Original Article

Design, synthesis and biological evaluation of novel histone deacetylase inhibitors based on virtual screening

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Key Words
Histone deacetylases; Virtual screening; Non-hydroxamate; MS-275

Abstract
Ligand- and structure-based virtual screening methods were employed to identify novel non-hydroxamate histone deacetylase (HDAC) inhibitors. Based on the newly identified hit compound 17a, three series of compounds were synthesized and evaluated for both HDAC1 inhibitory activity and cytotoxicity. Binding modes of representative structures were analyzed using the docking method to explain the observed disparity in HDAC1 inhibitory activities.

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1. Introduction

Histone deacetylases (HDACs) have been widely recognized as promising targets for cancer treatment. The primary activity of HDACs is to remove acetyl groups from the ε-amino groups of lysine residues in the N-terminal extension of the core histones, which results in chromatin condensation and transcriptional repression1. Eighteen mammalian HDACs have been identified and categorized into four structural and functionally distinct classes2. Class I (HDAC1–3 and 8), class II (HDAC4–7, 9 and 10) and class IV (HDAC11) HDACs share conserved residues in the catalytic core regions and require zinc ion for deacetylation, while class III (SIRT1–7) HDACs are unrelated sirtuin deacetylases and require NAD$^+$ for their enzymatic activity3.

A vast number of HDAC inhibitors are currently under development and four representative structures are shown in Fig. 1. Vorinostat (SAHA) and romidepsin (FK228) have been approved by US FDA for the treatment of relapsed cutaneous T-cell lymphoma (CTCL)4, and there are at least fourteen HDAC inhibitors under more than eighty clinical trials5. Most of the HDAC inhibitors have three common features: cap group, zinc binding group (ZBG) and hydrophobic spacer6. The hydroxamic acid moiety has been widely used as zinc binding group. Although it has strong metal chelating capability, it displays little isoform selectivity among class I, II and IV HDACs and might result in the inhibition of other metallo-enzymes or sequestration of metal ions. Hydroxamic acids also suffer from metabolic and pharmacokinetic problems such as rapid glucuronidation and sulfation7.

Different types of non-hydroxamate functionalities have been reported, such as benzamides8, electrophilic ketones9, ketoamides10, phosphonates11, N-formylhydroxylamine12 and so on. The 2-aminobenzamide derivative MS-275 presents excellent HDAC inhibitory activity and has been in phase I/II clinical trials for various solid tumors and hematological malignancies8,8. Nevertheless, many of the non-hydroxamate HDAC inhibitors have either reduced potency or metabolic disadvantages. Thus, there remains a need to develop HDAC inhibitors with new non-hydroxamate zinc binding group.

2. Results and discussion

2.1. Molecular design

X-ray crystal structures of human HDACs$^{3,14,15}$ and two bacterial HDAC-like enzymes, namely HDLP from Aquifex aeolicus (related to class I HDACs)$^{16}$ and HDAH from Bordetella/Alcaligenes strain FB188 (related to class Iib HDACs)$^{17,18}$ have been resolved in 2000 s. HDAC8, which shares the same active site and zinc binding residues with HDAC1–3, was used in our virtual screening. The sequence homology between HDAC8 and HDAC1 is high with 31% sequence identity and 53% sequence similarity19. In 2010, a crystal structure of HDAC2 complexed with a benzamide inhibitor was solved20. HDAC2, which demonstrates 85.2% sequence identity with HDAC1, was used as the template for HDAC1 homology modeling to explicate the biological results.

We searched the database of NCI2000 (with 238,819 molecules) and MiniMaybridge (with about 2000 molecules) by pharmacophore-based virtual screening. Based on the crystal structure of HDAC8/TSA complex (PDB code: 1T64), the pharmacophore model was built manually by mapping the chemical features on the corresponding functional groups of TSA in its binding conformation (Fig. 2) using Catalyst as reported in the literature21. Particular spatial shape was generated based on the binding conformation of TSA using Catalyst default settings, which merged with pharmacophore features to form the query model for the first round virtual screening. 847 molecules were selected for the next round of screening.

