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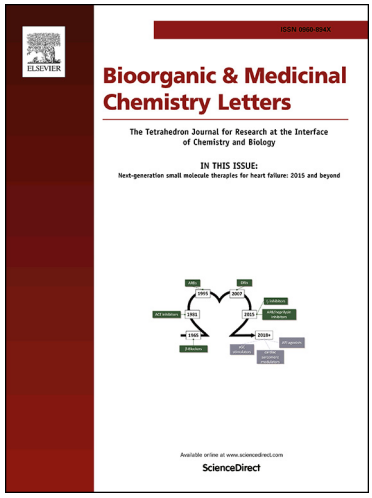

Development of hydroxamate-based histone deacetylase inhibitors containing 1,2,4-oxadiazole moiety core with antitumor activities

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PII: S0960-894X(18)30894-1
DOI: https://doi.org/10.1016/j.bmcl.2018.11.027
Reference: BMCL 26138

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 28 August 2018
Revised Date: 17 October 2018
Accepted Date: 13 November 2018

Please cite this article as: Yang, F., Shan, P., Zhao, N., Ge, D., Zhu, K., Jiang, C-s., Li, P., Zhang, H., Development of hydroxamate-based histone deacetylase inhibitors containing 1,2,4-oxadiazole moiety core with antitumor activities, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.11.027

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Development of hydroxamate-based histone deacetylase inhibitors containing 1,2,4-oxadiazole moiety core with antitumor activities

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ABSTRACT

Histone deacetylases (HDACs) has proved to be promising target for the development of antitumor drugs. In this study, we reported the design and synthesis of a class of novel hydroxamate-based bis-substituted aromatic amide HDAC inhibitors with 1,2,4-oxadiazole core. Most newly synthesized compounds displayed excellent HDAC1 inhibitory effects and significant anti-proliferative activities. Among them, compounds **11a** and **11c** increased acetylation of histone H3 and H4 in dose-dependent manner. Furthermore, **11a** and **11c** remarkably induced apoptosis in HepG2 cancer cells. Finally, the high potency of compound **11a** was rationalized by molecular docking studies.

Keywords:

1,2,4-oxadiazole, Hydroxamate, HDAC inhibitors, Structure-activity relationships, Antitumor

Epigenetics defines the heritable change of gene expression without modification of the DNA nucleotide sequence[1, 2]. Histone lysine acetylation level regulated by HDACs and histone acetyl-transferases (HATs) plays a key role in epigenetic modification of gene expression[3]. HDACs catalyze the removal of acetyl groups from the ε -amino lysine residues on histone tails, resulting in chromatin conformation condensation associated with down-regulation of tumor suppressor genes[4, 5]. In addition, HDACs also deacetylate many non-histone proteins, which influences the function, protein-protein interaction and stability of these proteins[6, 7]. To date, 18 HDAC enzymes have been identified and classified into four classes. Class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9 and 10) and class IV (HDAC11) HDACs are all belong to zinc-dependent enzymes, while class III (SIRT1-7) belong to NAD^+-dependent HDACs[8, 9]. The overexpression of HDACs have been found in

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various cancers, making them attractive targets for anticancer therapy[10, 11]. Several studies have demonstrated that Class I HDACs play a crucial role in regulating tumor growth, development and apoptosis.

It has been confirmed that HDAC inhibitors (HDACis) significantly repress cancer cell proliferation, angiogenesis, and metastasis while induce apoptosis, cell cycle arrest and DNA damage through multiple antitumor pathways[12, 13]. Up to now, five HDACis, vorinostat (**1** SAHA)[14], romidepsin (**2** FK-228)[15], belinostat (**3** PXD-101)[16], panobinostat (**4** LBH-589)[17] and chidamide (**5** Epidaza)[18], have been approved by FDA or CFDA for the treatment of cutaneous T-cell lymphoma (CTCL), peripheral T-cell lymphoma (PTCL) or multiple myeloma (MM), and more than 20 HDACis, such as compounds **6** and **7**, are at various stages of clinical trials as single agents or in combination with other chemotherapeutic agents for the treatment of various types of tumors[19, 20]. However, most of the investigated HDACis display relatively weak efficacy against solid tumors. The development of novel HDACis with improved potency against solid tumors is highly needed, to expand their application in broad spectrum of cancer[21].

