Design, Synthesis and Biological Evaluation of Nitro Oxide Donating N-Hydroxycinnamamide Derivatives as Histone Deacetylase Inhibitors

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Novel nitro oxide (NO)-donating N-hydroxycinnamamide derivatives 12a–j were designed and synthesized by coupling the carboxyl group of N-hydroxycinnamamides with phenylsulfonylfuroxan through various diols or alkyl amines, and their in vitro biological activities were evaluated. It was discovered that most of target compounds showed good histone deacetylases (HDACs) inhibition and anti-tumor activities, particularly for 12j, which had great HDACs inhibitory activities (IC_{50s}=0.15–0.26µM) and antiproliferative effects (IC_{50s}=3.21–7.12µM) comparable to suberoylanilide hydroxamic acid (SAHA) (IC_{50s}=0.16–1.41µM for HDACs, IC_{50s}=3.15–7.45µM for cancer cell inhibition). Furthermore, compound 12j with strong antitumor activities produced high levels of NO (up to 8.0µM of nitrites/nitrates) in colon cancer cells, and its antiproliferative activity was nearly half-diminished by hemoglobin (10µM), an NO scavenger. These results suggest that the strong antiproliferative activity of 12j could be attributed to the additive effects of high levels of NO production and inhibition of HDAC in the cancer cells.

Key words nitro oxide; histone deacetylase inhibitor; furoxan; N-hydroxycinnamamide; anti-tumor agent

Pharmacological targeting of proteins that regulate epigenetics has emerged as a promising therapeutic area of study. Epigenetic or chromatin modification is recognized by nonhistone proteins and is a code of gene expression. Among the various histone modifiers, Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are two reversible enzymes regulating histone acetylation status and executing the acetylation and deacetylation of the lysine residues at the amino terminal of histones. However, abnormal HDAC overexpression has been found to be involved in the development of several kinds of human cancers, including myeloid neoplasia and solid tumors. Recent studies have shown that acetylation of non-histone proteins is also relevant for tumorigenesis, cancer cell proliferation, and immune functions. Consequently, histone deacetylases are considered to be important targets in the development of anticancer agents, and in recent years considerable attention has been paid to HDAC inhibitors (HDACI) as anticancer agents.

There has been a high level of interest in developing small-molecule HDACI, and numerous structurally diverse HDACI have been developed as potential anticancer agents, which are grouped chemically into four classes: hydroxamic acids, benzamides, cyclic tetrapeptides, and short-chain fatty acids. The common pharmacophore of these HDACI consists of three domains: a zinc-binding group (ZBG), such as hydroxamic acid; a cap group, generally a hydrophobic and aromatic group; a saturated or unsaturated linker domain, composed of linear or cyclic structures that connect the ZBG and the cap group. Up to now, two of these HDACI, suberoylanilide hydroxamic acid (SAHA, Fig. 1) and cyclic peptide Romidepsin (FK228), have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL), validating HDACs’ potential as an important target for anticancer therapy. Other compounds in clinical trials include hydroxamic acids such as belinostat (PXD101), panobinostat (LBH589), and pracinostat (SB-939) with an N-hydroxycarboxamide moiety exhibiting excellent HDAC inhibitory activity, such as entinostat (MS-275), mocetinostat (MGCD-0103), and aliphatic acids, such as valproic acid. In addition, the selective HDAC6 inhibitor, ACY-1215, is currently undergoing human clinical trials for the treatment of multiple myeloma. Among these approved or clinical HDACI, PXD101, LBH589, and SB-939 share a common active fragment, N-hydroxycinnamamide. This fragment could not only form bichelation with the active site Zn^{2+} by its hydroxamic acid group but also form a sandwichlike π–π interaction by inserting its vinyl benzene group into two parallel phenylalanine residues of HDAC.

