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Synthesis and biological evaluation of novel histone deacetylases inhibitors with nitric oxide releasing activity

Wenwen Duan [a], Jinning Hou [a], Xiaojing Chu [b], Xiaoqian Li [c], Jian Zhang [d], Jin Li [a], Wenfang Xu [a], Yingjie Zhang [a,*]

[a] Department of Medicinal Chemistry, School of Pharmacy, Shandong University, Ji'nan, Shandong 250012, People's Republic of China
[b] Weifang Bochuang International Biological Medicinal Institute, Weifang, Shandong 261061, People's Republic of China
[c] Color Ultrasonic Room, The People's Hospital of Shouguang, Weifang, Shandong 262700, People's Republic of China
[d] Department of Medical Chemistry, School of Pharmacy, Weifang Medical University, West Baotong Street, Weifang, Shandong 261053, People's Republic of China

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ABSTRACT

A novel series of histone deacetylases inhibitors (HDACIs) containing benzofuroxan pharmacophore as nitric oxide (NO) donor were designed based on the combination principle and 'multifunctional drugs' theory. As a novel study on embedding NO donor into the structure of HDACIs, all designed hybrid compounds, especially **19d** and **24d**, displayed remarkable HDACs inhibitory activity and outstanding antiproliferative activity on tumor cells. Besides, they could produce high levels of NO in HCT-116 cells; furthermore, their antiproliferative activity on HCT-116 cells could be diminished by pretreatment with hemoglobin, as the NO scavenger, in a dose-dependent manner. All in all, our designed compounds displayed great inhibitory activities and might offer a prospective avenue to discover novel anti-cancer drugs.

1. Introduction

Epigenetic changes, such as DNA methylation, histone modifications, chromatin remodeling and non-coding RNA regulation, have been recognized being associated with cancers. Histone acetylation, a dynamic and reversible process, is regulated by both histone acetyltransferases (HATs) and histone deacetylases (HDACs).[1] HATs add acetyl groups to lysine residues of histone tails to form a permissive chromatin structure allowing transcription, while HDACs, catalyzing the removal of acetyl groups from *N*-acetyl lysine residues of histone, are found in all modern eukaryotic organisms and play fundamental roles on cellular proliferation, differentiation and homeostasis.[2] HDACs family includes eighteen enzymes and they are grouped into four classes based on their homology to the respective yeast transcriptional control factor sequence. Class I (HDACs 1-3, 8), Class IIa (HDACs 4, 5, 7, 9), Class IIb (HDACs 6, 10) and Class IV (HDAC 11) are Zn^{2+} dependent enzymes,[3] while Class III HDACs (sirtuins) are NAD^{+} dependent, belonging to sirtuins family.[4] Overexpression of HDACs can arrest gene transcription which is associated with a variety of disease states including cancer, cellular metabolism disorders, and inflammation. Therefore, it is no doubt that the design and synthesis of histone deacetylases inhibitors (HDACIs) is an effective way to adjust the dysregulation of HDACs. Currently, more and more HDACIs (Fig. 1) have entered into clinical trials, among which, SAHA (**1**, Fig. 1), romidepsin (Istodax), PXD-101 (**2**, Fig. 1) and LBH589 (**3**, Fig. 1) have been approved by Food and Drug Administration (FDA) for the treatment of cancers. Co-crystals of Zn^{2+} dependent HDACs with inhibitors demonstrated a common molecular model for HDACIs: a Zn^{2+}-binding group (ZBG), such as hydroxamic acid, can bind the Zn^{2+} of the active site; a big hydrophobic cap group, which can occupy the rim of the HDAC active pocket; and a saturated or unsaturated linker can connect the ZBG and cap group[5] (Fig. 1). This special structure is the important design direction to discover new HDACIs as anti-cancer drugs.

Nitric oxide (NO) is a key signaling molecule involved in regulating the numerous physiologic and pathologic processes,[6] such as tumor cells proliferation, angiogenesis, metastasis and accelerate tumor cells apoptosis by regulating functional proteins through S-nitrosylation, nitration.[7] Recently, many synthesized NO-releasing compounds with strong cytotoxicity against human carcinoma cells in vitro and anti-cancer growth and anti-metastasis activity in vivo have been reported.[8,9] High concentration of NO could be generated not only by inducible nitric oxide synthase (iNOS), stimulated by cytokines, but also by NO donor effectively. Recently, the

* Corresponding author. Tel./fax: +86 531 88382009.
E-mail address: zhangyingjie@sdu.edu.cn (Y. Zhang).

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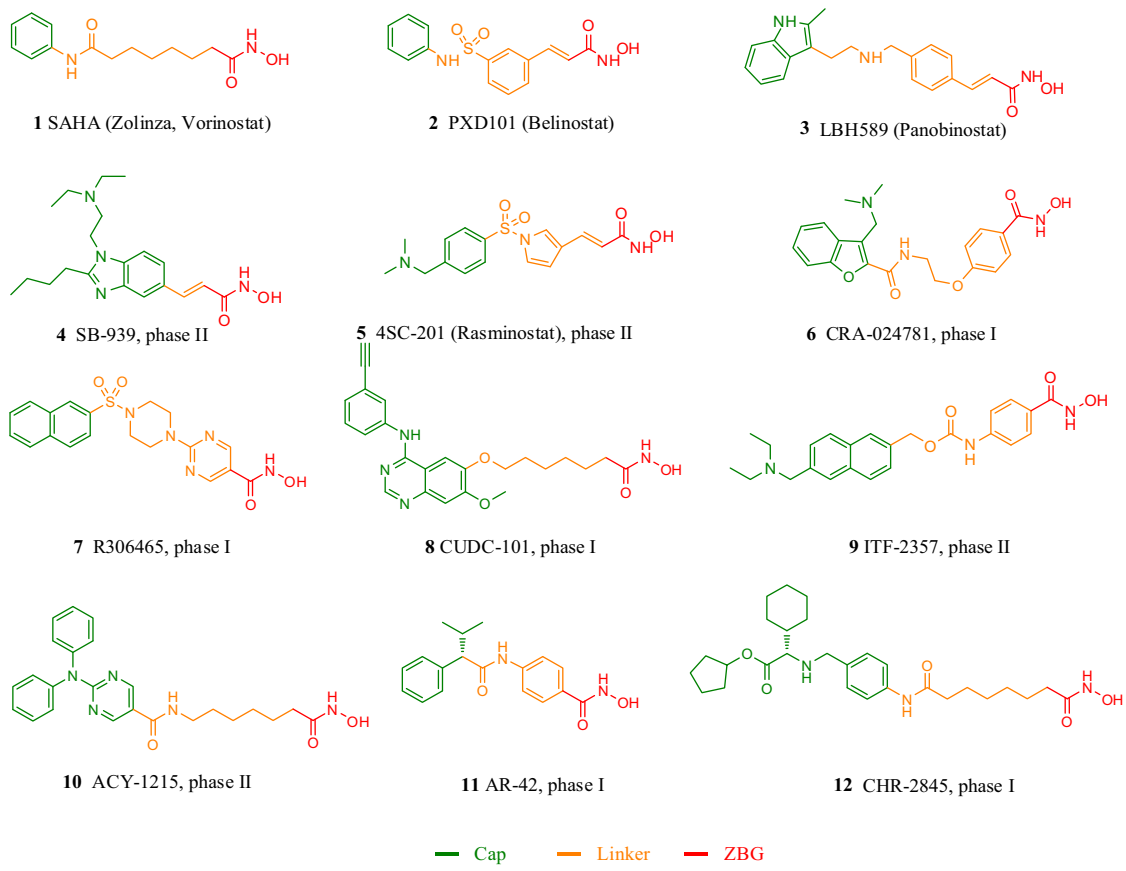


