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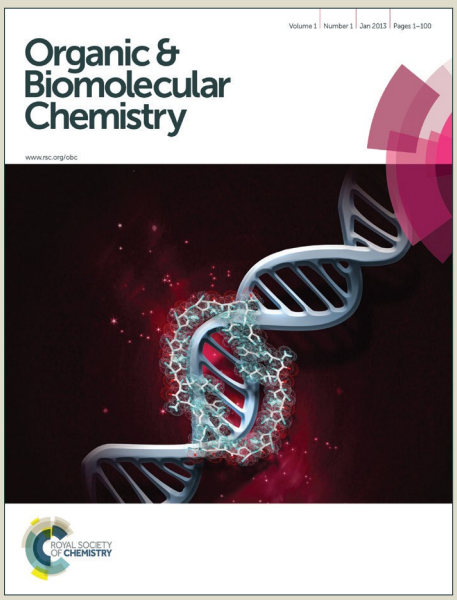

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ARTICLE

Hybrid of Thiazolidinone with Hydroxamate Scaffold for Developing Novel Histone Deacetylase Inhibitors with Antitumor Activities

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A series of novel histone deacetylase (HDAC) inhibitors were designed, synthesized and evaluated based on the strategies of hybrid of classic pharmacophore of HDAC inhibitors with thiazolidione scaffold. Some of compounds showed potent HDAC1 inhibition with nM IC_{50} values, More importantly, compound **12i** displayed much better anti-metastatic effects than Vorinostat (SAHA) against migration of A549 cell line. Further mechanism exploration implied that compound **12i** may inhibit tumor metastasis via modulating the epithelial-mesenchymal transition (EMT) and upregulating the acetylation of α-tubulin.

Introduction

Epigenetic regulations, involving modifications of chromatin structure, were one of the main causes of disordered gene expressions, which may be responsible for the occurrence and development of tumors, characterized with uncontrolled cell growth, proliferation, migration and so on.[1, 2] Histone deacetylases (HDACs) along with histone acetyl transferases (HATs) have been proven to regulate the acetylation states of histones and some other key non-histone proteins.[3, 4] For example, HDACs were involved in remodelling of chromatin and down-regulating many gene expressions,[5] while HATs were involved in opening chromatin structure and activation of transcription.[6] Global hyper-deacetylation of histones is also considered as a common hallmark of many kinds of cancers.[7, 8] Therefore inhibition of HDACs activities has been largely validated as one of the viable therapeutic strategies for cancer treatment.[9, 10]

Up to now, 18 human HDACs have been found and divided into four distinct classes.[11, 12] Class I (HDACs 1, 2, 3 and 8), II (HDACs 4, 5, 6, 7, 9 and 10), and IV (HDAC 11) are zinc-dependent metalloproteinases, whereas class III HDACs (sirtuins 1–7) are nicotinamide adenine dinucleotide (NAD^+) dependent.[7, 13] Different HDACs contribute to numerous specific processes during tumorigenesis and development.[14] HDAC inhibitors, **1** (Vorinostat, suberoylanilide hydroxamic acid, SAHA) and **2** (Romidepsin, FK-228) have been approved by the FDA for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006 and 2009 respectively (Figure 1),[15-17] Recently the third and fourth HDAC inhibitors, **3** (Belinostat) and **4** (Panobinostat), were also approved by the FDA for the treatment of relapsed or refractory peripheral T-cell lymphoma (PTCL) and multiple myeloma (MM) respectively.[17] In addition, many other HDAC inhibitors are in clinical trials, which validate that design and discovery of novel HDAC inhibitors are still one of effective strategies for cancer therapies.[18-21]

Various clinical trials of combining HDAC inhibitors with other anticancer agents have been proven as an effective strategy for broadening the spectrum in the treatment of solid tumor fields.[22] Several dual or multi- action HDAC inhibitors (Figure 1) were developed based on this successful strategy. For example, Compound **5** was equipped with HDAC inhibition as well as estrogen receptor modulation.[23] Compound **6** targeted HDAC and 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) for cancer treatment.[24] Moreover, Compounds **7** and **8** were also reported by different research groups.[25, 26] These results indicated that conjugation of the classic HDAC inhibitor moiety with other pharmacophores could promote the potency of HDAC inhibitors in the treatment of solid tumors. Tumor metastasis is regarded as a major contributor to the mortality of cancer patients,[27-29] therefore discovery of novel dual or

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multi-functional HDAC inhibitors bearing antitumor growth as well as metastasis activities is still our priority for solid tumor treatments.

