

Research Article

Design, Synthesis, Molecular Docking, and Anticancer Activity Evaluation of Chalcone Derivatives as HDAC6 Inhibitors

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Abstract

Background: Histone deacetylase 6 (HDAC6) is a pivotal regulator of multiple cellular pathways in tumors, thereby rendering HDAC6 a potential target for anticancer therapy. **Methods:** A series of HDAC6 inhibitors containing chalcone scaffolds was designed based on the principle of molecular hybridization. The antiproliferative activities of K562 and SiHa cells were assessed using the Cell Counting Kit-8 (CKK-8) assay. Fluorogenic assay kits were used to evaluate the enzyme activities of HDAC1 and HDAC6 *in vitro*. Western blot analysis was employed to assess the acetylation levels of α -tubulin (Ac- α -tubulin) and histone H3. Molecular docking was performed to elucidate the binding modes of the compounds with HDAC1 and HDAC6. **Results:** In total, 23 novel HDAC6 inhibitors containing a chalcone scaffold were synthesized. Most compounds demonstrated low nanomolar IC₅₀ values against HDAC6, with compound **3v** exhibiting the greatest potency, an IC₅₀ value of 4.3 nM and approximately 215-fold selectivity for HDAC6 over HDAC1. All target compounds exhibited strong antiproliferative effects against K562 and SiHa cells, with low micromolar IC₅₀ values. Nonetheless, compound **3v** demonstrated strong antiproliferative effects against both cell lines, with IC₅₀ values of 1.16 μ M in K562 cells and 1.28 μ M in SiHa cells, respectively. In addition, compound **3v** significantly upregulated the level of acetylated α -tubulin in K562 cells in a dose-dependent manner. Molecular docking analysis further revealed that compound **3v** exhibited a stronger binding affinity for HDAC6 than for HDAC1. Additionally, flow cytometry analysis revealed that compound **3v** induced apoptosis and the cell cycle arrest. **Conclusions:** Hydroxamic acid derivatives containing a chalcone fragment are potent HDAC6 inhibitors. These results indicate that chalcone substituents significantly affect enzyme and antiproliferative activities. Further studies are required to evaluate the anticancer potential and safety of these compounds.

Keywords: HDAC6 inhibitor; chalcone; molecular docking; chronic myeloid leukemia; cervical cancer

1. Introduction

Lysine acetylation is a major and reversible post-translational modification of proteins, regulated by two enzyme families with opposing activities: histone deacetylases (HDACs) and histone acetyltransferases (HATs) [1]. Protein lysine acetylation or deacetylation serves as a critical regulatory pathway in various cellular processes such as transcription, cell cycle, and cellular metabolism. HDACs have been recognized as effective therapeutic targets for the treatment of cancer, inflammation, and neurological disorders [2,3,4]. To date, 18 HDACs have been characterized, with 11 belonging to zinc (Zn²⁺)-dependent HDACs (classes I, II, and IV), and the remaining 7 classified as nicotinamide adenine dinucleotide (NAD⁺)-dependent HDACs (class III) [5]. Recent research indicates that pan-histone deacetylase inhibitors (HDACis) may be associated with adverse effects, including fatigue, nausea, diarrhea, vomiting, cardiotoxicity, hematotoxicity, and thrombocytopenia [6]. It is generally believed that subtype-selective HDACis have advantages over pan-HDAC inhibitors [7,8]. HDAC6 plays a crucial role in inducing angiogenesis, activating oncogenic proliferation signaling pathways and tu-

mor growth, activating antiapoptotic pathways through substrate deacetylation, activating invasive pathways, and enhancing cell migration and metastasis, making it a good target for anticancer drugs [9,10,11,12,13]. Moreover, HDAC6 is overexpressed in acute myeloid leukemia in humans, cervical cancer, ovarian cancer, colon cancer, breast cancer, gastrointestinal cancer and other cancers [11,14,15,16,17,18]. Overexpression of HDAC6 is associated with advanced tumors and increased tumor invasiveness, with lower survival rates [19].

To date, multiple HDAC6 inhibitors have been reported, such as vorinostat (SAHA, **1**), Tubastatin A (**2**), and Nexturastat A (**3**) [20,21,22]. The pharmacophore of common HDAC6 inhibitors consists of three domains: zinc-binding group (ZBG), linker, and the cap group (cap) [23]. However, the development of highly active and selective HDAC6 inhibitors for clinical treatment remains urgent. Chalcone is widely present in many natural plant products, including vegetables, fruits, spices and teas, and exhibits a broad range of physiological activities, including anticancer, anti-inflammatory, and antibacterial activities [24,25,26]. Chalcone can be easily modified to change its



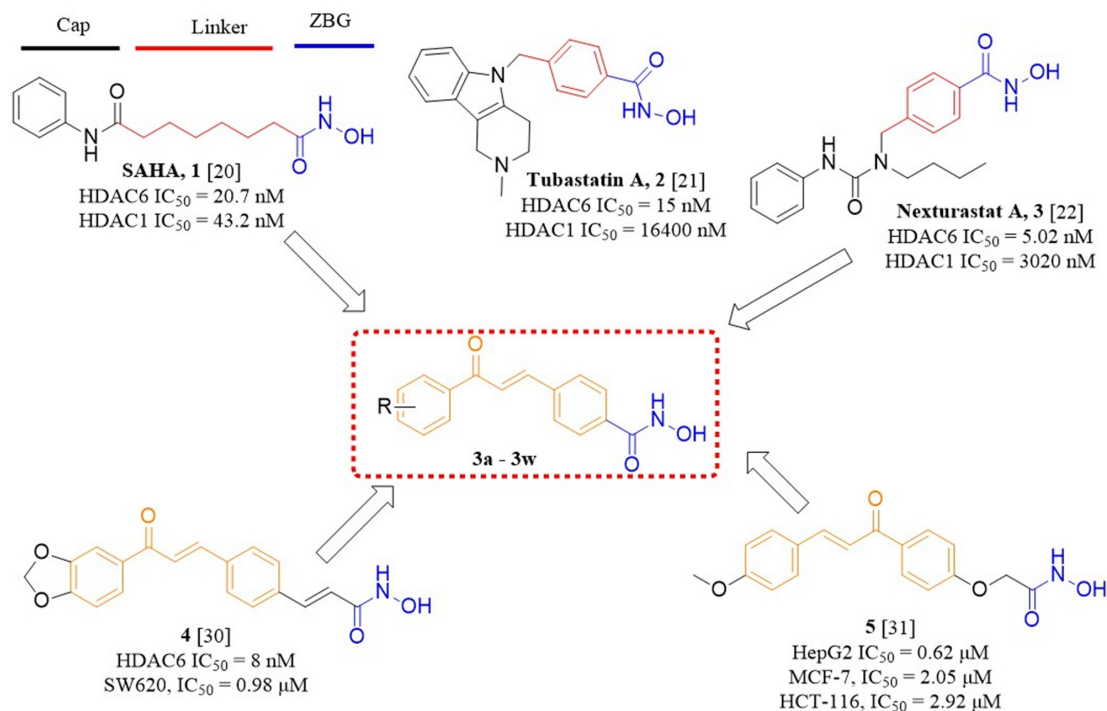


Fig. 1. Reported HDAC6 inhibitors and design strategies for target compounds. (Ref. [20,21,22,30,31]).

biological activity, and it is a useful template for drug development [27]. However, natural chalcone compounds still exhibit multiple limitations including poor absorption, low bioavailability, poor distribution characteristics and hepatotoxicity [28,29]. Recently, several HDAC6 inhibitors containing a chalcone fragment have been reported (Fig. 1, Ref. [20,21,22,30,31]). Compound **4** demonstrated a strong inhibitory effect on the human colon cancer cell line SW620 and exhibited high affinity for HDAC6 [30]. Compound **5** exhibited strong antiproliferative activity against HepG2, MCF-7, and HCT-116 cell lines [31]. In this study, the novel HDAC6 inhibitors were designed by hybridizing the ZBG with a chalcone fragment using the molecular hybridization method. The target compounds were synthesized and evaluated for their antiproliferative activity against leukemia cells (K562) and cervical cancer cells (SiHa), as well as their HDAC6 inhibitory activity *in vitro*. Additionally, the anticancer mechanism of action was explored using a cell apoptosis assay and Western blot analysis. Ultimately, this study aims to develop novel, highly selective, and potent HDAC6 inhibitors capable of overcoming the pharmacological limitations of natural chalcones, thereby providing promising therapeutic candidates for cancer treatment.

