Supplementary Information (SI) for ChemComm.
This journal is © The Royal Society of Chemistry 2025

Supplementary Information for

A pyrene-based HDACs inhibitor for dual-action therapy of breast cancer

Qizhang Li[a], Xing Lu [a], Yuanli Yang [a], Tianyu Li[b], Xuehua Chen[a], Rongmiao Yuan[a], Xin Ding[b, *], Silong Zhang[a, *], and Huan He[a, *]

[a] Key laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), Guizhou Provincial Key Laboratory of Innovation and Manufacturing for Pharmaceuticals, Guizhou University, Guiyang 550025, Guizhou Province, P. R. China

[b] Hubei Key Laboratory of Pollutant Analysis & Reuse Technology, Hubei Normal University, Huangshi 435002, P. R. China

* Corresponding authors. Email: dingx@hbnu.edu.cn, silongzhang@whu.edu.cn, ivy@whu.edu.cn.

Table of Contents

1. Experimental methods

Materials

Unless otherwise noted, all the chemicals and reagents were purchased from Bide Pharmatech Ltd. Solvents for chemical reactions were distilled before use.

Measurements

^{1}H NMR and ^{13}C NMR spectra were recorded on an Agilent DD2 600 spectrometer (600 MHz, ^{1}H NMR; 101 MHz, ^{13}C NMR) at room temperature. NMR spectra were calibrated to the solvent signals of $CDCl_3$ (δ 7.26 and 77.16) and tetramethylsilane (TMS) as reference. The chemical shifts are provided in ppm and the coupling constants in Hz. The following abbreviations for multiplicities are used: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet. Glassware was oven-dried, assembled while hot, and cooled under an inert atmosphere. Unless otherwise noted, all reactions were conducted in an atmosphere. Reaction progress was monitored using analytical thin-layer chromatography (TLC). Visualization was achieved by UV light (254 nm and 365nm). Chromatography was performed with silica gel (0.040-0.063 mm) packing. The UV−vis absorption spectra were performed using a SHIMADZU UV2550 spectrophotometer.

HDAC6 enzyme inhibition assay

The experiment on enzyme activity inhibition was collaboratively completed by Shanghai ChemPartner Co., Ltd. HDAC activity was quantified in vitro using the

Fluorogenic HDAC6 Assay Kit (BPS Bioscience, CA), adhering strictly to the manufacturer's guidelines. To initiate the enzymatic reaction, a mixture containing 15 microliters of enzyme solution, 10 microliters of substrate peptide solution, and differing concentrations of the compounds under investigation was prepared at a temperature of 37 °C. A microplate reader was employed to monitor the emission at 460 nm (with an excitation wavelength of 355 nm) over a period of 30 minutes.

$^1O_2$ detection assay by ABDA

9,10-Anthracenediylbis(methylene)dimalonic acid (ABDA) was used as an indicator to evaluate the $^1O_2$ generation ability under light irradiation. ABDA (50 μM) solution was mixed with the nanoparticle suspensions of **PySAHA** in water (2 μM) and then was exposed to LED light irradiation (20 mW/cm^2, 460 ± 10 nm). The absorbance decrease of ABDA at 400 nm was recorded at a time interval of 2.5 min.

Cell proliferation assay

The ATCC served as the source for the MCF-7 breast cancer cell line. Cells in the exponential growth phase were then seeded into 96-well plates, with each well containing 10,000 cells. These plates were further incubated for 48 hours to monitor any variations in cell proliferation. To assess the viability and proliferation of the cells, we utilized the MTT assay, which stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and is a commonly used method.

In vitro ROS determination by DCFH-DA

MCF-7 cells were incubated with **PySAHA** for 4 h, and then exposed to light irradiation (20 mW/cm^2, 460 ± 10 nm) for 5 min. Then the cells were collected and suspended in PBS containing 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 0.5 μM). After 15 min, the fluorescence of each cell were recorded with a flowcytometry.

Cell apoptosis assay

MCF-7 cells were incubated with **PySAHA** for 4 h, and then exposed to light irradiation (20 mW/cm^2, 460 ± 10 nm) for 5 min. MCF-7 cells were resuspended in phosphate buffer saline and stained with 2 μL Annexin V-FITC (50 μg/mL) for 15 min and 1 μL PI (50 μg/mL) for 5 min. The signal of FITC and PI was recorded by a flow cytometry.

Western blot analyses

MCF-7 cells were subjected to **PySAHA** treatment for a duration of 24 hours. Post-treatment, cellular proteins were extracted to prepare cell lysates. These lysates were then fractionated using 8% SDS-PAGE gels. The fractionated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane through the process of electroblotting. The PVDF membrane was incubated with specific primary antibodies: Rabbit anti-HDAC1 (diluted 1:5000, HUABIO, Hangzhou, China), anti-HDAC2 (diluted 1:40000, HUABIO, Hangzhou, China), anti-HDAC6 (diluted 1:1000, CST),

and Mouse Anti-β-actin (diluted 1:5000, Pumeike). To eliminate unbound antibodies and excess reagents, the membrane was washed with Tris-Buffered Saline. Subsequently, the membrane was incubated with Goat anti-Rabbit IgG (diluted 1:6000, Wuhan Kerui) and Goat anti-Mouse IgG (diluted 1:6000, Wuhan Kerui) secondary antibodies. After washing with TBS containing 0.1% Tween-20 to remove unbound secondary antibodies, the protein bands were visualized using enhanced chemiluminescence (ECL). Finally, the ImageJ software was utilized to conduct a quantitative analysis of the detected protein bands.

