Design, synthesis and biological evaluation of novel hydroxamates and 2-aminobenzamides as potent histone deacetylase inhibitors and antitumor agents

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ABSTRACT:

Many studies have indicated that histone deacetylase (HDAC) inhibitors are promising agents for the treatment of cancer. With the aim to search for novel potent HDAC inhibitors, we designed and synthesized two series of hydroxamates and 2-aminobenzamides compounds as HDAC inhibitors and antitumor agents. Those compounds were investigated for their HDAC enzymatic inhibitory activities and in vitro anti-proliferation activities against diverse cancer cell line (A549, HepG2, MGC80-3 and HCT116). Most of the synthesized compounds displayed potent HDAC inhibitory activity and antiproliferative activity. In particular, Compound 12a, N-(2-aminophenyl)-4-[(4-fluorophenoxy)methyl]benzamide, was shown to have the most HDAC inhibitory activity (70.6% inhibition at 5 μ M) and antitumor activity with IC₅₀ value of as low as 3.84 μ M against HepG2 human liver hepatocellular carcinoma cell line, more than 4.8-fold lower than CS055 and 5.9-fold lower than CI994. HDAC isoform selectivity assay indicated 12a is a potent HDAC2 inhibitor. Docking study of 12a suggested that it bound tightly to the binding pocket of HDAC2. Further investigation showed that 12a could inhibit the migration and colony formation of A549 cancer cells. Furthermore, 12a remarkably induced apoptosis and G2/M phase cell cycle arrest in A549 cancer cells. Those results indicated that compound 12a could be a promising candidate for treatment of cancer.

Keywords: Histone deacetylase inhibitor; hydroxamates; 2-aminobenzamides; Antiproliferation; Molecular Docking;

1. Introduction

Cancer is a widespread disease with high mortality. Traditional antitumor drugs usually result in seriously side effect because of lacking targeted therapies[1]. Histone deacetylase (HDACs) have become promising targets in recent years for cancer therapy. Histone lysine acetylation and deacetylation are important post-translational covalent modifications[2]. The acetylation of histones is balanced by histone acetyltransferases (HATs) and histone deacetylase (HDACs)[3-9]. Overexpressed HDACs catalyze the removal of acetyl groups from histones, resulting in chromatin condensation and silencing tumor suppressor genes[10-15]. HDAC inhibitors have been shown to cause differentiation and apoptosis of tumor cells[16]. Therefore, HDAC inhibitors are promising agents for the treatment of cancer. 18 HDAC isozymes have been identified so far, which can be divided into 4 classes: class I (HDACs 1,2,3 and 8), class II (HDACs 4,5,6,7,9 and 10), class IV (HDACs 11), and class III (sirtuins 1-7). Class I, class II, and class IV are zinc-dependent HDACs,

while class III are NAD-dependent sirtuins. It has been shown that the dysregulation of class I and II HDACs, especially class I isozymes, has been associated with the process of tumor cells proliferation, angiogenesis, invasion and metastasis[17-22].

To date, many HDAC inhibitors reached clinical trials, and five of them were approved for clinical uses: Vorinostat (SAHA, 1) and Romidepsin (FK228,2) for use in cutaneous T-cell lymphoma, Belinostat (PXD101,3) and Chidamide (CS055,4) both for use in peripheral T-cell lymphoma, and panobinostat (LBH589, 5) for multiple myeloma. Many HDAC inhibitors such as Tacedinaline (CI994, 6) , (MS-275, 7), (MGCD-0103, 8) are under clinical trials for the treatment of different kinds of cancers(Fig.1)[23-29]. The common pharmacophore of these HDAC inhibitors consists of 3 portions(Fig.1): (1) ZBG, a zinc binding group chelating Zinc at the bottom of HDACs active site; (2) a CAP group, interacting with the surface of HDACs; (3) a linker connecting the ZBG and the CAP group[30].

Fig. 1. FDA approved and clinical HDAC inhibitors.

In our previous studies, we have reported novel phenyl substituted SAHA-based hydroxamic acid derivatives[31]. From our examples, we found that the CAP group has a crucial impact on the antitumor activities of SAHA-based hydroxamic acid derivatives. In this report, we modified the CAP group and the hydrophobic linker of our compounds, in order to obtain novel potential HDAC inhibitors and search for more valuable candidates for the tumor therapy. Two series of hydroxamates, and 2-aminobenzamides were synthesized and evaluated for their inhibitory activities against HDACs and cancer cells. Docking studies of selected compounds binding to HDAC were also discussed.

2. Results and discussion

2.1. Chemistry

The synthetic route of target molecules is described in Schemes 1. The key intermediate 1a or 1b was synthesized by starting compound A (amine or ether). Then, 1a or 1b were directly converted to hydroxamic acids 2a, 3a, 2b or 3b by treating with hydroxylamine. In route (c) 1a or 1b was hydrolyzed and coupled with benzene-1,2-diamine to obtain compounds 5a-7a, 11a-19a, 5b-8b.

Scheme 1. Conditions: (a) K₂CO₃, KI/Butanone, reflux ; (b) KOH, NH₂OH. HCl/MeOH, rt; (c) 1.NaOH/MeOH, 2.HCl, 0°C; (d) benzene-1,2-diamine, BOP, Et₃N/DMF, rt;

2.2. Biological tests

2.2.1 HeLa Nuclear HDACs inhibition and antiproliferative assay

HDAC enzymatic inhibitory activities of the target compounds were evaluated using the *Fluor de Lys*[®]-Green HDAC Assay kit (BML-AK530, Enzo[®] Life Sciences) with CS055 and CI994 as positive control. Hela cell nuclear extracts were used as the source of the HDAC activity. Additionally, the antiproliferative effects of the targeted compounds against A549 human lung cancer cells, MGC80-3 human gastric cancer cells, and HepG2 human liver cancer cells were tested. The results are listed in Table 1-2. According to the results, we found that both the hydrophobic linker and cap group played a significant role in the inhibitory activities against HDACs and antiproliferative activity. In general, compounds owning potent HDAC inhibitory activity, also have better antiproliferative activity against diverse cancer cells. For hydroxamates, the compound 2b with longer linker showed better enzyme inhibitory activity and intro antiproliferative activity compared with shorter linker compound 2a. While 2-aminobenzamides, the compounds with shorter linker (5a-7a) showed better enzyme inhibitory activity and intro antiproliferative activity compared with longer linker compounds (5b-8b). In order to obtain more potent compounds, we also modified R₁ or R₂ to other groups (3a,6a,7a,3b,6b,7b,8b), as a result, all of them is less

potent than compound 5a. Compound 5a is more potent than control compounds (CS055, CI994) against HeLa nuclear HDACs and all three cancer cells. Therefore, further investigation of 5a as a lead compound is needed.

Table 1

HDAC enzyme inhibition and antiproliferative activity data (IC₅₀, μM) of compounds 2a-3a, 5a-7a

a

Compd.	R1	R2	% Inhibition ^a of HeLa nuclear HDACs		IC ₅₀ (μM) ^b		
			0.625 μM	5 μM	A549	MGC80-3	HepG2
CS055			39.7%	58.5%	11.02 ± 0.81	6.44 ± 1.17	22.65 ± 3.09
CI994			32.5%	49.4%	20.10 ± 0.76	11.60 ± 1.04	18.51 ± 1.77
2a		-OH	3.0%	55%	27.99 ± 3.06	/ ^c	29.55 ± 8.04
3a		-OH	/	81.7%	8.93 ± 0.70	/	7.77 ± 0.86
5a			40.3%	61.8%	6.76 ± 0.20	5.67 ± 0.82	6.70 ± 0.73
6a			26.0%	41.0%	8.34 ± 0.74	6.02 ± 0.34	7.02 ± 0.76
7a			8.8%	27.0%	/	7.39 ± 0.48	10.71 ± 1.46

^a The values represent the means of three independent experiments with SD less than 10%.

^b IC₅₀ values were obtained based on at least two independent experiments and expressed as means ± SD.

^c Not tested

Table 2HDAC enzyme inhibition and antiproliferative activity data (IC₅₀, μ M) of compounds 2b-3b, 5b-8b. **b**

Compd.	R1	R2	% Inhibition ^a of HeLa nuclear HDACs			IC ₅₀ (μ M) ^b	
			0.625 μ M	5 μ M	A549	MGC80-3	HepG2
CS055			39.7%	58.5%	11.02 $\pm$ 0.81	6.44 $\pm$ 1.17	22.65 $\pm$ 3.09
CI994			32.5%	49.4%	20.10 $\pm$ 0.76	11.60 $\pm$ 1.04	18.51 $\pm$ 1.77
2b		-OH	54.2%	92.7%	5.77 $\pm$ 0.34	/ ^c	8.40 $\pm$ 0.72
3b		-OH	56.4%	89.1%	5.48 $\pm$ 0.53	/	14.91 $\pm$ 1.17
5b			No inhibition	No inhibition	>32	>32	>32
6b			No inhibition	No inhibition	>32	>32	>32
7b			1.6%	33.6%	/	14.21 $\pm$ 0.7	13.50 $\pm$ 1.67
8b			No inhibition	8.5%	13.43 $\pm$ 0.64	>32	>32

^a The values represent the means of three independent experiments with SD less than 10%.^b IC₅₀ values were obtained based on at least two independent experiments and expressed as means $\pm$ SD.^c Not tested

To optimize the lead compound 5a further, we performed a SAR study on the cap moiety by introducing different substituents to phenyl group. Target compounds 11a-19a were prepared using the similar synthetic procedure of 5a, with replacement of the starting material by corresponding substituted phenol. HDAC enzyme inhibition activity against HeLa nuclear HDACs and antiproliferative activity against 4 kinds of cancer cells (A549 human lung cancer cells, MGC80-3 human gastric cancer cells, HepG2 human liver cancer cells and HCT116 human colorectal cancer cells) of these compounds were evaluated (Table 3). As shown in Table 3, Most of the compounds exhibited stronger HDAC inhibition and anti-proliferative activities against CS055 and CI994, which means that the substituents in phenyl ring of Cap group played a significant role in the inhibitory activities against HeLa nuclear HDACs and cancer cells. Among the compounds tested, 12a, 16a and 18a exhibited even better activities than 5a. However, substitution with nitro, chloro, bromo or isopropyl in phenyl ring of Cap group leads to a loss of activity. The rim of the active pocket of HDAC is hydrophobic, however, nitro, chloro and bromo are hydrophilic groups, which may be the reason why the substitutions with nitro, chloro, or bromo in phenyl ring of Cap group lead to a loss of activity.

Table 3

HDAC enzyme inhibition and antiproliferative activity data(IC₅₀, μ M) of compounds 11a-19a

Compd.	R	% Inhibition ^a of HeLa nuclear		IC ₅₀ (μ M) ^b			
		HDACs		A549	MGC80-3	HepG2	HCT116
		0.625 μ M	5 μ M				
CS055		39.7%	58.5%	11.02 $\pm$ 0.81	6.44 $\pm$ 1.17	22.65 $\pm$ 3.09	2.28 $\pm$ 0.77
CI994		32.5%	49.4%	20.10 $\pm$ 0.76	11.60 $\pm$ 1.04	18.51 $\pm$ 1.77	7.71 $\pm$ 0.86
5a	-H	40.3%	61.8%	6.76 $\pm$ 0.20	5.67 $\pm$ 0.82	6.70 $\pm$ 0.73	4.89 $\pm$ 0.29
11a	-NO ₂	No inhibition	No inhibition	>32	>32	>32	>32
12a	-F	46.5%	70.6%	5.37 $\pm$ 0.21	4.97 $\pm$ 0.65	3.84 $\pm$ 0.54	3.86 $\pm$ 0.39
13a	-Cl	18.7%	26.3%	>32	>32	>32	17.33 $\pm$ 2.74
14a	-Br	/ ^c	/	24.40 $\pm$ 2.76	>32	>32	>32
15a	-CH ₃	45.4%	64.1%	5.43 $\pm$ 0.25	6.53 $\pm$ 0.63	6.59 $\pm$ 0.60	4.86 $\pm$ 0.43
16a	-CH ₃ O	46.9%	69.4%	5.53 $\pm$ 0.21	3.48 $\pm$ 0.33	4.20 $\pm$ 0.51	2.02 $\pm$ 0.23
17a	-CH ₂ CH ₃	/	/	6.42 $\pm$ 0.36	5.88 $\pm$ 0.59	6.96 $\pm$ 1.31	4.83 $\pm$ 0.55
18a	-CH ₂ CH ₂ CH ₃	/	/	5.21 $\pm$ 1.27	4.42 $\pm$ 0.67	4.75 $\pm$ 0.89	3.23 $\pm$ 0.73
19a	-CH(CH ₃) ₂	No inhibition	1.8%	7.46 $\pm$ 0.97	>32	>32	4.57 $\pm$ 0.94

^a The values represent the means of three independent experiments with SD less than 10%.

^b IC₅₀ values were obtained based on at least two independent experiments and expressed as means $\pm$ SD.

^c Not tested

2.2.2 HDAC isoform selectivity of 12a

To further investigate the HDAC isoforms selectivity, compound 12a with the most potent HDAC inhibitory activity and antiproliferative activity was chosen to perform enzyme inhibitory assays against a series of HDAC isoenzymes. We determined the IC₅₀ values of compound 12a for class I representatives HDAC1 and HDAC2, and class II representative HDAC6 with CI994 as positive control (Table4). Results presented in Table 4 demonstrated that 12a is a potent HDAC2 inhibitor with IC₅₀ of 0.57 μ M.

Table 4

In vitro inhibition of HDACs isoforms of 12a

Compd.	IC ₅₀ ^a (μ M)		
	Class I	Class II	
	HDAC1	HDAC2	HDAC6
CI994	1.62 $\pm$ 0.25	1.20 $\pm$ 0.23	>40
12a	1.27 $\pm$ 0.20	0.57 $\pm$ 0.09	>40

^aIC₅₀ values were obtained based on two independent experiments and expressed as means $\pm$ SD.

2.2.3 Molecular docking

The proposed binding modes of compounds 12a and CI994 (Fig.2) in the active site of HDAC2 (PDB code 4LXZ) were investigated by docking analysis. Both 12a and CI994 could form 5 hydrogen bonds with amino acid residues GLY154, HIS145, HIS146, ASP181, and TYR308 in the HDAC2 active site. The cap group of 12a could be well accommodated in the hydrophobic S pocket of HDAC2, while CI994 could hardly achieve the same effect. Docking results of 12a to HDAC2 could well explain its potent HDAC2 inhibitory activity.

Fig. 2. Predicted binding modes of compounds 12a and CI994 on HDAC2 (PDB id:4LXZ). Interactions between the protein and the ligand are shown as yellow dotted lines. The ligands are shown as stick models in purple(CI994) and red(12a). (A) Molecular surface of the HDAC2 binding pocket (B) Compound 12a interacted with the actives of HDAC2. (C) Compound CI994 interacted with the actives of HDAC2.

2.2.4 Migration, colony formation, apoptosis and cell cycle analysis of 12a

Fig. 3. Migration of A549 cancer cells. A549 cancer cells were treated with compounds 12a, 16a, CS055, CI994 or vehicle for 48h, and their migration was determined using the scratch test. One representative of three independent experiments is shown. Cells treated with vehicle (DMSO) were used as controls.

We selected compounds 12a and 16a to evaluate their effects on the migration and colony formation of A549 cancer cells. As shown in Fig.3, compound 12a or 16a could inhibit the migration of A549 cancer cells and even more potent than CS055 and CI994. As shown in Fig.4, compound 12a or 16a could also inhibit the colony formation of A549 cancer cells in a dose dependent manner, and remarkably more potent than CI994.

To investigate whether the antiproliferative activities of 12a are accompanied by enhanced cancer cell apoptosis, flow cytometry assay were determined. As shown in Fig.5, 12a-treated A549 cells exhibited a dose-dependent increase of apoptosis by 25.3% and 64.4% at 4 and 8 μ M, respectively. The 64.4% induction of A549 cells apoptosis with incubation with 8 μ M of 12a was significantly higher than that of CS055 (22.42% apoptotic cells at the same concentration) and CI994 (12.58% apoptotic cells at the same concentration). These results showed that 12a is able to remarkably induce apoptosis of the A549 cancer cells.

We further investigated the effect of compound 12a on cell cycle progression of A549 cancer cells(Fig.6). In comparison with the control cells, 12a clearly arrested A549 cells in G2/M phase with a concentration dependent manner. Compound 12a caused G2/M fraction increased from 9.1% in control samples to 37.74% in treated samples. In the meanwhile, the G0/G1 fraction decreased from 75.77% to 54.03% and the S-phase fraction decreased from 15.13% to 8.23%, While CS055 and CI994 showed no obvious effect on the cell cycle of A549 cancer cells at 2 μ M or 16 μ M. These data suggested that compound 12a may induce G2/M phase arrest in A549 cancer cells.

Fig. 4. Compound 12a and 16a inhibit A549 cancer cell colony formation.

Fig. 5. Induction of apoptosis at 48h by compounds 12a, CS055 and CI994 in different concentrations in A549 cancer cells.

Fig. 6. Effect of CS055, CI994, and 12a in A549 cell cycle progression.

3. Conclusions

In conclusion, two series of hydroxamates and 2-aminobenzamides derivatives have been designed and synthesized as HDAC inhibitors. All targeted compounds were investigated for their Hela cell nuclear extracts HDAC enzymatic inhibitory activities and in vitro antiproliferative activities against diverse cancer cell lines. Most compounds exhibited potent HDAC inhibitory activity and antiproliferative activities. Especially, compound 12a, which showed more potent HDAC inhibition activity to CS055 and CI994, exhibited highly potent anti-proliferation effect, particularly 5.9-fold enhancement compared with CS055 and 4.8-fold enhancement compared with CI994 against HepG2 cancer cell. **HDAC isoform inhibition assay indicated 12a is a potent HDAC2 inhibitor.** Docking results of 12a to HDAC2 could well explain its potent HDAC2 inhibitory activity. Further investigation showed that 12a could inhibit the migration and colony formation of A549 cancer cells and remarkably induced the G2/M phase cell cycle arrest and apoptosis in

A549 cancer cells. By far, the existing data indicated that 12a is a potentially promising histone deacetylase inhibitor for the therapy of cancer. The in vivo antitumor activity evaluation and mechanism study of 12a are undertaken in our lab.

4. Experimental

4.1. Chemistry

All starting materials were obtained commercially and were used without further purification. All reactions were carried out in oven-dried glassware. Dried nitrogen was used to purge the reactor before the reaction and to protect the reaction during the entire operation. Melting points were recorded on a XT5, manufactured by Beijing Keyiecopti Instrument Factory and uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker AV-400 spectrometer (400MHz). The chemical shifts were reported in ppm relative to Me_4Si as internal standard. Mass spectra were obtained with a Waters Xevo G2 Qtof mass spectrometer. Thin layer chromatography (TLC) on pre-coated plates with silica gel F254, purchased from Qingdao Haiyang Chemical Co. Ltd., was employed to monitor the progress of the reaction. The purities of final compounds were established to be above 95% pure using a Shimadzu LC-20AT system with LC-20AT two-solvent delivery units: Solvent A: methanol. Solvent B: 0.1% H_3PO_4 aqueous solution. A SPD-M20A DAD detector was set at $\lambda = 254$ nm. The temperature was 30°C . The system included a SIL-20A auto sampler, a CTO-10ASVP column oven, a LC solution workstation Shimadzu, Kyoto, Japan and an analytical reversed phase C18 column (4.6×250 mm, $5\mu\text{m}$, Diamodsil). The flow rate of solvent A with the gradient started at 20% of methanol and increased to 90% over 35 min.

4.1.1. General method for the synthesis of 2a,3a,2b,3b

Step-1: To an oven-dried, 100-mL, three-necked, round-bottomed flask equipped with a thermometer, a vacuum-jacketed Dean-Stark trap topped with a reflux condenser fitted with a T-joint inlet, glass stoppers, and a Teflon-coated magnetic stirring bar was sequentially charged with compound A (8.7 mmol, 1 equiv.), compound B_1 or B_2 (8.7mmol), K_2CO_3 (17.4mmol), KI (8.7mmol) and 30mL of 2-Butanone. The reaction was heated at reflux for 6-12h in an oil bath. TLC analysis, using ethyl acetate: petroleum ether (1:12) as the eluent, indicated the complete reaction. The reaction mixture was allowed to cool to room temperature and then poured into 200mL of ethyl acetate with vigorous stirring. Stirring was continued for an additional 10min, and the resulting solid was then filtered off. The filtrate was concentrated to provide 1a or 1b as a solid.

Step-2: To a 100-mL beaker equipped with a Teflon-coated magnetic stirring bar were sequentially placed potassium hydroxide (74.3 mmol, 18equiv.) and methanol (20mL). The mixture was stirred until a complete dissolution was obtained. Hydroxylamine hydrochloride (82.6 mmol, 20 equiv.) was added. After an additional 30 min of stirring at 35°C , the reaction mixture was cooled to ambient temperature. 1a or 1b (4.1mmol, obtained from the step A) was added. Stirring was continued for 30 min. TLC was employed to monitor the progress of the reaction. After the complete reaction, the reaction mixture was poured into 600mL of ice-water mixture with vigorous stirring leading to the formation of a solid. The resulting solid was collected by vacuum filtration, and successively washed with water (2 X 10mL) and ether (2 X 10mL). After having been dried in vacuo, the crude product was further purified by recrystallization from acetonitrile to afford 2a,2b or 3b as a solid.

4.1.1.1 *N*-hydroxy-4-(phenoxymethyl)benzamide (2a)

White solid ; Yield 84%; HPLC *R*_t=23.19min; mp 130.0-131.4 °C; ¹H NMR (400MHz, DMSO-*d*₆): δ=7.77(d, *J*=8.0Hz, 2H), 7.45(d, *J*=8.0Hz, 2H), 7.30(t, *J*=7.6Hz, 2H), 7.02(d, *J*=8.1Hz, 2H), 6.95(t, *J*=7.3Hz, 1H), 5.13(s, 2H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=68.68, 114.75, 120.73, 126.57, 127.10, 129.47, 133.97, 138.85, 158.20, 163.62 ppm; C₁₄H₁₃NO₃, MS (ES⁺) m/z: 244.09 (M+H)⁺.

4.1.1.2. *N*-hydroxy-4-({4-[(1*E*)-*N*-hydroxyethanimidoyl]phenoxy}methyl)benzamide (3a)

White solid; Yield 75%; HPLC *R*_t=21.72min; mp 176.7-178.2 °C; ¹H NMR (400MHz, DMSO-*d*₆): δ=11.01(s, 1H), 7.78(d, *J*=7.8Hz, 2H), 7.60(d, *J*=8.5Hz, 2H), 7.51(d, *J*=7.8Hz, 2H), 7.03(d, *J*=8.5Hz, 2H), 5.19(s, 2H), 2.12(s, 3H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=11.42, 68.65, 114.61, 126.83, 126.93, 127.27, 129.75, 132.54, 139.83, 152.30, 158.51, 163.76 ppm; C₁₆H₁₆N₂O₄, MS (ES⁺) m/z: 301.19 (M+H)⁺.

4.1.1.3. (2*E*)-*N*-hydroxy-3-[4-(phenoxymethyl)phenyl]acrylamide(2b)

White solid; Yield 89%; HPLC *R*_t=27.46min; mp 140.4-141.7 °C; ¹H NMR (400MHz, DMSO-*d*₆): δ=7.59(d, *J*=7.4Hz, 2H), 7.48(m, 3H), 7.30(t, *J*=7.4Hz, 2H), 7.01(d, *J*=7.8Hz, 2H), 6.95(t, *J*=7.0Hz, 1H), 6.5(d, *J*=15.8Hz, 1H), 5.12(s, 2H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=68.62, 114.75, 119.18, 120.76, 127.52, 128.03, 129.48, 134.33, 137.71, 138.43, 158.16, 162.60 ppm; C₁₆H₁₅NO₃, MS (ES⁺) m/z: 270.13 (M+H)⁺.

4.1.1.4. (2*E*)-3-[4-(2,3-dihydro-1*H*-indol-1-ylmethyl)phenyl]-*N*-hydroxyacrylamide (3b)

Gray solid; Yield 57%; HPLC *R*_t=27.23min; mp 142.3-144.1 °C; ¹H NMR (400MHz, DMSO-*d*₆): δ=7.55(d, *J*=7.3Hz, 2H), 7.40(m, 3H), 7.05(d, *J*=6.8Hz, 1H), 6.99(t, *J*=7.4Hz, 1H), 6.58(m, 2H), 6.46(d, *J*=15.8Hz, 1H), 4.29 (s, 2H), 3.27(t, *J*=8.0Hz, 2H), 2.91(t, *J*=8.0Hz, 2H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=27.93, 52.27, 52.85, 106.94, 117.28, 118.77, 124.24, 127.03, 127.50, 128.43, 129.49, 133.67, 137.77, 139.84, 152.15, 162.63 ppm; C₁₈H₁₈N₂O₂, MS (ES⁺) m/z: 295.1449 (M+H)⁺.

4.1.2. General method for the synthesis of 5a-7a, 5b-8b, 11a-19a

Step-1: Following the same procedure of 4.1.1. to afford 1a or 1b

Step-2: To a solution of 1a or 1b (1equiv.) in CH₃OH, NaOH aqueous solution (2mol/L, 10 equiv.) was added. The reaction was heated at reflux for 2h in an oil bath. The reaction mixture was allowed to cool to room temperature. The reaction mixture was poured into 300mL of ice-water mixture with vigorous stirring. Then, add hydrochloric acid aqueous solution (2mol/L, 15 equiv.) to the mixture, leading to the formation of a solid. The resulting solid was collected by vacuum filtration, successively washed with water (5 X 10mL) and ether (2 X 10mL) and dried in vacuo to afford 4a or 4b as a solid.

Step-3: To a solution of acid 4a or 4b (1 equiv.) and (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) (1.2 equiv.) in anhydrous DMF was added triethylamine (4 equiv.), and the mixture was stirred at 20 °C under nitrogen for 10 min. Phenylene-1,2-diamine (1.2 equiv.) was then added and the solution stirred at room temperature under nitrogen for 4h. The mixture was diluted with ethyl acetate , then washed with water, saturated aqueous sodium hydrogen carbonate and last with brine. The organic layer was dried over MgSO₄, filtered, washed with MeOH, purified by recrystallization from acetonitrile to afford 5a-7a, 5b-8b as a solid.

4.1.2.1. *N*-(2-aminophenyl)-4-(phenoxymethyl)benzamide(5a)

Gray solid; Yield 51%; HPLC *R*_t=25.20min; mp 176.2-178.2 °C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.70 (s, 1H), 8.03(d, *J*=7.8Hz, 2H), 7.60(d, *J*=7.8Hz, 2H), 7.32(t, *J*=7.8Hz, 1H), 7.21(d, *J*=7.7Hz, 1H), 7.01(m, 4H), 6.82(m, *J*=7.8Hz, 1H), 6.63(t, *J*=7.2Hz, 1H), 5.22(s, 2H), 4.94(s, 2H); ¹³C NMR

(400MHz, DMSO- d_6): δ =68.50, 114.84, 116.11, 116.23, 120.83, 123.28, 126.47, 126.66, 127.15, 127.93, 129.50, 134.03, 140.58, 143.12, 158.12, 165.05 ppm; $C_{20}H_{18}N_2O_2$, MS (ES+) m/z : 319.1442 (M+H) $^+$.

4.1.2.2. *N*-(2-amino-4-fluorophenyl)-4-(phenoxyethyl)benzamide (6a)

Gray solid; Yield 59%; HPLC R_t =29.07min; mp 158.8-160.0°C; 1H NMR (400MHz, DMSO- d_6): δ =9.61 (s, 1H), 8.01(d, J =6.7Hz, 2H), 7.59(d, J =6.7Hz, 2H), 7.32(m, 2H), 7.05(m, 4H), 6.56(d, J =10.7Hz, 1H), 6.38(m, 1H), 5.24(m, 4H); ^{13}C NMR (400MHz, DMSO- d_6): δ =68.48, 101.28, 102.09, 114.83, 119.19, 120.83, 127.12, 127.94, 128.48, 128.58, 129.50, 133.89, 140.60, 145.41, 145.53, 158.10, 159.83, 162.20, 165.30 ppm; $C_{20}H_{17}FN_2O_2$, MS (ES+) m/z : 337.1347 (M+H) $^+$.

4.1.2.3. *N*-(2-aminophenyl)-4-[(5-bromo-2,3-dihydro-1H-indol-1-yl)methyl]benzamide (7a)

Gray solid; Yield 81%; HPLC R_t =32.20min; mp 171.0-171.9°C; 1H NMR (400MHz, DMSO- d_6): δ =9.66(s, 1H), 7.99(d, J =7.9Hz, 2H), 7.47(d, J =8.1Hz, 2H), 7.16(m, 3H), 6.99(t, J =7.5Hz, 1H), 6.80(d, J =7.7Hz, 1H), 6.62(t, J =7.5Hz, 1H), 6.53(d, J =8.4Hz, 1H), 4.91(s, 2H), 4.36(s, 2H), 3.35(m, 2H); 2.95(t, J =8.4Hz, 2H); ^{13}C NMR (400MHz, DMSO- d_6): δ =27.69, 51.84, 52.78, 107.86, 108.38, 116.10, 116.22, 123.32, 126.43, 126.64, 127.01, 127.69, 127.95, 129.45, 132.52, 133.46, 141.46, 143.11, 151.38, 165.05; $C_{22}H_{20}BrN_3O$, MS (ES+) m/z : 422.0866 (M+H) $^+$.

4.1.2.4. (2E)-*N*-(2-aminophenyl)-3-[4-(phenoxyethyl)phenyl]acrylamide (5b)

Gray solid; Yield 64%; HPLC R_t =29.06min; mp 202.7-204.2°C; 1H NMR (400MHz, DMSO- d_6): δ =9.41(s, 1H), 7.60(m, 5H), 7.32(m, 3H), 7.05(d, J =7.8Hz, 2H), 6.95(m, 3H), 6.77(d, J =7.8Hz, 1H), 6.60(t, J =7.3Hz, 1H), 5.16(s, 2H), 4.97(s, 2H); ^{13}C NMR (400MHz, DMSO- d_6): δ =68.67, 114.77, 116.00, 116.27, 120.78, 122.41, 123.50, 125.75, 127.71, 128.10, 129.50, 134.37, 138.68, 139.15, 141.53, 158.22, 163.44 ppm; $C_{22}H_{20}N_2O_2$, MS (ES+) m/z : 345.1604 (M+H) $^+$.

4.1.2.5. (2E)-*N*-(3-aminopyridin-4-yl)-3-[4-(phenoxyethyl)phenyl]acrylamide (6b)

Yellow solid; Yield 73%; HPLC R_t =24.17min; mp 176.8-179.3°C; 1H NMR (400MHz, DMSO- d_6): δ =9.53(s, 1H), 8.07(s, 1H), 7.79(d, J =5.3Hz, 1H), 7.70(m, 4H), 7.54(d, J =7.9Hz, 2H), 7.32(t, J =7.9Hz, 2H), 7.03(d, J =7.9Hz, 2H), 6.97(m, 2H), 5.23(s, 2H), 5.16(s, 2H); $C_{21}H_{19}N_3O_2$, MS (ES+) m/z : 346.1553 (M+H) $^+$.

4.1.2.6.

(2E)-*N*-(2-aminophenyl)-3-[4-(3,4-dihydroisoquinolin-2(1H)-ylmethyl)phenyl]acrylamide (7b)

Gray solid; Yield 87%; HPLC R_t =12.55min; mp 148.4-150.7°C; 1H NMR (400MHz, DMSO- d_6): δ =9.56(s, 1H), 7.60(m, 3H), 7.43(m, 3H), 7.11(m, 3H), 7.01(m, 2H), 6.93(t, J =7.4Hz, 1H), 6.78(d, J =7.9Hz, 1H), 6.59(t, J =7.4Hz, 1H), 5.03(s, 2H), 3.68(s, 2H), 3.56(s, 2H); 2.83(m, 2H), 2.69(m, 2H); ^{13}C NMR (400MHz, DMSO- d_6): δ =28.68, 50.26, 55.42, 61.48, 115.96, 116.18, 122.04, 123.59, 124.54, 125.44, 125.61, 125.95, 126.33, 127.60, 128.42, 129.22, 133.69, 134.05, 134.72, 139.28, 140.23, 141.46, 163.55; $C_{25}H_{25}N_3O$, MS (ES+) m/z : 384.2077 (M+H) $^+$.

4.1.2.7. (2E)-*N*-(2-aminophenyl)-3-[4-(2,3-dihydro-1H-indol-1-ylmethyl)phenyl]acrylamide (8b)

Yellow solid; Yield 67%; HPLC R_t =30.30min; mp 151.4-152.9°C; 1H NMR (400MHz, DMSO- d_6): δ =9.59 (s, 1H), 7.60 (m, 3H), 7.42 (m, 3H), 7.02 (m, 3H), 6.92 (d, J =7.4Hz, 1H), 6.77 (d, J =7.7Hz, 1H), 6.59(m, 3H), 5.04(s, 2H), 4.30(s, 2H); ^{13}C NMR (400MHz, DMSO- d_6): δ =27.96, 52.33, 52.91, 106.95, 115.95, 116.16, 117.30, 122.13, 123.60, 124.25, 124.55, 125.60, 127.06, 127.72, 128.47, 129.49, 133.72, 139.18, 140.11, 141.48, 152.17, 163.54 ppm; $C_{24}H_{23}N_3O$, MS (ES+) m/z : 370.1920 (M+H) $^+$.

4.1.2.8. *N*-(2-aminophenyl)-4-[(4-nitrophenoxy)methyl]benzamide (11a)

Yellow solid; Yield 59%; HPLC R_t =24.85min; mp 201.8-204.1°C; 1H NMR (400MHz, DMSO- d_6): δ =9.81(s, 1H), 8.24(d, J =9.2Hz, 2H), 8.06(d, J =8.1Hz, 2H), 7.60(d, J =8.1Hz, 2H), 7.27(m, 2H), 7.19(d, J =7.7Hz, 1H), 6.97(m, 1H), 6.79(d, J =8.1Hz, 1H), 6.60(t, J =7.6Hz, 1H), 5.39(s, 2H), 4.96(s, 2H); ^{13}C NMR (400MHz, DMSO- d_6): δ =69.55, 115.46, 116.13, 123.28, 125.88, 126.40, 126.70, 127.38, 128.09, 134.34, 139.28, 141.03, 143.13, 163.43, 164.92, ; $C_{20}H_{17}N_3O_4$, MS (ES+) m/z : 364.1299 (M+H) $^+$.

4.1.2.9. *N*-(2-aminophenyl)-4-[(4-fluorophenoxy)methyl]benzamide (12a)

White solid; Yield 62%; HPLC *R*_t=25.94min; mp 196.0-197.8°C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.69 (s, 1H), 8.02(d, *J*=8.0Hz, 2H), 7.58(d, *J*=8.0Hz, 2H), 7.20(m, 1H), 7.15(m, 2H), 7.06(m, 2H), 6.99(d, *J*=7.8Hz, 1H), 6.81(m, 1H), 6.62(t, *J*=7.5Hz, 1H), 5.20(s, 2H), 4.93(s, 2H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=69.18, 115.72, 115.95, 116.11, 116.19, 116.22, 126.48, 126.67, 127.19, 127.93, 134.07, 140.37, 143.12, 154.44, 157.79, 165.02; C₂₀H₁₇FN₂O₂, MS (ES+) *m/z*: 337.1347 (M+H)⁺.

4.1.2.10. *N*-(2-aminophenyl)-4-[(4-chlorophenoxy)methyl]benzamide(13a)

Gray solid; Yield 61%; HPLC *R*_t=28.96min; mp 191.6-193°C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.68(s, 1H), 8.01(d, *J*=8.1Hz, 2H), 7.58(d, *J*=8.1Hz, 2H), 7.36(m, 2H), 7.19(d, *J*=7.7Hz, 2H), 7.07(m, 2H), 6.99(t, *J*=7.4Hz, 1H), 6.80(d, *J*=7.8Hz, 1H), 6.61(t, *J*=7.6Hz, 1H), 5.22(s, 2H), 4.92(s, 2H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=68.94, 116.08, 116.20, 116.65, 123.23, 124.55, 126.47, 126.66, 127.21, 127.94, 129.25, 134.12, 140.13, 143.12, 156.97, 164.99; C₂₀H₁₇ClN₂O₂, MS (ES+) *m/z*: 353.1051 (M+H)⁺.

4.1.2.11. *N*-(2-aminophenyl)-4-[(4-bromophenoxy)methyl]benzamide(14a)

Gray solid; Yield 62%; HPLC *R*_t=29.80min; mp 201.4-202.6°C; ¹H NMR(400MHz, DMSO-*d*₆): δ=9.71(s, 1H), 8.03(d, *J*=8.0Hz, 2H), 7.58(d, *J*=8.0Hz, 2H), 7.48(m, 2H), 7.21(d, *J*=7.6Hz, 1H), 7.01(m, 3H), 6.82(m, 1H), 6.63(d, *J*=7.3Hz, 1H), 5.22(s, 2H), 4.93(s, 2H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=68.88, 112.28, 116.11, 116.23, 117.18, 123.26, 126.49, 126.67, 127.21, 127.96, 132.15, 134.13, 140.10, 143.13, 157.41, 165.01; C₂₀H₁₇BrN₂O₂, MS (ES+) *m/z*: 397.0549 (M+H)⁺.

4.1.2.12. *N*-(2-aminophenyl)-4-[(4-methylphenoxy)methyl]benzamide (15a)

White solid; Yield 53%; HPLC *R*_t=28.31min; mp 183.6-185.0°C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.68 (s, 1H), 8.01(d, *J*=8.1Hz, 2H), 7.57(d, *J*=8.1Hz, 2H), 7.20(d, *J*=7.7Hz, 1H), 7.11(d, *J*=8.3Hz, 2H), 6.99(m, 1H), 6.93(d, *J*=8.5Hz, 2H), 6.81(m, 1H), 6.62(t, *J*=7.5Hz, 1H), 5.18(s, 2H), 4.92(s, 2H), 2.24(s, 3H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=20.05, 68.57, 114.70, 116.10, 116.22, 123.27, 126.46, 126.66, 127.09, 127.89, 129.49, 129.82, 133.95, 140.75, 143.12, 155.99, 165.04; C₂₁H₂₀N₂O₂, MS (ES+) *m/z*: 333.1607 (M+H)⁺.

4.1.2.13. *N*-(2-aminophenyl)-4-[(4-methoxyphenoxy)methyl]benzamide (16a)

White solid; Yield 64%; HPLC *R*_t=24.84min; mp 173.2-174.7°C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.68 (s, 1H), 8.01(d, *J*=8.0Hz, 2H), 7.57(d, *J*=8.0Hz, 2H), 7.20(d, *J*=7.6Hz, 1H), 6.98(m, 3H), 6.88(m, 2H), 6.81(m, 1H), 6.62(t, *J*=7.5Hz, 1H), 5.15(s, 2H), 4.92(s, 2H), 3.71(s, 3H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=55.32, 69.10, 114.59, 115.79, 116.10, 116.22, 123.27, 126.46, 126.66, 127.11, 127.88, 133.93, 140.83, 143.12, 152.09, 153.54, 165.04 ppm; C₂₁H₂₀N₂O₃, MS (ES+) *m/z*: 349.1555 (M+H)⁺.

4.1.2.14. *N*-(2-aminophenyl)-4-[(4-ethylphenoxy)methyl]benzamide(17a)

Yellow solid; Yield 46%; HPLC *R*_t=30.78min; mp 171.9-173.8°C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.68(s, 1H), 8.01(d, *J*=8.1Hz, 2H), 7.57(d, *J*=8.1Hz, 2H), 7.19(d, *J*=7.7Hz, 1H), 7.14(d, *J*=8.5Hz, 2H), 6.96(m, 3H), 6.81(m, 1H), 6.62(t, *J*=7.5Hz, 1H), 5.19(s, 2H), 4.98(s, 2H), 2.54(m, 2H), 1.56(m, 2H), 1.16(t, *J*=7.5Hz, 3H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=15.87, 27.26, 68.57, 114.71, 116.10, 116.23, 123.28, 126.46, 126.66, 127.08, 127.90, 128.64, 133.95, 136.04, 140.77, 143.08, 156.17, 165.03; C₂₂H₂₂N₂O₂, MS (ES+) *m/z*: 347.1756 (M+H)⁺.

4.1.2.15. *N*-(2-aminophenyl)-4-[(4-propylphenoxy)methyl]benzamide(18a)

White solid; Yield 78%; HPLC *R*_t=33.20min; mp 171.5-173.0°C; ¹H NMR (400MHz, DMSO-*d*₆): δ=9.68(s, 1H), 8.01(d, *J*=8.0Hz, 2H), 7.58(d, *J*=8.0Hz, 2H), 7.20(d, *J*=7.7Hz, 1H), 7.12(d, *J*=8.4Hz, 2H), 6.97(m, 3H), 6.81(d, *J*=7.8Hz, 1H), 6.62(t, *J*=7.5Hz, 1H), 5.18(s, 2H), 4.92(s, 2H), 2.48(d, *J*=7.7Hz, 2H), 1.56(m, 2H), 0.88(t, *J*=7.4Hz, 3H); ¹³C NMR (400MHz, DMSO-*d*₆): δ=13.56, 24.27, 36.35, 68.57, 114.60, 116.09, 116.21, 123.27, 126.45, 126.65, 127.09, 127.90, 129.22, 133.96, 134.39, 140.76, 143.11, 156.21, 165.03; C₂₃H₂₄N₂O₂, MS (ES+) *m/z*: 361.1911 (M+H)⁺.

4.1.2.16. *N*-(2-aminophenyl)-4-[(4-isopropylphenoxy)methyl]benzamide(19a)

Gray solid; Yield 53%; HPLC R_t =32.71min; mp 178.3-179.9°C; ^1H NMR (400MHz, DMSO- d_6): δ =9.70(s, 1H), 8.03(d, J =7.8Hz, 2H), 7.58(d, J =8.1Hz, 2H), 7.18(m, 3H), 6.97(m, 3H), 6.82(d, J =8.1Hz, 1H), 6.62(t, J =7.6Hz, 1H), 5.19(s, 2H), 4.93(s, 2H), 2.84(m, 1H); 1.18(d, J =6.9Hz, 6H); ^{13}C NMR (400MHz, DMSO- d_6): δ =24.08, 32.57, 68.58, 114.64, 116.11, 116.23, 123.29, 126.46, 126.65, 127.07, 127.14, 127.92, 133.97, 140.73, 140.80, 143.12, 156.22, 165.03 ; $\text{C}_{23}\text{H}_{24}\text{N}_2\text{O}_2$, MS (ES+) m/z :361.1910 (M+H) $^+$.

4.2. Biological materials and methods

4.2.1. HDAC Inhibition Assay

The *HeLa Nuclear Extract* HDAC inhibition assay was performed using *Fluor de Lys[®]-Green* HDAC Assay kit (BML-AK530, Enzo[®] Life Sciences). HDAC1, 2 and 6 inhibition assay was performed using *Fluor de Lys[®]* HDAC1 Assay kit (BML-AK511, Enzo[®] Life Sciences), *Fluor de Lys[®] -Green* HDAC2 Assay kit (BML-AK512, Enzo[®] Life Sciences) and *Fluor de Lys[®]* HDAC6 Assay kit (BML-AK516, Enzo[®] Life Sciences). 15 μL of HDAC was mixed with 10 μL of tested compounds. Allow diluted *Fluor de Lys[®]-Green* Substrate and the microtiter plate to equilibrate to assay temperature (37°C) for 5 minutes. add 25 μL diluted substrate to each well and mix thoroughly. Incubate the plate at 37°C for enough time and then add 50 μL *Fluor de Lys[®]* Developer to each well to stop the HDAC reaction. Plate was Incubated at 25°C for another 10 min and fluorescence measurements were obtained using an EnSpire multimode plate reader (PerkinElmer, USA).

4.2.2. Antiproliferative assay

Cell lines A549, MGC80-3, HepG2 and HCT116 were obtained from the Cell Resource Center, Peking Union Medical College. A549 and HCT116 cells were cultured in RPMI1640 (Corning, USA) contained 10% fetal bovine serum (FBS) (ThermoFisher, USA) and 1% penicillin/streptomycin. MGC80-3 and HepG2 cells were cultured in DMEM (Corning, USA) contained 10% fetal bovine serum (FBS) (ThermoFisher, USA) , and 1% penicillin/streptomycin. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells in logarithmic phase were seeded in 96-well culture plates at a density of 3000-4000 cells/well. After 12h, cells were treated with various concentrations of compounds or solvent control. After 72h of incubation, 10 μL of CCK-8 reagent was added to each well, and the cells were incubated for an additional 1h. Absorbance was measured at 450nm using an EnSpire multimode plate reader (PerkinElmer, USA). IC₅₀ values were calculated using percentage of growth versus untreated control.

4.2.3. Scratch test.

The scratch test method was used to estimate cell migration. The cells were seeded onto 12-well plates and treated with DMSO or compounds CS055, CI994, 12a and 16a. After tracing the bottom of the wells, the cells were allowed to migrate. The image were captured after 48h using an OPTIC BDS200 microscope.

4.2.4. Colony formation assay

A549 cells were seeded in 6-well plates at a density of 3000 cells per well. After 24h, cells were treated with DMSO, CS055, CI994, 12a or 16a. After 7 days, Colonies were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet.

4.2.5. Cell apoptosis analysis

Cell apoptosis was determined using Vybrant Apoptosis Assay kit, (Invitrogen). Briefly, A549 cancer cells (1.6×10^5 /well) were incubated in 6-well plates for 24h and then treated with DMSO, CS055, CI994 or 12a. After 48h, cells were harvested and washed three times with prechilled PBS. A 100 μ L volume of 1 X annexin-binding buffer, 5 μ L of Alexa Fluor 488 annexin and 1 μ L of propidium iodide (100 μ g/ml) were added. The cells were incubated for 15min in dark. After staining, 400 μ L of 1 X annexin-binding buffer was added, mixed gently and kept on ice. The samples were detected with a MoFlo XDP flow cytometer (Beckman Coulter, Inc.).

4.2.6. Cell cycle analysis.

A549 cancer cells (1.6×10^5 /well) were incubated in 6-well plates for 24h and then treated with DMSO, CS055, CI994 or 12a. After 48h, cells were harvested, and fixed with 70% ethanol in phosphate buffer at -20°C overnight. Cells were incubated with 200 μ L of DNase-free RNase (1mg/mL) for 30min, and stained by propidium iodide(PI) staining solution (100 μ g/ml). DNA content was measured by a MoFlo XDP flow cytometer (Beckman Coulter, Inc.).

4.2.7. Molecular docking

Molecular docking calculations were carried out with Surflex-dock in Sybyl-X 2.0. (Tripos Inc.) The three-dimensional structure of HDAC2 (PDB id:4LXZ) was taken from the Protein Data Bank. For protein preparation, the water molecular and ligand were removed and hydrogen atoms were added. The structures of CI994, 12a were optimized using the conjugated gradient method with the Tripos force field with convergence criterion set at 0.001kcal/(A.mol). Other parameters accepted the default values.

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Hela nuclear HDAC inhibitory activity: 70.6% inhibition at 5 μ M
 HDAC2 inhibitor with IC₅₀ of 0.57 μ M
 antiproliferative activity: IC₅₀ 5.37 μ M against A549 cell;
 IC₅₀ 4.97 μ M against MGC80-3 cell;
 IC₅₀ 3.84 μ M against HepG2 cell;
 IC₅₀ 3.86 μ M against HCT116 cell