2. Materials and Methods

2.1 Chemistry

All reagents and solvents were purchased from Shanghai Adamas Reagent Co., Ltd. (Shanghai, China) and used directly. The structures of the target compounds

and intermediates were characterized by ¹H nuclear magnetic resonance (¹H NMR), ¹³C nuclear magnetic resonance (¹³C NMR) and mass spectrometry (MS). The NMR spectra were recorded in dimethyl sulfoxide (DMSO-*d*₆) using a 600 MHz NMR spectrometer (Bruker). Mass spectra were obtained using an Agilent 6200 electrospray ionization quadrupole time-of-flight (ESI-qTOF) liquid chromatography-mass spectrometry (LC-MS) instrument (Agilent Technologies, California, USA). Thin-layer chromatography (TLC) was performed on HSGF254 silica gel plates, and the spots were visualized under ultraviolet light at 254 nm.

2.1.1 General Procedure for the Synthesis of Intermediates (1a–1w)

A substituted acetophenone (2.0 mmol), 4-formylbenzoic acid (0.30 g, 2.0 mmol) and potassium hydroxide (0.34 g, 6.0 mmol) were added to methanol (20 mL) and stirred at 20–30 °C overnight. After the reaction, the pH of the mixture was adjusted to below 5 with 5% hydrochloric acid, and the precipitate was then filtered, washed with methanol (30% in water), and dried in a vacuum oven to give intermediates **1a–1w** with more than 85% yield.

(E)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**1d**)

Off-white solid, yield, 91.2%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 13.12 (s, 1H), 8.22–8.17 (m, 2H), 8.04 (d, J = 15.7 Hz, 1H), 8.02–7.97 (m, 4H), 7.79 (d, J = 15.6 Hz, 1H), 7.67–7.63 (m, 2H).

(E)-4-(3-(4-(dimethylamino)phenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**1q**)

Off-white solid, yield, 88.7%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 13.10 (s, 1H), 8.08–8.05 (m, 2H), 8.02 (d, *J* = 15.6 Hz, 1H), 7.97 (d, *J* = 1.9 Hz, 4H), 7.67 (d, *J* = 15.6 Hz, 1H), 6.77 (d, *J* = 9.1 Hz, 2H), 3.05 (s, 6H).

2.1.2 General Synthetic Procedure for the Preparation of Intermediates (**2a–2w**)

The appropriate intermediate **1** (1.0 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine (THP-ONH₂, 0.13 g, 1.1 mmol), anhydrous dichloromethane (20 mL) and triethylamine (0.5 mL, 3.5 mmol) were mixed, followed by the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 0.23 g, 1.2 mmol) and 1-hydroxybenzotriazole (HOBt, 15 mg, 0.11 mmol). The mixture was stirred at 20–30 °C overnight and monitored by TLC until completion. The reaction was quenched and washed with water. The organic layer was concentrated to obtain the crude product. The crude product was purified by column chromatography over silica gel to obtain the intermediates **2a–2w** with over 70% yield.

(E)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (**2d**)

Off-white solid, yield, 81.3%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.77 (s, 1H), 8.24–8.17 (m, 2H), 8.04 (d, *J* = 15.7 Hz, 1H), 8.01 (d, *J* = 8.3 Hz, 2H), 7.85 (d, *J* = 8.3 Hz, 2H), 7.78 (d, *J* = 15.6 Hz, 1H), 7.69–7.63 (m, 2H), 5.02 (s, 1H), 4.10 (dd, *J* = 18.0, 12.7 Hz, 1H), 3.54 (dd, *J* = 6.7, 4.5 Hz, 1H), 1.74 (s, 3H), 1.63–1.50 (m, 3H).

(E)-4-(3-(4-(dimethylamino)phenyl)-3-oxoprop-1-en-1-yl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (**2q**)

Off-white solid, yield, 75.6%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.75 (s, 1H), 8.11–8.04 (m, 2H), 8.01 (d, *J* = 15.3 Hz, 1H), 7.95 (d, *J* = 8.3 Hz, 2H), 7.83 (d, *J* = 8.3 Hz, 2H), 7.66 (d, *J* = 15.5 Hz, 1H), 6.77 (d, *J* = 9.1 Hz, 2H), 5.02 (s, 1H), 4.07 (s, 1H), 3.54 (dd, *J* = 6.7, 4.5 Hz, 1H), 3.05 (s, 6H), 1.73 (s, 3H), 1.63–1.48 (m, 3H).

2.1.3 General Procedure for the Synthesis of Target Compounds (**3a–3w**)

To a round-bottom flask (25 mL) were added the appropriate intermediate (**2a–2w**) (0.5 mmol), isopropanol (5 mL), and 10% hydrochloric acid (3 mL) at room temperature, and the mixture was stirred for 1 h. The precipitate was filtered, washed with methanol (20% in water), and dried in a vacuum oven to give target compounds **3a–3w** with 39.7–82.9% yield.

(E)-N-hydroxy-4-(3-oxo-3-phenylprop-1-en-1-yl)benzamide (**3a**)

Off-white solid, yield 68.4%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.37 (s, 1H), 9.16 (s, 1H), 8.18 (d, *J* = 7.7 Hz, 2H), 8.04 (d, *J* = 15.6 Hz, 1H), 7.99 (d, *J* =

8.0 Hz, 2H), 7.83 (d, *J* = 8.0 Hz, 2H), 7.77 (d, *J* = 15.6 Hz, 1H), 7.68 (t, *J* = 7.3 Hz, 1H), 7.58 (t, *J* = 7.5 Hz, 2H). ¹³C NMR(151 MHz, DMSO-*d*₆) δ 189.14, 163.50, 142.86, 137.41, 137.26, 134.18, 133.36, 128.89, 128.88, 128.65, 127.40, 123.49. HRMS(ESI), [M+H]⁺ calculated 268.0968, found 268.0976.

(E)-N-hydroxy-4-(3-(4-nitrophenyl)-3-oxoprop-1-en-1-yl)benzamide (**3b**)

Yellow solid, yield 78.6%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.36 (s, 1H), 9.14 (s, 1H), 8.38 (d, *J* = 1.7 Hz, 4H), 8.04 (d, *J* = 15.7 Hz, 1H), 8.00 (d, *J* = 8.3 Hz, 2H), 7.83 (t, *J* = 12.0 Hz, 3H). ¹³C NMR(151 MHz, DMSO-*d*₆) δ 188.33, 163.35, 149.92, 144.28, 142.14, 136.92, 134.50, 129.97, 129.07, 127.38, 123.88, 123.24. HRMS(ESI), [M+H]⁺ calculated 313.0819, found 313.0850.

(E)-4-(3-(3,4-dimethoxyphenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3c**)

Yellow solid, yield 69.5%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.37 (s, 1H), 9.20 (s, 1H), 8.05 (d, *J* = 15.6 Hz, 1H), 7.98 (d, *J* = 8.0 Hz, 2H), 7.95 (d, *J* = 8.4 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 2H), 7.73 (d, *J* = 15.5 Hz, 1H), 7.62 (s, 1H), 7.11 (d, *J* = 8.4 Hz, 1H), 3.87 (s, 3H), 3.86 (s, 3H). ¹³C NMR(151 MHz, DMSO-*d*₆) δ 187.27, 163.44, 153.40, 148.86, 141.94, 137.43, 133.97, 130.37, 128.80, 127.35, 123.63, 123.41, 110.92, 110.73, 55.85, 55.65. HRMS(ESI), [M+H]⁺ calculated 328.1179, found 328.1193.

(E)-4-(3-(4-chlorophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3d**)

Off-white solid, yield 75.6%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.37 (s, 1H), 9.14 (s, 1H), 8.20 (d, *J* = 8.4 Hz, 2H), 8.03 (d, *J* = 15.6 Hz, 1H), 7.99 (d, *J* = 8.1 Hz, 2H), 7.83 (d, *J* = 8.1 Hz, 2H), 7.78 (d, *J* = 15.6 Hz, 1H), 7.65 (d, *J* = 8.4 Hz, 2H). ¹³C NMR(151 MHz, DMSO-*d*₆) δ (ppm) 188.04, 163.39, 143.32, 138.33, 137.15, 136.06, 134.27, 130.58, 128.98, 127.38, 123.15. HRMS(ESI), [M+H]⁺ calculated 302.0578, found 302.0591.