In vivo antitumor studies

Female Balb/c mice were sourced from Beijing Vital River Laboratory Animal Technology Co., Ltd., with all experimental procedures conducted in strict adherence to the ethical standards stipulated by the Principles of Laboratory Animal Care and Use Committee of Guizhou University (Approval No. EAE-GZU-2025-T033). The mice were implanted with MCF-7 cells on their dorsal regions, and upon reaching a tumor diameter of approximately 4 mm, they were randomly assigned to five groups, each comprising five mice. The groups were categorized as follows: Group I (vehicle control), Group II (only light group), Group III (10 mpk **PySAHA**), and Group IV (10 mpk **PySAHA** and light). The compounds were dissolved in saline and administered to the mice through intravenous injection. Tumor volumes were carefully monitored over a period of 12 days, with tumor length and width recorded every three days. Tumor volumes were calculated using the formula: length × width × width × 0.5. The tumor

growth inhibition (TGI) rate was determined using the formula: %TGI = (1-($V_{t(treat\ group)}$-$V_{0(treat\ group)}$)/($V_{t(vehicle\ group)}$-$V_{0(vehicle\ group)}$))×100%, where V_t is the average tumor volume of a group on the specific day, and V_0 is the average tumor volume of a group on the initial day. At the conclusion of the study, the tumors and vital organs were excised for further analysis.

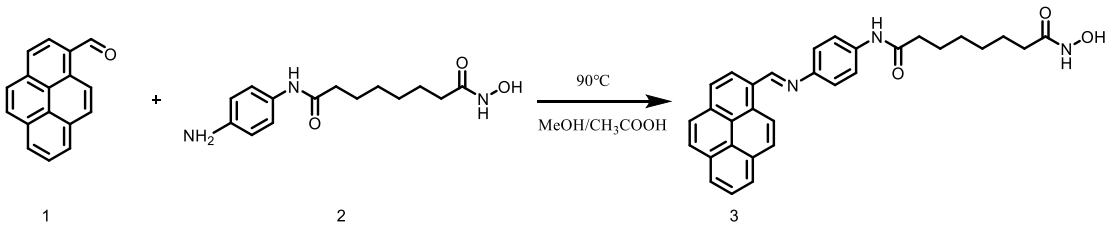
Molecular docking simulations

The simulations were calculated on Sybyl 8.0 software in a Molecular Simulation Workstation (Guizhou Qianshuo Technology Co., Ltd) equipped with Intel Xeon 8369B CPUs. The three dimensional structure of HDAC6 was obtained from RCSB databank (PDB ID5WGL). The docking box were defined around the existing molecule which occupied the ligand site. A threshold value of 0.4 and a bloat value of 5 were set for defining the box. The structure of the ligands was constructed in SYBYL 8.0 software. A total of 20 docking pose were generated and the one that has a highest docking score were selected for analysis. The binding poses were visualized by VMD 1.9.3 software.

Statistical analysis

Data were shown as mean ± standard deviation. Significance was calculated by Student's t-test by using Origin software.

2. Synthetic procedures and characterization data



1 2 3

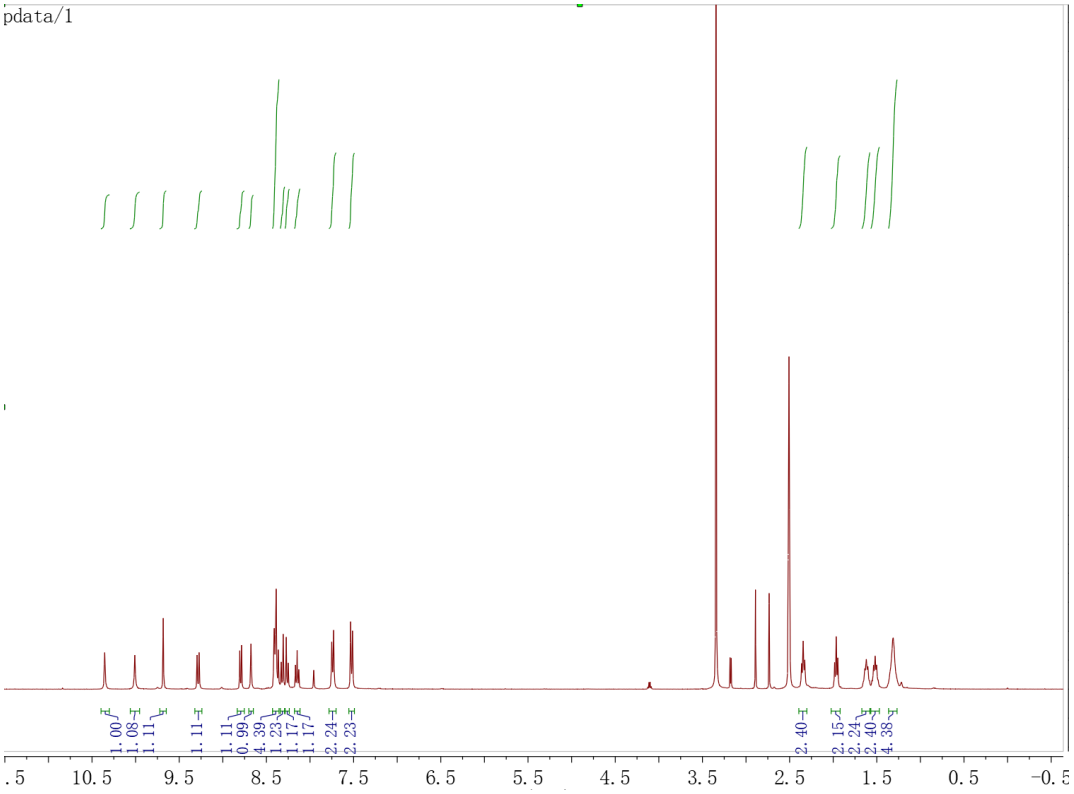
Synthesis of (*E*)-N^1-hydroxy-N^8-(4-((pyren-1-ylmethylene)amino)phenyl)octane diamide (3)

