Electronic Supplementary Information

Quantitative Measurement of Intracellular HDAC1/2 Occupancy Using a trans-cyclooctene Largazole Thiol Probe

Hua Xu,*a Lee Roberts,a Song Chou,b Betsy Pierce,c Arjun Narayanan,a and Lyn H. Jonesa

1. Cell culture
Peripheral blood mobilized CD34+ cells were purchased from Lonza (2M-101A). The erythroid culture and differentiation method is adapted from Sankaran et al.¹ Specifically, cells were washed twice in StemSpan medium (Stem Cells technologies) after thawing and cultured in StemSpan supplemented with cytokine CC100 cocktail (Stem Cell Technologies) and penicillin-streptomycin at 10⁵ cells/ml concentration in a 37 °C cell culture incubator. On day 7, cells are pelleted and cultured in erythroid differentiation medium consisting of StemSpan medium supplemented with 20 ng/ml SCF (R&D systems), 5 ng/ml IL-3 (R&D systems), 1 U/ml EPO (R&D systems), 2 μM dexamethasone (Sigma), 1 μM β-estradiol and penicillin-streptomycin. Cell concentration was maintained at between 10⁵ and 10⁶ cells/ml for additional 4 days before compound treatment.

2. Biochemical assay
The HDAC inhibition assay was performed using assay components from BPS Bioscience: HDAC assay buffer (BPS catalog number 50031), HDAC developer (BPS catalog number 50030), HDAC substrate 3 (BPS number 50037), HDAC class 2a substrate 1 (BPS number 50040). The reactions were conducted at 37 °C for 30 minutes in a 50μl mixture containing 50 mM HEPES, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, 200 μM TCEP, 5 μg BSA, a substrate (10 μM HDAC substrate 3 for HDAC1, 2, 3 and 2 μM HDAC class 2a substrate 1), an HDAC enzyme (10 ng HDAC1, 5 ng HDAC2, 2 ng HDAC3, or 10 ng HDAC8) and varying concentrations of the compound (0.001 – 10000 nM). After enzymatic reactions, 50 μl of HDAC Developer was added to each well and the plate was incubated at room temperature for an additional 20 minutes. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 460 nm using a Tecan Infinite M1000 microplate reader. The data were analyzed using Graphpad Prism. In the absence of the compound, the fluorescent intensity (Ft) in each data set was defined as 100% activity. In the absence of HDAC, the fluorescent intensity (Fb) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity = (F-Fb)/(Ft-Fb), where F = the fluorescent intensity in the presence of the compound.

The values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation Y=B+(T-B)/(1+10((LogEC50-X)×Hill Slope)), where Y=percent activity, B=minimum percent activity, T=maximum percent activity, X= logarithm of compound and Hill Slope=slope factor or Hill coefficient. The IC₅₀ value was determined by the concentration causing a half-maximal percent activity.
3. **Occupancy assay**

Erythroid progenitor cells were incubated with DMSO or 1–100 nM Dacinostat at 37 degree for 2 hours or 24 hrs, and then incubated with 3 μM TCO largazole thiol at 37 °C for 10 minutes. Cells were then washed with PBS, resuspended in PBS supplemented with protease inhibitors (Sigma), and then lysed by sonication. Lysate were collected after centrifugation, and clicked with 50 μM tetrazine-PEG4-biotin at r.t. for 10 min. The reaction mixture was then incubated with 100 μl streptavidin beads (Thermo Fisher) at room temperature for 30 minutes. After washing thrice with 500 μl TBST (0.1% tween-20), HDAC1/2 was eluted off the beads by LDS sample dye and analyzed by SDS-PAGE and immunoblot. Target occupancy was calculated by comparing HDAC1/2 signals to the vehicle sample, and then fitted into the following equation to obtain OC₅₀:

\[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\left(\frac{X - \log OC_{50}}{\log 10}\right)}} \]

where Y represents the percent occupancy at inhibitor concentration X.

4. **mRNA analysis**

1 ml of erythroid progenitors after 11 days of culture were treated with indicated concentrations of Dacinostat for three days. On day 14, cells were pelleted and their RNAs were extracted using an RNeasy plus RNA extraction kit (Qiagen). Reverse transcription was performed using a Quantitech reverse Transcription Kit (Qiagen). 6 μl of total RNA was used for each 20 μl reverse transcription reaction. The cDNAs were diluted 40 fold and 1 μl of diluted cDNA was used for each real-time PCR reaction. Real-time PCR reactions were performed in 384 well format using Power SYBR Green master mix (Life Technologies) and HBB (Qiagen QT00244489) and HBG (Qiagen QT01884064) primers.
5. Compound synthesis

The synthetic route for TCO largazole thiol was described in Scheme S1.

