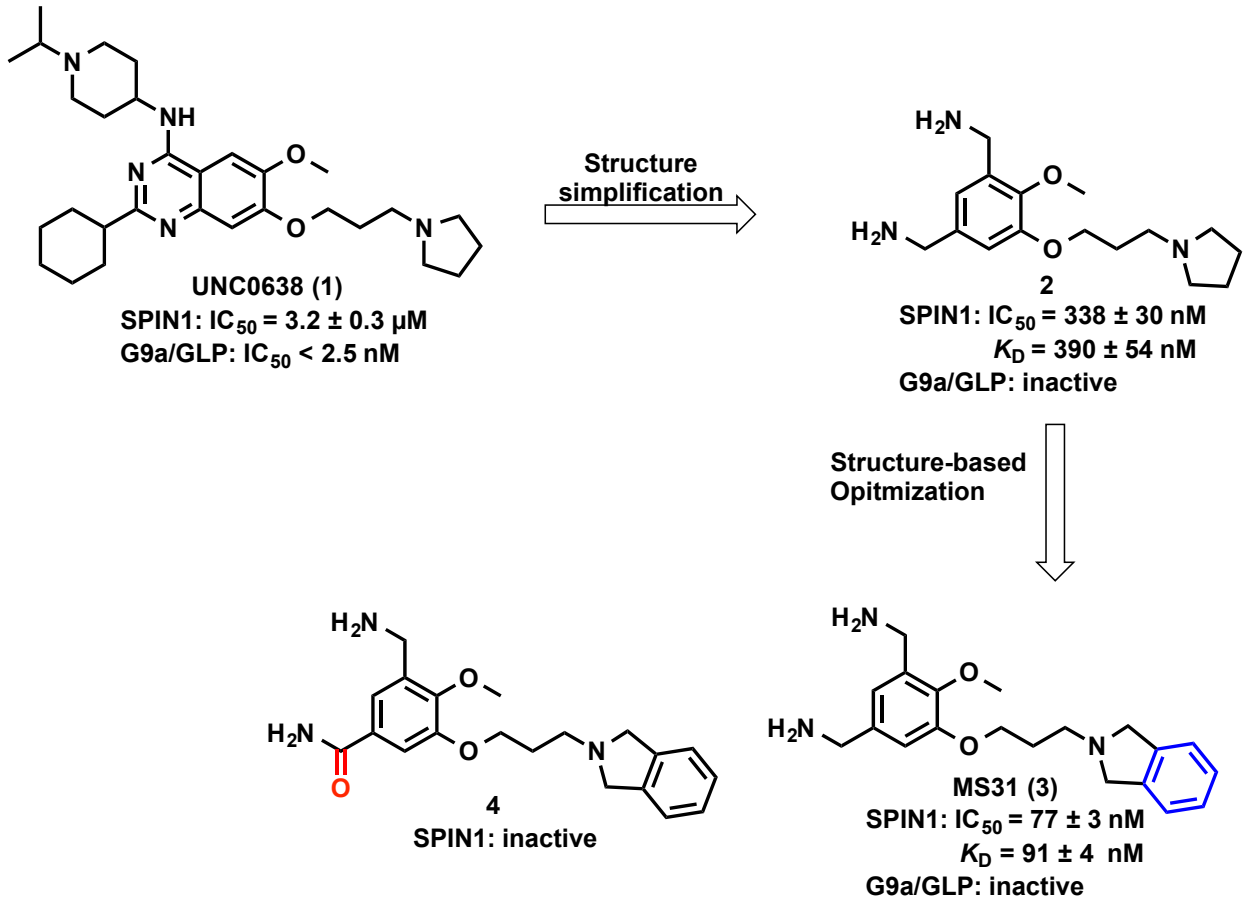


Figure 2. Discovery of compound **3** and its inactive control compound **4**.

