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Novel cinnamohydroxamic acid derivatives as HDAC inhibitors with anticancer activity *in vitro* and *in vivo*

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ABSTRACT

A novel series of cinnamohydroxamic acid derivatives were synthesized and their biological activities against HDAC were assessed. Our results showed that the compound with more strong inhibitory activity to HDAC would exhibited more significant anti-proliferative effect on tumor cells. Among these compounds, 7e displayed clearly inhibitory effects on HDAC and tumor cell growth. Furthermore, HDAC isoforms enzyme data indicated that, compared to HDAC pan-inhibitor SAHA, 7e owned an enhanced inhibitory effect on HDAC1, 3 and 6 isoforms. Meanwhile, it also significantly suppressed cell growth of lung cancer cells compared to SAHA, but with lower toxicity in normal cells. Mechanistically, 7e prompted acetylation of histone3 and histone4, led to up-regulation of p21, and then mediated cell cycle arrest and pro-apoptosis. Moreover, the *in vivo* study indicated that compound 7e could retard tumor growth of A549 xenograft models. These findings support the further investigation on the anti-tumor potential of this class of compounds as HDAC inhibitor.

1. Introduction

Epigenetic aberrations, which are considered as crucial drivers of various human cancers, are often caused by genetic defects that contributes to functional deregulation of epigenetic proteins, their altered expression and/or their atypical recruitment to certain gene promoters [1,2]. Due to the reversibility of epigenetic changes, epigenetic enzymes and regulatory proteins are recognized as ideal targets for small molecules [2–4].

Histone deacetylases (HDACs), that deacetylate lysines on core histones and other cellular proteins, plays the key roles in the epigenetic regulation of gene transcription and controlling cellular functions, such as cell-cycle, terminal differentiation, apoptosis, migration, invasion and angiogenesis [5,6]. Until now, there were 18 mammalian HDAC isoforms have been identified in humans, which could be divided into four classes according to their homology to yeast prototypes [7]. Class I HDAC proteins includes HDAC-1, 2, 3 and 8, whereas class II includes HDAC-4, 5, 6, 7, 9 and both these classes are zinc-dependent enzymes. The class III HDACs called Sirtuins are the NAD+-dependent HDACs. Class IV has only one member, HDAC11. Among all of the HDACs, class I, II and IV have been revealed to be closely related to tumorigenesis [8–10]. Our previous and others reports demonstrated that HDACs were overexpressed in a multitude of human cancers [11–13], further suggesting its important role in the development and progression of cancer.

Currently, several small synthetic molecules and natural products of HDACIs have been advanced into clinical trials [14,15]. Importantly, anti-tumor drugs vorinostat (SAHA), romidepsin (FK228), belinostat (PXD101) and panbinostat have been approved by FDA [16,17], thus providing clinical validation of this therapeutic strategy. However, the limited efficacy in solid tumor [18] and undesirable adverse reactions [19] of the approved HDACIs promote us to make extensive efforts to generate numerous HDACIs

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belonging to diverse chemical classes.

Because HDACs are enzymes with zinc ion, various series of compounds with the hydroxamic acid and *N*-(2-aminophenyl) amide as zinc-chelating moiety were reported and showed powerful HDAC inhibition activities [20]. Here we disclosed some cinnamoyl compounds including the two moieties and evaluated their activity in order to obtain potential HADC inhibitors.

2. Materials and methods

2.1. Chemistry

2.1.1. General

Melting points were determined with a X-4 apparatus and were uncorrected. The NMR spectra were recorded on Bruker Ascend 400 or Bruker UltraShield 600. All ^{1}H-NMR spectra were run at 400 MHz or 600 MHz in deuterated dimethylsulphoxide (DMSO-d_6). Chemical shifts (δ_H) are reported relative to TMS as internal standard. All coupling constant (J) values are given in hertz. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. Mass spectra were measured on Agilent 1100 Series MSD Trap (SL) spectrometer. Analytical thin layer chromatography (TLC) on silica gel plates containing UV indicator was routinely employed to follow the course of reactions and to check the purity of products. All reagents and solvents were purified and dried by standard techniques. The synthesis was shown in Supplementary Materials and Methods, the purities of all the target compounds were more than 98.0% by high performance liquid chromatography (HPLC).

2.2. Biology

2.2.1. Cell lines and cell culture

Human promyelocytic leukemia cells HL60, human cutaneous T cell lymphoma cells HuT102, human lung cancer cells A549 and human normal mammary epithelial cell line MCF-10A were obtained from the American Type Culture Collection (MFanassas, VA). Primary human umbilical vascular endothelial cells (HUVECs) were purchased from Life Technology. The cells were routinely cultured in RPMI-1640 or MEM medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C in a humidified incubator with 5% CO_2. All cell lines used were between passages 3 and 8 for each experiment.