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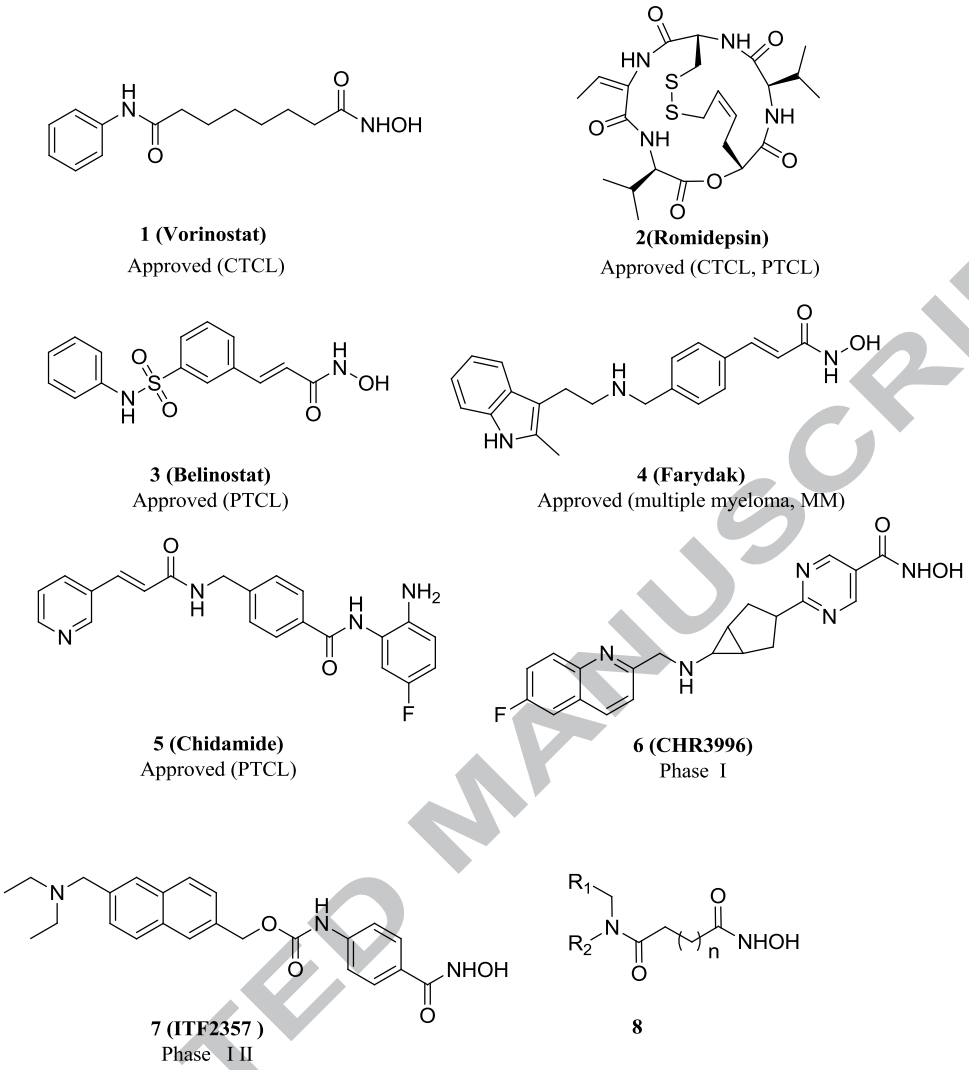

Figure 1. Structures of FDA approved HDACis and representative examples in clinical trials.

Most HDACis share the common three-motif pharmacophore model, comprising an aromatic amide surface recognition group (usually called CAP group), a zinc binding group (ZBG), and a linker part connecting ZBG and CAP[22]. Hydroxamic acid-based HDACis with branched CAP region (**8**) have been demonstrated to have anticancer effects[23-25]. In previous report, we have identified a series of novel hydroxamate-based HDACis of bis-substituted aromatic amides, with potent activities against tumor growth and metastasis[26]. Detailed SAR analysis revealed that the branched CAP region containing a *p*-substituted phenyl and two linked heteroaromatic groups is important for increasing potency. Particularly, compound **9** containing pyrimidinylthiopheneyl exhibited nanomolar IC_{50}

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values toward HDACs as well as submicromolar activity against proliferation and migration of breast cancer cells both *in vitro* and *in vivo*. To investigate whether the pyrimidinylthiopheneyl functionality is essential for the anti-cancer activities of these compounds, we designed a series of phenyloxadiazole containing bis-substituted aromatic amides hydroxamate based HDACis (Figure 2).

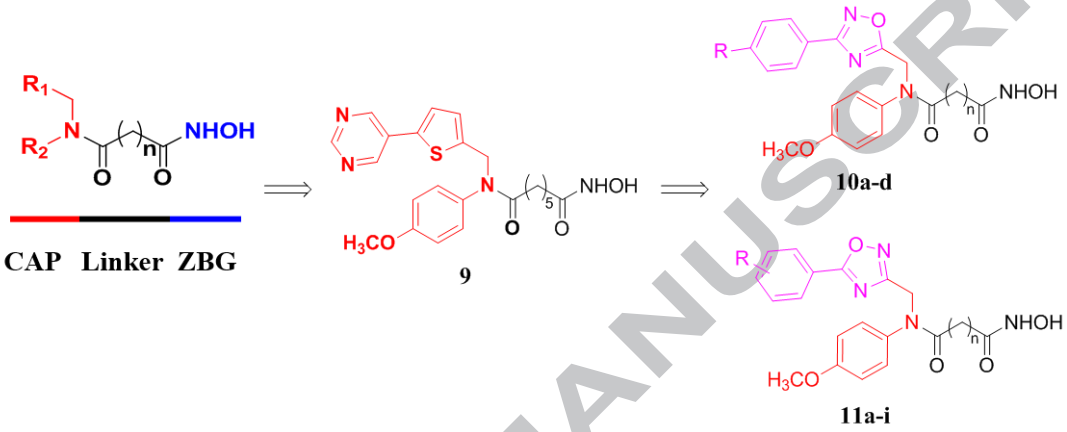

Figure 2. The design of novel 1,2,4-oxadiazole containing hydroxamate based HDACis

