A Selenium-Containing Selective Histone Deacetylase 6 Inhibitor for Targeted in vivo Breast Tumor Imaging and Therapy

Chu Tang, Yang Du, Qian Liang, Zhen Cheng, Jie Tian

*Engineering Research Center of Molecular and Neuro Imaging, Ministry of Education, School of Life Science and Technology, Xidian University, Xi’an, Shaanxi 710126, China

CAS Key Laboratory of Molecular Imaging, The State Key Laboratory of Management and Control for Complex Systems, Institute of Automation, Chinese Academy of Sciences, Beijing 100190, China;

Beijing Key Laboratory of Molecular Imaging, Beijing, 100190, China

University of Chinese Academy of Sciences, Beijing, 100080, China

Molecular Imaging Program at Stanford (MIPS), Department of Radiology, and Bio-X Program, Canary Center at Stanford for Cancer Early Detection, Stanford University, Stanford, California, 94305-5344
Materials and methods

1 Materials. All chemicals and solvents were purchased from commercial sources and were used without further purification. IRDye800CW-NHS ester was purchased from LI-COR, Inc. Fluorescein isothiocyanate (FITC), 17β-estradiol tamoxifen were obtained from Sigma-Aldrich. 6-Bromohexanoyl chloride, potassium selenocyanate, aniline, N-Boc-p-phenylenediamine and trifluoroacetic acid (TFA) were purchased from J&K Scientific Ltd. Dichloromethane, acetonitrile and triethylamine were dried over CaH₂ and distilled prior to use.

1H NMR and 13C NMR spectra were recorded on a Bruker Biospin AV400 (400 MHz) instrument. Chemical shifts are reported in ppm (parts per million) and are referenced to either tetramethylsilane or the solvent. The purification of the SelSA, FITC-SelSA and IRDye800CW-SelSA were carried out on a semipreparative reversed-phase HPLC system equipped with a diode array UV-vis absorbance detector (Agilent 1200 HPLC).

2 Synthesis of 7-bromoheptanoic acid phenylamide (3). To a 0 °C cooled solution of aniline (1.1457 g, 12.3 mmol) in dry CH₂Cl₂ (50 mL), triethylamine (1.8721 mg, 11.7 mmol), and 6-bromohexanoyl chloride (2.8822 g, 13.5 mmol) were added under Ar atmosphere. The reaction solution was allowed to warm to room temperature and was stirred for 24 h. Subsequently, the reaction mixture was poured into water and extracted with CH₂Cl₂ (3 × 20 mL). The extracts were dried (Na₂SO₄) and evaporated. The crude product was purified by silica gel column chromatography (Petroleum ether-EtOAc, 4:1) to yield bromo amide 3 as a yellow oil (2.9518 g, 89.2%). 1H NMR (400 MHz, CDCl₃) δ 7.52 (d, J = 7.6 Hz, 2H), 7.33 (t, J = 8.0 Hz, 2H), 7.12 (m, 2H), 3.53 (t, J = 7.2 Hz, 2H), 2.37 (t, J = 7.2 Hz, 2H), 1.84 (m, 2H), 1.75 (m, 2H), 1.51 (m, 2H).

3 Synthesis of 5-phenylcarbamoylpentyl selenocyanide (SelSA, 4). To a solution of bromo amide 3 (1.2563 g, 4.65 mmol) in dry acetonitrile (30 mL), KSeCN (670.1 mg, 4.65 mmol) was added. The reaction solution was stirred at room temperature for 24 h under Ar atmosphere. The solvent was removed by rotary evaporation to yield crude product that was purified by silica gel column chromatography (Petroleum ether-EtOAc, 1:1). The product SelSA was isolated (936.3 mg, 68.2% yield) as a pale yellow solid. 1H NMR (400 MHz, CDCl₃) δ 7.52 (d, J = 8.0 Hz, 2H), 7.34 (t, J = 7.6 Hz, 2H), 7.09 (br s, 1H, NH), 7.11 (t, J = 7.2 Hz, 1H), 3.06 (t, J = 7.2 Hz, 2H), 2.41 (t, J = 7.6 Hz, 2H), 2.00 (m, 2H), 1.81 (m, 2H), 1.56 (m, 2H). 13C NMR (100 MHz, CDCl₃) δ 170.84, 138.01, 129.21, 124.49, 119.95, 101.70, 37.35, 30.65, 29.37, 28.61, 24.67. MS (ESI): m/z 319.05 ([M+Na]⁺).

