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Title:

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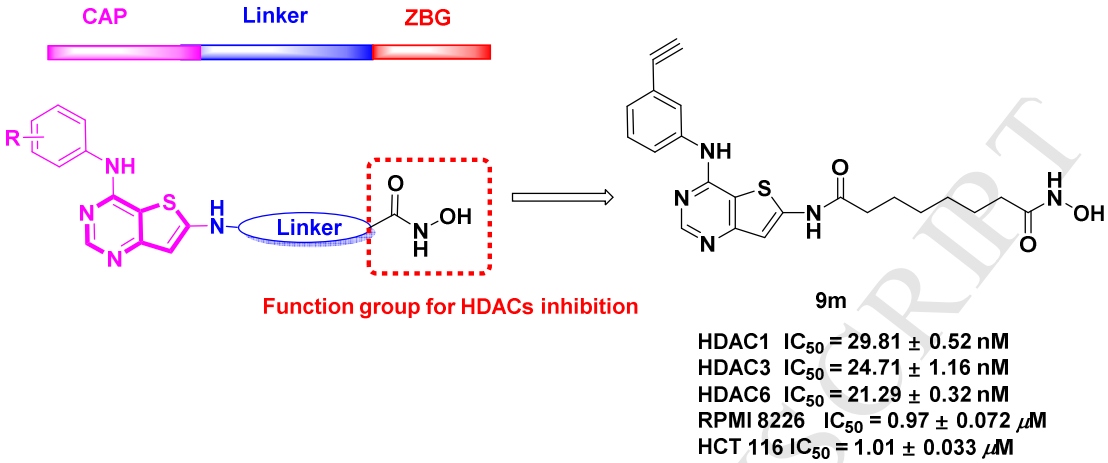
Graphical Abstract



By rational design and modification, compound **9m** showed excellent HDACs inhibitory activity, strong anti-proliferative activity against human cancer cell lines including RMPI 8226 and HCT 116. Moreover, compound **9m** noticeably up-regulated the level of histone H3 acetylation at the low concentration of 0.3 μM.

Highlights:

- Novel thienopyrimidine hydroxamic acid based derivatives were designed and synthesized.
- Most of these compounds displayed good to excellent inhibitory activity against HDACs with the IC_{50} values below 50 nM.
- Some compounds significantly up-regulated the levels of Histone H3 acetylation and α-tubulin acetylation as well as exhibited powerful anti-proliferative activities against cancer cell lines *in vitro*.

Design, Synthesis and Biological Evaluation of Thienopyrimidine Hydroxamic Acid Based Derivatives as Structurally Novel Histone Deacetylase (HDAC) Inhibitors

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Author Contributions

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Abstract:

New thienopyrimidine hydroxamic acid derivatives as HDACs inhibitors were designed, synthesized and evaluated. All compounds were evaluated for their ability to inhibit recombinant human HDAC1, HDAC3, and HDAC6 isoforms and *in vitro* anti-proliferative activity on tumor cell lines RMPI 8226 and HCT 116. Most of these compounds displayed good to excellent inhibitory activities against HDACs. The IC_{50} values of compound **9m** against HDAC1, HDAC3, and HDAC6 was 29.81 ± 0.52 nM, 24.71 ± 1.16 nM, and 21.29 ± 0.32 nM. Most of the compounds showed strong anti-proliferative activity against human cancer cell lines including RMPI 8226 and HCT 116. The IC_{50} values of compound **9m** against RPMI 8226 and HCT 116 proliferation were 0.97 ± 0.072 μM and 1.01 ± 0.033 μM, respectively. In addition, compound **9m** noticeably up-regulated the level of histone H3 acetylation at the low concentration of 0.3 μM.

Keywords: HDAC inhibitor, anti-proliferation, Western blot, thienopyrimidine, Structure-activity relationship

1. Introduction

Histone acetylation and deacetylation, catalyzed by multisubunit complexes, play a key role in the regulation of eukaryotic gene expression.[1] The acetylation status of histones is determined by two sets of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs).[2] HDACs also interact with retinoblastoma tumor-suppressor protein and this complex is a key element in the control of cell proliferation and differentiation. Together with metastasis-associated protein-2 MTA2, HDACs deacetylates p53 and modulates its effect on cell growth and apoptosis. HDAC inhibitors demonstrated prominent antitumor efficacy on broad spectrum neoplasms in preclinical and clinical studies. [3-5] This concept has been well validated by the approval of HDAC inhibitors vorinostat (suberoylanilide hydroxamic acid, SAHA) [6] and romidepsin (depsipeptide) [7] for the treatment of cutaneous T-cell lymphoma.

The thienopyrimidine fragment is widely present in antitumor agents, [8] anti-inflammatory agents, [9] and anti-diabetic agents. [10] In our previous studies, we reported 4-anilinothieno[2,3-*d*]pyrimidine derivatives as irreversible EGFR inhibitors that displayed excellent inhibitory activities against wild type and mutant EGFR (Fig. 1). [11] CUDC-101 has been reported as a multi inhibitors against HDAC, EGFR and HER2 for the treatment of cancer, which contains a quinazoline moiety within its structure. [12] On the basis of these observations, we attempted using substituted 4-anilinothieno[3,2-*d*]pyrimidine moiety as a cap group (CAP) for protein surface interactions, fixed carbon chain as a linker region, and hydroxamic acid group

as a zinc binding group (ZBG) (Fig. 1). Herein, we presented the design, synthesis and biological evaluation of thienopyrimidine hydroxamic acid based derivatives as novel HDACs inhibitors. Most of these compounds showed excellent potencies against HDACs and displayed powerful anti-proliferative activities against cancer cell lines *in vitro*.

