Supporting information for article:

Inhibition of zinc-dependent histone deacetylases with a chemically triggered electrophile

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SI Figure 3. Evaluation of assay positives from SMM screen using an enzymatic assay using a high dose (~30 – 100 μM) cherry pick (left), followed up by a low dose (2 – 50 μM) cherry pick (right).

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SI Figure 5. Time-course inhibition profiles for BRD4354 (A) and TSA (B).

SI Figure 6. TSA inhibits HDAC5 in a reversible manner. HDAC5 was incubated with 10 μM or 1 mM TSA for 1 h at room temperature. The solutions were diluted 100x and activity was measured; final concentrations of TSA after dilution were 0.1 μM and 10 μM.

SI Figure 7. Thermal stability of HDAC5 monitored by CD. Changes of CD signal at 222 nm for HDAC5 was monitored as a function of temperature, with BRD4354 (red) and without compound (blue).

SI Figure 8. Isothermal calorimetry of BRD4354 and HDAC5 and controls. (A) 2 μM BRD4354 was injected into 5 μM HDAC5. (B) Overlay of injection of 2 μM BRD4354 and 5 μM HDAC5 (black), 30 μM BSA with 100 μM BRD4354 (blue), and 100 μM BRD4354 into Tris buffer (red).

SI Figure 9. Changes in bulk acetylation of histones and tubulin in response to treatment with TSA and BRD4354.

SI Figure 10. Changes in acetylation of p53 (target of acetyltransferase domain of p300 and deacetylase HDAC5) in response to panabinostat (known HDACi), BRD4354, and inactive analog **26**.

SI Figure 11. Retest a subset of large intestine and haematopoietic and lymphoid lineages for cellular viability profiling. BRD4354 and TSA were incubated with each cell line in dose. The viability was measured using Cell Titer Glo and the fluorescence signal was plotted versus concentration.

SI Figure 12. Top 50 upregulated and top 50 downregulated genes from landmark 1000 genes (L1000) in response to treatment of A549 cells with BRD4354 (10 μM) for 24 hours. Three leftmost columns correspond to compound treatment.

Statistical analysis and heatmaps

Data analysis and graphical representation was performed with R language.[1] Briefly, data was downloaded from the ChemBank repository (chembank.broadinstitute.org), by searching for SMM project titled "Commercial HDACs Screening." From this data, values from "CompositeZ" column were plotted without partitioning for particular assays and this distribution was shown in Figure 1A. "Composite Z" values were compared within each assay and across all 17,163 compounds and compounds were assigned a rank in each assay, such that highest ranking compound was furthest removed from the normal distribution in the positive sense and lowest ranking compound was furthest removed in the negative sense. The rankings of all 17,163 compounds were used to generate the heatmap in Figure1B. Clustering of both HDACs and compounds was derived by calculating Euclidean distances of each pair of rankings. This was practically achieved by using "dist" and "hclust" functions within "heatmap.2" package developed by Andy Liaw (original) and revised by R. Gentleman, M. Maechler, W. Huber, and G. Warnes. Similarly, distances and clustering in Figure 2C were calculated by using the corresponding IC_{50} values for compounds in biochemical assays with a given enzyme.

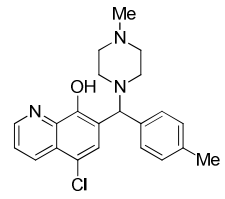
Chemistry

Dry solvents were purchased from Sigma-Aldrich. Unless otherwise stated, all reagents were obtained from commercial sources and used without further purification. ^{1}H NMR spectra were recorded on Varian Unity/Inova 500 (500 MHz), or Bruker Ultrashield 300 (300 MHz) spectrometers. ^{1}H NMR data are reported as follows: chemical shift in parts per million (ppm) relative to tetramethylsilane, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; br, broadened), coupling constant (Hz), and integration. ^{13}C NMR spectra were recorded on Varian Unity/ Inova 500 (126 MHz) or Bruker Ultrashield 300 (75 MHz) spectrometers. ^{13}C NMR chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane. All ^{13}C NMR spectra were determined with broadband decoupling. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using E. Merck silica gel 60 F254 precoated plates (0.25 mm). Flash chromatography was performed using a CombiFlash companion system (Teledyne ISCO, Inc.) with pre-packed FLASH silica gel columns (Biotage, Inc.). Purity of all used compounds was assayed with ^{1}H NMR to be at least 95%.

BRD4354 was originally purchased from Enamine (Monmouth Jct., NJ), Cat # T0509-4226. Compound **24** (cat # T0505-8724), **18** (cat # T5473172), **13** (cat # T5473149), **12** (cat # T5330115), **23** (cat # T5474714), **15** (cat # T0510-2559), and **10** (cat # T5477639) were purchased from Enamine (Monmouth Jct., NJ). Compound **5** (cat # PB56981454), was purchased from UORSY (Kyiv, Ukraine). Compounds **7** (cat #

A1101-1810) and **8** (cat # A1105-0003) were purchased from AllLab (Kyiv, Ukraine). Compound **31** (cat # 6404139) was purchased from Chembridge (San Diego, CA).

Structures of compounds represented in Figure 2C heatmap



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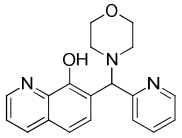

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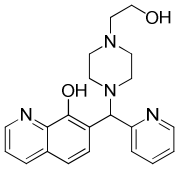

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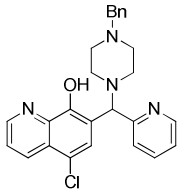

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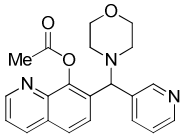

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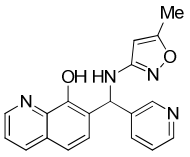

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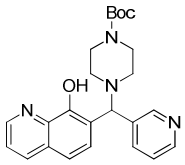

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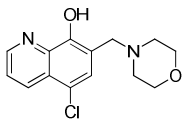

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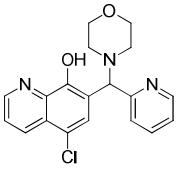

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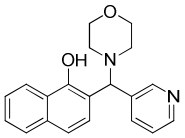

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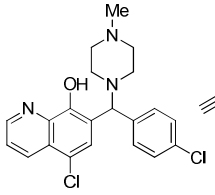

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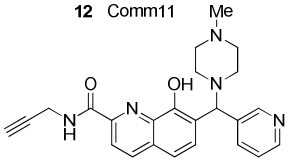

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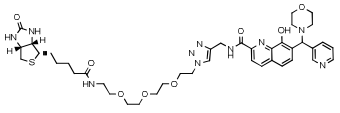

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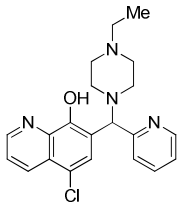

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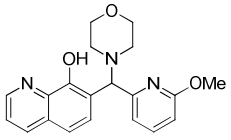

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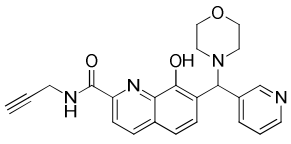

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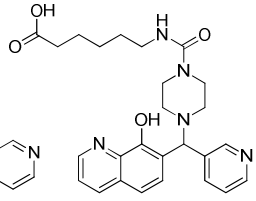

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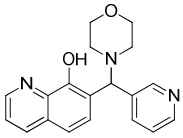

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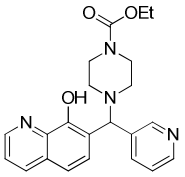

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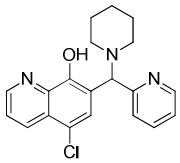

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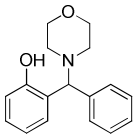

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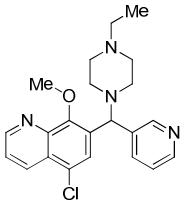

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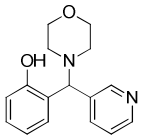

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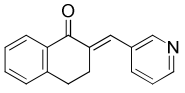

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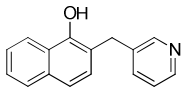

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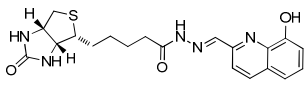

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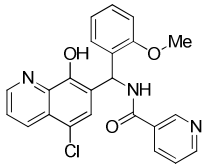
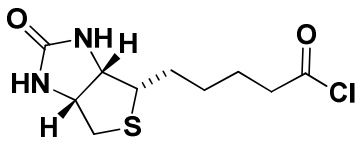


31 Comm20

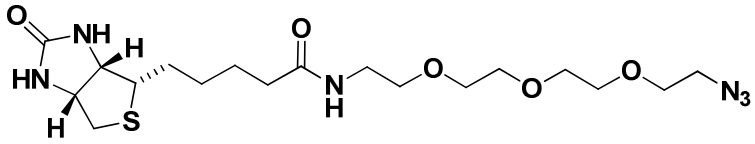
Compound synthesis and spectral data

AF30



A solution of biotin (200 mg, 0.82 mmol, 1 equiv) in 5 mL (69 mmol, 84 equiv) of $SOCl_2$ was stirred at room temperature for 1 hour. The volatiles were evaporated *in vacuo* and then co-evaporated with toluene (3 × 15 mL) to yield AF30 that was directly used in the subsequent reaction.

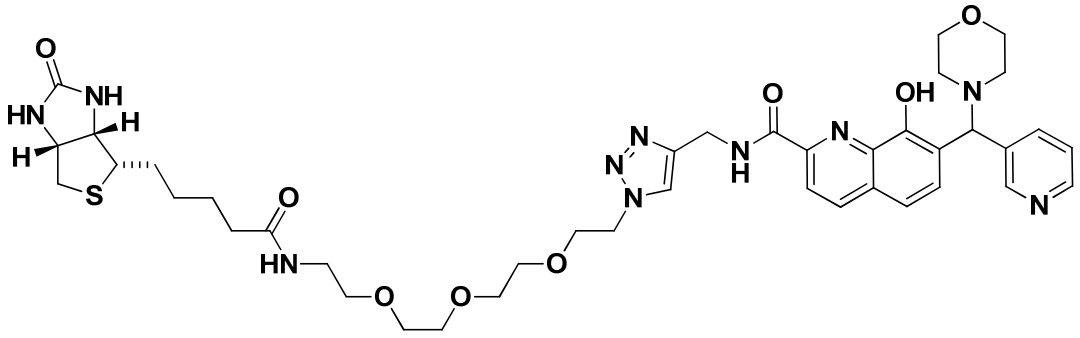
AF31



2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethanamine (0.146 mL, 0.736 mmol, 0.9 equiv) and triethylamine (0.34 ml, 2.46 mmol, 3 equiv) were pre-mixed for 10 min at room temperature in 10 mL of THF. AF30 (215 mg, 0.82 mmol, 1 equiv) solution in THF (15 mL) was then added to this mixture and stirred at room temperature for 20 min. After 20 min, product formation was observed on LC-MS $(M+H)^+$ = 445 Da and $(M+HCO2)^-$ = 489. Product was then purified by column chromatography using methanol in dichloromethane (0 to 10%). Solvent was removed *in vacuo* and product was dried under high vacuum to yield AF31 (110.6 mg, 30.4%).

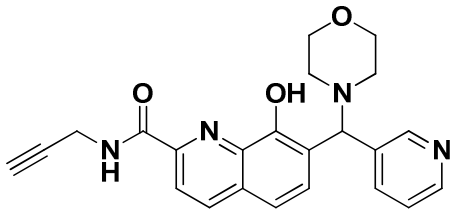
^{1}H NMR (300 MHz, CD_3OD) δ = 8.01 (br. s., 1 H), 4.49 (td, *J* = 4.0, 7.8 Hz, 1 H), 4.30 (td, *J* = 4.0, 8.0 Hz, 1 H), 3.70 - 3.58 (m, 7 H), 3.57 - 3.50 (m, 2 H), 3.42 - 3.26 (m, 6 H), 3.20 (tt, *J* = 3.7, 7.3 Hz, 3 H), 2.97 - 2.85 (m, 1 H), 2.70 (dd, *J* = 3.8, 12.6 Hz, 1 H), 2.26 - 2.12 (m, 2 H), 1.80 - 1.50 (m, 4 H), 1.49 - 1.37 (m, 2 H), 1.36 - 1.26 (m, 3 H); ^{13}C NMR (75MHz, CD_3OD) δ = 176.3, 71.8, 71.8, 71.7, 71.4, 71.3, 70.8, 63.5, 61.8, 57.1, 54.9, 52.0, 41.2, 40.5, 36.9, 29.9, 29.6, 27.0.