Molecular docking program DOCK5.0 was employed for subsequent screening based on the crystal structure of HDAC8 (PDB code: 1T64). The protocol was verified by docking TSA back into HDAC8. The RMSD between the docking pose and the binding conformation in the crystal structure is 0.91 Å. The small molecules obtained were ranked according to their scores calculated by the energy scoring function in DOCK program. The top 300 molecules with best scores were subjected to drug-likeness analysis. Thirty compounds were finally chosen as candidate molecules for further investigation.

![Figure 1](image-url)  
Examples of clinically tested HDAC inhibitors.
Among the 30 candidate molecules, compound 17a, which is featured by its unique structure, was identified as a new hit compound with weak HDAC1 inhibitory activity. Compounds 5a–e were designed by combining the cap group and spacer of MS-275 derivatives with the zinc binding group of 17a to validate the potential of 2,5-dimethoxybenzamide as zinc binding group. While compounds 13a and 13b were designed by integrating the spacer and zinc binding group of MS-275 with cap group from 17a to examine the feasibility of 4-(2-methoxyphenyl)piperidine as a new cap group. Compounds 17b–e were derivatives of 17a with various substituted benzamides to explore the effects of different substitutions on the inhibitory activity against HDACs (as shown in Fig. 3).

2.2. Chemistry

The general approach to synthesize compounds 5a–e is outlined in Scheme 1. Condensation of 3-(hydroxymethyl)pyridine or substituted phenyl methanol (1) with 4-(aminomethyl)benzoic acid (3) using 1,1′-carbonyldiimidazole (2) gave carboxylic acids 4, which underwent an amide coupling reaction with 2,5-dimethoxyaniline to afford 5.

The synthetic route of compounds 13a–b is outlined in Scheme 2. Grignard Reaction of tert-butyl-4-oxopiperidine-1-carboxylate (6) and 1-bromo-2-methoxybenzene (7), followed by dehydration and deprotection in 6 N HCl and reduction under 50 psi, gave 4-(2-methoxyphenyl)piperidine hydrochloride (10).
Treatment of 10 and methyl 4-(aminomethyl)benzoate with triphosgene gave urea 11a. Reductive amination of 10 with methyl 4-formylbenzoate and NaBH4 provided 11b. The intermediates 11 were hydrolyzed and condensed with 1,2-phenylenediamine to afford amides 13.

The synthesis of compounds 17a–e is presented in Scheme 3. Nucleophilic aromatic substitution of 4-bromo-3-nitrobenzaldehyde (14) with 10, followed by Knoevenagel Reaction produced intermediate 16. In the last steps, condensation of 16 with different substituted anilines and successive deprotection led to 17.

2.3. Biological evaluation

2.3.1. HDAC1 enzyme inhibition assay

Binding modes of benzamide HDAC inhibitors have been demonstrated in HDAC2/benzamides complex. The hydrogen atoms of the ortho-NH2 group are involved in hydrogen bonds with the side chains of histidines 145 and 146, the nitrogen of the ortho-NH2 group chelates the zinc, and the carbonyl oxygen interacts with both the tyrosine side chain hydroxyl and the zinc.

Compounds 5a–e are MS-275 derivatives with 2-aminobenzamide replaced by 2,5-dimethoxybenzamide from 17a. Compounds 5a–e were evaluated using an HDAC1 enzyme inhibition assay and the results were shown in Table 1. As the oxygen atom is more electronegative than the nitrogen atom, it might result in weaker coordination with the zinc ion, and this fact might explain the observation that none of these compounds showed significant HDAC1 inhibitory activities. The results also implicates that a potent zinc binding group is crucial for HDAC inhibitory activity.

Compounds 13a and 13b are MS-275 derivatives with 4-(2-methoxyphenyl)piperidine as the cap group. The percentage inhibition of both compounds is slightly higher than that of MS-275 (Table 2), which suggests favorable interactions of 4-(2-methoxyphenyl)piperidine with the enzyme.