(Figure 1 Insert here)

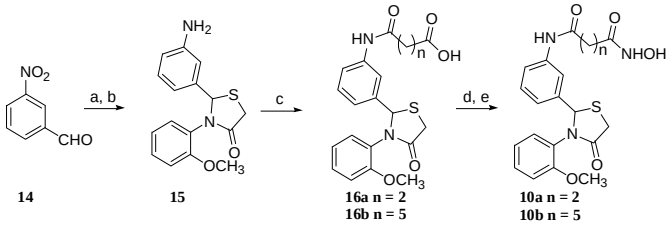
The classic structural scaffold of HDAC inhibitors consists of three distinct structural motifs,[30] a recognition cap group (CAP), a linker domain (Linker) and a zinc-binding group (ZBG, Figure 2).[31-34] The cap group presents a potential opportunity to discover more potent HDAC inhibitors.[32] One small molecule **9** (CK-548) and its derivatives, with thiazolidinone scaffold, have been reported to regulate the cellular movements and strongly inhibit tumor growth and metastasis.[35, 36] In addition, a series of novel hydroxmate-based HDAC inhibitors was also designed and optimized in our previous research work and displayed excellent antitumor growth and metastasis activities,[8] therefore one type of novel HDAC inhibitors was herein designed and modified with hybrid of hydroxamate scaffold and thiazolidinone structure as the CAP region (Figure 2).[24, 37-40] Their biological activities were further explored against tumor cell proliferation and migration as well as possible mechanisms.

(Figure 2 Insert here)

Chemistry

With the goal of discovering more novel HDAC inhibitors with high potent antitumor activities against solid tumors, two conjugated compounds **10a–b** were firstly synthesized according to the procedures described in **Scheme 1**. The intermediate 2-(3-nitrophenyl) -3-(2-methoxyphenyl)-4-thiazolidinone **15** was constructed from 2-methoxyaniline, 3-nitro-benzaldehyde and thioglycolic acid in toluene,[36, 41] which was then reduced to corresponding amine. Condensation of this amine intermediate **15** with succinic or pimelic acid anhydride in 1,4-dioxane and with subsequent esterification to afford esters compounds, and then the ester groups were treated with NH_2OH•HCl in methanol to get the compounds **10a–b.**[42]

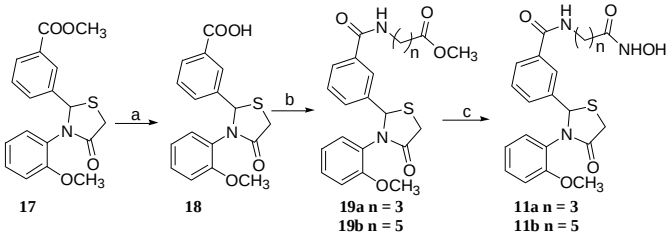
Scheme 1. Synthesis of compounds **10a**–**b**[a]



[a]Reagents and conditions: (a) *o*-anisidine, thioglycolic acid, toluene, reflux, 82%; (b) Zn, CH_3COOH, $EtOH/H_2O$, 85%; (c) Succinic or pimelic acid anhydride, 1,4-dioxane, reflux, 60-80%; (d) MeOH, $SOCl_2$, reflux, 80-90%; (e) NH_2OH•HCl, MeOH, KOH, 30-70%.

Compounds **11a–b** were prepared following the methods described in **Scheme 2**. The starting material **17** was synthesized in a similar manner of step 1 of scheme 1, then the ester group was hydrolyzed under basic condition to yield acid analogue **18**, which was coupled with amino acid esters (n = 3 or 5) and the target compounds were achieved by transforming ester to hydroxamate group.