![Scheme S1. Synthetic route of TCO largazole thiol](image-url)
Compound A (0.2 g, 0.4 mmol) was combined with FMOC-Lys(BOC)-OH, compound B (0.25 g, 0.53 mmol), diluted with 25 mL of DCM, placed over ice, and treated sequentially with EDC (0.10 g, 0.53 mmol) and DMAP (0.004 g, 0.036 mmol). The reaction remained stirring over ice for an additional 10 minutes before it was warmed to ambient temperature and stirred for 2 hours. The reaction solution was then concentrated under reduced pressure, with the resulting residue adsorbed onto silica gel and purified by normal phase silica gel chromatography eluting with 0-10% methanol in DCM to give compound C (0.24 g, 60%) as a yellow solid. \( ^1H \text{ NMR (400 MHz, DMSO)} \delta 7.91 (d, J=7.41 Hz, 2H), 7.66-7.74 (m, 2H), 7.43 (s, 2H), 7.28-7.37 (m, 14H), 7.18-7.27 (m, 3H), 5.57-5.67 (m, 1H), 5.47-5.57 (m, 1H), 5.35-5.45 (m, 1H), 4.97-5.06 (m, 1H), 4.19-4.33 (m, 3H), 4.03 (s, 1H), 3.93-3.98 (m, 1H), 3.54-3.67 (m, 2H), 3.42 (dd, J=8.20, 16.78 Hz, 1H), 3.32 (s, 1H), 3.22 (d, J=11.71 Hz, 1H), 2.84-2.93 (m, 2H), 2.13-2.23 (m, 1H), 2.03-2.10 (m, 2H), 1.86-1.97 (m, 2H), 1.51-1.64 (m, 2H), 1.38 (s, 9H), 1.33 (br. s., 3H), 0.93 (d, J=7.02 Hz, 3H), 0.84 (d, J=7.02 Hz, 3H). \( ^{13}C \text{ NMR (101 MHz, DMSO)} \delta 144.9, 144.3, 144.2, 141.2, 129.6, 128.9, 128.5, 128.1, 127.6, 127.1, 125.7, 120.6, 66.6, 66.2, 47.1, 31.2, 30.9, 28.8, 19.1, 17.6. \text{ LRMS (ESI), m/z 1013.4 [M+H]}.\)

Compound C (0.18 g, 0.61 mmol) was combined with DMAP (0.15 g, 1.2 mmol), diluted with 5 mL of dry DMF, and stirred at ambient temperature for 30 minutes before a solution of Compound D (0.60 g, 0.59 mmol) in 5 mL of dry DMF was added. The resultant reaction solution stirred at ambient temperature overnight. The reaction solution was then concentrated under reduced pressure and the residue recovered adsorbed onto silica gel and purified by normal phase silica gel chromatography eluting with 0-20% methanol in DCM to give compound E (0.42 g, 64%) as a thick, pale yellow oil. \( ^1H \text{ NMR (400 MHz, MeOD)} \delta 8.15 (s, 1H), 7.81 (d, J=7.41 Hz, 2H), 7.67 (t, J=8.59 Hz, 2H), 7.35-7.45 (m, 8H), 7.25-7.35 (m,
8H), 7.17-7.24 (m, 3H), 5.57-5.73 (m, 2H), 5.43 (dd, J=6.44, 15.41 Hz, 1H), 4.62 (s, 2H), 4.30-4.44 (m, 2H), 4.22 (t, J=6.83 Hz, 1H), 4.15 (dd, J=5.07, 8.98 Hz, 1H), 3.89 (d, J=11.71 Hz, 1H), 3.37 (d, J=15.41 Hz, 1H), 3.01 (t, J=6.63 Hz, 2H), 2.61-2.70 (m, 1H), 2.52-2.61 (m, 1H), 2.13-2.21 (m, 2H), 1.98 (q, J=6.76 Hz, 2H), 1.77 (d, J=6.63 Hz, 1H), 1.63 (s, 3H), 1.44 (s, 1H), 1.30-1.40 (m, 3H).