Pyrene-1-carbaldehyde (200 mg, 1mmol) and N^1-(4-aminophenyl)-N^8-hydrox yoctamidide (242 mg, 1mmol) were dissolved in 30 mL of MeOH, and 5 mL of glacial acetic acid was added. The reaction mixture is stirred at 70 °C for 5h. After the reaction was completed, the solvent was evaporated, and the crud e product was purified by silica gel column chromatography in CH_2Cl_2/MeOH (20-40%), and 3 was a yellow solid (141 mg, 67.5%).

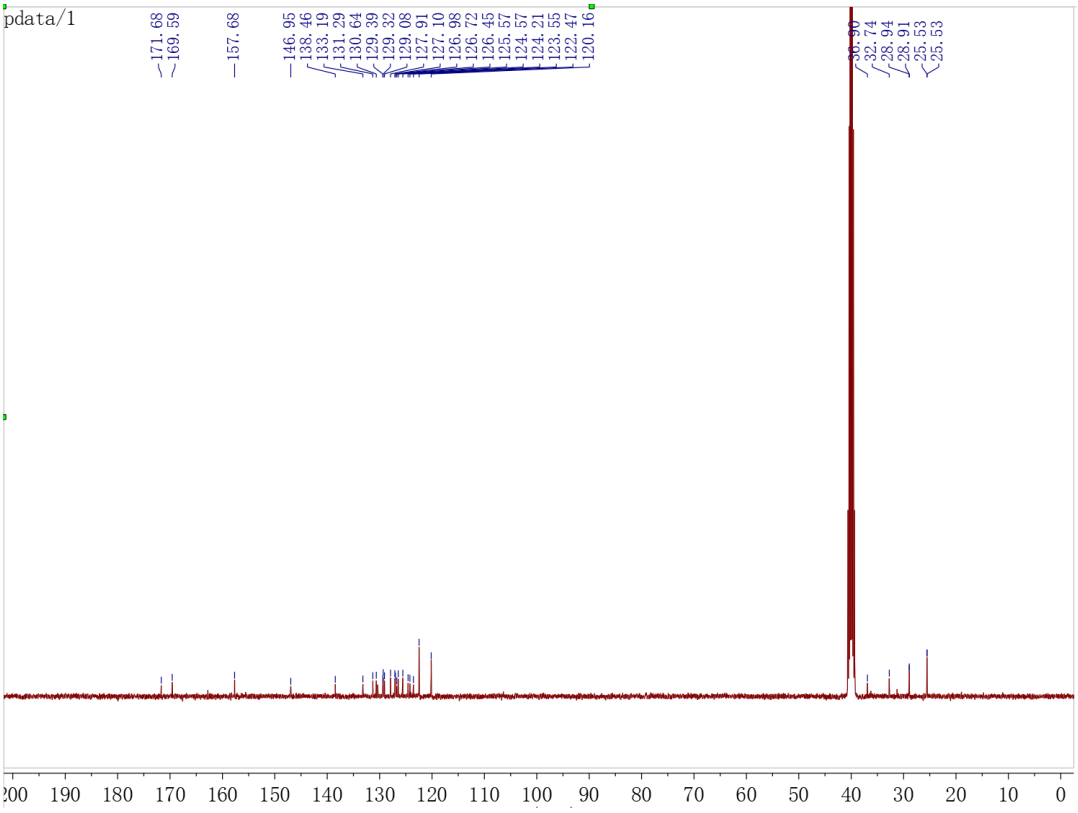
^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.35 (s, 1H), 10.01 (s, 1H), 9.68 (s, 1H), 9.28 (d, *J* = 9.3 Hz, 1H), 8.80 (d, *J* = 8.1 Hz, 1H), 8.68 (s, 1H), 8.43 – 8.37 (m, 4H), 8.32 (d, *J* = 8.9 Hz, 1H), 8.26 (d, *J* = 8.9 Hz, 1H), 8.15 (t, *J* = 7.6 Hz, 1H), 7.74 (d, *J* = 8.4 Hz, 2H), 7.52 (d, *J* = 8.5 Hz, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.97 (t, *J* = 7.4 Hz, 2H), 1.61 (q, *J* = 7.2 Hz, 2H), 1.52 (p, *J* = 7.3 Hz, 2H), 1.34 – 1.29 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 171.68, 169.59, 157.68, 146.95, 138.46, 133.19, 131.29, 130.64, 129.39, 129.32, 129.08, 127.91, 127.10, 126.98, 126.72, 126.45, 125.57, 124.57, 124.21, 123.55, 122.47, 120.16, 36.90, 32.74, 28.94, 28.91, 25.53, 25.53. ESI-HRMS m/z calcd for $C_{31}H_{29}N_3O_3H^+$ 491.22089, found 492.2281 [M + H] $^+$. HPLC purity ＞95%.