17 (AF52)



394 µL of 10 mM TBTA and 328 µL of 10 mM $Cu(PF_6)(MeCN)_4$ were pre-mixed at room temperature. After 10 min of pre-mixing, this solution was added to AF31 (29.2 mg, 0.066 mmol) in 6.5 mL of THF, to which 8-hydroxy-7-(morpholino(pyridin-3-yl)methyl)-N-(prop-2-ynyl)quinoline-2-carboxamide (26.4 mg, 0.066 mmol) was added. After 5 hours, product was visualized by LC-MS (M = 846). After purification by ISCO column chromatography and evaporation of the solvent, 7.6 mg of compound **17** (14%) was isolated as a mixture of diastereomers.

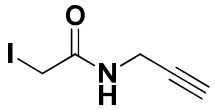
20 (ZBV73)



8-Hydroxy-*N*-(prop-2-ynyl)quinolone-2-carboxamide (170.2 mg, 0.752 mmol, 1 equiv), nicotinaldehyde (70.6 µL, 0.752 mmol, 1 equiv) and morpholine (64.9 µL, 0.752 mmol, 1 equiv) were stirred in ethanol (3.76 mL, 0.2 M) at 60 °C. The product was isolated via ISCO flash chromatography using methanol in dichloromethane (0% to 10% gradient), in 36% yield (109 mg); starting propargyl amide was recovered in 48% yield (81.5 mg).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 11.23 - 10.11 (m, 1 H), 8.77 (d, *J* = 2.0 Hz, 1 H), 8.59 - 8.46 (m, 2 H), 8.34 - 8.29 (m, 1 H), 8.24 (d, *J* = 8.8 Hz, 1 H), 8.01 (br. s., 1 H), 7.62 (br. s., 1 H), 7.37 (d, *J* = 8.3 Hz, 1 H), 7.30 (dd, *J* = 4.9, 7.8 Hz, 1 H), 5.03 - 4.87 (m, 1 H), 4.31 (dd, *J* = 2.4, 5.9 Hz, 2 H), 3.95 - 3.69 (m, 4 H), 2.78 - 2.48 (m, 4 H), 2.23 (t, *J* = 2.4 Hz, 1 H); ^{13}C NMR (126MHz, $CDCl_3$) δ = 164.4, 150.9, 149.4, 148.6, 147.7, 137.2, 136.5, 135.7, 129.0, 128.1, 123.9, 122.9, 119.6, 118.2, 79.4, 71.2, 71.1, 66.7, 52.3, 29.0.

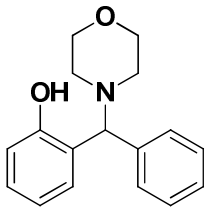
SFL52



2-Iodoacetic acid (200 mg, 1.08 mmol, 1 equiv), propargylamine (68.9 uL, 1.08 mmol, 1 equiv), EDCI (206 mg, 1.08 mmol, 1 equiv), and DMAP (13.14 mg, 0.11 mmol, 0.1 equiv) were dissolved in dichloromethane (3.59 mL, 0.3 M) and stirred at room temperature. The crude product was purified by flash column chromatography with ethyl acetate in hexanes (product elutes at 35% ethyl acetate in hexanes), yielding 58.2 mg of purified material (24.3%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 6.20 (br. s., 1 H), 4.09 (dd, *J* = 2.4, 5.4 Hz, 2 H), 3.78 - 3.66 (m, 2 H), 2.29 (t, *J* = 2.7 Hz, 1 H).

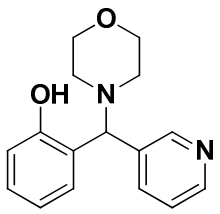
25 (SFL1)



2-Hydroxybenzaldehyde (244 mg, 2 mmol, 1 equiv), morpholine (174 µL, 2 mmol, 1 equiv) and phenylboronic acid (244 mg, 2 mmol, 1 equiv) were mixed in a 10 mL microwave vial and heated to 100 °C with microwave irradiation. The product was isolated in 51.6% yield (278 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.76 (br. s., 1 H), 7.51 - 7.38 (m, 2 H), 7.37 - 7.22 (m, 3 H), 7.15 (t, *J* = 7.6 Hz, 1 H), 6.97 (d, *J* = 7.5 Hz, 1 H), 6.89 (d, *J* = 8.1 Hz, 1 H), 6.75 (t, *J* = 7.4 Hz, 1 H), 4.43 (s, 1 H), 3.78 (d, *J* = 3.6 Hz, 4 H), 2.61 (br. s., 2 H), 2.53 - 2.36 (m, 2 H).

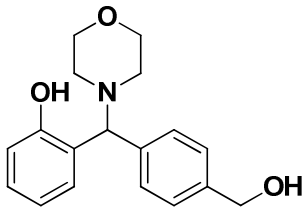
27 (SFL2)



2-Hydroxybenzaldehyde (244 mg, 2 mmol, 1 equiv), morpholine (174 µL, 2 mmol, 1 equiv) and pyridine-3-ylboronic acid (246 mg, 2 mmol, 1 equiv) were mixed in a 10 mL microwave vial and heated to 100 °C with microwave irradiation. The product was isolated in 7% yield (38 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.45 (br. s., 1 H), 8.81 - 8.36 (m, 2 H), 7.85 (d, *J* = 7.5 Hz, 1 H), 7.32 - 7.21 (m, 1 H), 7.15 (t, *J* = 7.6 Hz, 1 H), 6.95 (d, *J* = 7.5 Hz, 1 H), 6.88 (d, *J* = 8.3 Hz, 1 H), 6.81 - 6.69 (m, 1 H), 4.46 (s, 1 H), 3.77 (br. s., 4 H), 2.63 (br. s., 2 H), 2.51 - 2.37 (m, 2 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 155.9, 149.7, 149.6, 135.7, 135.0, 129.1, 124.0, 123.8, 119.8, 117.2, 73.8, 66.7, 52.2.

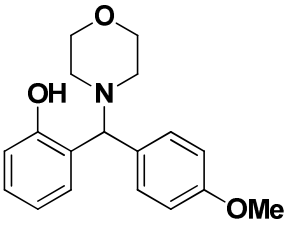
SFL3



2-Hydroxybenzaldehyde (244 mg, 2 mmol, 1 equiv), morpholine (174 uL, 2 mmol, 1 equiv) and 4-(hydroxymethyl)phenylboronic acid (304 mg, 2 mmol, 1 equiv) were mixed in a 10 mL microwave vial and heated to 100 °C with microwave irradiation. The product was isolated in 64% yield (384 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.72 (br. s., 1 H), 7.48 - 7.37 (m, 2 H), 7.32 (d, *J* = 7.9 Hz, 2 H), 7.17 - 7.07 (m, 1 H), 6.95 (d, *J* = 7.0 Hz, 1 H), 6.89 - 6.81 (m, 1 H), 6.73 (t, *J* = 7.4 Hz, 1 H), 4.65 (br. s., 2 H), 4.42 (s, 1 H), 3.76 (br. s., 4 H), 2.76 - 2.33 (m, 4 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 156.0, 140.8, 138.6, 129.3, 128.7, 128.7, 127.5, 124.7, 119.7, 117.0, 76.4, 66.8, 64.8, 52.2.

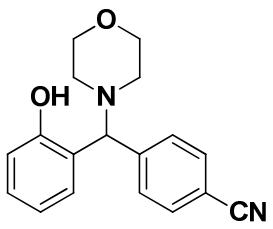
SFL17



2-Hydroxybenzaldehyde (244 mg, 2 mmol, 1 equiv), morpholine (174 uL, 2 mmol, 1 equiv) and 4-methoxyphenylboronic acid (304 mg, 2 mmol, 1 equiv) were mixed in a 10 mL microwave vial and heated to 100 °C with microwave irradiation. The product was isolated in 81% yield (484 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.81 (br. s., 1 H), 7.34 (d, *J* = 8.3 Hz, 2 H), 7.17 - 7.09 (m, 1 H), 6.94 (d, *J* = 7.3 Hz, 1 H), 6.89 - 6.80 (m, 3 H), 6.74 (dt, *J* = 0.9, 7.4 Hz, 1 H), 4.39 (s, 1 H), 3.83 - 3.68 (m, 7 H), 2.59 (br. s., 2 H), 2.50 - 2.39 (m, 2 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 159.4, 156.1, 131.2, 130.2, 129.8, 129.3, 128.6, 125.1, 119.6, 117.0, 114.2, 76.0, 66.9, 55.2.

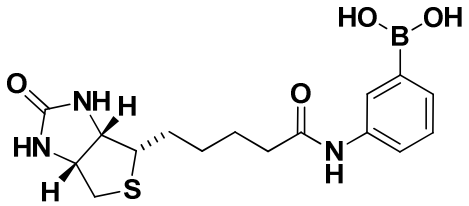
SFL18



2-Hydroxybenzaldehyde (244 mg, 2 mmol, 1 equiv), morpholine (174 uL, 2 mmol, 1 equiv) and 4-cyanophenylboronic acid (294 mg, 2 mmol, 1 equiv) were mixed in a 10 mL microwave vial and heated to 100 °C with microwave irradiation. The product was isolated in 75% yield (442 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.34 (br. s., 1 H), 7.70 - 7.42 (m, 3 H), 7.23 - 7.11 (m, 1 H), 7.04 - 6.67 (m, 4 H), 4.43 (s, 1 H), 3.75 (d, *J* = 13.0 Hz, 4 H), 2.74 - 2.28 (m, 4 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 157.5, 155.7, 144.8, 132.8, 129.4, 129.1, 129.1, 123.6, 120.0, 119.4, 117.4, 66.7, 52.9, 52.4.

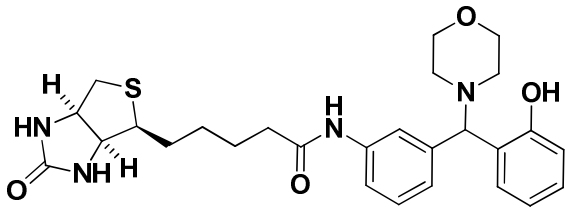
SFL27



3-Aminophenylboronic acid hemisulfate (95 mg, 0.514 mmol, 1 equiv) and *N*-hydroxysuccinimide biotin ester (175 mg, 0.514 mmol, 1 equiv) were dissolved in DMF (2.56 mL, 0.2 M) and sodium bicarbonate (129 mg, 1.541 mmol, 3 equiv) was added to the mixture. The product was isolated with silica gel flash chromatography on ISCO with methanol (0% to 30%) in dichloromethane, in 37% yield (69 mg).

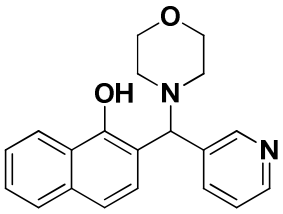
^{13}C NMR (75 MHz, CD_3OD) δ = 174.6, 166.2, 139.2, 135.4, 131.4, 130.5, 129.2, 125.6, 63.5, 61.8, 57.0, 41.1, 37.7, 29.8, 29.6, 26.9.

SFL29



SFL27 (68.5 mg, 0.189 mmol, 1 equiv), 2-hydroxybenzaldehyde (23 mg, 0.189 mmol, 1 equiv) and morpholine (16 µL, 1 equiv) were heated to 100 °C. Product was isolated through column chromatography with methanol in dichloromethane (0% to 10%), in 18% yield (17.2 mg).

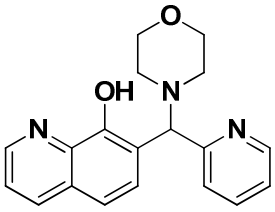
14 (SFL30)



Solution of 1-naphthol (115 mg, 0.8 mmol, 1 equiv), nicotinaldehyde (86 mg, 0.8 mmol, 1 equiv) and morpholine (69.7 µL, 0.8 mmol, 1 equiv) in ethanol (4 mL, 0.2 M) was stirred at 85 °C. The product was isolated in 64% yield (256 mg).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 11.89 (br. s., 1 H), 8.27 (s, 1 H), 8.17 - 8.05 (m, 1 H), 7.95 - 7.85 (m, 1 H), 7.47 (d, *J* = 7.3 Hz, 1 H), 7.37 - 7.27 (m, 1 H), 7.14 - 7.00 (m, 2 H), 6.90 - 6.78 (m, 2 H), 6.70 - 6.55 (m, 1 H), 4.23 - 3.98 (m, 1 H), 3.57 - 3.20 (m, 4 H), 2.52 - 1.94 (m, 4 H).