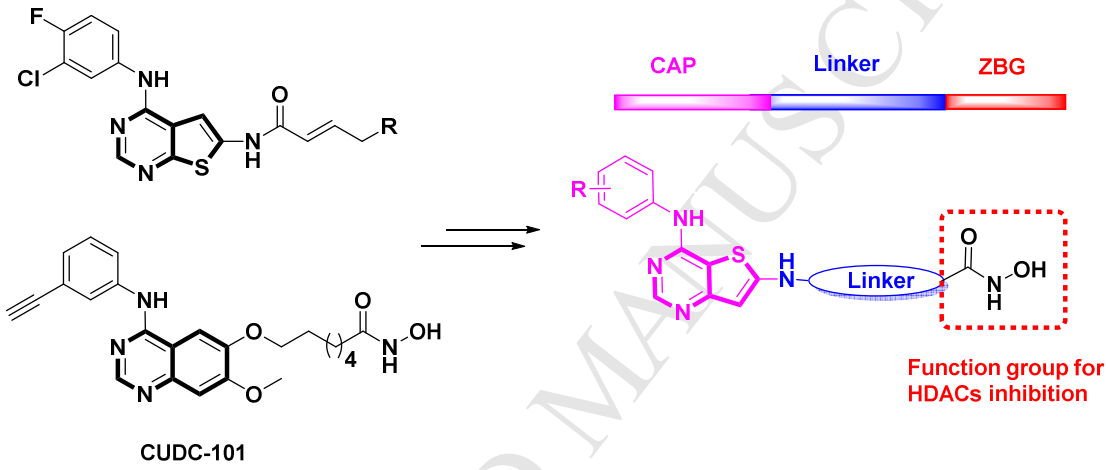


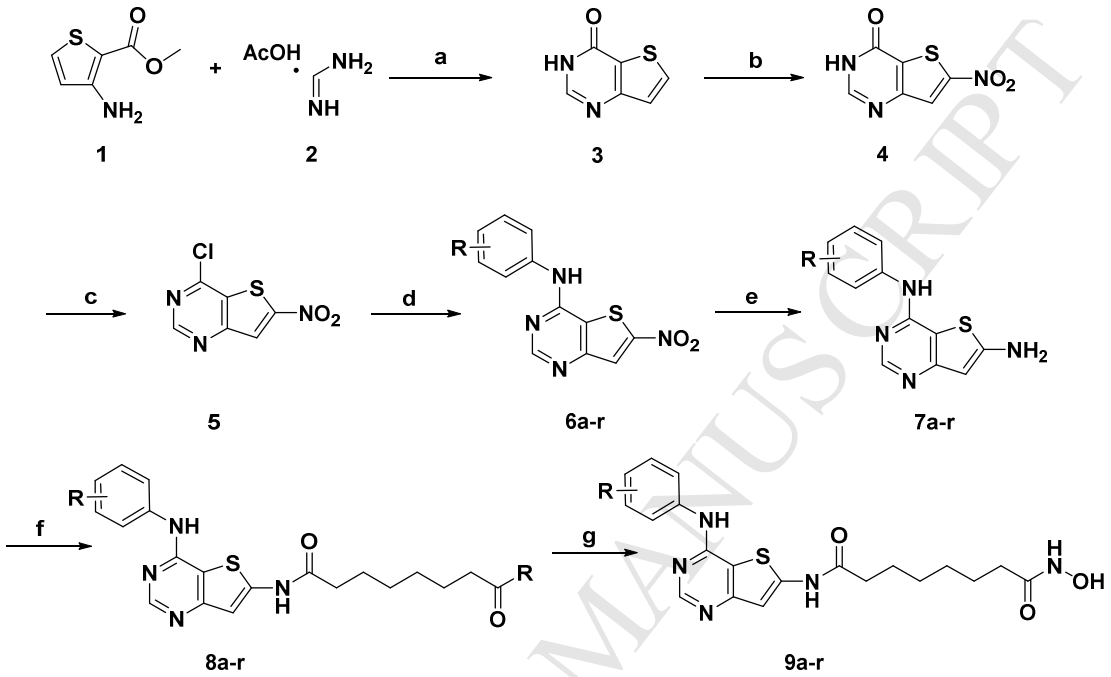
Fig. 1. Design strategy and modification of novel HDACs inhibitors.

2. Results and discussion

2.1. Chemistry

The synthetic route to obtain the desired thienopyrimidine-based hydroxamic acid derivatives **9a-r** are shown in Scheme 1. Heated by the microwave, the thieno[3,2-*d*]pyrimidin-4(3H)-one **3** was obtained *via* the cyclization of methyl 3-aminothiophene-2-carboxylate with formamidine acetate. Then, the nitration of intermediate **3**, followed by chlorination to afford **5**. Reduction of nitro group using iron/NH_4Cl, then coupling with various substituted anilines afforded the key

intermediate **7**. Treatment with different acyl chlorides to form amide **8**. Finally, the amide **8** was coupled with freshly prepared hydroxylamine hydrochloride to generate the targeted products **9a-r**.



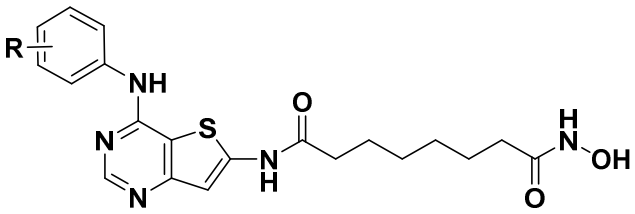
Scheme 1. Reagents and conditions: a) microwave, 4-5 mins; b) H_2SO_4/HNO_3, 0 ^oC-r.t; c) $POCl_3$, TEA, CH_3CN, 85 ^oC; d) various substituted aniline, 1,4-dioxane, 85 ^oC; e) Fe, $NH_4Cl_{(aq)}$, EtOH, 50 ^oC, 2 h; f) $ROCO(CH_2)_4COCl$, DIPEA, THF, R = Methyl or Ethyl; g) $NH_2OH{\cdot}HCl$, KOH, MeOH-THF, 0 ^oC-r.t.

2.2. *In vitro* HDAC inhibitory activity

Firstly, in order to assess the efficacy of compounds **9a-r** against recombinant human HDAC activity, biochemical assays were performed as described previously. [13] Compound potency against the recombinant human HDAC1, HDAC3, and HDAC6 isoforms was interrogated using SAHA as the positive control. The effects of regiochemical substitution and electronics on the phenyl ring were explored. The

results are presented in Table 1. Most of the compounds exhibited excellent inhibitory activity against HDAC1, HDAC3, and HDAC6 isoforms which indicated the thienopyrimidine scaffold was an outstanding capping group possessing strong binding affinity with the HDACs surface. All of these inhibitors occupied a narrow potency range, indicating tolerability for a wide variety of substituents on the phenyl ring. A tendency in enzymatic potency of *para*-substitution < *ortho*-substitution < *meta*-substitution was noted as shown in Table 1. Then, whether the introduced halogen groups affect the inhibitory activity against HDACs is tested. We found that most of these compounds (**9c-9e** and **9g-9i**) showed strong potential activities against HDACs with IC_{50} values < 100 nM. Both electron-withdrawing and electron-donating groups on the phenyl ring were tolerated for HDACs Inhibition. For example, compounds **9n** (3-CN) and **9o** (3-OMe) exhibited good inhibitory activities against HDAC1, 3, and 6 with IC_{50} values of 45.02, 19.80, 20.05 and 62.25, 21.37, 20.90 nM, respectively. Additionally, introducing steric hindrance at the 3-position and 4-position of the phenyl ring (**9j-9l** and **9p-9r**) decreased HDACs inhibitory activities. Eventually, the SAR from this small group of compounds found compound **9m** as a potent HDACs inhibitor. The IC_{50} values of compound **9m** against HDAC1, HDAC3, and HDAC6 was 29.81 ± 0.52 nM, 24.71 ± 1.16 nM, and 21.29 ± 0.32 nM.