Nitro oxide (NO), which is naturally generated from l-arginine by the action of NO synthase (NOS), is a key signaling and effector molecule and plays a pivotal role in diverse physiological and pathophysiological processes. It is well-known that high concentration of NO, generated from synthetic NO-releasing compounds, could not only have strong cytotoxicity against human carcinoma cells and induce the apoptotic of tumor cells, but also prevent tumors from metastasizing and sensitize tumor cells to chemotherapy, radiation and immunotherapy in vitro and in vivo. As an important class of NO donors, furoxans such as phenylsulfonylfuroxans are thermally stable compounds and can produce high levels of NO in vitro and inhibit the growth of tumors in vivo. Furthermore, it was reported that tumor cells are more sensitive to relative high concentration of NO than normal cells and a variety of phenylsulfonylfuroxan-based NO releasing compounds have been investigated to show selective antitumor activity in vitro and in vivo.

It is known that the Class I enzyme HDAC2 can be directly or indirectly modified by NO, undergoing either Tyr-nitration or S-nitrosylation. One example of NO-donor HDACI, which is able to simultaneously release NO and regulate HDACs, affects a number of micro-RNAs and synergistically promotes myogenic differentiation. It is therefore of high interest to investigate whether introduction of phenylsulfonylfuroxan moiety to the N-hydroxycinnamamides would provide

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a hitherto unknown class of NO donating HDAC inhibitors that may effectively develop inhibitory effect of HDACs and release high levels of NO with an additive effect for treatment of cancer. As part of our ongoing research program, a series of phenylsulfonylfuroxan-based N-hydroxycinnamamide derivatives 12a–j were designed and synthesized by coupling the carboxyl of N-hydroxycinnamamides with phenylsulfonylfuroxan through different carbochain length substituted diols or alkylol amines.

Chemistry

The synthesis of 12a–j was described in Chart 1. The substituted furoxans 6a–j were prepared in a four step sequence. Thiophenol 1 as the starting material was converted to 2-(phenylthio)acetic acid 3 by treatment with chloroacetic acid 2 in 97% yield. Oxidation of 3 using 30% H₂O₂ aqueous solution in acetic acid afforded 2-(phenylsulfonyl)acetic acid 4, which was directly reacted with fuming nitric acid to form diphenylsulfonylfuroxan 5. Compound 5 was then converted to various monophenylsulfonyl furoxans 6a–j by treatment with corresponding diol or alkylol amines. On the other hand, methyl 4-formylbenzoate 7 was reacted with propanedioic acid in the presence of piperidine to give (E)-3-(4-(methoxycarbonyl) phenyl) acrylic acid 8, which was further treated with tetrahydro-2H-pyran-2-ol to form compound 9 in the presence of N-methylmorpholine and ethyl chloroformate.
slightly weaker than those of amide compounds –12f by IR, 1H-NMR, MS, and elemental analyses.umn chromatography, and their structures were characterizedvide suggested the amide bond of 12f –HDACs inhibitory activities of ester derivatives –12a were measured and presented in Table 1. The results indicated the the target compounds against HeLa cell nuclear HDACs werements.

Fig. 2. NO Release Behaviors of the Indicated Compounds and Their Correlation with in Vitro Antiproliferative Activities

(A) Quantitative measurement of intracellular NO production. HCT116 cells were treated with various compounds (50 μM) for 6 h, and the amounts of intracellular nitrite/nitrate were determined using a colorimetric Griess reaction assay. Data are expressed as means±S.D. from three separate experiments. (B) HCT116 cells were pre-treated with, or without, the indicated concentrations of NO scavenger hemoglobin (Hb) for 1 h and then treated with 5 or 10 μM of 12j for 24 h. The cell growth was determined by the MTT. Data are expressed as mean% of inhibition on the growth of HCT116 cells from three independent experiments. The cells treated with different concentrations of hemoglobin alone did not affect their growth (data not shown).