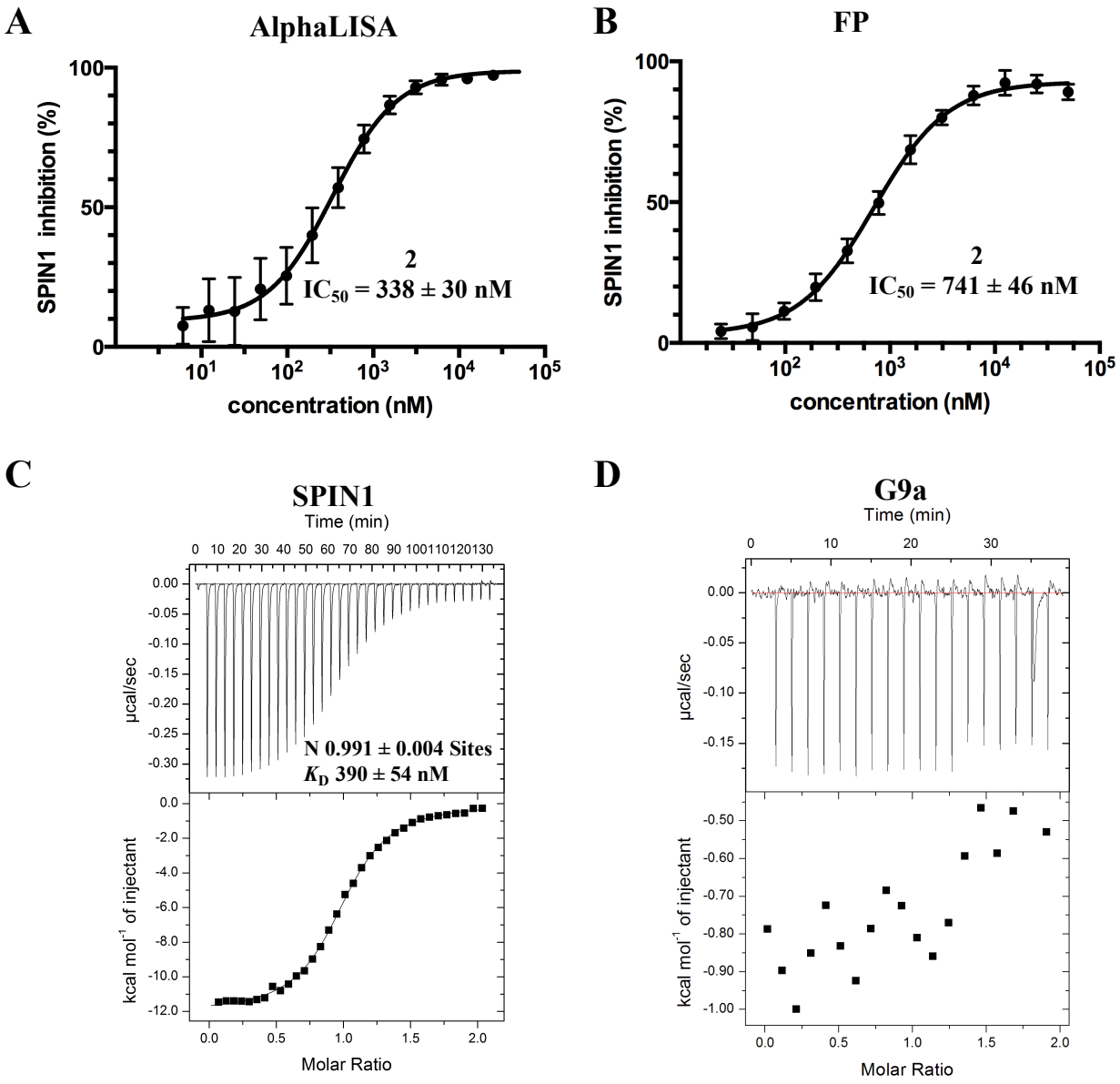


Figure 3. Compound **2** is a selective SPIN 1 inhibitor. Compound **2** inhibited the interactions between SPIN1 and H3K4me3 in a concentration dependent manner with IC_{50} values of (A) 338 ± 30 nM in AlphaLISA assay (n = 3) and (B) 741 ± 46 nM in FP assay (n = 3). (C) Compound **2** bound SPIN1 with a K_D value of 390 ± 54 nM in ITC assay (n = 3). (D) Compound **2** did not bind G9a.