(E)-N-hydroxy-4-(3-oxo-3-(*p*-tolyl)prop-1-en-1-yl)benzamide (**3e**)

Off-white solid, yield 73.5%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.36 (s, 1H), 9.15 (s, 1H), 8.09 (d, *J* = 7.8 Hz, 2H), 8.03 (d, *J* = 15.6 Hz, 1H), 7.98 (d, *J* = 7.9 Hz, 2H), 7.83 (d, *J* = 7.9 Hz, 2H), 7.75 (d, *J* = 15.6 Hz, 1H), 7.38 (d, *J* = 7.8 Hz, 2H), 2.40 (s, 3H). ¹³C NMR(151 MHz, DMSO-*d*₆) δ 188.52, 163.49, 143.84, 142.49, 137.32, 134.92, 134.09, 129.44, 128.84, 128.80, 127.37, 123.50, 21.26. HRMS(ESI), [M+H]⁺ calculated 282.1125, found 282.1132.

(E)-N-hydroxy-4-(3-(4-methoxyphenyl)-3-oxoprop-1-en-1-yl)benzamide (**3f**)

Off-white solid, yield 59.4%. ¹H NMR(600 MHz, DMSO-*d*₆) δ 11.36 (s, 1H), 9.07 (s, 1H), 8.19 (dd, *J* = 9.3, 2.3 Hz, 2H), 8.04 (d, *J* = 15.6 Hz, 1H), 7.97 (d, *J* = 8.3 Hz, 2H), 7.83 (d, *J* = 8.3 Hz, 2H), 7.73 (d, *J* = 15.6 Hz, 1H), 7.09 (dd, *J* = 9.4, 2.3 Hz, 2H), 3.87 (s, 3H). ¹³C NMR(151 MHz, DMSO-*d*₆) δ 187.29, 163.46, 163.38,

141.99, 137.42, 133.98, 131.08, 130.32, 128.76, 127.36, 123.50, 114.10, 55.64. HRMS(ESI), $[M+H]^+$ calculated 298.1074, found 298.1086.

(E)-4-(3-(2,4-dichlorophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3g**)

Off-white solid, yield 81.2%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.35 (s, 1H), 9.09 (s, 1H), 7.87 (d, J = 8.0 Hz, 2H), 7.80 (d, J = 7.8 Hz, 3H), 7.65 (d, J = 8.2 Hz, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.48 (d, J = 16.2 Hz, 1H), 7.36 (d, J = 16.2 Hz, 1H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 192.32, 163.28, 145.52, 137.20, 136.59, 135.81, 134.62, 131.32, 130.87, 129.77, 128.97, 127.72, 127.44, 127.33. HRMS(ESI), $[M+H]^+$ calculated 336.0189, found 336.0188.

(E)-4-(3-(4-fluorophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3h**)

Off-white solid, yield 51.8%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.37 (s, 1H), 9.12 (s, 1H), 8.28 (dd, J = 8.8, 5.6 Hz, 2H), 8.04 (d, J = 15.6 Hz, 1H), 7.98 (d, J = 8.3 Hz, 2H), 7.84 (d, J = 8.3 Hz, 2H), 7.77 (d, J = 15.6 Hz, 1H), 7.46–7.37 (m, 2H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.64, 165.14 (d, J = 252.3 Hz), 161.37, 142.97, 137.15, 134.19, 134.08, 131.64 (d, J = 9.5 Hz), 128.87, 127.35, 123.25, 115.86 (d, J = 21.8 Hz). HRMS(ESI), $[M+H]^+$ calculated 286.0874, found 286.0897.

(E)-4-(3-(3,5-difluorophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3i**)

Off-white solid, yield 45.4%. ^1H NMR(600 MHz, DMSO- d_6) δ (ppm) 11.36 (s, 1H), 9.15 (s, 1H), 8.03 (t, J = 11.8 Hz, 3H), 7.93–7.88 (m, 2H), 7.83 (t, J = 11.6 Hz, 3H), 7.61 (tt, J = 8.9, 2.3 Hz, 1H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 186.71, 162.70 (d, J = 248.24 Hz), 162.62 (d, J = 248.2 Hz), 161.40, 144.23, 140.59 (t, J = 7.8 Hz), 137.47, 134.47, 129.18, 127.37, 122.67, 111.86 (d, J = 20.69 Hz), 111.82 (d, J = 20.54 Hz), 108.70 (t, J = 26.0 Hz). HRMS(ESI), $[M+H]^+$ calculated 304.0780, found 304.0761.

(E)-4-(3-(4-ethylphenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3j**)

Yellow solid, yield 73.8%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.37 (s, 1H), 9.16 (s, 1H), 8.11 (d, J = 8.0 Hz, 2H), 8.03 (d, J = 15.6 Hz, 1H), 7.98 (d, J = 8.1 Hz, 2H), 7.83 (d, J = 8.1 Hz, 2H), 7.75 (d, J = 15.6 Hz, 1H), 7.41 (d, J = 8.0 Hz, 2H), 2.70 (q, J = 7.6 Hz, 2H), 1.21 (t, J = 7.6 Hz, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 188.59, 163.50, 149.85, 142.48, 137.32, 135.19, 134.09, 128.90, 128.82, 128.27, 127.39, 123.55, 28.27, 15.25. HRMS(ESI), $[M+H]^+$ calculated 296.1281, found 296.1286.

(E)-N-hydroxy-4-(3-oxo-3-(*m*-tolyl)prop-1-en-1-yl)benzamide (**3k**)

Pale yellow solid 57.9%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.35 (s, 1H), 9.13 (s, 1H), 8.00 (dt, J = 14.1, 10.8 Hz, 5H), 7.83 (d, J = 8.3 Hz, 2H), 7.76 (d, J = 15.6 Hz, 1H), 7.52–7.45 (m, 2H), 2.43 (s, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 189.13, 161.39, 142.69, 138.31, 137.43, 133.97, 129.05, 128.86, 128.75, 127.35, 125.86, 123.55,

122.78, 20.93. HRMS(ESI), $[M+H]^+$ calculated 282.1125, found 282.1128.

(E)-4-(3-(3-fluorophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3l**)

Off-white solid, yield 53.8%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.37 (s, 1H), 9.15 (s, 1H), 8.04 (dd, J = 11.5, 7.9 Hz, 2H), 8.00 (t, J = 7.1 Hz, 3H), 7.84 (d, J = 8.1 Hz, 2H), 7.80 (d, J = 15.6 Hz, 1H), 7.64 (dd, J = 14.0, 7.6 Hz, 1H), 7.54 (t, J = 8.4 Hz, 1H). ^{13}C NMR(151 MHz, DMSO- d_6) δ (ppm) 187.92, 163.45, 162.39 (d, J = 245.1 Hz), 143.55, 139.63 (d, J = 6.3 Hz), 137.11, 134.32, 131.06 (d, J = 7.8 Hz), 129.04, 127.37, 124.82, 123.10, 120.26 (d, J = 21.4 Hz), 115.19 (d, J = 22.3 Hz). HRMS(ESI), $[M+H]^+$ calculated 286.0874, found 286.0872.

(E)-4-(3-(4-butoxyphenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3m**)

Off-white solid, yield 55.8%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.35 (s, 1H), 9.39 (s, 1H), 8.17 (d, J = 8.7 Hz, 2H), 8.03 (d, J = 15.6 Hz, 1H), 7.96 (d, J = 7.8 Hz, 2H), 7.83 (d, J = 8.0 Hz, 2H), 7.72 (d, J = 15.5 Hz, 1H), 7.25–6.96 (m, 2H), 4.2–4.01 (m, 2H), 1.72 (d, J = 6.2 Hz, 2H), 1.44 (dd, J = 14.1, 6.9 Hz, 2H), 1.15–0.79 (m, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.23, 162.86, 141.93, 137.42, 133.96, 131.06, 130.15, 128.74, 127.35, 126.47, 123.49, 114.47, 67.64, 30.62, 18.71, 13.71. HRMS(ESI), $[M+H]^+$ calculated 340.1543, found 340.1579.

(E)-4-(3-(4-(4-chlorobutoxy)phenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3n**)

Off-white solid, yield 63.7%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 9.05 (s, 1H), 8.27–8.12 (m, 2H), 8.03 (d, J = 15.6 Hz, 1H), 7.96 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 8.3 Hz, 2H), 7.72 (d, J = 15.6 Hz, 1H), 7.12–7.07 (m, 2H), 4.21–4.08 (m, 2H), 3.72 (t, J = 6.2 Hz, 2H), 1.98–1.81 (m, 4H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.23, 162.68, 161.38, 141.93, 137.39, 133.95, 131.04, 130.25, 128.71, 127.32, 123.48, 114.49, 67.21, 45.13, 28.80, 26.02. HRMS(ESI), $[M+H]^+$ calculated 374.1154, found 374.1159.