Table 1. Inhibition of HeLa Nuclear HDACs by 12a–j

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IC50 (μM)</th>
<th>Compd.</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAHA</td>
<td>0.17</td>
<td>12f</td>
<td>0.18</td>
</tr>
<tr>
<td>12a</td>
<td>0.55</td>
<td>12g</td>
<td>0.21</td>
</tr>
<tr>
<td>12b</td>
<td>0.86</td>
<td>12h</td>
<td>0.59</td>
</tr>
<tr>
<td>12c</td>
<td>&gt;1</td>
<td>12i</td>
<td>0.25</td>
</tr>
<tr>
<td>12d</td>
<td>&gt;1</td>
<td>12j</td>
<td>0.15</td>
</tr>
<tr>
<td>12e</td>
<td>&gt;1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) The data are the mean values of IC50 from at least three independent experiments.

Compound 9 was subsequently hydrolyzed to obtain interme- diate 10, which were directly treated with 6a–j in CH2Cl2 in the presence of 1-(3-dimethyl aminopropyl)-3-ethylcarbodi-imide hydrochloride (EDCI) and 4-dimethylamino pyridine (DMAP) to generate 11a–j, respectively. Finally, O-protected groups of 10 and 11a–j were removed by CF3COOH to pro- vide 10a and 12a–j. The products 12a–j were purified by column chromatography, and their structures were characterized by IR, 1H-NMR, MS, and elemental analyses.

Results and Discussion

All synthesized compounds 12a–j were evaluated for their ability to inhibit HeLa cell nuclear HDACs (which consist of mostly HDAC1–3), with SAHA as a reference compound. The values of half inhibitory concentration (IC50) about all the target compounds against HeLa cell nuclear HDACs were measured and presented in Table 1. The results indicated the HDACs inhibitory activities of ester derivatives 12a–e were slightly weaker than those of amide compounds 12f–j, which suggested the amide bond of 12f–j could be beneficial for them to bind to the active site. Moreover, among these amide compounds, compound 12j possessed the strongest HeLa nuclear HDACs inhibition comparable to that of SAHA.

Furthermore, the active compounds 12f and g, 12i and j, and NO donor moiety 6j (the intermediate of 12j) was further tested for their in vitro HDACs inhibitory activities toward HDAC2, HDAC6 and HDAC8 in order to know whether active compounds possessed strong HDACs inhibitory effects and isoform selectivity. Results listed in Table 2 showed that 12f and g and 12i and j exhibited significant HDACs inhibitory activities against HDACs 2, 6, and 8. Both 12j and NO donor moiety 6j demonstrated significant effects on HDAC2 comparable to SAHA, which may partially be due to NO re-lease from 12j or 6j inducing S-nitrosylation of HDAC2.29,30 Furthermore, compounds 12f and g and 12i and j also showed stronger inhibitory potency than SAHA for HDAC8. However, the active compounds 12f and g and 12i and j did not exhibit any isoform selectivity in the HDACs inhibitory assay.

Next, we tested whether the levels of NO production upon treating HCT116 cells with target compounds were associated with their antitumor activities. The levels of nitrite/nitrate produced in the cell lysates of different types of cells were characterized using the Griess assay. As can be seen in Fig. 2A, treatment with SAHA resulted in little nitrite/nitrate. In contrast, treatment with 12f–j produced high levels of nitrite/nitrate in HCT116 cells. The most potent compound 12j released the highest levels of NO, and the derivatives with lower antiproliferative activity released less NO under the same conditions. These results indicate that the amounts of intra-cellular NO release were well associated with their in vitro antiproliferative activity. To further determine the contribution of NO to the inhibitory activities of NO-donating N-hydroxycinnamamide derivatives, 12j was selected for testing its anti-proliferative activity in the presence or absence of NO scavenger hemoglobin (Hb). HCT116 cells were pre-treated with, or without, different concentrations of Hb for 1 h and then