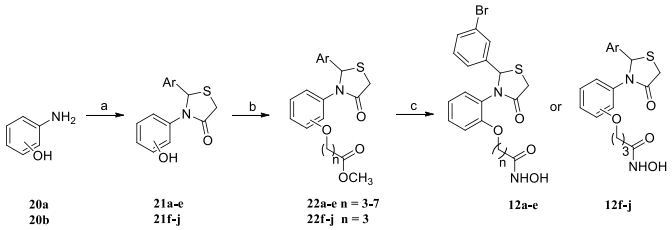
Scheme 2. Synthesis of compounds **11a**–**b**



Reagents and conditions: (a) LiOH•H_2O, $MeOH/H_2O$, 85%; (b) $NH_2(CH_2)nCOOCH_3$, EDC, HOBt, DMF, 60-80%; (c) NH_2OH•HCl, MeOH, KOH, 30-70%.

Target compounds **12a–j** were synthesized according to the procedures described in **Scheme 3**. The intermediate 2,3-diaryl-4-thiazolidinone derivatives **21a-j** were constructed from aminophenol, a series of aromatic aldehydes and thioglycolic acid, which was alkylated with brominated fatty acid esters (n = 3-7) to yield esters compounds **22a-j**, and the ester groups were treated with NH_2OH•HCl in methanol to get the compounds **12a–j**.

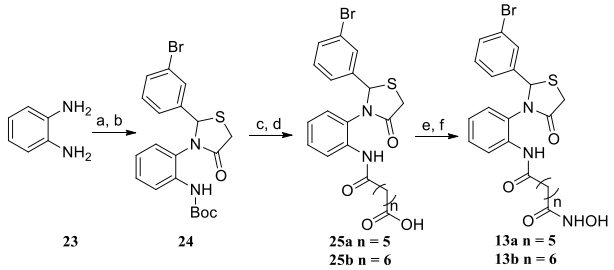
Scheme 3. Synthesis of compounds **12a**–**j**



Reagents and conditions: (a) ArCHO, thioglycolic acid, toluene, reflux, 40-50%; (b) $Br(CH_2)nCOOCH_3$, K_2CO_3, DMF, 80-90%; (c) NH_2OH•HCl, MeOH, KOH. 30-70%

The compounds **13a–b** were synthesized according to the methods described in **Scheme 4**. The starting compound **23** was firstly protected by Boc group, and was converted to the 2,3-diaryl-4-thiazolidinone compound **24**, then the Boc group was cleaved in acidic condition, and subsequent route was the same as step c-e in **Scheme 1**.

Scheme 4. Synthesis of compounds **13a**–**b**



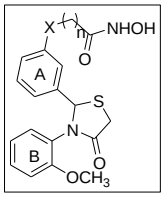
Reagents and conditions: (a) $(Boc)_2O$, $NaHCO_3$, Et_2O, 46%; (b) 3-bromobenzaldehyde， thioglycolic acid, toluene, reflux, 75%; (c) TFA, DCM, 80%; (d) anhydride, 1,4-dioxane, reflux, 60-80%; (e) MeOH, $SOCl_2$, reflux; (f) NH_2OH•HCl, MeOH, KOH, 30-70%.

Results and discussion

HDAC1 Inhibition Assay.

All the target compounds were firstly evaluated for their inhibitory activities against HDAC1 considering that class I HDACs isozymes are mainly involved in tumorigenesis and development. Compound **1** (Vorinostat) was selected as a positive control. In order to validate the location of linker and ZBG region in the scaffold, compounds **10a–b** and **11a–b** were synthesized with the linker and ZBG region located on the *meta*-position of A ring. The results in Table 1 indicated that there was a linker-length-dependent HDAC1 inhibition, compounds **10b** and **11b** containing five-methylene in the linker part showed better activities than analogues with two or three-methylene (**10a** and **11a** respectively), which was consistent with classical feature of HDAC inhibitors. In addition, the inversion of amide bond listed in the linker part seemed to show little difference against HDAC1 activity (**10b** *vs* **11b**).

