

# Supporting Information

## Design and Optimization of Novel Hydroxamate-Based HDAC Inhibitors of Bis-substituted Aromatic Amides Bearing Potent Activities against Tumor Growth and Metastasis

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### Contents:

A. The Docking studies of selected compounds with HDAC2 and 8.	S2-S4
B. HDAC activity assay.	S4
C. Histone acetylation analysis.	S4-S5
D. <i>In vitro</i> proliferation assay.	S5
E. <i>In vitro</i> migration assay.	S5
F. <i>In Vitro</i> anti-invasion assay.	S5-S6
G. Immunofluorescence assay.	S6
H. Western blot.	S6
I. <i>In vivo</i> MDA-MB-231 xenograft model.	S7
J. <i>In vivo</i> MDA-MB-231 metastasis model.	S7
K. Pharmacokinetic study of <b>11b</b> in rats.	S7-S8
L. Additional references	S8-S9

**The Docking studies of selected compounds with HDAC2 and 8.** To briefly explore the possible reasons that the linker of this series of compounds with five-methylene showed better activities than the one with six-methylene, docking assays were performed by using Autodock Vina<sup>1</sup> and the graphics were obtained by using UCSF Chimera 1.9.<sup>2</sup> For the docking assays, the center of box was the zinc finger zone and the size of box was 40\*40\*40. The number of conformation docking was 50. The docking assays were carried out by using the two crystals of HDAC2 and HDAC8 with **1** complex (HDAC2-**1**, PDB ID: 4LXZ; HDAC8-**1**, PDB ID: 1T69) through searching the RCSB Protein Data Bank ([www.rcsb.org](http://www.rcsb.org)). Compounds **8d** and **8e** were selected as examples to be docked with HDAC crystals. Through analyzing the binding mode of HDAC2-**8d** and HDAC2-**8e**, the results showed that one hydrogen bond was formed by the carbonyl of compound **8d** with the N-H group of imidazolyl group of His183. However, the hydrogen bond was broken despite the CAP group of **8e** occupying the active pocket which was similar with compound **8d** (Figure S1). This binding mode might explain to some extent why compound **8e** with a six-methylene linker would decrease the interaction with HDAC2 than the analogue with a five-methylene linker.

The docking assays of **8d** and **8e** with HDAC8 (Figure S2) indicated similar results as those with HDAC2. One hydrogen bond was formed by the carbonyl of compound **8d** with the N-H group of imidazolyl group of His180. However, the hydrogen bond between the carbonyl of compound **8e** and the N-H group of imidazolyl group of His180 was also broken while the CAP group of **8e** was bound to the same active

pocket of HDAC8 which was similar with compound **8d** (Figure S2). This docking results also suggested that the hydrogen bond was broken in those compounds with the longer linker.

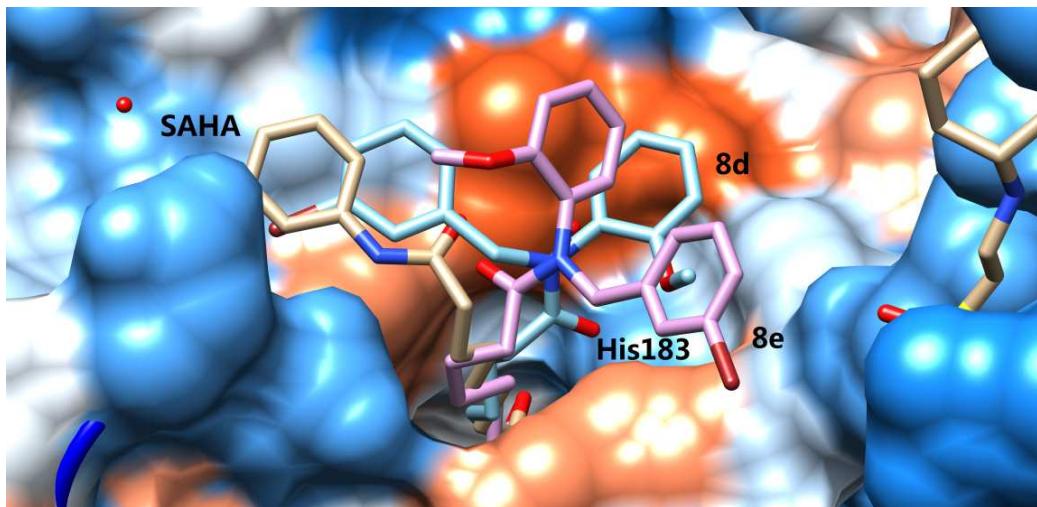


Figure S1. The binding mode of Compound **8d**, **8e** and **1** with HDAC2. **1** was colored with the grey, **8d** is colored with the blue and **8e** with the purple.