Oxadiazole derivatives generally display specific and high pharmacological properties, such as anti-inflammatory[27], antimicrobial[28], anticonvulsant[29] and anti-cancer[30] activities. Researchers mainly focus on 1,2,4-oxadiazole for its multi-applicability in medicinal chemistry [31]. Recently, a number of 1,2,4-oxadiazole derivations have been reported to display anticancer activities, and the results demonstrated that substituents at C-3 and C-5 of 1,2,4-oxadiazole ring were necessary for the good activity[32, 33]. In this study, we used 1,2,4-oxadiazole to replace thiophene of compound **9**, then a series of 1,2,4-oxadiazole containing bis-substituted aromatic amides HDACis were synthesized and evaluated for their antitumor activies.

The synthesis of compounds **10a-d** was outlined in Scheme 1. Appropriately substituted nitriles **12a-c** were converted to **13a-c,** which were then cyclized with bromoacetyl bromide in refluxing tetrahydrofuran to obtain 1,2,4-oxadiazole compounds **14a-c**. Intermediates **14a-c** were

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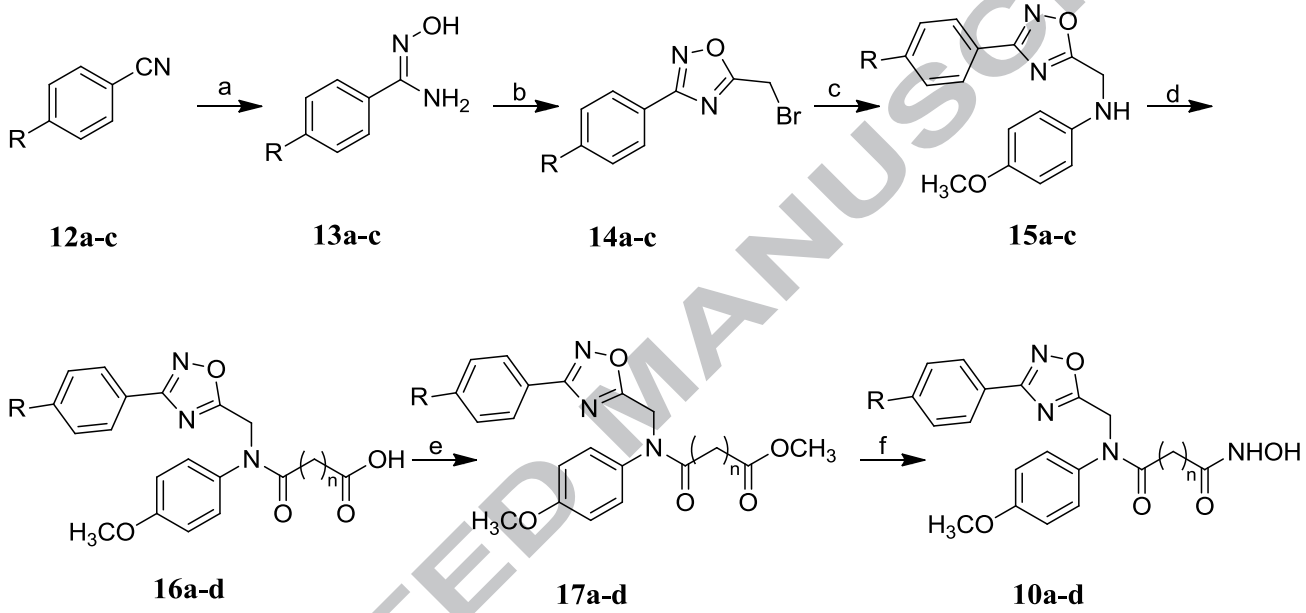
subsequently condensed with *p*-anisidine to produce compounds **15a-c**, which were further condensed with different anhydrides in 1,4-dioxane to yield acids **16a-d.** Compounds **16a-d** then underwent esterification using catalytic amounts of $SOCl_2$ in methanol to produce methyl esters **17a-d**, Finally, treatment of methyl esters with hydroxylamine to afford the target compounds **10a-d**.