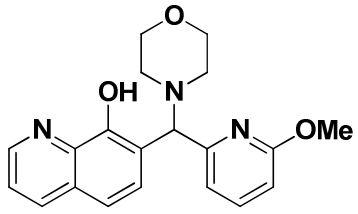
6 (SFL39)



8-Hydroxyquinoline (116 mg, 0.8 mmol, 1 equiv), picolinaldehyde (86 mg, 0.8 mmol, 1 equiv) and morpholine (69.7 µL, 0.8 mmol, 1 equiv) were dissolved in ethanol (4 mL, 0.2 M) and stirred at 85 °C. The product was isolated by flash column chromatography using methanol in dichloromethane (0% to 15%), in 24% yield (257 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.91 - 8.73 (m, 1 H), 8.58 (d, *J* = 4.9 Hz, 1 H), 8.06 (d, *J* = 8.3 Hz, 1 H), 7.72 - 7.56 (m, 2 H), 7.51 (d, *J* = 8.1 Hz, 1 H), 7.39 (dd, *J* = 4.1, 8.3 Hz, 1 H), 7.25 (s, 1 H), 7.19 - 7.08 (m, 1 H), 4.98 (s, 1 H), 3.89 - 3.70 (m, 4 H), 2.66 (br. s., 2 H), 2.53 (br. s., 2 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 151.3, 149.4, 148.7, 139.3, 137.0, 135.7, 128.2, 127.7, 123.1, 122.6, 121.6, 118.0, 66.9, 52.4.

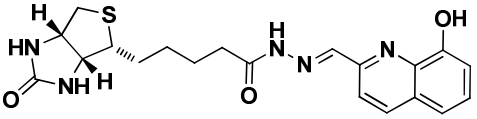
19 (SFL42)



8-Hydroxyquinoline (116 mg, 0.8 mmol, 1 equiv), 6-methoxypicolinaldehyde (110 mg, 0.8 mmol, 1 equiv) and morpholine (70 µL, 0.8 mmol, 1 equiv) were stirred in ethanol (4 mL, 0.2 M) at 85 °C. The product was isolated by flash column chromatography using methanol in dichloromethane (0% to 15%), in 31% yield (109.5 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.82 (dd, *J* = 1.5, 4.1 Hz, 1 H), 7.97 (dd, *J* = 1.3, 8.3 Hz, 1 H), 7.47 - 7.37 (m, 1 H), 7.35 - 7.23 (m, 2 H), 7.16 (d, *J* = 8.5 Hz, 1 H), 7.00 (d, *J* = 7.3 Hz, 1 H), 6.53 (dd, *J* = 0.6, 8.3 Hz, 1 H), 5.23 (s, 1 H), 4.66 (s, 1 H), 3.90 (s, 3 H), 3.84 - 3.67 (m, 4 H), 2.61 (br. s., 2 H), 2.53 - 2.41 (m, 2 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 163.7, 156.6, 151.8, 148.6, 139.3, 139.0, 135.4, 128.2, 127.8, 121.2, 120.6, 117.5, 115.4, 109.6, 75.1, 66.9, 53.5, 53.3, 51.9.

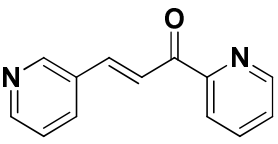
30 (SFL49)



8-Hydroxyquinoline-2-carbaldehyde (335 mg, 1.935 mmol, 1 equiv) and biotinyl hydrazine (500 mg, 1.935 mmol, 1 equiv) were stirred in DMF (19.4 mL, 0.1 M). Acetonitrile (50 mL) was added to the mixture and upon standing in the freezer (-20 °C) for several hours, the product precipitated from solution as an off-white solid. The product was additionally purified with flash chromatography using methanol in dichloromethane (0% to 10%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 11.73 (br. s., 1 H), 8.49 - 8.32 (m, 1 H), 8.26 (s, 1 H), 8.13 - 7.98 (m, 1 H), 7.55 - 7.33 (m, 2 H), 7.15 (d, *J* = 6.1 Hz, 1 H), 4.31 (br. s., 1 H), 4.21 - 4.07 (m, 1 H), 3.13 (br. s., 1 H), 2.82 (dd, *J* = 5.3, 11.9 Hz, 1 H), 2.72 (t, *J* = 7.0 Hz, 1 H), 2.58 (d, *J* = 12.5 Hz, 1 H), 2.29 (t, *J* = 6.1 Hz, 1 H), 1.65 (t, *J* = 6.6 Hz, 3 H), 1.56 - 1.32 (m, 3 H).

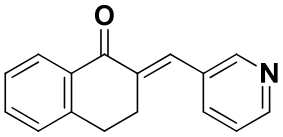
ZBV28



Nicotinaldehyde (107 mg, 1 mmol, 1 equiv), 2-acetylpyridine (121 mg, 1 mmol, 1 equiv) and diazabicycloundecene (152 mg, 1 mmol, 1 equiv) were dissolved in THF (0.2 M) and stirred at room temperature overnight. The product was isolated with flash silica gel column chromatography with methanol (0% to 5%) in dichloromethane, in 24% yield (50 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.87 (d, *J* = 1.7 Hz, 1 H), 8.72 (td, *J* = 0.8, 4.8 Hz, 1 H), 8.60 (dd, *J* = 1.3, 4.7 Hz, 1 H), 8.36 (d, *J* = 16.0 Hz, 1 H), 8.20 - 8.14 (m, 1 H), 8.03 (td, *J* = 1.7, 7.8 Hz, 1 H), 7.93 - 7.82 (m, 2 H), 7.49 (ddd, *J* = 1.2, 4.8, 7.6 Hz, 1 H), 7.33 (dd, *J* = 4.9, 7.9 Hz, 1 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 188.9, 151.0, 150.4, 148.9, 140.6, 137.0, 134.6, 130.9, 127.1, 123.6, 122.9, 122.8.

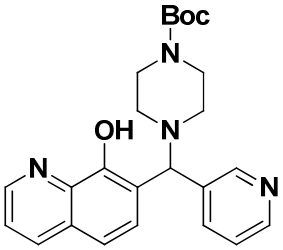
28 (ZBV27)



3,4-Dihydronaphthalen-1(2H)-one (5.9 g, 40.3 mmol, 1 equiv), nicotinaldehyde (6.5 g, 60.4 mmol, 1.5 equiv), piperidine (0.8 g, 9.5 mmol, 0.23 equiv) and acetic acid (0.81 g, 13.42 mmol, 0.33 equiv) were stirred at room temperature. The product of the reaction was isolated with silica gel flash column chromatography with ethyl acetate (0% to 80%, product elutes at 60%) in hexanes, in 26% yield (2.48 g).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.70 (d, *J* = 1.5 Hz, 1 H), 8.58 (dd, *J* = 1.3, 4.9 Hz, 1 H), 8.13 (d, *J* = 7.9 Hz, 1 H), 7.80 (s, 1 H), 7.74 (d, *J* = 7.9 Hz, 1 H), 7.56 - 7.46 (m, 1 H), 7.37 (td, *J* = 4.0, 7.9 Hz, 2 H), 7.29 - 7.22 (m, 1 H), 3.15 - 3.06 (m, 2 H), 3.04 - 2.85 (m, 2 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 187.2, 150.4, 149.1, 143.1, 137.5, 136.8, 133.5, 133.1, 132.4, 131.7, 128.3, 128.2, 127.1, 123.3, 28.7, 27.2.

11 (ZBV10)

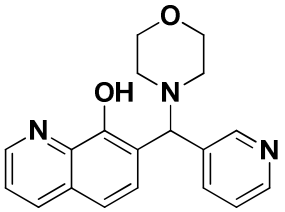


tert-Butyl piperazine-1-carboxylate (257 mg, 1.38 mmol, 1 equiv), nicotinaldehyde (148 mg, 1.38 mmol, 1 equiv), and 8-hydroxyquinoline (200 mg, 1.38 mmol, 1 equiv) were dissolved in ethanol (3 mL, 0.46 M) and stirred at 100 °C in a sealed vial in the

microwave reactor. The product was isolated with silica gel flash column chromatography with methanol (0% to 5%) in dichloromethane, in 53% yield (306 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.82 (dd, *J* = 1.6, 4.2 Hz, 1 H), 8.77 (d, *J* = 2.1 Hz, 1 H), 8.48 (dd, *J* = 1.7, 4.7 Hz, 1 H), 8.09 (dd, *J* = 1.6, 8.2 Hz, 1 H), 7.89 (d, *J* = 7.7 Hz, 1 H), 7.53 (d, *J* = 8.7 Hz, 1 H), 7.41 (dd, *J* = 4.2, 8.2 Hz, 1 H), 7.30 (d, *J* = 8.7 Hz, 1 H), 7.22 (dd, *J* = 4.9, 7.9 Hz, 1 H), 4.96 (s, 1 H), 3.50 (br. s., 4 H), 2.61 - 2.38 (m, 4 H), 1.45 (s, 9 H).

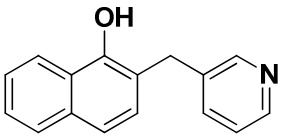
22 (ZBV57)



Morpholine (0.9 mL, 10.3 mmol, 1 equiv), nicotinaldehyde (1.11 g, 10.33 mmol, 1 equiv), and 8-hydroxyquinoline (1.5 g, 10.33 mmol, 1 equiv) were dissolved in ethanol (52 mL, 0.2 M) and stirred at room temperature. The product was isolated with silica gel flash column chromatography with methanol (0% to 10%) in dichloromethane, in 28.2% yield (936 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.88 - 8.68 (m, 2 H), 8.46 (dd, *J* = 1.5, 4.7 Hz, 1 H), 8.06 (dd, *J* = 1.3, 8.3 Hz, 1 H), 7.89 (d, *J* = 7.9 Hz, 1 H), 7.52 (d, *J* = 8.5 Hz, 1 H), 7.38 (dd, *J* = 4.1, 8.3 Hz, 1 H), 7.28 (d, *J* = 8.5 Hz, 1 H), 7.21 (dd, *J* = 4.9, 7.7 Hz, 1 H), 4.91 (s, 1 H), 3.85 - 3.67 (m, 4 H), 2.67 - 2.42 (m, 4 H).

29 (ZBV58)

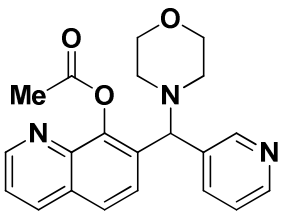


Compound **28** (ZBV27) (100 mg, 0.425 mmol, 1 equiv),and potassium *tert*-butoxide (47.7 mg, 0.425 mmol, 1 equiv) were dissolved in *tert*-butanol (1mL) and stirred at room temperature. The product was isolated with silica gel flash column chromatography with ethyl acetate (0% to 75%) in hexanes, in 36.5% yield (36.5 mg).

^{1}H NMR (500 MHz, DMSO-d_6) δ = 9.39 (s, 1 H), 8.53 (d, *J* = 2.4 Hz, 1 H), 8.37 (dd, *J* = 1.2, 4.6 Hz, 1 H), 8.27 - 8.18 (m, 1 H), 7.83 - 7.75 (m, 1 H), 7.60 (d, *J* = 7.8 Hz, 1 H), 7.49 - 7.41 (m, 2 H), 7.38 (d, *J* = 8.3 Hz, 1 H), 7.29 (d, *J* = 8.3 Hz, 1 H), 7.28 - 7.24 (m, 1

H), 4.21 - 4.06 (m, 2 H); ^{13}C NMR (126MHz, DMSO-d_6) δ = 149.7, 149.5, 147.0, 136.9, 135.9, 133.2, 128.7, 127.5, 125.5, 125.4, 124.9, 123.4, 121.9, 121.2, 119.5, 32.6.

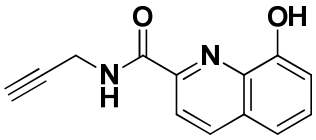
9 (ZBV64)



Compound **22** (ZBV57) (100 mg, 0.31 mmol, 1 equiv), acetic anhydride (152 µL, 1.56 mmol, 5 equiv), and triethyl amine (217 µL, 1.56 mmol, 5 equiv) were dissolved in THF (3.11 mL, 0.1 M). The product was isolated with silica gel flash column chromatography with methanol (0% to 5%) in dicholoromethane in quantitative yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.81 - 8.64 (m, 2 H), 8.37 (br. s., 1 H), 8.06 - 7.95 (m, 1 H), 7.86 - 7.74 (m, 1 H), 7.71 - 7.57 (m, 1 H), 7.31 (dt, *J* = 4.4, 8.1 Hz, 1 H), 7.23 - 7.16 (m, 1 H), 7.16 - 7.10 (m, 1 H), 5.01 - 4.55 (m, 1 H), 3.80 - 3.49 (m, 4 H), 2.55 - 2.47 (m, 3 H), 2.45 - 2.30 (m, 4 H).