Table 2. IC50 Value for the Inhibition of HDACs 2, 6, and 8 by 12f, g and 12i, j

<table>
<thead>
<tr>
<th>Compd.</th>
<th>HDAC2</th>
<th>HDAC6</th>
<th>HDAC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAHA</td>
<td>0.18</td>
<td>0.16</td>
<td>1.41</td>
</tr>
<tr>
<td>6j</td>
<td>0.43</td>
<td>ND(1)</td>
<td>ND</td>
</tr>
<tr>
<td>12f</td>
<td>0.16</td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td>12g</td>
<td>ND</td>
<td>0.23</td>
<td>0.46</td>
</tr>
<tr>
<td>12i</td>
<td>ND</td>
<td>0.28</td>
<td>0.42</td>
</tr>
<tr>
<td>12j</td>
<td>ND</td>
<td>0.16</td>
<td>0.26</td>
</tr>
</tbody>
</table>

a) The data are the mean values of IC50 from at least three independent experiments. b) Not detected.
treated with the indicated concentrations of 12j. The effects of different treatments on the growth of HCT116 cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Fig. 2B). Treatment with 12j alone greatly inhibited the growth of HCT116 cells, while the inhibitory effects of 12j on the growth of HCT116 cells were dramatically reduced by pre-treatment with Hb, which appeared to be dose-dependent. Similar patterns of reduced levels of nitrate/nitrite in the cell lysates were observed. These data clearly demonstrated that high levels of NO production by the compound contributed to its antiproliferative activity against human cancer cells in vitro.

Next, synthetic compounds 11j, 12a–j were also assessed for anti-proliferative activities against five human cancer cells HCT116, SW620, Lovo (human colon carcinoma cells), MCF-7 (human breast adenocarcinoma cells), and Hela were evaluated by MTT assays in vitro, with SAHA as positive control. The values of half inhibitory concentration (IC_{50}) about all the target compounds against each tumor cell line were measured and presented in Table 3. For most of the compounds tested, the cell growth inhibition results were consistent with the HDAC inhibition profiles. Most of the target compounds displayed good to moderate antiproliferative activities. The antitumor activities of ester derivatives 12a–e were slightly weaker than those of amide compounds 12f–j. What’s more, compound 12j exhibited strongest antiproliferative activities with IC_{50} values of 3.21–7.12 μM against each tested cancer cell, which were comparable to SAHA (IC_{50}=3.15–7.45 μM). Interestingly, compound 11j (the intermediate of 12j, IC_{50}=9.12–11.4 μM) displayed weak cancer inhibitory activities, which were 2–3-fold less than those of 12j. Such phenomenon suggested that the hydroxamic acid moiety has a positive influence on the cytotoxicity of this series of N-hydroxyferulicamide derivatives.

As active compound 12j consists of NO donor moiety (6j) and the N-hydroxycinnamamide moiety (10a) (Chart 1), the inhibitory effects of these two moieties on HCT116 cells were examined at two different concentrations. As shown in Fig. 3, the NO-donating moiety 6j retained a certain degree of inhibitory potency, which was similar to the N-hydroxycinnamamide moiety 10a. And both of them were obviously less potent than 12j. However, the combination of 10a with 6j significantly induced tumor cell antiproliferative effect comparable to 12j. These results suggest that the antitumor activity of 12j may be attributed to the additive effects of phenylsulfonylfuroxan and the N-hydroxycinnamamide moiety and that both of them may play crucial roles in the antiproliferative activity of 12j.

Structure–Activity Relationship (SAR) Analysis of SAR revealed that 12a–j with different linkers displayed variable antitumor activities. The compounds 12f–j linked with alkanalamin exhibited stronger inhibitory effects than the ester derivatives 12a–e linked with diol against human cancer cells. It is likely that the ester compounds can usually be metabolized into the corresponding acid and alcohol, which may lead to the partial loss of their inhibitory activity. In addition, variations in the carbocycle lengths of linkers significantly affected the in vitro anticancer activity of these derivatives. For example, the compounds 12a, 12f and 12j with a two-carbon diol or alkylol amine showed relatively stronger anticancer activity than compounds with a three- or four-carbon linker. However, further investigation about the precise SAR of these compounds is ongoing.