4 Synthesis of tert-butyl 4-(6-bromohexanamido)phenylcarbamate (6). The process was performed as in the preparation of 3 using N-Boc-1,4-phenylenediamine 5 instead of aniline to afford selenocyanide 6. A white solid was obtained with 80.9% yield; 1H NMR (400 MHz, DMSO-d₆) δ 9.71 (s, 1H), 9.21 (s, 1H), 7.43 (d, J = 8.8 Hz, 2H), 7.32 (d, J = 8.8 Hz, 2H), 3.37 (m, 2H), 2.24 (t, J = 7.6 Hz, 2H), 2.16 (t, J = 7.6 Hz, 2H), 1.55 (m, 2H), 1.45 (s, 9H), 1.28 (m, 2H).

5 Synthesis of tert-butyl 4-(6-selenocyanatohexanamido)phenylcarbamate (7). The process was performed as in the preparation of SelSA using bromo amide 6 instead of 3 to afford N-Boc protected selenocyanide 7. A yellow solid was obtained with 71.5% yield; 1H NMR (400 MHz, DMSO-d₆) δ 9.74 (s, 1H), 9.21 (s, 1H), 7.34 (d, J = 8.8 Hz, 2H), 7.34 (d, J = 8.8 Hz, 2H), 3.55 (t, J = 6.8 Hz, 2H), 2.28 (t, J = 7.6 Hz, 2H), 1.82 (m, 2H), 1.61 (m, 2H), 1.46 (s, 9H), 1.42 (m, 2H). 13C NMR (100 MHz, DMSO-d₆) δ 171.02, 153.09, 134.81, 133.41, 120.19, 119.42, 101.96, 78.97, 37.12, 30.67, 29.40, 28.63, 28.52, 24.24.

6 Synthesis of 5-(4-aminophenyl)carbamoylpentyl selenocyanide (8). Trifluoroacetic acid (1 mL) was added slowly to a solution of selenocyanide 7 (430.9 mg, 1.05 mmol) in CH₂Cl₂ (15 mL) at 0 °C. Subsequently, the mixture was
stirred at 0 °C for 4 h, diluted with water (30 mL), and then extracted with ethyl acetate (3 × 30 mL). The extracts were dried (Na₂SO₄) and evaporated. The residue was purified by silica gel column chromatography (methylene chloride-methanol, 4:1) to yield 8 as a yellow solid (291.9 mg, 89.6% yield). ¹H NMR (400 MHz, CD₃OD) δ 7.28 (d, J = 8.8 Hz, 2H), 6.714 (d, J = 8.8 Hz, 2H), 3.10 (t, J = 7.6 Hz, 2H), 2.36 (t, J = 7.6 Hz, 2H), 1.93 (m, 2H), 1.751 (m, 2H), 1.56 (m, 2H). ¹³C NMR (100 MHz, CD₃OD) δ 172.51, 143.83, 143.83, 129.41, 121.94, 115.47, 102.79, 36.15, 30.59, 29.12, 28.28, 24.84.

7 Synthesis of FITC-SelSA conjugate (9). To a solution of FITC (0.8 mg, 2.1 μmol) in dry DMSO (2 mL), 8 (0.9 mg, 3.1 μmol) and Et₃N (50 μL) were added and stirred in the dark at room temperature for 24 h. Subsequently, the crude product was purified by high performance liquid chromatography (HPLC) on a C-18 column (250 mm × 10 mm) using gradient elution with CH₃CN and H₂O as solvents A and B, respectively, and flow rate of 5 mL/min. The fractions containing FITC-SelSA were collected and lyophilized. The purified FITC-SelSA was obtained as a yellow solid with 81.9% yield and over 98% purity by analytical HPLC. MS (ESI): m/z 699.64 ([M]+).

8 Synthesis of IRDye800CW-SelSA Conjugate (10). To a solution of IRDye800CW-NHS (0.5 mg, 0.43 μmol) in dry DMSO (2 mL), 8 (0.2 mg, 0.64 μmol) and Et₃N (30 μL) were added and stirred in the dark at room temperature for 24 h, then quenched with trifluoroacetic acid (50 μL). Subsequently, the crude product was purified by HPLC on a C-18 column (250 mm × 10 mm) using gradient elution with CH₃CN (0.1% TFA) and H₂O as solvents A and B, respectively, and flow rate of 5 mL/min. The fractions containing IRDye800CW-SelSA were collected and lyophilized. The purified IRDye800CW-SelSA was obtained as a green solid with 82.4% yield and over 98% purity by analytical HPLC. MS (ESI): m/z 1294.42 ([M-H]-).

9 Cell lines. Breast cancer MCF-7 and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC). MCF-7-fLuc and MDA-MB-231-fLuc were obtained from GeneChem, Inc., China. Human normal breast epithelial cells (MCF-10A) and healthy kidney epithelial cells (VERO) were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China).

10 HDAC inhibition assay. HDAC-inhibition activity was measured using an HDAC Assay Kit (BPS Bioscience) according to previously described methods. IC₅₀ values were calculated by the dose-response curve using Origin software (OriginLab, Inc.).