Figure 1. Pharmacophore model and structures of representative HDACIs.

oxadiazole moiety (**13**, Fig. 2) has increasingly attracted medicinal chemists' attention and been applied in many studies because it is stable against acid and base, its broad and interesting bioactivities and it can produce high levels of NO in vitro and inhibit the growth of tumors in vivo.[10] Benzofuroxan (benzofurzan oxide; benzo[1,2-c]1,2,5-oxadiazole *N*-oxide, **14**, Fig. 2) is an important motif, which was exploited previously by numerous researchers for its important biological activities, showed a wide spectrum of relevant pharmacological properties, such as antiprotozoal, antifungal and anti-platelet aggregation activities. Additionally, it has the potential for the treatment of cardiovascular diabetic complications due to its prominent NO-releasing activity.[11]

It is worth noting that several reports have displayed that NO-dependent regulation of HDACs functions.[12] NO, being a key regulator of HDAC function in mammalian neurons,[13] could regulate chromatin remodel in neuronal development[14] and skeletal muscle homeostasis[15] through S-nitrosylation of HDAC2. Furthermore, NO is also an upstream signal that controls the balance between HATs and HDACs.[16] Besides, some documents have shown that HDACIs and NO are synergistic in many diseases such as cardiac hypertrophy and wound healing.[17]

Enlightened by these findings, together with the theory of 'multifunctional drugs' and our previous studies on HDACIs equipped with phenylsulfonylfuroxan module as NO donor,[18] we thereof designed a novel series of HDACIs containing NO donor to improve their antiproliferative activities on tumor cells. In the structures of novel NO-HDACIs, benzofuroxan, as the NO donor, was designed to be the 'cap group' of HDACIs. What's more, the saturated aliphatic chains without any branched chains were designed as the 'linker' in the structures of HDACIs (Fig. 3). In this paper, we described the synthesis and relevant biological evaluation of all compounds and we hope this novel study uniting HDACIs and NO donor could provide and develop a prospective direction on the discovery of anticancer drugs.

2. Results and discussion

2.1. Chemistry

The synthetic routes of compounds **19a–19e** and **24a–24e** are outlined in Schemes 1 and 2. To synthesize the target compounds,

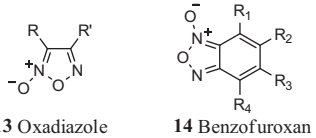


Figure 2. The chemical structure of oxadiazole and benzofuroxan.

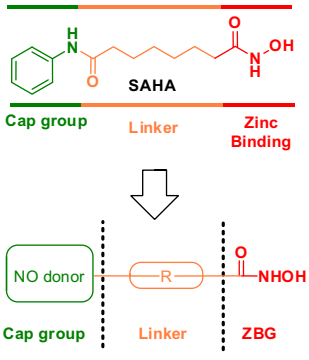
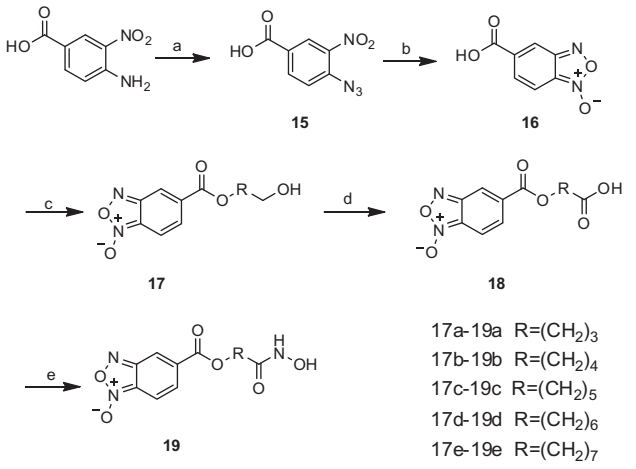
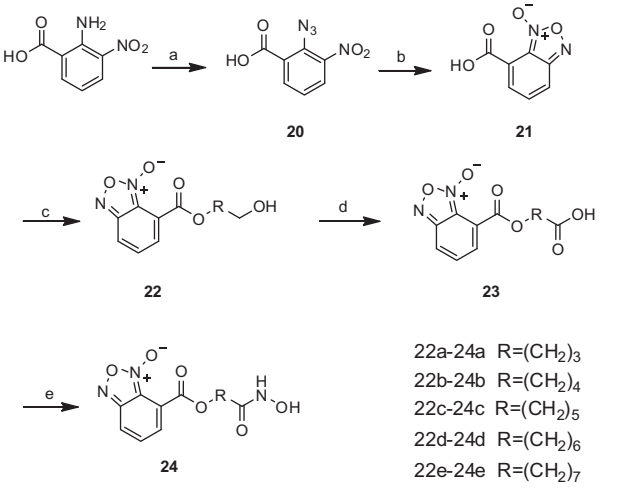


Figure 3. Design strategy of the target compounds.



Scheme 1. The synthesis of compounds **19a**–**19e**. Reagents and conditions: (a) 33% $NaNO_2$, 35% HCl, NaN_3; (b) toluene; (c) $OH(CH_2)_nOH$, PyBOP, TEA, DCM; (d) *Jones* agent, acetone; (e) isobutyl chlorocarbonate, TEA, THF; $NH_2OH{\cdot}HCl$, CH_3OH.



Scheme 2. The synthesis of compounds **24a**–**24e**. Reagents and conditions: (a) 33% $NaNO_2$, 35% HCl, NaN_3; (b) toluene; (c) $OH(CH_2)_nOH$, PyBOP, TEA, DCM; (d) *Jones* agent, acetone; (e) isobutyl chlorocarbonate, TEA, THF; $NH_2OH{\cdot}HCl$, CH_3OH.

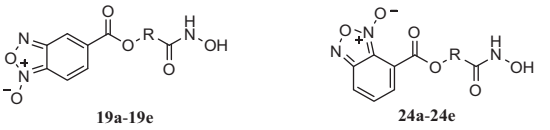
the key intermediate **16** or **21** should be firstly prepared from the matching benzoic acid substituted with nitryl and amino (purchased from Adamas-beta, China). Take the Scheme 1 for example, one-pot reaction of the 4-Amino-3-nitrobenzoic acid by diazo-reaction with sodium azide gave compound **15**, then refluxed after changing the solvent to toluene without purification to get compound **16**. The compound **16** was treated with benzotriazol-1-oxytripyrrolidinophosphoium and the corresponding diols to get intermediates **17a**–**17e**, which were oxidized under *Jones* agent to get compounds **18a**–**18e**. The target compounds **19a**–**19e** were obtained from **18a**–**18e** in the conditions of hydroxylamine hydrochloride. The whole preparation processes of target compounds **24a**–**24e** were similar as that of **19a**–**19e**.