We further examined 17a derivatives with different substituted benzamides (Table 3) to investigate the effects of different substitutions on the inhibition of HDACs. Compound 17b with 2-aminobenzamide showed noticeable HDAC1 inhibitory activity (35.99% inhibition at the concentration of 50 μmol/L). Replacing the 2-aminobenzamide (17b) with 2-methoxybenzamide (17e) resulted in decreased inhibitory activity, which is consistent with the above result that the oxygen atom of methoxyl group might have weaker affinity with the zinc ion. Introduction of 5-phenyl substituent on the aniline ring (17d) increased the potency remarkably, while incorporation of 5-methoxyl substituent (17e) had no effects on potency. These results supported the recognition that the phenyl moiety may be optimal for binding in the narrow entry to the internal cavity of the zinc active site, while methoxyl group is probably too bulky to fit the internal cavity22–24.

2.3.2. Molecular docking

To obtain information on the structural basis of the observed disparity in HDAC1 inhibitory activities, we docked three 2-aminobenzamide containing compounds MS-275, 13a and 17a into HDAC1 homology model using a validated molecular dock program (GOLD)25,26. The homology model was constructed as described in the literature19. Independent docking of MS-275 (89.64% inhibition), 13a (95.36% inhibition) and 17a (35.99% inhibition) revealed that these compounds exhibited preferences for different binding modes at the pocket rim (as shown in Fig. 4). For MS-275 and 13a, pyridine and 4-(2-methoxyphenyl)piperidine interact with Tyr 195 at the
Table 1  Biological activities of MS-275 derivatives 5a-e containing 2,5-dimethoxybenzamide as potential zinc binding group.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>X</th>
<th>Clog P</th>
<th>% Inhibition of HDAC1 at 50 μmol/L</th>
<th>Cytotoxicity (IC₅₀, μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17a</td>
<td>-</td>
<td>-</td>
<td>5.048</td>
<td>25.76 ± 0.82</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>5a</td>
<td>H</td>
<td>N</td>
<td>1.559</td>
<td>21.07 ± 1.23</td>
<td>19.43</td>
</tr>
<tr>
<td>5b</td>
<td>3-OCH₃</td>
<td>C</td>
<td>2.975</td>
<td>25.63 ± 2.42</td>
<td>35.13</td>
</tr>
<tr>
<td>5c</td>
<td>4-OCH₃</td>
<td>C</td>
<td>2.975</td>
<td>ND*</td>
<td>14.67</td>
</tr>
<tr>
<td>5d</td>
<td>4-Cl</td>
<td>C</td>
<td>3.769</td>
<td>10.92 ± 0.98</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>5e</td>
<td>4-Ph</td>
<td>C</td>
<td>4.944</td>
<td>16.20 ± 12.27</td>
<td>16.83</td>
</tr>
<tr>
<td>MS-275</td>
<td>-</td>
<td>-</td>
<td>0.822</td>
<td>89.64 ± 2.84</td>
<td>1.32</td>
</tr>
</tbody>
</table>

ND*: not determined.

Table 2  Biological activities of MS-275 derivatives 13a and 13b with 4-(2-methoxyphenyl)piperidine as cap group.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Y</th>
<th>Clog P</th>
<th>% Inhibition of HDAC1 at 50 μmol/L</th>
<th>Cytotoxicity (IC₅₀, μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13a</td>
<td>CONHCH₂</td>
<td>2.677</td>
<td>95.36 ± 1.62</td>
<td>1.26</td>
</tr>
<tr>
<td>13b</td>
<td>CH₂</td>
<td>3.377</td>
<td>93.98 ± 0.33</td>
<td>2.12</td>
</tr>
<tr>
<td>MS-275</td>
<td>-</td>
<td>0.822</td>
<td>89.64 ± 2.84</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Table 3  Biological activities of 17a derivatives with different substituted benzamides as zinc binding groups.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₂</th>
<th>Clog P</th>
<th>% Inhibition of HDAC1 at 50 μmol/L</th>
<th>Cytotoxicity (IC₅₀, μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17a</td>
<td>2, 5-CH₃O</td>
<td>5.048</td>
<td>25.76 ± 0.82</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>17b</td>
<td>2-NH₂</td>
<td>4.311</td>
<td>35.99 ± 2.29</td>
<td>2.48</td>
</tr>
<tr>
<td>17c</td>
<td>2-CH₃O</td>
<td>5.023</td>
<td>25.76 ± 2.63</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>17d</td>
<td>2-NH₂-5-Ph</td>
<td>6.199</td>
<td>47.79 ± 0.76</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>17e</td>
<td>2-NH₂-5-CH₃O</td>
<td>4.447</td>
<td>30.92 ± 0.20</td>
<td>4.94</td>
</tr>
<tr>
<td>MS-275</td>
<td>-</td>
<td>0.822</td>
<td>89.64 ± 2.84</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Figure 4  GOLD docking solutions for MS-275 (blue), 13a (yellow) and 17a (green) at HDAC1; (a) Up view of the surface of HDAC1 near the active site and (b) Side view of the benzamides–HDAC1 interaction.
rim of the binding pocket. For the binding mode of 17a, the structure at the entrance of the active site is not flexible enough to enable the interaction between 4-(2-methoxyphenyl)piperidine and Tyr 195. This docking result explains the low potency of compounds 17, and suggests that interaction between the cap group and the enzyme surface is crucial for HDAC inhibitory activity and the rigidity of the spacer could affect potency by defining the orientation of the cap group.

