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Research Letter

Synthesis and Evaluation of 2-Alkylthio-4-(*N*-substituted sulfonamide)pyrimidine Hydroxamic Acids as Anti-myeloma Agents

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A series of pyrimidine hydroxamic acids with a sulfide substituent at the second position and a sulfonamide substituent at the fourth position have been synthesized and evaluated for their activity against human myeloma cell line RPMI 8226. Several compounds exhibited significant anti-cancer potency. It was found that representative compound 6a selectively killed cancerous but not normal cells. Moreover, compound 6a was effective in causing apoptosis in RPMI 8226 cells and exhibited promising HDAC-inhibitory activities.

Key words: anti-cancer activity, apoptosis, histone acetylation, multiple myeloma, pyrimidine hydroxamic acid, sulfide substituent

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Cancer is a group of different diseases characterized by uncontrolled growth and spread of abnormal cells (1). Multiple myeloma accounts for approximately 1% of all cancers and 10% of hematologic malignancies (2). Despite the fact that the significant advances have achieved in the last two decade with the discovery of novel agents such as proteasome inhibitors (bortezomib) and immunomodulatory drugs (thalidomide and lenalidomide), the disease remains incurable (3). The continued commitment to the arduous task of discovering non-traditional, efficient, and safe anti-myeloma agents remains critically important.

Pyrimidine moiety, as a structural component of several key biomolecules, has attracted great attention from organic and medicinal chemists (4–7). Recently, we have designed and synthesized a series of novel pyrimidine-based compounds (8–16). To explore our compounds as potential anti-myeloma agents, we have screened our pyrimidine-based library and identified 2-alkylthio-4-(*N*-substituted sulfonamide)pyrimidine hydroxamic acids with anti-myeloma activity *in vitro*. Moreover, the presence of *N*-substituted sulfonamido substituent in the pyrimidine ring is essential to achieve activity. These results are different from the previously reported 2-aminopyrimidine hydroxamic acids as anti-cancer HDAC inhibitors (such as JNJ-16241199, Figure 1) (17–20). To the best of our knowledge, there is no report of 2-alkylthiopyrimidine hydroxamic acids as anti-cancer agents. Herein, the synthesis and evaluation of the novel 2-alkylthio-4-(*N*-substituted sulfonamide)pyrimidine hydroxamic acids as anti-myeloma agents are presented.

R^1 substituted pyrimidine hydroxamic acids **6a–i** were synthesized by the method shown in Scheme 1. The compound **1** was reacted with *N*-benzyl-4-methoxybenzenesulfonamide in the presence of K_2CO_3 as a base to afford ester **2**. Hydrolysis of **2** afforded acid **3**. Intermediate **3** was converted to hydroxamic acid **4** in a two-step process involving formation of an activated acyl chloride followed by addition of hydroxylamine (21). Oxidation of **4** with *m*-CPBA gave the sulfoxide intermediate **5**. Final pyrimidine hydroxamic acids **6a–i** were obtained by reacting sulfoxide **5** with various nucleophiles (R^1H).

R^2 substituted pyrimidine hydroxamic acids **6j–s** were prepared by the method shown in Scheme 2. Compound **7** was reacted with thiourea in the presence of NaOEt as a base to afford pyrimidine **8** (22). Condensation of **8** with 1-iodopentane formed **9**. Chlorination of **9** using phosphoryl chloride yielded the desired **10**. Coupling of **10** with nucleophile (R^2H) gave intermediate **11**. Intermediate **11l** (R^2=H) could be obtained from starting material **1**. Reduction of **1** with Zn-NH_4Cl furnished **12**. Oxidation of **12** with *m*-CPBA gave the sulfone **13**. Condensation of **13** with pentane-1-thiol formed intermediate **11l**. Hydrolysis of **11** afforded acid **14**. Intermediate **14** was converted to hydroxamic acid **6** (**6k–p** and **6r**) in a two-step process involving formation of