Table 1. Structures and *in vitro* inhibitory activity against HDACs of compounds **9a-r**



9a-r

Compd.	R	IC_{50} (nM) ± SD [a]		
		HDAC1	**HDAC3**	**HDAC6**
9a	2-Me	137.85 ± 9.58	98.79 ± 15.83	87.60 ± 1.39
9b	2-Me, 4-F	246.57 ± 4.92	196.62 ± 3.13	119.94 ± 20.93
9c	2-F, 4-F	91.99 ± 0.46	99.87 ± 5.47	19.17 ± 1.07
9d	2-F,4-Cl	54.27 ± 21.44	133.06 ± 11.79	36.98 ± 8.16
9e	2-F,5-CH_3	75.12 ± 2.90	67.54 ± 39.36	29.56 ±1.43
9f	2-CH_3,4-OCH_3	117.28 ± 7.70	144.07 ± 5.25	111.04 ± 9.50
9g	3-F	73.73 ± 1.43	64.51 ± 7.23	42.52 ±1.88
9h	3-Cl	53.80 ± 5.37	45.55 ± 1.88	33.84 ± 7.48
9i	3-Br	54.46 ± 5.67	33.77 ± 8.70	16.87 ± 0.15
9j	3-ethyl	116.23 ± 5.09	147.00 ± 0.90	58.54 ± 4.16
9k	3-isopropyl	99.91 ± 2.75	65.05 ± 26.99	35.79 ±0.54
9l	3-tertbutyl	122.49 ± 8.49	50.27 ± 8.03	56.02 ±0.11
9m	3-enthynyl	29.81 ± 0.52	24.71 ± 1.16	21.29 ± 0.32
9n	3-CN	45.02 ± 0.94	19.80 ± 4.31	20.05 ±1.25
9o	3-OCH_3	62.25 ± 1.95	21.37 ± 5.91	20.90 ± 0.77
9p	4-ethyl	257.88 ± 47.48	356.90 ± 9.93	98.27 ± 1.36

9q	4-isopropyl	143.25 ± 40.11	152.46 ± 5.46	36.61 ± 0.81
9r	4-OCH_3	174.73 ± 0.91	215.48 ± 24.33	71.38 ± 5.99
SAHA	-	195.00 ± 16.12	181.05 ± 28.92	105.10 ± 25.46

[a] IC_{50} values were obtained based on three separate experiments and expressed as means ± SD.

2.3. Anti-proliferation of all compounds against RPMI 8226 and HCT 116 cells

Furthermore, the anti-proliferative activities of the new target compounds were tested on human cancer cell lines RPMI 8226 and HCT 116. As summarized in Table 2, most of the compounds exhibited good anti-proliferative activity against both RPMI 8226 and HCT 116 cell lines. Compounds **9e** (IC_{50} = 1.06 ± 0.193 μM) and **9o** (IC_{50} = 1.26 ± 0.086 μM) showed similar anti-proliferative activities against RPMI 8226 cells with the positive control SAHA, while the other compounds displayed less anti-proliferative activity than SAHA. Compared to the RPMI 8226 cancer cells, the HCT 116 cells appeared to be similar sensitive to our synthesized compounds. Compounds **9e** (IC_{50} = 1.33 ± 0.283 μM) and **9o** (IC_{50} = 1.33 ± 0.255 μM) showed similar anti-proliferative activities against HCT 116 cells with the reference compound SAHA. Compound **9m** possessed a slightly higher activity against both RPMI 8226 and HCT 116 cells than SAHA, with the IC_{50} value of 0.97 ± 0.072 and 1.01 ± 0.033 μM, respectively.

Table 2. *In vitro* anti-proliferative activity against RPMI 8226 and HCT 116 cells of all compounds.

Compd.	IC_{50} (μM) ± SD [a]	
	RPMI 8226	**HCT 116**
9a	1.75 ± 0.116	2.56 ± 0.386
9b	1.95 ± 0.141	2.64 ± 0.290
9c	1.74 ± 0.115	1.94 ± 0.334
9d	1.90 ± 0.108	1.79 ± 0.144
9e	1.06 ± 0.193	1.33 ± 0.283
9f	1.98 ± 0.069	2.48 ± 0.172
9g	1.63 ± 0.114	1.78 ± 0.351
9h	1.52 ± 0.104	1.82 ± 0.409
9i	1.75 ± 0.118	1.74 ± 0.170
9j	1.57 ± 0.119	1.44 ± 0.045
9k	2.08 ± 0.191	1.76 ± 0.339
9l	3.55 ± 0.403	2.93 ± 0.058
9m	0.97 ± 0.072	1.01 ± 0.033
9n	1.58 ± 0.066	2.09 ± 0.375
9o	1.26 ± 0.086	1.33 ± 0.255
9p	2.54 ± 0.135	2.36± 0.404
9q	2.79 ± 0.360	2.07 ± 0.442
9r	1.84 ± 0.129	2.18 ± 0.196
SAHA	1.25 ± 0.150	1.31 ± 0.085

[a] IC_{50} values were obtained based on three separate experiments and expressed as

means ± SD.

2.4. Analysis Ac-tubulin and Ac-histone H3 levels in RPMI 8226 cells by Western boltting

With the cell proliferation data in hand for both RPMI 8226 and HCT 116 cells, three representative compounds **9m**, **9n**, and **9o** were selected to validate the observed biochemical potencies by assessing their effects on the levels of tubulin and histone H3 acetylation in RPMI 8226. For this assay, suberoylanilide hydroxamic acid（SAHA）was used as the positive control. As shown in Fig. 2, Western blot analysis revealed that 0.3 μM compound **9m** induced significantly more acetylated tubulin and acetylated histone H3 compared to that of 0.3 μM compounds **9n** and **9o**, which was consistent with their strong inhibitory activities against HDAC and cancer cells. Even 0.3 μM compound **9m** can dramatically increase tubulin acetylation, which is much better than that of SAHA. Additionally, the western blot analysis displayed that our designed compounds, like the reference compound SAHA, were pan-HDACs inhibitors.

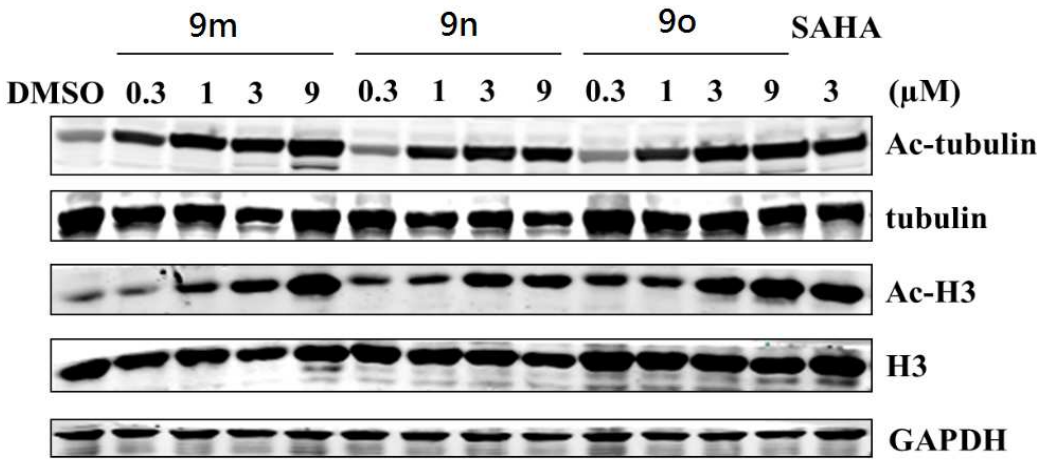


Fig. 2. Western blot analysis of Ac-tubulin and Ac-histone H3 from RPMI 8226 cell cultured for 24 h with DMSO control and compounds **9m**, **9n**, **9o**, and SAHA.

