Supporting Information

Modulation of Activity Profiles for Largazole-Based HDAC Inhibitors through Alteration of Prodrug Properties

Lilibeth A. Salvador,[†,⊥] Heekwang Park,[§] Fatma H. Al-Awadhi,[†] Yanxia Liu,[†,‡] Bumki Kim,[§] Sabrina L. Zeller,[§] Qi-Yin Chen,[†,‡] Jiyong Hong,[§,∥] Hendrik Luesch[†,‡,*]

[†]Department of Medicinal Chemistry, University of Florida, Gainesville, Florida 32610, United States, [‡]Center for Natural Products, Drug Discovery and Development (CNPD3), University of Florida, Gainesville, Florida, United States, [§]Department of Chemistry, Duke University, Durham, North Carolina 22708, United States, [∥]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, United States, [⊥]Marine Science Institute, College of Science, University of the Philippines, Diliman, Quezon City 1101, Philippines

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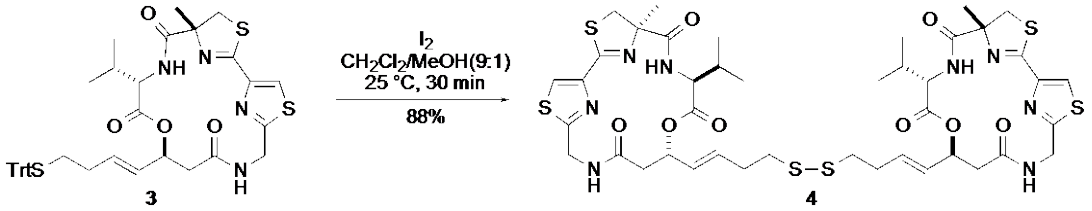
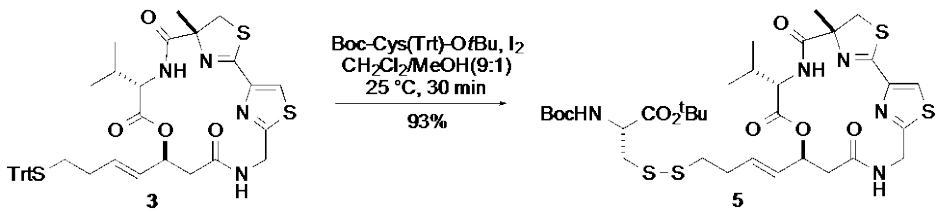


Scheme S1. Preparation of homodimer (**4**) from trityl-protected largazole thiol (**3**)

To a solution of **3** (110 mg, 0.149 mmol) in CH_2Cl_2/MeOH (15 mL, 9:1) was added I_2 (76 mg, 0.298 mmol) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was quenched by the addition of saturated $Na_2S_2O_3$ solution. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/MeOH, 5/5/1) to afford **4** (135 mg, 88%): $[\alpha]^{25}_D$= +23.1 (*c* 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (s, 2 H), 7.17 (d, *J* = 9.5 Hz, 2 H), 6.64 (dd, *J* = 9.0, 3.0 Hz, 2 H), 5.89 (ddd, *J* = 16.0, 7.5, 7.0 Hz, 2 H), 5.69 (m, 2 H), 5.54 (dd, *J* = 15.5, 7.0 Hz, 2 H), 5.25 (dd, *J* = 17.5, 9.5 Hz, 2 H), 4.61 (dd, *J* = 9.5, 3.5 Hz, 2 H), 4.21 (dd, *J* = 17.5, 3.5 Hz, 2 H), 4.02 (d, *J* = 11.5 Hz, 2 H), 3.27 (d, *J* = 11.0 Hz, 2 H), 2.88 (dd, *J* = 16.5, 10.5 Hz, 2 H), 2.73 (m, 2 H), 2.71 (dd, *J* = 7.0, 7.0 Hz, 4 H), 2.43 (m, 4 H), 2.10 (m, 2 H), 1.86 (s, 6 H), 0.70 (d, *J* = 7.0 Hz, 6 H), 0.53 (d, *J* = 7.0 Hz, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.7, 169.4, 169.1, 168.2, 164.7, 147.6, 132.9, 128.5, 124.3, 84.6, 72.1, 57.9, 43.4, 41.2, 40.6, 37.8, 34.3, 31.9, 24.4, 19.0, 16.8; HRMS (ESI) *m/z* 991.2456 [$(M+H)^+$, $C_{42}H_{54}N_8O_8S_6$ requires 991.2462].