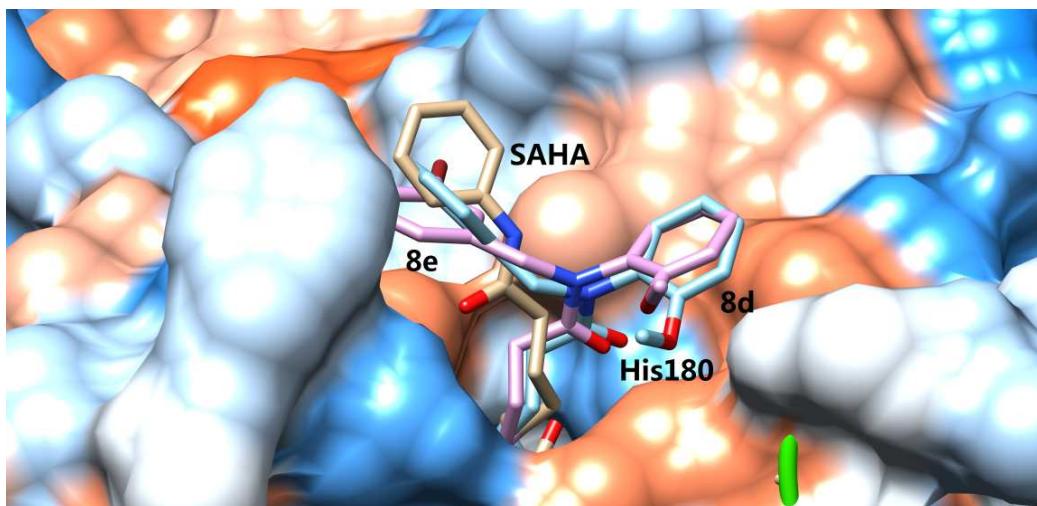


Figure S2. The binding mode of Compound **8d**, **8e** and **1** with HDAC8. **1** was colored with the grey, **8d** is colored with the blue and **8e** with the purple.

**HDAC activity assay.** HDAC activity assays were performed as previously reported.<sup>7</sup>

Briefly, HDAC enzyme solution (HeLa nuclear extract, HDACs 1-11) was incubated with compounds at different concentrations in the presence of HDAC substrate (Boc-Lys (Ac)-AMC) at 37 °C for 60 min. Then the lysine developer was added to stop the reaction. After 30 min, the data was recorded in a fluorescence plate reader with excitation at 355 nm wavelength and emission at 460 nm wavelength.

**Histone acetylation analysis.** The acetylation status of histone 3 and 4 was determined by immunoblot analysis. Briefly, after exposure to **1** or other compounds, cells were lysed using RIPA buffer [(50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 1 % sodium deoxycholic acid, 0.1 % SDS, 2 mM phenylmethylsulfonyl fluoride (PMSF), 30 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and protease or phosphatase inhibitor cocktail (Sigma-Aldrich, Inc. Shanghai, China)]. Samples were run on 15% SDS-PAGE gels. Gels were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) and incubated overnight using indicated antibodies. Actin expression as determined by Western blot using anti-actin antibody (Sigma) was used as loading control.

**In vitro proliferation assay.** MTT proliferation assay was performed as described previously.<sup>3</sup> Human breast cancer cells (MDA-MB-231, MCF-7 and T47D) and mouse breast cancer cells (4T1) were seeded at a density of  $5 \times 10^3$  in 96-well plates, and treated with indicated concentrations of compounds. After incubation for 48 h, Aqueous One Solution (Promega, Madison, WI) was added, and the absorption was measured at 490 nm.

***In vitro* migration assay.** The inhibitory effect of compounds on tumor cell migration was detected as described previously.<sup>4</sup> Tumor cells (MDA-MB-231) were seeded on a six-well plate. Upon reaching about 90% confluence, the cell monolayer was scraped by a sterile 0.1 mL pipette tip to create wounds, and fresh medium was added containing different concentrations of compound samples. After 10 h incubation at 37 °C, 5% CO<sub>2</sub>, cells were fixed and photographed. The migrated cells were manually quantified. The percentage inhibition of migration was expressed using 100% as the number of migratory cells in the untreated control group.