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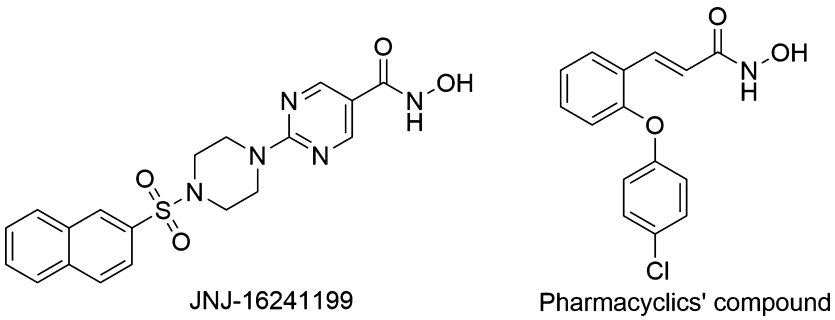
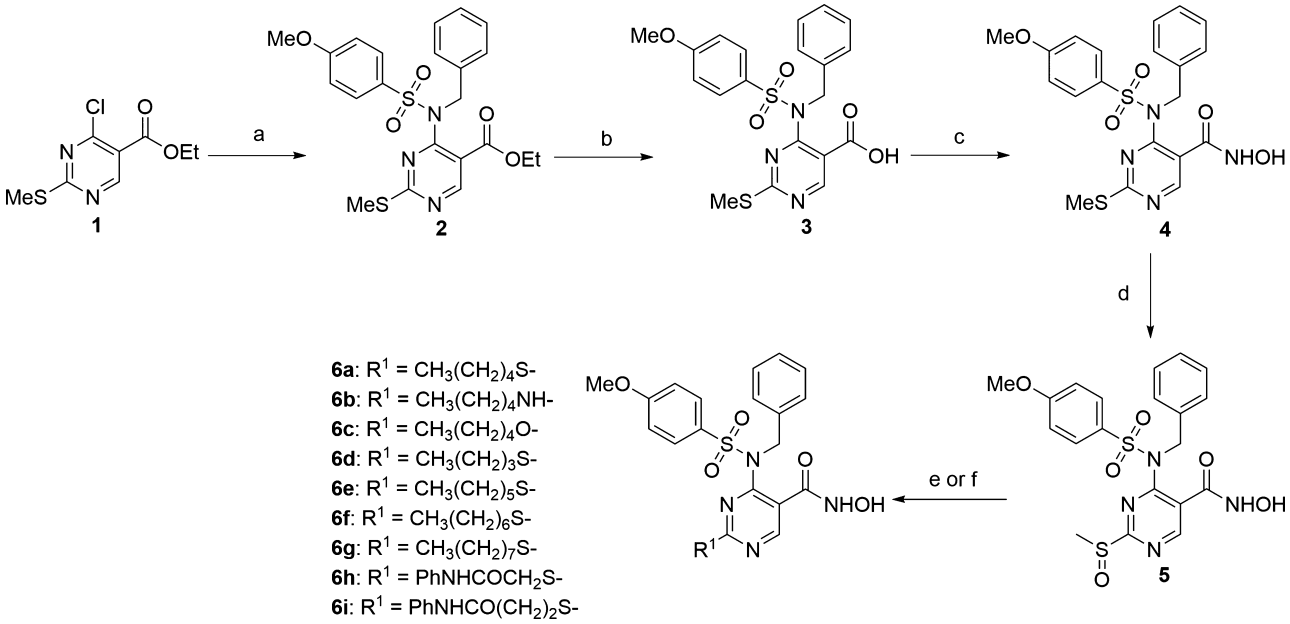


Figure 1: Hydroxamic acid derivatives as anti-cancer HDAC inhibitors.