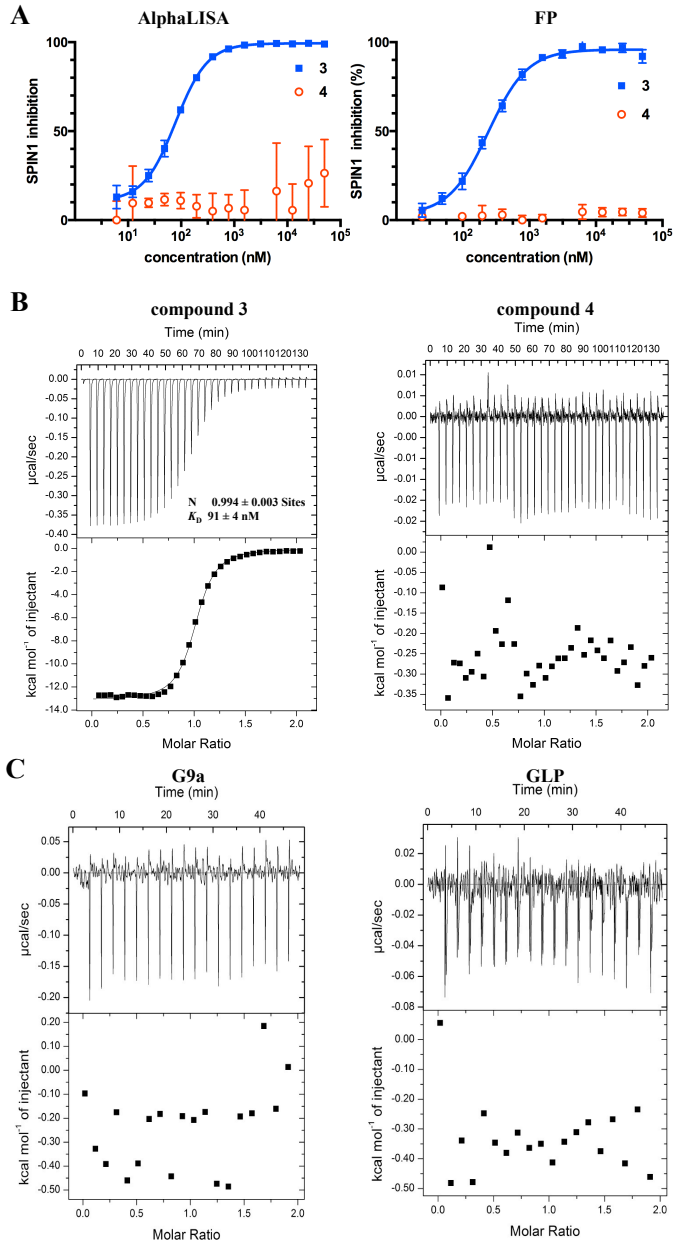


Figure 4. Compound **3** is a potent SPIN1 inhibitor. (A) Compound **3**, but not compound **4**, potently inhibited the interactions between SPIN1 and H3K4me3 with IC_{50} values of 77 ± 3 nM in AlphaLISA assay (n = 3) and 243 ± 10 nM in FP assay (n = 3). (B) Compound **3** bound SPIN1 with a K_D value of 91 ± 4 nM (n = 3) while compound **4** did not bind SPIN1 in ITC experiments. (C) Compound **3** did not bind either G9a or GLP in ITC experiments.