3. ^{1}H NMR and ^{13}C NMR Spectra of final compounds

^{1}H NMR of compound **PySAHA**



^{13}C NMR of compound **PySAHA**


pdata/1
171.68
169.59
157.68
146.95
138.46
133.19
131.29
130.64
129.39
129.32
129.08
127.91
127.10
126.98
126.72
126.45
125.57
124.57
124.21
123.55
122.47
120.16
36.90
32.74
28.94
28.91
25.53
25.53
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0

4. **HPLC and HRMS result**

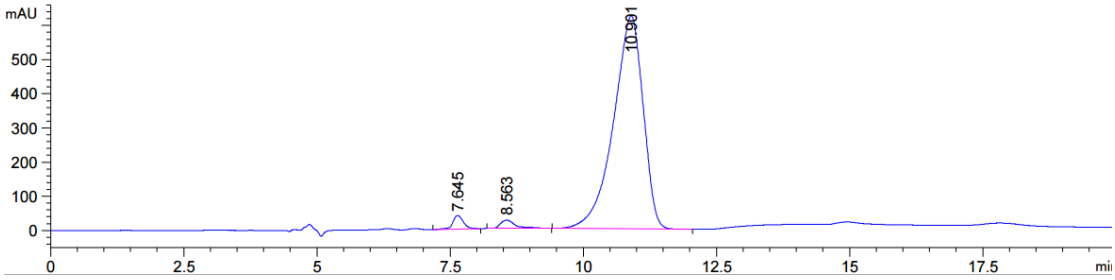
HPLC result and spectra

Table S1. HPLC result of final compound

Cmpd	Reverse Phase	Ret. Time (min)	Purity(%)
FT-30	95:5 MeCN:H_2O	10.901	95.8923

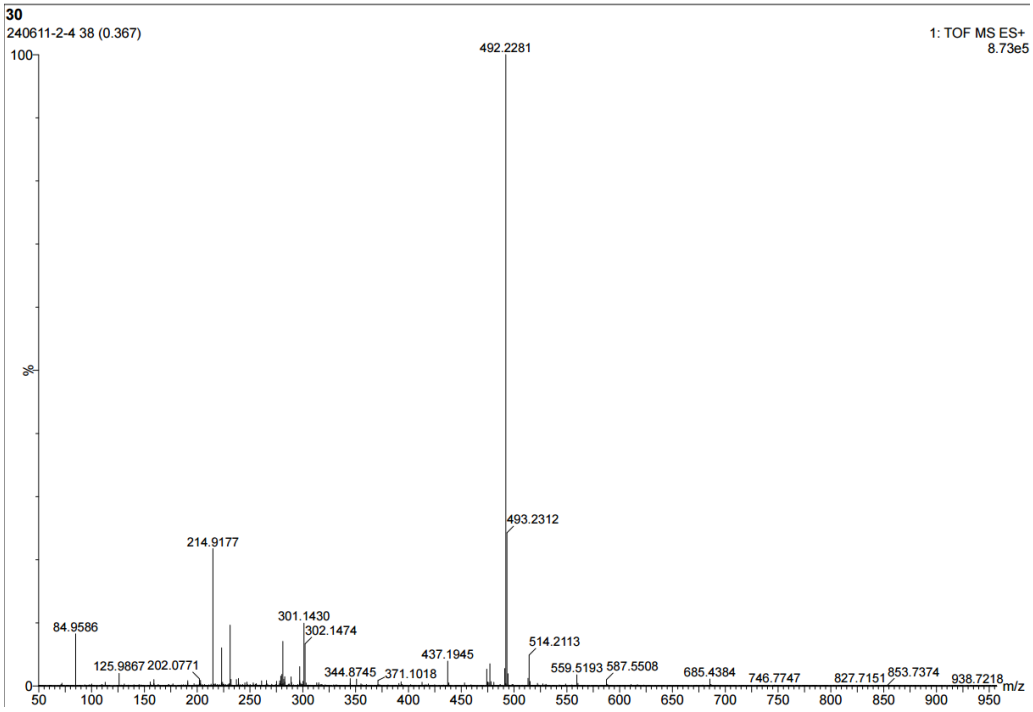
The HPLC was conducted on Agilent 1100 Series and all the results were obtained under the condition of UV 254nm.