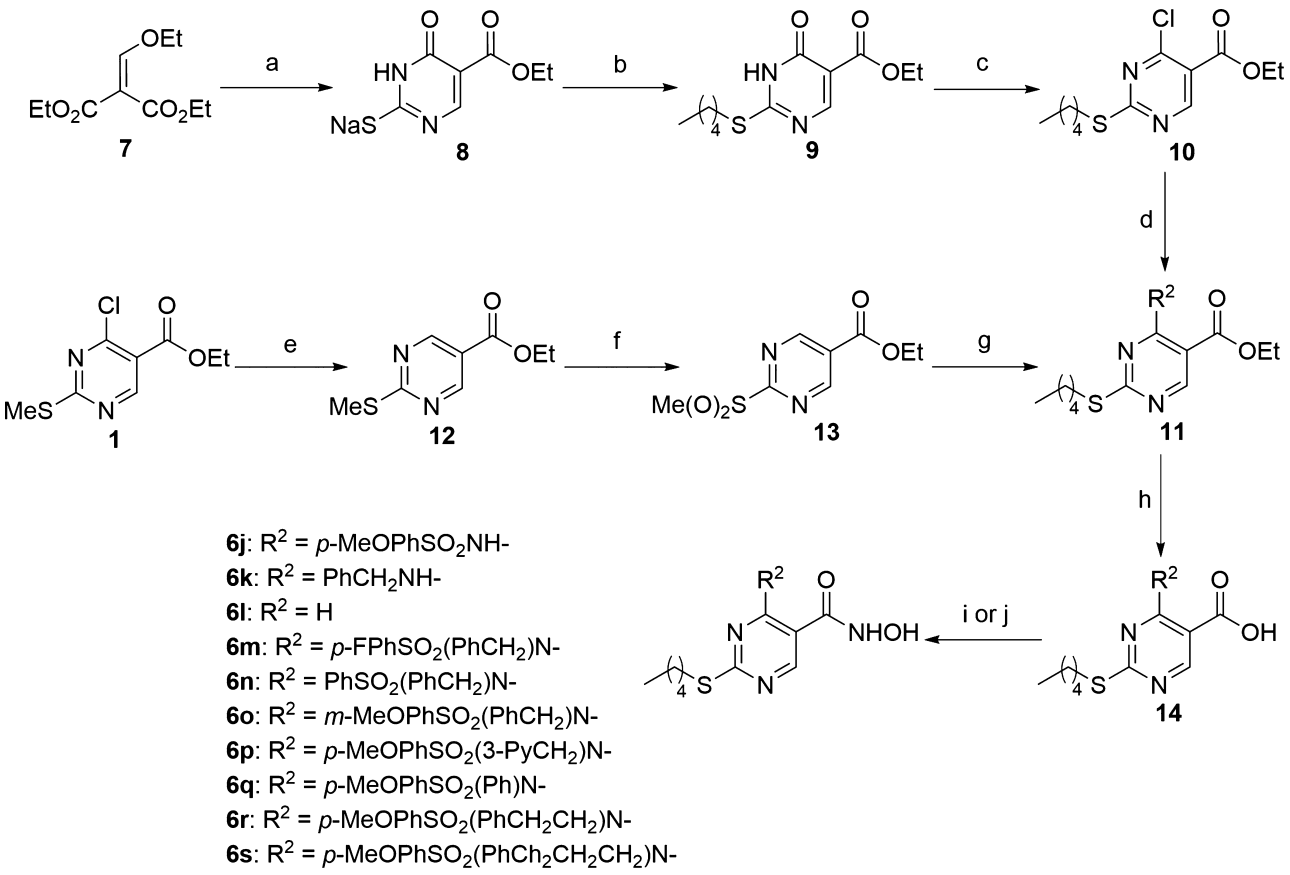


Scheme 1: Synthesis of compounds **6a–i**. Reagents and conditions: (a) *N*-benzyl-4-methoxybenzenesulfonamide, K_2CO_3, CH_3CN, reflux, 64%; (b) NaOH aq, 1,4-dioxane, 50 °C, 98%; (c) (i) $(COCl)_2$, DMF, CH_2Cl_2, rt, (ii) NH_2OH•HCl, Et_3N, DMF, CH_2Cl_2, 0 °C to rt, 60% for two steps; (d) *m*-CPBA, CH_2Cl_2, rt, 65%; (e) thiol or pentan-1-amine, Et_3N, CH_3CN, rt, 32–60%; (f) pentan-1-ol, NaH, rt, 28%.

an activated acyl chloride followed by addition of hydroxylamine. Alternatively, amidation of **14** with commercially available NH_2OTHP and removal of the tetrahydropyranyl (THP) protecting moiety in acidic conditions led to the final pyrimidine hydroxamic acid **6** (**6j**, **6q** and **6s**) (20).

The α,β-unsaturated hydroxamic acid **6t** was prepared by the method shown in Scheme 3. Initially, reduction of **10** with $LiAlH_4$ gave the alcohol **15** in only 14% yield. Treatment of **10** with DIBAL-H formed the alcohol **15** in good yield. Oxidation of **15** with MnO_2 gave the aldehyde **16**. Coupling of **16** with *N*-benzyl-4-methoxybenzenesulfonamide followed by Wittig olefination yielded the ester **18** (23). Hydrolysis of **18** afforded acid **19**. Intermediate **19** was converted to hydroxamic acid **6t** in a two-step process involving formation of an activated acyl chloride followed by addition of hydroxylamine.