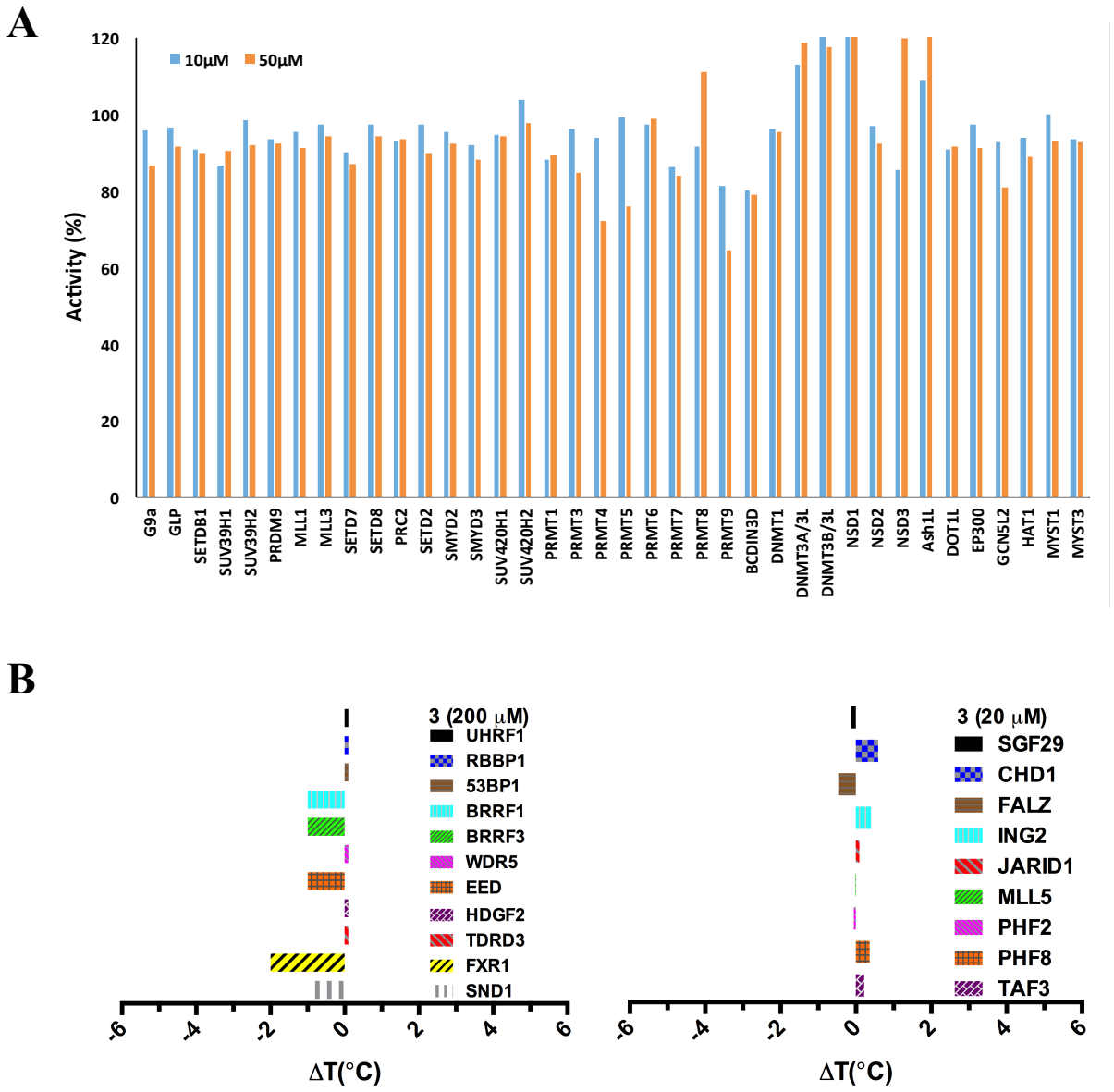


Figure 5. Compound **3** is a selective SPIN1 inhibitor. (A) Compound **3** did not significantly inhibit the enzymatic activity of 33 methyltransferases and 5 acetyltransferases at 10 μM (blue) and 50 μM (red). (B) Compound **3** did not induce significant thermal shifts for 20 methyllysine, methylarginine and acetyllysine reader proteins at 20 μM or 200 μM in thermal shift assays (n = 2).