$^{13}$C NMR (101 MHz, MeOD) $\delta$ 174.5, 171.6, 170.7, 169.6, 157.2, 147.4, 144.9, 144.0, 143.8, 141.2, 132.9, 129.4, 127.9, 127.5, 127.4, 126.9, 126.8, 126.4, 124.9, 124.9, 122.7, 119.6, 83.5, 78.5, 72.0, 66.6, 66.4, 54.2, 40.6, 40.4, 39.7, 31.0, 30.9, 29.1, 27.5, 22.9, 22.7. LRMS (ESI), m/z 1108.1 [M+H]$^+$. 

Compound E (0.81 g, 0.73 mmol) was diluted with 25 mL of dry acetonitrile and 5 mL of methanol at ambient temperature and treated with excess diethylamine (4 mL, 40 mmol). The resultant reaction solution stirred at ambient temperature for an hour before it was concentrated under reduced pressure and the residue triturated with 2 x 25 mL ether to give compound F (0.65 g, 100%, crude). LRMS (ESI), m/z 886.3 [M+H]$^+$. 

Compound F (0.34 g, 0.35 mmol) in 100 mL of DCM was added drop-wise over 30 minutes to a solution of HATU (0.14 g, 0.37 mmol) and DIEA (0.06 mL, 0.36 mmol) in 100 mL ACN and 100 mL DCM at ambient temperature. The reaction continued for an additional hour post addition before it was concentrated under reduced pressure. The residue recovered was adsorbed onto silica gel and purified by normal
phase silica gel chromatography eluting with 0-100% ethyl acetate in heptane to give compound G (0.265 g, 43%) as a pale yellow, crispy solid. ¹H NMR (400 MHz, DMSO) δ 8.60-8.68 (m, 1H), 8.27 (s, 1H), 7.31-7.39 (m, 13H), 6.38-6.46 (m, 1H), 5.42-5.63 (m, 3H), 5.04 (dd, J=8.78, 17.37 Hz, 1H), 4.44-4.51 (m, 1H), 4.32-4.42 (m, 1H), 3.94 (d, J=11.32 Hz, 1H), 3.27-3.31 (m, 1H), 2.78-2.93 (m, 1H), 2.71 (s, 3H), 2.11-2.17 (m, 2H), 2.02-2.08 (m, 2H), 1.68 (s, 4H), 1.51-1.62 (m, 1H), 1.38 (s, 9H), 0.79-1.02 (m, 2H), 0.65 (d, J=5.46 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 156.0, 146.9, 144.8, 129.6, 128.8, 128.0, 127.9, 127.8, 127.6, 127.3, 126.7, 66.7, 42.6, 41.2, 38.7, 36.1, 31.4, 31.2, 28.7. HRMS (ESI) Calculated for C₄₆H₅₄N₅O₆S₃ [M+H]⁺ m/z: 868.3231; found, 868.3229.

Compound G (0.10 g, 0.12 mmol) was diluted with 10 mL of DCM, placed over ice, and degassed by bubbling through with nitrogen for 10 minutes. TFA (0.4 mL, 5 mmol) was then added drop-wise and after 10 minutes over ice, the reaction solution was stirred at ambient temperature for an additional hour. The reaction solution was adsorbed onto silica gel and purified by normal phase silica gel chromatography eluting with 0-30% methanol in DCM to give compound H (0.077 g, 76%) as a white gum. ¹H NMR (400 MHz, CDCl₃) δ 8.31 (s, 1H), 7.43 (d, J=7.41 Hz, 5H), 7.12-7.36 (m, 10H), 5.75-5.86 (m, 1H), 5.65 (t, J=8.39 Hz, 1H), 5.41 (dd, J=7.81, 15.61 Hz, 1H), 5.20 (dd, J=8.39, 18.15 Hz, 1H), 4.64-4.67 (m, 1H), 4.45 (d, J=11.71 Hz, 1H), 4.38 (dd, J=4.10, 18.15 Hz, 1H), 3.62 (d, J=11.71 Hz, 1H), 3.10-3.25 (m, 1H), 2.94 (s, 3H), 2.70 (dd, J=1.95, 17.17 Hz, 1H), 2.07-2.14 (m, 2H), 1.86-1.96 (m, 2H), 1.30 (d, J=7.41 Hz, 1H), 1.19-1.28 (m, 2H), 0.96-1.09 (m, 1H), 0.84-0.96 (m, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 171.7, 170.2, 169.9, 168.7, 160.1, 159.7, 159.3, 146.6, 144.8, 135.6, 129.6, 128.0, 128.0, 127.9, 127.4, 126.7, 126.6, 119.2, 116.4, 113.5, 73.6, 66.7, 53.5, 42.2, 41.3, 40.4, 39.2, 38.9, 31.4, 31.0, 29.1, 25.3, 21.7, 20.4. HRMS (ESI) Calculated for C₄₆H₅₄N₅O₆S₃ [M+H]⁺ m/z: 769.2736; found, 769.2738.
Compound H (0.075 g, 0.085 mmol) was diluted with 5 mL of DCM at ambient temperature and treated sequentially with the (E)-cyclooct-4-enyl-2,5-dioxo-1-pyrrolidinyl carbonate, compound I (0.025 g, 0.094 mmol), and DIEA (0.05 mL, 0.3 mmol). The resultant reaction solution stirred for 30 minutes at ambient temperature before it was adsorbed onto silica gel and purified by normal phase silica gel chromatography eluting with 0-100% ethyl acetate in heptane to give compound J (0.0461 g, 59%) as a white solid. 

