Supporting Information

PET imaging demonstrates HDAC target engagement and clarifies brain penetrance of known and novel small molecule inhibitors in rat.

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General materials and methods

Fifty-five male Sprague-Dawley rats (250-550 g, Charles River Labs) were used for the study. Animals were pair-housed on a diurnal 12:12 light/dark cycle with free access to food and water and weighed immediately prior to blocking dose administration or daily treatment. Animals were anesthetized using isoflurane/oxygen (2-3% in 1.5 L/min induction ~ 5-10 min.; 1.8-2.5% in 1.5 L/min for the duration of setup and PET/CT scanning, ~80 min.) Under anesthesia, rat tails were warmed with tap water, dried, and swabbed with 70% isopropyl alcohol. A lateral tail vein was catheterized (24 GA 0.75in, #381700, BD Biosciences), secured with tape and flushed with ~400 µL of heparinized saline (Hospira; 2 units / mL in 0.9% saline).

Chemicals

All chemicals were purchased from commercial vendors or synthesized in house according to published schemes, or structures (**see Fig. 1, main text**), with purity >99% as evaluated by HPLC and/or proton NMR: Martinostat (in house, see Scheme S1 and chemical synthesis); SAHA (Sigma, SML0061); Givinostat (in house, see ref[1]); PK5 and PK6 (in house, see ref[2]); CN54 (in house, see ref[3]); CI-994 and Cpd60 (in house, see ref[4, 5]); RGFP966 (in house; see

ref[6]); Valproate (Sigma, P4543); CN147 (in house, see Scheme S2 and chemical synthesis). Chemicals were formulated for intravenous (i.v.) delivery in a vehicle containing 10% DMSO, 10% Tween 80, and 80% saline and injected in a volume of 1-2mL/kg. Chemicals were formulated for i.p. delivery in the above vehicle or in 10% DMSO, 45% PEG400 and 45% saline and administered via injection in a volume of 2-10mL/kg. Vehicle-treated control (i.e baseline) animals received the same solution and injected volume without solubilized chemical. Pretreatment times >30 min were administered via i.p. injection under manual restraint. Pretreatment times < 30 min were administered via i.v. or i.p. injection as indicated, under stable anesthesia.

Chemical Synthesis of Martinostat and CN147

(*E*)-methyl 3-(4-((((3r,5r,7r)-adamantan-1-ylmethyl)amino)methyl)phenyl)acrylate (3)

Adamantan-1-ylmethanamine (1) (1g, 6.0 mmol) and (*E*)-methyl 3-(4-formylphenyl)acrylate (2) (1g, 5.3 mmol) was dissolved in MeOH (30 mL) and the mixture was stirred at room temperature for 2 h. Sodium borohydride (0.61g, 16 mmol) was then added, and the suspension was stirred overnight at room temperature. The white precipitate was filtered and dried to obtain the product 3 (1.35 g, yield: 75%).^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.69 (d, *J* = 16 Hz, 1H), 7.48 (d, *J* = 7 Hz, 2H), 7.35 (d, *J* = 7 Hz, 2H), 6.43 (d, *J* = 16 Hz, 1H), 3.81 (s, 2H), 3.80 (s, 3H), 2.23 (s, 3H), 1.96 (s, 3H), 1.63-1.73 (m, 6H), 1.53 (s, 6H); ^{13}C-NMR (125 MHz, $CDCl_3$): 167.55, 144.78, 143.81, 132.87, 128.35 (2C), 128.08 (2C), 117.10, 62.15, 54.28, 51.65, 40.85 (3C), 37.24 (3C), 33.49, 28.48 (3C). LC-MS calculated for $C_{22}H_{29}NO_2$ expected [M]: 339.2; Found $[M+H]^+$: 340.3.