Anti-proliferative activities of the pyrimidine hydroxamic acids were evaluated using the human myeloma cell line RPMI 8226 as an experimental model. As shown in Table 1, our data demonstrated that among 20 compounds tested, nine compounds (**6a**, **6e–i**, **6m**, **6o** and **6s)** exhibited significant anti-proliferative activity with IC_{50} below 10 μM. To our knowledge, this is the first example of 2-alkylthiopyrimidine hydroxamic acids as anti-cancer agents. It was observed that compound **6a** is more potent than both nitrogen and oxygen analog (entry 1 versus entries 2 and 3), indicating that the thiol linker at the C-2 position is essential to enhance growth inhibitory effects. Moreover, compounds **6a** (entry 1) and **6e–i** (entries 5–9) showed better growth inhibition compared with **6d** (entry 4), suggesting that the length of R^1-group had effect on activities. It was noteworthy that the presence of a *N*-substituted sulfonamido substituent (R^2) in the pyrimidine ring

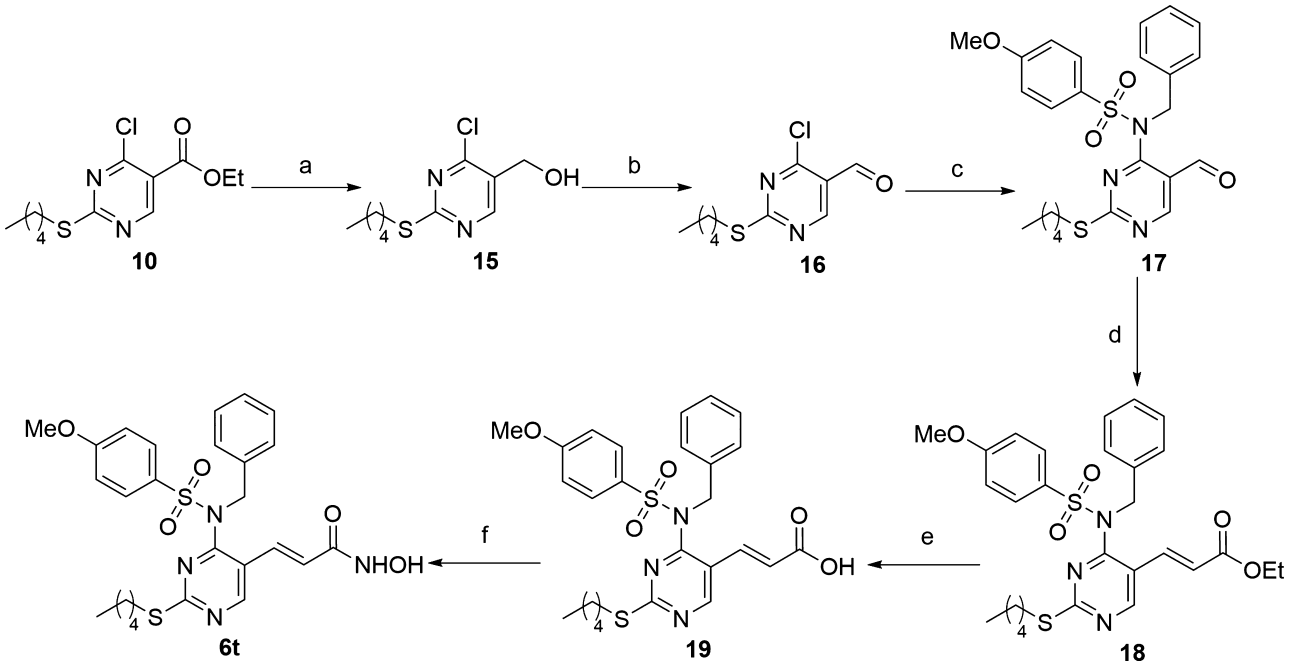


Scheme 2: Synthesis of compounds **6j**–**s**. Reagents and conditions: (a) thiourea, EtONa, EtOH, reflux, 98%; (b) $CH_3(CH_2)_4I$, NaI, tetrabutyl ammonium bromide, CH_3Cl_3, H_2O, 50 °C, 53%; (c) $POCl_3$, reflux, 89%; (d) nucleophile (R^2H), K_2CO_3, CH_3CN, reflux, 34–83%; (e) Zn powder, NH_4Cl aq, toluene, 80 °C, 56%; (f) *m*-CPBA, CH_2Cl_2, rt, 76%; (g) $CH_3(CH_2)_4SH$, Et_3N, CH_3CN, rt, 89%; (h) NaOH aq, 1,4-dioxane, 50 °C, 72–99%; (i) (i) $(COCl)_2$, DMF, CH_2Cl_2, rt, (ii) NH_2OH•HCl, Et_3N, THF, H_2O, 0 °C to rt, 7–93% for two steps; (j) (i) NH_2OTHP [*O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine], EDC•HCl, Et_3N, HOBt, CH_2Cl_2, THF, rt, (ii) CF_3CO_2H, CH_2Cl_2, MeOH, rt, 28–35% for two steps.