Reverse Phase (Method 95:5MeCN:H_2O, Flow rate 1.0 mL/min)



No.	Retention Time (min)	Area (mAU*s)	Peak height (mAU)	Percent
1	7.645	551.08801	40.04818	2.1918
2	8.563	481.7236	24.13468	1.9159
3	10.901	24110.7	623.8396	95.8923
Total				100.0000

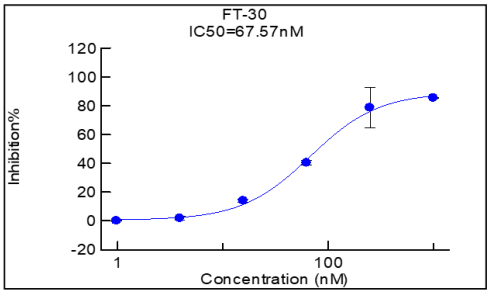
HRMS spectra of compound **PySAHA**



5. **Supplementary Figures**

A

Compound ID	Concentration (nM)	Inhibition%		Mean	SD
PySAHA	1000	85.2	85.6	85.4	0.3
	250	88.6	68.6	78.6	14.1
	63	41.3	39.4	40.4	1.4
	16	14.7	13.1	13.9	1.2
	3.9	1.0	2.6	1.8	1.2
	1.0	-0.2	0.2	0.0	0.3



B

Compound ID	Concentration (nM)	Inhibition%		Mean	SD
SAHA	10000	99.7	99.7	99.7	0.0
	2500	99.1	98.5	98.8	0.4
	625	95.6	96.1	95.8	0.3
	156	89.9	89.6	89.7	0.2
	39	73.3	71.9	72.6	0.9
	10	46.9	47.1	47.0	0.1
	2	18.0	17.1	17.5	0.6
	0.6	7.7	5.8	6.8	1.4
	0.15	4.2	4.9	4.5	0.5
	0.04	3.4	1.4	2.4	1.4

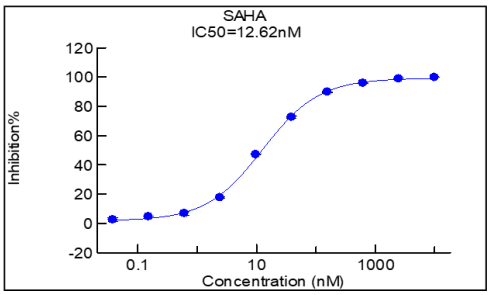


Figure S1. Inhibitory data for **PySAHA** (A) and **SAHA** (B) against HDAC6 determined by a fluorescent method.

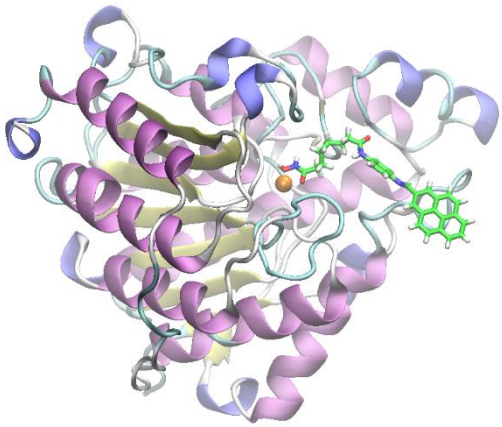


Figure S2. Binding pose of **PySAHA** with HDAC6 (PDB ID: 5wgl) computed by molecular docking simulation. The protein is represented by ribbons, and the catalytic Zn^{2+} is represented by an orange bead. **PySAHA** is represented by green sticks.

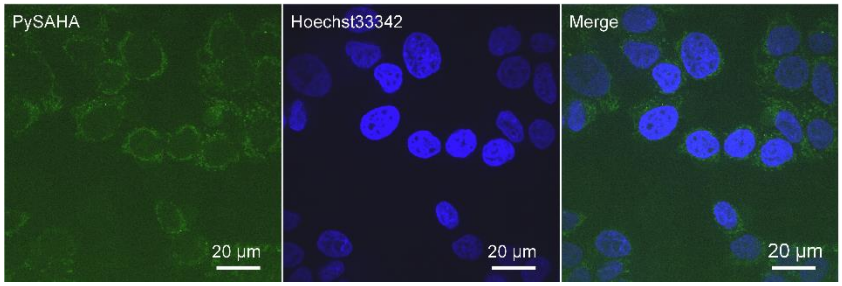


Figure S3. Fluorescent images of **PySAHA** (2 μM) in MCF-7 cells with/without the presence of **SAHA** (20 μM). Cells were treated with **PySAHA** and **SAHA** for 4 h, and the cell nucleus was stained with Hoechst33342 (0.5 μM).

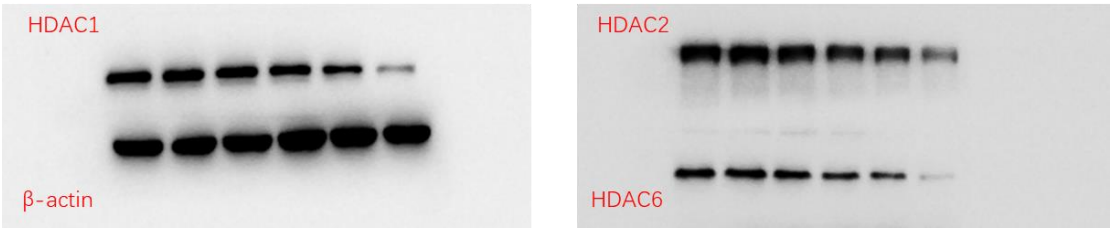


Figure S4. Uncropped raw data for immunoblotting studies in Figure 3A.