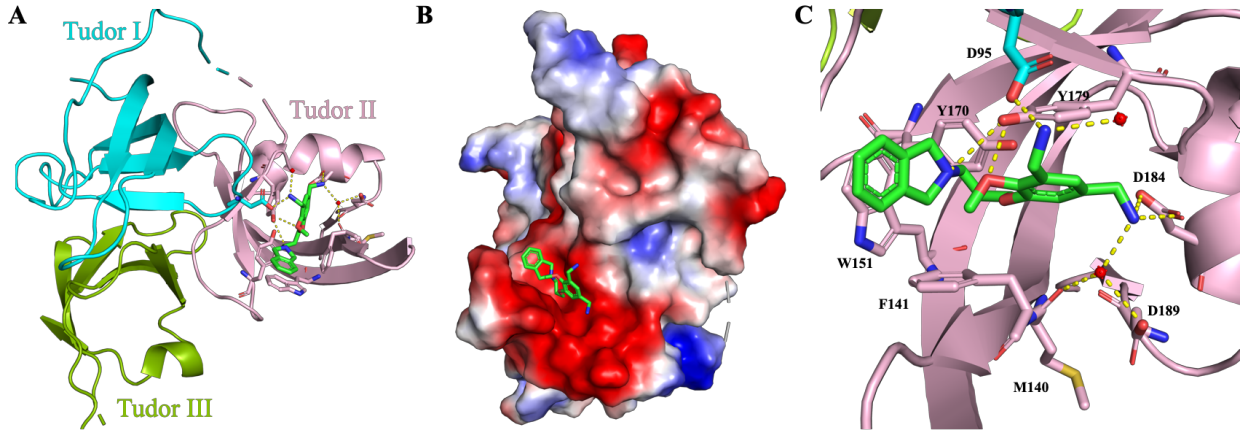


Figure 6. Crystal structure of the SPIN1-compound **3** complex. (A) Compound **3** (green) occupies the Tudor domain II (pink) of SPIN1. (B) Electrostatic potential surface view of the structure, ranging from −6 kT/e (red) to +6 kT/e (blue). (C) Close-up view of the SPIN1-compound **3** complex structure with key ligand-protein interactions. Yellow dashes, hydrogen bonds. Red ball, water molecules.