Conclusion

In summary, a series of novel NO-donating HDAC in-

Table 3. The IC_{50} Values of 11j and 12a–j against Five Human Cancer Cell Lines

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Hela</th>
<th>HCT116</th>
<th>SW620</th>
<th>Lovo</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAHA</td>
<td>3.15</td>
<td>5.83</td>
<td>4.72</td>
<td>5.56</td>
<td>7.45</td>
</tr>
<tr>
<td>11j</td>
<td>12.5</td>
<td>9.69</td>
<td>11.3</td>
<td>12.6</td>
<td>14.1</td>
</tr>
<tr>
<td>12a</td>
<td>9.51</td>
<td>9.47</td>
<td>9.12</td>
<td>10.5</td>
<td>11.4</td>
</tr>
<tr>
<td>12b</td>
<td>15.2</td>
<td>14.7</td>
<td>13.9</td>
<td>16.8</td>
<td>18.3</td>
</tr>
<tr>
<td>12c</td>
<td>25.8</td>
<td>21.6</td>
<td>20.5</td>
<td>24.6</td>
<td>27.5</td>
</tr>
<tr>
<td>12d</td>
<td>21.0</td>
<td>17.9</td>
<td>19.2</td>
<td>19.4</td>
<td>22.7</td>
</tr>
<tr>
<td>12e</td>
<td>15.8</td>
<td>13.6</td>
<td>15.0</td>
<td>18.6</td>
<td>16.4</td>
</tr>
<tr>
<td>12f</td>
<td>5.02</td>
<td>4.67</td>
<td>5.53</td>
<td>6.65</td>
<td>6.28</td>
</tr>
<tr>
<td>12g</td>
<td>6.71</td>
<td>5.78</td>
<td>6.14</td>
<td>8.17</td>
<td>7.18</td>
</tr>
<tr>
<td>12h</td>
<td>13.9</td>
<td>12.7</td>
<td>11.9</td>
<td>14.5</td>
<td>15.0</td>
</tr>
<tr>
<td>12i</td>
<td>7.27</td>
<td>6.89</td>
<td>7.46</td>
<td>8.75</td>
<td>9.59</td>
</tr>
<tr>
<td>12j</td>
<td>4.18</td>
<td>3.21</td>
<td>4.43</td>
<td>5.03</td>
<td>7.12</td>
</tr>
</tbody>
</table>

\( ^a \) The inhibitory effects of individual compounds on the proliferation of cancer cell lines were determined by the MTT assay. The data are the mean values of IC_{50} from at least three independent experiments.
hitors 12a–j were designed and synthesized by coupling the carboxyl group of N-hydroxycinnamamide with phenylsulfonylfuroxan through various carbochain length substituted diols or alkylol amines. The in vitro assay of their inhibitory activities against five human cancer cell lines showed that the phenylsulfonyl-furoxan-based N-hydroxycinnamamide derivatives displayed good to moderate antiproliferative activities. Especially, compound 12j had greater antiproliferation and HDACs inhibitory activities, comparable to SAHA, in human colon carcinoma cells. What's important, the compounds with strong HDACs inhibitory activities, comparable to SAHA, in human HCT116 cells. Our findings suggest that the NO-donating this compound may play an important role in antiproliferation a dose-dependent manner, suggesting that NO released by

Experimental
Melting points of individual compounds were determined on a Mel-TEMP II melting point apparatus and uncorrected. Infrared (IR) spectra (KBr) were recorded on a Nicolet Impact 410 instrument (KBr pellet). 1H-NMR was recorded with a Bruker Avance 300 MHz spectrometer at 303 K, with chemical shift in parts per million (ppm, δ) downfield from tetramethylsilane, or trimethylsilyl (TMS) as an internal standard. MS spectra were recorded on a Mariner Mass Spectrum (electrospray ionization (ESI)) and HR-MS on Agilent technologies LC/MSD time-of-flight (TOF). Element analysis were performed on an Eager 300 instrument. All compounds were routinely checked by TLC and 1H-NMR. TLCs and preparative thin-layer chromatography were performed on silica gel GF/UV 254, and the chromatograms were conducted on silica gel (200–300 mesh, Merck) and visualized under UV light at 254 and 365 nm. Thiophenol 1 and compounds 7 were commercially available, and compounds 3–5 and 6a–j were prepared according the literatures.24–26 All solvents were reagent grade and, when necessary, were purified and dried by standards methods. Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate.