Scheme 1. Synthesis of compounds 10a-d



12a-c **13a-c** **14a-c** **15a-c**

16a-d **17a-d** **10a-d**

Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, Et_3N, MeOH, 12h, 80-90%; (b) $BrCH_2COBr$, THF, reflux, 12h, 50-55%; (c) *p*-Anisidine, K_2CO_3, DMF, 3h, 35-40%; (d) anhydride, 1,4-dioxane, reflux, 5h, 70-80%; (e) MeOH, Cat.$SOCl_2$, reflux, 1h, 90-95%; (f) $NH_2OH{\cdot}HCl$, KOH, MeOH, 2h, 20-40%.

The preparation route for compounds **11a-i** was depicted in Scheme 2. Coupling of *p*-anisidine and hydroxylamine hydrochloride gave **19**, which was further condensed with pimelic acid anhydride to yield acid **20**. Subsequent transformations were carried out using similar protocols as outlined in Scheme 1. At last, the ester compounds **23a-i** were converted to corresponding **11a-i** by reacting with NH_2OH in methanol.

Scheme 2. Synthesis of compounds 11a-i

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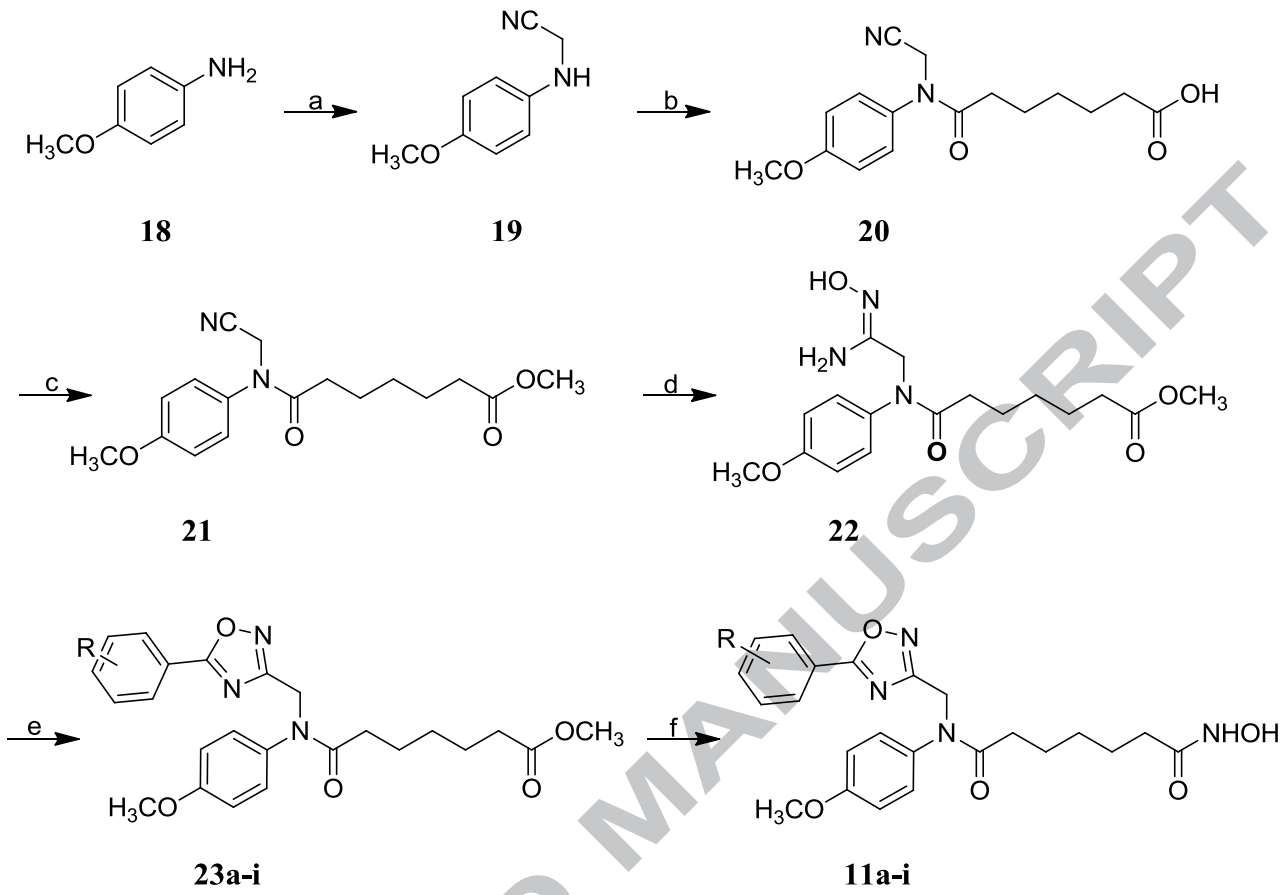

Reagents and conditions: (a) Bromoacetonitrile， K_2CO_3, DMF, 5h, 92.6%; (b) Pimelic acid anhydride， 1,4-dioxane, reflux, 4h, 92.1%; (c) MeOH, Cat.$SOCl_2$, reflux, 1h, 90.5%; (d) $NH_2OH{\cdot}HCl$, Na_2CO_3, $EtOH/H_2O$, 12h, 65.8%; (e) RC_6H_4COCl, THF, reflux, 12h, 80-90%; (f) $NH_2OH{\cdot}HCl$, KOH, MeOH, 2h, 20-40%.