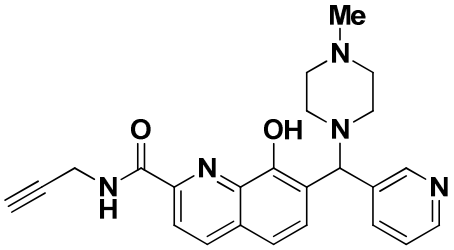
ZBV69



8-Hydroxyquinoline-2-carbonyl chloride (113 mg, 0.54 mmol, 1 equiv), propargylamine (41.8 µL, 0.65 mmol, 1.2 equiv), and triethylamine (228 µL, 1.63 mmol, 3 equiv) were dissolved in dichloromethane (5.4 mL, 0.1 M) and stirred at room temperature. The product was isolated by flash silica gel column chromatography with ethyl acetate in hexanes.

^{1}H NMR (500 MHz, DMSO-d_6) δ = 10.18 (s, 1 H), 10.00 (t, *J* = 5.9 Hz, 1 H), 8.53 - 8.49 (m, 1 H), 8.14 (d, *J* = 8.3 Hz, 1 H), 7.60 - 7.54 (m, 1 H), 7.48 (d, *J* = 8.3 Hz, 1 H), 7.18 (d, *J* = 7.3 Hz, 1 H), 4.26 - 4.16 (m, 2 H), 3.26 - 3.21 (m, 1 H).

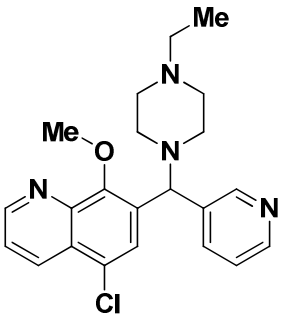
16 (ZBV74)



ZBV69 (29.9 mg, 0.132 mmol, 1 equiv), nicotinaldehyde (12.4 µL, 0.132 mmol, 1 equiv), and 1-methylpiperazine (14.66 µL, 0.132 mmol, 1 equiv) were dissolved in ethanol (661 µL, 0.2 M) and stirred at 70 °C. The product was isolated with silica gel column chromatography with methanol (0% to 20%) in dichloromethane, in 52.4% yield (28.8 mg).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.38 - 10.45 (m, 1 H), 8.71 (d, *J* = 1.9 Hz, 2 H), 8.50 (dd, *J* = 1.5, 4.7 Hz, 1 H), 8.29 (d, *J* = 8.5 Hz, 1 H), 8.20 (d, *J* = 8.7 Hz, 1 H), 7.84 (td, *J* = 1.7, 8.0 Hz, 1 H), 7.46 (d, *J* = 8.5 Hz, 1 H), 7.31 (d, *J* = 8.7 Hz, 1 H), 7.22 (dd, *J* = 4.9, 7.9 Hz, 1 H), 4.84 (s, 1 H), 4.26 (dd, *J* = 2.6, 5.8 Hz, 2 H), 2.57 (br. s., 8 H), 2.34 (s, 3 H), 2.19 (t, *J* = 2.4 Hz, 1 H); ^{13}C NMR (75MHz, $CDCl_3$) δ = 164.3, 151.5, 149.7, 149.3, 148.0, 137.6, 137.3, 136.0, 135.6, 129.4, 128.5, 123.9, 122.9, 119.6, 118.3, 79.5, 71.3, 54.9, 51.5, 45.5, 29.1.

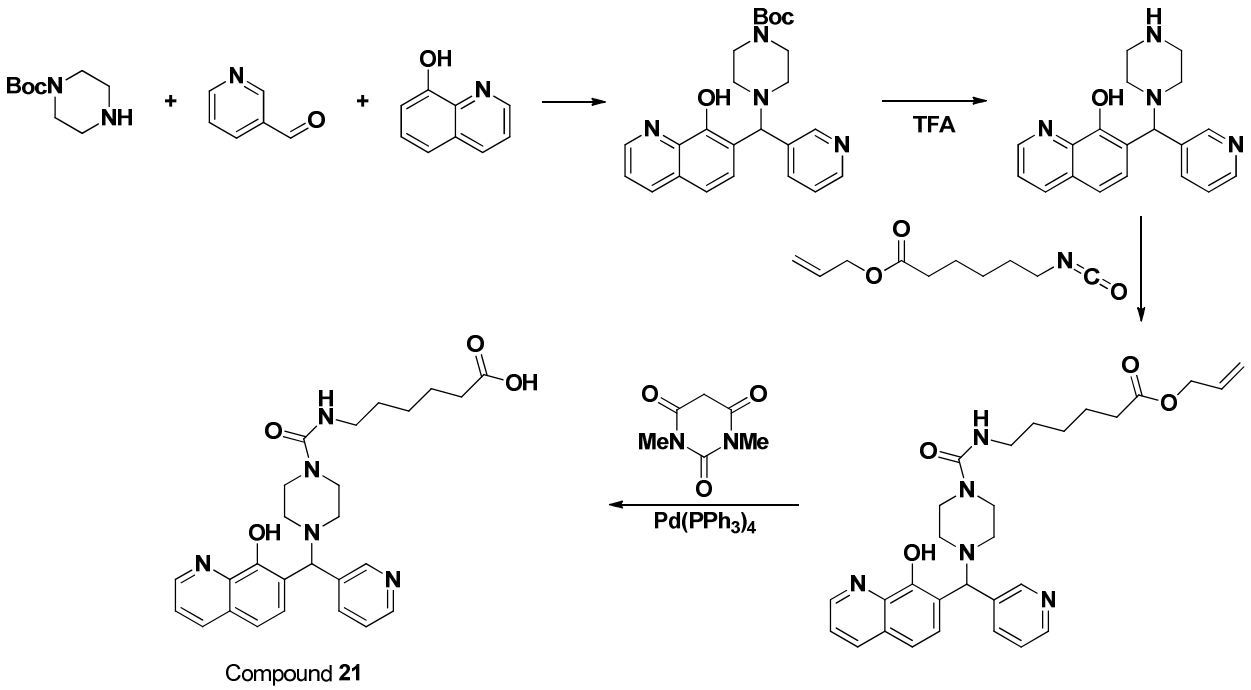
26 (MK1)



BRD4354 (10 mg, 26 µmol, 1 equiv) and cesium carbonate (9.4 mg, 28.6 µmol, 1.1 equiv) was added to anhydrous DMF (0.1 M), followed by the addition of dimethyl sulfate (2.7 µL, 28.6 µmol, 1.1 equiv). The solution was allowed to stir overnight till product formed. The reaction mixture was purified by silica flash chromatography.

^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.99 (dd, *J* = 1.5, 3.9 Hz, 1 H), 8.68 (s, 1 H), 8.50 (dd, *J* = 1.5, 8.3 Hz, 1 H), 8.44 (d, *J* = 3.9 Hz, 1 H), 7.94 (s, 1 H), 7.86 (td, *J* = 2.0, 7.8 Hz, 1 H), 7.72 - 7.67 (m, 1 H), 7.36 (dd, *J* = 4.9, 7.8 Hz, 1 H), 4.06 (s, 3 H), 3.37 (s, 1 H), 3.33 (s, 2 H). piperazine peaks overlapping with DMSO-d6 signal; ^{13}C NMR (126 MHz, DMSO-d_6) δ = 152.4, 150.5, 149.3, 148.7, 142.8, 135.4, 132.8, 125.8, 125.3, 124.9, 124.0, 122.8, 62.3, 52.8.

21 (ZBV25)



Nicotinaldehyde (148 mg, 1.4 mmol), *tert*-butyl piperazine-1-carboxylate (257 mg, 1.4 mmol), and quinolin-8-ol (200 mg, 1.4 mmol) were dissolved in ethanol (volume: 3 ml, density: 0.81 g/ml) and heated to 100 °C in a sealed microwave vial for 1 hour. The solvent was evaporated and the mixture was separated with ISCO column chromatography with methanol in dichloromethane (0 - 5%), yielding product (306 mg, 53%).

tert-Butyl 4-((8-hydroxyquinolin-7-yl)(pyridin-3-yl)methyl)piperazine-1-carboxylate (171 mg, 0.4 mmol) was dissolved in dichloromethane (volume: 2033 µl, density: 1.325 g/ml) to which was slowly added 2,2,2-trifluoroacetic acid (311 µl, 4.0 mmol) and stirred at room temperature. The reaction mixture was partitioned between NaHCO3 and additional dichloromethane and purified using ISCO column chromatography (methanol in dichloromethane 0 - 5%).

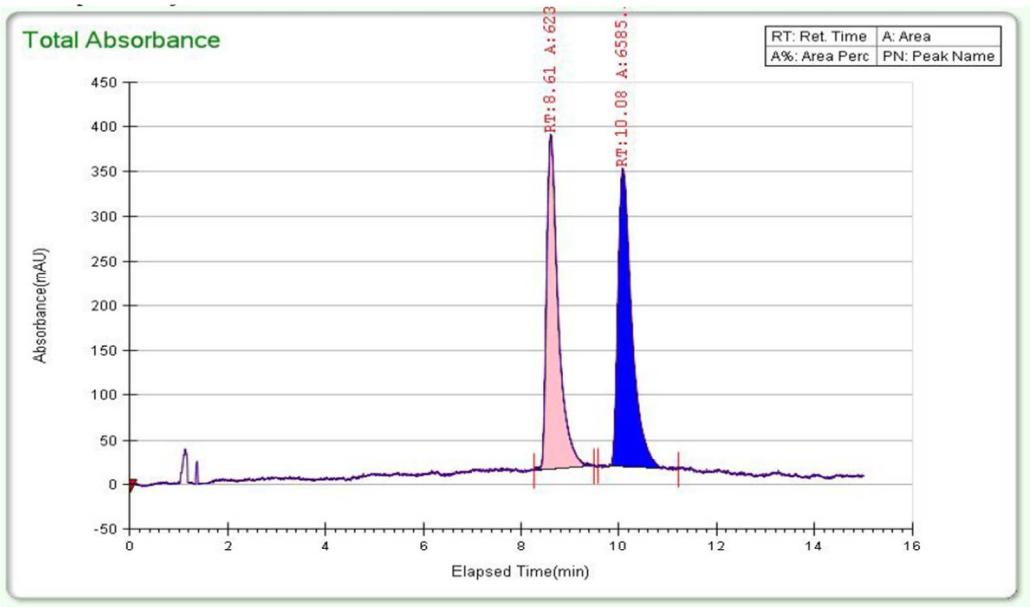
7-(Piperazin-1-yl(pyridin-3-yl)methyl)quinolin-8-ol (50 mg, 0.16 mmol) and allyl 6-isocyanatohexanoate (30.8 mg, 0.16 mmol) were mixed in DMF (volume: 1561 µl, density: 0.944 g/ml) and stirred until starting material was consumed. The mixture was partitioned between water and dichloromethane, extracted and dried with brine and sodium sulfate, yielding 54 mg (67%) of product.

Allyl 6-(4-((8-hydroxyquinolin-7-yl)(pyridin-3-yl)methyl)piperazine-1-carboxamido)hexanoate (38 mg, 0.073 mmol) and 1,3-barbituric acid (34.4 mg, 0.220 mmol) were dissolved in dichloromethane (volume: 734 µl, density: 1.325 g/ml). To this was added tetrakis(triphenylphosphine)palladium(0) (8.48 mg, 7.34 µmol) and the reaction was stirred until deallylation was completed. Product was isolated with ISCO column chromatography yielding 15.2 mg (43%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.34 (d, J=6.59 Hz, 2 H) 1.41 - 1.54 (m, 2 H) 1.54 - 1.70 (m, 2 H) 2.31 (t, J=7.06 Hz, 2 H) 2.37 - 2.56 (m, 3 H) 3.20 (d, J=3.58 Hz, 2 H) 3.33 - 3.47 (m, 4 H) 4.90 - 5.02 (m, 2 H) 7.22 (dd, J=7.54, 4.52 Hz, 1 H) 7.40 (dd, J=8.10, 4.14 Hz, 1 H), 7.49 (d, J=8.67 Hz, 1 H) 7.88 (d, J=7.54 Hz, 1 H) 8.07 (d, J=7.54 Hz, 1 H) 8.45 (br. s., 1 H) 8.78 (d, J=8.48 Hz, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 24.3, 26.3, 29.6, 34.0, 40.5, 43.8, 51.4, 53.4, 67.1, 118.3, 121.8, 124.0, 126.6, 127.8, 136.0, 136.3, 137.2, 138.6, 148.0, 148.4, 148.9, 150.2, 157.9, 176.9.