(E)-3-(4-(Methoxycarbonyl)phenyl)acrylic Acid 8 To a flask was added methyl 4-formylbenzoate 7 (1.64 g, 10 mmol), propanedioic acid (1.56 g, 15 mmol), piperidine (0.08 mL, 0.81 mmol), and pyridine (10 mL) at room temperature. The reaction mixture was heated to 85°C for 3 h under a steady flow of nitrogen gas, cooled to room temperature, and poured into 2 mL aqueous HCl (100 mL). The resulting mixture was cooled to 0°C and filtered. The filter cake washed with acetonitrile (2×10 mL), and dried in vacuo to afford 8 as white solid, yield 85%, mp: 140–142°C.

(E)-4-(3-Oxo-3-((tetrahydro-2H-pyran-2-yl)oxy)aminoprop-1-en-1-yl)benzoic Acid 10 To a solution of 8 (1.65 g, 8 mmol) in 50 mL anhydrous tetrahydrofuran (THF) at 0°C were added N-methylmorpholine (1.05 g, 12 mmol) and ethyl carbonochloridate (0.87 g, 8 mmol), and the mixture was stirred at room temperature for 1 h, which was then added dropwise to a solution of tetrahydro-2H-pyran-2-ol (0.94 g, 8 mmol) in 10 mL anhydrous THF. After the reaction was completed, the resulting mixture was allowed to pour into ice-water, and extracted with ethyl acetate (30 mL×3). The organic phase was washed with water and brine, then dried over anhydrous sodium sulfate, filtered and evaporated to afford the crude product, which was poured into 5 mL methanol containing 1.5 mL 2 N NaOH and continuously stirred for 2 h. After the mixture being cooled, the solvent was evaporated, and the residue was neutralized to pH=3 with 1 mL HCl. The precipitate was filtered, washed with water, and dried in vacuo to afford the crude, which was purified by column chromatography on silica gel to give compound 10 as pale yellow waxy solid, yield 56%, MS (ESI) m/z=292 [M+H]+.

(E)-4-(3-(Hydroxamino)-3-oxoprop-1-en-1-yl)benzoic Acid (10a) A solution of 10 (0.23 g, 0.80 mmol) and CF3COOH (1 mL) in 4 mL dry CH2Cl2 was stirred at room temperature for 2 h. The solvent was removed under reduced pressure. The crude residue was dissolved in 15 mL dichloromethane and 2 mL Et3N was slowly added to the solvent, and the crude product was purified by column chromatography (MeOH–CH2Cl2=1:1–1:10) to yield 10a as white waxy solid, yield 88%. Analytical data for 10a: MS (ESI) m/z=208 [M+H]+; 1H-NMR (DMSO-d6, 300 MHz, δ ppm): 10.33 (br, 1H), 8.57 (br, 1H), 7.86 (d, J=7.8 Hz, 2H, Ar-H), 7.66 (d, J=7.8 Hz, 2H, Ar-H), 7.50 (d, J=16.2 Hz, 1H, CH=), 6.55 (d, J=16.2 Hz, 1H, CH=).