2.3.3. Cytotoxic activity in vitro

All compounds were evaluated for cytotoxicity to human colon cancer HCT-116 cells, human breast adenocarcinoma MCF-7 cells and human umbilical vein endothelial cells (HUVEC) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. As shown in Table 1, compounds 5a–e showed modest cytotoxicity to HCT-116 and MCF-7 cells in concert with their low HDAC1 inhibitory activities. Compounds 13a and 13b exhibited excellent anti-proliferative activity to HCT-116 cells. Nevertheless, they also showed cytotoxicity to HUVEC (Table 2). As shown in Table 3, compounds 17a and 17e exhibited similar anti-proliferative activity against HCT-116 cells with MS-275 and have no cytotoxicity to HUVEC. Though 17d presented the highest potency against HDAC1 in compounds 17, it showed no anti-proliferative activity to HCT-116 cells. ClogP values were calculated using Chemdraw. Compounds 17a, 17c and 17d which showed no anti-proliferative activity to HCT-116 cells all have ClogP values greater than 5.

3. Conclusions

In summary, ligand- and structure-based virtual screening was employed to identify novel non-hydroxamate HDAC inhibitors. Based on the newly identified hit compound 17a, three series of compounds were synthesized and evaluated on both HDAC1 inhibitory activity and cytotoxicity. Binding modes of representative structures were analyzed using the docking method. MS-275 derivatives 5a–e with 2,5-dimethoxybenzamide as zinc binding group showed weak HDAC1 inhibitory activities suggesting methoxyl as a poor zinc binding group. Replacement of pyridine in MS-275 with 4-(2-methoxyl)phenyl as zinc binding group showed weak HDAC1 inhibitory activities suggesting methoxyl as a poor zinc binding group.

4. Experimental

4.1. Chemical synthesis

Melting points were determined on Thomas Hoover apparatus. Infrared spectra were acquired on Nicolet Impact 410 spectrophotometer using a KBr film. The absorption band is given in cm⁻¹. ¹H NMR spectra were recorded on a Bruker ACF-300 spectrometer (300 MHz). Chemical shifts are presented in ppm relative to tetramethylsilane. Mass spectra were obtained on a Mariner Mass Spectrum, or a GC-2010 mass spectrometer. Elemental analyses were determined on a Carlo Erba 1106 elementary analysis apparatus.

4.2. General procedures to prepare amides

Method A: A mixture of benzoic acid (1 eq.), PyBOP (1 eq.) and triethylamine (1.2 eq.) in dichloromethane was stirred at room temperature for 15 min. Substituted phenylamine (1.2 eq.) was then added. The mixture was stirred at room temperature for 8 h. The solvent was removed under reduced pressure, and the crude product so-obtained was purified by column chromatography.

Method B: A mixture of benzoic acid (1 eq.), BOP (1.2 eq.) and triethylamine (1.2 eq.) in N,N-dimethylformamide (DMF) was stirred at room temperature for 10 min. Substituted phenylamine (1.2 eq.) was then added. The mixture was stirred at room temperature overnight. Aqueous sat. NH₄Cl (15 mL) was added to the mixture and the crude product was collected by filtration and purified by column chromatography.
methoxyphenyl)piperidine-1-carboxamide (72.56; H, 5.68; N, 5.64. Found: C, 72.31; H, 5.93; N, 5.63.