(*E*)-methyl 3-(4-((((3r,5r,7r)-adamantan-1-ylmethyl)(methyl)amino)methyl)phenyl)acrylate (4)

To a solution of 3 (0.5 g, 1.5 mmol) in MeOH (30 mL) was added formaldehyde (33 % aq. solution, 2 mL) followed by acetic acid (0.1 mL). The mixture was stirred at room temperature

for 2 h. Sodium borohydride (0.61g, 16 mmol) was then added, and the suspension was stirred overnight at room temperature. The white precipitate was filtered and purified by flash chromatography in hexanes:ethyl acetate (4:1) to obtain the product 4 (0.29 g, yield: 55%) as white solid. ^{1}H-NMR (500 MHz, MeOH-d_4): δ 7.55 (d, *J* = 16 Hz, 1H), 7.52 (d, *J* = 7.5 Hz, 2H), 7.37 (d, *J* = 7.5 Hz, 2H), 6.47 (d, *J* = 16 Hz, 1H), 3.77 (s, 2H), 2.21 (s, 2H), 1.94 (s, 3H), 1.66-1.76 (m, 6H), 1.54-1.55 (m, 6H); ^{13}C-NMR (125 MHz, MeOH-d_4): 164.93, 141.32, 139.71, 133.76, 128.62 (2C), 127.43 (2C), 116.8901, 61.08, 53.32, 40.42 (3C), 36.78 (3C), 32.84, 28.48 (3C). LC-MS calculated for $C_{21}H_{28}N_2O_2$ expected [M]: 340.2; Found $[M+H]^+$: 341.3.

(*E*)-3-(4-((((3r,5r,7r)-adamantan-1-ylmethyl)(methyl)amino)methyl)phenyl)-N-hydroxyacrylamide (Martinostat)

To a solution of 5 (0.5 g, 1.4 mmol) in MeOH/THF (3 mL/3 mL) at 0 °C was added NH_2OH (50 % aq. solution, 3 mL) followed by 1 M NaOH (2 mL). The mixture was stirred at 0 °C for 2 h, warmed to room temperature, and stirred for 2 h. Acidification with 1 M HCl to pH 7-8 (pH paper) resulted in product precipitation. The precipitate was filtered and dried to obtain Martinostat (0.21 g, 42 %) as white solid. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 7.49 (d, *J* = 7.5 Hz, 2H), 7.42 (d, *J* = 16 Hz, 1H), 7.33 (d, *J* = 7.5 Hz, 2H), 6.45 (d, *J* = 16 Hz, 1H), 3.48 (s, 2H), 2.11 (s, 3H), 2.06 (s, 2H), 1.89 (s, 3H), 1.55-1.65 (m, 6H), 1.46 (s, 6H); δ ^{13}C-NMR (125 MHz, DMSO-d_6): 163.15, 142.00, 138.43, 139.91, 129.36 (2C), 127.77 (2C), 119.06, 70.12, 64.57, 45.71, 41.01 (3C), 37.21 (3C), 35.27, 28.33 (3C). LC-MS calculated for $C_{22}H_{30}N_2O_2$ expected [M]: 354.2; Found $[M+H]^+$: 355.3. Elemental analysis: Calculated: C: 74.54%, H: 8.53%, O: 7.90%; found: C: 73.91%, H: 8.58%, O: 7.69%.

(*E*)-3-(4-(((((3r,5r,7r)-adamantan-1-yl)methyl)(methyl)amino)methyl)phenyl)-N-(2-aminophenyl) acrylamide (CN147)

A mixture of compound 4 (2.3 g, 6.5 mmol) and 1M NaOH (26 mL, 26 mmol) was dissolved in methanol and water (25:5 mL). The reaction mixture was heated at rt overnight. The solvent was removed & water was added & acidified with 1N HCl (pH-4-5). The precipitated solid was filtered to afford 1.98 g of compound 5 (yield: 90%). To a stirred solution of compound 5 (0.2 g, 0.59 mmol) in DMF (5 mL) was added EDC.HCl (0.17 g, 0.89 mmol) and DMAP (cat.) followed by tert-butyl (2-aminophenyl)carbamate (0.15 g, 0.71 mmol) at room temperature under nitrogen atmosphere. The resultant reaction mixture was stirred overnight at rt. Reaction monitored by TLC, after aqueous work up the obtained organic layer was dried over Na2SO4 and concentrated under vacuum to afford crude 220 mg of compound 7 (yield : 55%).