On the basis of our previous results[26], the series of bis-substituted aromatic amides hydroxamate-based HDAC inhibitors were pan-HDAC inhibitors, and showed more potent inhibition against class I HDAC isoforms especially HDAC1, so we chose HDAC1 for enzyme inhibition assay. All synthesized compounds and the reference compound SAHA were detected for their ability to inhibit HDAC1 at 20 nM. Results in Table 1 were shown as inhibition rates, indicating that the four derivations (**10a-d**) showed weaker inhibitory activities than SAHA, compound **10d** with five methylene linker possessed stronger inhibitory rate than compound **10a, 10b** and **10c** with six

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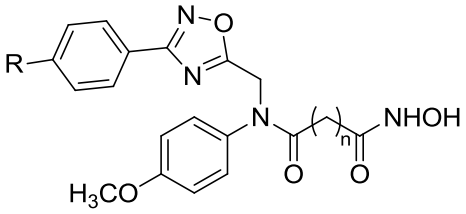
methylene linker, which was in line with our previous reported work[26]. Thus, the five methylene linker length was retained in subsequent structural modifications.

Table 1 HDAC inhibition activity of compounds **10a-c**



Compd.	R	n	20 nM
			%inhibition on HDAC1
10a	OCH_3	6	35
10b	CH_3	6	30
10c	H	6	40
10d	H	5	50
SAHA			60

Assays were performed in replicate ($n \geq 3$), the SD values are < 20% of the mean

Upon further modification, 3-aryl-5-alkyl-1,2,4-oxadiazole was converted to 5-aryl-3-alkyl-1,2,4-oxadiazole to afford **11a**. To further investigate how electron-withdrawing groups (EWGs) or electron-donating groups (EDGs) of the benzene ring would affect potency, compounds **11b-i** were then synthesized and screened. The results in Table 2 displayed that all derivations (**11a-i**) showed better inhibitory activities than SAHA. However, the potency of HDAC1 inhibition was only moderately affected by addition of EWGs or EDGs relative to that of **11a**, with no particular substituent-dependent trend being observed. To explore the optimal substitution position, analysis of the activities of fluorine-substituted (**11b-d**) and methyl-substituted (**11g-i**) series revealed

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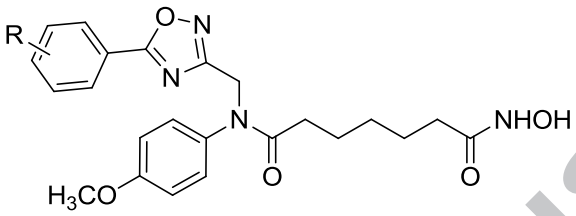
that compounds with *m*-substitution (**11c** and **11h**) showed better inhibition ratio than those with *o*- or *p*-substitution.

Table 2 HDAC inhibition activity of compounds **11a-i**



Compd.	R	20 nM
		%inhibition on HDAC1
11a	H	90
11b	2′-F	85
11c	3′-F	90
11d	4′-F	86
11e	4′-Br	86
11f	4′-NO_2	85
11g	2′-CH_3	83
11h	3′-CH_3	90
11i	4′-CH_3	85
SAHA		60

Assays were performed in replicate ($n \geq 3$), the SD values are < 20% of the mean

Three compounds (**11a**, **11c**, **11h**) with good HDAC1 inhibitory rates were further evaluated for the IC_{50} values against HDAC1. All the three compounds showed promising activities with IC_{50}

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values in the nanomolar range (Table 3). Compound **11a** was the most potent one, displaying IC_{50} value of 8.2 nM, which was nearly two fold lower than SAHA (IC_{50} = 15.0 nM). Compound **11c** and **11h** showed IC_{50} values of 10.5 and 12.1 nM, also lower than that of SAHA.

Table 3 IC_{50} values of HDAC1 inhibition of representative compounds

Compd.	**IC_{50} (nM)**
11a	8.2
11c	10.5
11h	12.1
SAHA	15.0

Assays were performed in replicate ($n \geq 3$), the SD values are < 20% of the mean

Compounds **11a**, **11c** and **11h** were further tested for their anti-proliferative activities against human hepatocellular carcinoma cells (HCCLM3 and HepG2), SAHA was used as the reference compound. The results are summarized in Table 4. All of these compounds showed excellent anti-proliferative activities with the IC_{50} values in micromolar range (1.07 - 6.83 μM) in both two cell lines. Moreover, the results indicated that the inhibitory activities of these compounds on HepG2 are more sensitive than HCCLM3 cells. Compounds **11a** and **11c** showed the better growth inhibition with IC_{50} value of 1.07 μM and 1.03 μM in HepG2 cells, which was superior to SAHA obviously.