3.36–3.40 (2H, m), 3.74 (3H, s), 3.77 (3H, s), 3.81 (3H, s), 6.43 (1H, d, J = 15.3 Hz), 6.53 (1H, dd, J = 9.0 Hz, J = 3.0 Hz), 6.75 (1H, d, J = 9.0 Hz), 6.81 (1H, d, J = 8.1 Hz), 6.87 (1H, m), 7.06 (1H, d, J = 8.7 Hz), 7.16 (2H, d, J = 7.5 Hz), 7.52 (1H, dd, J = 8.7 Hz, J = 2.1 Hz), 7.58 (1H, d, J = 15.3 Hz), 7.90 (1H, s), 7.94 (1H, d, J = 2.1 Hz), 8.19 (1H, d, J = 2.7 Hz). IR (KBr) cm⁻¹: 2955, 2833, 1679, 1650, 1520, 1280, 1101. MS (ESI) m/z: 518.3 [M+H]^+. Anal. Caled for C_{28}H_{29}N_{3}O_{5}: C, 69.02; H, 6.02; N, 11.15. Found: C, 66.47; H, 5.89; N, 11.43.

4.2.10. (E)-N-(2-methoxyphenyl)-3-(4-(4-(2-methoxyphenyl)piperidin-1-yl)-3-nitrophenoil)acrylamide (17c)

'H NMR (DMSO-d_{6}) δ: 1.88–1.93 (4H, m), 3.05–3.16 (3H, m), 3.43–3.47 (2H, m), 3.84 (3H, s), 3.93 (3H, s), 6.51 (1H, d, J = 15.6 Hz), 6.87–7.14 (6H, m), 7.18–7.26 (2H, m), 5.75–7.68 (2H, m), 7.95–8.02 (2H, m), 8.50 (1H, d, J = 7.4 Hz). IR (KBr) cm⁻¹: 3374, 1676, 1608, 1529, 1458. MS (ESI) m/z: 488.2 [M+H]^+. Anal. Caled for C_{33}H_{32}N_{4}O_{4}: C, 76.89; H, 6.00; N, 8.62. Found: C, 69.02; H, 6.02; N, 8.57.

4.2.11. (E)-N-(4-aminobiphenyl-3-yl)-3-(4-(4-(2-methoxyphenyl)piperidin-1-yl)-3-nitrophenoil)acrylamide (17d)

'H NMR (DMSO-d_{6}) δ: 1.87–1.88 (4H, m), 3.00–3.14 (3H, m), 3.36–3.40 (2H, m), 3.83 (3H, s), 6.51 (1H, d, J = 15.6 Hz), 6.82–7.02 (4H, m), 7.18–7.37 (6H, m), 7.42–7.62 (5H, m), 7.89–7.94 (2H, m). IR (KBr) cm⁻¹: 3377, 1608, 1526, 1490, 1237. MS (ESI) m/z: 549.4 [M+H]^+. Anal. Caled for C_{32}H_{31}N_{3}O_{2}: C, 72.24; H, 5.88; N, 10.21. Found: C, 72.24; H, 6.00; N, 10.23.

4.3. Biological evaluation

4.3.1. Fluorimetric HDAC1 assay

The HDAC1 fluorescent activity assay was based on the Fluor of Lys Substrate and Developer combination (BioMol), carried out according to the supplier’s instructions. First, the Fluor of Lys Substrate, which comprises an acetylated lysine side chain, was incubated with purified recombinant HDAC1 enzymes in the presence or the absence of test compounds. Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the Developer produces a fluorophore. Fluorescence was quantified with a TECAN inphinite M200 station.

4.3.2. In vitro cytotoxicity assay

All compounds were dissolved in DMSO at a stock concentration of 10 mg/mL, and diluted with fresh medium before assays. Cell lines were seeded into 96-well flat bottom plates at density of 6000 cells/well. Twelve hours after seeding, each compound dilution was added in duplicate and incubation continued at 37 °C in a humidified atmosphere containing 5% CO₂. After 72 h, 20 μL MTT at 5 mg/mL in PBS (filter sterilized, light protected, and stored at 4 °C) was added to each well, and after 4 h of incubation at 37 °C, the fluorescence was measured at 570 nm using Thermo Multiskan Spectrum.
References