3. Conclusion

In conclusion, based on the previous work in our laboratory, a series of thienopyrimidine-based hydroxamic acid derivatives **9a-r** were designed, synthesized and evaluated as novel HDAC inhibitors. The *in vitro* biological evaluations indicated that most compounds exhibited excellent inhibition against recombinant HDAC enzyme activities and multiple cancer cells lines proliferation. Especially, the compounds **9e**, **9m**, and **9o** could significantly inhibit the proliferation of RPMI 8226 and HCT 116 cancer cell lines with low IC_{50} values. Western blot analysis revealed that compounds **9m**, **9n**, and **9o** caused a substantial increase in the level of acetylated α-tubulin and histone H3 in a dose-dependent manner. The present results suggested that thienopyrimidine-based hydroxamic acid derivatives could be lead compounds for further optimization to develop novel HDAC inhibitors.

4. Experiment Section

4.1. Chemistry

The reagents (chemicals) were commercially available and used without further purification. Analytical thin-layer chromatography (TLC) was performed on HSGF 254 (0.15-0.2 mm thickness). Column chromatography was performed on silica gel 300-400 mesh to purify the compounds. Nuclear magnetic resonance (NMR) spectra were performed on a Brucker AMX-400 (TMS as IS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric, electrospray and matrix-assisted laser desorption ionization (EI, ESI and MALDI) produced by Finnigan MAT-95, LCQ-DECA spectrometer and IonSpec4.7 T.

4.2 General procedures for synthesis of HDACs inhibitors

4.2.1. Thieno[3,2-*d*]pyrimidin-4(3H)-one (3)

A mixture of compound **1** (20 g, 0.127 mol) and formimidamide acetate **2** (16 g, 0.155 mol) was reacted in the condition of microwave for 4-5 mins. After cooled to room temperature, water was added and the solid was precipitate, then filtered, and the cake was washed with water, dried under infrared light to obtain the desired compound **3**, 14.8 g as gray solid in 76% yield. ESI-MS m/z 153.0 $[M+H]^+$.

4.2.2. 6-Nitrothieno[3,2-d]pyrimidin-4(3H)-one (4)

To a mixture of H_2SO_4 (20 mL) and HNO_3 (15 mL) was added compound **3** (9.7 g,

0.064 mol) slowly under ice-bath condition. Then, the reaction mixture was heated to room tempreture. When the staring material was consumed, the mixture was poured into the ice-water carefully, and the yellow solid was formed, followed by filtration. The cake was washed with water for three times, dried to afford the desired compound **4** , 10.8 g as yellow solid in 86% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.00 (s, 1H), 9.33 (s, 1H), 8.35 (s, 1H); MS (ESI) *m/z* 195.9 $[M-H]^-$.

4.2.3. 4-Chloro-6-nitrothieno[3,2-*d*]pyrimidine (5)

To a solution of compound **4** (2 g, 0.01 mol) in CH_3CN (20 mL) was added $POCl_3$ (2.04 mL, 0.022 mol) and TEA (2.82 mL, 0.02 mol). Then the mixture was heated to 85 ^{o}C. After the start material was completed, the mixture was poured into ice-water, and extracted with EA, washed by $NaHCO_3$ solution, brine, dried by Na_2SO_4, concentrated and purified by flash silica gel column (0-100% EA in PE gradient) to obtain desired compound **5**, 1.7 g in 78% yield. ESI-MS *m/z* 216.0 $[M+H]^+$.

4.3. General process for synthesis of intermediates 6a-r

4.3.1. 6-Nitro-*N*-(*o*-tolyl)thieno[3,2-d]pyrimidin-4-amine 6a

To a solution of compound **5** (1.7 g, 0.008 mol) in 1,4-dioxane (20 mL) was added *o*-toluidine (1.1 mL, 0.01 mol) at room temperature. Subsequently, the mixture was heated to 85 ^{o}C. When the start material was consumed monitored by TLC, the reaction was cooled to room temperature. After removal of the solvent, the residue was suspended in ethyl acetate, filtered and washed with ethyl acetate to afford the intermediate **6a**, 2.05 g as yellow solid in 83% yield. ESI-MS *m/z* 287.0 $[M+H]^+$.

The other substituted aniline was treated with intermediate **5** for preparation of

intermediates **6b-r** according to the same procedure described for **6a**.

4.4. General process for synthesis of intermediates 7a-r

4.4.1. N^4-(*o*-tolyl)thieno[3,2-d]pyrimidine-4,6-diamine 7a

To a solution of intermediate **6a** (2.05 g, 0.007 mol) in ethanol (30 mL) was added iron powder (4 g, 0.07 mol) and saturated NH_4Cl solution (30 mL). The mixture was stirred at 50 ^oC for 2 h. Then, the mixture was filtered through celite, washed with ethanol followed by the water was added to the mixture to give the solid. Then the solid was filtered, washed and dried to afford the intermediate **7a**, 1.32 g light green solid in 61% yield. ESI-MS m/z 257.1 $[M+H]^+$.

The other nitro intermediates **6b-r** was reduced to intermediates **7b-r** according to the same procedure described for **7a**.

4.5. General process for synthesis of intermediates 8a-r

4.5.1. Methyl 7-oxo-7-((4-(*o*-tolylamino)thieno[3,2-d]pyrimidin-6-yl)amino) heptanoate 8a

To a solution of 7-methoxy-7-oxoheptanoic acid (165 mg, 0.9 mmol) in $SOCl_2$ (2 mL) at 50 ^oC, the mixture was stirred 2 h. After removal of the solvent, **7a** (150 mg, 0.59 mmol) in dry THF was added, and DIPEA (193 μL, 1.18 mmol) at 0 ^oC. Then the mixture was stirred at room temperature and was monitored by TLC. After an overnight time, the reaction was stopped and quenched by slowly addition of saturated NH_4Cl solution. The mixture was then extracted by ethyl acetate three times. The combined organic layer was washed with water and brine, respectively. dried over anhydrous Na_2SO_4, concentrated in *vacuo* and purified by flash silica gel column

(0-45% EA in PE gradient) to obtain desired production **8a** as yellow solid in 71% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.31 (s, 1H), 9.67 (s, 1H), 8.69 (s, 1H), 8.52 (s, 1H), 8.33 (s, 1H), 7.48 – 7.26 (m, 4H), 3.96 (s, 3 H), 2.60 – 2.54 (m, 2H), 2.36 (s, 3H), 1.88 (t, *J* = 7.4 Hz, 2H), 1.68 (p, *J* = 7.2 Hz, 2H), 1.51 (p, *J* = 7.2 Hz, 2H), 1.39 – 1.33 (m, 4H). ESI-MS *m/z* 427.1 $[M+H]^+$.