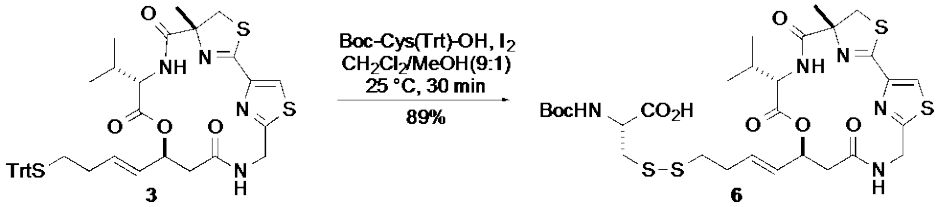
Preparation of Heterodimer 1 (5)



Scheme S2. Preparation of heterodimer 1 (**5**) from trityl-protected largazole thiol (**3**)

To a solution of **3** (32 mg, 0.043 mmol) in CH_2Cl_2/MeOH (4 mL, 9:1) was added *N*-Boc-Cys(Trt)-O^{t}Bu (223 mg, 0.43 mmol) and I_2 (220 mg, 0.87 mmol) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was quenched by the addition of saturated $Na_2S_2O_3$ solution. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/MeOH, 10/10/1) to afford **5** (31 mg, 93%): $[\alpha]^{25}_D$= –9.7 (*c* 1.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1 H), 7.13 (d, *J* = 9.2 Hz, 1 H), 6.53 (m, 1 H), 5.82 (ddd, *J* = 15.6, 7.2, 6.8 Hz, 1 H), 5.62 (m, 1 H), 5.49 (dd, *J* = 15.6, 6.8 Hz, 1 H), 5.30 (d, *J* = 8.0 Hz, 1 H), 5.23 (dd, *J* = 17.6, 9.6 Hz, 1 H), 4.55 (dd, *J* = 9.2, 3.2 Hz, 1 H), 4.40 (m, 1 H), 4.23 (dd, *J* = 17.6, 3.2 Hz, 1 H), 3.98 (d, *J* = 11.2 Hz, 1 H), 3.23 (d, *J* = 11.6 Hz, 1 H), 3.12 (dd, *J* = 13.6, 4.8 Hz, 1 H), 3.03 (dd, *J* = 13.6, 6.0 Hz, 1 H), 2.81 (dd, *J* = 16.4, 10.0 Hz, 1 H), 2.68 (dd, *J* = 7.2, 7.2 Hz, 2 H), 2.65 (dd, *J* = 13.6, 2.4 Hz, 1 H), 2.39 (ddd, *J* = 7.2, 7.2, 7.2 Hz, 2 H), 2.06 (m, 1 H), 1.82 (s, 3 H), 1.43 (s, 9 H), 1.40 (s, 9 H), 0.65 (d, *J* = 6.8 Hz, 3 H), 0.47 (d, *J* = 6.8 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.6, 169.7, 169.3, 168.9, 168.0, 164.6, 155.1, 147.5, 132.5, 128.5, 124.3, 84.4, 82.7, 79.9, 72.0, 57.8, 53.8, 43.3, 41.8, 41.1, 40.4, 37.6, 34.2, 31.7, 28.4, 28.0, 24.3, 18.9, 16.7; HRMS (ESI) *m/z* 772.2537 [$(M+H)^+$, $C_{33}H_{49}N_5O_8S_4$ requires 772.2537].

Preparation of Heterodimer 2 (6)



Scheme S3. Preparation of heterodimer 2 (**6**) from trityl-protected largazole thiol (**3**)

To a solution of **3** (22 mg, 0.030 mmol) in CH_2Cl_2/MeOH (4 mL, 9:1) was added *N*-Boc-Cys(Trt)-OH (138 mg, 0.298 mmol) and I_2 (151 mg, 0.596 mmol) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was quenched by the addition of saturated $Na_2S_2O_3$ solution. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was

purified by column chromatography (silica gel, EtOAc/hexanes/MeOH, 10/10/1) to afford **6** (19 mg, 89%): $[\alpha]^{25}_{D}$= –375.1 (*c* 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1 H), 7.65 (m, 1 H), 7.06 (d, *J* = 9.6 Hz, 1 H), 5.78 (dd, *J* = 16.0, 3.6 Hz, 1 H), 5.60 (m, 2 H), 5.25 (dd, *J* = 17.6, 9.2 Hz, 1 H), 5.00 (d, *J* = 7.2 Hz, 1 H), 4.71 (m, 1 H), 4.64 (dd, *J* = 9.2, 3.2 Hz, 1 H), 4.16 (dd, *J* = 17.6, 3.2 Hz, 1 H), 4.04 (d, *J* = 11.6 Hz, 1 H), 3.33 (d, *J* = 11.6 Hz, 1 H), 3.22 (m, 2 H), 3.14 (dd, *J* = 14.0, 2.4 Hz, 1 H), 2.84 (dd, *J* = 17.2, 2.4 Hz, 1 H), 2.57 (m, 3 H), 2.18 (m, 2 H), 1.83 (s, 3 H), 1.41 (s, 9 H), 0.75 (d, *J* = 6.8 Hz, 3 H), 0.53 (d, *J* = 6.8 Hz, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 175.8, 172.2, 170.3, 170.0, 168.2, 157.9, 148.0, 133.5, 130.0, 126.9, 85.0, 80.6, 79.5, 73.9, 59.0, 54.3, 43.7, 41.8, 41.5, 40.6, 38.6, 35.4, 32.7, 28.8, 24.3, 19.7, 17.1; HRMS (ESI) *m/z* 716.1927 [$(M+H)^+$, $C_{29}H_{41}N_5O_8S_4$ requires 716.1911].