\[ ^1H \text{ NMR (400 MHz, DMSO)} \delta \ 8.61-8.65 (m, 1H), 8.26 (s, 1H), 7.30-7.39 (m, 11H), 7.17-7.29 (m, 4H), 6.55-6.60 (m, 1H), 5.38-5.65 (m, 6H), 4.93-5.05 (m, 1H), 4.45-4.49 (m, 1H), 4.34 (d, J=16.39 Hz, 2H), 4.16-4.21 (m, 1H), 3.93 (d, J=11.71 Hz, 1H), 3.28-3.31 (m, 1H), 2.82 (dd, J=11.12, 16.98 Hz, 2H), 2.62-2.71 (m, 2H), 2.20-2.32 (m, 4H), 2.10-2.18 (m, 2H), 2.01-2.08 (m, 2H), 1.82-1.95 (m, 3H), 1.67 (s, 3H), 1.47-1.65 (m, 3H), 0.93-0.99 (m, 2H), 0.79-0.89 (m, 1H), 0.55-0.71 (m, 1H). ^13C NMR (101 MHz, CDCl\textsubscript{3}) \delta \ 173.4, 169.8, 169.5, 156.3, 147.4, 144.8, 133.6, 133.5, 129.6, 127.9, 127.8, 126.7, 124.8, 84.4, 80.2, 72.1, 66.7, 60.4, 52.9, 42.7, 41.3, 41.2, 40.0, 38.8, 38.6, 34.4, 32.6, 31.6, 31.3, 31.2, 31.0, 24.5, 21.4, 21.1, 14.2. \text{LRMS (ESI), m/z 942.4 [M+Na]}. 

Compound J (0.045 g, 0.04 mmol) was diluted with 5 mL of DCM, placed over ice, and degassed by bubbling through with nitrogen for 10 minutes. The triisopropylsilane (0.01 mL, 0.049 mmol) was then added, followed by TFA (0.15 mL, 1.96 mmol). The resultant reaction solution stirred over ice for an additional 10 minutes before warming to ambient temperature and stirring an additional hour. The reaction solution was directly adsorbed onto silica gel and purified by normal phase silica gel
chromatography eluting with 0-20% methanol in DCM to give compound K (0.023 g, 69%) as a thick, colorless oil. $^1$H NMR (400 MHz, DMSO) $\delta$ 8.76 (d, $J$=4.68 Hz, 1H), 8.28 (s, 1H), 7.21 (d, $J$=8.20 Hz, 1H), 5.40-5.82 (m, 5H), 4.97 (dd, $J$=8.78, 17.37 Hz, 1H), 4.47-4.56 (m, 2H), 4.33-4.43 (m, 1H), 3.92 (d, $J$=11.32 Hz, 1H), 2.83-2.97 (m, 1H), 2.74 (d, $J$=14.83 Hz, 2H), 2.52-2.56 (m, 1H), 2.18-2.34 (m, 5H), 2.10-2.21 (m, 2H), 2.01-2.08 (m, 4H), 1.85-1.96 (m, 3H), 1.68 (s, 3H), 1.54-1.65 (m, 3H), 1.12-1.18 (m, 3H), 0.80-0.97 (m, 1H), 0.56-0.74 (m, 1H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 172.8, 171.6, 171.5, 166.7, 157.1, 145.8, 132.3, 132.3, 132.1, 130.6, 122.9, 75.0, 71.2, 52.3, 41.2, 39.5, 37.9, 35.1, 34.6, 32.8, 31.2, 31.1, 31.0, 30.5, 24.5, 24.0, 23.2. HRMS (ESI) Calculated for C$_{31}$H$_{44}$N$_5$O$_6$S$_3$ [M+H]$^+$ m/z: 679.2556; found, 679.2561.

Reference