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Design, Synthesis, and Biological Evaluation of 2,4-Imidazolinedione Derivatives as HDAC6 Isoform-Selective Inhibitors

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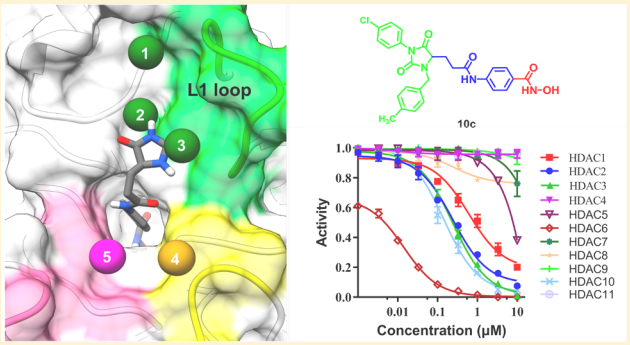
Supporting Information

ABSTRACT: Histone deacetylase 6 (HDAC6) has emerged as a promising drug target for various human diseases, including diverse neurodegenerative diseases and cancer. Herein, we reported a series of 2,4-imidazolinedione derivatives as novel HDAC6 isoform-selective inhibitors based on structure-based drug design. Most target compounds exhibit good profiles in a preliminary screening concerning HDAC6 inhibitory activities. Moreover, the most active compound **10c** increases the acetylation level of α-tubulin with little effect on the acetylation of histone H3. Further biological evaluation suggested that potent compound **10c**, which possesses good antiproliferative activity, could induce apoptosis in HL-60 cell by activating caspase 3.



KEYWORDS: *HDAC6, isoform-selective, 2,4-imidazolinedione, antiproliferative, apoptosis*

Many human diseases related to epigenetic etiology have stimulated the development of "epigenetic" therapies.[1] As epigenetic eraser, histone deacetylases (HDACs), together with histone acetyltransferases (HATs), maintain the homeostasis of the acetylation on histones and other proteins.[2] However, the aberrant acetylated state of histone mediated by HDACs overexpression is associated with many diseases including cancer.[3] Inhibition of HDACs could induce apoptosis as well as antiproliferation of diverse cancer cells in vivo or in vitro, and HDACs have emerged as promising targets for cancer treatment.[4,5] Efforts of two decades have developed five approved HDAC inhibitors (vorinostat, romidepsin, panobinostat, belinostat, and chidamide) for the treatment of cutaneous T-cell lymphomas (CTCL), multiple myeloma (MM), and peripheral T-cell lymphomas (PTCL).[6−10] Despite the success of HDAC inhibitors in cancer therapy, the indiscriminate inhibition on HDACs causes various side effects, such as diarrhea, fatigue, neutropenia, and so on.[11−14] HDAC isoform-selective inhibitors, which target specific HDAC isoform, provide a blueprint for the development of HDAC inhibitors with no or less side effects.[15] As a member of HDAC family, HDAC6 plays a pivotal role in myriad eukaryotic biological processes.[16,17] The overexpression of HDAC6 has been confirmed in many tumor cell lines,[18,19] and HDAC6 is required for efficient oncogenic cell transformation.[20] Although some HDAC6 selective inhibitors exhibited good antiproliferative activities,[21,22] other HDAC6 inhibitors displayed minor antiproliferative activities.[23,24] Indiscriminate inhibition on HDACs or off-target effect may be caused by the controversy in the antiproliferative activity of HDAC6 selective inhibitors. Therefore, the antiproliferative activities remained to be explored. Here, we sought to develop HDAC6 isoform-selective inhibitors and explore their antiproliferative activities.

HDAC inhibitors have the well accepted pharmacophore: zinc binding group (ZBG), linker, and cap group (SI Figure 1). Modifying cap, altering the linker or varying ZBG have become important strategies to develop HDAC isoform-selective inhibitors.[25] Recently, our group developed purine derivatives as HDAC inhibitors, which exhibit nanomolar inhibitory activities against HDAC6, whereas the selectivity remained to be improved.[26]