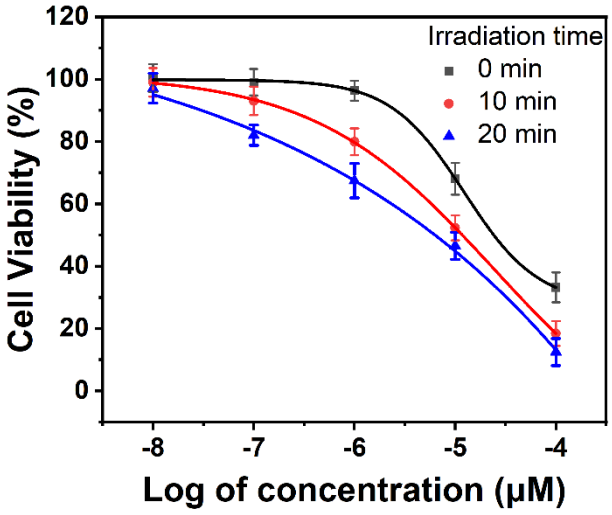


Figure S5. Viability of MCF-10A cells treated with **PySAHA** and light (460 ± 20 nm, 20 mW/cm^2). The IC_{50} for inhibiting the proliferation of MCF10A cells was 25.7 ± 4.4 μM. When the cells was irradiated with light for 10 and 20 min, the IC_{50} dereased to 12.5 ± 2.7 μM, and 6.8 ± 1.8 μM.

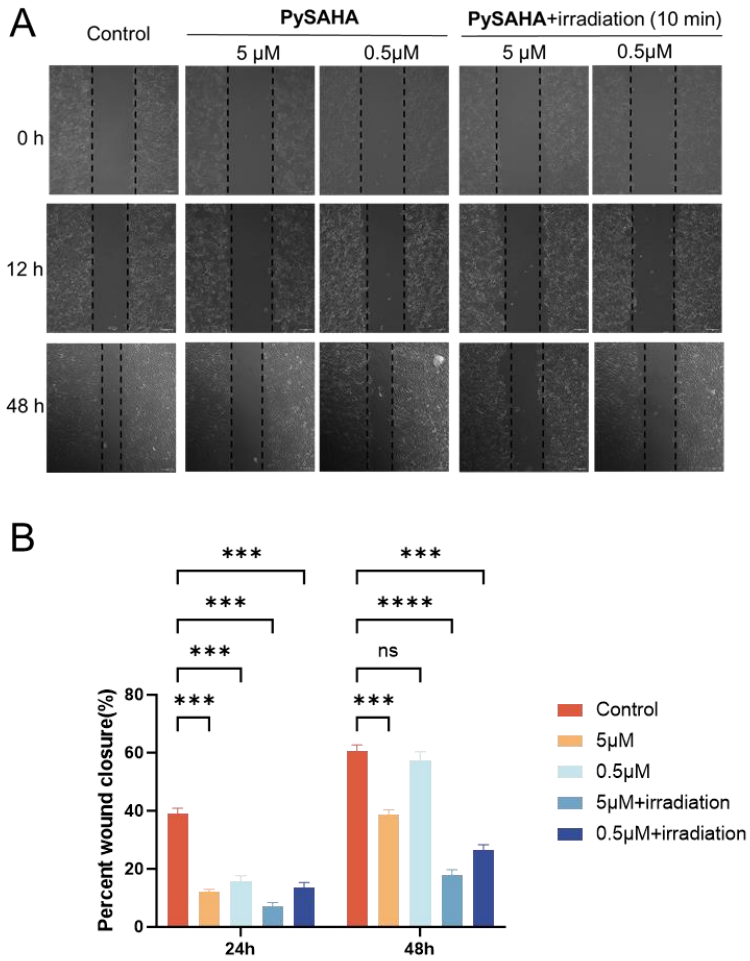


Figure S6. (A) Cell migration experiments for MCF-7 cells treated with **PySAHA** and light. (B) Qualitatively analysis of wound closure (%).