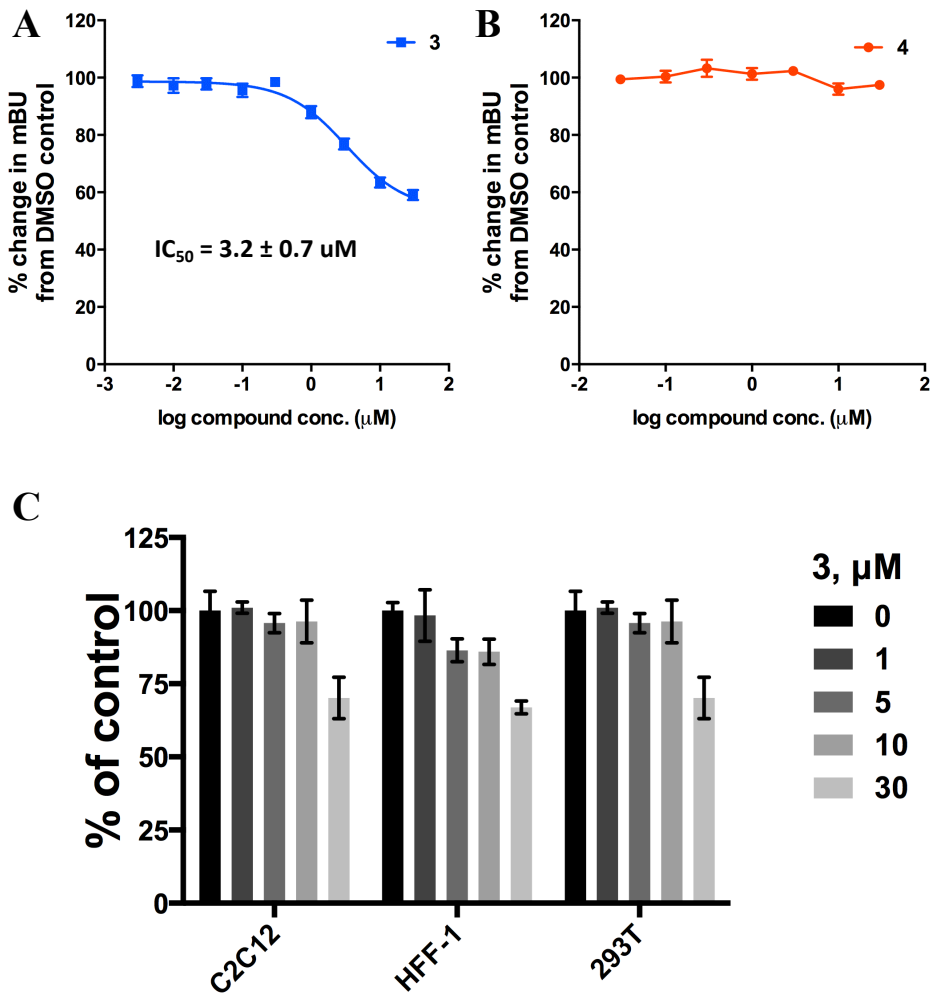
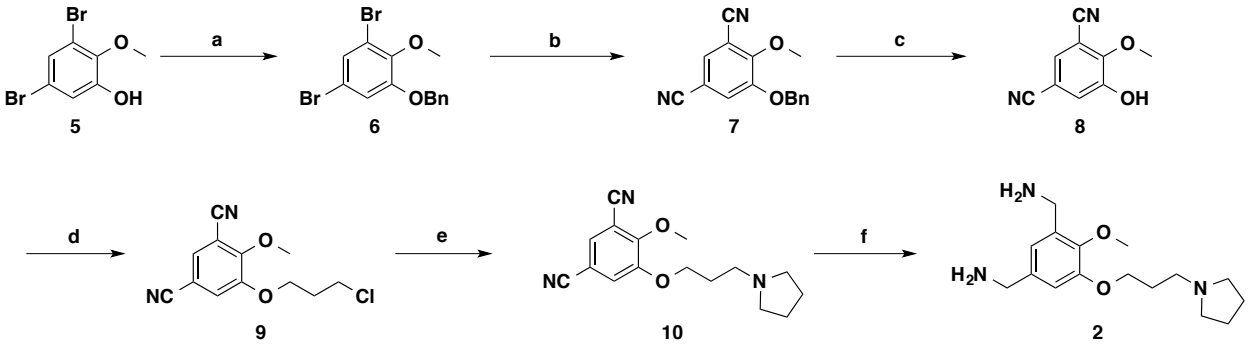


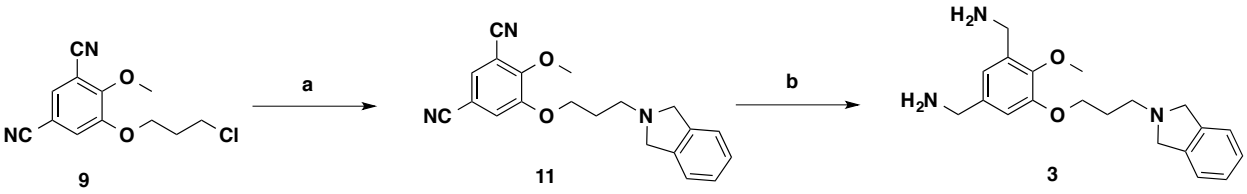
Figure 7. Compound **3** binds SPIN1 in cells and shows no cytotoxicity. (A) Compound **3** disrupted the interaction between SPIN1 and histone H3 (IC_{50} = 3.2 ± 0.7 μM (n = 4)) in U2OS cells in a NanoBRET assay. (B) Compound **4** was inactive in the NanoBRET assay (n = 3). (C) Compound **3** was not toxic to C2C12 and 293T cell lines or primary fibroblasts HFF1, at up to 30 μM (n = 3). Indicated cell lines were cultured in the presence of indicated concentrations of compound **3** for 6 days. Cell viability was measured using Alamar blue.