It has been widely recognized that cap group plays an important role in isoform selectivity of HDAC inhibitors.[25] We analyzed the binding pockets in HDAC6 using *AlphaSpace*[27] and found several hydrophobic pockets in the rim of lysine binding channel, including L1 loop pockets 1, 2, and 3 (Figure 1A). A recent study suggested that L1 loop pocket of HDAC6 provides a conserved binding site to achieve HDAC6 isoform selectivity.[28] Moreover, some 3-aryl-2,4-imidazolinedione derivatives were mentioned as HDAC inhibitors in 2015,[29] whereas these structures with only one aromatic substitution in R_1 position did not exhibit HDAC6 selective inhibition. Molecular docking of 2,4-imidazolinedione-based lead compound **I** (Figure 1A,B), which possesses no substituent at R_1 and R_2 positions, suggested that proper modification of 2,4-

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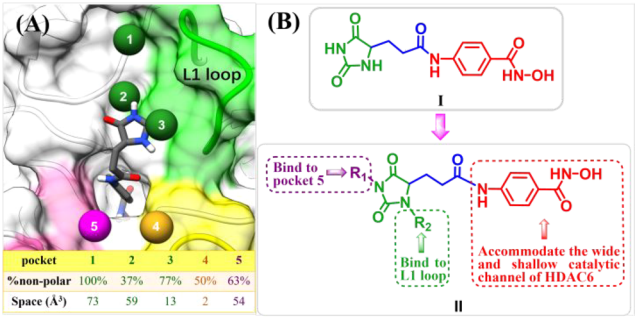


Figure 1. (A) Pocket analysis of HDAC6 was performed using *AlphaSpace*.[27] (B) Design of 2,4-imidazolinedione derivatives as HDAC6 selective inhibitors.

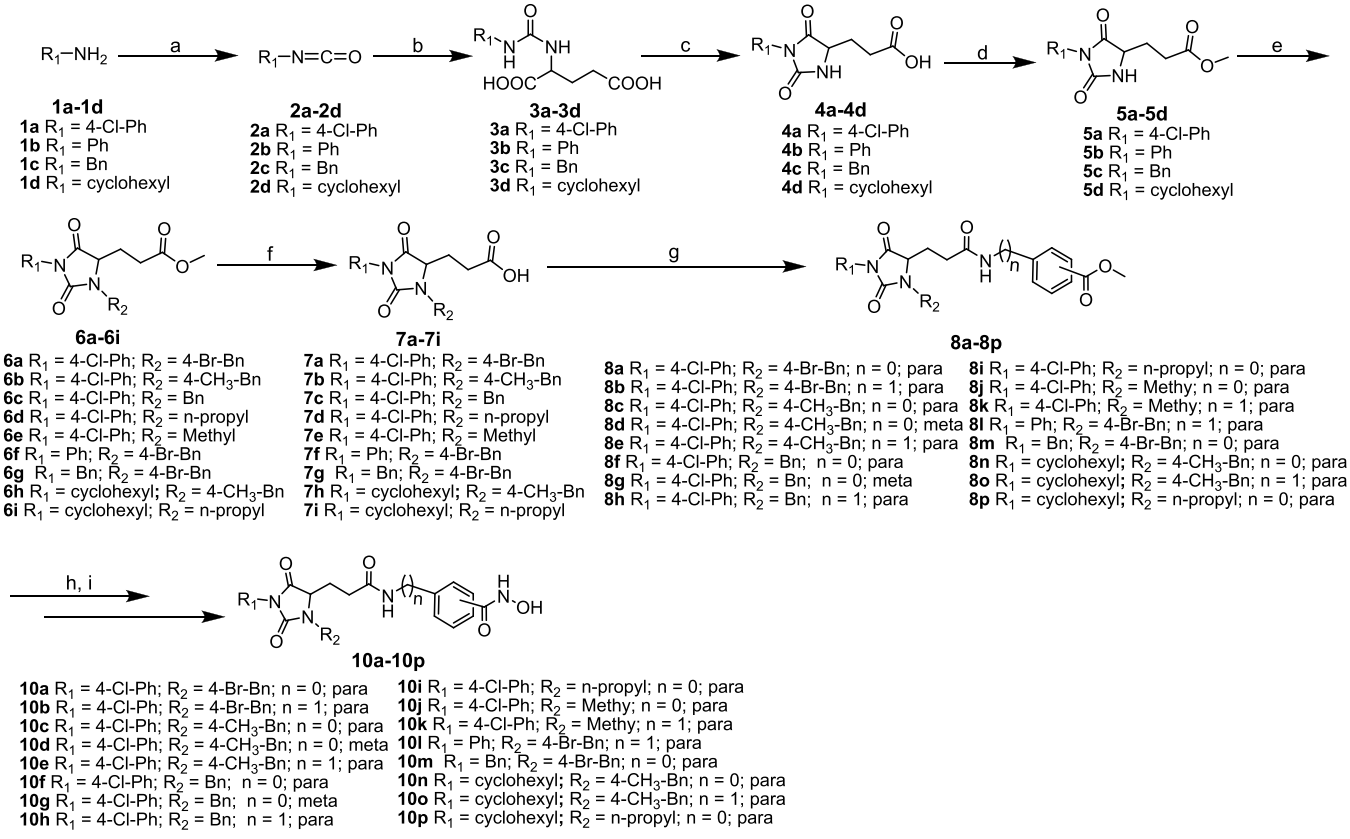
imidazolinedione scaffold would be helpful to accommodate unoccupied pocket spaces and improve binding affinity to HDAC6. Considering the hydrophobicity and size of these unoccupied pockets (Figure 1A), different aromatic rings were introduced into 2,4-imidazolinedione at R_1 and R_2 positions, which serves as cap group in our newly designed HDAC6 inhibitors (Figure 1B). However, bulky and aromatic linkers accommodate the wide and shallow catalytic channel of HDAC6 well,[30,31] which indicated that *N*-hydroxybenzamide could be used as linker and ZBG (Figure 1B).