(E)-N-hydroxy-4-(3-(4-(3-methoxypropoxy)phenyl)-3-oxoprop-1-en-1-yl)benzamide (**3o**)

Brown solid, yield 71.7%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 9.12 (s, 1H), 8.17 (d, J = 8.8 Hz, 2H), 8.03 (d, J = 15.6 Hz, 1H), 7.97 (d, J = 8.3 Hz, 2H), 7.83 (d, J = 8.3 Hz, 2H), 7.72 (d, J = 15.6 Hz, 1H), 7.08 (d, J = 8.9 Hz, 2H), 4.14 (t, J = 6.4 Hz, 2H), 3.48 (t, J = 6.3 Hz, 2H), 3.25 (s, 3H), 1.98 (p, J = 6.3 Hz, 2H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.24, 163.53, 162.73, 141.95, 137.43, 133.97, 131.08, 130.25, 128.74, 127.36, 126.47, 123.49, 114.48, 68.36, 65.10, 57.99, 28.84. HRMS(ESI), $[M+H]^+$ calculated 356.1492, found 356.1483.

(E)-N-hydroxy-4-(3-(3-(3-methoxypropoxy)phenyl)-3-oxoprop-1-en-1-yl)benzamide (**3p**)

Yellow solid, yield 57.4%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 9.12 (s, 1H), 8.00 (dd, J

= 14.6, 12.0 Hz, 3H), 7.83 (d, J = 8.4 Hz, 2H), 7.79–7.72 (m, 2H), 7.66–7.63 (m, 1H), 7.49 (t, J = 7.9 Hz, 1H), 7.27–7.23 (m, 1H), 4.12 (t, J = 6.4 Hz, 2H), 3.50 (t, J = 6.3 Hz, 2H), 3.26 (s, 3H), 1.98 (p, J = 6.3 Hz, 2H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 188.81, 163.46, 158.91, 142.88, 138.83, 137.24, 134.15, 129.99, 128.89, 127.32, 123.49, 121.06, 119.64, 68.44, 64.91, 57.96, 28.92. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 356.1492, found 356.1502.

(E)-4-(3-(4-(dimethylamino)phenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3q**)

Yellow solid, yield 39.7%. ^1H NMR(600 MHz, DMSO- d_6) δ (ppm) 11.32 (s, 1H), 9.14 (s, 1H), 8.11–8.04 (m, 2H), 7.99 (d, J = 15.6 Hz, 1H), 7.93 (d, J = 8.3 Hz, 2H), 7.81 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 15.5 Hz, 1H), 6.82–6.72 (m, 2H), 3.05 (s, 6H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 185.93, 163.54, 153.44, 140.45, 137.74, 133.62, 130.82, 128.48, 127.31, 124.99, 123.89, 110.86, 39.63. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 311.1390, found 311.1393.

(E)-4-(3-(4-acetamidophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3r**)

Yellow solid, yield 58.9%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 10.34 (s, 1H), 9.12 (s, 1H), 8.16 (d, J = 8.8 Hz, 2H), 8.02 (d, J = 15.6 Hz, 1H), 7.96 (d, J = 8.3 Hz, 2H), 7.83 (d, J = 8.3 Hz, 2H), 7.77 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 15.6 Hz, 1H), 2.09 (d, J = 18.5 Hz, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.40, 169.00, 163.47, 143.87, 142.08, 137.36, 134.00, 131.95, 129.99, 128.71, 127.34, 123.45, 118.29, 24.22. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 325.1183, found 325.2019.

(E)-N-hydroxy-4-(3-(4-((4-methylphenyl)sulfonamido)phenyl)-3-oxoprop-1-en-1-yl)benzamide (**3s**)

Off-white solid, yield 73.8%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.32 (s, 1H), 10.88 (s, 1H), 9.11 (s, 1H), 8.10–8.04 (m, 2H), 7.99–7.90 (m, 3H), 7.81 (d, J = 8.3 Hz, 2H), 7.74 (d, J = 8.3 Hz, 2H), 7.69 (d, J = 15.6 Hz, 1H), 7.38 (d, J = 8.1 Hz, 2H), 7.28–7.24 (m, 2H), 2.33 (s, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.43, 163.46, 143.76, 142.54, 142.24, 137.26, 136.41, 134.41, 134.05, 132.34, 130.28, 129.90, 128.71, 127.33, 126.75, 123.33, 117.89. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 437.1166, found 437.1141.

(E)-N-hydroxy-4-(3-oxo-3-(4-propylphenyl)prop-1-en-1-yl)benzamide (**3t**)

Off-white solid, yield 81.8%, ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 9.12 (s, 1H), 8.10 (d, J = 8.3 Hz, 2H), 8.02 (d, J = 15.6 Hz, 1H), 7.97 (d, J = 8.3 Hz, 2H), 7.83 (d, J = 8.3 Hz, 2H), 7.75 (d, J = 15.6 Hz, 1H), 7.39 (d, J = 8.3 Hz, 2H), 2.70–2.63 (m, 2H), 1.63 (dd, J = 15.0, 7.5 Hz, 2H), 0.90 (t, J = 7.3 Hz, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 188.57, 163.48, 148.22, 142.41, 137.30, 135.20, 134.07, 128.79, 128.77, 128.75, 127.35, 123.55, 37.19,

23.76, 13.60. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 310.1438, found 310.1456.

(E)-4-(3-(2,4-difluorophenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3u**)

Off-white solid, yield 43.6%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 9.12 (s, 1H), 7.93 (dt, J = 8.6, 4.3 Hz, 1H), 7.88 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 8.3 Hz, 2H), 7.68 (d, J = 15.8 Hz, 1H), 7.56 (dd, J = 15.9, 2.3 Hz, 1H), 7.49–7.43 (m, 1H), 7.27 (td, J = 8.4, 2.3 Hz, 1H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 187.44, 164.63 (dd, J = 253.4, 12.4 Hz), 163.41, 161.17 (dd, J = 255.9, 13.3 Hz), 143.43, 136.78, 134.44, 128.77, 128.58, 127.44, 126.56 (d, J = 4.0 Hz), 112.33 (dd, J = 21.6, 3.4 Hz), 105.17 (t, J = 26.4 Hz). HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 304.078, found 304.0729.

(E)-4-(3-(3,5-dimethylphenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3v**)

Off-white solid, yield 82.9%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.35 (s, 1H), 9.12 (s, 1H), 7.99 (dd, J = 17.1, 12.0 Hz, 3H), 7.83 (d, J = 8.3 Hz, 2H), 7.79 (s, 2H), 7.74 (d, J = 15.6 Hz, 1H), 7.30 (s, 1H), 2.38 (s, 6H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 189.47, 163.47, 142.47, 138.07, 137.47, 137.29, 134.65, 134.09, 128.80, 127.33, 126.31, 123.63, 20.80. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 296.1281, found 296.1284.

(E)-4-(3-(3,4-dimethylphenyl)-3-oxoprop-1-en-1-yl)-N-hydroxybenzamide (**3w**)

Yellow solid, yield 76.6%. ^1H NMR(600 MHz, DMSO- d_6) δ 11.34 (s, 1H), 9.12 (s, 1H), 8.02 (d, J = 15.6 Hz, 1H), 7.99–7.94 (m, 3H), 7.92 (dd, J = 7.9, 1.4 Hz, 1H), 7.83 (d, J = 8.3 Hz, 2H), 7.74 (d, J = 15.6 Hz, 1H), 7.33 (d, J = 7.9 Hz, 1H), 2.33 (s, 3H), 2.31 (s, 3H). ^{13}C NMR(151 MHz, DMSO- d_6) δ 188.58, 163.49, 142.62, 142.26, 137.34, 136.94, 135.26, 134.04, 129.86, 129.54, 128.76, 127.34, 126.34, 123.56, 19.63, 19.33. HRMS(ESI), $[\text{M}+\text{H}]^+$ calculated 296.1281, found 296.1288.

2.2 In Vitro Antiproliferative Assays

The *in vitro* antiproliferative effects of compounds **3a**–**3w** on the human cervical squamous cell carcinoma cell line SiHa and the human chronic myeloid leukemia cell line K562 were evaluated using the Cell Counting Kit-8 (CCK-8) assay, following the method detailed in reference [32].