Resolution of rac-BRD4354 with SFC

Enantiomers of BRD4354 were separated by Shanghai ChemPartner Co. Ltd., Shanghai 201203, China, using supercritical fluid chromatography (SFC). Mobile phase consisted of supercritical CO_2 (flow rate 2.4 ml/min) with isopropyl alcohol as a cosolvent (20%, 0.6 ml/min), on a chiral AD-H column (4.6*250 mm, 5 µm) kept at a constant temperature of 37.4 °C.



Peak Info

Number	RT (min)	Area %	Area
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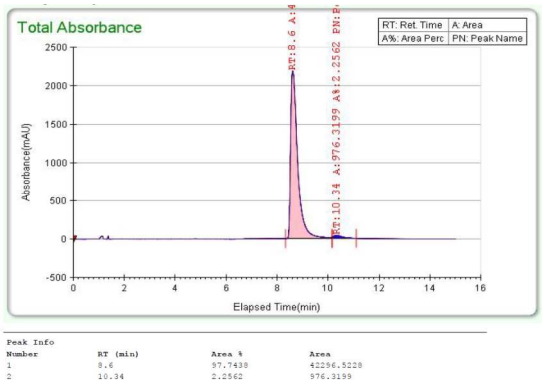
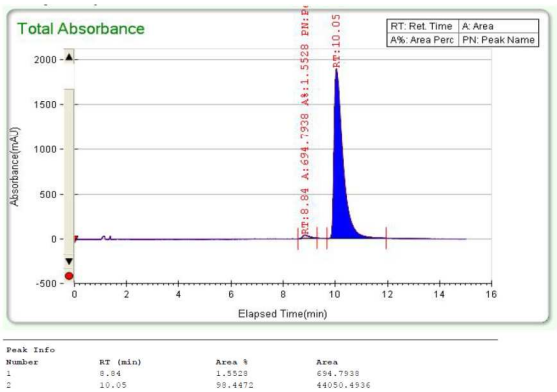
2	10.08	51.3521	6585.5429





SI Figure 1. Racemic BRD4354 and SFC separated enantiomers.

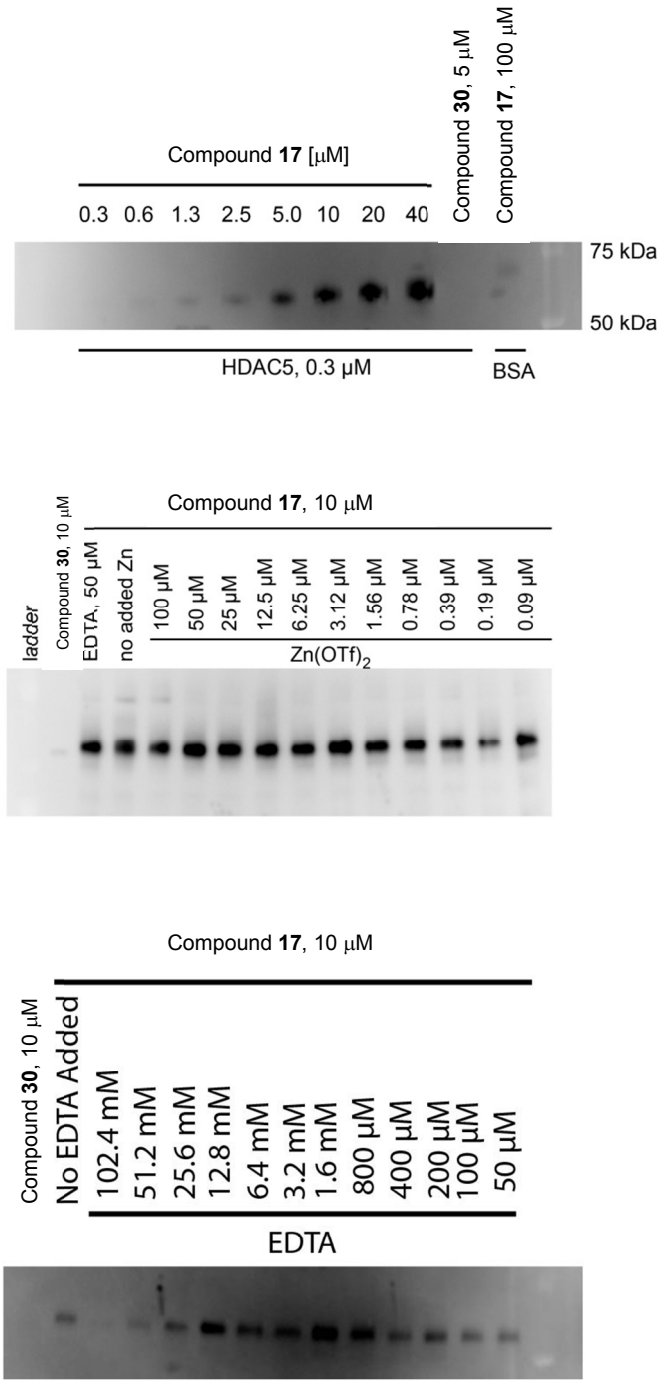
Covalent adduct formation

Kinetics of *N*-Ac cysteine adduct formation (Figure 4A)

The stock solutions of 0.1 M $Zn(OTf)_2$ (72.72 mg, 2 mL H_2O), 0.01 M $Zn(OTf)_2$ (7.27 mg, 2 mL H_2O), 0.1 M triflic acid (30 mg, 17 µL, 2 mL DMSO), 0.01 M triflic acid (3 mg, 1.7 µL, 2 mL DMSO), 0.1 M *N*-acetyl-L-cysteine (Fluka, 65.28 mg, 4 mL DMSO), 0.1 M BRD4354 (76.4 mg, 2 mL DMSO), were prepared in 1-dram glass vials. 100 µL of BRD4354 and NAC solutions were mixed in 10 different Eppendorf tubes. Subsequently, 100 µL of each additive was added to appropriate tubes and the tubes were shaken at room temperature and sampled by removing 5 µL from each reaction and diluting it with 45 µL of DMSO at regular time periods with more frequent sampling early in the reaction. The extent of the reaction was determined by LC-MS (Alliance 2795, Waters, Milford, MA). Ratio of reactant and adduct was measured by UV absorbance at 210 nm. Identity was determined on an SQ mass spectrometer by positive and negative electrospray ionization. Mobile phase A consisted of 0.01% formic acid in water, while mobile phase B consisted of 0.01% formic acid in acetonitrile. The gradient ran from 5% to 95% mobile phase B over 6.75 minutes at 1.75 mL/min. An Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0x30 mm column was used with column temperature maintained at 40 °C. 2.1 µL of sample solution were injected. The peak corresponding to BRD4354 at mass of 382.16 and peak corresponding to adduct at 431.07 were identified. The diode array curves in the region corresponding to these masses were integrated. The percent adduct formation is calculated from the ratio of integrated area corresponding to adduct and the sum of integrated areas of adduct and the starting material.

Western blot visualization of covalent interaction between biotin-labeled analog and HDAC5 (Figure 4B)

Recombinant HDAC5 was diluted from stock solutions into phosphate buffer saline to obtain 50 µL solutions of 2.95 µM HDAC. Biotinylated active compound **17** (AF52) was added to each solution to give final concentrations ranging from 0.3 to 40 µM. A biotinylated inactive compound **30** (SFL49) was also evaluated at 5 µM as well as the DMSO vehicle control. These protein-compound mixtures were incubated at room temperature with shaking for 1 hour. 10 µL aliquots were supplemented with 2 µL of 6X Laemmli sample buffer and heated to 95 °C for 10 minutes, followed by SDS-PAGE for 45 minutes at 200 V. The proteins were transferred to a PVDF membrane followed by blocking with 5% BSA in TBST and incubation with streptavidin labeled-horseradish peroxidase (Cell Signaling, 1000x dilution in TBST, 5% BSA). The membrane was washed 3 times 5 minutes in TBST and bands were visualized using SuperSignal® West Femto Maximum Sensitivity Substrate, (Thermo Scientific, Cat # 34096).



SI Figure 2. Immunoblots of HDAC5 treated with biotinylated analog, compound **17**, and probed with streptavidin-horseradish peroxidase aimed at evaluating the effect of additional zinc or EDTA.

Mass spectrometric (Electrospray ionization / time of flight) determination of covalent interaction between intact HDAC9 and BRD4354 (Figure 4C)

Recombinant HDAC9 (2 µM), and recombinant HDAC9 treated with BRD4354 (20 µM) for 30 minutes at room temperature were analyzed on an Agilent 6210 ESI-TOF mass spectrometer using a short reverse phase separation for protein cleanup and separation. The mass spectrometer was operated in positive ion mode with a fragmentor voltage of 170 V, a nebulizer pressure of 40 psi, and a countercurrent drying gas of 12 L/min. The column was Agilent PLRP-S, 5 cm column, 1000 angstrom pore size, 8 micron particle size. A short 10 minute gradient elution was done at 0.250 ml/min. After a 2 minute wash using 95% of a 0.1 % formic acid in water (5% acetonitrile), a gradient elution was performed to 95% acetonitrile for 10 minutes and maintained for 5 minutes at 95%. The column was set to 95% H_2O after 0.1 minutes and maintained for another 5 minutes. The multiple charge state distribution observed was deconvoluted using MaxEnt (Maximum Entropy Algorithm) for deconvolution with a signal to noise threshold set to 30, and de-convoluted between (5,000 and 100,000 Da).

Determination of specific covalently labeled residues through LC-MS/MS analysis of peptide mixture obtained through tryptic digest of HDAC5 and HDAC9 (Figure 4D)

Sequence analysis was performed at the Harvard Mass Spectrometry and Proteomics Resource Laboratory by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (µLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer.

The sequences summarized in Figure 4D are the result of analysis by microcapillary reverse-phase HPLC, directly coupled to the nano-electrospray ionization source of an LTQ-Orbitrap Velos or LTQ-Oritrap XL mass spectrometer. These instruments acquire individual sequence (MS/MS) spectra on-line at high mass accuracy (<2 ppm) and sensitivity (<<<1 femtomole) for multiple peptides in the chromatographic run. These MS/MS spectra were correlated with known sequences using the algorithm Sequest developed at the Univ. of Washington (Eng *et al*, 1994), and programs developed in Harvard Microchemistry Facility (Chittum *et al*, 1998). MS/MS peptide sequences were next reviewed manually for consensus with known proteins and the results were compared to Sequest analysis.

Legend:

HDAC9, 15 min treatment

M*:+15.99490 C#:+57.02146 C@:+268.04547

Peptide	Position	Peptide	Position
THSSPAASVLPHPAMDR	6-22	SSLPC#GGLGVDSDTIWNELHSSGAAR	124-149
THSSPAASVLPHPAM*DR	6-22	GGLGVDSDTIWNELHSSGAAR	129-149
THSSPAASVLPHPAM*DRPLQPG	6-27	GLGVDSDTIWNELHSSGAAR	130-149