General Procedure for the Synthesis of 12a–j Compound 10 (0.23 g, 0.80 mmol) was mixed with 6a–j (0.80 mmol), EDCI (153 mg, 0.8 mmol), DMAP (97 mg, 0.80 mmol). The mixture was stirred at room temperature for 8–12 h. After filtration, the filtrate was evaporated to dryness in vacuo, and poured into 4 mL dry CH2Cl2 containing 1 mL CF3COOH in was stirred at room temperature for 1–2 h. The solvent was removed under reduced pressure. The crude residue was dissolved in 15 mL dichloromethane and 2 mL Et3N was slowly added to the solvent, and the crude product was purified by column chromatography (MeOH–CH2Cl2=1:6–1:10) to yield 12. (E)-4-(2-((4-(3-(Hydroxamino)-3-oxoprop-1-en-1-yl)-benzoyloxy)ethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-Oxide (12a) The title compound was obtained starting from 10 and 6a. White waxy solid, yield: 62%. Analytical data for 12a: IR (KBr, cm−1): 3433, 2925, 1746, 1622, 1446, 1211, 1018; MS (ESI) m/z=476 [M+H]+; 1H-NMR (DMSO-d6, 300 MHz, δ ppm): 8.63 (br, 1H), 8.04 (d, 2H, J=7.8 Hz, Ar-H), 7.84 (d, J=7.8 Hz, 2H, Ar-H), 7.73 (m, 1H, Ar-H), 7.52–7.63 (m, 4H, Ar-H), 7.48 (d, J=16.2 Hz, 1H, CH=), 6.55 (d, J=16.2 Hz, 1H, CH=), 4.59 (t, 2H, J=4.81 Hz, OCH2), 4.49 (t, 2H, J=4.81 Hz, OCH2); ESI-HR-MS (m/z): [M+H]+ Calcd for C29H24N4O9S: 476.0764; Obsd: 476.0770. Anal. Calcd for C29H24N4O9S: C, 50.53%; H, 3.60%; N, 8.84%. Found: C, 50.36; H, 3.69; N, 8.62.

(E)-4-(3-(Hydroxamino)-3-oxoprop-1-en-1-yl)benzoyloxy)propoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-Oxide (12b) The title compound was obtained starting from 10 and 6b. White waxy solid, yield: 65%. Analytical data for 12b: IR (KBr, cm−1): 3441, 2930, 1732, 1615, 1450, 1323, 1035; MS (ESI) m/z=490 [M+H]+; 1H-NMR (DMSO-d6, 300 MHz, δ ppm): 10.27 (br, 1H), 8.56 (br, 1H), 8.00 (d, 2H, J=7.8 Hz, Ar-H), 7.81 (d, J=7.8 Hz, 2H, Ar-H), 7.70 (m, 1H, Ar-H), 7.51–7.65 (m, 4H, Ar-H), 7.46 (d, J=16.2 Hz, 1H, CH=), 6.52 (d, J=16.2 Hz, 1H, CH=), 4.61 (t, 2H, J=4.8 Hz, OCH2),
(E)-4-(4-(4-(3-(Hydroxyamino)-3-oxoprop-1-en-1-yl)benzoyl)oxy)butan-2-yl)oxy)-3-(phenylsulfonyl)-1,2,5-oxidazole 2-Oxide (12d) The title compound was obtained starting from 10 and 6d. Pale yellow waxy solid, yield: 52%. Analytical data for 12d: IR (KBr, cm⁻¹): 3420, 2944, 1724, 1620, 1441, 1220, 1032; MS (ESI) m/z: [M + H]⁺: 490.1077; [M + H⁺-H₂O]: 464.0982. 

(E)-4-(4-(4-(3-(Hydroxyamino)-3-oxoprop-1-en-1-yl)benzoyl)oxy)butan-2-yl)oxy)-3-(phenylsulfonyl)-1,2,5-oxidazole 2-Oxide (12e) The title compound was obtained starting from 10 and 6e. Pale yellow waxy solid, yield: 53%. Analytical data for 12e: IR (KBr, cm⁻¹): 3420, 2931, 1700, 1615, 1450, 1170, 1020; MS (ESI) m/z: [M + H]⁺: 489.1077; [M + H⁺-H₂O]: 463.0982. 

(E)-4-(4-(4-(3-(Hydroxyamino)-3-oxoprop-1-en-1-yl)benzoyl)oxy)butan-2-yl)oxy)-3-(phenylsulfonyl)-1,2,5-oxidazole 2-Oxide (12f) The title compound was obtained starting from 10 and 6f. Pale yellow waxy solid, yield: 61%. Analytical data for 12f: IR (KBr, cm⁻¹): 3432, 2939, 2928, 1717, 1615, 1425, 1235, 1052; MS (ESI) m/z: [M + H]⁺: 489.1077; [M + H⁺-H₂O]: 463.0982. 