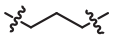
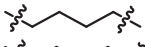
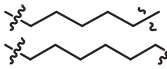
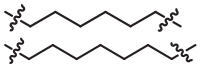
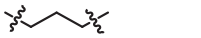
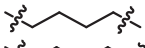
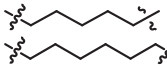
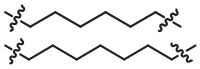
2.2. Biological evaluation

2.2.1. HDACs inhibition of target compounds

Based on the fact that all zinc ion dependent HDACs were highly conserved in their active sites, the HDACs inhibitory activities of all these target compounds were evaluated against Hela cell nuclear extracts (primarily containing HDAC1 and HDAC2) with SAHA as positive control according to the previous method in our lab.[18]

Table 1
The inhibitory activities of compounds against Hela nuclear extracts



Compd	R	IC_{50} (nM) ± SD[a]
19a		5569.7 ± 2181.0
19b		2207.0 ± 486.2
19c		437.0 ± 222.8
19d		18.4 ± 5.8
19e		180.4 ± 59.4
24a		6807.2 ± 2975.5
24b		257.6 ± 27.8
24c		17.0 ± 1.5
24d		12.8 ± 0.4
24e		137.3 ± 65.7
16	–	$>10^6$
21	–	$>10^6$
SAHA	–	107.8 ± 19.9

[a] Results expressed as the mean ± standard deviation of at least three separate determinations.

The result listed in the Table 1 revealed that the IC_{50} values of compounds **19e** (180.4 nM) and **24e** (137.3 nM) were comparable to SAHA (107.9 nM), and compounds **19d** (18.4 nM), **24c** (17.0 nM), **24d** (12.8 nM) were more potent than SAHA clearly. This result revealed that the inhibitory activities of HDACIs were affected dramatically by the characteristics of cap group and linker length. When the ring of furoxan was at the *ortho*-position of the benzoic acid, the compounds were more potent than those of being at the *meta*-position with the same linker length; in addition, when the linker was saturated aliphatic chains without any branched chains, the linker was longer, the activity was more potential, and the length of linker should not more than eight carbons.

2.2.2. Antiproliferative activity of tumor cells in vitro

The ten novel synthesized compounds **19a**–**19e** and **24a**–**24e** were subjected to in vitro cytotoxicity evaluation against U937 (human Histiocytic leukemia cell), HCT-116 (human colorectal carcinoma cell), B16 (rat melanoma cell), PC-3 (human prostate carcinoma cell), Hela (human cervical carcinoma cell), HEL (human erythrocyte leukemia cell), KG1 (human leukemia cell) and ES-2 (ovarian clear cell carcinoma cell) cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, respectively, using SAHA as the positive control. Table 2 displayed that most of our synthesized compounds demonstrated similar activity as SAHA and the combination of SAHA and NO donor **16** or **21** with mole ratio of 1:1 exhibited dramatic additive effect with compounds **19d** and **24d** being more potent than SAHA against the proliferation of HCT-116 cell lines.

2.2.3. NO generation measurement

Aside from the test of inhibitory activity against the extracts of HELA cells and the antiproliferative effect of tumor cells, the levels of the NO generated by these compounds were also measured (Fig. 4) HCT-116 cells were respectively exposed to 100 μM of compounds **19c**, **19d**, **19e**, **24c**, **24d**, **16**, **21** and SAHA in the same incubation time 5 h. The levels of NO released in the cell lysates were determined and presented as that of nitrite using a Griess assay (purchased from Beyotime Biotechnology, China). From the

Table 2
The inhibitory activities of compounds **19a–19e**, **24a–24d** and SAHA against several tumor cells

Compd	IC_{50} (μM)[a]							
	HCT-116	U937	B16	PC-3	Hela	HEL	KG1	ES-2
19a	2.06 ± 0.08	13.04 ± 0.68	22.23 ± 4.56	6.58 ± 0.65	17.01 ± 2.54	2.09 ± 0.13	4.02 ± 0.25	27.06 ± 0.57
19b	2.63 ± 0.33	19.87 ± 5.58	23.97 ± 4.63	12.70 ± 2.38	15.87 ± 2.42	1.21 ± 0.63	8.19 ± 0.37	25.04 ± 3.01
19c	3.36 ± 0.28	33.40 ± 0.86	30.25 ± 3.49	12.44 ± 2.91	17.33 ± 2.42	11.55 ± 0.95	3.69 ± 0.64	20.60 ± 6.95
19d	1.09 ± 0.27	12.36 ± 2.87	4.97 ± 0.17	0.95 ± 0.28	9.18 ± 2.40	1.26 ± 0.02	0.37 ± 0.17	5.07 ± 2.29
19e	2.29 ± 0.35	11.86 ± 3.89	7.99 ± 1.56	1.39 ± 0.21	5.31 ± 1.33	1.50 ± 0.20	5.21 ± 0.63	9.23 ± 1.24
24a	16.23 ± 0.48	53.86 ± 3.23	>80	13.42 ± 1.24	71.98 ± 3.65	6.60 ± 0.03	12.40 ± 9.21	73.79 ± 0.81
24b	2.73 ± 0.41	11.27 ± 1.53	30.48 ± 6.31	14.20 ± 0.97	20.25 ± 7.73	3.14 ± 0.30	6.69 ± 0.71	44.39 ± 6.79
24c	2.81 ± 0.20	21.61 ± 3.56	27.89 ± 1.26	16.89 ± 2.38	42.11 ± 6.19	3.74 ± 0.83	12.37 ± 0.50	66.35 ± 0.92
24d	1.09 ± 0.03	34.65 ± 2.67	18.25 ± 5.52	9.37 ± 0.44	27.17 ± 9.48	3.71 ± 0.56	2.98 ± 2.09	21.18 ± 3.07
24e	>80	>80	>80	>80	34.25 ± 2.88	12.11 ± 1.87	0.53 ± 0.02	>80
16	55.78 ± 7.28	ND[b]	ND	ND	ND	ND	ND	ND
21	63.96 ± 8.78	ND	ND	ND	ND	ND	ND	ND
SAHA + **16**	11.49 ± 4.71[c]	ND	ND	ND	ND	ND	ND	ND
SAHA + **21**	8.52 ± 3.12[d]	ND	ND	ND	ND	ND	ND	ND
SAHA	4.25 ± 1.36	4.82 ± 2.85	4.44 ± 1.22	2.78 ± 1.28	12.28 ± 0.98	2.42 ± 0.15	0.68 ± 0.03	2.95 ± 0.24

[a] Results expressed as the mean ± standard deviation of at least three separate determinations.
[b] Not determined.
[c] This data means that 11.49 μM SAHA plus 11.49 μM compound **16**.
[d] This data means that 8.52 μM SAHA plus 8.52 μM compound **21**.