K562 cells and SiHa cells were purchased from Procell Life Science & Technology (Wuhan, Hubei, China). Short tandem repeat (STR) profiling confirmed the identities of the K562 and SiHa cells, and the mycoplasma test results were negative. Briefly, the human chronic myeloid leukemia cell line K562 and the human cervical squamous cell carcinoma cell line SiHa were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a humidified incubator with 5% CO₂. Cells ($2\text{--}3 \times 10^4$ cells/well) were plated in 96-well plates and incubated for 12 h and then treated with different concentrations of the tested compounds (**3a**–**3w**)

for 48 h. After that, CCK-8 solution (10 μ L/well) was added and incubated for another 2 h. The absorbance was read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were performed in triplicate. The IC₅₀ value was calculated using GraphPad Prism 8.0 software (GraphPad Software, Boston, Massachusetts, USA).

2.3 *In Vitro* HDAC Inhibitory Activity Assays

The inhibitory activities of HDAC1 and HDAC6 were evaluated using HDAC1 and HDAC6 Fluorogenic Assay Kits (BPS Bioscience) according to the method provided by the supplier. The compounds were dissolved in DMSO containing 90% water and tested at a minimum of six concentrations for IC₅₀ determination using a 10-fold serial dilution. The diluted fluorogenic HDAC substrate, 0.1% bovine serum albumin (BSA) solution, tested compounds, recombinant human HDAC1 and HDAC6 enzymes, and HDAC detection buffer solution were added to a black 96-well plate and incubated at 37 °C for 30 min. The fluorescence intensity was read using a microplate reader with a fluorescence detector (SpectraMax iD3) at an excitation wavelength of 350 nm and a detection wavelength of 450 nm. The IC₅₀ value was calculated using GraphPad Prism 8.0 software.

2.4 Cell Apoptosis Assay

Flow cytometric analysis was performed using Annexin V staining combined with Fixable Viability Dye eFluor 780 in accordance with the method described in the RayBio Annexin V Apoptosis Detection Kit (RayBright, Violet 450) [33]. Briefly, K562 cells were cultured in a 6-well plate for 12 h, subsequently treated with compound 3v (1.25 μ M, 2.5 μ M and 5 μ M), and cultured for another 24 h. The cells were harvested, washed with phosphate-buffered saline (PBS), centrifuged, and treated with Ray-Bright 780live/dead maker (RayBio, Lot, 062424). Incubated on ice for 20 min, washed with Annexin V binding buffer, then treated with V450-Annexin-V. Incubated for 15 min, washed with Annexin V binding buffer. Singlet cell populations were gated using FSC-A/FSC-H, and fluorescence compensation was calibrated with unstained and single-stained controls. Quadrant gating was established on the basis of the cell populations from the unstained and single-stained controls. Finally, the cells were analyzed using a flow cytometer (Cytek Northern Light 3 Laser 16V-14B-8R, Wuxi, Jiangsu, China).

2.5 Cell Cycle Assay

Flow cytometry was used to assess the effect of compound 3v on cell-cycle distribution according to a previous method [33]. The harvested K562 cells were fixed with 75% ethanol at 4 °C overnight, followed by centrifugation at 2000 rpm for 5 min to remove the supernatant. The cells were then stained with PI for 30 min in the dark before be-

ing analyzed by flow cytometry (Cytek Northern Light 3 laser 16V-14B-8R, Wuxi, Jiangsu, China).

2.6 Western Blotting Analysis

K562 cells were treated with compound 3v for 24 h. Subsequently, the cells were harvested, washed with PBS, and then lysed with radioimmunoprecipitation assay (RIPA) lysis buffer for 30 min on ice. After centrifugation, the protein concentration in the supernatant was determined using a bicinchoninic acid (BCA) assay kit. The protein samples (20 μ g/sample) were loaded onto sodium dodecyl sulfate-polyacrylamide gels and resolved by electrophoresis. The proteins were then transferred to polyvinylidene difluoride membranes. Membranes were probed with specific primary antibodies (Acetyl- α -Tubulin (Lys40) (D20G3) XP® Rabbit mAb (Cell Signaling Technology, Danvers, Massachusetts, USA, Lot: 5335T, 1:2000), Acetyl-Histone H3 (Lys27) (D5E4) XP® Rabbit mAb (Cell Signaling Technology, Danvers, Massachusetts, USA, Lot: 8173T, 1:1000)) overnight at 4 °C. Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized with enhanced chemiluminescence (ECL) reagent.

2.7 Molecular Docking

The X-ray crystal structures of HDAC6 (Protein Data Bank [PDB] ID: 6PYE, resolution 1.48 Å) and HDAC1 (PDB ID: 4BKX, resolution 1.75 Å) were retrieved from the Protein Data Bank (<https://www.rcsb.org/>). Molecular docking was conducted according to the method reported in reference [34]. Briefly, energy minimization of the three-dimensional structures of the target compounds was performed using the AMBER 10: EHT force field. The protein structure was prepared with the QuickPrep module of Molecular Operating Environment (MOE) 2022 using the default parameters before docking. The cocrystallized ligand of the protein was utilized to define the active site, and the docking protocol embedded in MOE was then employed to perform the docking. The triangle matcher docking placement methodology and induced fit post-placement refinement were employed. The GBVI/WSA dG docking scoring function was employed to accurately predict the activity of the compounds. To validate the reliability of docking predictions, the cocrystallized ligand was used as a reference.

3. Results and Discussion

3.1 Chemistry

The synthetic route for the preparation of compounds 3a–3w is shown in (Fig. 2). A commercially available substituted benzaldehyde was mixed with 4-formylbenzoic acid in methanol in the presence of potassium hydroxide at room temperature to obtain intermediates 1a–1w with more than 85% yield. Subsequently, the intermediates 1a–1w were reacted with THP-ONH₂ in the presence of EDCI,

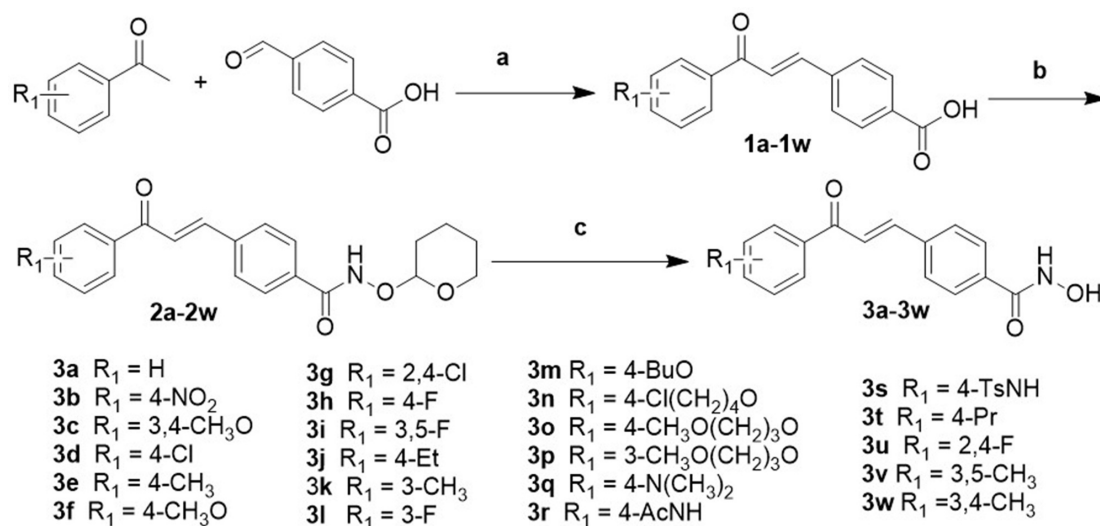


Fig. 2. The synthesis route for compounds **3a–3w**. Reaction conditions: (a) KOH, methanol, rt; (b) THP-ONH₂, EDCI, HOBT, Et₃N, anhydrous CH₂Cl₂; (c) 10% HCl, isopropanol, rt.

HOBT and triethylamine in anhydrous dichloromethane to give intermediates **2a–2w** with over 70% yield. Finally, the intermediates **2a–2w** were deprotected using 10% hydrochloric acid in isopropanol to afford the target compounds **3a–3w** with 39.7%–82.9% yield.

3.2 In Vitro Biological Evaluations

3.2.1 In Vitro Antiproliferative Activity

The *in vitro* antiproliferative activity of target compounds **3a–3w** was evaluated using the CCK-8 assay against K562 and SiHa cell lines, with SAHA employed as the positive control. The results were summarized in Table 1. The CCK-8 antiproliferative assays demonstrated that all tested compounds exhibited strong antiproliferative activity against the K562 and SiHa cell lines, with IC₅₀ values ranging from 1.16 to 8.51 μM. Among them, compounds **3a**, **3c**, **3d**, **3e**, **3f**, **3i**, **3j**, **3k**, **3o**, **3p**, **3q**, **3u**, **3v**, and **3w** (IC₅₀, 1.16–3.51 μM) exhibited higher cytotoxicity against K562 cells than SAHA (IC₅₀, 4.82 μM). Additionally, compound **3v** (IC₅₀, 1.28 μM) also demonstrated higher cytotoxicity against SiHa cells compared to SAHA (IC₅₀, 1.55 μM), while compounds **3k** and **3w** exhibited comparable cytotoxicity to SAHA.