In our ongoing study, we report the synthesis and biological evaluation of the disubstituted (R_1 and R_2) 2,4-imidazolinedione *N*-hydroxybenzamides derivatives as HDAC6 selective inhibitors.

The synthetic methods for compounds **10a−10p** are shown in Scheme 1. The amines listed were transformed into isocyanates through triphosgene and intermediate urea (**3a−3d**) and obtained from the isocyanates coupled with the amino acid. The cyclization reactions were simply mediated by concentrations of HCl to obtain intermediates **4a−4d**. Then, the key intermediates **6a−6i** were prepared by *N*-alkylation at R_2 of 2,4-imidazolinedione. Compounds **7a−7i** were obtained by deprotecting with LiOH, and they were used to prepare **8a−8p** by amide coupling. After deprotecting, intermediates **9a−9p** were converted to target compounds **10a−10p** according to literature procedures.[32] It should be noted that intermediates **6a−6i**, **7a−7i**, and **8a−8p** and target compounds **10a−10p** are racemates.

We evaluated HDAC6 inhibitory activities of all target compounds with vorinostat (SAHA) as the positive control. As shown by the results summarized in Table 1, most target compounds have good HDAC6 inhibitory activities. Structure−activity relationships suggested that compounds with *para*-substituted *N*-hydroxybenzamides exhibit good HDAC6 inhibitory activities, whereas compounds with *meta*-substituted *N*-hydroxybenzamides are less active. Molecule docking study of compound **10c** (SI Figure 15) indicated that the

Scheme 1[a]



[a]Reagents and conditions: (a) triphosgene, $NaHCO_3$, CH_2Cl_2; (b) (i) toluene, 2 M NaOH, 0 °C, 4 h, (ii) 6 M HCl, 44−72%; (c) hydrochloric acid, reflux, 3 h, 88−92%; (d) CH_3COCl, MeOH, reflux, 5 h, 95−97%; (e) K_2CO_3, KI, DMF, overnight, 45−93%; (f) LiOH·H_2O, THF/H_2O, rt, 6 h, 78−97%; (g) HATU, DIPEA, CH_2Cl_2, rt, overnight, 40−95%; (h) LiOH·H_2O, THF/H_2O, rt, 6 h; (i) isobutyl chlorocarbonate, 4-methylmorpholine, THF, NH_2OH·HCl, KOH, MeOH, rt, 6 h, 17−65%.

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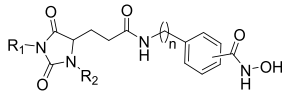
Table 1. HDAC6 Inhibitory Activities of Target Compounds