The other intermediates **8b-r** was prepared according to the same procedure described for **8a**.

4.6. General process for synthesis of the targeted compounds 9a-r

4.6.1.

***N*^{1}-hydroxy-*N*^{7}-(4-(*o*-tolylamino)thieno[3,2-d]pyrimidin-6-yl)heptanediamide 9a**

To a solution of **8a** (178 mg, 0.43 mmol) in anhydrous THF was added hydroxylamine hydrochloride (5 mL). Then, the mixture was stirred at room temperature and monitored by TLC. After 24 hours, the reaction was stopped and water was added into the mixture, filtered the formed solid and washed with water. The crude product was purified by flash silica gel column (0-10% MeOH in DCM) to give the targeted compound **9a** as white solid in 57% yield. Mp 176.8-177.5 ^{o}C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.36 (s, 1H), 10.11 (s, 1H), 9.57 (s, 1H), 8.74 (s, 1H), 8.49 (s, 1H), 8.22 (s, 1H), 7.44 – 7.16 (m, 4H), 2.50 – 2.43 (m, 2H), 2.17 (s, 3H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.57 (p, *J* = 7.6, 7.1 Hz, 2H), 1.48 (p, *J* = 7.2 Hz, 2H), 1.34 – 1.20 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.6, 169.0, 156.7, 153.8, 152.5, 136.1, 136.0, 130.5, 130.2, 128.7, 127.2, 126.3, 115.8, 111.9, 35.3, 32.3, 28.4, 25.2, 25.1, 17.9. LRMS (ESI) *m/z* 428.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{21}H_{25}N_5O_3S$ $[M-H]^-$

calcd 426.1605, found 426.1594.

4.6.2.

***N*[1]-(4-((4-fluoro-2-methylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*[8]-hydroxy octanediamide 9b**

Compound **9b** was prepared from **8b** according the same process described for **9a** in 51% yield. Mp 196.1-197.4 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.32 (s, 1H), 10.09 (s, 1H), 9.51 (s, 1H), 8.65 (s, 1H), 8.49 (s, 1H), 8.23 (s, 1H), 7.34 (dd, *J* = 8.7, 5.6 Hz, 1H), 7.20 (dd, *J* = 9.7, 3.0 Hz, 1H), 7.09 (td, *J* = 8.5, 3.0 Hz, 1H), 2.46 (t, *J* = 7.4 Hz, 2H), 2.16 (s, 3H), 1.93 (t, *J* = 7.4 Hz, 2H), 1.56 (p, 2H), 1.48 (p, 2H), 1.35 – 1.19 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.1, 169.5, 161.2 (d, *J* = 243.3 Hz), 157.3, 154.2, 152.9, 139.4 (d, *J* = 8.52 Hz), 132.9, 131.1 (d, *J* = 9.57 Hz), 130.7, 117.2 (d, *J* = 22.2 Hz), 116.3, 113.4 (d, *J* = 22.3 Hz), 112.3, 40.5, 35.7, 32.7, 28.9, 25.6, 25.5, 18.4. LRMS (ESI) *m/z* 446.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{21}H_{24}FN_5O_3S$ $[M-H]^-$ calcd 444.1511, found 444.1516

4.6.3.

***N*[1]-(4-((2,4-difluorophenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*[8]-hydroxyoctane diamide 9c**

Compound **9c** was prepared from **8c** according the same process described for **9a** in 40% yield. Mp 206.7-208.3 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 10.13 (s, 1H), 9.69 (s, 1H), 8.63 (s, 1H), 8.51 (s, 1H), 8.30 (s, 1H), 7.61 – 7.48 (m, 1H), 7.44 – 7.30 (m, 1H), 7.22 – 7.06 (m, 1H), 1.92 (t, *J* = 7.4 Hz, 2H), 1.56 (p, *J* = 6.4 Hz, 2H), 1.47 (p, *J* = 7.2 Hz, 2H), 1.34 – 1.18 (m, 6H). ^{13}C NMR (125 MHz,

DMSO-d_6) δ 172.2, 169.5, 160.8 (dd, *J* = 246.7, 11.1 Hz), 158.0 (dd, *J* = 249.5, 12.7 Hz), 156.6, 154.1, 152.9, 130.8, 130.7 (d, *J* = 10.0 Hz), 122.7(dd, *J* =13.0, 2.8 Hz), 111.9 (d, *J* = 22.7 Hz), 116.3, 113.1, 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 450.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{20}H_{21}ClFN_5O_3S$ $[M-H]^-$ calcd 464.0965, found 464.0962.

4.6.4.

***N*^{1}-(4-((4-chloro-2-fluorophenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*^{8}-hydroxyoctanediamide 9d**

Compound **9d** was prepared from **8d** according the same process described for **9a** in 42% yield. Mp 209.1-211.0 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 1H), 10.15 (s, 1H), 9.78 (s, 1H), 8.63 (s, 1H), 8.53 (s, 1H), 8.32 (s, 1H), 7.56 (t, *J* = 8.5 Hz, 2H), 7.33 (d, *J* = 9.7 Hz, 1H), 1.92 (t, *J* = 7.4 Hz, 2H), 1.56 (p, *J* = 7.5 Hz, 2H), 1.47 (p, *J* = 8.1, 7.5 Hz, 2H), 1.35 – 1.16 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.5, 157.3 (d, *J* = 251.0 Hz), 156.2, 154.0, 152.9, 131.0 (d, *J* = 7.6 Hz), 130.8, 130.1, 125.8 (d, *J* = 10.2 Hz), 125.1, 117.1 (d, *J* = 24.0 Hz).116.3, 113.5, 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 466.0 $[M+H]^+$, HRMS (ESI) m/z for $C_{20}H_{21}ClFN_5O_3S$ $[M-H]^-$ calcd 464.0965, found 464.0962.

4.6.5.

***N*^{1}-(4-((2-fluoro-5-methylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*^{8}-hydroxyoctanediamide 9e**

Compound **9e** was prepared from **8e** according the same process described for **9a** in 56% yield. Mp 200.0-201.8 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 10.32 (s, 1H),

10.15 (s, 1H), 9.73 (s, 1H), 8.68 (s, 1H), 8.53 (s, 1H), 8.30 (s, 1H), 7.32 (dd, *J* = 7.7, 2.2 Hz, 1H), 7.20 (dd, *J* = 10.1, 8.4 Hz, 1H), 7.17 – 7.08 (m, 1H), 2.48 (d, *J* = 8.0 Hz, 2H), 2.32 (s, 3H), 1.94 (t, *J* = 7.3 Hz, 2H), 1.59 (p, *J* = 7.2 Hz, 2H), 1.49 (p, *J* = 7.2 Hz, 2H), 1.35 – 1.20 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.1, 169.6, 156.0 (d, *J* = 245.5 Hz), 156.6, 154.10, 152.9, 134.1 (d, *J* = 3.5 Hz), 130.8, 129.6, 128.6 (d, *J* = 7.5 Hz), 125.5 (d, *J* = 12.6 Hz), 116.2, 116.1 (d, *J* = 20.2 Hz), 113.1, 35.7, 32.7, 28.9, 25.6, 25.5, 20.7. LRMS (ESI) *m/z* 446.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{21}H_{24}FN_5O_3S$ $[M-H]^-$ calcd 444.1511, found 444.1504.