Structure-activity relationship (SAR) analysis revealed that substitution with a strong electron-withdrawing group at the para-position (**3b**, IC₅₀, 4.83 μM) decreased the cytotoxicity against K562 cells, compared with compound **3a** (IC₅₀, 1.95 μM). Compounds bearing simple electron-donating substituents at the para-position (**3e**, **3f**, **3j**, **3q**; IC₅₀ = 1.55–2.90 μM) had little effect on the cytotoxicity of K562 cells, whereas those with bulkier electron-donating groups at the para-position (**3m**, **3n**, **3r**, **3s**, **3t**; IC₅₀ = 5.20–8.51 μM) significantly reduced cytotoxicity. The introduction of methyl and fluoro substituents at the para or meta positions exerted no significant influence on the cytotoxic ac-

Table 1. Antiproliferative activity of compounds **3a–3w** against K562 and SiHa Cells.

Comp.	R	IC ₅₀ , μM	
		K562	SiHa
3a	H	1.95 ± 0.11	3.38 ± 0.32
3b	4-NO ₂	4.83 ± 0.21	5.12 ± 0.28
3c	3,4-CH ₃ O	3.51 ± 0.24	4.64 ± 0.31
3d	4-Cl	2.13 ± 0.15	6.19 ± 0.32
3e	4-CH ₃	1.55 ± 0.22	2.41 ± 0.18
3f	4-CH ₃ O	2.90 ± 0.24	6.57 ± 0.34
3g	2,4-Cl	4.78 ± 0.25	4.20 ± 0.24
3h	4-F	5.62 ± 0.33	4.39 ± 0.21
3i	3,5-F	3.06 ± 0.27	4.67 ± 0.23
3j	4-Et	2.49 ± 0.39	2.47 ± 0.20
3k	3-CH ₃	2.35 ± 0.25	1.98 ± 0.09
3l	3-F	5.52 ± 0.31	4.25 ± 0.34
3m	4-BuO	5.68 ± 0.34	5.95 ± 0.36
3n	4-Cl(CH ₂) ₄ O	8.51 ± 0.52	7.59 ± 0.59
3o	4-CH ₃ O(CH ₂) ₃ O	1.82 ± 0.12	4.77 ± 0.37
3p	3-CH ₃ O(CH ₂) ₃ O	3.37 ± 0.21	5.07 ± 0.24
3q	4-N(CH ₃) ₂	2.44 ± 0.24	5.28 ± 0.30
3r	4-AcNH	7.98 ± 0.44	6.12 ± 0.38
3s	4-TsNH	5.77 ± 0.53	5.89 ± 0.24
3t	4-Pr	5.20 ± 0.27	4.61 ± 0.38
3u	2,4-F	2.02 ± 0.18	4.84 ± 0.18
3v	3,5-CH ₃	1.16 ± 0.13	1.28 ± 0.08
3w	3,4-CH ₃	1.36 ± 0.09	1.81 ± 0.11
SAHA		4.82 ± 0.35	1.55 ± 0.17

tivity against K562 and SiHa cells (**3e** vs **3k**, **3h** vs **3l**). The majority of bisubstituted compounds exhibited enhanced cytotoxicity against K562 and SiHa cells (**3e** vs **3v**, **3w**, **3h** vs **3i**, **3u**).

Table 2. Inhibitory effect of compounds 3a–3w against HDAC6 and HDAC1.

Comp.	R	IC ₅₀ (nM)		Selectivity (HDAC1/6)
		HDAC6	HDAC1	
3a	H	14.4 ± 0.8	594 ± 21	41.2
3b	4-NO ₂	86.5 ± 3.9	ND	ND
3c	3,4-CH ₃ O	26.0 ± 1.1	186 ± 11	7.2
3d	4-Cl	16.9 ± 0.9	525 ± 29	31.1
3e	4-CH ₃	47.4 ± 2.1	585 ± 33	12.3
3f	4-CH ₃ O	29.3 ± 1.6	ND	ND
3g	2,4-Cl	35.7 ± 2.6	ND	ND
3h	4-F	18.1 ± 0.7	ND	ND
3i	3,5-F	4.6 ± 0.2	984 ± 56	213.9
3j	4-Et	39.1 ± 1.7	NA	ND
3k	3-CH ₃	15.9 ± 0.6	387 ± 17	24.3
3l	3-F	95.8 ± 5.2	ND	ND
3m	4-BuO	155 ± 8.8	ND	ND
3n	4-Cl (CH ₂) ₄ O	126 ± 6.3	ND	ND
3o	4-CH ₃ O(CH ₂) ₃ O	27.6 ± 1.1	ND	ND
3p	3-CH ₃ O(CH ₂) ₃ O	58.1 ± 2.5	ND	ND
3q	4-N(CH ₃) ₂	76.2 ± 3.7	ND	ND
3r	4-AcNH	12.3 ± 0.6	352 ± 19	28.6
3s	4-TsNH	26.4 ± 0.5	489 ± 35	18.5
3t	4-Pr	75.3 ± 4.1	ND	ND
3u	2,4-F	11.7 ± 0.3	476 ± 16	40.7
3v	3,5-CH ₃	4.3 ± 0.2	926 ± 68	215.3
3w	3,4-CH ₃	9.2 ± 0.3	684 ± 32	74.3
Tubastatin A		17.6 ± 0.6	15,762 ± 934	895.6

ND, Not determined. Selectivity (SI): IC₅₀(HDAC1)/IC₅₀(HDAC6).

3.2.2 HDAC6 and HDAC1 Inhibition Study of the Target Compounds

The inhibitory activities of target compounds **3a–3w** against HDAC6 and HDAC1 were evaluated, with **Tubastatin A** serving as the positive control. The results are shown in Table 2. The majority of synthesized compounds exhibited potent inhibitory activity against HDAC6, with IC₅₀ values below 100 nM, except for compounds **3m** and **3n**. SAR analysis indicated that mono-substitution at the para or meta position markedly attenuated the inhibitory activity against HDAC6 compared to compound **3a** (IC₅₀ of 14.4 nM), except for the 4-acetamino substitution (**3r**, 12.3 nM). The 3,5-disubstituted compounds **3i** and **3v** significantly enhanced the inhibitory activity against HDAC6, with IC₅₀ values of 4.6 nM and 4.3 nM, respectively. Additionally, they increased the selectivity for HDAC6 over HDAC1, with the selectivity increasing from 41.2 to 213.9 and 215.3, respectively.

3.2.3 Compound 3v Induced Apoptosis of K562 Cells

Flow cytometric analysis was employed to evaluate apoptosis in K562 cells following treatment with compound **3v**. The results are shown in Fig. 3. The number of normal cells (Q4) was decreased in a dose-dependent manner, while the number of early apoptotic cells (Q3) and late

apoptotic cells (Q2) increased in a dose-dependent manner. Nevertheless, as the dose increased, the proportion of early and late apoptotic cells was increased to 97.4%, while necrotic cells (Q1) was merely 0.39%. Therefore, compound **3v** induced apoptosis in a dose-dependent manner.

3.2.4 Compound 3v Induced K562 Cell Cycle Arrest

The excessive proliferation of cancer cells originates from the loss of normal cell differentiation and apoptotic functions, which is attributed to abnormalities in the cell-cycle regulatory mechanisms. To investigate the effect of compound **3v** on cancer cell cycle progression, we performed flow cytometric analysis on K562 cells following propidium iodide (PI) staining. As demonstrated in Fig. 4, following 24 h of drug treatment, compound **3v** induced a reduction in the proportion of G1 and G2 phase cells from 56.82% and 5.78% to 38.71% and 2.67%, respectively, whereas the percentages of S phase cells were increased from 37.40% to 57.62%. These results demonstrated that compound **3v** can induce cell cycle arrest and apoptosis, suggesting that disruption of the cell cycle may be associated with its pro-apoptotic effect.