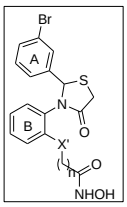
Table 1. IC_{50} values of HDAC1 inhibition of compounds **10a–b** and **11a–b**



Cpd.	X	n	IC_{50} (nM)
10a	-$NHCOCH_2$-	1	350 ±70
10b	-$NHCOCH_2$-	4	17 ±2
11a	-$CONHCH_2$-	2	4930 ±440
11b	-$CONHCH_2$-	4	37 ±1
1	---	---	10 ±3

On the other hand, the linker and ZBG region were transferred to the *ortho*-position of B ring and a series of compounds **12a–e** and **13a–b** were synthesized with different linker length in order to further demonstrate the structure activity relationship between the different linker length and HDAC1 inhibitory activities. As shown in Table 2, the activities of target compounds were improved with the elongation of linker, for example, compound **12e,** containing the longest linker length, showed the most potent activity (IC_{50} = 29 ±8 nM) with the n = 7 in this series of analogues (**12a–e**). In addition, when the oxygen atom was then replaced by amide, the inhibitory activities were found to be similar to that with the same linker length (**12d** *vs* **13a** and **12e** *vs* **13b**).

Table 2. IC_{50} values of HDAC1 Inhibition of compounds **12a–e** and **13a–b**

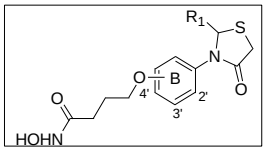


Cpd.	X’	n	IC_{50} (nM)
12a	O	3	1860 ±110
12b	O	4	530 ±20
12c	O	5	260 ±5
12d	O	6	50 ±4
12e	O	7	29 ±8
13a	-$NHCOCH_2$-	4	29 ±0
13b	-$NHCOCH_2$-	5	52 ±7
1	---	---	10 ±3

Further modifications focused on A ring while keeping the linker region with three-methylene linker length on the *ortho*–position of B ring in order to maintain suitable molecular weights of target compounds. The A ring was replaced with heterocycles for increasing activities, which were also reported in our previous research work for obtaining increased activities.[8] Unfortunately, compounds **12g** and **12h** displayed decreased inhibitory activities. However, when the linker and ZBG region were introduced to *para*–position of B ring, the enzymatic inhibitory activities were similar to that of the

corresponding analogues (**12i** and **12j**). The results suggested that substituted positions of the linker and ZBG region of B ring impacted the efficacy of compounds definitely. Inhibition activity of compound **12i** with *para*–position substitution was approximately 20-fold better than its counterpart compound **12g** with *ortho*–position.

Table 3. IC_{50} values of HDAC1 Inhibition of compounds **12f–j**



Cpd.	position	R_1	IC_{50} (nM)
12f	2'-	4-Cl-Ph$(CH_2)_2$NHCOPh-	320 ±70
12g	2'-	5-bromothiopheneyl	1360 ±230
12h	2'-	5-(5-pyrimidinyl)thiopheneyl	1440 ±190
12i	4'-	5-bromothiopheneyl	73 ±14
12j	4'-	5-(5-pyrimidinyl)thiopheneyl	91 ±1
1	---	---	10 ±3

Anti-proliferative activities against four different HDAC1 highly-expressed cell lines in vitro.

In order to evaluate the activities of those compounds against tumor cells with high HDAC1 expression, compounds **10b**, **11b**, **12d–e**, **12i** and **13a** were selected based on their potent inhibition against HDAC1 enzyme to test their anti-proliferative effect against a panel of four different tumor cell lines (HeLa, MCF-7, LNCaP and A549) through SRB assay with their highly expression of HDAC1.[43] And compound **1** was also chosen as the positive control, as listed in table 4. It is surprising for us that although compounds (**10b** and **11b**) with the linker and ZBG region located on A ring showed potent HDAC1 inhibition with low nM IC_{50} values, both of them lost most of proliferative effects against LNCaP and A549 cell lines compared to the analogues with located in B ring or positive control **1**. These results indicated that the introduction of the linker and ZBG to A ring may not be suitable for increasing their potential as novel anticancer agents. However, compounds with the linker and ZBG to B ring exhibited comparable activities as **1**, especially for **12i** and **13a**, which showed almost equivalent activities compared to **1** against four different cancer cell lines, with the IC_{50} values ranging from 2.1 ±0.5 to 5.4 ±0.5 μM.