11 Western blot analysis for α-tubulin. MDA-MB-231 cells were treated with SelSA or the HDAC inhibitor SAHA for 48 h and then lysed in RIPA buffer. Total protein was electrophoretically separated using tris-glysine gradient gels and then analyzed by Western blot. The primary antibody was α-tubulin (Pierce, 1:1000) and secondary antibody was anti-goat IgG-HRP or anti-rabbit antibodies (Pierce, 1:10000).

12 Molecular modeling. The crystal structures of HDAC1 (PDB: 4BKX), HDAC6 (PDB: SEDU), and HDAC8 (PDB: 2V5X) were obtained from the protein data bank and all water molecules were removed. Crystallographic coordinates of SAHA and SelSA were prepared using Biochemoffice. Preparation of the ligands and proteins were performed with AutoDockTools (ADT), and molecular docking experiments were performed using AutoDock software (version 4.2). The figures were prepared using PyMOL (Erwin Schrödinger).

13 In vitro cellular uptake assays. The flow cytometry was used to qualitatively assess the effect of FITC-SelSA was ingested by breast tumor cells. MCF-7, MDA-MB-231, MCF-10A (5 × 10⁵) were incubated in 6-well culture plates for 24 h and treated with/without various concentrations (0, 0.25, 0.5, 1, 5, 10 μM) of FITC-E₂ or FITC for 3 h, followed were washed with ice-cold PBS and harvested. Flow cytometry was use to collect the signals from the
cells. All data were analyzed using BD CFlow® Plus software, the mean fluorescence intensity and percentage of cell uptake the probe were used to evaluate the targeting effect of FITC-SelSA.

14 ERα expression reactivation assay. To evaluate the effects of SelSA on ERα re-expression, MDA-MB-231 cells were treated with SelSA (0.5 μM) for 3 days and then ERα mRNA and protein expression was examined using quantitative real-time PCR (qPCR) and Western blot assays as described.3 The primers used were ERα-ligand binding domain (LBD) forward (5'-ATAGGATCCATCAACGCTCCTAAGAAAG-3') and reverse (5'-ATATCGAGGCTAGGCGCATGTA-3').

To investigate whether SelSA could enable the responsiveness of TNBC cells to estradiol (E2) and tamoxifen by inducing ERα re-activation, SelSA-pretreated MDA-MB-231 cells were treated with 10 nM E2 or 1 μM tamoxifen for an extra 2 days, and then cell viability was evaluated using a standard MTS assay (Biovision Inc.). The expression of progesterone receptor (PGR), the ERα targeted gene, was also detected using real-time qPCR, with the oligonucleotide sequences: forward (5'-AATGCCCAGCATGCTGCCT-3') and reverse (5'-GGAGCTGCCGACTGTCGG-3').

15 Proliferation assays. First, the anti-proliferative effects of SelSA and SAHA were tested using the MTS assay. Subsequently, the impact of SelSA and SAHA was further examined by bioluminescence imaging (BLI) using the IVIS Imaging Spectrum System (PerkinElmer) on MCF-7-fLuc cells the MDA-MB-231-fLuc cells. Furthermore, apoptotic cells were tested using the Apoptosis Detection Kit (Cat # 640922 BioLegend).

16 Flow cytometry analysis of apoptotic Cells. MCF-7 and MDA-MB-231 cells were cultured in six-well plates and treated with/without different concentrations of SelSA or SAHA (0.25, 0.5 μM) for 48 h. After that, cells were washed, harvested and stained with 7-amino-actinomycin D (7-AAD) and FITC-Annexin-V using the FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, Inc.). The percentages of apoptotic cells were analyzed by BD Accuri C6 flow cytometer (BD Biosciences, USA).

17 Establishment of tumor-bearing animal model. Female Balb/c mice (six-week old, 15-18 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All procedures regarding animal studies were approved by the Institutional Animal Care and Use Committee at Peking University (Permit No: 2011-0039). MCF-7 and MDA-MB-231-fLuc cells were transplanted subcutaneously in the axillary region of mice.

18 Hematoxylin and eosin (H&E) staining. After treatment, tumors and major organs were dissected and fixed in formalin. The fixed tissues were then embedded in paraffin. Sections (4-μm thick) were cut and stained with H&E, then were imaged using a DMI3000 bright-field microscope (Leica).

19 In vitro confocal microscopy images. MCF-7 and MDA-MB-231 cells were treated with 0.5 μM FITC-SelSA or 100-fold excess SAHA as the blocking agent for 3 h. Subsequently, cells were stained with rhodamine phalloidin (or HDAC6 antibody) for cytoskeleton staining and DAPI for nuclear staining. The fluorescence signals were recorded using a Zeiss710 confocal laser scanning microscope.