2.2.2. HDAC activity assay

The A549 nuclear protein concentration was measured by BCA protein assay (Beyontime, CHN). The *in vitro* HDAC assay was performed with an HDAC fluorescent activity assay kit (Biovison, USA) as our previous reported. Briefly, proteins were incubated with different concentrations of compounds and SAHA (Sigma, USA) at 37 °C for 30 min in the presence of an HDAC fluorimetric substrate. The HDAC assay developer (which produces a fluorophore in reaction mixture) was added, and the fluorescence was measured using a microplate reader (Molecular Devices). HDAC activity is presented as the means ± SD of three determinants.

2.2.3. HDAC isoforms activity assay

The HDAC1, 3, 6, and 8 activity was assessed using commercial kits (BPS bioscience, USA). Briefly, the purified HDACs proteins were incubated with different concentrations of 7e and SAHA at 37 °C for 30 min in the presence of an HDAC fluorimetric substrate containing an acetylated lysine side chain. The HDAC assay developer (which produces a fluorophore in reaction mixture) was added, and the fluorescence was measured using a microplate reader (Molecular Devices). HDAC isoforms activity is presented as the means ± SD of three determinants.

2.2.4. Anti-proliferation assay

The *in vitro* anti-proliferation effects of compounds were determined by MTT assay. The cells (1×10^5 cells/ml) were seeded into 96-well culture plates. After overnight incubation, the cells were treated with various concentrations of agents for 48 h. Then 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (2.5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. After centrifugation (2500 rpm, 10 min), the medium with MTT was aspirated, followed by the addition of 100 μl DMSO. The optical density of each well was measured at 570 nm with a microplate reader (Molecular Devices).

2.2.5. Western blot analysis

The western blot analysis was performed as our previous reported [11]. The cells were lysed in lysis buffer (1% NP40, 20 mM Tris-HCl, pH 8, 130 mM NaCl, 10 mM NaF, 10 μg/ml aprotinin, 40 μM leupeptin, 1 mM DTT, 1 mM Na_3VO_4 and 1 mM PMSF) by incubating on ice for 30 min. Then, denatured proteins were electrophoresed on SDS-PAGE, transferred to PVDF membranes, and incubated at room temperature for 1 h or at 4 °C overnight with anti-Ac-H3 antibody (1:400; Millipore, USA), anti-Ac-H4 antibody (1:400; Millipore, USA), anti-H3 antibody (1:500; Cell Signaling Technology, USA), anti-H4 antibody (1:500; Cell Signaling Technology, USA), and anti-β-actin antibody (1:2000; Santa Cruz Biotechnology, USA). After washing in Tris buffered saline with 0.05% Tween 20 (TBS-Tween), blots were incubated with horseradish peroxidase-conjugated antibodies: goat-anti-mouse IgG (1:5000; Santa Cruz Biotechnology). Finally, blots were developed using the enhanced chemiluminescence system (ECL Plus, Amersham Pharmacia Biotech). Results were normalized to the internal control β-actin.

2.2.6. Flow cytometry assay

The flow cytometry assay was performed as our previous reported [21]. Briefly, about 1×10^6 HL60 and A549 cells were harvested at room temperature after pre-treatment with 7e for 24 h. The supernatant was removed, and ice-cold 70% ethanol was added. Ethanol-fixed cells were re-suspended in PBS containing 0.1 mg/ml RNase and incubated at 37 °C for 30 min. The pelleted cells were suspended in 1.0 ml of 40 μg/ml propidium iodide (PI) and analyzed by using a flow cytometer (Becton Dickinson, San Jose, CA). The cell cycle distribution was estimated according to standard procedures. The percentage of cells in the different cell cycle phases (G0/G1, S, or G2/M phase) was calculated using CELLQuest (Becton Dickinson) software. The cells of sub-G1 peak were considered as apoptosis.

2.2.7. In vivo antitumor efficacy studies

To determine the *in vivo* anti-tumor activity of 7e, viable A549 cells (5×10^6/100 μl PBS per mouse), as confirmed by trypan blue staining, and then subcutaneously (s.c.) injected into the right flank of male NOD/SCID mice. When the average s.c. tumor volume reached 50 mm^3, the mice were randomly divided into various treatment and control group (4 mice per group). Tumor size was measured once every three days with a caliper (calculated volume = shortest diameter^2 × longest diameter/2). Body weight, diet consumption and tumor size were recorded once every three days. After 14 days, the mice were sacrificed. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Shenyang Pharmaceutical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Shenyang Pharmaceutical University.