To a solution of compound 7 (145 mg, 2.78 mmol) in DCM (5 mL) was added TFA (10 eq) at °C. The mixture was stirred at room temperature for 4 h. After completion of reaction, the obtained reaction mixture was concentrated under vacuum and the residue was basified by 5%Aq.K_2CO_3 solution (pH = 9). Filtered the obtained solid and washed with water and dried under vacuum to afford pure desired CN147 (100 mg, yield: 85%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.37 (s, 1H), 7.51-7.58 (m, 3H), 7.33-7.41(m, 3H), 6.85-6.73 (m, 2H), 6.75(d, *J* = 7.2 Hz, 1H), 6.58 (t, *J1=J2* = 7.2 Hz, 1H), 4.94 (s, 2H), 3.53 (s, 2H), 2.16 (s, 3H), 2.10 (s, 2H), 1.92 (s, 3H), 1.59-1.69 (m, 6H), 1.50 (s, 6H); δ ^{13}C-NMR (100 MHz, DMSO-d_6): 163.51, 141.81, 141.55, 139.46, 133.41, 129.00 (2C), 127.48 (2C), 125.69, 124.66, 123.51, 121.65, 116.22, 115.97, 70.22, 64.09, 45.30 (2C), 40.53 (3C), 36.72 (2C), 34.78 (3C). LC-MS calculated for $C_{22}H_{30}N_2O_2$ expected [M]: 429.3; Found $[M+H]^+$: 430.4. Elemental analysis: Calculated: C: 78.28%, H: 8.21%, O: 9.78%; found: C: 78.05%, H: 8.17%, O: 9.73%.

[^{11}C]Martinostat preparation and delivery:

Radiolabeled Martinostat was prepared on 17 separate occasions as previously described and eluted in a solution containing 10% Ethanol, 90% saline with a specific activity of 1nCi/ mMol. A dose-calibrator was used to draw radiotracer (0.3-1.3 mCi) in an injected volume of 0.5-1.1 mL. In each experiment, rats received radiotracer doses (i.v.) matched for activity and injected volume (± 15%).

Imaging and reconstruction

Concomitant with radiotracer administration, PET data were collected for up to 60min using dedicated small animal imaging systems: a GammaMedica Triumph PET/CT/SPECT scanner or a Siemens R4 PET scanner. PET data were reconstructed using an MLEM-3D method with 16 iterations and the following histogramming: 8 x 15 sec; 8 x 1 min; 10 x 2 min; 6 x 5 min. Attenuation correction was applied during data reconstruction using CT data (Triumph datasets) or from attenuation scans using a Germanium (Ge) 68 line source (R4 datasets).

Image analysis

Reconstructed dynamic datasets, with the injected activity decay corrected to the start of scan, were analyzed using AMIDE software with elliptical regions of interest (ROI) placed around the whole brain, as identified from overlaid CT datasets, anatomical landmarks visible in each PET dataset (spine, lachrymal glands, nasal cavity), and radiotracer uptake intensity. Raw time-activity datasets were normalized for injected radiotracer dose (drawn dose minus post-injection residual radioactivity in syringe) and weight. To facilitate comparison between subjects, [^{11}C] signal was expressed as a percent relative to uptake in whole brain at time = 600 sec. HDAC target binding at time = 3600 sec was determined for baseline and blocking conditions by the trend in the time activity curve slope from time 10 min until the end of scan. Comparison of

blocked binding (n=1/condition unless noted) was made relative to the comprehensive set of baseline replicates (n=9).

Behavioral testing

Behavioral testing with CN147 and controls was applied to a cohort of 12 rats. Following 7 days of daily i.p. treatment with i) vehicle (10% DMSO, 10% Tween 80, 80% saline; 2mL/kg); or solubilized ii) CN147 (0.025 mg/kg) or iii) CN147 (0.1 mg/kg), rats were exposed to the modified forced swim test (FST), exactly as described[7]. Briefly, FST exposure (15 min exposure, 25 ºC tap water, 30 cm water depth) was performed 18-24 h after the previous i.p. treatment, after which rats were returned to their home cages to dry for approximately 2-3 hrs, then given daily i.p. treatment as before. FST testing was performed one day later (5 min test, 25 ºC tap water, 30 cm water depth) with behaviors digitally recorded for subsequent analysis of immobility by a trained evaluator blinded to the identity of the treatment groups. Rats were returned to home cages and monitored without treatment for 72 hours. Rats were then treated for 7 additional days with i) vehicle, ii) CN147 (0.5 mg/kg) or iii) CN147 (2.0 mg/kg) via daily i.p. injection; treatment assignments for each rat remained the same with a 20x increase in CN147 dose. Animals were then evaluated for locomotor activity in an open field (60 cm square) using automated tracking software (MedAssociates). We confirmed the sensitivity of our FST apparatus and immobility scoring using an independent cohort of rats; following i.p. treatment 24, 13 and 1hr before FST testing, rats injected with imipramine (20 mg/kg) demonstrated lower immobility (104 ± 3.2 sec, mean ± s.e.m) compared to rats injected with vehicle (185.7 ± 19.8 sec); n = 3 rats / treatment group; Student's t-test p = 0.014.