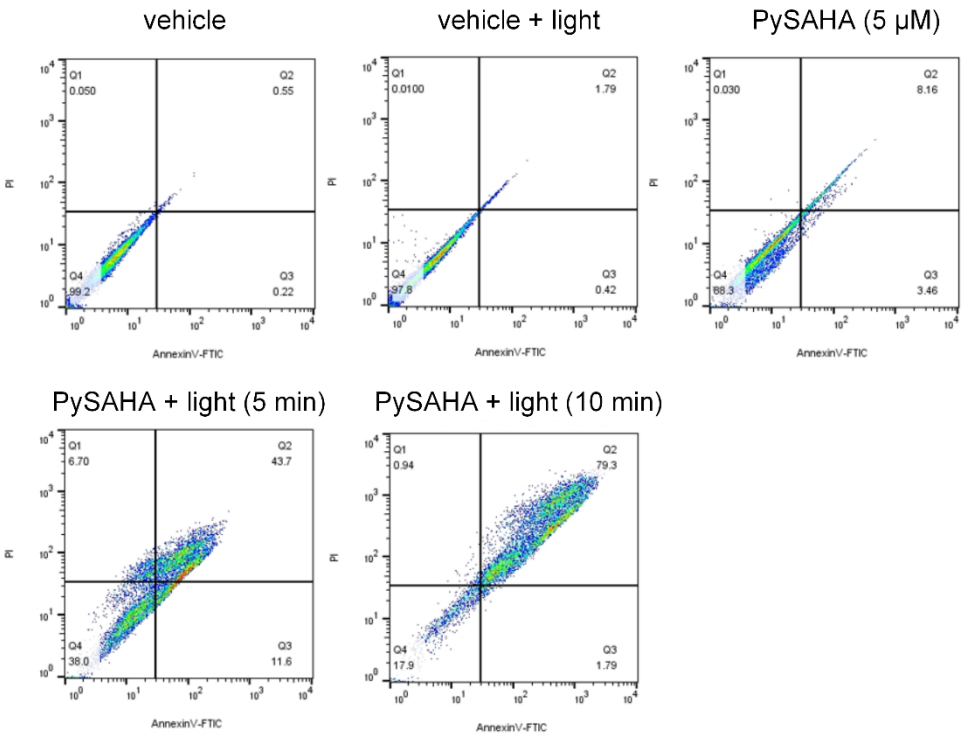


Figure S7. Cell apoptosis analysis determined by an Annexin V-FITC/PI Apoptosis Kit.

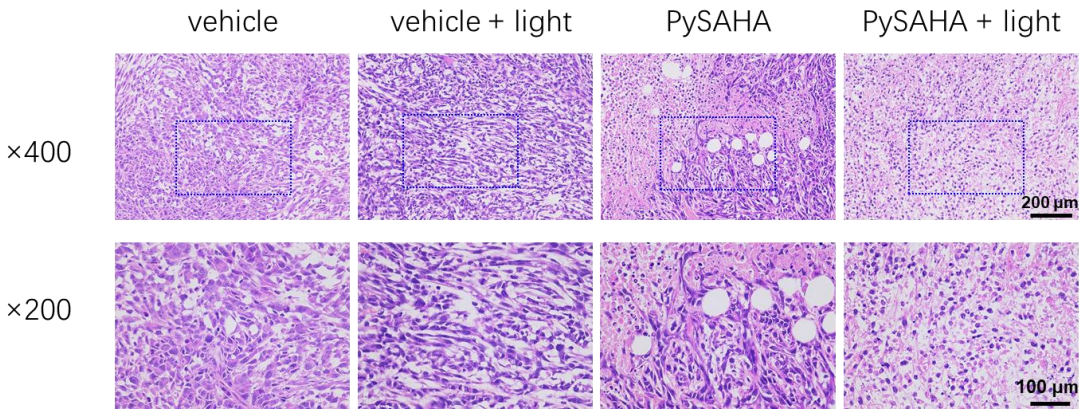


Figure S8. H&E (Hematoxylin and Eosin) staining results of dissected tumors in each group.

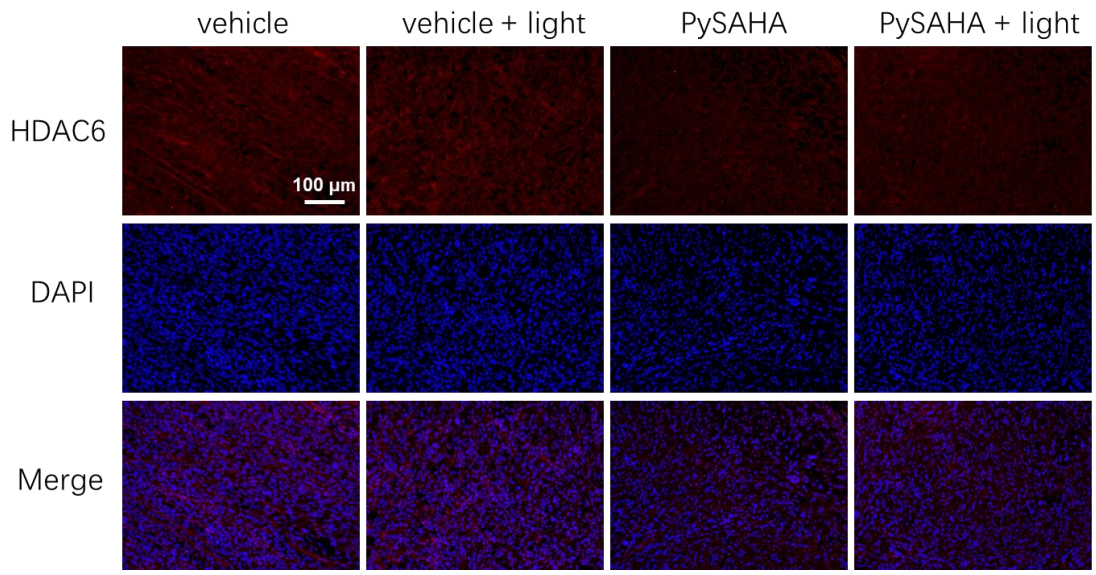


Figure S9. Immunofluorescent results of the HDAC6 levels in the dissected tumors.