appears to be essential to achieve activity (entry 1 versus entries 10–12). These results are different from the previously reported 2-aminopyrimidine hydroxamic acids as anti-cancer HDAC inhibitors where the R^2-group is hydrogen atom (such as JNJ-16241199, Figure 1) (17–20). Further studies were focused on the effects of the sulfonamide moiety at the C-4 position. Compound **6m** with a *p*-fluoro group (entry 13) showed slightly more active than compound **6a**. Removal of *p*-methoxy of compound **6a** led to decreased inhibitory activity (**6n**, entry 14). Among all the tested compounds, compound **6o** with an *m*-methoxy group was found to be the most active with the IC_{50} value of 5.6 μM (entry 15). Replacement of the phenyl ring by a pyridyl group led to decreased inhibitory activity (**6p** versus **6a**, entry 16). Removal (**6q** versus **6a**, entry 17) or insertion (**6r** versus **6a**, entry 18) of a methylene spacer between the amino and phenyl ring reduced the activity; however, the insertion of a longer chain, as in **6s** (entry 19), enhanced the activity. It was unexpected that the belinostat and panobinostat (6) analog **6t** is not substantially toxic to RPMI 8226 cells (entry 20). In consistent to the data published by others (24), the positive control compound SAHA has an IC_{50} values of 1.0 μM.

In addition, the anti-cancer activity of representative compound **6a** against MM1S was further tested, and the preliminary result indicated that the IC_{50} value was 7.5 μM. It was interesting to note that representative compound **6a** did not present any detectable cytotoxicity against normal peripheral blood mononuclear cell (PBMC) for the tested concentrations from 0.078 to 20 μM (Figure 2), suggesting that compound **6a** is a potent anti-myeloma agent that selectively kill cancerous but not normal cells.

To elucidate the possible molecular mechanism of compounds **6**'s anti-myeloma activity, we further analyzed the apoptotic cell death induced by representative compound **6a**. As shown in Figure 3, compound **6a** induced RPMI 8226 cell apoptosis in drug dose-dependent way, confirming that apoptosis contributes to the anti-myeloma effect of compound **6a**.



Scheme 3: Synthesis of compound **6t**. Reagents and conditions: (a) DIBAL-H, THF, 50 °C, 76%; (b) MnO_2, $CHCl_3$, reflux, 70%; (c) *N*-benzyl-4-methoxybenzenesulfonamide, K_2CO_3, CH_3CN, rt, 53%; (d) ethyl 2-(diethoxyphosphoryl)acetate, NaH, THF, reflux, 66%; (e) NaOH aq, 1,4-dioxane, 50 °C, 98%; (f) (i) $(COCl)_2$, DMF, CH_2Cl_2, rt, (ii) NH_2OH•HCl, Et_3N, THF, H_2O, 0 °C to rt, 16% for two steps.

Table 1: Anti-proliferative activities of compounds **6** in RPMI 8226 cells. RPMI 8226 cells were cultured in the presence of compounds **6** at a serial concentration for 48 h. IC_{50} were determined by MTS assay[a]

Entry	Compd.	IC_{50} (μM)	Entry	Compd.	IC_{50} (μM)
1	**6a**	8.6 ± 0.4	12	**6l**	>10
2	**6b**	>10	13	**6m**	7.0 ± 0.1
3	**6c**	>10	14	**6n**	>10
4	**6d**	>10	15	**6o**	5.6 ± 0.6
5	**6e**	7.8 ± 0.4	16	**6p**	>10
6	**6f**	9.1 ± 0.3	17	**6q**	>10
7	**6g**	9.8 ± 0.2	18	**6r**	>10
8	**6h**	9.3 ± 0.5	19	**6s**	6.8 ± 0.1
9	**6i**	8.8 ± 0.4	20	**6t**	>10
10	**6j**	>10	21	SAHA	1.0 ± 0.1
11	**6k**	>10			

[a]Data were obtained from four independent experiments.