DRPLQPGSATGIAYDPLM*LK	21-40	GVDSDTIWNELHSSGAAR	132-149
PLQPGSATGIAYDPLMLK	23-40	GVDSDTIWNELHSSGAARM*AVGCVIELASK	132-161
PLQPGSATGIAYDPLM*LK	23-40	VDSDTIWNELHSSGAAR	133-149
QPGSATGIAYDPLM*LK	25-40	VDSDTIWNELHSSGAARMAVGC@VIELASK	133-161
SATGIAYDPLMLK	28-40	DSDTIWNELHSSGAARM*AVGCVIELASK	134-161
GIAYDPLMLKHQCVCGNSTTHPEHAGR	31-57	TIWNELHSSGAARMAVGCVIELASK	137-161
IAYDPLMLKHQC#VCGNSTTHPEHAGR	32-57	WNELHSSGAARMAVGC@VIELASK	139-161
IAYDPLM*LKHQCVC#GNSTTHPEHAGR	32-57	WNELHSSGAARMAVGCVIELASKVASGELK	139-168
AYDPLMLK	33-40	WNELHSSGAARM*AVGC#VIELASKVASGELK	139-168
LMLKHQCVC#GNSTTHPEHAGR	37-57	LHSSGAARMAVGC@VIELASK	142-161
LMLKHQC#VC#GNSTTHPEHAGR	37-57	LHSSGAARM*AVGC#VIELASK	142-161
LKHQC#VC#GNSTTHPEHAGR	39-57	AARM*AVGC@VIELASKVASGELK	147-168
KHQC#VC#GNSTTHPEHAGR	40-57	AARM*AVGC@VIELASKVASGELKNGFAVVR	147-175
KHQC#VC#GNSTTHPEHAGRIQSIWSR	40-64	ARMAVGC@VIELASKVASGELKNGFAVVR	148-175
HQC#VC#GN	41-47	ARM*AVGC@VIELASKVASGELKNGFAVVR	148-175
HQC#VCGNSTTHPEHAGR	41-57	MAVGCVIELASK	150-161
HQC#VC#GNSTTHPEHAGR	41-57	MAVGC#VIELASK	150-161
HQCVCGNSTTHPEHAGRI	41-58	MAVGC@VIELASKVASGELK	150-168
HQC#VCGNSTTHPEHAGRIQS	41-60	MAVGC#VIELASKVASGELK	150-168
HQC#VC#GNSTTHPEHAGRIQSIWSR	41-64	MAVGCVIELASKVASGELKN	150-169
HQCVCGNSTTHPEHAGRIQSIWSRLQET	41-68	AVGC#VIELASK	151-161
HQCVCGNSTTHPEHAGRIQSIWSRLQETGLL	41-71	VGC#VIELASK	152-161
CGNSTTHPEHAGRIQSIWSRLQETGLLNK	45-73	GCVIELASK	153-161
GNSTTHPEHAGR	46-57	GC#VIELASK	153-161
NSTTHPEHAGR	47-57	KVASGELK	161-168
STTHPEHAGR	48-57	VASGELKN	162-169
TTHPEHAGR	49-57	VASGELKNGF	162-171
TTHPEHAGRIQSIWSRLQETGLLNKCER	49-76	VASGELKNGFAVVRPP	162-177
THPEHAGRIQSIWSRLQETGLLNKC#ER	50-76	VASGELKNGFAVVRPPGHHAEESTAM*GF	162-189
AGRIQSIWSR	55-64	GELKNGFAVVR	165-175
IQSIWSR	58-64	NGFAVVRPPGH	169-179
IWSRLQETGLLNKC#ERIQGR	61-80	NGFAVVRPPGHHA	169-181
SRLQETGLLNKC#ER	63-76	NGFAVVRPPGHHAE	169-182
LQETGLLN	65-72	VRPPGHHAEESTAMGFCFFNSVAITAK	174-200
LQETGLLNK	65-73	VRPPGHHAEESTAM*GFC#FFNSVAITAKYLR	174-203
LQETGLLNKC#	65-74	PPGHHAEESTAM*GFCFFNSVA	176-196
LQETGLLNKCER	65-76	PPGHHAEESTAMGFC#FFNSVAITAKYL	176-202
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LQETGLLNKC#ERIQGRK	65-81	STAM*GFCFFNSVAITAKYLRDQLNISK	184-210
QETGLLNK	66-73	FCFFNSVAITAK	189-200
QETGLLNKC#ER	66-76	FNSVAITAK	192-200
ETGLLNK	67-73	NSVAITAK	193-200

TGLLNKC#ER	68-76	YLRDQLN	201-207
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NKCERIQGR	72-80	DQLNISK	204-210
CERIQGRKASLEEIQLVHSE	74-93	QLNISK	205-210
CERIQGRKASLEEIQLVHSEHHSLLYGTNP	74-103	ILIVDLDVHHGN	211-222
IQGRKASLE	77-85	ILIVDLDVHHGNGT	211-224
IQGRKASLEEIQ	77-88	VDLDVHHGNGTQQAFYADPSILYISLHR	214-241
KASLEEI	81-87	HGNGTQQAFYADPSILYISLHR	220-241
KASLEEIQ	81-88	YADPSILYISLHR	229-241
KASLEEIQLV	81-90	YDEGNFFPGSGAPNEVGTGLGEGY	242-265
KASLEEIQLVHS	81-92	YDEGNFFPGSGAPNEVGTGLGEGYNIN	242-268
KASLEEIQLVHSEH	81-94	GLGEGYNINIAWTGGLDPPMGDVEYLEAFR	260-289
KASLEEIQLVHSEHH	81-95	GYNINIAWTGGLDPPMGDVEYLEAFR	264-289
KASLEEIQLVHSEHHS	81-96	GDVEYLEAFR	280-289
KASLEEIQLVHSEHHSLL	81-98	TIVKPVA	290-296
KASLEEIQLVHSEHHSLLY	81-99	TIVKPVAK	290-297
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KASLEEIQLVHSEHHSLLYGTNPLDGQKLD	81-110	EFDPDMVLVSA	298-308
ASLEEIQLVHSEHHS	82-96	EFDPDMVLVSAGFDALEGHTPPLGGYK	298-324
ASLEEIQLVHSEHHSLLY	82-99	EFDPDM*VLVSAGFDALEGHTPPLGGYK	298-324
ASLEEIQLVHSEHHSLLYGT	82-101	EFDPDMVLVSAGFDALEGHTPPLGGYKVTAK	298-328
ASLEEIQLVHSEHHSLLYGTN	82-102	EFDPDM*VLVSAGFDALEGHTPPLGGYKVTAK	298-328
ASLEEIQLVHSEHHSLLYGTNPLDGQK	82-108	DPDM*VLVSAGFDALEGHTPPLGGYKVTAK	300-328
ASLEEIQLVHSEHHSLLYGTNPLDGQKLD	82-110	PDM*VLVSAGFDALEGHTPPLGGYK	301-324
ASLEEIQLVHSEHHSLLYGTNPLDGQKLDPR	82-112	VLVSAGFDALEGHTPPLGGYK	304-324
SLEEIQLVHSEHHSLLYGTNPLDGQK	83-108	VLVSAGFDALEGHTPPLGGYKVTAK	304-328
LEEIQLVHSEHHSLLYGTNPLDGQK	84-108	SAGFDALEGHTPPLGGYKVTAK	307-328
QLVHSEHHSLLYGTNPLDGQK	88-108	AGFDALEGHTPPLGGYK	308-324
HSEHHSLLYGTNPLDGQK	91-108	AGFDALEGHTPPLGGYKVTAKC#FGHLTK	308-335
SEHHSLLYGTNPLDGQK	92-108	GFDALEGHTPPLGGYK	309-324
EHHSLLYGTNPLDGQK	93-108	FDALEGHTPPLGGYKVTAKC@FGHLTK	310-335
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YGTNPLDGQK	99-108	TPPLGGYKVTAKC@FGHLTKQLMTLADGR	317-344
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LDPRILLGDDSQKFFSSLPCGGLGVDSDTIW	109-139	TAKC#FGHLTK	326-335

RILLGDDSQK	112-121	CFGHLTK	329-335
ILLGDDSQK	113-121	CFGHLTKQLM*TLADGRVVLALEGGHDLTAIC	329-359
ILLGDDSQKF	113-122	FGHLTK	330-335
ILLGDDSQKFF	113-123	QLMTLADGR	336-344
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ILLGDDSQKFFSSLPC#GGLGVD	113-134	VVLALEGGHDLTAI	345-358
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FFSSLPC#GGLGVDSDTIWNELHSSGAARM*	122-150	NAVISLQK	389-396

Legend:

HDAC5; 15 min treatment

M*:+15.99490	C#:+57.02146	C@:+268.04547	
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RTQSSPAAPGGM*K	1-13	GIGVDSDTVWNEMHSSSAVR	126-145
RTQSSPAAPGGMKSPPDQPV	1-20	SDTVWNEM*HSSSAVRMAVGCLLELAFK	131-157
RTQSSPAAPGGMKSPPDQPVK	1-21	TVWNEM*HSSSAVRMAVGC@LLELAFK	133-157
RTQSSPAAPGGM*KSPPDQPVK	1-21	TVWNEM*HSSSAVRMAVGC#LLELAFK	133-157
RTQSSPAAPGGMKSPPDQPVKHLFTTG	1-27	VWNEMHSSSAVRMAVGCLLELAFK	134-157
TQSSPAAPGGMK	2-13	VWNEMHSSSAVRM*AVGC@LLELAFK	134-157
TQSSPAAPGGM*K	2-13	WNEM*HSSSAVRM*AVGC@LLELAFK	135-157
TQSSPAAPGGMKSPPDQPV	2-20	EMHSSSAVRM*AVGC@LLELAFK	137-157
TQSSPAAPGGMKSPPDQPVK	2-21	EM*HSSSAVRMAVGC@LLELAFK	137-157
SSPAAPGGMK	4-13	MHSSSAVRMAVGCLLELAFKVAAGELK	138-164
SPAAPGGMK	5-13	SSSAVRM*AVGCLLELAFK	140-157
PGGM*KSPPDQPVK	9-21	AVRMAVGC#LLELAFKVAAGELK	143-164
GGM*KSPPDQPVKHLFTTGVVYDTFMLK	10-36	VRM*AVGC@LLELAFKVAAGELK	144-164
SPPDQPVK	14-21	MAVGC#LLELAFK	146-157
SPPDQPVKHL	14-23	AVGC#LLELAFK	147-157
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SPPDQPVKHLFTTGV	14-28	NGFAIIRPPGHHAEESTAM*GFC#F	165-187
SPPDQPVKHLFTTGVVYDTF	14-33	NGFAIIRPPGHHAEESTAM*GFCFFN	165-189
SPPDQPVKHLFTTGVVYDTFM*L	14-35	NGFAIIRPPGHHAEESTAMGFCFFNS	165-190

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SPPDQPVKHLFTTGVVYDTFM*LK	14-36
SPPDQPVKHLFTTGVVYDTFM*LKHQC#	14-39
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PPDQPVK	15-21
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HLFTTGVVYDTFMLK	22-36
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TVVMPIAHEFSPDVVLVSA	286-304
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SAGFDAVEGHLSPLGGYSVTAR	303-324
AGFDAVEGHLSPLGGYSVTAR	304-324
GFDAVEGHLSPLGGYSVTAR	305-324
VEGHLSPLGGYSVTAR	309-324
LSPLGGYSVTAR	313-324
GGYSVTAR	317-324
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CFGHLTRQLMTLAG	325-338
FGHLTR	326-331

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WSC#VQK	401-406
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ETVSAMALLSVGAEQAQAAAAR	427-448
SAM*ALLSVGAEQAQAAAAR	430-448
ALLSVGAEQAQAAAAR	433-448
LSVGAEQAQAAAAR	435-448
SVGAEQAQAAAAR	436-448
VGAEQAQAAAAR	437-448
PRPAEEPMEQEPAL	452-465

Enzyme activity data and reversibility studies

Materials

Recombinant HDACs were purchased from BPS Bioscience, Inc. HDACs 1, 2, 3, 6, and 8 were supplied as full length proteins. HDACs 4, 5, 7, and 9 were supplied as the corresponding catalytic domains. The anti-acetylated lysine antibody was purchased from Cell Signaling (cat # 9441L), while anti-acetylated α-tubulin (cat # T6793-.5ml) and DAPI stain (cat # D8417) were purchased from Sigma. Alexa Fluor 488 anti-mouse (cat # A21202) and Alexa Fluor 647 anti-rabbit (cat # A21244) were purchased from Invitrogen. Cell Titer-Glo reagent kit was purchased from Promega (cat # G7571).

Recombinant expression of HDAC5 catalytic domain

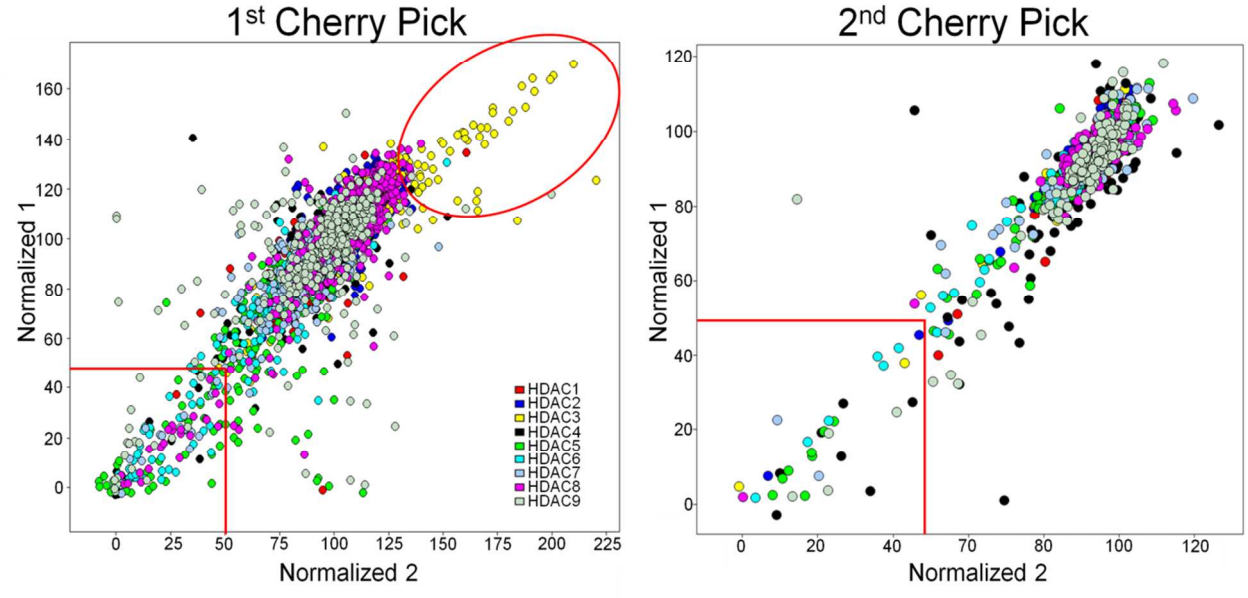
Recombinant HDAC5 catalytic domain was expressed in Dr. Steven Almo's lab at Albert Einstein College of Medicine. To produce HDAC5, cDNA corresponding to residues 1-466 of HDAC5 was cloned into baculovirus transfer vector pIEx (EMD Millipore) for cytosolic expression. Transfection and virus amplification was then performed with Bacmagic-2 (EMD Millipore) in suspension cultures of Sf9 insect cells. For protein production, suspension cultures of insect High Five cells were cultured in Express Five SFM (Life Technologies) and grown to a density of 2 x 10^6 cells/mL before being infected at a multiplicity of infection of ~10. After 72 hours cells were centrifuged (800 x g; 20 minutes), resuspended in 25 mM Hepes pH 7.6, 250 mM NaCl, 500 mM L-Arg, 10% glycerol, and lysed by sonication (Sonic Dismembrator 500, Fisher Scientific). Soluble HDAC5 was then recovered from cell lysate by affinity chromatography using Ni-IDA resin (Clontech). The HDAC5 protein was then subject to size exclusion chromatography over a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare) into a final buffer condition of 25 mM Hepes pH 7.6 with 250 mM NaCl and 10% glycerol.