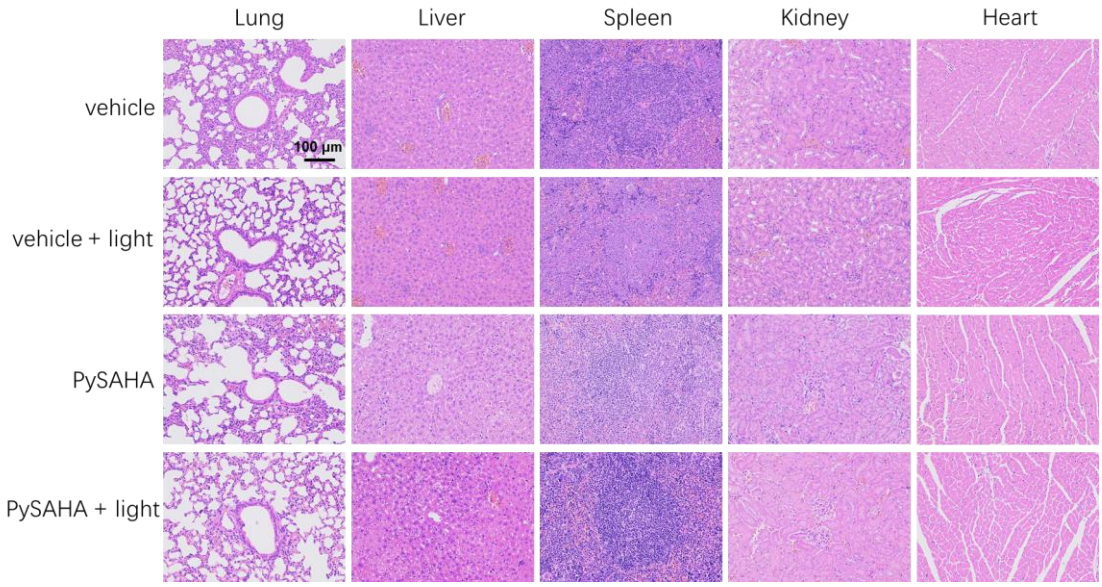


Figure S10. H&E (Hematoxylin and Eosin) staining results of major organs in each group.

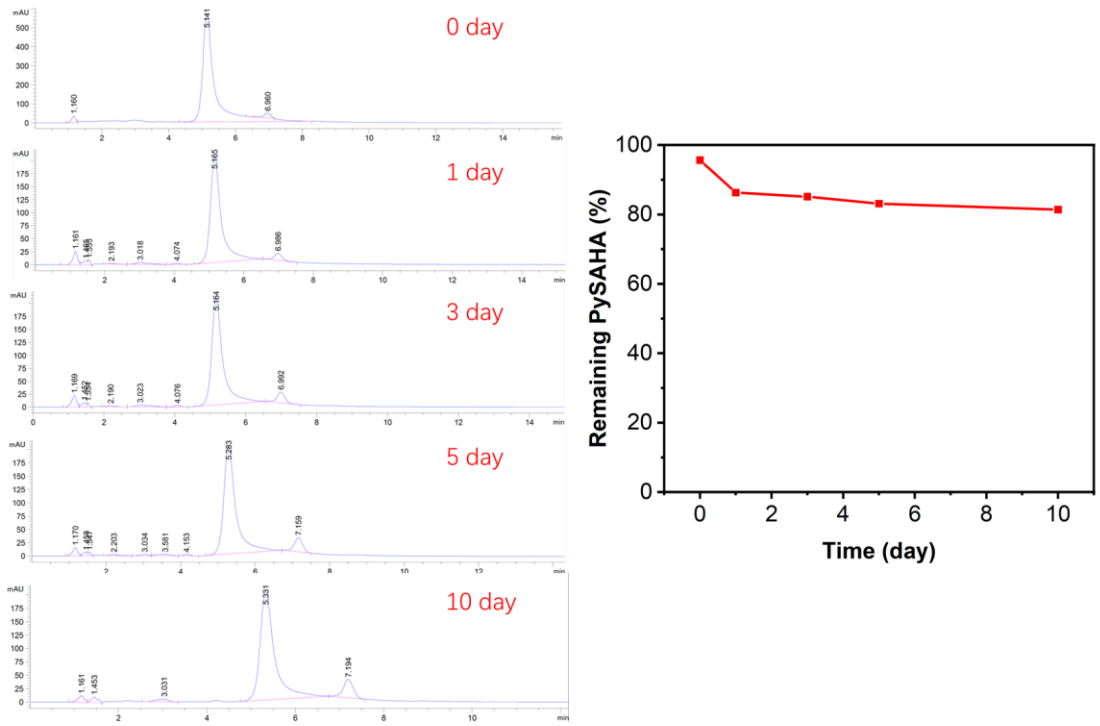


Figure S11. Determination of stability of **PySAHA** in PBS buffer by HPLC analysis.