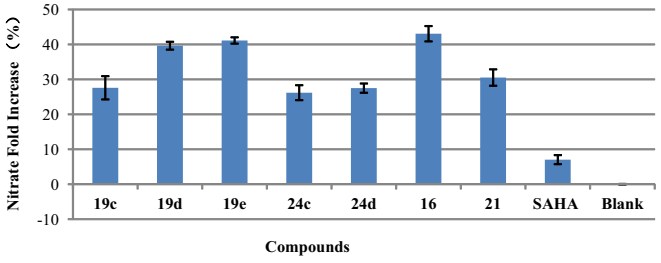


Figure 4. NO production by indicated compounds and SAHA in HCT-116 cells. HCT-116 cells were treated with a 100 μM concentration of each compound for 5 h.

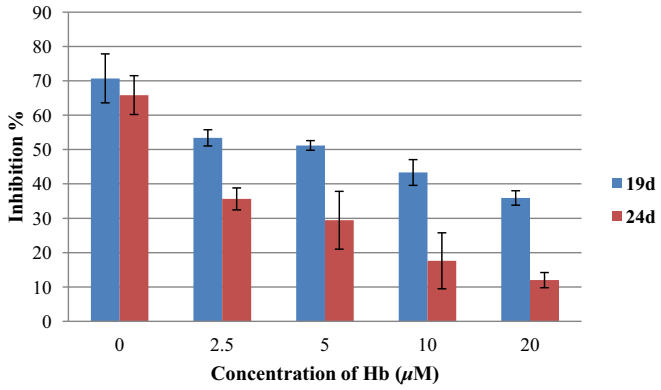


Figure 5. Effects of hemoglobin on the antiproliferative effect of **19d** and **24d** in HCT-116 cells. HCT-116 cells were pretreated with the indicated concentrations of hemoglobin (0, 2.5, 5, 10 or 20 μM) for 1 h and then treated with 2 μM of **19d** or **24d** for 24 h.

results showed in Figure 4 we could find that the compounds we designed and synthesized, especially **19d** and **19e**, could release NO efficiently.

2.2.4. The inhibitory activity of HCT-116 in vitro with hemoglobin

In order to verify the point that the antiproliferative activity on tumor cells of our compounds came from the synergism of HDACs inhibition and NO release, we went a further step to test the antiproliferative effects of **19d** and **24d** in the presence or absence of the NO scavenger, hemoglobin (purchased from Beyotime Biotechnology, China). HCT-116 cells were pretreated with the indicated concentrations of NO scavenger hemoglobin (Hb) (0, 2.5, 5, 10 or 20 μM) for 1 h and then treated with 2 μM of **19d** or **24d** for 24 h. The results were presented as the percentage of cell growth inhibition relative to control cells in Figure 5. It was observed that the treatment with **19d** and **24d** remarkably inhibited the growth of HCT-116 cells and the inhibitory effects were diminished by pretreatment with hemoglobin in a dose-dependent manner. These results demonstrated that NO produced by **19d** and **24d** substantially contributed to their inhibition on tumor cell proliferation in vitro.

3. Conclusion

In the light of the combination principle and the 'multifunctional drugs' theory, ten novel HDACIs coupling with benzofuroxan, as NO-donor, were synthesized and characterized. Among them, **19d**, **19e**, **24c**, **24d**, **24e** displayed potential HDACs inhibition against the Hela cell nuclear extract (mainly containing HDAC1 and HDAC2), and our primary structure activity relationships study mainly focused on the length of linker and the position of furoxan ring on the phenyl group that played crucial roles in HDACs inhibition. All target compounds were evaluated their antiproliferative activities against tumor cells by MTT assay using SAHA as control, most of which, especially **19d** and **24d** shown potent inhibition to the proliferation of tumor cells. What's more, high levels of NO could be generated from our synthesized compounds in HCT-116 cells. Besides, the inhibitory activities of **19d** and **24d** against HCT-116 cells could be partially diminished by the NO scavenger hemoglobin, which confirmed our designed compounds were 'multifunctional drugs' both on the inhibitory activities of HDACs and NO release. Further structural optimization and in vivo activity evaluation of these promising antitumor agents are underway in our lab.

4. Experimental section

4.1. Chemistry

All commercially available starting materials, reagents, and solvents were used without further purification unless otherwise

stated. All reactions were monitored by TLC with 0.25 mm silica gel plates (60GF-254). UV light, iodine stain, and ferric chloride were used to visualize the spots. Silica gel or C18 silica gel was used for column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX spectrometer at 600 MHz, δ in parts per million and J in hertz, using TMS as an internal standard. High-resolution mass spectra were conducted by Shandong Analysis and Test Center in Ji'nan, China. ESI-MS spectra were recorded on an API 4000 spectrometer. Melting points were determined uncorrected on an electrothermal melting point apparatus. All tested compounds are >95% pure by HPLC analysis, performed on a Agilent 1100 HPLC instrument using an 5 μM ODS HYPERSIL column (4.6 mm × 250 mm) according to the following method. Compounds **19c**, **19d**, **19e**, **24c** and **24d** were eluted with 30% acetonitrile/70% water (containing 0.1% acetic acid) over 20 min, with detection at 254 nm and a flow rate of 1.0 mL/min.

4.1.1. Synthesis of compound 5-carboxybenzo[*c*][1,2,5]oxadiazole 1-oxide (16)

To a solution of 4-Amino-3-nitrobenzoic acid (3.6 g, 20 mmol) in 100 mL 35% (v/v) HCl at ice bath atmosphere (0–5 °C), 4.4 mL 33% sodium nitrite solution was added slowly. After stirring 1 h at 0–5 °C, sodium azide solution (1.3 g in 5 mL H_2O) was added into the slurry drop by drop and keep the inner temperature lower than 15 °C. Additional 1.5 h stirred later, the mixture was filtered and the filter cake was washed with saturated sodium hyposulfite. After vacuum dried, the compound **15** was obtained as yellow solid. Then, the compound **15** was resolved in 100 mL toluene and refluxed at 80 °C for another 1 h. The mixture was filtered to get yellow solid, which was recrystallized with ethanol to give the compound **16** as yellow solid, yield 80%. ^{1}H NMR (600 MHz, DMSO) δ 13.80 (s, 1H), 8.21 (d, J = 182.0 Hz, 1H), 7.86 (s, 2H).

4.1.2. Synthesis of compound 17

To a solution of compound **16** (0.9 g, 5 mmol) in 50 mL dried DCM, 1.2 mL 1,4-butanediol, PyBOP (3.1 g, 6 mmol) and triethylamine (1.6 mL, 6.5 mmol) were added and the mixture was stirred at room temperature for 10 h. DCM was removed and the residue was extracted with EtOAc (3 × 50 mL) and washed twice with water and brine. The organic portion was dried by anhydrous Na_2SO_4. The crude product was obtained by removing EtOAc and purified by silica chromatography column (P:E = 3:1) to get the product.