compd	R_1	R_2	n	position	IC_{50} (nM)[a]
10a	4-Cl-Ph	4-Br-Bn	0	para	9.7 ± 0.6
10b	4-Cl-Ph	4-Br-Bn	1	para	16.5 ± 0.4
10c	4-Cl-Ph	4-CH_3-Bn	0	para	4.4 ± 0.4
10d	4-Cl-Ph	4-CH_3-Bn	0	meta	>40
10e	4-Cl-Ph	4-CH_3-Bn	1	para	18.9 ± 0.4
10f	4-Cl-Ph	Bn	0	para	7.6 ± 0.8
10g	4-Cl-Ph	Bn	0	meta	>40
10h	4-Cl-Ph	Bn	1	para	12.7 ± 2.7
10i	4-Cl-Ph	*n*-propyl	0	para	12.6 ± 2.3
10j	4-Cl-Ph	methyl	0	para	7.0 ± 1.3
10k	4-Cl-Ph	methyl	1	para	11.8 ± 3.2
10l	Ph	4-Br-Bn	1	para	13.6 ± 2.1
10m	Bn	4-Br-Bn	0	para	9.8 ± 1.8
10n	cyclohexyl	4-CH_3-Bn	0	para	10.3 ± 1.3
10o	cyclohexyl	4-CH_3-Bn	1	para	17.0 ± 0.78
10p	cyclohexyl	*n*-propyl	0	para	>40
SAHA					39.9 ± 9

[a]All compounds were assayed at least two times, and the results are expressed with standard deviations.

hydroxamic acid of compound **10c** chelated with zinc ion properly and formed a series of hydrogen bond interactions with key residues (His 610, Tyr 782). This may help us to understand the reason why *para*-substituted *N*-hydroxybenzamides are more active than *meta*-substituted *N*-hydroxybenzamides (Table 1). As for the linker, compounds without spacer (n = 0) possess suitable linkers, and these linkers allow the cap groups to reach and interact with L1 loop, while allowing the ZBG to chelate with zinc ion properly (SI Figure 15). However, compounds **10b**, **10e**, **10h**, **10k**, **10l**, and **10o**, which possess one methylene spacer (n = 1), exhibit lower inhibition against HDAC6 due to the longer linkers. Moreover, docking study of compound **10c** suggested that the phenyl in the linker of (*S*)-**10c** formed $\pi-\pi$ interactions with Phe 680, whereas the phenyl in the linker of (*R*)-**10c** formed $\pi-\pi$ interactions with Phe 620. Specially, the amide in the linkers of (*S*)-**10c** and (*R*)-**10c** both formed hydrogen bond interaction with Ser 568, which may explain the reason why compounds with no spacer (n = 0) between amide group and phenyl in the linker are more active than those with one methylene spacer (n = 1).

In our studies, different substituents attached to 2,4-imidazolinedione in R_1 and R_2 position also show impacts on HDAC6 inhibitory activities. Except compound **10j**, compounds with phenyl and substituted benzyl on cap region show better HDAC6 inhibitory activities compared to other compounds. Compound **10p**, which possesses no aromatic rings at R_1 and R_2 positions, exhibits poor HDAC6 inhibitory activity.

For potent compounds **10a**, **10c**, and **10f**, we subsequently evaluated their HDAC inhibitory profile. IC_{50} values calculated from the dose−effect curve (SI Figure 11) indicated that all tested compounds exhibit good HDAC6 isoform selectivity (Table 2). It is worth mentioning that compound **10c** possesses better HDAC6 selectivity compared to the positive control SAHA. Potent molecule **10c** exhibits 218-fold selectivity against HDAC1 and more than 53-fold selectivity against HDAC2 and 3 as well as over 20000-fold selectivity against HDAC4, 7, 8, 9, and 11.

Research evidence revealed that HDAC6 inhibition induces apoptosis and suppresses the growth of carcinoma cells.[23,24,33] Therefore, potent compounds (**10a**, **10c**, and **10f**) were chosen to evaluate their antiproliferative activities. The results (Table 3) suggested that compounds **10a**, **10c**, and **10f** exhibit good antiproliferative activities against several tumor cell lines and that molecule **10c** possesses better antiproliferative activities against HL-60 cell (IC_{50} = 0.25 μM) as well as RPMI-8226 cells (IC_{50} = 0.23 μM). Moreover, the antiproliferative activities of compound **10c** against K562, HCT-116, and A549 cell lines were approximately three times that of the positive control SAHA.