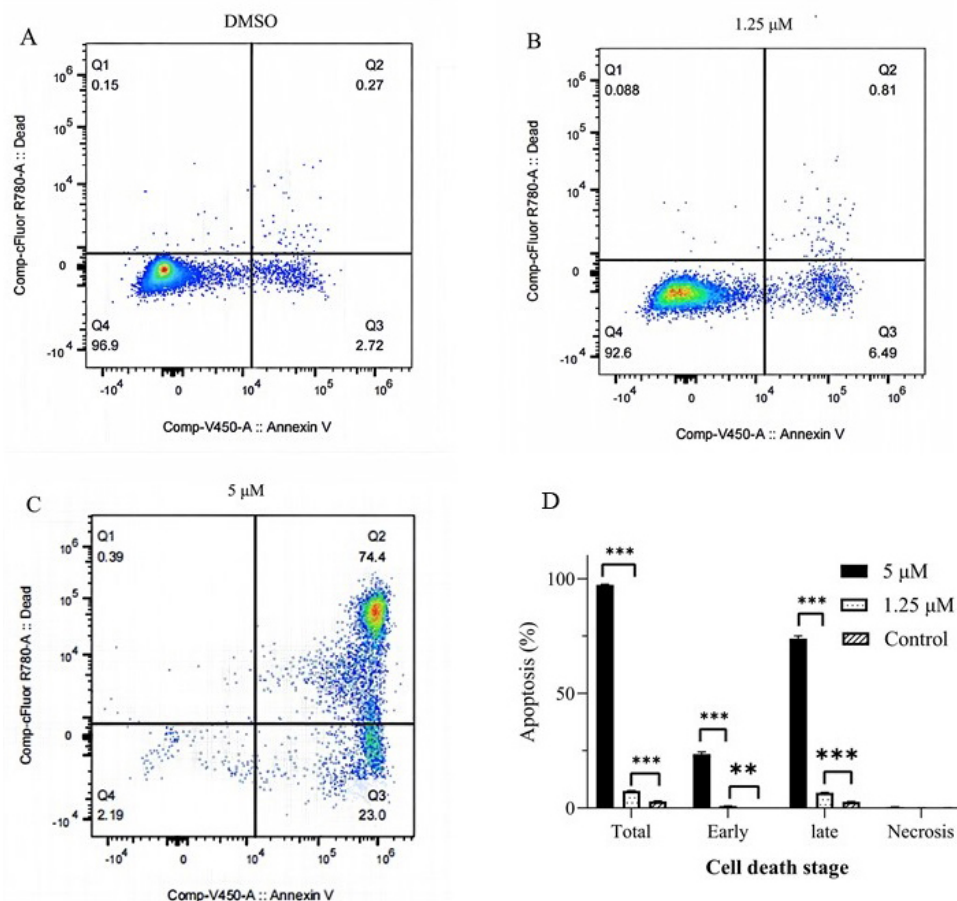


Fig. 3. Compound 3v induced apoptosis in K562 cells. (A) K562 cells were treated with DMSO and then analyzed by flow cytometry using Annexin V/Fixable Viability Dye eFluor 780 staining. (B) K562 cells were treated with 1.25 μ M **3v**. (C) K562 cells were treated with 5 μ M **3v**. (D) Total cell death represents early cell death + late cell death. The data represent the mean $\pm$ standard deviation of three independent experiments. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively.

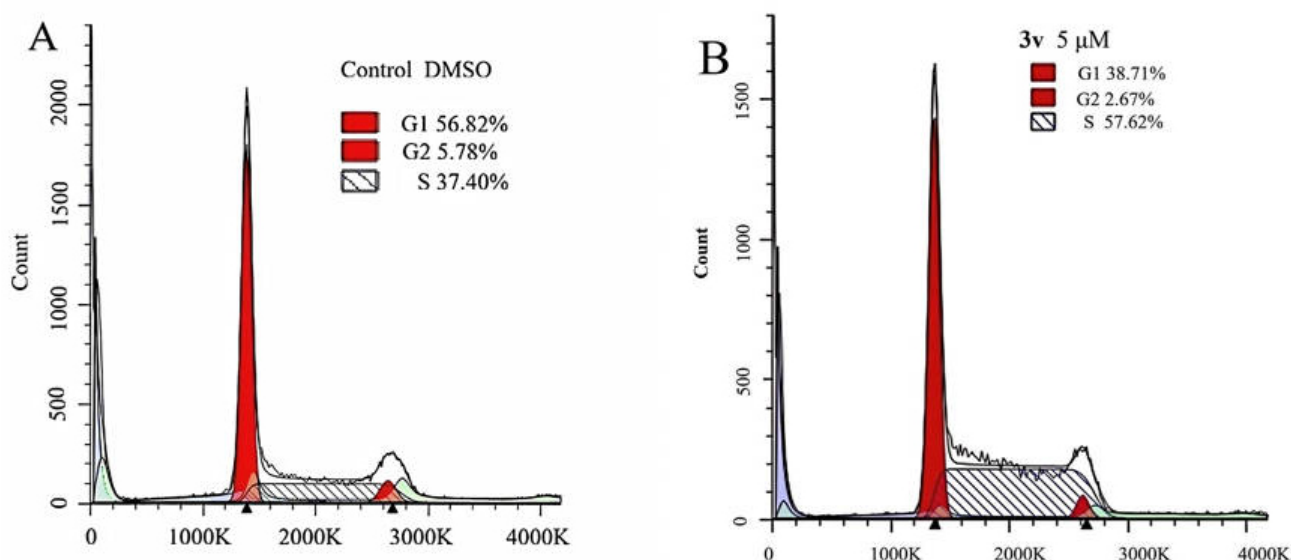


Fig. 4. Effect of compound 3v on the cell-cycle distribution of K562 cells. (A) K562 cells were treated with DMSO, and the cell cycle was then analyzed using a flow cytometer after propidium iodide (PI) staining. (B) K562 cells were treated with 5 μ M **3v**.

3.2.5 Western Blot Assay

As shown in Fig. 5, compound **3v** significantly enhanced the accumulation of acetylated α -tubulin (Ac- α -tubulin) and acetylation of histone H3 in a dose-dependent manner. Moreover, at a concentration of 5 μ M, the acetylation level of α -tubulin was significantly higher than that of H3. Therefore, the results indicate that **3v** preferentially inhibited HDAC6 in K562 cells.

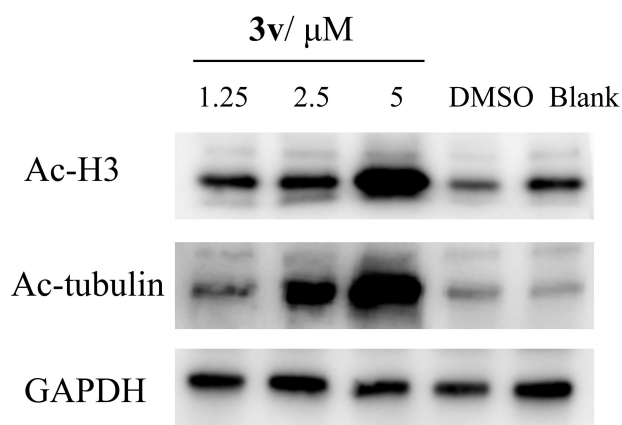


Fig. 5. Western blot analysis of acetylated histone H3 (Ac-H3) and acetylated α -tubulin (Ac- α -tubulin) in K562 cells after treatment with compound **3v**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the loading control.

3.2.6 ADMET Prediction

SwissADME prediction website and ADMETlab 3.0 were used to predict the key physicochemical properties of compounds **3i** and **3v** [35,36]. The predicted parameters associated with Lipinski's rule of five are shown in Table 3. Both compounds **3i** and **3v** adhered to Lipinski's rule of five with zero violations, thereby exhibiting favorable drug-like properties. The predicted absorption, distribution, metabolism, excretion, and toxicity (ADMET) parameters are summarized in Table 4. Compounds **3i** and **3v** exhibited moderate solubility and high human intestinal absorption and were predicted to be readily absorbed by the gastrointestinal tract. Compounds **3i** and **3v** also exhibited low drug-induced neurotoxicity, but had high Ames toxicity and high carcinogenicity. Rat oral acute toxicity testing predicted that **3v** exhibited low toxicity, but **3i** exhibited moderate toxicity. Given the defective ADMET properties of compound **3v** in silico, further optimization of the lead compound is needed.

3.3 Molecular Docking Study

Molecular docking was employed to elucidate the interactions between the target compounds and proteins and guide the SAR study. The docking results are shown in **Supplementary Tables 1,2**. The results showed that

Table 3. Parameters of Lipinski's rule of five for compounds **3i** and **3v**.