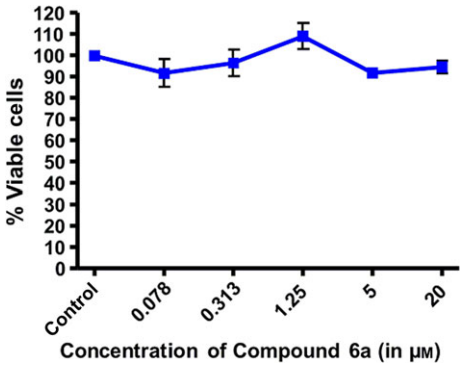


Figure 2: Effects of Compound **6a** on human PBMC proliferation. PBMCs from healthy donor were cultured in the presence of compound **6a** at the indicated concentration for 24 h. Cell viability was measured by MTS assay.

Compounds **6** contain hydoxamic functional group, the active motif of one type of HDAC inhibitors. To explore possible HDAC-inhibitory activities of compounds **6**, we treated RPMI 8226 cells with representative compound **6a**. Histone acetylation was determined by Western blot analysis using antibody against acetylated histone H4. As shown in Figure 4, compound **6a** has the potent close to SAHA. Histone acetylation signal induced by 10 μM of compound **6a** is stronger than that induced by 5 μM of SAHA but weaker than that induced by 10 μM of SAHA, suggesting that compound **6a** could be a potential lead compound for further development in anti-myeloma therapy. Moreover, it was noteworthy that cinnamoyl HDAC inhibitors with a bulky substituent at the orthoposition of the phenyl ring have been reported recently (Pharmacyclics' compound, Figure 1) (25), indicating that compound **6a** appears to be capable of binding to HDACs.

In summary, the synthesis and evaluation of anti-cancer activity of a series of pyrimidine hydroxamic acids with various sulfide substituents at the second position and

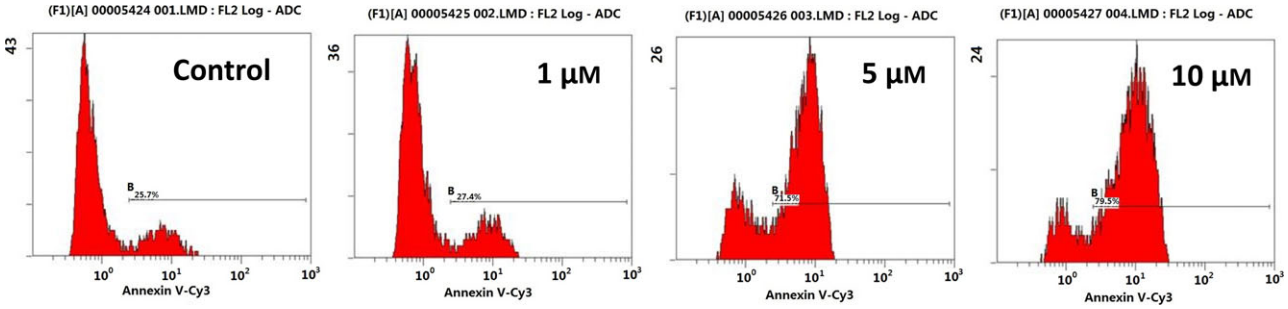


Figure 3: Compound **6a** induced apoptosis in RPMI 8226 cells. RPMI 8226 cells were cultured in the presence of compound **6a** at the indicated concentration for 24 h. Apoptosis was determined by flowcytometry following Annexin V-FITC staining.

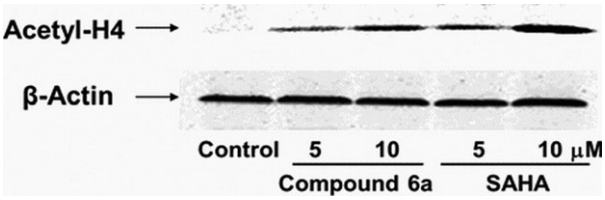


Figure 4: Compound **6a** induced histone acetylation in RPMI 8226 cells. RPMI 8226 cells were cultured in the presence of compound **6a** at the indicated concentration for 24 h. Histone H4 acetylation was determined by Western blotting with antibody against acetyl-histone H4. β-Actin was determined for loading control.

sulfonamide substituents at the fourth position have been conducted. Several compounds exhibited potent anti-cancer activity against selected human myeloma cell line RPMI 8226. It was found that representative compound **6a** selectively killed cancerous but not normal cells. Moreover, compound **6a** was effective in causing apoptosis in RPMI 8226 cells and exhibited promising HDAC-inhibitory activities. Based on current investigation, compound **6a** blocks the HDAC activity either directly or indirectly. Further study on the binding mode of compound **6a** with HDAC is currently underway.

Acknowledgments

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Competing Interest

The authors declare no competing financial interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Experimental section.