20 In vivo and ex vivo fluorescence molecular imaging (FMI). FMI was carried out to examine the in vivo biodistribution and tumor-targeting ability of IRDye800CW-SelSA. For in vivo FMI studies, IRDye800CW-SelSA was intravenously injected into the breast of tumor-bearing mice (n = 3). Then, fluorescence images were recorded dynamically from 0 to 48 h postinjection using the IVIS Imaging Spectrum System (PerkinElmer). For the blocking studies, tumor-bearing mice (n = 3) were co-injected with 15 nmol IRDye800CW-SelSA and unlabeled HDACi SAHA (dosage = 20 mg/kg). Regions of interest (ROIs) of tumors and adjacent normal tissues were measured, and the
tumor to normal tissue (T/N) fluorescence intensity ratios were further calculated. After 48 h in vivo imaging, the tumor and major organs were dissected out for ex vivo FMI signal acquisition. The tumor to normal tissue (T/N) ratio was calculated as follows:

\[
\text{Tumor to normal tissue (T/N) ratio} = \frac{\text{Fluorescence intensity}_{\text{tumor}}}{\text{Fluorescence intensity}_{\text{adjacent tissues}}}
\]

21 **In vivo bioluminescent imaging (BLI).** To evaluate the therapeutic effects in vivo, BLI was dynamically implemented on the MDA-MB-231-fLuc breast tumor bearing mice using the IVIS Imaging Spectrum System (PerkinElmer, USA). Ten minutes prior to the initiation of BLI, the tumor bearing mice in the supine position received an intraperitoneal injection of D-luciferin solution (40 mg/mL, 100 μL) under isoflurane anaesthesia. The region of interest (ROI) of the tumors in each mouse at different time points was quantitatively analyzed based on BLI light intensity using IVIS Living Imaging 3.0 software (PerkinElmer, USA).

22 **In vivo antitumor treatment.** Breast tumor-bearing mice were treated with 25 mg/kg test compounds (SelSA or SAHA) or the same volume of vehicle (1:1:8 of DMSO/Tween/PBS) daily for 18 days by gavage. The tumor volume was measured every 3 days using the formula: volume (mm\(^3\)) = (a × b\(^2\))/2, where a = long dimension and b = short dimension. The tumor growth inhibition rate was determined according the formula:

\[
\text{inhibition} (%) = (1 - \frac{T}{C}) \times 100
\]

where T and C are the tumor weights of the treated group and the vehicle control group at the end point, respectively.

23 **Statistics.** Data are shown as the means ± SD from three independent experiments. One-way ANOVA with a Tukey test was performed for comparing differences between groups, and a two-tailed Student’s t test was performed to compare two groups. *P < 0.05 was considered statistically significant.

**References**

**Figure S1.** HDAC6-targeted specificity of FITC-SelSA to MDA-MB-231 cells in vitro. Cells were stained with HDAC6 antibody (red) and cell nuclei (DAPI, blue). (A) Confocal laser microscopic imaging of MDA-MB-231 cells incubated in FITC-SelSA. (B) Blocking study of MDA-MB-231 cells incubated in FITC-SelSA with 100-fold excess SAHA.

**Figure S2** Absorbance and fluorescent emission of IRDye800CW-SelSA, demonstrating an absorbance peak at ~774 nm and an emission peak at ~803 nm.
Figure S3. Inhibitory effect of SelSA and SAHA on MCF-7 breast tumor xenograft models. (A) Average body weight changes of MCF-7 xenografts after different treatments. (B) Average tumor weight of MDA-MB-231-fLuc xenografts after different treatments. (C) Average body weight changes of MDA-MB-231-fLuc tumor-bearing mice for 18 days with different treatments. *\( P < 0.05 \), **\( P < 0.01 \) vs the control. #\( P < 0.05 \) SelSA treatment group vs. the SAHA group.

Table S1. Acute toxicity of SelSA in mice

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of mice</th>
<th>Mouse mortality</th>
<th>Total mortality</th>
<th>Survival (%) on day 18</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>843</td>
<td>8</td>
<td>1 3 4 0</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>674</td>
<td>8</td>
<td>0 1 6 0</td>
<td>7</td>
<td>12.5</td>
<td>554.0</td>
</tr>
<tr>
<td>539</td>
<td>8</td>
<td>0 0 3 0</td>
<td>3</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td>431</td>
<td>8</td>
<td>0 0 1 0</td>
<td>1</td>
<td>87.5</td>
<td></td>
</tr>
<tr>
<td>344</td>
<td>8</td>
<td>0 0 0 0</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>95% confidence limits: 359.83-584.25 mg/kg.