4.6.6.

***N*^{1}-hydroxy-*N*^{8}-(4-((4-methoxy2-methylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)octanediamide 9f**

Compound **9f** was prepared from **8f** according the same process described for **9a** in 34% yield. Mp 190.0-191.8 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.31 (s, 1H), 10.03 (s, 1H), 9.39 (s, 1H), 8.64 (d, *J* = 1.7 Hz, 1H), 8.45 (s, 1H), 8.15 (s, 1H), 7.19 (d, *J* = 8.6 Hz, 1H), 6.89 (d, *J* = 2.9 Hz, 1H), 6.80 (dd, *J* = 8.6, 2.9 Hz, 1H), 3.77 (s, 3H), 2.44 (t, *J* = 7.4 Hz, 2H), 2.10 (s, 3H), 1.91 (t, *J* = 7.4 Hz, 2H), 1.53 (p, *J* = 7.2 Hz, 2H), 1.45 (p, *J* = 7.1 Hz, 2H), 1.32 – 1.20 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.0, 169.6, 158.9, 157.8, 154.3, 152.9, 138.6, 130.9, 130.5, 129.0, 116.5, 115.9, 112.0, 55.7, 35.7, 32.7, 28.9, 25.6, 25.5, 18.5. LRMS (ESI) *m/z* 458.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{22}H_{27}N_5O_4S$ $[M-H]^-$ calcd 456.1711, found 456.1713.

4.6.7.

***N*^{1}-(4-((3-fluorophenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*^{8}-hydroxyoctanedia**

mide 9g

Compound **9g** was prepared from **8g** according the same process described for **9a** in 56% yield. Mp 213.1-214.8 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (d, 1H), 10.24 (s, 1H), 9.95 (s, 1H), 8.72 (s, 1H), 8.68 (d, *J* = 1.7 Hz, 1H), 8.38 (s, 1H), 7.91 (dt, *J* = 12.0, 2.3 Hz, 1H), 7.62 (ddd, *J* = 8.3, 2.0, 0.9 Hz, 1H), 7.40 (td, *J* = 8.3, 6.9 Hz, 1H), 6.93 (tdd, *J* = 8.4, 2.6, 0.9 Hz, 1H), 2.48 (d, *J* = 4.2 Hz, 2H), 1.93 (t, *J* = 7.4 Hz, 2H), 1.58 (p, *J* = 7.3 Hz, 2H), 1.48 (p, *J* = 7.2 Hz, 2H), 1.34 – 1.22 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 162.5 (d, *J* = 240.9 Hz), 155.3, 153.7, 152.9, 141.5 (d, *J* = 11.3 Hz), 130.9, 130.5 (d, *J* = 9.6 Hz), 117.6, 117.6, 116.4, 114.3, 110.2 (d, *J* = 21.1 Hz), 108.7 (d, *J* = 26.2 Hz), 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 432.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{20}H_{22}FN_5O_3S$ $[M-H]^-$ calcd 430.1355, found 430.1358.

4.6.8.

***N*^{1}-(4-((3-chlorophenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*^{8}-hydroxyoctanedia**

mide 9h

Compound **9h** was prepared from **8h** according the same process described for **9a** in 56% yield. Mp 191.8-193.0 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 10.21 (s, 1H), 9.92 (s, 1H), 8.72 (s, 1H), 8.66 (s, 1H), 8.38 (s, 1H), 8.09 (t, *J* = 2.1 Hz, 1H), 7.77 (ddd, *J* = 8.3, 2.1, 1.0 Hz, 1H), 7.39 (t, *J* = 8.1 Hz, 1H), 7.22 – 7.04 (m, 1H), 2.50 (t, 2H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.58 (p, *J* = 7.2 Hz, 2H), 1.49 (p, *J* = 7.2 Hz, 2H), 1.37 – 1.22 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.2, 153.7, 152.9, 141.2, 133.3, 130.9, 130.6, 123.4, 121.4, 120.3, 116.4, 114.3, 35.7, 32.7,

28.9, 25.6, 25.5. LRMS (ESI) *m/z* 448.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{20}H_{22}ClN_5O_3S$ $[M-H]^-$ calcd 446.1059, found 446.1066.

4.6.9.

N^1-(4-((3-bromophenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-N^8-hydroxyoctanediamide 9i

Compound **9i** was prepared from **8i** according the same process described for **9a** in 59% yield. Mp 204.8-206.0 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H), 10.22 (s, 1H), 9.91 (s, 1H), 8.72 (s, 1H), 8.67 (s, 1H), 8.38 (s, 1H), 8.22 (t, *J* = 2.0 Hz, 1H), 7.87 – 7.77 (m, 1H), 7.34 (t, *J* = 8.0 Hz, 1H), 7.28 (dt, *J* = 8.1, 1.4 Hz, 1H), 2.49 (t, *J* = 7.2 Hz, 2H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.64 – 1.54 (m, 2H), 1.53 – 1.43 (m, 2H), 1.33 – 1.22 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.2, 153.7, 152.9, 141.3, 130.9, 126.3, 124.2, 121.7, 120.7, 116.4, 114.3, 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 492.0 $[M+H]^+$, HRMS (ESI) m/z for $C_{20}H_{23}BrN_5O_3S$ $[M+H]^+$ calcd 492.0699, found 492.07134.

4.6.10.

N^1-(4-((3-ethylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-N^8-hydroxyoctanediamide 9j

Compound **9j** was prepared from **8j** according the same process described for **9a** in 70% yield. Mp 184.9-186.1 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ 10.33 (d, *J* = 1.8 Hz, 1H), 10.16 (s, 1H), 9.72 (s, 1H), 8.66 (d, *J* = 1.7 Hz, 1H), 8.63 (s, 1H), 8.32 (s, 1H), 7.66 – 7.61 (m, 1H), 7.59 (t, *J* = 1.9 Hz, 1H), 7.28 (t, *J* = 7.8 Hz, 1H), 6.99 (dt, *J* = 7.5, 1.3 Hz, 1H), 2.48 (t, *J* = 7.4 Hz, 2H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.58 (p, 2H), 1.49

(p, 2H), 1.35 – 1.24 (m, 4H), 1.21 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.7, 153.9, 152.7, 144.6, 139.3, 130.9, 128.9, 123.8, 122.2, 120.4, 116.0, 113.8, 35.7, 32.7, 28.9, 28.7, 25.6, 25.5, 16.0. LRMS (ESI) *m/z* 442.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{22}H_{27}N_5O_3S$ $[M-H]^-$ calcd 440.1762, found 440.1761.

4.6.11.