Scheme 1. Synthesis of Compound 2



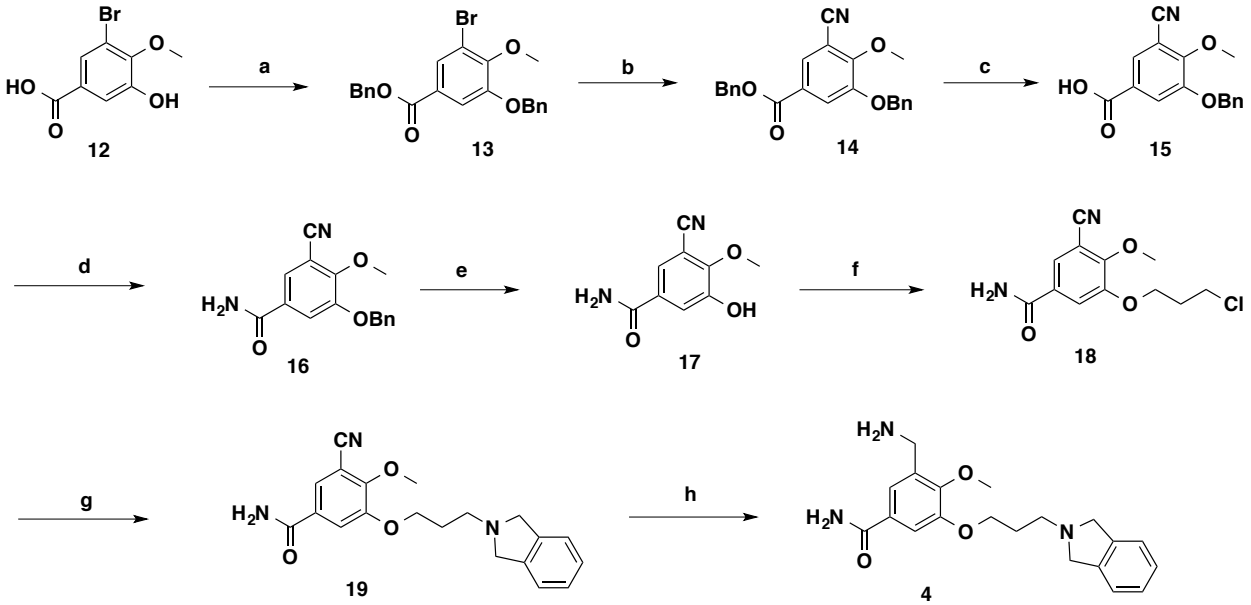
Reagent and conditions: (a) BnBr, K_2CO_3, DMF, rt, 12 h, 90%; (b) CuCN, NMP, 170 °C, 18 h, 40%; (c) H_2 (1 atm), 10% Pd/C, EtOAc, rt, 5 h; (d) 1-bromo-3-chloropropane, DMF, rt, 12 h, 60% in 2 steps; (e) pyrrolidine, K_2CO_3, KI, DMF, 60 °C, 12 h, 80%; (f) H_2 (1 atm), Raney Ni, NH_4OH, MeOH, rt, 12 h, 70%.

Scheme 2. Synthesis of Compound 3



Reagent and conditions: (a) isoindoline, K_2CO_3, KI, DMF, 60 °C, 12 h, 60%; (b) H_2 (1 atm), Raney Ni, NH_4OH, MeOH, rt, 12 h, 65%.

Scheme 3. Synthesis of Compound 4



Reagent and conditions: (a) BnBr, K_2CO_3, DMF, rt, 12 h, 91%; (b) CuCN, NMP, 170 °C, 18 h, 54%; (c) NaOH, MeOH, H_2O, rt, 2 h. (d) $(COCl)_2$, DMF, CH_2Cl_2, rt, 1 h; NH_4OH, 0 °C, 1 h, 41% in 3 steps; (e) H_2 (1 atm), 10% Pd/C, EtOAc, rt, 5 h; (f) 1-bromo-3-chloropropane, DMF, rt, 12 h, 55% in 2 steps; (g) isoindoline, K_2CO_3, KI, DMF, 60 °C, 12 h, 61%; (h) H_2 (1 atm), Raney Ni, NH_4OH, MeOH, rt, 12 h, 72%.

TOC Graphic:

