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Novel Histone Deacetylase Inhibitors with Enhanced Enzymatic Inhibition Effects and Potent in vitro and in vivo Anti-tumor Activities

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Abstract

In the present work, a series of small molecules were designed and synthesized based on structural optimization. Significant improvement in the enzymatic inhibition activity of the synthesized compounds was discovered. Moreover, tested compounds have moderate preference for class I HDACs over HDAC6 proved by enzymatic selectivity assay. The in vitro anti-proliferation assay reveals that representative compounds can selectively inhibit the growth of the non-solid lymphomatous cells and leukemic cells such as U937, K562 and HL60 cell lines. In the in vivo anti-tumor assay, molecule **D17** showed better performance than SAHA in inhibition of U937 tumor growth. The western blot analysis revealed that representative molecules can block the function of both class I HDACs and HDAC6. More importantly, our western blot results revealed that the levels of some oncogenic proteins (p-Akt in the PI3K/AKT/mTOR signal pathway, c-Raf and p-Erk in the MAPK signal pathway) were dramatically down-regulated by our compounds in U937 cell line rather than MDA-MB-231 cells. This cellular mechanism difference might be an important reason why U937 cell line was more sensitive to our HDACs inhibitors than MDA-MB-231 cell line.

Keywords

histone deacetylase; inhibitors; anti-tumor; structural optimization

Introduction

Histone deacetylases (HDACs) are a class of enzymes responsible for removing acetyl groups from the ε-N-acetyl lysine on a histone. 18 members of HDACs have been discovered until now, and they are divided into 4 classes. ^[1] Class I HDACs include HDAC 1, 2, 3 and 8, within this class, HDAC 1, 2 and 8 are primarily found in nucleus, HDAC 3 is

found in both nucleus and cytoplasm. Class II HDACs (IIa: HDAC4, 5, 7 and 9; IIb: HDAC6 and 10) are able to shuttle in and out of the nucleus. Class I and II HDACs are also called “classical zinc-dependent” HDACs. Class III HDACs (known as sirtuins, Sirt1–7) are a set of NAD-dependent enzymes. Class IV HDAC (HDAC11) which also exists in both nucleus and cytoplasm is considered as an atypical category of its own based on sequence similarity to the others.

Acetylation and deacetylation of histones and non-histone proteins by histone acetylases (HATs) and HDACs play important role in the regulation of gene expression and transcription.^[2] The effects include signal transduction, protein phosphorylation, cell cycle, proliferation, apoptosis, cardiac development and so on.^[3] Overexpression and aberrant recruitment of HDACs have a significant role in tumorigenesis.^[4]

Histone deacetylase inhibitors (HDACIs) have been proved to alter gene expression by blocking the action of HDACs and inducing hyperacetylation of histones.^[5] A number of structurally diverse HDACIs have shown potent antitumor efficacy in various stages of clinical trials. Suberoyl anilide hydroxamic acid (SAHA)^[6] and FK228^[7] have been approved by FDA for treatment of advanced cutaneous T-cell lymphoma (CTCL).

In order to develop novel HDACIs as antitumor agents, a series of small molecules were designed and synthesized based on virtual screening results.^[8] A phenylglycine containing structure (**4h**) was discovered to have good performance in the enzyme inhibition assays and tumor cell growth inhibition assays. Structural modifications were performed in the present work in discovery of more potent molecules. To improve the activities of synthesized compounds, aromatic groups introduced to substitute with the Boc group of **4h**, were designed to interact with hydrophobic residues (Phe155, Phe210 and Tyr308) in the active site of HDAC2 (Figure 1 and 2).

Chemistry

Based on the lead structure **4h**, a series of phenylglycine containing hydroxamic acid derivatives were synthesized according to the procedures described in scheme 1. The starting material (S)-2-amino-2-phenylacetic acid was protected by Boc group; the other material 4-aminobenzoic acid was protected by methyl ester. Intermediate (S)-methyl 4-(2-((tert-butoxycarbonyl)amino)-2-phenylacetamido)benzoate (**a**) was derived by coupling these two products. subsequent N-deprotection of intermediate **a** and condensation with various acyl chloride sulfonyl chloride and arylamines afforded **c1-c19**. Target compounds **D1-D19** were derived by treating the corresponding intermediates (**c1-c19**) with NH₂OK in methanol.

Results and Discussion

The enzymatic inhibition assay was performed against Hela cell nucleus extract which is a mixture of HDAC isoforms. Significant improvement in the inhibitory activity of the derived molecules (compared with **4h**), indicates that aromatic R groups enhance the inhibitor-receptor interactions by binding to hydrophobic residues (Phe155, Phe210 and Tyr308) (Table 1). A carbamido group containing molecule **D18** has the best performance in the enzymatic inhibition assay with IC₅₀ value of 7.5 nM. Molecules with bigger aromatic groups (naphthalene ring) have predominant inhibitory activities such as **D3** (14.3 nM), **D5** (15.6 nM), **D16** (23.5 nM) and **D17** (9.7 nM). The chlorine substituted in the phenyl ring reduces the activity apparently compared with **D7**, such as **D1** (348 nM), **D2** (95.5 nM) and **D10** (125 nM). However, the bromine substituted in the phenyl group can improve the activity obviously (**D13**: 13.0 nM). The fatty group (such as tert-butyl group) and strong

electrophilic group (such as trifluoro-methyl group) make negative effect to the inhibitory activity (**D6**: 105 nM, **D8**: 85.5 nM; **D18**: 7.5 nM, **D19**: 47.7 nM). As to fluorine and methoxy group substituents, the para position is better than the ortho position (**D4** vs **D9**, **D11** vs **D12**), although the difference is not significant. The position also plays important role in the naphthalene sulfonyl groups, the 1-position (**D5**) has comparative better performance than the 2-position (**D16**). Furthermore, polar group in the naphthalene ring improves the enzymatic inhibitory activity obviously (**D17**). From the above findings, we can conclude that bigger aromatic ring in the R group is beneficial to the HDAC inhibitory activity. And to the substituent groups of the aromatic ring, polar groups (not strong electrophilic groups) are favored and the positions of the substituent groups also affect the activity.

To characterize the isoform selectivity of these molecules, representative compounds **D3**, **D5**, **D13**, **D17** and **D18** were tested against HDAC1, HDAC2, HDAC3 and HDAC6 (Table 3). The result shows that these four molecules display preference for HDAC1, HDAC2 and HDAC3 over HDAC6. As to class I HDACs, the tested molecules show modest selectivity for HDAC1 and HDAC3 over HDAC2. Molecule **D18** has the most potent activity in the selectivity assay with IC₅₀ values of 4.8 nM, 19.5 nM, 5.9 nM and 294.2 nM against HDAC1, HDAC2, HDAC3 and HDAC6, respectively.

Docking process was performed to predict the binding patterns of derived molecules in the active site of HDAC2 (Figure 2). There is a narrow tunnel in the active site of HDAC2, and the zinc ion locates at the end of the tunnel. An open pocket which was surrounded by hydrophobic residues (Phe155, Phe210 and Tyr308) lies under the opening of the gap (Figure 2a). Molecules **D5** (Figure 2b) and **D17** (Figure 2d) have similar binding patterns in the active site of HDAC2. The hydroximic acid group chelating to the zinc ion makes the molecule bind tightly to the receptor. The linker locating in the tunnel has strong hydrophobic interactions with residues around the gap. The benzene ring of the phenylglycine group interacting with the hydrophobic area of the opening also makes contributions to the binding. The external motif binding to the pocket under the opening has good spatial matching with the surface of the active site. All these effects in combination enhanced the ligand-receptor interactions. However, molecule **D16** has a different binding mode (Figure 2c); both the phenyl ring and the naphthalene ring in the external motif locate in the opening of the gap. The H-bond interactions formed between the ligands and HDAC2 also have a significant role in the binding. The NH group of the hydroximic acid group of all these molecules has H-bond interaction with NE2 of His146. Asp104 which is also a key residue for H-bond binding has H-bond interaction with NH1 of **D5** and NH1 of **D17**. Molecule **D16** has no H-bond interactions with Asp104. Lack of interactions with the pocket and H-bond bindings was supposed to be the reason for comparative low activity of **D16** in the enzyme inhibition assay.

To evaluate the anti-proliferation activity of the synthesized molecules, MTT assays were performed using U937, K562, HL60, MCF7 PC3 and MDA-MB-231 cell lines. The derived compounds were firstly screened by U937 cells at the concentration of 2 μ M, molecules have >50% inhibition were selected for the anti-proliferation assay and their IC₅₀ values will be calculated (Table 2). On the whole, lymphomatous and leukemic cells lines (U937, K562 and HL60) are more sensitive to these molecules than the solid tumor cell lines (MCF7, PC3 and MDA-MB-231), which is in line with the published preclinical and clinical research results that the therapeutic efficacy of HDACIs is restricted predominantly to hematological malignancies.^[9] Inhibition the growth of U937 cells, **D3** (1.32 μ M) and **D17** (1.24 μ M) have smaller IC₅₀ values than SAHA. As to K562 cells most of the derived compounds (except **D11** and **D12**) have better performances than SAHA, and **D17** is the most active molecule (IC₅₀=0.70 μ M). Only **D11** has better IC₅₀ values than SAHA in inhibition HL60 cell

growth (**D11**: 0.78; SAHA: 1.44). Molecule **D3** (2.92 μ M) and **D4** (2.04 μ M) have better performance than SAHA (5.09 μ M) in the MCF7 cell growth inhibition assays. Only three compounds have bigger IC₅₀ values than SAHA in the PC3 cell assays, and **D18** (IC₅₀=1.51 μ M) is the most active molecule. Molecule **D3** (IC₅₀=4.23 μ M) has the best performance in inhibition the growth of MDA-MB-321 cells.

Human leukemic monocyte lymphoma cell line (U937) was the most sensitive to the tested molecules (mean IC₅₀ value of 1.51 μ M). Therefore, U937 cell line was selected for the in vivo anti-tumor assay. The most effective U937 cell growth inhibitor **D17** which also has the best water solubility was selected for the in vivo anti-proliferation assay. To preliminarily investigate the in vivo anti-proliferation ability of molecule **D17**, only one concentration (100 mg/kg/d) was applied and SAHA was used as a positive control. After 16 days of intragastric administration, the mice were executed, and the tumor weight was measured. The mean weight of tumors in the negative control group is 2.66 g (SD 37%), and the value of SAHA groups and **D17** group is 2.00 g (SD 26%) and 1.24 g (SD 18%), respectively (Figure 3a). The inhibitory ratio of molecule **D17** is 53.57% compared with SAHA with a value of 24.97% (Figure 3b). The result showed that **D17** exhibited improved anti-tumor activity compared with SAHA. The tumor volume was measured every two days, and the result has correlation with the tumor weight (Figure 3c and Figure 4). All these results confirm molecule **D17** is more statistically significant than SAHA. No evidence of mice body weight (measured every two days) loss reveals that molecule **D17** is safe (Figure 3d). Moreover, no obvious toxic signs in liver and spleen were detected.

Western blot studies were performed to evaluate the intracellular functions of representative molecules (Figure 5). The results show that these investigated molecules (**D3**, **D5**, **D13**, **D17** and **D18**) are cell permeable and can inhibit intracellular and even nuclear HDACs by monitoring the acetylation levels of tubulin (target of HDAC6), histone H3 and H4 (targets of HDAC1 and HDAC2), and p21 which is substrate of class I HDACs. All the tested molecules increase the acetylated level of tubulin in the 6h treatment, indicating that the tested molecule can efficiently block the function of HDAC6 in U937 cells. After 24h, the effect of **D3**, **D5** and **D13** in the increase of acetylated tubulin level is still significant, but the action of **D17** and **D18** reduced. All the tested molecules have stronger abilities than SAHA in the increase of the cyclin-dependent kinase (CDK) inhibitor p21 levels in both time points. It indicates that the representative molecules are effective and stable class I HDAC inhibitor. The tested molecules have a manner of more effective in the induction of the acetylated levels of histone H3 than histone H4 which correlates with the enzymatic selectivity assay results.

HDAC inhibitors have the limitation of ineffective low concentrations in solid tumors. [9] The US FDA approved drug SAHA and FK228 have no obvious efficacy in treating relapsed or refractory breast, colorectal, non-small cell lung cancer, metastatic head and neck cancer and so on proved by various clinical trials. [10] In the present work, in order to explore the reason why hematological tumor cell lines were more sensitive to HDACIs than solid tumor cell lines, we performed a series of western blot analysis to investigate the different effects of molecules **D3** and **D17** on the signaling cascades between non-solid (U937) and solid (MDA-MB-231) tumor cell lines (Figure 6). The c-Raf and Erk proteins are two crucial kinases in the activation of MAPK pathway. The phosphorylated Erk (p-Erk) is the active form of Erk. MDA-MB-231 has hyper-active MAPK pathway and the selected molecules cannot lower the signal. However, the tested molecules can obviously decrease the levels of c-Raf and p-Erk protein without reduction the expression of Erk protein in U937 cells. For the PI3K/AKT/mTOR cell proliferation pathway, although MDA-MB-231 cells have high-level of Akt protein, the p-Akt level is very low, which indicates that MDA-MB-231 cells do not depended on PI3K/AKT/mTOR signal pathway to grow and to survive.

On the contrary, although U937 cells have lower Akt level, the p-Akt level is very high, which could be significantly decreased by compounds **D3** and **D17**. Our results contribute the sensitivity of U937 cells to the tested inhibitors to their effects on these two oncogenic pathways, which are critical for cancer cell survivals. To the best of our knowledge, the present work constitutes the first report of HDACs inhibitors antitumor mechanism difference between non-solid (U937) and solid (MDA-MB-231) tumor cell lines. Further detailed mechanism studies which are performed to support our findings are still in progress.

Conclusions

A series of HDAC inhibitors were synthesized by structural modification of lead compound and evaluated by enzymatic inhibition assays, in vitro and in vivo anti-proliferation assays, isoform selectivity test and western blot analysis. Significant improvement in the enzymatic inhibition activity was discovered by changing the Boc group to be substituted aromatic groups. The MTT assays show that the derived molecules are potent anti-proliferation agents especially against non-solid tumor cell lines such as U937, K562, and HL60 cells. In the in vivo anti-tumor assays using athymic nude mice inoculated with U937 cells, molecule **D17** shows better performance than the positive control SAHA. The evidence indicates molecule **D17** is valuable for further development. The isoform selectivity study shows that the representative molecules are relatively more prone to bind to class I HDACs (HDAC1, HDAC2 and HDAC3) rather than HDAC6. The western blot assay reveals that the representative molecules can block the action of both class I HDACs and HDAC6. The anti-tumor mechanism difference between non-solid (U937) and solid tumor cells (MDA-MB-231) was investigated by western blot analysis. The MAPK and PI3K/AKT/mTOR signal pathways were discovered to make U937 cells rather than MDA-MD-231 cells sensitive to the tested molecules.

EXPERIMENT SECTION

Materials and methods

All commercially available starting materials, reagents and solvents were used without further purification. All reactions were monitored by TLC with 0.25 mm silica gel plates (60GF-254). UV light and ferric chloride were used to visualize the spots. ¹H NMR spectra were recorded on a Bruker DRX spectrometer at 600 MHz, using TMS as an internal standard. High-resolution mass spectra were performed in Shandong Analysis and Test Center in Ji'nan, China. Melting points were determined uncorrected on an electrothermal melting point apparatus. Optical rotation values were measured at room temperature using a modular circular polarimeter 200 operating at $\lambda = 589$ nm. Maximum absorption wave lengths of the target compounds were detected for HPLC analysis. The molecules were dissolved in methanol at a concentration of 1 mg per 100 mL and scanned from 500 nm to 200 nm using a UV-2102PCS spectrophotometer. All the derived target compounds are > 95% pure proved by HPLC analysis, performed on a Agilent 1100 HPLC instrument using a Phenomenex Synergi 4 μ Hydro-RP 80A column (250mm $\times$ 4.6mm). All the target compounds were eluted with acetonitrile and water (containing 0.1% formic acid) in various proportions detected at their maximum absorption wave lengths.

(S)-methyl 4-(2-((tert-butoxycarbonyl)amino)-2-phenylacetamido)benzoate (**a**) have been synthesized and described in our previous work. [8b]

(S)-methyl 4-(2-amino-2-phenylacetamido)benzoate hydrochloride (**b**)

To a solution of compound **e** (3.84 g, 10 mmol) in EtOAc (30 mL), a solution of EtOAc (30 mL) saturated by dry HCl gas was added. The reaction solution was stirred at room

temperature for 5 h. Precipitates appeared and were filtered, with the filter being washed with ether, to give desired compound **b** (2.18 g, 68% yield). ESI-MS m/z : 285.3 $[M+H]^+$.

General Procedure for the Preparation of c1 and its Analogues. (S)-methyl 4-(2-(4-chlorobenzamido)-2-phenylacetamido)benzoate (c1)

To a solution of **b** (0.96 g, 3.0 mmol) in THF (50 mL) / H₂O (1.0 mL), NaHCO₃ (1.01 g, 12 mmol) was added. After 10 min, 4-chlorobenzoyl chloride (0.58 g, 3.3 mmol) was added. The reaction solution was stirred at room temperature for 8 h. Then, the solvent was evaporated with the residue being taken up in EtOAc (50 mL). the EtOAc solution was washed with saturated citric acid (3 × 20 mL), NaHCO₃ (3 × 20 mL), and brine (3 × 20 mL), dried over MgSO₄, and evaporated under vacuum. The desired compound **c1** (0.89 g, 70% yield) was derived by crystallization in EtOAc as white powder. ¹H NMR (DMSO-*d*₆) δ 10.76 (s, 1H), 9.20 (d, *J* = 6.6Hz, 1H), 7.97 (d, *J* = 7.8Hz, 2H), 7.92 (d, *J* = 8.4Hz, 2H), 7.76 (d, *J* = 8.4Hz, 2H), 7.58 (d, *J* = 7.2Hz, 2H), 7.54 (d, *J* = 8.4Hz, 2H), 7.42-7.39 (m, 2H), 7.36-7.34 (m, 1H), 5.83 (d, *J* = 7.2Hz, 1H), 3.82 (s, 3H).

(S)-methyl 4-(2-(2-chlorobenzamido)-2-phenylacetamido)benzoate (c2)

White powder, 74% yield. ¹H NMR (DMSO-*d*₆) δ 10.80 (s, 1H), 9.34 (d, *J* = 7.2Hz, 1H), 7.93 (d, *J* = 7.8Hz, 2H), 7.76 (d, *J* = 7.8Hz, 2H), 7.58 (d, *J* = 7.8Hz, 2H), 7.50-7.44 (m, 3H), 7.40-7.38 (m, 3H), 7.35-7.32 (m, 1H), 5.85 (d, *J* = 7.2Hz, 1H), 3.82 (s, 3H).

(S)-methyl 4-(2-(2-naphthamido)-2-phenylacetamido)benzoate (c3)

White powder, 77% yield. ¹H NMR (DMSO-*d*₆) δ 10.79 (s, 1H), 9.23 (d, *J* = 7.2Hz, 1H), 8.61 (s, 1H), 8.04 (d, *J* = 7.8Hz, 1H), 8.02-7.98 (m, 3H), 7.93 (d, *J* = 9.0Hz, 2H), 7.78 (d, *J* = 8.4Hz, 2H), 7.64-7.59 (m, 4H), 7.44-7.41 (m, 2H), 7.36 (t, *J* = 7.2Hz, 1H), 5.91 (d, *J* = 7.2Hz, 1H), 3.82 (s, 3H).

(S)-methyl 4-(2-(4-fluorobenzamido)-2-phenylacetamido)benzoate (c4)

White powder, 75% yield. ¹H NMR (DMSO-*d*₆) δ 10.72 (s, 1H), 9.12 (d, *J* = 7.2Hz, 1H), 8.03-8.01 (m, 2H), 7.92 (d, *J* = 9.0Hz, 2H), 7.75 (d, *J* = 9.0Hz, 2H), 7.57 (d, *J* = 7.8Hz, 2H), 7.42-7.39 (m, 2H), 7.36-7.33 (m, 1H), 7.31-7.28 (m, 2H), 5.82 (d, *J* = 7.2Hz, 1H), 3.82 (s, 3H).

(S)-methyl 4-(2-(naphthalene-1-sulfonamido)-2-phenylacetamido)benzoate (c5)

White powder, 79% yield. ¹H NMR (DMSO-*d*₆) δ 10.47 (s, 1H), 9.15 (d, *J* = 9.6Hz, 1H), 8.73 (d, *J* = 8.4Hz, 1H), 8.13 (d, *J* = 7.2Hz, 1H), 8.06 (d, *J* = 8.4Hz, 1H), 7.95 (d, *J* = 8.4Hz, 1H), 7.83 (d, *J* = 9.0Hz, 2H), 7.67-7.65 (m, 1H), 7.61-7.58 (m, 1H), 7.53-7.50 (m, 1H), 7.41 (d, *J* = 8.4Hz, 2H), 7.29-7.28 (m, 2H), 7.17-7.16 (m, 3H), 5.20 (d, *J* = 9.6Hz, 1H), 3.81 (s, 3H).

(S)-methyl 4-(2-(4-(tert-butyl)phenylsulfonamido)-2-phenylacetamido)benzoate (c6)

White powder, 74% yield. ¹H NMR (DMSO-*d*₆) δ 10.52 (s, 1H), 8.73 (d, *J* = 9.6Hz, 1H), 7.84 (d, *J* = 8.4Hz, 2H), 7.66 (d, *J* = 7.8Hz, 2H), 7.50 (d, *J* = 8.4Hz, 2H), 7.37-7.36 (m, 4H), 7.27-7.23 (m, 3H), 5.17 (d, *J* = 9.6Hz, 1H), 3.80 (s, 3H), 1.17 (s, 9H).

(S)-methyl 4-(2-benzamido-2-phenylacetamido)benzoate (c7)

White powder, 72% yield. ¹H NMR (DMSO-*d*₆) δ 10.72 (s, 1H), 9.07 (d, *J* = 7.2Hz, 1H), 7.94 (d, *J* = 9.0Hz, 2H), 7.92 (d, *J* = 9.0Hz, 2H), 7.75 (d, *J* = 9.0Hz, 2H), 7.58 (d, *J* = 7.8Hz, 2H), 7.56-7.54 (m, 1H), 7.47 (t, *J* = 7.8Hz, 2H), 7.42-7.39 (m, 2H), 7.36-7.33 (m, 1H), 5.84 (d, *J* = 7.2Hz, 1H), 3.82 (s, 3H).

(S)-methyl 4-(2-phenyl-2-(4-(trifluoromethyl)phenylsulfonamido)acetamido)benzoate (c8)

White powder, 75% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.61 (s, 1H), 9.14 (d, $J = 9.6\text{Hz}$, 1H), 7.91 (d, $J = 8.4\text{Hz}$, 2H), 7.85 (d, $J = 9.0\text{Hz}$, 2H), 7.75 (d, $J = 8.4\text{Hz}$, 2H), 7.48 (d, $J = 8.4\text{Hz}$, 2H), 7.36 (d, $J = 7.2\text{Hz}$, 2H), 7.26-7.22 (m, 3H), 5.24 (d, $J = 9.6\text{Hz}$, 1H), 3.81 (s, 3H).

(S)-methyl 4-(2-(2,6-difluorophenylsulfonamido)-2-phenylacetamido)benzoate (c9)

White powder, 76% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.66 (s, 1H), 9.37 (d, $J = 9.6\text{Hz}$, 1H), 7.88 (d, $J = 9.0\text{Hz}$, 2H), 7.57 (d, $J = 9.0\text{Hz}$, 2H), 7.55-7.53 (m, 1H), 7.46 (d, $J = 9.0\text{Hz}$, 2H), 7.31-7.29 (m, 2H), 7.26 (t, $J = 7.2\text{Hz}$, 1H), 7.11 (t, $J = 9.0\text{Hz}$, 2H), 5.38 (d, $J = 9.6\text{Hz}$, 1H), 3.81 (s, 3H).

(S)-methyl 4-(2-(4-chlorophenylsulfonamido)-2-phenylacetamido)benzoate (c10)

White powder, 77% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.59 (s, 1H), 8.97 (d, $J = 9.6\text{Hz}$, 1H), 7.88 (d, $J = 8.4\text{Hz}$, 2H), 7.71 (d, $J = 8.4\text{Hz}$, 2H), 7.52 (d, $J = 8.4\text{Hz}$, 2H), 7.46 (d, $J = 8.4\text{Hz}$, 2H), 7.37 (d, $J = 6.6\text{Hz}$, 2H), 7.29-7.25 (m, 3H), 5.20 (d, $J = 9.6\text{Hz}$, 1H), 3.81 (s, 3H).

(S)-methyl 4-(2-(2-methoxybenzamido)-2-phenylacetamido)benzoate (c11)

White powder, 78% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.83 (s, 1H), 9.18 (d, $J = 7.2\text{Hz}$, 1H), 7.93 (d, $J = 8.4\text{Hz}$, 2H), 7.74 (d, $J = 8.4\text{Hz}$, 2H), 7.56 (d, $J = 7.8\text{Hz}$, 2H), 7.54 (d, $J = 8.4\text{Hz}$, 1H), 7.41 (t, $J = 7.8\text{Hz}$, 2H), 7.33 (t, $J = 7.2\text{Hz}$, 1H), 7.24 (d, $J = 9.0\text{Hz}$, 1H), 7.08 (t, $J = 7.2\text{Hz}$, 1H), 5.87 (d, $J = 7.2\text{Hz}$, 1H), 4.00 (s, 3H), 3.82 (s, 3H).

(S)-methyl 4-(2-(4-methoxybenzamido)-2-phenylacetamido)benzoate (c12)

White powder, 82% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.70 (s, 1H), 8.89 (d, $J = 7.2\text{Hz}$, 1H), 7.94 (d, $J = 9.0\text{Hz}$, 2H), 7.92 (d, $J = 7.8\text{Hz}$, 2H), 7.75 (d, $J = 7.2\text{Hz}$, 2H), 7.57 (d, $J = 7.2\text{Hz}$, 2H), 7.40 (t, $J = 7.8\text{Hz}$, 2H), 7.34 (t, $J = 7.8\text{Hz}$, 1H), 6.99 (d, $J = 8.4\text{Hz}$, 2H), 5.82 (d, $J = 7.2\text{Hz}$, 1H), 4.02 (s, 3H), 3.81 (s, 3H).

(S)-methyl 4-(2-(3-bromobenzamido)-2-phenylacetamido)benzoate (c13)

White powder, 76% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.72 (s, 1H), 9.27 (d, $J = 6.6\text{Hz}$, 1H), 8.16 (s, 1H), 7.93-7.91 (m, 3H), 7.76-7.74 (m, 3H), 7.57 (d, $J = 7.2\text{Hz}$, 2H), 7.45-7.40 (m, 3H), 7.35 (t, $J = 7.2\text{Hz}$, 1H), 5.81 (d, $J = 6.6\text{Hz}$, 1H), 3.82 (s, 3H).

(S)-methyl 4-(2-phenyl-2-(2-propylpentanamido)acetamido)benzoate (c14)

White powder, 77% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.66 (s, 1H), 8.65 (d, $J = 7.2\text{Hz}$, 1H), 7.91 (d, $J = 8.4\text{ Hz}$, 2H), 7.72 (d, $J = 8.4\text{ Hz}$, 2H), 7.48 (d, $J = 7.8\text{ Hz}$, 2H), 7.40-7.37 (m, 2H), 7.33-7.31 (m, 1H), 5.65 (d, $J = 7.2\text{Hz}$, 1H), 3.81 (s, 3H), 1.45-1.40 (m, 2H), 1.28-1.21 (m, 4H), 1.19-1.13 (m, 2H), 0.85 (m, 3H), 0.80 (t, $J = 7.8\text{ Hz}$, 3H).

(S)-methyl 4-(2-phenyl-2-(2-phenylacetamido)acetamido)benzoate (c15)

White powder, 73% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.77 (s, 1H), 9.02 (d, $J = 7.8\text{Hz}$, 1H), 7.90 (d, $J = 9.0\text{ Hz}$, 2H), 7.72 (d, $J = 9.0\text{ Hz}$, 2H), 7.50 (d, $J = 7.8\text{ Hz}$, 2H), 7.40-7.37 (m, 2H), 7.33-7.31 (m, 1H), 7.30-7.28 (m, 4H), 7.22-7.19 (m, 1H), 5.63 (d, $J = 7.8\text{Hz}$, 1H), 3.81 (s, 3H), 3.59 (m, 2H).

(S)-methyl 4-(2-(naphthalene-2-sulfonamido)-2-phenylacetamido)benzoate (c16)

White powder, 78% yield. ^1H NMR ($\text{DMSO}-d_6$) δ 10.55 (s, 1H), 8.94 (d, $J = 9.6\text{Hz}$, 1H), 8.34 (s, 1H), 7.96-7.94 (m, 2H), 7.90 (d, $J = 8.4\text{Hz}$, 1H), 7.79-7.77 (m, 1H), 7.74 (d, $J =$

9.0Hz, 2H), 7.61-7.59 (m, 1H), 7.56-7.53 (m, 1H), 7.39-7.36 (m, 4H), 7.23 (t, $J = 7.2$ Hz, 2H), 7.19-7.17 (m, 1H), 5.25 (d, $J = 8.4$ Hz, 1H), 3.80 (s, 3H).

(S)-methyl 4-(2-(5-(dimethylamino)naphthalene-1-sulfonamido)-2-phenylacetamido)benzoate (c17)

White powder, 75% yield. ^1H NMR (DMSO- d_6) δ 10.40 (s, 1H), 9.06 (d, $J = 9.6$ Hz, 1H), 8.37 (d, $J = 8.4$ Hz, 1H), 8.28 (d, $J = 7.8$ Hz, 1H), 8.12 (d, $J = 7.2$ Hz, 1H), 7.81 (d, $J = 8.4$ Hz, 2H), 7.55-7.52 (m, 1H), 7.49 (t, $J = 7.8$ Hz, 1H), 7.40 (d, $J = 8.4$ Hz, 2H), 7.30-7.29 (m, 2H), 7.18-7.16 (m, 4H), 5.17 (d, $J = 9.6$ Hz, 1H), 3.80 (s, 3H), 2.71 (s, 6H).

General Procedure for the Preparation of c18 and c19. (S)-methyl 4-(2-(3-(2-methoxyphenyl)ureido)-2-phenylacetamido)benzoate (c18)

To a solution of triphosgene (1.5 g, 5.0 mmol) in dioxane (40 mL), 2-methoxyaniline (1.23 g, 10 mmol) was added. The reaction solution was stirred at 110 °C for 8 h. Then, the solvent was evaporated sufficiently without further purification.

The residue was dissolved in DCM, compound **b** (1.6 g, 5.0 mmol) and Et₃N (0.61 g, 6.0 mmol) was added. The reaction solution was stirred at room temperature for 5 h. Then, the solvent was evaporated with the residue being taken up in EtOAc (50 mL). The EtOAc solution was washed with saturated citric acid (3 × 20 mL), NaHCO₃ (3 × 20 mL), and brine (3 × 20 mL), dried over MgSO₄, and evaporated under vacuum. The desired compound **c18** (0.97 g, 45% yield) was derived by crystallization in EtOAc as white powder. ^1H NMR (DMSO- d_6) δ 10.75 (s, 1H), 8.39 (s, 1H), 8.05 (d, $J = 6.6$ Hz, 1H), 7.91 (d, $J = 8.4$ Hz, 2H), 7.89 (d, $J = 7.8$ Hz, 1H), 7.74 (d, $J = 8.4$ Hz, 2H), 7.52 (d, $J = 7.2$ Hz, 2H), 7.42-7.39 (m, 2H), 7.34-7.32 (m, 1H), 6.97 (d, $J = 7.8$ Hz, 1H), 6.89-6.87 (m, 1H), 6.82 (t, $J = 7.8$ Hz, 1H), 5.56 (d, $J = 7.8$ Hz, 1H), 4.02 (s, 3H), 3.81 (s, 3H).

(S)-methyl 4-(2-(3-(2,6-diisopropylphenyl)ureido)-2-phenylacetamido)benzoate (c19)

White powder, 43% yield. ^1H NMR (DMSO- d_6) δ 10.76 (s, 1H), 7.92 (d, $J = 8.4$ Hz, 2H), 7.74 (s, 2H), 7.52 (d, $J = 6.6$ Hz, 2H), 7.40 (t, $J = 6.6$ Hz, 2H), 7.33-7.31 (m, 1H), 7.17 (d, $J = 7.8$ Hz, 2H), 7.10-7.18 (m, 2H), 5.59 (d, $J = 8.4$ Hz, 1H), 3.82 (s, 3H), 3.33 (s, 2H), 1.10 (d, $J = 6.6$ Hz, 12H).

General Procedure for the Preparation of D1–D19. (S)-4-chloro-N-(2-((4-(hydroxycarbamoyl)phenyl)amino)-2-oxo-1-phenylethyl)benzamide (D1)

Compound **g1** (0.5 g, 1.18 mmol) was dissolved in 14 mL of NH₂OK (0.56 g, 24 mmol) methanol solution. After 2 h, the solvent was evaporated under vacuum. The residue was acidified with saturated citric acid, and then extracted with EtOAc (3 × 20 mL). The organic layers were combined, washed with brine (3 × 20 mL) and dried over MgSO₄. The desired compound **D1** (0.23 g, 54% yield) was derived by crystallization in EtOAc as white powder. Mp: 250–252 °C. $[\alpha]_D^{20}$ –6.88 (c 1.0, MeOH). ^1H NMR (DMSO- d_6) δ 11.10 (s, 1H), 10.58 (s, 1H), 9.15 (d, $J = 7.8$ Hz, 1H), 8.93 (s, 1H), 7.98 (d, $J = 8.4$ Hz, 2H), 7.72 (d, $J = 8.4$ Hz, 2H), 7.67 (d, $J = 9.0$ Hz, 2H), 7.58 (d, $J = 7.2$ Hz, 2H), 7.55 (d, $J = 7.2$ Hz, 1H), 7.42-7.39 (m, 2H), 7.36-7.34 (m, 1H), 5.84 (d, $J = 7.8$ Hz, 1H). HRMS (AP-ESI) m/z calcd for C₂₂H₁₉ClN₃O₄ $[M + H]^+$ 424.1064, found 424.1057. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 58.56, 118.96, 127.96, 128.18, 128.34, 128.59, 128.60, 129.03, 132.88, 136.76, 137.60, 141.77, 164.26, 166.04, 169.51. HPLC retention time: 9.6 min, eluted by CH₃CN: H₂O = 45: 55.

(S)-2-chloro-N-(2-((4-(hydroxycarbamoyl)phenyl)amino)-2-oxo-1-phenylethyl)benzamide (D2)

White powder crystallized in EtOAc, 45% yield. Mp: 250–252 °C. $[\alpha]_D^{20}$ –8.20 (c 1.0, MeOH). ^1H NMR (DMSO- d_6) δ 11.11 (s, 1H), 10.62 (s, 1H), 9.29 (d, J = 7.8 Hz, 1H), 8.93 (s, 1H), 7.73 (d, J = 9 Hz, 2H), 7.67 (d, J = 8.4 Hz, 2H), 7.58 (d, J = 7.8 Hz, 2H), 7.48 (d, J = 6.6 Hz, 1H), 7.47 (d, J = 4.8 Hz, 1H), 7.45 (d, J = 8.4 Hz, 1H), 7.41–7.40 (m, 2H), 7.38 (t, J = 8.4 Hz, 1H), 7.35–7.32 (m, 1H), 5.86 (d, J = 7.8 Hz, 1H). HRMS (AP-ESI) m/z calcd for $\text{C}_{22}\text{H}_{19}\text{ClN}_3\text{O}_4$ $[\text{M} + \text{H}]^+$ 424.1064, found 424.1057. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 58.06, 118.97, 127.35, 127.97, 128.25, 128.46, 128.97, 129.76, 129.93, 131.34, 136.55, 137.70, 141.71, 164.24, 166.78, 169.20. HPLC retention time: 7.5 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-N-(2-((4-(hydroxycarbamoyl)phenyl)amino)-2-oxo-1-phenylethyl)-2-naphthamide (D3)

White powder crystallized in EtOAc, 47% yield. Mp: 238–240 °C. $[\alpha]_D^{20}$ –7.01 (c 1.0, MeOH). ^1H NMR (DMSO- d_6) δ 11.13 (s, 1H), 10.77 (s, 1H), 9.22 (d, J = 7.2 Hz, 1H), 8.93 (s, 1H), 8.63 (s, 1H), 8.05 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 9.0 Hz, 1H), 8.02 (d, J = 8.4 Hz, 1H), 8.00 (d, J = 9.0 Hz, 1H), 7.74–7.71 (m, 2H), 7.65 (d, J = 7.8 Hz, 2H), 7.63–7.62 (m, 1H), 7.62–7.60 (m, 1H), 7.44–7.41 (m, 2H), 7.37–7.35 (m, 1H), 5.95 (d, J = 7.8 Hz, 1H). HRMS (AP-ESI) m/z calcd for $\text{C}_{26}\text{H}_{22}\text{N}_3\text{O}_4$ $[\text{M} + \text{H}]^+$ 440.1610, found 440.1607. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 58.66, 119.01, 125.07, 127.13, 127.92, 128.04, 128.16, 128.38, 128.40, 128.65, 128.70, 128.94, 129.36, 131.41, 132.50, 134.71, 137.96, 141.91, 164.25, 166.96, 169.69. HPLC retention time: 8.8 min, eluted by CH_3CN : H_2O = 45: 55.

(S)-4-fluoro-N-(2-((4-(hydroxycarbamoyl)phenyl)amino)-2-oxo-1-phenylethyl)benzamide (D4)

White powder crystallized in EtOAc, 46% yield. Mp: 240–242 °C. $[\alpha]_D^{20}$ –5.64 (c 1.0, MeOH). ^1H NMR (DMSO- d_6) δ 11.12 (s, 1H), 10.65 (s, 1H), 9.10 (d, J = 7.2 Hz, 1H), 8.93 (s, 1H), 8.05–8.03 (m, 2H), 7.72 (d, J = 8.4 Hz, 2H), 7.69 (d, J = 9 Hz, 2H), 7.59 (d, J = 9.0 Hz, 2H), 7.42–7.39 (m, 2H), 7.35 (t, J = 7.2 Hz, 1H), 7.30 (t, J = 9.0 Hz, 1H), 5.86 (d, J = 7.2 Hz, 1H). HRMS (AP-ESI) m/z calcd for $\text{C}_{22}\text{H}_{19}\text{FN}_3\text{O}_4$ $[\text{M} + \text{H}]^+$ 408.1359, found 408.1355. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 58.61, 115.58, 118.98, 127.90, 128.17, 128.43, 128.91, 130.60, 130.62, 137.79, 141.86, 163.66, 164.27, 165.93, 169.61. HPLC retention time: 7.7 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-N-hydroxy-4-(2-(naphthalene-1-sulfonamido)-2-phenylacetamido)benzamide (D5)

White powder crystallized in EtOAc and hexane (1: 1), 53% yield. Mp: 152–154 °C. $[\alpha]_D^{20}$ –7.60 (c 1.0, MeOH). ^1H NMR (DMSO- d_6) δ 11.07 (s, 1H), 10.33 (s, 1H), 9.09 (d, J = 9 Hz, 1H), 8.92 (s, 1H), 8.74 (d, J = 8.4 Hz, 1H), 8.14–8.13 (m, 1H), 8.08 (d, J = 7.8 Hz, 1H), 7.96 (d, J = 8.4 Hz, 1H), 7.68–7.66 (m, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.60 (d, J = 7.8 Hz, 2H), 7.52 (t, J = 7.8 Hz, 2H), 7.34 (d, J = 9 Hz, 2H), 7.29 (d, J = 7.8 Hz, 1H), 7.18–7.16 (m, 1H), 5.21 (d, J = 8.4 Hz, 1H). HRMS (AP-ESI) m/z calcd for $\text{C}_{25}\text{H}_{22}\text{N}_3\text{O}_5\text{S}$ $[\text{M} + \text{H}]^+$ 476.1280, found 476.1273. HPLC retention time: 13.0 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-4-(2-(4-(tert-butyl)phenyl)sulfonamido)-2-phenylacetamido)-N-hydroxybenzamide (D6)

White powder crystallized in EtOAc and hexane (1: 1), 48% yield. Mp: 155–157 °C. $[\alpha]_D^{20}$ –7.77 (c 1.0, MeOH). ^1H NMR (DMSO- d_6) δ 11.08 (s, 1H), 10.39 (s, 1H), 8.93 (s, 1H), 8.67 (d, J = 9.6 Hz, 1H), 7.67 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 9 Hz, 2H), 7.39–7.27 (m, 4H), 7.27 (d, J = 8.4 Hz, 2H), 7.25–7.23 (m, 1H), 5.17 (d, J = 10.2 Hz, 1H), 1.14 (s, 9H). HRMS (AP-ESI) m/z calcd for $\text{C}_{25}\text{H}_{28}\text{N}_3\text{O}_5\text{S}$ $[\text{M} + \text{H}]^+$ 482.1749, found 482.1743. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 60.19, 118.99, 125.88, 126.96, 127.46, 128.01,

128.04, 128.26, 128.76, 137.24, 138.07, 141.19, 155.62, 164.07, 168.17. HPLC retention time: 10.6 min, eluted by CH₃CN: H₂O = 45: 55.

(S)-4-(2-benzamido-2-phenylacetamido)-N-hydroxybenzamide (D7)

White powder crystallized in EtOAc, 42% yield. Mp: 229–230 °C. $[\alpha]_D^{20}$ –6.60 (c 1.0, MeOH). ¹H NMR (DMSO-*d*₆) δ 11.10 (s, 1H), 10.59 (s, 1H), 9.02 (d, *J* = 7.8 Hz, 1H), 8.9 (s, 1H), 7.95 (d, *J* = 7.2 Hz, 2H), 7.72 (d, *J* = 9 Hz, 2H), 7.67 (d, *J* = 9 Hz, 2H), 7.59 (d, *J* = 7.8 Hz, 1H), 7.56 (t, *J* = 7.2 Hz, 2H), 7.48 (t, *J* = 7.8 Hz, 2H), 7.46–7.36 (m, 2H), 7.35 (t, *J* = 7.2 Hz, 1H), 5.86 (d, *J* = 7.2 Hz, 1H). HRMS (AP-ESI) *m/z* calcd for C₂₂H₂₀N₃O₄ [M + H]⁺ 390.1454, found 390.1449. ¹³C NMR (75 MHz, DMSO-*d*₆) δ: 58.49, 118.95, 127.93, 128.17, 128.27, 128.34, 128.60, 128.93, 131.94, 134.13, 137.84, 141.82, 164.27, 166.97, 169.94. HPLC retention time: 9.9 min, eluted by CH₃CN: H₂O = 35: 65.

(S)-N-hydroxy-4-(2-phenyl-2-(4-(trifluoromethyl)phenylsulfonamido)acetamido)benzamide (D8)

White powder crystallized in EtOAc and hexane (1: 1), 40% yield. Mp: 200–202 °C. $[\alpha]_D^{20}$ –7.20 (c 1.0, MeOH). ¹H NMR (DMSO-*d*₆) δ 11.10 (s, 1H), 10.48 (s, 1H), 9.09 (d, *J* = 9.6 Hz, 1H), 8.94 (s, 1H), 7.92 (d, *J* = 8.4 Hz, 2H), 7.76 (d, *J* = 8.4 Hz, 2H), 7.67 (d, *J* = 9 Hz, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.37 (d, *J* = 7.8 Hz, 2H), 7.26 (d, *J* = 8.4 Hz, 2H), 7.23–7.22 (m, 1H), 5.24 (d, *J* = 9 Hz, 1H). HRMS (AP-ESI) *m/z* calcd for C₂₂H₁₉F₃N₃O₅S [M + H]⁺ 494.0997, found 494.0993. ¹³C NMR (75 MHz, DMSO-*d*₆) δ: 60.18, 119.02, 127.52, 127.54, 127.99, 128.07, 128.79, 128.82, 128.86, 136.72, 140.99, 144.96, 164.08, 167.83. HPLC retention time: 8.6 min, eluted by CH₃CN: H₂O = 45: 55.

(S)-4-(2-(2,6-difluorophenylsulfonamido)-2-phenylacetamido)-N-hydroxybenzamide (D9)

White powder crystallized in EtOAc and hexane (1: 1), 47% yield. Mp: 215–217 °C. $[\alpha]_D^{20}$ –8.40 (c 1.0, MeOH). ¹H NMR (DMSO-*d*₆) δ 11.09 (s, 1H), 10.52 (s, 1H), 9.29 (d, *J* = 9 Hz, 2H), 8.93 (s, 1H), 7.68 (d, *J* = 9 Hz, 2H), 7.56 (m, 1H), 7.49 (d, *J* = 8.4 Hz, 2H), 7.46 (d, *J* = 7.8 Hz, 2H), 7.32–7.9 (m, 2H), 7.27 (d, *J* = 7.2 Hz, 1H), 7.12 (t, *J* = 9 Hz, 2H), 5.37 (d, *J* = 9.6 Hz, 1H). HRMS (AP-ESI) *m/z* calcd for C₂₁H₁₈F₂N₃O₅S [M + H]⁺ 462.0935, found 462.0931. ¹³C NMR (75 MHz, DMSO-*d*₆) δ: 60.70, 113.45, 113.60, 118.85, 127.60, 128.18, 128.53, 128.87, 135.62, 136.91, 141.25, 158.16, 159.87, 164.18, 167.92. HPLC retention time: 8.3 min, eluted by CH₃CN: H₂O = 40: 60.

(S)-4-(2-(4-chlorophenylsulfonamido)-2-phenylacetamido)-N-hydroxybenzamide (D10)

White powder crystallized in EtOAc and hexane (1: 1), 45% yield. Mp: 228–230 °C. $[\alpha]_D^{20}$ –7.60 (c 1.0, MeOH). ¹H NMR (DMSO-*d*₆) δ 11.10 (s, 1H), 10.46 (s, 1H), 8.93 (d, *J* = 7.2 Hz, 1H), 8.92 (s, 1H), 7.72 (d, *J* = 8.4 Hz, 2H), 7.68 (d, *J* = 9.0 Hz, 2H), 7.47 (d, *J* = 7.2 Hz, 2H), 7.44 (d, *J* = 8.4 Hz, 2H), 7.38 (d, *J* = 7.8 Hz, 2H), 7.28 (d, *J* = 8.4 Hz, 2H), 7.26–7.25 (m, 1H), 5.20 (d, *J* = 7.2 Hz, 1H). HRMS (AP-ESI) *m/z* calcd for C₂₁H₁₉ClN₃O₅S [M + H]⁺ 460.0734, found 460.0725. ¹³C NMR (75 MHz, DMSO-*d*₆) δ: 60.20, 119.05, 128.21, 128.85, 128.98, 129.13, 129.25, 129.30, 136.97, 137.60, 140.03, 141.10, 164.14, 168.04. HPLC retention time: 10.3 min, eluted by CH₃CN: H₂O = 40: 60.

(S)-N-(2-((4-(hydroxycarbamoyl)phenyl)amino)-2-oxo-1-phenylethyl)-2-methoxybenzamide (D11)

White powder crystallized in EtOAc, 46% yield. Mp: 156–158 °C. $[\alpha]_D^{20}$ –7.50 (c 1.0, MeOH). ¹H NMR (DMSO-*d*₆) δ 11.11 (s, 1H), 10.69 (s, 1H), 9.17 (d, *J* = 7.8 Hz, 1H), 8.93 (s, 1H), 7.91 (d, *J* = 7.8 Hz, 1H), 7.73 (d, *J* = 9 Hz, 2H), 7.65 (d, *J* = 8.4 Hz, 2H), 7.7–7.54 (m, 3H), 7.42–7.39 (m, 2H), 7.34 (d, *J* = 7.2 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 1H), 7.09 (t, *J* = 7.2 Hz, 1H), 5.87 (d, *J* = 7.2 Hz, 1H), 4.01 (s, 3H). HRMS (AP-ESI) *m/z* calcd for

$C_{23}H_{22}N_3O_5$ $[M + H]^+$ 420.1559, found 420.1553. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 56.83, 17.75, 112.89, 119.15, 121.32, 121.37, 127.30, 128.27, 128.43, 129.18, 131.44, 133.67, 138.88, 141.43, 157.94, 164.08, 164.21, 169.28. Retention time: 8.2 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-N-hydroxy-4-(2-(4-methoxybenzamido)-2-phenylacetamido)benzamide (D12)

White powder crystallized in EtOAc, 49% yield. Mp: 240–241 °C. $[\alpha]_D^{20}$ –7.80 (*c* 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.09 (s, 1H), 10.55 (s, 1H), 8.91 (s, 1H), 8.83 (d, *J* = 7.8 Hz, 1H), 7.95 (d, *J* = 7.2 Hz, 2H), 7.72 (d, *J* = 9.0 Hz, 2H), 7.67 (d, *J* = 9.0 Hz, 2H), 7.57 (d, *J* = 7.2 Hz, 2H), 7.41–7.39 (m, 2H), 7.35–7.33 (m, 2H), 7.00 (d, *J* = 8.4 Hz, 2H), 5.84 (d, *J* = 7.2 Hz, 1H), 3.82 (s, 3H). HRMS (AP-ESI) *m/z* calcd for $C_{23}H_{22}N_3O_5$ $[M + H]^+$ 420.1559, found 420.1554. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 55.73, 58.42, 113.81, 118.95, 126.31, 127.94, 128.21, 128.35, 128.92, 130.17, 137.96, 141.82, 162.24, 164.27, 166.42, 169.79. HPLC retention time: 10.1 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-3-bromo-N-(2-((4-(hydroxycarbamoyl)phenyl)amino)-2-oxo-1-phenylethyl)benzamide (D13)

White powder crystallized in EtOAc, 48% yield. Mp: 234–236 °C. $[\alpha]_D^{20}$ –6.80 (*c* 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.10 (s, 1H), 10.58 (s, 1H), 9.23 (d, *J* = 7.2 Hz, 1H), 8.93 (s, 1H), 8.17 (s, 1H), 7.94 (d, *J* = 7.8 Hz, 1H), 7.76 (d, *J* = 7.8 Hz, 1H), 7.72 (d, *J* = 9.0 Hz, 2H), 7.67 (d, *J* = 8.4 Hz, 2H), 7.58 (d, *J* = 7.2 Hz, 2H), 7.45 (d, *J* = 7.8 Hz, 1H), 7.41 (d, *J* = 9.0 Hz, 1H), 7.37–7.34 (m, 1H), 5.84 (d, *J* = 7.8 Hz, 1H). HRMS (AP-ESI) *m/z* calcd for $C_{22}H_{19}BrN_3O_4$ $[M + H]^+$ 468.0559, found 468.0554. HPLC retention time: 11.5 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-N-hydroxy-4-(2-phenyl-2-(2-propylpentanamido)acetamido)benzamide (D14)

White powder crystallized in EtOAc, 47% yield. Mp: 267–269 °C. $[\alpha]_D^{20}$ –7.20 (*c* 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.09 (s, 1H), 10.52 (s, 1H), 8.92 (s, 1H), 8.61 (d, *J* = 7.2 Hz, 1H), 7.71 (d, *J* = 8.4 Hz, 1H), 7.64 (d, *J* = 8.4 Hz, 2H), 7.48 (d, *J* = 7.2 Hz, 2H), 7.40–7.37 (m, 2H), 7.33–7.30 (m, 1H), 5.67 (d, *J* = 7.8 Hz, 1H), 1.47–1.43 (m, 1H), 1.28–1.21 (m, 4H), 1.19–1.12 (m, 6H). HRMS (AP-ESI) *m/z* calcd for $C_{23}H_{30}N_3O_4$ $[M + H]^+$ 412.2236, found 412.2231. HPLC retention time: 11.9 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-N-hydroxy-4-(2-phenyl-2-(2-phenylacetamido)acetamido)benzamide (D15)

White powder crystallized in EtOAc, 46% yield. Mp: 225–226 °C. $[\alpha]_D^{20}$ –6.70 (*c* 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.09 (s, 1H), 10.56 (s, 1H), 8.92 (s, 1H), 8.91 (d, *J* = 8.4 Hz, 1H), 7.71 (d, *J* = 9.0 Hz, 2H), 7.63 (d, *J* = 9.0 Hz, 2H), 7.50 (d, *J* = 7.2 Hz, 2H), 7.40–7.37 (m, 2H), 7.33 (d, *J* = 7.2 Hz, 1H), 7.29–7.28 (m, 4H), 7.22–7.20 (m, 1H), 5.65 (d, *J* = 7.8 Hz, 1H), 3.60 (s, 2H). HRMS (AP-ESI) *m/z* calcd for $C_{23}H_{22}N_3O_4$ $[M + H]^+$ 404.1610, found 404.1607. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 42.10, 60.20, 118.96, 126.76, 128.22, 128.38, 129.00, 129.52, 136.77, 138.26, 141.68, 163.01, 169.59, 170.61. HPLC retention time: 9.8 min, eluted by CH_3CN : H_2O = 36: 64.

(S)-N-hydroxy-4-(2-(naphthalene-2-sulfonamido)-2-phenylacetamido)benzamide (D16)

White powder crystallized in EtOAc and hexane (1: 1), 53% yield. Mp: 196–198 °C. $[\alpha]_D^{20}$ –6.90 (*c* 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.07 (s, 1H), 10.43 (s, 1H), 8.94 (s, 1H), 8.33 (s, 1H), 7.96 (d, *J* = 8.4 Hz, 1H), 7.94 (d, *J* = 9.0 Hz, 1H), 7.90 (d, *J* = 7.8 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 1H), 7.60 (t, *J* = 7.2 Hz, 1H), 7.57 (s, 1H), 7.55 (d, *J* = 8.4 Hz, 2H), 7.38 (d, *J* = 7.2 Hz, 2H), 7.30 (d, *J* = 8.4 Hz, 2H), 7.22 (t, *J* = 7.2 Hz, 2H), 7.17 (d, *J* = 7.2 Hz, 1H), 5.23

(s, 1H). HRMS (AP-ESI) m/z calcd for $C_{25}H_{22}N_3O_5S$ $[M + H]^+$ 476.1280, found 476.1276. HPLC retention time: 9.6 min, eluted by CH_3CN : H_2O = 43: 57.

(S)-4-(2-(5-(dimethylamino)naphthalene-1-sulfonamido)-2-phenylacetamido)-N-hydroxybenzamide (D17)

White powder crystallized in EtOAc and hexane (1: 1), 49% yield. Mp: 152–154 °C. $[\alpha]_D^{20}$ –6.78 (c 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.08 (s, 1H), 10.28 (s, 1H), 9.04 (d, J = 9.6 Hz, 1H), 8.94 (s, 1H), 8.37 (d, J = 8.4 Hz, 1H), 8.29 (d, J = 8.4 Hz, 1H), 8.12 (d, J = 7.2 Hz, 1H), 7.60 (d, J = 9.0 Hz, 2H), 7.55–7.53 (m, 1H), 7.51–7.48 (m, 1H), 7.31–7.29 (m, 4H), 7.18–7.16 (m, 4H), 5.16 (d, J = 9.6 Hz, 1H), 2.71 (s, 6H). HRMS (AP-ESI) m/z calcd for $C_{27}H_{27}N_4O_5S$ $[M + H]^+$ 519.1702, found 519.1699. HPLC retention time: 10.2 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-N-hydroxy-4-(2-(3-(2-methoxyphenyl)ureido)-2-phenylacetamido)benzamide (D18)

White powder crystallized in EtOAc, 33% yield. Mp: 201–203 °C. $[\alpha]_D^{20}$ –6.40 (c 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.09 (s, 1H), 10.61 (s, 1H), 8.92 (s, 1H), 8.39 (s, 1H), 8.07 (s, 1H), 8.06 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 7.8 Hz, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.66 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 7.8 Hz, 2H), 7.42–7.39 (m, 2H), 7.33 (t, J = 7.2 Hz, 1H), 6.97 (d, J = 7.8 Hz, 1H), 6.90–6.87 (m, 1H), 6.84–6.82 (m, 1H), 5.57 (d, J = 7.8 Hz, 1H), 3.84 (s, 3H). HRMS (AP-ESI) m/z calcd for $C_{23}H_{23}N_4O_5$ $[M + H]^+$ 435.1668, found 435.1662. ^{13}C NMR (75 MHz, DMSO- d_6) δ : 57.98, 60.19, 111.09, 118.47, 120.88, 121.63, 121.72, 127.11, 127.89, 129.02, 129.06, 129.62, 126.75, 139.06, 141.73, 147.90, 154.98, 164.27, 167.60. HPLC retention time: 8.7 min, eluted by CH_3CN : H_2O = 40: 60.

(S)-4-(2-(3-(2,6-diisopropylphenyl)ureido)-2-phenylacetamido)-N-hydroxybenzamide (D19)

White powder crystallized in EtOAc, 42% yield. Mp: 252–254 °C. $[\alpha]_D^{20}$ –8.40 (c 1.0, MeOH). 1H NMR (DMSO- d_6) δ 11.12 (s, 1H), 10.67 (s, 1H), 8.95 (s, 1H), 7.77 (s, 1H), 7.71 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 7.8 Hz, 2H), 7.52 (d, J = 7.8 Hz, 2H), 7.39 (d, J = 7.2 Hz, 2H), 7.32 (d, J = 7.2 Hz, 1H), 7.18 (s, 2H), 7.10 (s, 2H), 5.58 (d, J = 8.4 Hz, 1H), 3.16–3.13 (m, 2H), 1.11 (d, J = 7.2 Hz, 12H). HRMS (AP-ESI) m/z calcd for $C_{28}H_{33}N_4O_4$ $[M + H]^+$ 489.2502, found 489.2497. HPLC retention time: 8.8 min, eluted by CH_3CN : H_2O = 48: 52.

In Vitro HDAC Enzyme Inhibition Assay

In vitro activity assay against Hela cell nucleus extract has been described in our previous work.^[11] Briefly, Hela cell nucleus extract solution (10 μ L) was mixed with various concentrations of compound sample (50 μ L). Fluorogenic substrate (40 μ L) was added, and after 30 min of incubation at 37 °C, the reaction was stopped by addition of developer (100 μ L) containing trypsin and TSA. Fluorescence intensity was measured after 20 min of reaction using a microplate reader at excitation and emission wavelengths of 390 nm and 460 nm. The IC_{50} values were determined based on the fluorescence intensity readings. The procedures of HDAC1, 2, 3 and 6 assays were similar to that of Hela nucleus extract.

Molecular Docking Study

The docking procedure was performed using Sybyl-X 2.0 and Maestro v7.5 (schrodinger Inc, supported by Shanghai Institute of Materia Medica Chinese Academy of Sciences). All the molecules sketched and minimized by Sybyl-X were ionized and assigned OPLS 2005 force field by LigPrep. The structure of human HDAC2 derived from the protein database (PDB entry: 3MAX) was used for the docking study. The HDAC2 structure was prepared by removal of waters and ligands, assignment of OPLS 2005 force field using Maestro. The active site was defined as a cubic box centered by zinc ion by Glide dock, and the side

length of the box is 20 Å. Extra precision was applied in the docking process, other parameters were set as default.