To explore and verify the mechanism of the antiproliferative activity of target compounds, we further performed apoptotic assay (AnnexinV/PI staining), cell cycle analysis (PI staining), and caspase 3 activation in HL-60 cell. As shown in Figure 2A, compounds **10a** and **10c** exhibit mild apoptosis induction at 0.25 μM but exhibit strong apoptosis induction at 0.45 μM, whereas the positive control SAHA exhibits approximately no ability to induce apoptosis at different concentrations (0.25 μM, 0.45 μM). Cell cycle analysis (Figure 2B) showed that both **10a** and **10c** could induce cell cycle arrest at G1 phase and subG1 phase compared to the control (DMSO). Subsequently, caspase 3 activation assay was performed to explore the mechanism of apoptosis induction by potent

Table 2. HDACs Inhibitory Profiles of 10a, 10c, and 10f

	IC_{50} (nM)[a]			
HDAC isoform	**10a**	**10c**	**10f**	SAHA
HDAC1	194 ± 13	959 ± 206	111 ± 30	46 ± 1
HDAC2	249 ± 6	277 ± 17	203 ± 14	120 ± 7
HDAC3	126 ± 13	235 ± 33	96 ± 16	35 ± 1
HDAC4	>100000	>100000	27000 ± 6000	>100000
HDAC5	4600 ± 400	14900 ± 1700	4600 ± 600	41500 ± 10000
HDAC6	9.7 ± 0.6	4.4 ± 0.4	7.6 ± 0.8	39.9 ± 9
HDAC7	6000 ± 200	>100000	8800 ± 1300	41800 ± 500
HDAC8	6700 ± 700	>100000	3600 ± 100	1800 ± 300
HDAC9	9100 ± 800	>100000	15200 ± 2900	64000 ± 8100
HDAC10	268 ± 23	164 ± 10	114 ± 26	80 ± 8
HDAC11	>100000	>100000	>100000	40000 ± 3000

[a]Assays were performed in replicate ($n \geq 2$).

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Table 3. Antiproliferative Activities of 10a, 10c, and 10f

	IC_{50} (μM)[a]				
compd	K562	HL-60	RPMI-8226	HCT-116	A549
10a	0.69 ± 0.21	0.34 ± 0.05	0.36 ± 0.06	1.25 ± 0.07	5.86 ± 0.34
10c	0.49 ± 0.10	0.25 ± 0.01	0.23 ± 0.04	0.83 ± 0.03	0.79 ± 0.07
10f	0.83 ± 0.13	0.61 ± 0.03	0.64 ± 0.06	2.25 ± 0.12	9.45 ± 1.07
SAHA	1.45 ± 0.13	0.52 ± 0.08	0.57 ± 0.06	1.81 ± 0.17	2.42 ± 0.02

[a]IC_{50} values are expressed as the mean ± standard deviation of three separate determinations.