HDAC enzymatic assay

The trypsin-coupled, kinetic HDAC activity assay used in this study has been described previously.[2] Briefly, this fluorogenic assays uses an acetylated lysine tripeptide substrate, amide-linked to a fluorescently quenched aminocoumarin (AMC). When HDACs deacetylated the lysine residue, trypsin is able to cleave the AMC, allowing for increase of fluorescent signal. HDAC inhibitors prevent the deacetylation of the lysine, thus preventing the increase of fluorescence.

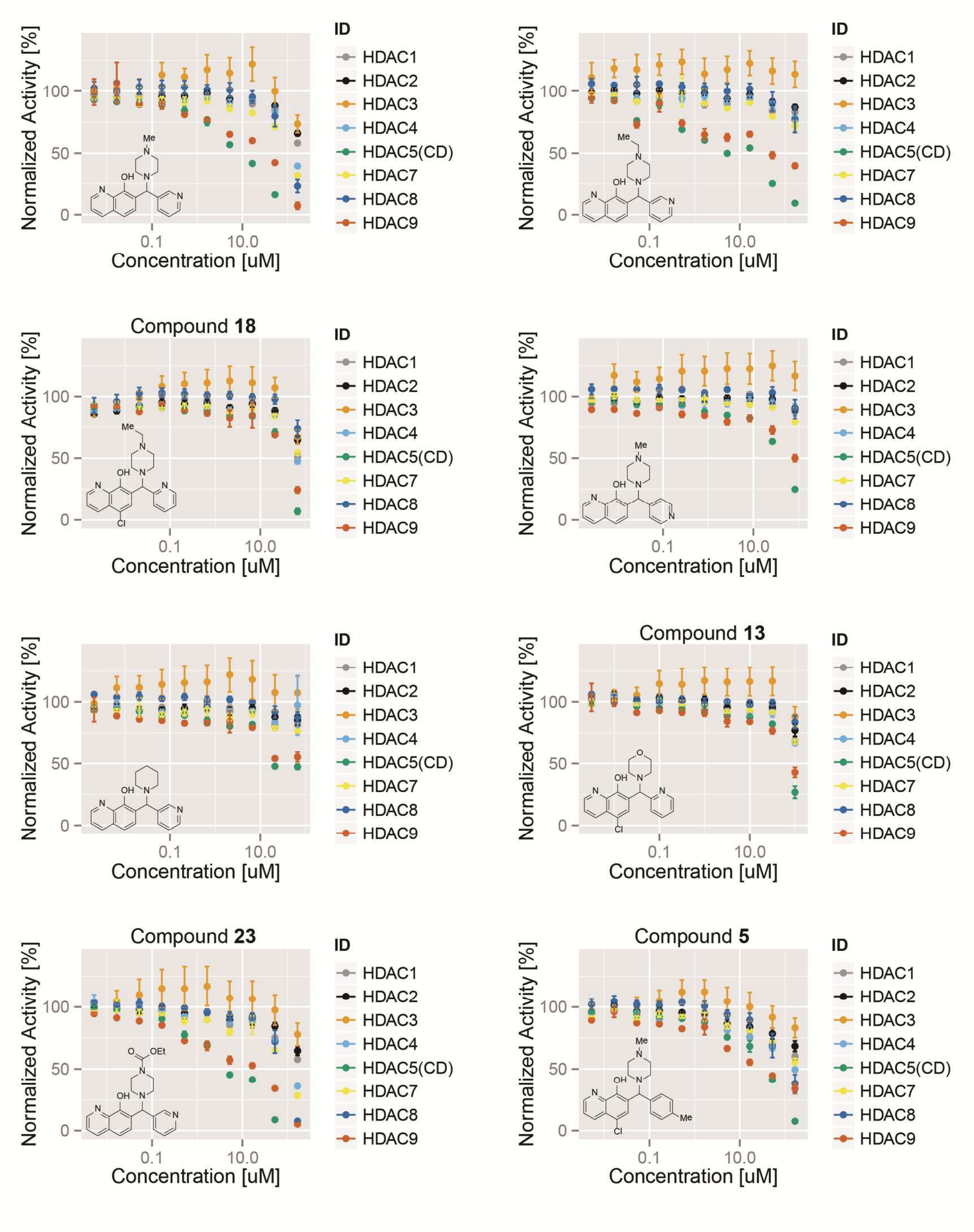
HDACs were purchased from BPS Bioscience, Inc. or recombinantly expressed. Enzyme reactions were run in 50 mM HEPES, 100 mM KCl, 0.001% (v/v) Tween-20, 0.05% (w/v) BSA, pH 7.4. The appropriate substrate concentration for each HDAC was determined using their corresponding Km values. Km values were determined by monitoring the increase of fluorescence of each HDAC at varying substrate concentrations. The increase in fluorescence units was plotted versus substrate

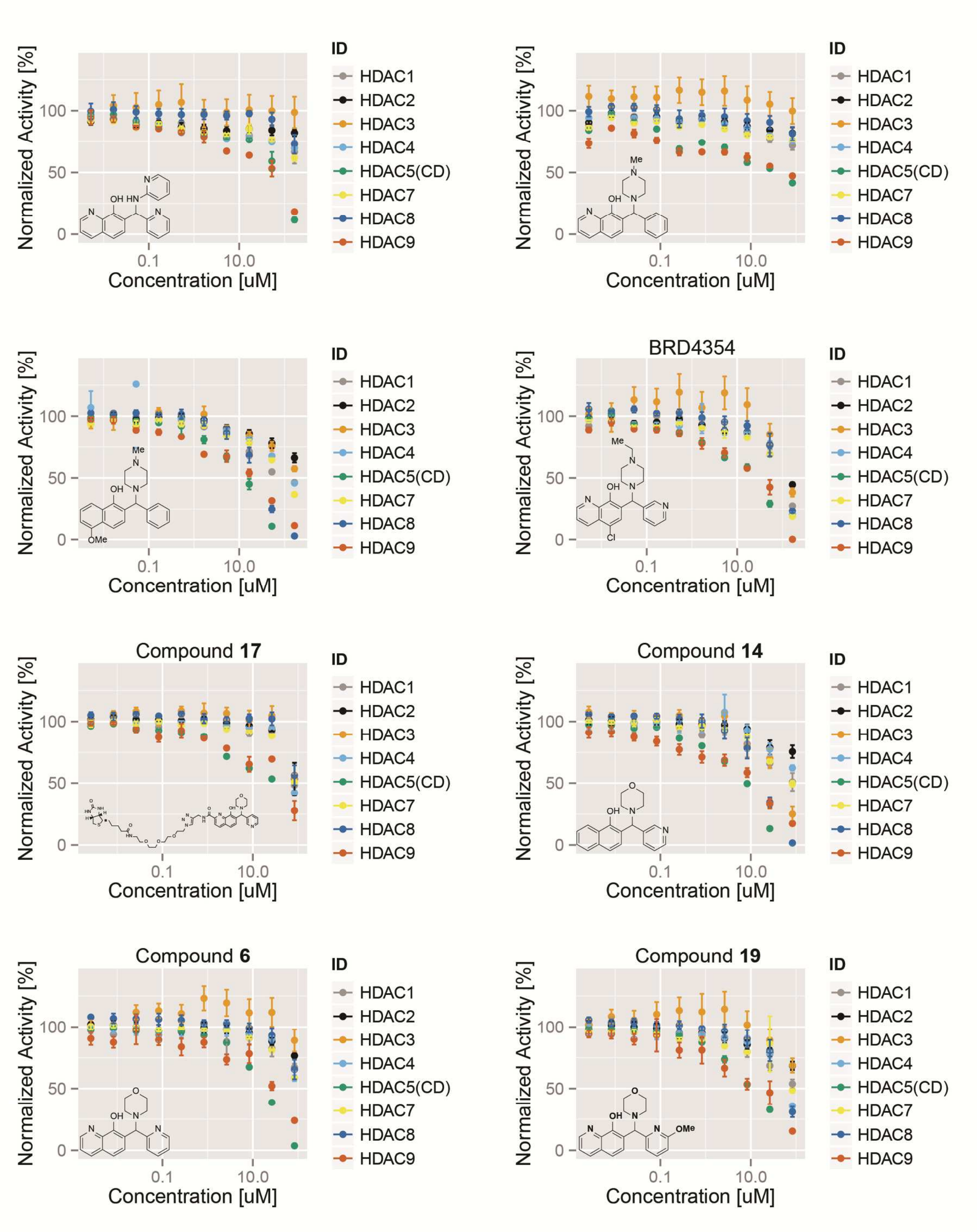
concentration. The graphs were fit to a Michaelis-Menten model using GraphPad Prism. The substrate Km was calculated based on the concentration at 50% of maximum fluorescence. Assays corresponding to HDACs 4, 5, 7, 8, 9 involved a trifluoro-acetylated lysine substrate as described in Bradner *et al,* while HDACs 1, 2, 3, and 6 used an acetyl-Leu-Gly-acetyl-Lys tripeptide as the substrate.[2] Reactions were carried out in 384-well plates and fluorescence was measured using a multi-label plate reader and plate stacker (Envision, Perkin-Elmer). The increase of fluorescence, resulting from the cleavage by trypsin of the fluorescently quenched AMC,was plotted over time, and the slope was calculated analyzed and plotted using Spotfire DecisionSite and GraphPad Prism. The assay was used to evaluate SMM primary assay positives at single dose and in dose-response for selected active compounds. The assay was also used to evaluate analogs.



SI Figure 3. Evaluation of assay positives from SMM screen using an enzymatic assay using a high dose (~30 – 100 μM) cherry pick (left) and followed up by a low dose (2 – 50 μM) cherry pick (right).