***N*^{1}-hydroxy-*N*^{8}-(4-((3-isopropylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)octane diamide 9k**

Compound **9k** was prepared from **8k** according the same process described for **9a** in 74% yield. Mp 182.8-184.2 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 10.16 (s, 1H), 9.73 (s, 1H), 8.72 – 8.55 (m, 2H), 8.32 (s, 1H), 7.68 (d, *J* = 7.7 Hz, 1H), 7.58 (s, 1H), 7.29 (t, *J* = 7.8 Hz, 1H), 7.02 (d, *J* = 7.4 Hz, 1H), 2.97 – 2.81 (m, 1H), 1.94 (t, *J* = 7.2 Hz, 2H), 1.66 – 1.54 (m, 2H), 1.54 – 1.41 (m, 2H), 1.37 – 1.12 (m, 12H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.7, 153.9, 152.7, 149.2, 139.2, 130.9, 128.8, 122.4, 120.8, 120.5, 116.0, 113.8, 35.7, 33.9, 32.7, 28.9, 25.6, 25.5, 24.3. LRMS (ESI) *m/z* 456.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{23}H_{29}N_5O_3S$ $[M-H]^-$ calcd 454.1918, found 454.1924.

4.6.12.

***N*^{1}-(4-((3-*tert*-butylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-*N*^{8}-hydroxyoctane diamide 9l**

Compound **9l** was prepared from **8l** according the same process described for **9a** in 71% yield. Mp 159.6-160.3 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H), 10.16 (s, 1H), 9.73 (s, 1H), 8.65 (d, *J* = 14.4 Hz, 2H), 8.33 (s, 1H), 7.84 – 7.57 (m,

2H), 7.30 (t, *J* = 7.9 Hz, 1H), 7.17 (d, *J* = 7.8 Hz, 1H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.65 – 1.54 (m, 2H), 1.54 – 1.44 (m, 2H), 1.39 – 1.19 (m, 15H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.7, 153.9, 152.7, 151.5, 139.0, 130.9, 128.6, 121.2, 120.1, 119.8, 116.0, 113.8, 35.7, 34.9, 32.7, 31.6, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 470.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{24}H_{31}N_5O_3S$ $[M-H]^-$ calcd 468.2075, found 468.2079.

4.6.13.

N^1-(4-((3-ethynylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-N^8-hydroxyoctanediamide 9m

Compound **9m** was prepared from **8m** according the same process described for **9a** in 50% yield. Mp 214.6-216.1 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.32 (s, 1H), 10.19 (s, 1H), 9.83 (s, 1H), 8.68 (s, 1H), 8.65 (d, *J* = 1.4 Hz, 1H), 8.35 (d, *J* = 0.5 Hz, 1H), 8.03 (t, *J* = 1.8 Hz, 1H), 7.81 (ddd, *J* = 8.3, 2.2, 1.0 Hz, 1H), 7.37 (dd, *J* = 8.3, 7.5 Hz, 1H), 7.20 (dt, *J* = 7.7, 1.3 Hz, 1H), 4.19 (s, 1H), 2.47 (t, *J* = 4.9 Hz, 2H), 1.92 (t, *J* = 7.4 Hz, 2H), 1.63 – 1.53 (m, 2H), 1.52 – 1.42 (m, 2H), 1.35 – 1.20 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.4, 153.8, 152.8, 139.8, 130.9, 129. 5, 127.1, 125.0, 122.8, 122.3, 116.3, 114.2, 84.0, 81.1, 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 438.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{22}H_{23}N_5O_3S$ $[M-H]^-$ calcd 436.1449, found 436.1447.

4.6.14.

N^1-(4-((3-cyanophenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-N^8-hydroxyoctanediamide 9n

Compound **9n** was prepared from **8n** according the same process described for **9a** in 48% yield. Mp 199.1-200.8 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H), 10.25 (s, 1H), 10.08 (s, 1H), 8.75 (s, 1H), 8.69 (s, 1H), 8.43 (d, *J* = 12.8 Hz, 2H), 8.10 (d, *J* = 7.8 Hz, 1H), 7.65 – 7.48 (m, 2H), 1.95 (t, *J* = 7.7 Hz, 2H), 1.60 (p, *J* = 7.5 Hz, 2H), 1.50 (p, *J* = 7.4 Hz, 2H), 1.39 – 1.18 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.5, 155.2, 153.7, 153.0, 140.6, 131.0, 130.4, 127.1, 126.4, 124.6, 119.3, 116.6, 114.4, 111.8, 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 437.1 $[M-H]^-$, HRMS (ESI) m/z for $C_{21}H_{22}N_6O_3S$ $[M-H]^-$ calcd 437.1401, found 437.1409.

4.6.15.

N^1-hydroxy-N^8-(4-((3-methoxyphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)octanediamide 9o

Compound **9o** was prepared from **8o** according the same process described for **9a** in 60% yield. Mp 188.6-189.7 ^{o}C; ^{1}H NMR (400 MHz, Methanol-d_4) δ 10.33 (s, 1H), 10.17 (s, 1H), 9.75 (s, 1H), 8.66 (s, 2H), 8.34 (s, 1H), 7.62 – 7.46 (m, 1H), 7.46 – 7.37 (m, 1H), 7.28 (t, *J* = 8.1 Hz, 1H), 6.78 – 6.63 (m, 1H), 3.78 (s, 3H), 2.48 (t, 2H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.59 (p, *J* = 7.2 Hz, 2H), 1.49 (p, 2H), 1.38 – 1.21 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 169.6, 155.7, 153.9, 152.7, 139.2, 138.2, 130.9, 128.8, 125.0, 123.4, 120.1, 116.0, 113.9, 35.7, 32.7, 28.9, 25.6, 25.5, 21.7. LRMS (ESI) *m/z* 444.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{21}H_{25}N_5O_4S$ $[M-H]^-$ calcd 442.1554, found 442.1551.

4.6.16.

N^1-(4-((4-ethylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)-N^8-hydroxyoctanediam

ide 9p

Compound **9p** was prepared from **8p** according the same process described for **9a** in 71% yield. Mp 180.4-181.6 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 10.14 (s, 1H), 9.70 (s, 1H), 8.65 (s, 1H), 8.59 (s, 1H), 8.31 (s, 1H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.21 (d, *J* = 8.5 Hz, 2H), 2.61 (q, *J* = 7.6 Hz, 2H), 2.50 – 2.45 (m, 2H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.64 – 1.54 (m, 2H), 1.52 – 1.43 (m, 2H), 1.34 – 1.24 (m, 4H), 1.20 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.1, 169.6, 155.7, 153.9, 152.7, 139.9, 136.8, 130.9, 128.2, 123.2, 115.9, 113.7, 35.7, 32.7, 28.9, 28.1, 25.6, 25.5, 16.2. LRMS (ESI) *m/z* 442.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{22}H_{27}N_5O_3S$ $[M-H]^-$ calcd 440.1762, found 440.1762.

4.6.17.