Table 4. Anti-proliferative activities of representative compounds (The values are averaged from 3 independent experiments)

Cpd.	Anti-proliferation activities IC_{50} (μM)			
	HeLa	**MCF-7**	**LNCaP**	**A549**
10b	21 ±3	21 ±3	> 50	> 50
11b	7.3 ±0.8	10 ±2	27 ±2	47 ±3
12d	3.2 ±0.4	4.1 ±0.4	4.0 ±0.7	6.7 ±0.2
12e	6.4 ±1.1	6.0 ±0.6	31 ±3	>50
12i	3.2 ±0.5	2.1 ±0.5	2.9 ±0.3	4.6 ±0.8
13a	2.1 ±0.4	5.2 ±0.8	2.5 ±0.9	5.4 ±0.5
1	2.8 ±0.2	1.3 ±0.3	3.6 ±1.0	7.2 ±0.1

*Compounds **12i** and **13a** inhibited the colony formation of A549 cell line.*

In order to further evaluate the efficacy of these compounds against tumor cell proliferation and development, the colony formation assay on A549 cell line was performed to confirm the anti-proliferative activities of compounds **12i** and **13a** with compound 1 as the positive control. As one of the gold standards for measuring the effects of cytotoxic agents on cancer cells *in vitro*, it closely mimics the pathological process from individual tumor cell development to macroscopic cell clones *in vivo*.[44, 45] As shown in Figure 3, the data indicated that both of compounds **12i** and **13a** significantly inhibited colony formation of A549 cells in a dose-dependent manner, at meantime, they also showed better efficacy compared to **1** concerning the number and size of colony. All of these results suggested that both of **12i** and **13a** may partly block the *in vivo* growth and development of A549 cancer cell line.

(Figure 3 Insert here)

*Compounds **12i** and **13a** induced the level of acetylated histone on A549 cell line.*

Growing evidences indicated a strong correlation among overexpression of HDACs activities, deacetylation of the histone tails (particularly histone H3 and H4 tails) and chromatin architecture in tumors.[46] Thus the anticancer effect of HDAC inhibitors could be also assessed by their potency in inducing hyper-acetylation of histone H3 or H4,[14] In order to confirm the HDAC inhibitory activity of these compounds, **12i** and **13a** were chosen to examine their efficacy for

histone acetylation at two different concentrations (2.0 and 5.0 μM) and with compound **1** as a positive control. As listed in Figure 4, the results demonstrated that compounds **12i** and **13a** significantly upregulated the level of acetylated histone H3 and H4 in a dose-dependent manner as well as positive control **1**, which also indicated that both of **12i** and **13a** can increase the acetyl level of histone of cancer cells through inhibiting the activities of HDACs.

(Figure 4 Insert here)

*Compounds **12i** and **13a** induced cell cycle arrest and enhanced apoptosis on A549 cell line*

Previous results of cellular cytotoxicity assay and colony formation assay indicated that **12i** and **13a** inhibited cancer cell proliferation. Next, whether these compounds affected the cancer cell cycle arrest and induction of apoptosis was explored by flow cytometry. The results showed that **12i** and **13a** caused cell apoptosis in A549 cell line in a dose-dependent manner, and early apoptosis induction was especially evident (Figure 5A). And **12i** and **13a** were also found to increase cell numbers at G2/M phase after 24 h treatment with raising concentration, accompanied with decreased cell numbers at S phase and G1 phase in A549 cell line (Figure 5B). These results indicated that **12i** and **13a** inhibited cell proliferation by inducing cell cycle arrest and enhancing apoptosis on cell line.