4.1.2.1. 5-((4-Hydroxybutoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (17a). Weak yellow solid, yield: 72%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 8.39–8.05 (m, 1H), 7.95–7.69 (m, 2H), 4.35 (t, J = 6.6 Hz, 2H), 3.47 (t, J = 6.4 Hz, 2H), 1.82–1.76 (m, 2H), 1.58 (dt, J = 13.4, 6.5 Hz, 2H). ^{13}C NMR (125 MHz, d_6-DMSO) δ 166.0, 159.9, 139.8, 132.4, 131.4, 121.7, 66.7, 62.4, 30.2, 27.4. ESI-MS m/z 253.2 $[M+H]^+$.

4.1.2.2. 5-(((5-Hydroxypentyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (17b). Weak yellow solid, yield: 80%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.77 (dd, J = 294.4, 137.8 Hz, 3H), 4.36 (t, J = 6.6 Hz, 2H), 3.67 (t, J = 6.4 Hz, 2H), 1.81 (dd, J = 14.8, 7.1 Hz, 2H), 1.67–1.59 (m, 2H), 1.56–1.47 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.2, 164.1, 66.3, 62.6, 32.3, 28.5, 22.4. ESI-MS m/z 267.3 $[M+H]^+$.

4.1.2.3. 5-(((6-Hydroxyhexyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (17c). Weak yellow solid, yield: 70%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 7.88 (m, 3H), 4.32 (t, J = 6.3 Hz, 2H), 3.40 (dd, J = 11.2, 6.2 Hz, 2H), 1.77–1.71 (m, 2H), 1.47–1.38 (m, 4H), 1.39–1.33 (m, 2H). ^{13}C NMR (131 MHz, d_6-DMSO) δ 166.0, 159.9, 139.8, 132.4, 131.4, 121.7, 66.7, 62.4, 33.0, 29.3, 26.6, 26.2. ESI-MS m/z 281.3 $[M+H]^+$.

4.1.2.4. 5-(((7-Hydroxyheptyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (17d). Weak yellow solid, yield: 77%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19 (s, 1H), 7.83 (s, 1H), 7.54 (s, 1H), 4.35 (t, J = 6.7 Hz, 2H), 3.63 (t, J = 6.6 Hz, 2H), 1.81–1.75 (m, 2H), 1.56 (dd, J = 13.5, 6.7 Hz, 2H), 1.45 (dt, J = 14.4, 7.2 Hz, 2H), 1.41–1.36 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 163.9, 130.6, 120.2, 116.6, 66.3, 62.8, 32.6, 29.0, 28.5, 25.9, 25.6. ESI-MS m/z 295.3 $[M+H]^+$.

4.1.2.5. 5-(((8-Hydroxyoctyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (17e). Weak yellow solid, yield: 75%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.67 (s, 1H), 7.38 (s, 1H), 4.43 (t, J = 6.5 Hz, 2H), 3.71 (t, J = 6.4 Hz, 2H), 1.84–1.70 (m, 2H), 1.61 (dd, J = 13.2, 6.6 Hz, 2H), 1.42–1.37 (m, 2H), 1.31 (dd, J = 17.1, 8.1 Hz, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 145.3, 134.9, 133.5, 128.1, 127.9, 125.6, 66.5, 62.8, 32.7, 29.2, 29.1, 28.5, 25.8, 25.6. ESI-MS m/z 309.3 $[M+H]^+$.

4.1.3. Synthesis of compound 18

To a solution of **17a** (800 mg, 3.2 mmol) in 10 mL acetone, Jones reagent (3 mL) was added at 0–5 °C, after that, this mixture was stirred at rt for 10 h. The precipitate was filtered off and acetone was removed in vacuo, and then EtOAc (3 × 50 mL) was added to extract and the organic portion was washed with water and brine, dried by anhydrous Na_2SO_4. The crude product was obtained by removing EtOAc and purified by silica chromatography column (P:E = 3:1) to get the product.

4.1.3.1. 5-((3-Carboxypropoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (18a). Weak yellow solid. Yield: 68%. ^{1}H NMR (600 MHz, DMSO) δ 12.16 (s, 1H), 8.31 (d, J = 183.7 Hz, 1H), 7.98–7.68 (m, 2H), 4.34 (t, J = 6.3 Hz, 2H), 2.43 (t, J = 7.2 Hz, 2H), 2.01–1.95 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 177.2, 166.0, 159.9, 139.8, 132.4, 131.4, 121.7, 65.7, 31.8, 24.4. ESI-MS m/z 267.2 $[M+H]^+$.

4.1.3.2. 5-((4-Carboxybutoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (18b). Weak yellow solid. Yield: 79%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 12.06 (s, 1H), 8.58–7.50 (m, 3H), 4.33 (t, J = 6.2 Hz, 2H), 2.30 (t, J = 7.3 Hz, 2H), 1.79–1.70 (m, 2H), 1.65 (dt, J = 14.3, 7.3 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 174.7, 164.2, 65.9, 33.6, 28.0, 21.5. ESI-MS m/z 281.2 $[M+H]^+$.

4.1.3.3. 5-(((5-Carboxypentyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (18c). Weak yellow solid. Yield: 72%. ^{1}H NMR (600 MHz, DMSO) δ 11.98 (s, 1H), 8.51–7.56 (m, 3H), 4.32 (t, J = 6.5 Hz, 2H), 2.24 (t, J = 7.3 Hz, 2H), 1.74 (dd, J = 14.6, 6.9 Hz, 2H), 1.57 (dd, J = 15.1, 7.5 Hz, 2H), 1.44 (dd, J = 15.3, 8.0 Hz, 2H).^{13}C NMR (151 MHz, DMSO) δ 177.2, 166.0, 159.9, 139.8, 132.4, 131.4, 121.7, 66.7, 34.5, 29.3, 25.9, 24.9. ESI-MS m/z 295.3 $[M+H]^+$.

4.1.3.4. 5-(((6-Carboxyhexyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (18d). Weak yellow solid. Yield: 81%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.55 (s, 1H), 8.33–7.50 (m, 3H), 4.36 (t, J = 6.6 Hz, 2H), 2.37 (t, J = 7.4 Hz, 2H), 1.83–1.75 (m, 2H), 1.71–1.64 (m, 2H), 1.48–1.39 (m, 4H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 174.4, 159.2, 76.0, 61.4, 29.0, 23.9, 23.6, 20.9, 19.7. ESI-MS m/z 309.3 $[M+H]^+$.

4.1.3.5. 5-(((7-Carboxyheptyl)oxy)carbonyl)benzo[*c*][1,2,5]-oxadiazole 1-oxide (18e). Weak yellow solid. Yield: 77%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.58 (s, 1H), 7.78 (m, 3H), 4.35 (t, J = 6.5 Hz, 2H), 2.35 (t, J = 7.4 Hz, 2H), 1.88–1.74 (m, 2H),

1.70–1.59 (m, 2H), 1.41 (dd, J = 32.3, 5.2 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 180.1, 164.1, 66.4, 34.0, 28.9, 28.9, 28.6, 25.8, 24.6. ESI-MS m/z 323.3 $[M+H]^+$.