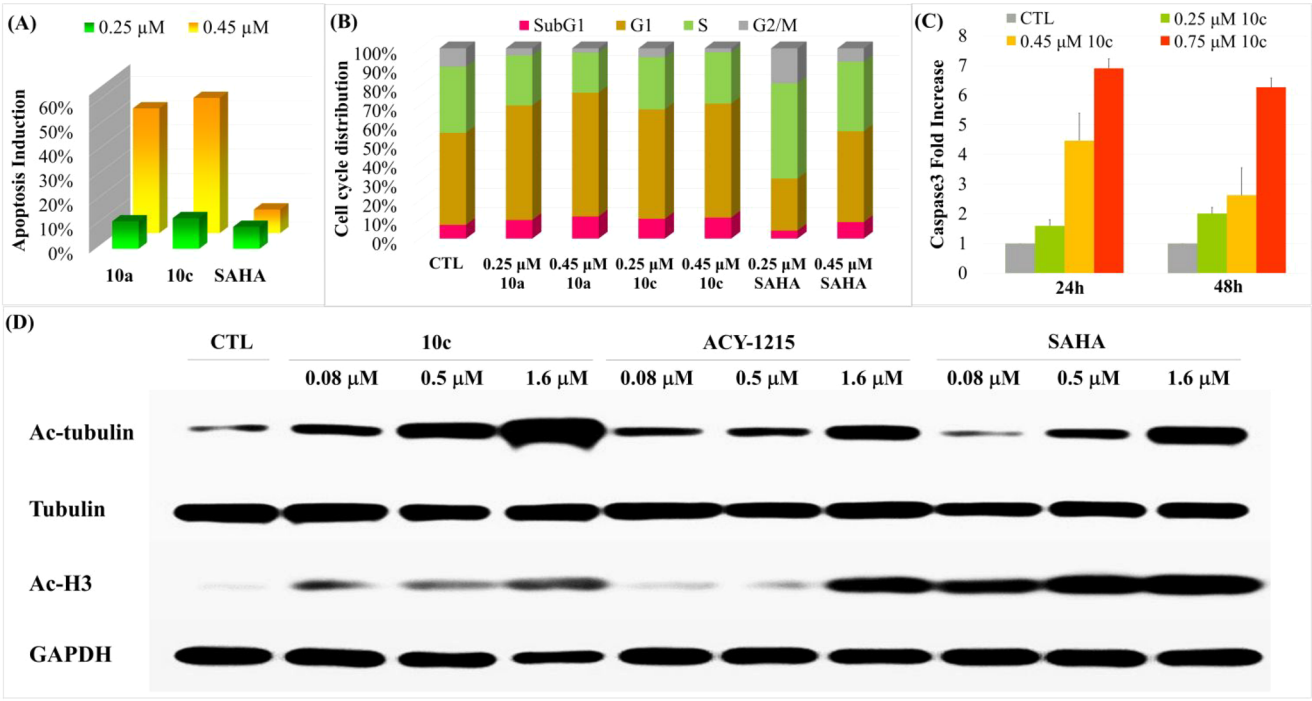

Figure 2. (A) Inducing apoptosis of HL-60 by **10a**, **10c**, and SAHA. (B) Cell cycle analysis for **10a**, **10c**, and SAHA against HL-60. (C) Fold increase of activated caspase 3 after treatment with compound **10c** for 24 and 48 h in HL-60 cell. Data are expressed as mean ± SD of at least three independent experiments. Significant differences between treated cells with respect to control (untreated cells) are indicated as $p < 0.05$. (D) Levels of the acetylation of α-tubulin and H3 after treatment with compound **10c**, ACY-1215, and SAHA in HL-60 cells at different concentrations for 24 h, respectively.

compound **10c** in HL-60 cell. After incubating HL-60 cells with various concentrations (0.25, 0.45, and 0.75 μM) for 24 and 48 h (Figure 2C), the amount of activated caspase 3 is significantly increased compared to control (untreated cells). Above research evidence indicated that compound **10c** could induce apoptosis via activating caspase 3 in HL-60 cell.

To confirm whether potent molecule **10c** targets HDAC6 to exert antiproliferative activity, Western blot was performed. As shown in Figure 2D, SAHA increases the acetylation of α-tubulin (substrate for HDAC6) and the acetylation of H3 (a major substrate of class I HDACs), whereas ACY-1215 and compound **10c** strongly increased the acetylation of α-tubulin with slight effect on H3 at low concentrations.

In summary, a series of 1,3-disubstituted 2,4-imidazolinedione derivatives were developed as HDAC6 selective inhibitors. Biological evaluations show that some compounds (**10a**, **10c**, and **10f**), especially compound **10c**, exhibit good HDAC6 inhibitory activities, isoform-selectivities, and antiproliferative activities. Further studies revealed that potent molecule **10c** could induce apoptosis by activating caspase 3 and significantly increase the acetylation of α-tubulin with little effect on the acetylation of H3. It is necessary to be noted that all target compounds are racemic and that developing a method for the separation of enantiomers is needed in future work. In addition, compound **10c** may be used as a lead compound for the development of more potent and selective HDAC6 inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00084.

Approved HDAC inhibitors and the well accepted pharmacophore of HDAC inhibitors; experimental methods; synthesis and characterization of target compounds **10a**−**10p**; ^{1}H NMR, ^{13}C NMR, and HRMS spectra of representative compounds; HPLC analysis; HDAC6 and HDAC isoform inhibitory assay; in vitro antiproliferative assay (MTT assay); apoptosis and cell cycle analysis; caspase 3 activation assay; Western blot assay; molecular docking (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

HDACs, histone deacetylase; HATs, histone acetyltransferases; ZBG, zinc binding group; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; HATU, 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexa-fluorophosphate; DIPEA, *N*,*N*-diisopropylethylamine; HeLa, human cervical cancer; SAHA (vorinostat), suberanilohydroxa-mic acid; TSA, trichostatin A; BSA, albumin from bovine serum; His, histidine; Tyr, tyrosine; Phe, phenylalanine; Ser, serine; MTT, methylthiazolyldiphenyl-tetrazolium bromide; K562, chronic myelogenous leukemia cell; HL-60, human promyelocytic leukemia cell; RPMI-8226, human multiple myeloma cell line; HCT-116, human colon cancer cells; A549, human lung cancer cells; PBS, phosphate-buffered saline; ACY-1215, rocilinostat; PI, propidium iodide

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