In Vitro Antiproliferative Assay

Tumor cell inhibition assay was determined using MTT method. Briefly, 2,000 cells were seeded into each well of 96-well plates which were incubated at 37 °C, 5% CO₂ for overnight. Then the cells were treated with compound sample in various concentrations for 48 h. After that, a 0.5% MTT solution was added to each well. After 4 h of incubation, formazan formed from MTT was extracted by adding DMSO (200 µL) for 5 min. Optical density values were then detected at 570 nm on a microplate reader.

In Vivo Antitumor Assay against U937 Xenograft

For in vivo antitumor efficacy study, 1.8×10^7 U937 cells were inoculated subcutaneously in the right shoulder of male athymic nude mice (5–6 weeks old, Slac Laboratory Animal, Shanghai). Then days after injection, tumors were palpable and mice were randomized into treatment and control groups (5 mice per group). The treatment groups were administrated with 100 mg/kg/d intragastrically, the control group was administrated with equal volume of PBS solution. During treatment, the body weight of mice was monitored regularly. After 16 days of administration, the mice were executed and the tumor weight was measured by an electronic balance.

Western blot analysis

For western blot analysis, total protein extracts were separated on a polyacrylamide gel, transferred onto PVDF membranes and blotted as previously described.^[12] Briefly, U937 and MDA-MB-231 cells with different treatment were collected and lysed with lysis buffer for 30 min, then centrifuged for 15 min at 4000 rpm at 4 °C, the supernatant was the whole-cell extracts. Total protein extracts were separated by 12% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Cat. IPVH00010, Millipore). Membrane was blocked with 5% milk in TBS-T for 1 h at room temperature, then incubated with a 1:1000 or 1:2000 dilution of primary antibody overnight at 4 °C including tubulin (Sigma), acetylated tubulin (Sigma), histone H3 (Sigma), acetylated histone H3 (Sigma), acetylated histone H4 (Sigma), p21 (Cell Signaling), c-Raf (Sigma), p-Erk (Sigma), Erk (Sigma), p-Akt (Sigma) and Akt (Sigma). Then, the membrane was washed three times and incubated at 1:2000 dilution of anti-mouse or anti-rabbit goat-HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the membrane was washed another three times and developed by enhanced chemiluminescence (ECL, Cat. WBKL S0050, Millipore).

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Figure 1.

Design of new molecules based on the structure of **4h**. X group is CO, SO₂ or CONH, R group is substituted aromatic rings.

Figure 2.

a: molecule **D5**, **D16** and **D17** in the surface of HDAC2, the phenyl ring of **D16** was colored cyan, the naphthalene ring of **D16** was colored green; the Binding pattern of molecule **D5** (b), **D16** (c) and **D17** (d) in the active site of HDAC2.

Figure 3.

a: tumor weight plot of negative control group, positive control group (SAHA) and **D17** group; b: inhibitory ratio plot of SAHA and **D17**; c: tumor volume plot of these experimental groups (negative control, SAHA and **D17**); d: mice body weight plot of these experimental groups (negative control, SAHA and **D17**).

Figure 4.
Picture of dissected tumor tissues.

Figure 5.

Western blot analysis of acetylated histone H3, acetylated histone H4, p21, and acetylated tubulin in U937 cell line after 6h and 24h of treatment with compounds at 1 μ M. Tubulin was used as a loading control.

Figure 6.

Western blot analysis of c-Raf, p-Erk, Erk, p-Akt, Akt and histone H3 in U937 and MDA-MB-231 cell line after 24h of treatment with compounds (**D3** and **D17**) at 1 μ M. Histone H3 was used as a loading control.

Scheme 1.

Synthesis of compounds **D1-D19**. Reagents and conditions: (1) $(\text{Boc})_2\text{O}$, Et_3N , $\text{MeOH}/\text{H}_2\text{O}$; (2) CH_3COCl , CH_3OH ; (3) TBTU, Et_3N , THF; (4) EtOAc , HCl ; (5) RSO_2Cl , RCOCl , NaHCO_3 , $\text{THF}/\text{H}_2\text{O}$; RNH_2 , triphosgene, dioxane, Et_3N , DCM; (6) NH_2OK , MeOH .

Table 1

The enzyme inhibitory activities of the derived molecules

Compound	R	IC ₅₀ (nM) ^a
D1		348.6 ± 21.2
D2		95.5 ± 4.6
D3		14.3 ± 0.9
D4		25.5 ± 1.6
D5		15.6 ± 1.1
D6		105.5 ± 9.7

Compound	R	IC ₅₀ (nM) ^a
D7		28.7 ± 2.2
D8		85.5 ± 6.7
D9		60.2 ± 4.7
D10		125.5 ± 10.6
D11		33.6 ± 2.3
D12		20.5 ± 1.1
D13		13.7 ± 0.7

Compound	R	IC ₅₀ (nM) ^a
D14		22.8 ± 1.8
D15		34.6 ± 2.4
D16		23.5 ± 1.7
D17		9.7 ± 0.3
D18		7.5 ± 0.2
D19		47.7 ± 3.3
4h		144.4 ± 9.7

Compound	R	IC ₅₀ (nM) ^a
SAHA		145.6 ± 7.6

^a Each value is the mean of at least three experiments.

Table 2The tumor cell growth inhibitory activities of synthesized molecules (IC₅₀: μ M)^a

Compounds	U937	K562	HL60	MCF7	PC3	MDA-MB-231
D3	1.32 $\pm$ 0.07	1.19 $\pm$ 0.04	3.15 $\pm$ 0.13	2.92 $\pm$ 0.15	3.77 $\pm$ 0.27	4.23 $\pm$ 0.33
D4	1.64 $\pm$ 0.08	1.59 $\pm$ 0.06	4.55 $\pm$ 0.21	2.04 $\pm$ 0.17	5.88 $\pm$ 0.36	4.78 $\pm$ 0.44
D5	1.63 $\pm$ 0.09	0.93 $\pm$ 0.02	2.88 $\pm$ 0.16	8.66 $\pm$ 0.73	10.1 $\pm$ 0.57	5.12 $\pm$ 0.34
D7	1.77 $\pm$ 0.06	1.47 $\pm$ 0.07	2.02 $\pm$ 0.15	10.8 $\pm$ 0.93	5.67 $\pm$ 0.35	6.22 $\pm$ 0.24
D11	1.71 $\pm$ 0.03	3.16 $\pm$ 0.11	0.78 $\pm$ 0.02	8.23 $\pm$ 0.32	20.5 $\pm$ 1.73	6.67 $\pm$ 0.25
D12	1.55 $\pm$ 0.04	1.93 $\pm$ 0.12	1.80 $\pm$ 0.09	6.28 $\pm$ 0.33	3.00 $\pm$ 0.15	4.65 $\pm$ 0.24
D13	1.39 $\pm$ 0.03	1.58 $\pm$ 0.06	1.53 $\pm$ 0.04	9.71 $\pm$ 0.44	31.6 $\pm$ 2.33	4.47 $\pm$ 0.31
D17	1.24 $\pm$ 0.02	0.70 $\pm$ 0.01	3.66 $\pm$ 0.13	5.34 $\pm$ 0.22	3.31 $\pm$ 0.16	4.46 $\pm$ 0.28
D18	1.31 $\pm$ 0.07	1.55 $\pm$ 0.05	3.83 $\pm$ 0.29	5.14 $\pm$ 0.43	1.51 $\pm$ 0.05	4.34 $\pm$ 0.26
Av. ^b	1.51 $\pm$ 0.05	1.57 $\pm$ 0.06	2.69 $\pm$ 0.14	6.57 $\pm$ 0.41	9.48 $\pm$ 0.66	4.99 $\pm$ 0.30
SAHA	1.38 $\pm$ 0.09	1.86 $\pm$ 0.07	1.44 $\pm$ 0.06	5.09 $\pm$ 0.21	6.33 $\pm$ 0.22	4.70 $\pm$ 0.35

^a Each value is the mean of three experiments.^b The mean IC₅₀ value of tested molecules in each column.

Table 3

HDACs isoform selectivity of representative molecules (IC₅₀, nM) ^a

Compound	HDAC1	HDAC2	HDAC3	HDAC6
D3	28.7 ± 1.9	243.5 ± 11.7	16.8 ± 1.2	375.8 ± 19.7
D5	30.7 ± 1.3	170.6 ± 9.7	23.1 ± 1.1	294.5 ± 11.7
D13	11.8 ± 0.9	89.2 ± 4.7	8.5 ± 0.3	195.8 ± 11.9
D17	24.7 ± 1.4	106.7 ± 8.9	24.4 ± 1.5	365.9 ± 20.6
D18	4.8 ± 0.3	19.5 ± 1.4	5.9 ± 0.4	294.2 ± 22.3
SAHA	76.1 ± 2.6	256.3 ± 11.4	28.1 ± 1.8	118.2 ± 10.8

^a Each value is the mean of three experiments.