Biological Materials and Methods

Enzymatic Assays. Enzyme inhibitory assays were carried out by BPS Bioscience. In brief, compounds **2**, **4** and **5** were incubated with an HDAC enzyme (HDACs 1–11), an appropriate HDAC substrate, bovine serum albumin and HDAC buffer with or without DTT. Duplicate reactions were carried out at 37 °C for 30 min, except for HDAC11, done at room temperature for 3 h. The reactions were quenched at the end of the incubation period with the addition of HDAC developer. Reactions were further incubated for 15 min at room temperature prior to fluorescence measurement (ex 360 nm/em 460 nm). The % inhibitory activity was calculated according to the equation $(F-F_b)/(F_t-F_b)$, where F- fluorescent intensity of compound treated wells, F_b- fluorescent intensity of blank wells, F_t- fluorescent intensity of solvent control wells. IC_{50} values were calculated using GraphPad Prism.

Cell culture. HCT116 colorectal adenocarcinoma cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) under a humidified environment with 5% CO_2 at 37 °C.

Cell viability assay. HCT116 cells were plated in 96-well plates (10,000 cells/well) and 24 h later treated with various concentrations of largazole (**1**), largazole homodimer (**4**), largazole heterodimer 1 (**5**) and largazole heterodimer 2 (**6**) or solvent control. After another 48 h of incubation, cell viability was measured using MTT according to the manufacturer's instructions (Promega).

Immunoblot analysis. HCT116 cells (400,000 cells/well) were seeded in 6-well plates and 24 h later treated with various concentrations of compounds or solvent control. Following incubation for 8 or 24 h, whole-cell protein lysates were prepared using PhosphoSafe lysis buffer (Novagen) and protein concentration measured using the BCA Protein Assay kit (Pierce). Cell lysates containing 20 μg of protein were separated by SDS-PAGE, transferred to PVDF membranes, probed with antibodies and detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Anti-acetyl-histone H3 (Lys9/18) antibody was obtained from Millipore, anti-β-actin and anti-rabbit antibody were from Cell Signaling.

***In vivo* studies.** Female nude mice (nu/nu), 3 to 5 weeks old, were obtained from Charles River Laboratories Inc. (Wilmington, MA) and used for human tumor xenografts. Tumors were established by subcutaneous injection of 1 x 10^6 HCT116 cells on the right rear flank of a nude mouse in a volume of 100 μL of sterile saline. When the tumor reached the expected volume (250 – 300 mm^3), mice were treated with largazole (**1**), largazole homodimer (**4**), largazole heterodimer 2 (**6**) by intraperitoneal injection (in DMSO, 25 μL/mouse) or oral gavage (in 60% polyethylene glycol, 15% glycerol, 15% ethanol, 10% DMSO, 100 μL/mouse). Tumors were harvested at 4, 12, and 24 h after the treatment. All studies were carried out under the protocol approved by the Institutional Animal Care and Use Committee at the University of Florida.

Tumor samples were homogenized through sonication in PhosphoSafe lysis buffer, centrifuged, and the supernatants were collected and used for immunoblot analysis probing with acetyl histone H3 antibody. Immunoblot analyses of the tumor samples were carried out similar to the procedure stated previously.

Metabolite Analyses

General. HPLC-MS was done on a 3200 QTRAP (Applied Biosystems) equipped with a Shimadzu UFLC System.

Cellular stability. Analysis of the cellular stability was performed based on the method of Liu et al,[1] with modifications. In brief, to the diluted HCT116 protein lysate solution (0. 75 mg/mL) was added 10 μL aliquot of **1**, **4** or **6** (25 μg/mL). The solutions were incubated for varying durations: 0.25, 30, 60, 120, 240, 480, 720, 1440 min. To prevent the auto-oxidation of largazole thiol during workup and accurately compare the stability of the prodrugs, the free largazole thiol was adducted to *N*-ethyl maleimide (NEM). A 100 μL aliquot of NEM (1.9 mg/mL) in ethyl

acetate was added to the incubation solution to quench the reaction and form the largazole thiol-NEM adduct. Additional ethyl acetate and internal standard harmine were added after adduct formation. Following workup, the ethyl acetate layer was collected and evaporated to dryness under nitrogen. Samples were reconstituted in 50 μL methanol. A volume of 10 μL of the reconstituted solution was injected into the HPLC-MS system.