4.1.4. Synthesis of compound 19

To a solution of compound **18a** (760 mg, 3.1 mmol) in dried THF, Isobutyl chlorocarbonate (0.4 mL, 2.8 mmol) was added by drop at 0 °C, after stirring 0.5 h, triethylamine (0.7 mL, 2.3 mmol) was added dropping at 0 °C. Then the precipitate was filtered to get the first filtrate after stirring additional 1 h at room temperature. Potassium hydroxide (270 mg, 3.4 mmol) and hydroxylamine hydrochloride (220 mg, 3.4 mmol) were dissolved in anhydrous menthol completely and the residue was filtered to get the second filtrate. The first filtrate was dropped into the second filtrate and the mixture was stirred for additional 4 h at room temperature and nitrogen atmosphere. Ferric trichloride was the color-producing reagent. THF and menthol were removed after the pH of the reacted solution was adjusted to 3.0 with 2 N HCl. And then the residue was extracted with EtOAc, and the organic portion was dried by anhydrous Na_2SO_4. The crude product was obtained by removing EtOAc and purified by silica chromatography column (P:E = 3:1) to get the products.

4.1.4.1. 5-((4-(Hydroxyamino)-4-oxobutoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (19a). Weak yellow solid. Yield: 76%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.46 (s, 1H), 8.74 (s, 1H), 8.54–8.05 (m, 1H), 8.02–7.50 (m, 2H), 4.31 (t, J = 5.6 Hz, 2H), 2.15 (t, J = 6.9 Hz, 2H), 2.04–1.92 (m, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 169.0, 164.2, 131.5, 118.9, 117.4, 65.7, 29.4, 24.6. HRMS (AP-ESI) m/z calcd for $C_{11}H_{11}N_3O_6Na$ $[M+Na]^+$: 304.0540; found: 304.0535.

4.1.4.2. 5-(((5-(Hydroxyamino)-5-oxopentyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (19b). Weak yellow solid. Yield: 72%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.40 (s, 1H), 8.71 (d, J = 5.1 Hz, 2H), 8.18 (d, J = 9.4 Hz, 1H), 7.97 (d, J = 9.4 Hz, 1H), 4.34 (t, J = 6.2 Hz, 2H), 2.03 (d, J = 7.4 Hz, 2H), 1.74–1.70 (m, 2H), 1.66 (dd, J = 14.7, 7.4 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 168.8, 164.1, 149.4, 148.8, 133.7, 131.0, 119.8, 117.0, 65.5, 65.4, 31.8, 27.6, 21.6. HRMS (AP-ESI) m/z calcd for $C_{12}H_{13}N_3O_6Na$ $[M+Na]^+$: 318.0697; found: 318.0935.

4.1.4.3. 5-(((6-(Hydroxyamino)-6-oxohexyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (19c). Weak yellow solid. Yield: 67%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.33 (s, 1H), 8.64 (s, 1H), 7.98 (m, 3H), 4.32 (t, J = 6.5 Hz, 2H), 1.99 (dd, J = 9.6, 5.1 Hz, 2H), 1.78–1.70 (m, 2H), 1.60–1.53 (m, 2H), 1.39 (dt, J = 15.0, 7.7 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 170.1, 166.0, 159.9, 139.8, 132.4, 131.4, 121.7, 66.7, 34.4, 29.3, 25.9, 23.2. HRMS (AP-ESI) m/z calcd for $C_{13}H_{15}N_3O_6Na$ $[M+Na]^+$: 332.0853; found: 332.0848.

4.1.4.4. 5-(((7-(Hydroxyamino)-7-oxoheptyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (19d). Weak yellow solid. Yield: 79%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.34 (s, 1H), 8.66 (s, 1H), 8.51–7.60 (m, 3H), 4.31 (t, J = 6.5 Hz, 2H), 1.96 (t, J = 7.3 Hz, 2H), 1.75–1.70 (m, 2H), 1.55–1.49 (m, 2H), 1.40 (dd, J = 14.8, 7.5 Hz, 2H), 1.30 (dt, J = 14.8, 7.5 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 169.5, 164.2, 131.4, 117.5, 66.2, 40.4, 40.3, 40.1, 40.0, 39., 39.7, 39.6, 32.7, 28.7, 28.3, 25.6, 25.4. HRMS (AP-ESI) m/z calcd for $C_{14}H_{17}N_3O_6Na$ $[M+Na]^+$: 346.1010; found: 346.1028.

4.1.4.5. 5-(((8-(Hydroxyamino)-8-oxooctyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (19e). Weak yellow solid. Yield: 70%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.33 (s, 1H), 8.66 (s, 1H), 8.49–7.56 (m, 3H), 4.30 (t, J = 6.4 Hz, 2H), 1.98–1.89 (m, 2H), 1.76–1.67 (m, 2H), 1.52–1.44 (m, 2H), 1.38 (dt, J = 14.6, 7.2 Hz, 2H), 1.34–1.28 (m, 2H), 1.25 (dt, J = 13.7, 6.9 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 169.1, 163.7, 65.8, 32.2, 28.5, 28.4, 28.0, 25.3, 25.1. HRMS (AP-ESI) m/z calcd for $C_{15}H_{19}N_3O_6Na$ $[M+Na]^+$: 360.1166; found: 360.1194.

4.1.5. Synthesis of 7-carboxybenzo[*c*][1,2,5]oxadiazole 1-oxide (21)

The method is as the same as the preparation of compound **16**. ^{1}H NMR (600 MHz, d_6-DMSO) δ 8.13 (ddd, J = 11.6, 8.0, 1.4 Hz, 2H), 7.53 (t, J = 8.0 Hz, 1H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 165.4, 144.7, 134.8, 132.0, 127.8, 127.7, 126.2.

4.1.6. Synthesis of compound 22

The method is as the same as the preparation of compound **17a**–**17e**.

4.1.6.1. 4-((4-Hydroxybutoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (22a). Weak yellow solid, yield: 71%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.07 (d, J = 7.7 Hz, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.38 (t, J = 7.9 Hz, 1H), 4.46 (t, J = 6.6 Hz, 2H), 3.74 (t, J = 6.3 Hz, 2H), 1.92 (dt, J = 14.3, 7.1 Hz, 2H), 1.73 (dd, J = 14.0, 7.0 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 134.9, 128.0, 125.6, 66.2, 62.2, 29.0, 25.2. ESI-MS m/z 253.3 $[M+H]^+$.

4.1.6.2. 4-(((5-Hydroxypentyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (22b). Weak yellow solid, yield: 75%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (dd, J = 7.8, 1.1 Hz, 1H), 7.93 (dd, J = 8.1, 1.0 Hz, 1H), 7.37 (t, J = 8.0 Hz, 1H), 4.40 (t, J = 6.7 Hz, 2H), 3.64 (t, J = 6.5 Hz, 2H), 1.84–1.77 (m, 2H), 1.60–1.57 (m, 2H), 1.47–1.44 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 145.3, 134.9, 133.5, 128.1, 127.9, 125.6, 66.4, 62.7, 32.5, 28.5, 25.7, 25.4. ESI-MS m/z 267.3 $[M+H]^+$.