***N*^{1}-hydroxy-*N*^{8}-(4-((4-isopropylphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)octane diamide 9q**

Compound **9q** was prepared from **8q** according the same process described for **9a** in 68% yield. Mp 154.0-156.3 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 10.13 (s, 1H), 9.70 (s, 1H), 8.65 (s, 1H), 8.59 (s, 1H), 8.31 (s, 1H), 7.69 – 7.61 (m, 2H), 7.28 – 7.21 (m, 2H), 2.94 – 2.85 (m, 1H), 2.50 – 2.46 (m, 2H), 1.94 (t, *J* = 7.4 Hz, 2H), 1.65 – 1.54 (m, 2H), 1.53 – 1.43 (m, 2H), 1.35 – 1.25 (m, 2H), 1.22 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.1, 169.5, 155.7, 153.9, 152.6, 144.5, 136.9, 130.9, 126.7, 115.9, 113.7, 35.7, 33.41, 32.7, 28.9, 25.6, 25.5, 24.5. LRMS (ESI) *m/z* 456.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{23}H_{29}N_5O_3S$ $[M-H]^-$ calcd 454.1918, found 454.1919.

4.6.18.

N^1-hydroxy-N^8-(4-((4-methoxyphenyl)amino)thieno[3,2-*d*]pyrimidin-6-yl)octanediamide 9r

Compound **9r** was prepared from **8r** according the same process described for **9a** in 59% yield. Mp 222.2-223.9 ^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (s, 1H), 10.12 (s, 1H), 9.65 (s, 1H), 8.75 (s, 1H), 8.55 (s, 1H), 8.28 (s, 1H), 7.69 – 7.46 (m, 2H), 7.11 – 6.78 (m, 2H), 3.77 (s, 3H), 2.50 – 2.42 (m, 2H), 1.93 (t, J = 7.4 Hz, 2H), 1.64 – 1.53 (m, 2H), 1.53 – 1.43 (m, 2H), 1.36 – 1.19 (m, 4H). ^{13}C NMR (125 MHz, DMSO) δ 172.1, 169.5, 156.7, 156.1, 154.0, 152.6, 131.8, 130.8, 125.5, 115.9, 114.2, 113.3, 55.7, 35.7, 32.7, 28.9, 25.6, 25.5. LRMS (ESI) *m/z* 444.1 $[M+H]^+$, HRMS (ESI) m/z for $C_{21}H_{24}N_5O_4S$ $[M-H]^-$ calcd 442.1554, found 442.1539.

4.7. Biological evaluation

4.7.1. Materials

The antibodies for Histone H_3, α-tubulin and GAPDH were purchased from Cell Signaling Technology. Acetyl-Histone H_3 was purchased from Merck Millipore. Acetyl-α-Tubulin was purchased from Sigma-aldrich. Both anti-Rabbit and anti-mouse IgG (H+L), DyLight 800 labeled secondary antibodies were purchased from KPL (Kirkegaard & Perry Laboratories. Inc), PVDF transfer membrane was purchased from Amersham Biosciences corporation

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4.7.2. Cell culture and Cell Viability Assay.

Human multiple myeloma (MM) cell line RPMI 8226 was gifted from Professor Jian Hou (Chang Zheng Hospital, Shanghai, China). RPMI 8226 cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS). Human HCT 116 colon cancer cells were obtained from the Cell Bank (Chinese Academy of Sciences, Shanghai, China). HCT 116 cells were cultured in 5A medium supplemented with 10% Fetal Bovine Serum (FBS). Cells were maintained in a humidified 37°C/5% CO_2 incubator.

4.7.3. *In vitro* HDAC activity assay

All three full-length recombinant human HDACs (rhHDACs)1, 3 and 6 were expressed in insect High5 cells using a baculoviral expression system, and all His_6-tagged and GST-fusion proteins was purified using Ni-NTA (QIAGEN). The deacetylase activity of rhHDACs 1 and 3 were assayed with a HDAC substrate (Ac-Lys-Tyr-Lys(ε-acetyl)-AMC), and HDAC6 was assayed with another HDAC substrate (Boc-Lys(ε-acetyl)-AMC). The total HDAC assay volume was 25 μL and all the assay components were diluted in Hepes buffer (25 mM Hepes, 137 mM NaCl, 2.7 mM KCl and 4.9 mM $MgCl_2$, pH 8.0). The reaction was carried out in black 384-well plates (OptiPlateTM-384F, PerkinElmer). In brief, the HDAC assay mixture contained the substrate (5-50 μM, 5 μL), rhHDAC isoforms (20-200 nM) and inhibitors (1 μL). Positive controls contained all the above components except the inhibitors. The negative controls contained neither enzymes nor inhibitors. The HDAC6 assay components were incubated at room temperature for 3 h, and HDAC1 or 3 were incubated for 24 h. The reaction was quenched with the addition of 25 μL.

Trypsin with the final concentration of 0.3125%. After 30 min incubation at room temperature, the 384 micro-well plates were read at wavelengths 355 nm(excitation) and 460 nm(emission) using Envision (PerkinElmer). Each experiment was done in triplicate.

4.7.4. Cell proliferation assay

RPMI 8226 cells were seeded in 96-well plates (5000 cells/well), then treated with compounds for 72 h. HCT 116 cells (3000 cells/well) were plated in a 96-well plate overnight, then treated with compounds for 72 h. Cells were measured with CellTiter 96® AQueous non-radioactive cell proliferation assay by Promega. Data analysis was performed with GraphPad Prism 5.0. Each experiment was done in triplicate.

4.7.5. Western blot analysis

1×10^6 RPMI 8226 cells were seeded in a 6-well plate. Then cells were treated with compounds for 24 h, cells were lysed by boiling in SDS buffer. Proteins were analyzed by Western blot.

20-100 μg of protein per lane was loaded onto a Tricine–SDS-PAGE and then transferred to a PVDF membrane. The membrane was incubated with the following primary antibodies: anti-tubulin, anti-ac tubulin, anti-GAPDH, anti-H_3 and anti-ac H_3 at 4°C overnight. Then, the membrane was washed with TBST three times and incubated with Anti-Rabbit or mouse IgG (H+L). DyLight 800 labeled secondary antibody for 60 min at room temperature. After three times washing with TBST, the immunoblots were visualized by Odyssey® Infrared Imaging System (LI-COR Biosciences).

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Table 1. Structures and *in vitro* inhibitory activity against HDACs of compounds **9a-r**

Table 2. *In vitro* anti-proliferative activity against RPMI 8226 and HCT 116 cells of all compounds.

Scheme 1. Reagents and conditions: a) microwave, 4-5 mins; b) H_2SO_4/HNO_3, 0 ^oC-r.t; c) $POCl_3$, TEA, CH_3CN, 85 ^oC; d) various substituted aniline, 1,4-dioxane, 85 ^oC; e) Fe, $NH_4Cl_{(aq)}$, EtOH, 50 ^oC, 2 h; f) $ROCO(CH_2)_4COCl$, DIPEA, THF, R = Methyl or Ethyl; g) $NH_2OH{\cdot}HCl$, KOH, MeOH-THF, 0 ^oC-r.t.

Fig. 1. Design strategy and modification of novel HDACs inhibitors.

Fig. 2. Western blot analysis of Ac-tubulin and Ac-histone H3 from RPMI 8226 cell cultured for 24 h with DMSO control and compounds **9m**, **9n**, **9o**, and SAHA.