(Figure 5 Insert here)

*Compound **12i** suppressed metastatic activities on A549 cell line.*

Based on our initial strategies for developing novel HDAC inhibitors with potent anti-proliferative and anti-migratory effects against tumor cells, it is valuable to investigate their anti-migratory effect against cancer cell line. Compounds **12i** and **13a** were also chosen and the results were shown in Figure 6A and 6B. In a transwell migration assay, compound **12i** obviously decreased A549 cell migration at the investigated concentration (2.0 and 5.0 μM), with the IC_{50} value about 3.2 μM, which was about 6-7 fold more potent than **1**, with IC_{50} value about 20 μM. These data suggested that compound **12i** possesses more anti-migration efficacy than **1** in the transwell migration model. To study the possible mechanism of **12i** on cell migration, some potential downstream effectors of tumor metastasis were chosen to be determined. The epithelial-mesenchymal transition (EMT), as a highly conserved cellular program that allows polarized, immotile epithelial cells to be converted to motile mesenchymal cells, has been widely implicated in promoting carcinoma invasion and metastasis.[47] Western blot analysis displayed the upregulation of epithelial markers (E-cadherin and β-catenin) and a downregulation of a mesenchymal marker (vimentin) after treatment with **12i** (Figure 6C). According to previous research, the regulation of tubulin acetylation was closely related to cell motility.[48] In our experiment，compound **12i** was found to induce the acetylation level of α-tubulin significantly (Figure 6D). These results implied that compound **12i** may inhibit tumor metastasis via modulating the EMT and upregulating the acetylation of tubulin.

(Figure 6 Insert here)

Conclusions

In summary, a series of hybrid of thiazolidinone and hydroxamic acid derivatives were designed and synthesized as potent HDAC1 inhibitors. Some of them showed comparable enzymatic activities as **1** against HDAC1. Among them, compound **12i** not only exhibited potent anti-proliferative activities against four different cancer cell lines and inhibited colony formation of cancer cells, but also showed better anti-metastasis activity via modulating the EMT and upregulating the acetylation of α-tubulin against A549 cell line. In addition, compound **12i** was found to induce cell cycle arrest and enhance cancer cell apoptosis. Preliminary mechanism studies also indicated that **12i** behaved according to multiple mechanisms including HDAC inhibition. All of these data led to a new promising direction of exploring novel HDAC inhibitors with dual functions. Continued efforts are in progress in our laboratory to develop more potent dual or multi-functional HDAC inhibitors.

Figure legends:

Figure 1. FDA approved HDAC Inhibitors and representative examples of dual-action HDAC inhibitors

Figure 2. Design of conjugated compounds based on HDAC and Arp2/3 complex inhibitors

Figure 3. *A.* Compounds **12i** and **13a** inhibited the colony formation of A549 cell lines**.** After treatment by various concentration of ***12i and 13a*** in 6-well plates for 7 days, cells were fixed and stained with crystal violet and the numbers of cell colonies were counted. ** $p < 0.01$, *** $p < 0.001$ versus the control group. ***B.*** Compounds **12i** and **13a** induced the level of acetylated histone in A549 cell lines.

Figure 4. Western blot analysis of the effects of compounds **12i** at different concentrations on tumor cell A549 acetylated histone levels. Treatment resulted in upregulated expression of acetyl-histone H3 and H4 levels in a dose-dependent manner.

Figure 5. *A. 12i* and ***13a*** mediated A549 cells apoptosis in concentration-dependent manner. Cells were treated with different

concentration of ***12i*** *and* ***13a*** for 48 h. Then the percentage of apoptosis cells was analyzed with flow cytometry by staining with PI and annexin V-FITC. ***B. 12i*** and ***13a*** induced cell cycle arrest of A549 cell line. Cells were stained with PI and analyzed by flow cytometry after treatment with various concentration of ***12i*** *and* ***13a*** for 24 h.

Figure 6. ***A***. Representive images of Compound **12i** inhibiting A549 cell migration in the transwell assay different concentrations. ***B***. Statistical analysis result of cell migration of **12i**, **13a** and **1**, ** $p < 0.01$, *** $p < 0.001$ versus the control group. *C. and D.* Compound **12i** inhibits tumor metastasis via modulating the EMT and upregulating the acetylation of tubulin. Cell lysates were extracted after treating A549 with **12i** or **1** for 48 h. Cell extracts were run on SDS−polyacrylamide gels and probed using indicated antibodies.

Acknowledgements

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