4.1.6.3. 4-(((6-Hydroxyhexyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (22c). Weak yellow solid, yield: 67%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (dd, J = 7.9, 1.6 Hz, 1H), 7.92 (dd, J = 8.1, 1.6 Hz, 1H), 7.37 (t, J = 8.0 Hz, 1H), 4.40 (t, J = 6.7 Hz, 2H), 3.66 (t, J = 6.4 Hz, 2H), 1.86–1.79 (m, 2H), 1.63 (dt, J = 14.6, 6.7 Hz, 2H), 1.52 (tt, J = 9.6, 6.1 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 145.3, 135.0, 133.4, 128.0, 127.9, 125.7, 117.1, 110.9, 66.3, 62.4, 32.1, 28.3, 22.2. ESI-MS m/z 281.3 $[M+H]^+$.

4.1.6.4. 4-(((7-Hydroxyheptyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (22d). Weak yellow solid, yield: 68%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (dd, J = 7.9, 1.4 Hz, 1H), 7.91 (dd, J = 8.1, 1.4 Hz, 1H), 7.36 (t, J = 8.0 Hz, 1H), 4.37 (t, J = 6.7 Hz, 2H), 3.60 (t, J = 6.6 Hz, 2H), 1.77 (dd, J = 14.7, 6.9 Hz, 2H), 1.54 (p, J = 6.8 Hz, 2H), 1.42 (dd, J = 14.6, 6.9 Hz, 2H), 1.37 (dd, J = 11.5, 8.0 Hz, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 145.2, 135.0, 133.5, 128.0, 125.7, 66.5, 62.8, 32.6, 29.0, 28.5, 25.9, 25.6. ESI-MS m/z 295.3 $[M+H]^+$.

4.1.6.5. 4-(((8-Hydroxyoctyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (22e). Weak yellow solid, yield: 77%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.07 (dd, J = 7.9, 1.6 Hz, 1H), 7.94 (dd, J = 8.1, 1.5 Hz, 1H), 7.38 (t, J = 8.0 Hz, 1H), 4.40 (t, J = 6.7 Hz, 2H), 3.63 (t, J = 6.6 Hz, 2H), 1.84–1.76 (m, 2H), 1.56 (dd, J = 13.8, 6.8 Hz, 2H), 1.48–1.42 (m, 2H), 1.39 (dd, J = 16.8, 8.3 Hz, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 164.2, 145.3, 134.9, 133.5, 128.1, 127.9, 125.6, 66.5, 62.8, 32.7, 29.2, 29.1, 28.5, 25.8, 25.6. ESI-MS m/z 309.3 $[M+H]^+$.

4.1.7. Synthesis of compound 23

The method is as the same as the preparation of compound **18a**–**18e**.

4.1.7.1. 4-((3-Carboxypropoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (23a). Weak yellow solid, yield: 68%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 8.07 (d, J = 7.7 Hz, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.38 (t, J = 7.8 Hz, 1H), 4.46 (d, J = 5.9 Hz, 2H), 2.55 (t, J = 6.5 Hz, 2H), 2.23–2.08 (m, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 173.6, 159.2, 130.3, 128.9, 123.5, 122.8, 120.9, 60.5, 25.7, 18.9. ESI-MS m/z 267.2 $[M+H]^+$.

4.1.7.2. 4-((4-Carboxybutoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (23b). Weak yellow solid, yield: 72%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (d, J = 7.7 Hz, 1H), 7.94 (d, J = 8.0 Hz, 1H), 7.38 (t, J = 7.9 Hz, 1H), 4.40 (t, J = 6.2 Hz, 2H), 2.39 (t, J = 7.1 Hz, 2H), 1.82 (dd, J = 13.3, 6.6 Hz, 2H), 1.75–1.66 (m, 2H), 1.55–1.45 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 179.7, 164.3, 145.3, 135.2, 128.2, 125.8, 66.2, 33.9, 28.3, 25.5, 24.3. ESI-MS m/z 281.2 $[M+H]^+$.

4.1.7.3. 4-(((5-Carboxypentyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (23c). Weak yellow solid, yield: 77%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (d, J = 10.0 Hz, 1H), 7.70 (d, J = 9.9 Hz, 1H), 7.42 (t, J = 9.0 Hz, 1H), 4.33 (t, J = 9.5 Hz, 2H), 2.21 (t, J = 16.1 Hz, 2H), 1.95–1.71 (m, 2H), 1.68–1.44 (m, 2H), 1.33 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 177.2, 167.2, 166.5, 133.4, 131.0, 130.3, 116.4, 66.7, 34.5, 29.3, 25.9, 24.9. ESI-MS m/z 295.3 $[M+H]^+$.

4.1.7.4. 4-(((6-Carboxyhexyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (23d). Weak yellow solid, yield: 71%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.18 (s, 1H), 8.07 (d, J = 7.8 Hz, 1H), 7.94 (d, J = 7.9 Hz, 1H), 7.38 (t, J = 7.9 Hz, 1H), 4.40 (t, J = 6.4 Hz, 2H), 2.37 (t, J = 7.2 Hz, 2H), 2.08–1.80 (m, 2H), 1.76–1.62 (m, 2H), 1.58–1.32 (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 179.4, 164.2, 145.3, 135.0, 133.6, 128.0, 125.6, 66.3, 33.8, 28.6, 28.4, 25.6, 24.5. ESI-MS m/z 309.3 $[M+H]^+$.

4.1.7.5. 4-(((7-Carboxyheptyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (23e). Weak yellow solid, yield: 79%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.01 (s, 1H), 8.08 (dd, J = 7.8, 1.5 Hz, 1H), 7.95 (dd, J = 8.1, 1.5 Hz, 1H), 7.39 (t, J = 8.0 Hz, 1H), 4.41 (t, J = 6.7 Hz, 2H), 2.37 (t, J = 7.4 Hz, 2H), 1.85–1.79 (m, 2H), 1.70–1.63 (m, 2H), 1.47 (dd, J = 14.1, 6.6 Hz, 2H), 1.41 (dd, J = 12.0, 8.3 Hz, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 179.7, 164.2, 134.9, 133.3, 128.1, 127.9, 125.6, 66.4, 33.9, 28.8, 28.7, 28.5, 25.7, 24.5. ESI-MS m/z 323.3 $[M+H]^+$.

4.1.8. Synthesis of compound 24

The method is as the same as the preparation of compound **19a–19e**.

4.1.8.1. 4-((4-(Hydroxyamino)-4-oxobutoxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (24a). Weak yellow solid, yield: 68%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.46 (s, 1H), 8.77 (s, 1H), 8.19 (d, J = 8.0 Hz, 1H), 8.14 (d, J = 7.8 Hz, 1H), 7.56 (t, J = 7.9 Hz, 1H), 4.33 (t, J = 6.1 Hz, 2H), 2.14 (t, J = 7.0 Hz, 2H), 2.00–1.94 (m, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 168.9, 164.3, 145.1, 135.4, 132.6, 128.7, 126.9, 65.8, 29.2, 24.6. HRMS (AP-ESI) m/z calcd for $C_{11}H_{11}N_3O_6Na$ $[M+Na]^+$: 304.0540; found: 304.0563.