Comp.	Parameters						
	M.W.	Log P	TPSA	nHBA	nHBD	nRB	nVL
3i	303.26	3.26	66.40	5	2	5	0
3v	295.33	3.24	66.40	3	2	5	0

Note: The data were calculated using SwissADME (<https://www.swissadme.ch/>); M.W., molecular weight, g/mol; Log P, calculated lipophilicity; TPSA, topological polar surface area, $\text{\AA}^2$; nHBA, number of H-bond acceptors; nHBD, number of H-bond donors; nRB, number of rotatable bonds; nVL, number of violations from Lipinski's rule.

the target compounds exhibited markedly lower molecular docking scores for HDAC6 than for HDAC1. As shown in Fig. 6A, compound **3v** fit into the hydrophobic channel of the binding pocket, and formed a hydroxamic acid- Zn^{2+} coordination interaction, with a distance of 1.78 $\text{\AA}$ between Zn^{2+} and the carbonyl oxygen of the hydroxamate group. The amino acid residues Gly582 and Cys584 formed strong hydrogen bonds with the hydroxylamine group. In Fig. 6B, compound **3v** also formed a coordination interaction between the hydroxamate group and the catalytic Zn^{2+} ion, but the distance between Zn^{2+} and the C=O group increased from 1.78 $\text{\AA}$ to 2.02 $\text{\AA}$. The binding energy between **3v** and HDAC6 was -8.5246 kcal/mol, which was much lower than that for HDAC1 (-7.8828 kcal/mol). These results indicated a lower affinity of **3v** for HDAC1 than for HDAC6.

4. Limitations of the Study

The present study has several limitations. First, cytotoxicity testing on normal cells was not conducted, so the *in vitro* toxicity of the target compounds is unknown. Second, the increase in Ac- α -tubulin relative to Ac-H3 was observed, which supports preferential HDAC6-associated cellular activity, but it does not by itself establish selective inhibition of HDAC6. Third, no *in vivo* activity or pharmacokinetic studies were conducted, and the *in vivo* activity of these compounds is unclear. Therefore, further cellular and *in vivo* studies are warranted to address these limitations and evaluate the therapeutic potential of these compounds against cancer.

5. Conclusion

In the present study, 23 novel HDAC6 inhibitors containing chalcone fragments were designed and synthesized. The target compounds were characterized using NMR and high-resolution mass spectrometry. All target compounds were evaluated for their *in vitro* antiproliferative activity and HDAC6 enzyme inhibitory activity. Some compounds were evaluated for their HDAC1 enzyme inhibitory activity. Compounds **3i** and **3v** demonstrated potent inhibitory

Table 4. The predicted data of ADMET for compounds 3i and 3v.

Comp.	Log S ^a	GI ^a	BBB ^a	AMES ^b	Carcinogenicity ^b	ROAT ^b	Neurotoxicity ^b
3i	-4.92	High	Yes	high	high	moderate	low
3v	-5.14	High	Yes	high	high	low	low

Note: a, the data were calculated using SwissADME; b, the data were calculated using ADMETlab 3.0: <https://admetlab3.scbdd.com/>; GI, gastrointestinal absorption; BBB, blood-brain barrier; ROAT, rat oral acute toxicity.

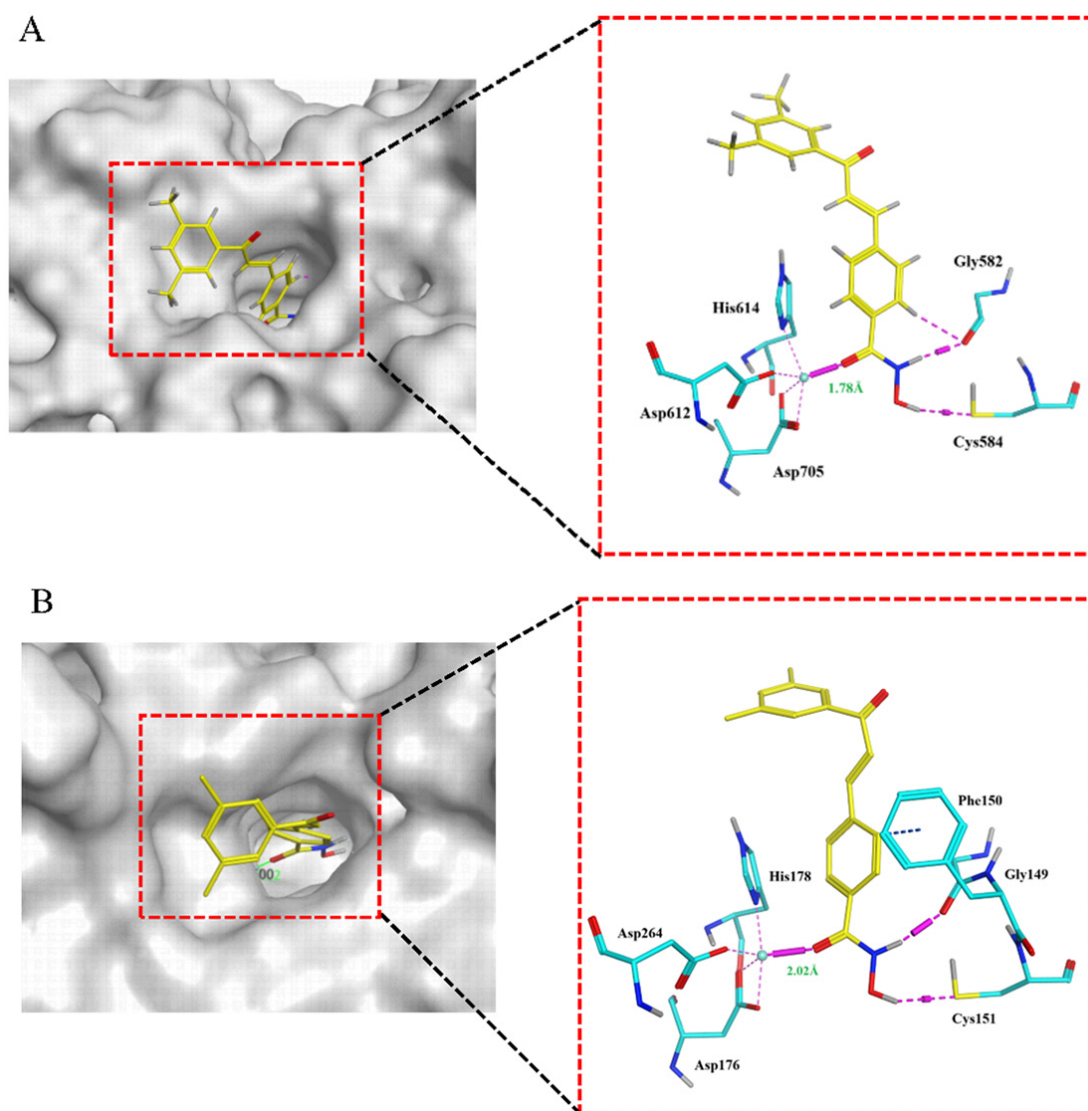


Fig. 6. The binding mode of 3v with HDAC1/6. (A) The binding mode of 3v (yellow) in complex with HDAC6 (PDB: 6PYE). (B) The binding mode of 3v (yellow) in complex with HDAC1 (PDB: 4BKX). The metal-coordination interaction and H-bonds are indicated by purple dashed lines, and the H-Pi bond is indicated by a blue dashed line.

activity against HDAC6, along with high selectivity over HDAC1, with IC_{50} values of 4.6 ± 0.2 nM and 4.3 ± 0.2 nM, respectively, corresponding to selectivity indices of 213.9-fold and 215.3-fold, respectively. Compound 3v also displayed potent antiproliferative activity against K562 cells and SiHa cells, with IC_{50} values of 1.16 ± 0.13 μ M and 1.28 ± 0.08 μ M, respectively. Flow cytometry analysis showed that compound 3v exerted antiproliferative activity by in-

ducing cell apoptosis. Western blot analysis demonstrated that compound 3v preferentially inhibited HDAC6 enzyme activity. Future studies should incorporate additional cellular experiments and *in vivo* studies to facilitate the further optimization of these lead compounds.

Availability of Data and Materials

All data in this study are available upon reasonable request by contacting the corresponding author.

Author Contributions

Conceptualization, MJY; methodology, YLZ, YTL, XLG and XQZ; validation: YLZ; formal analysis: YTL; writing, original draft preparation, YLZ and YTL; writing, review and editing, MJY; funding acquisition, MJY. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/Pharmazie55787>.

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