Table 4 Anti-proliferative activities of representative compounds against human hepatocellular carcinoma cells

Compd.	**IC_{50} (μM)**	
	HCCLM3	**HepG2**

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11a	5.19	1.07
11c	6.56	1.03
11h	6.83	1.68
SAHA	6.52	4.50

Assays were performed in replicate ($n \geq 3$), the SD values are < 20% of the mean

Given that the inhibition of HDACs by compounds **11a** and **11c** enhanced the tumor cell anti-proliferative activities, the HDAC inhibitory effects of compounds on the level of acetylation of histone H3 and H4 in HepG2 cells were determined by immunoblotting assays. HepG2 cells were incubated with SAHA and compounds **11a** and **11c** (1.25, 2.5 and 5.0 μM). As depicted in Figure 3, both compounds can markedly increase the level of acetylated histone H3 and H4 in a dose-dependent manner, which was consistent with the HDAC1 inhibition activities.

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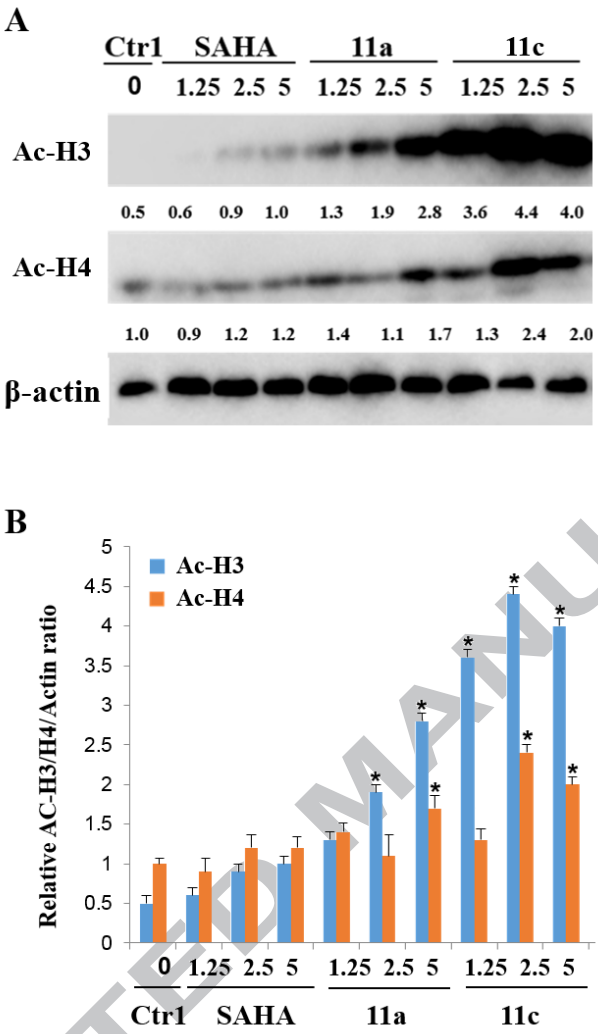

Figure 3. Western blot analysis of the effects of compounds **11a** and **11c** at different concentrations on acetylated histone levels in HepG2 cells. A) Treatment resulted in upregulated expression of acetylated H3 and H4 levels in dose dependent manner. B) Quantitative analysis. The levels of Ac-H3 and Ac-H4 relative to β-actin control were determined by densitometric scanning. (*P ≤ 0.05 *vs.* SAHA treated group, n = 3)

To determine whether the inhibitory effects of target compounds on liver cancer cell proliferation are accompanied by enhancing apoptosis, HepG2 cells were incubated with different concentrations of target compounds and SAHA for 48 h. FITC-annexin V/propidium iodide (PI) staining and flow cytometry assay were performed. As shown in Figure 4, compounds **11a** and **11c**

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dose dependently induced apoptosis in HepG2 cells and the apoptosis percentage was even higher than that of the positive control compound, SAHA.