***In vitro* anti-invasion assay.** A modified Boyden chamber (8- $\mu$ m pore size) with a Matrigel (-precoated membrane filter insert was used in this assay. Briefly, tumor cells (MDA-MB-231,  $5 \times 10^4$ ) in 100  $\mu$ L serum-free medium were added in the upper chamber and 600  $\mu$ L of medium (containing 10% FBS) was added in the bottom chamber. Different concentrations of compound samples were added to both chambers. Cells were allowed to migrate about 12 h. Non-invasive cells in the upper chamber were removed with a cotton swab, then fixed in 3.7% paraformaldehyde and stained with 0.1% crystal violet. Photographs were taken using an inverted microscope and the cells were quantified manually to calculate the number of invaded cells.<sup>5</sup>

**Immunofluorescence assay.** Immunofluorescence assay was performed as previously described.<sup>6</sup> MDA-MB-231 cells were plated on gelatin-coated glass coverslips. After treatment for indicated times, cells were fixed with 4% PFA in PBS for 30 min, permeabilized for 5 min with 0.5% Triton X-100 in PBS, and blocked with 1% BSA

in PBS for 20 min. Coverslips were incubated with anti - Paxillin at 4°C overnight and then washed with PBS and incubated for 1 h with Alexa 488 – conjugated secondary antibody. FITC-phalloidin and 4', 6-diamidino-2-phenylindole (DAPI) were further used to stain F-actin and nuclei respectively. Photographs were obtained with a confocal microscope.

**Western blot.** Western blot assay was performed as previously described.<sup>3</sup> Whole cell lysates were prepared in RIPA buffer supplemented with proteinase inhibitor cocktail (Roche, Basel, Switzerland). Cellular protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes and blocked with 5% BSA. The membranes were then probed with indicated primary antibodies followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody. The bound antibodies were detected using the ECL detection system.

**In vivo MDA-MB-231 xenograft model.** MDA-MB-231 (human breast tumor cell) xenograft model was prepared as described previously.<sup>7, 8</sup> To better picture the inhibitory effect on antitumor by target compounds, MDA-MB-231-Luciferased cells (encoding the luciferase reportor gene) were used in this experiment. MDA-MB-231-Luciferased cells ( $1 \times 10^6$ ) were implanted in the abdominal mammary fat pad of female nude mice (BALB/c-*nu*). On the day 4, the mice were randomized to six per group and dosed intraperitoneally with DMSO (control group), **11b** (10 mg/kg/day or 30 mg/kg/day) or **1** (30 mg/kg/day) for 30 days. Tumor growth inhibition was detected by a Xenogen IVIS 2000 Biophotonic Imager.

**In vivo MDA-MB-231 metastasis model.** These experiments were performed as described previously.<sup>6</sup> In brief,  $1 \times 10^7$  MDA-MB-231-Luciferased cells in 0.1 mL PBS were injected into the tail vein. The mice were divided into four groups (n = 6). DMSO or **11b** (10 mg/kg/day, 30 mg /kg/day) or **1** (30 mg/kg/day) was given by intraperitoneal injection from day 0 to 30. After treatment for 30 days, images of xenograft mice were taken using a Xenogen IVIS 2000 Biophotonic Imager.

**Pharmacokinetic study of 11b in rats.** Sprague-Dawley rats were fasted for 12 h with free access to water prior to the pharmacokinetic study. The rats were randomized into two groups for intravenous injection and oral administration. Blood samples were collected from the orbital plexus into heparinized centrifuge tubes at 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, 8 h and 12 h after oral administration, and 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h and 5 h after intravenous administration.

The concentration of **11b** in plasma was determined by an Agilent 1290 LC system coupled with 6460 triple-quadrupole mass spectrometer (Agilent Technologies, USA), which was equipped with an Agilent Jet Stream electrospray ionization ionization (ESI) source. Chromatography separation was performed on an Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8  $\mu$ m; USA). The mass spectrometer was operated in the negative ESI mode, and the detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 455.2 precursor [M + H]<sup>+</sup> to the m/z 175.1 product ion for **11b**. The lower limit of quantification (LLOQ) was 2 ng/mL with a linear range of 2 - 2000 ng/mL. The

pharmacokinetic parameters were calculated by WinNonlin software version 5.2.1 based on noncompartmental analysis.

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