Plasma Stability. Plasma stability of the prodrugs was analyzed based on the procedure by Liu et al,[1] with minor modifications. Prodrugs **1**, **4** or **6** were incubated with 100 μL of mouse serum for 0.25, 30, 60, 120, 240, 480, 720, 1440 min. The reaction was quenched by adding ethyl acetate spiked with NEM. Ethyl acetate workup and HPLC-MS analysis was carried out according to the procedure used for determining cellular stability, using harmine as internal standard.

HPLC-MS Parameters. Analysis of largazole (**1**), largazole thiol (**2**)-NEM adduct and heterodimer 2 (**6**) was done using HPLC-MS [column, Onyx Monolithic C18 (4.6 × 125 mm), Phenomenex; solvent, 0.1% aqueous formic acid (solvent A) – 0.1% formic acid in MeOH (solvent B); flow rate, 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan)]. A step-wise gradient elution was employed starting at 60% B and 40% A, then increased to 100% B at 5 min and maintained at this condition for 5 min. Parameters were optimized prior to analysis using direct syringe infusion. The retention times (t_R, min; MRM ion pair) of the analytes and internal standard are as follows: harmine (1.5; 213→170), thiol-NEM adduct (2.5; 622→79), largazole **1** (4.3; 623→497), heterodimer **6** (3.1; 716→497). Compound dependent parameters used were as follows: Largazole: DP 65, EP 7.0, CE 37, CXP 23, CEP 28, thiol-NEM adduct: DP 90, EP 4.0, CE 73, CXP 3.0, CEP 38, Harmine: DP 50, EP 8.0, CE 41, CXP 2.0, CEP 12.0. Source gas parameters used were as follows: CUR 10, CAD Low, IS 4500, TEM 450.0, GS1 50.0, GS2 40.0. Homodimer (**4**) (3.7; 989→461) was analyzed using the same chromatographic conditions in the negative ion mode, using harmine (1.5; 211→194) as internal standard. Compound-dependent parameters are as follows: homodimer **4**: DP -105, EP -11, CE -50, CXP -9.0, CEP -48, Harmine: DP -45, EP -6.0, CE -46, CXP -6.0, CEP 14. Source gas parameters used were as follows: CUR 10, CAD Medium, IS -3000, TEM 450.0, GS1 50.0, GS2 50.0.

***In vivo* largazole thiol levels in tumors.** Excised tumors were homogenized and extracted with methanol (1:10 w:v) spiked with internal standard harmine. The homogenates were incubated on ice for 10 min and centrifuged at 16000g (15 min at 4 °C). The supernatant was collected and the pellet was reextracted with methanol. All the supernatant was pooled, dried under nitrogen and partitioned with ethyl acetate-water. The ethyl acetate layer was collected, dried and reconstituted in 50 µL MeOH. A 10 µL aliquot was injected for HPLC-MS analysis according to the validated method of Yu et. al.[2]

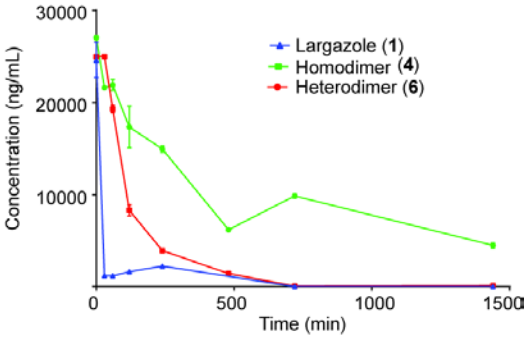


Figure S1. Plasma stability of prodrugs **1**, **4** and **6**.

References

[1] Liu, Y.; Salvador, L. A.; Byeon, S.; Ying, Y.; Kwan, J. C.; Law, B. K.; Hong, J.; Luesch, H. Anticolon cancer activity of largazole, a marine-derived tunable histone deacetylase inhibitor. *J. Pharmacol. Exp. Ther.* **2010**, *335*, 351–361.

[2] Yu, M.; Salvador, L. A.; Sy, S. K. B.; Tang, Y.; Singh, R. S. P.; Chen, Q.-Y.; Liu, Y.; Hong, J.; Derendorf, H.; Luesch, H. Largazole Pharmacokinetics in Rats by LC-MS/MS. *Mar. Drugs* **2014**, *12*, 1623–1640.