Biochemical Assays

HDAC activity was measured *in vitro* using recombinant human HDACs 1-9 (BPS Bioscience) using the Caliper EZ reader II system as previously described [8]. Enzymes and substrate were

incubated at room temperature for 60 min or, for HDAC1-3, 180 min to account for slow-binding, a characteristic of benzamide-based HDAC inhibitors. IC_{50} values were fit by Genedata Screener software using a four-parameter logistic dose response model. Inhibition of HDAC10 and 11 was not measured due to low purity/activity of available enzymes [8].

Pharmacokinetic Profile Determination

Pharmacokinetics of CN147 were evaluated in rats treated (i.v. and i.p.) with CN147 (1 mg/kg) in vehicle (10% DMSO, 10% Tween 80, 80% saline). Brains were collected at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hr post-treatment (*n* = 3 rats/group), and analyzed using LC-MS/MS as described [8]. Data acquisition and control system were created using Analyst 1.4 software (ABI Inc, Canada).

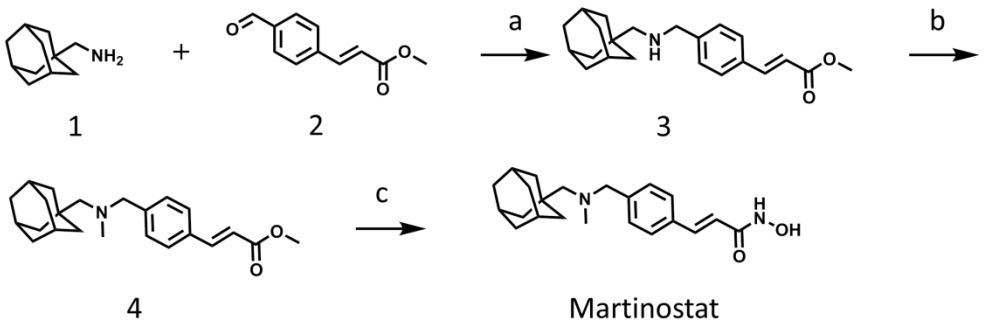
Table S1.

Table S1. Power analysis for blocked binding group sizes

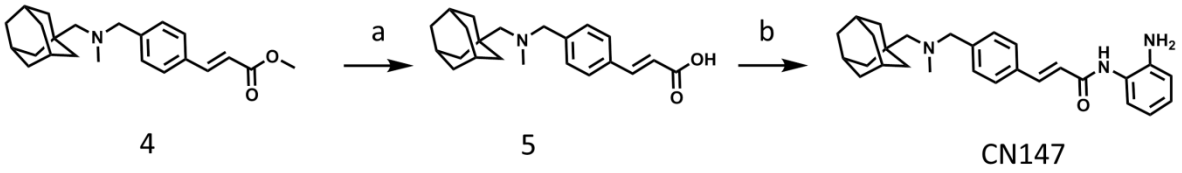
	Baseline:(μ0)	Blocked: (μ1):					
% Binding:	**97.1**	**92.5**	**90**	**85**	**80**	**75**	**50**
Calculated 'n'/group:		**14**	**6**	**3**	**2**	**1**	**1**

(stdev = 6.2; α = 0.05; power = 0.80; 2-sample z test)

Chemical Synthesis Schemes S1 and S2.



Scheme S1. **Synthesis of Martinostat**. Reagents and conditions: (a) $NaBH_4$, MeOH, overnight, rt, 75%; (b) formaldehyde, AcOH, $NaBH_4$, MeOH, rt, overnight, 55%. (c) NH_2OH (aq), 1M NaOH, MeOH/THF, 0 °C to rt, 4h, 42%.



Scheme S2. **Synthesis of CN147**. Reagents and conditions: (a) NaOH, MeOH, overnight, rt, 90%; (b) i: tert-butyl (2-aminophenyl)carbamate, EDC.HCl, DMAP, DMF, rt, overnight, 55%; ii: TFA, DCM, rt, 4h, 85%.

References for Supplemental Information & Methods

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