4.1.8.2. 4-(((5-(Hydroxyamino)-5-oxopentyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (24b). Weak yellow solid, yield: 72%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.40 (s, 1H), 8.71 (s, 1H), 8.12 (d, J = 8.2 Hz, 1H), 8.08 (d, J = 8.1 Hz, 1H), 7.48 (t, J = 7.4 Hz, 1H), 4.32 (t, J = 9.9 Hz, 2H), 2.14 (t, J = 16.1 Hz, 2H), 2.00–1.84 (m, 2H), 1.58–1.29 (m, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 168.8, 164.2, 145.1, 135.4, 132.5, 128.8, 126.9, 65.3, 34.4, 28.8, 23.1. HRMS (AP-ESI) m/z calcd for $C_{12}H_{13}N_3O_6Na$ $[M+Na]^+$: 318.0697; found: 318.0946.

4.1.8.3. 4-(((6-(Hydroxyamino)-6-oxohexyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (24c). Weak yellow solid, yield: 77%. ^{1}H NMR (500 MHz, d_6-DMSO) δ10.36 (s, 1H), 8.70 (s, 1H), 8.20 (d, J = 8.0 Hz, 1H), 8.11 (d, J = 7.7 Hz, 1H), 7.56 (t, J = 7.1 Hz, 1H), 4.34 (t, J = 9.7 Hz, 2H), 1.93 (t, J = 11.2 Hz, 2H), 1.78 (tt, J = 14.2, 9.7 Hz, 2H), 1.58–1.22 (m, 4H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 169.5, 164.4, 145.1, 135.2, 132.6, 128.7, 127.5, 126.9, 66.4, 32.7, 28.9, 25.7, 25.5. HRMS (AP-ESI) m/z calcd for $C_{13}H_{15}N_3O_6Na$ $[M+Na]^+$: 332.0853; found: 332.0856.

4.1.8.4. 4-(((7-(Hydroxyamino)-7-oxoheptyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (24d). Weak yellow solid, yield: 71%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.33 (s, 1H), 8.65 (s, 1H), 8.20 (d, J = 7.8 Hz, 1H), 8.12 (d, J = 7.3 Hz, 1H), 7.57 (t, J = 8.0 Hz, 1H), 4.34 (t, J = 6.5 Hz, 2H), 1.95 (t, J = 7.3 Hz, 2H), 1.78–1.68 (m, 2H), 1.55–1.48 (m, 2H), 1.40 (dt, J = 14.8, 7.5 Hz, 2H), 1.30 (dt, J = 14.6, 7.4 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 169.5, 164.4, 145.1, 135.3, 132.6, 128.7, 127.5, 126.9, 66.3, 32.7, 28.7, 28.4, 25.6, 25.4. HRMS (AP-ESI) m/z calcd for $C_{14}H_{17}N_3O_6Na$ $[M+Na]^+$: 346.1010; found: 346.1040.

4.1.8.5. 4-(((8-(Hydroxyamino)-8-oxooctyl)oxy)carbonyl)benzo[*c*][1,2,5]oxadiazole 1-oxide (24e). Weak yellow solid, yield: 79%. ^{1}H NMR (600 MHz, d_6-DMSO) δ 10.30 (s, 1H), 8.61 (s, 1H), 8.20 (dd, J = 8.1, 1.4 Hz, 1H), 8.12 (dd, J = 7.8, 1.4 Hz, 1H), 7.57 (t, J = 8.0 Hz, 1H), 4.35 (t, J = 6.6 Hz, 2H), 1.94 (t, J = 7.3 Hz, 2H), 1.76–1.71 (m, 2H), 1.49 (dd, J = 14.7, 7.3 Hz, 2H), 1.43–1.37 (m, 2H), 1.34–1.30 (m, 2H), 1.26 (dt, J = 13.5, 6.8 Hz, 2H). ^{13}C NMR (151 MHz, d_6-DMSO) δ 169.6, 164.4, 145.1, 135.2, 132.6, 128.7, 127.0, 66.4, 32.7, 28.9, 28.8, 28.4, 25.7, 25.5. HRMS (AP-ESI) m/z calcd for $C_{15}H_{18}N_3O_6Na$ $[M+Na]^+$: 360.1166; found: 360.3301.

4.2. Biological materials and methods

4.2.1. In vitro inhibition of Hela cells extracts fluorescence assay

In vitro HDACs inhibition assays were conducted as previously described in our group.[19] In brief, 10 μL of Hela nuclear extracts was mixed with 0.0097, 0.039, 0.39, 1.56, 6.25, 25 μM of target compounds (50 μL) and SAHA, and then they were incubated for 30 min, using 100% and none HDACs groups as control group. Five minutes later, fluorogenic substrate Boc-Lys (acetyl)-AMC (40 μL) 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 nm 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 IC_{50} values were calculated using a regression analysis of the concentration/inhibition data.

4.2.2. In vitro antiproliferative assay

In vitro antiproliferative assays were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as previously described.[17] Briefly, all cell lines were maintained in RPMI1640 medium containing 10% PBS at 37 °C in 5% CO_2 humidified incubator. Cells were passaged the day before dosing into a 96-well cell plate and allowed to grow for a minimum of 4 h prior to addition of compounds. These tested compounds were all dissolved in DMSO and then diluted in culture medium so that the effective DMSO concentration did not exceed 0.2%. After compounds addition, the plates were incubated for an additional 48 h, and then 0.5% MTT solution was added to each well. After further incubation for 4 h, formazan formed from MTT was extracted by adding 200 μL of DMSO for 15 min. Absorbance was then

determined using an ELISA reader at 490 nm and 630 nm, and the IC_{50} values were calculated according to the inhibition rations.

4.2.3. In vitro nitrite measurement

The nitrite measurement in vitro was tested using the reported method.[9] The levels of NO generated by individual compounds in the cells are presented as that of nitrite and were determined by the colorimetric assay using the colorimetric assay kit, according to the manufacturer's instruction. Briefly, HCT-116 cells (5×10^5/well) were treated with a 100 μM concentration of each compound for 5 h. Subsequently, the cells were harvested, and their cell lysates were prepared and then mixed with Griess for 10 min at 37 °C, followed by measurement at 540 nm by an enzyme-linked immunosorbent assay plate reader. The cells treated with DMSO were used as negative controls for the background levels of nitrite production, while sodium nitrite at different concentrations was prepared as the positive control for the standard curve.

4.2.4. In vitro antiproliferative assay with hemoglobin

This assay was as similar as the in vitro antiproliferative assay. HCT-116 cell line was maintained in RPMI1640 medium containing 10% PBS at 37 °C in 5% CO_2 humidified incubator. Cells were passaged the day before dosing into a 96-well cell plate and then treated with 2 μM **19d** and 2 μM **24d** for 24 h after pretreating indicated concentrations hemoglobin (Hb) (0, 2.5, 5, 10 or 20 μM) for 1 h. Later, 0.5% MTT solution was added to each well. After further incubation for 4 h, formazan formed from MTT was extracted by adding 200 μL of DMSO for 15 min. Absorbance was then determined using an ELISA reader at 490 nm and 630 nm, and the IC_{50} values were calculated according to the inhibition rations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.06.015.

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