(E)-4-(2-(4-(3-(Hydroxyamino)-3-oxoprop-1-en-1-yl)benzamido)ethoxy)-3-(phenylsulfonyl)-1,2,5-oxidazole 2-Oxide (12g) The title compound was obtained starting from 10 and 6g. Pale yellow waxy solid, yield: 58%. Analytical data for 12g: IR (KBr, cm⁻¹): 3425, 2946, 1718, 1617, 1431, 1234, 1165; MS (ESI) m/z: [M + H]⁺: 489.1077; [M + H⁺-H₂O]: 463.0982. 

(E)-4-(2-(4-(3-(Hydroxyamino)-3-oxoprop-1-en-1-yl)benzamido)ethoxy)-3-(phenylsulfonyl)-1,2,5-oxidazole 2-Oxide (12h) The title compound was obtained starting from 10 and 6h. Pale yellow waxy solid, yield: 52%. Analytical data for 12h: IR (KBr, cm⁻¹): 3429, 2944, 1724, 1620, 1441, 1220, 1032; MS (ESI) m/z: [M + H]⁺: 489.1077; [M + H⁺-H₂O]: 463.0982. 

Cell Culture SW620, HCT116, Lovo (human colon carcinoma cells), MCF-7 (human breast adenocarcinoma cells), and Hela cells were maintained in 10% fetal bovine serum.
Dulbecco’s modified Eagle’s medium (FBS DMEM) medium (Gibco, Invitrogen), which were supplemented with 10% fetal calf serum (PAA, Austria) and antibiotics [100 IU/mL penicillin and 100 µg/mL streptomycin (Amresco)]. All of the cell lines were purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and were grown at 37°C in a 5% CO₂ atmosphere.

**MTT Assay** The inhibitory effects on cell proliferation of test compounds were investigated by the MTT method. SW620, HCT116, Lovo, MCF-7, and Hela cells at a final density of 1.0 × 10⁴ cells/well were plated in 96-well plates overnight and treated with or without different concentrations of test compounds for various periods of time. During the last 4 h culture, the cells were exposed to MTT (5 mg/mL), and the resulting formazan crystals were dissolved in 150 µL of DMSO and measured using a spectrophotometer (Tecan) at a test wavelength of 570 nm. Experiments were conducted in triplicate. Inhibition rate (%) = [(Acontrol − Aexperimental) / Acontrol] × 100%.

**In Vitro HDACs Inhibition Fluorescence Assay** In vitro HDACs inhibition assays were conducted according to the literature. In brief, 10 µL of enzyme solution (HELa nuclear extract, HDAC2, HDAC6, or HDAC8) was mixed with various concentrations of tested compound. Five minutes later, fluorogenic substrate Boc-Lys (acetyl)–AMC (40 µM) was added, and the mixture was incubated at 37°C for 30 min and then stopped by addition of 100 µL of developer containing trypsin and TSA. After incubation at 37°C for 20 min, fluorescence intensity was measured using a microplate reader at excitation and emission wavelengths of 390 and 460 nm, respectively. The inhibition ratios were calculated from the fluorescence intensity readings of tested wells relative to those of control wells, and the IC₅₀ values were calculated using a regression analysis of the concentration/inhibition data.

**Nitrate/Nitrite Measurement in Vitro** The levels of nitrate/nitrite produced by individual compounds in the cells were determined by the colorimetric assay using the nitrate/nitrite colorimetric assay kit (Beyotime, China), according to the manufacturer’s instructions. Briefly, HCT116 cells (1 × 10⁶ well) were treated with the indicated concentrations of test compounds, and the nitrate/nitrite contents of the cell lysates were detected by the Griess assay. The absorbance was read at 540 nm on a spectrophotometer (Smart spec, Bio-Rad). The cells treated with diluent were used as negative controls for the background levels of nitrate/nitrite production, while with sodium nitrate at different concentrations was used as positive controls for the standard curve.

**References**