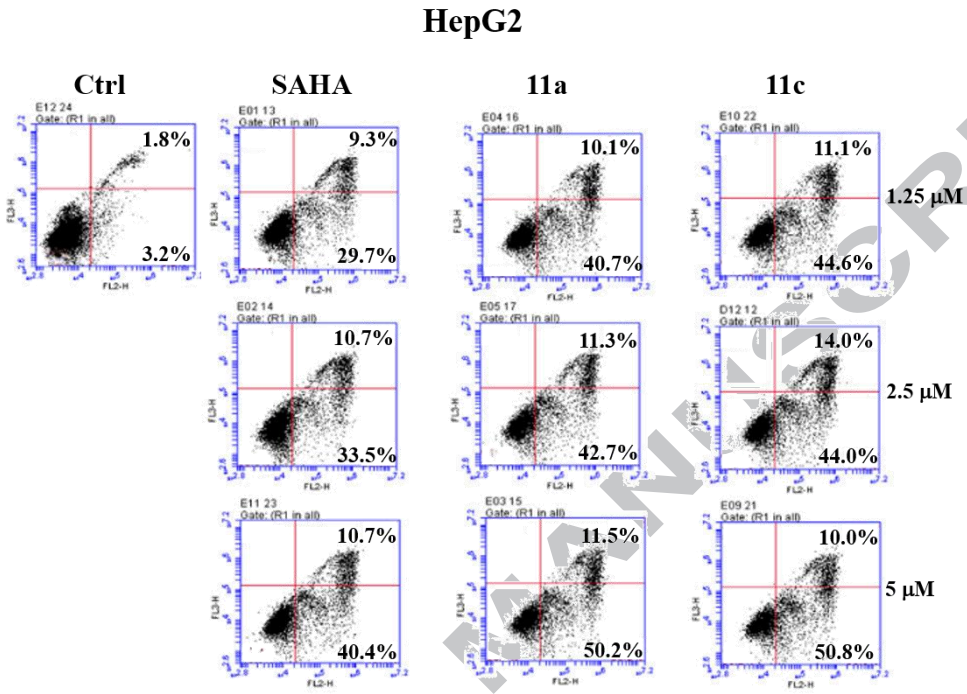

Figure 4. Flow cytometry analysis. Compounds **11a** and **11c** mediated HepG2 cells apoptosis in concentration dependent manner.

Reports suggest that compounds which meet the two criteria of (1) polar surface area (tPSA) less than 140 $Å^2$; (2) 12 or fewer H-bond donors and acceptors may have good oral bioavailability[34, 35]. Several parameters of target compounds were carried out for prediction of the ADME properties through the molinspiration program (http://www.molinspiration.com/cgi-bin/properties). As shown in Table 5, compounds **11a** and **11c** both have only nine hydrogen bond acceptors (n-ON) and two donors (n-OHNH). tPSA did not exceed 140 A^2. The calculated LogP (cLogP) in an acceptable range (–2 to 5). The above results indicated that compounds **11a** and **11c** are in a reasonable region for further development as potential drug candidates.

Table 5. Theoretical Prediction of the ADME Properties of representative compounds

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Compd.	cLogP	MW	tPSA	n-ON	n-OHNH
11a	2.77	438.48	117.79	9	2
11c	2.91	456.47	117.79	9	2
SAHA	2.47	264.32	78.42	5	3

To better explaining the effective HDAC1 inhibitory activity of compound **11a**, the docking assays (Autodock-4.27)[36] were employed to analyze the binding model between **11a** and HDAC1 (PDB code: 4BKX, chosen for its highest resolution). As shown in Figure 5, compound **11a** and SAHA shared the same binding mode (Figure 5A and 5B). The hydroxamate group of **11a** could chelate the zinc ion and could form two hydrogen bonds with GLY149 and HIS178 residues in the active site of HDAC1. Meanwhile, the phenyl of compound **11a** could also form π-π stacking interaction with PHE 150 in the surface region of HDAC1. It is worth pointing out that the 4-methoxy group on the phenyl of compound **11a** docked into the hydrophobic pocket of the surface region of HDAC1, which could preliminarily rationalize the better inhibitory activity of **11a** than SAHA (Figure 5C).

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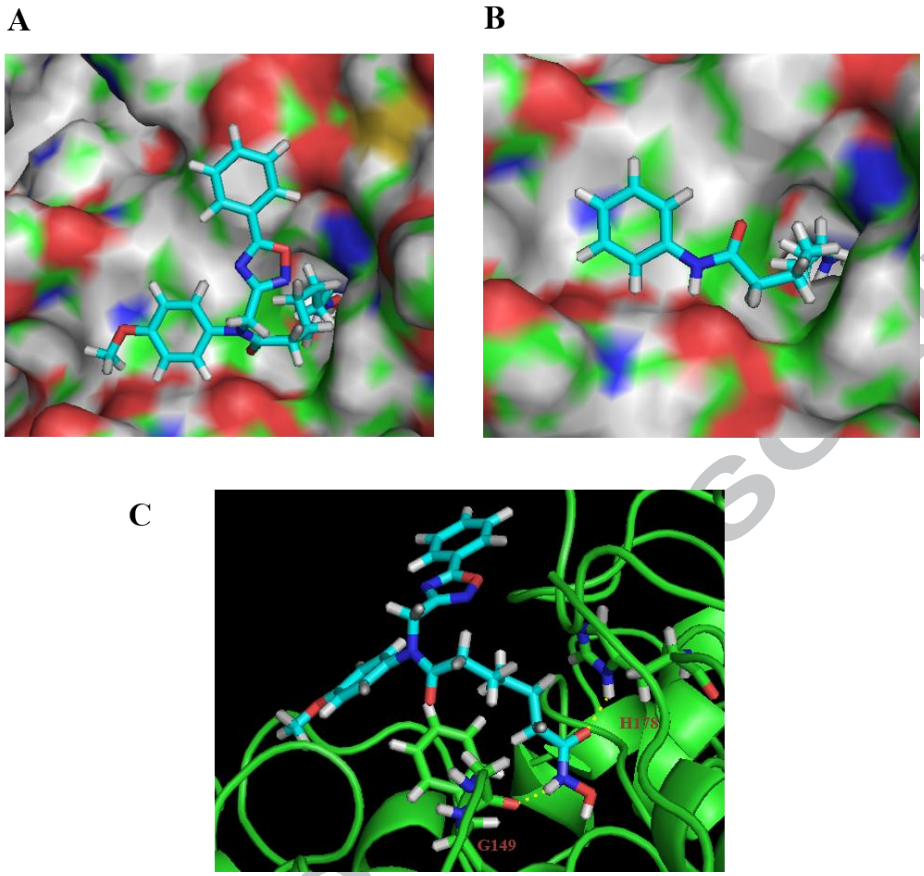

Figure 5. Proposed binding mode of compound **11a** and SAHA with HDAC1 (PDB 4BKX). A) Molecular surface of the HDAC1 binding pocket with **11a**. (B) Molecular surface of the HDAC1 binding pocket with SAHA. (C) **11a** interacted with the active site of HDAC1.

Hydroxamic acid based HDACis with branched CAP region has been demonstrated to possess anticancer effects. Based on previous research, a series of 1,2,4-oxadiazole containing bis-substituted aromatic amides HDACis were synthesized and evaluated for their antitumor activities. Most newly synthesized compounds displayed potent inhibitory activities against HDAC1. Several compounds exhibited super anti-proliferative activities in HepG2 cell line, with an IC_{50} value range of 1.07-1.68 μM, which were 2-4 fold lower than SAHA (IC_{50} = 4.50 μM). Immunoblot analysis showed that **11a** and **11c** increased the levels of acetylated histone H3 and H4 in dose dependent manner, confirming

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its HDAC inhibitory action. Furthermore, compounds **11a** and **11c** could significantly induce cell apoptosis. Finally, the high potency of compound **11a** was interpreted by molecular docking study. Taken together, these results suggest that these novel 1,2,4-oxadiazole containing HDAC inhibitors could be identified as promising agents for hepatic carcinoma treatment.

Acknowledgments

We acknowledge the financial support from National Natural Science Foundation of China (No. 81703360), the Natural Science Foundation of Shandong Province (No. ZR2016HB43, JQ2017021), the Young Taishan Scholars Program (tsqn20161037), and the Shandong Talents Team Cultivation Plan of University Preponderant Discipline (No. 10027).

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Highlights:

- Thirteen new hydroxamate-based bis-substituted aromatic amide HDAC inhibitors with 1,2,4-oxadiazole core were obtained.
- The structures were elucidated by comprehensive spectroscopic analyses.
- Three compounds displayed excellent HDAC1 inhibitory effects and significant anti-proliferative activities.
- Two compounds increased acetylation of histone H3 and H4 in dose-dependent manner.
- Two compounds remarkably induced apoptosis in HepG2 cancer cells.

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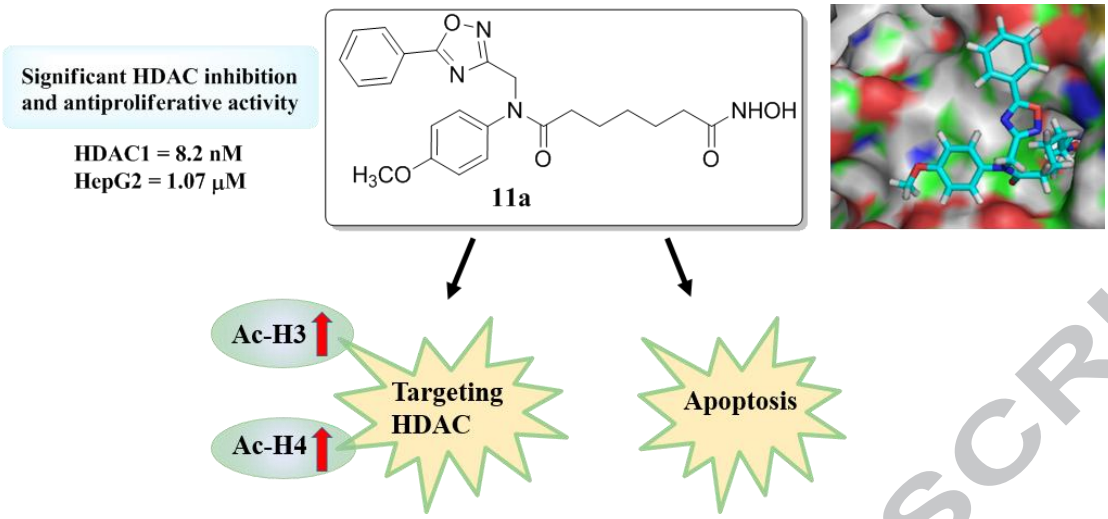

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