Selected dose-response curves in HDAC biochemical inhibition assay





Normalized Activity [%]
Concentration [uM]
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50
100
0.1
10.0
ID
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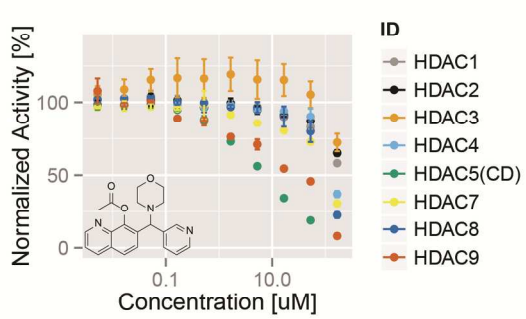
HDAC2
HDAC3
HDAC4
HDAC5(CD)
HDAC7
HDAC8
HDAC9
BRD4354
Compound 17
Compound 14
Compound 6
Compound 19


Normalized Activity [%]
100
50
0
0.1
10.0
Concentration [uM]
ID
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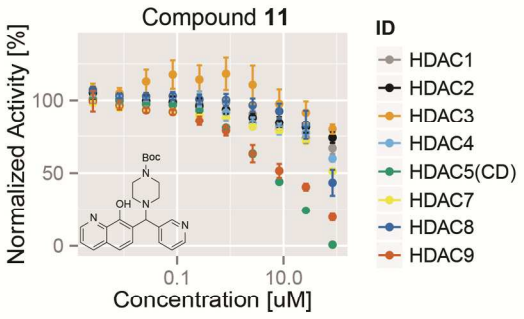
HDAC2
HDAC3
HDAC4
HDAC5(CD)
HDAC7
HDAC8
HDAC9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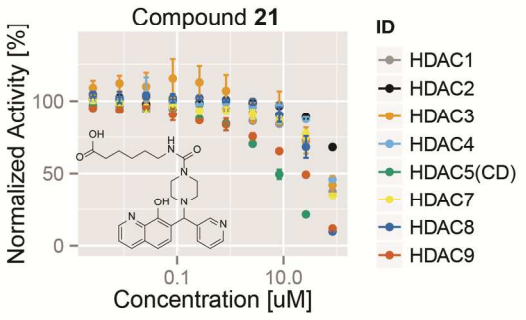
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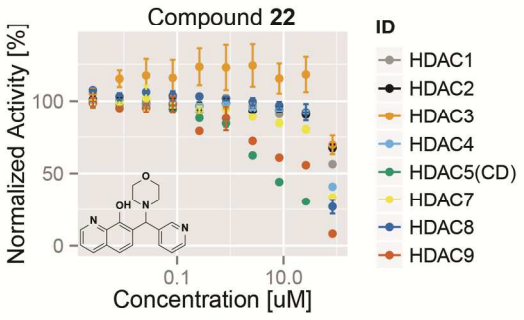
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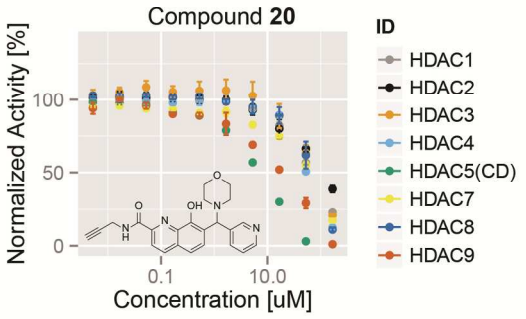
HDAC5(CD)
HDAC7
HDAC8
HDAC9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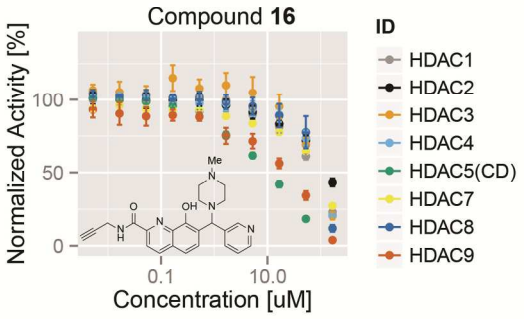
HDAC9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HDAC5(CD)
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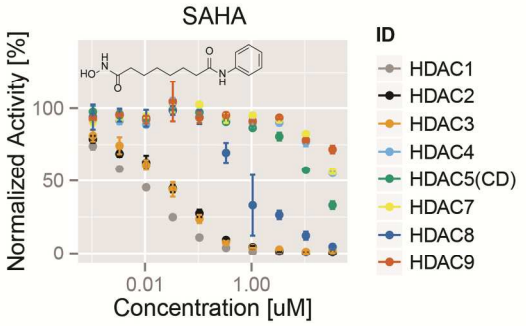
HDAC9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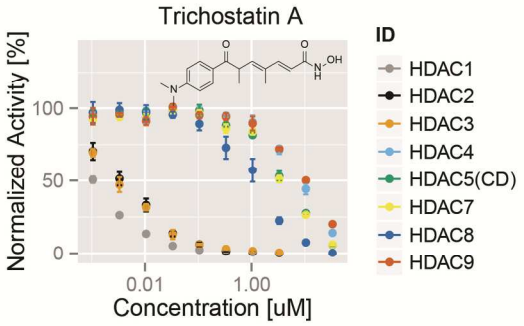
Trichostatin A
Normalized Activity [%]
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0.01
1.00
Concentration [uM]
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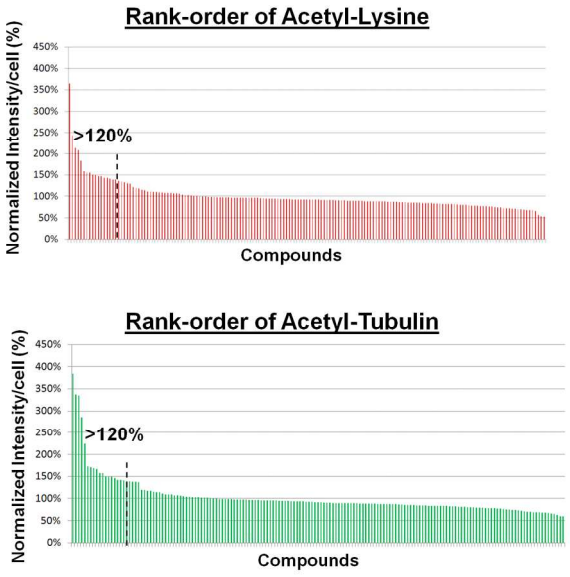
HDAC2
HDAC3
HDAC4
HDAC5(CD)
HDAC7
HDAC8
HDAC9

Immunofluorescence microscopy

Each compound was screened at a single dose in HeLa cells. The level of acetylated lysine and acetylated tubulin were evaluated per compound and ordered by increase of fluorescence. The compounds that exhibited greater than 120% increase over DMSO control were evaluated in dose response assays.

Immunofluorescence microscopy was used to evaluate the acetylation status of proteins as previously described.[3, 4] Briefly, HeLa cells were grown in DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum and 1% penicillin /streptomycin and plated in 384 well plate at a density of 2000 cells/well. The cells were allowed to recover overnight at 37 °C. The cells were then treated with compounds for 16 hours. After which the cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, followed by permeabilization with 1x PBS, 0.1% triton X-100 for 1 hour at room temperature, and blocked with 1x PBS, 0.1% triton X-100, 2% (w/v) BSA for 1 hour at room temperature. The cells were incubated overnight at 4 °C with 1:1000 of anti-acetylated lysine,1:1000 anti-acetylated α-tubulin in 1x PBS, 0.1% triton X-100, 2% BSA. The following day the cells were washed with 1x PBS, 0.1% triton X-100 and incubated with 1:1000 of Alexa Fluor 488 anti-mouse, 1:1000 Alexa Fluor 647 anti-mouse, 1:1000 DAPI, in the PBS, 0.1% triton X-100, 2% BSA, for 1 hour. The cells were washed and 30 µl 1x PBS was added to the wells. The plate was imaged at 20x using a high content image express microscope (IX5000, Molecular Devices). The images were analyzed using Molecular Devices' MetaXpress software and fluorescence was measured per cell and normalized to the DMSO wells.

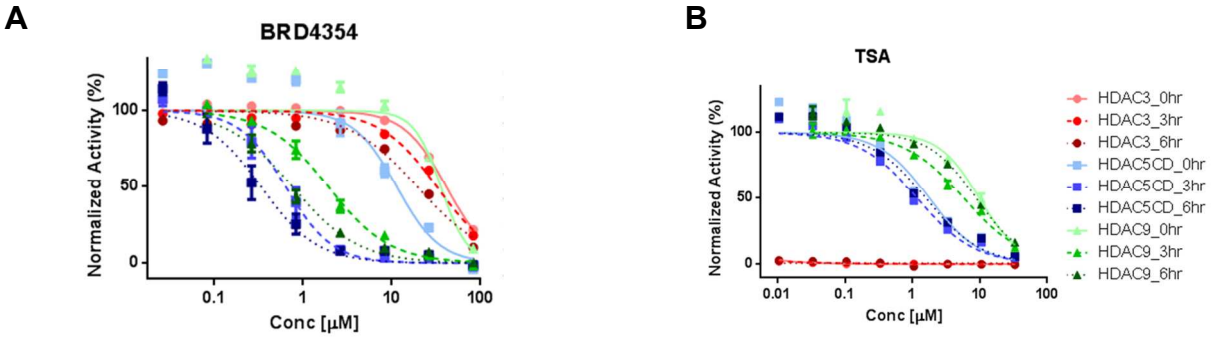
Compounds were followed up in dose response if they inhibited HDACs by at least 50% or if they showed to increase acetylation status.



SI Figure 4. Rank-order analysis of immunofluorescence of acetylated lysine and acetylated tubulin for cherry picked compounds.

Time-dependent inhibition studies (Figure 3A)

Following the general procedure for the biochemical assay, compounds were pin-transferred into 384-well plates and incubated for 0, 3 and 6 hours at room temperature. Afterwards, substrate and trypsin were added to the reaction and the fluorescence was measured over time. The data was analyzed with the same procedure.



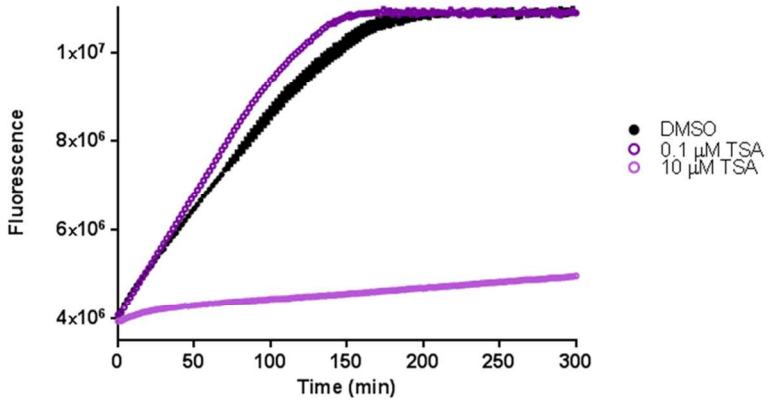
SI Figure 5. Time-course inhibition profiles for BRD4354 (A) and TSA (B).

Dialysis of HDAC5 after treatment with BRD4354 (Figure 3C)

HDAC5 was incubated with BRD4354 at concentrations of 100 nM and 50 µM, respectively, in 50 mM HEPES, 100 mM KCl, 0.001% (v/v) Tween-20, 0.05% (w/v) BSA, pH 7.4, 0.4% DMSO for 1 hour at room temperature. The mixture was transferred to 20 kDa MWCO, 0.1-0.5 ml Slide-A-Lyzer dialysis cassette (Pierce) and dialyzed against 1L of 50 mM HEPES, 100 mM KCl, 0.001% (v/v) Tween-20, 0.05% (w/v) BSA, pH 7.4. After 4 hours samples were taken and buffer was changed and dialyzed over night. Samples were taken before dialysis, 4 hours and 1 day after dialyzing. Activity was measured, as previously described, in triplicate.

Reversibility study of HDAC5 and BRD4354 (Figure 3B)

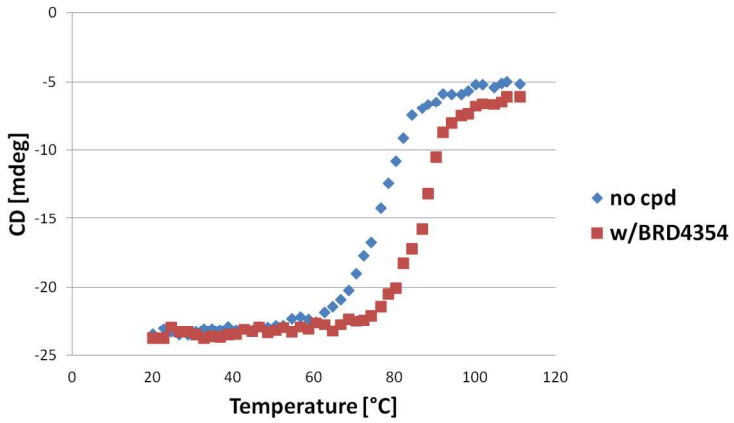
The appropriate concentration of substrate and HDAC5 was determined to be 100 µM 0.1 nM, respectively, to give a linear increase in signal. Titration of BRD4354 was done using 100 µM substrate and 0.1 nM HDAC5 to determine an appropriate IC_{50} concentration (0.8 µM) at those conditions. 100x the final concentration of HDAC5 (10 nM) was incubated with 10x the IC_{50} of BRD4354 (8 µM), or control, Trichostatin A (TSA) (10 µM) for one hour at room temperature. The reaction mixture was then diluted 100x by substrate and trypsin and activity was measured over time. To establish a baseline for a fully inhibited control, HDAC5 was incubated 800 µM BRD4354 or 1 mM TSA for 1 hour at room temperature, after dilution with trypsin and substrate the final concentration was 8 µM for BRD5435 and 10 µM for TSA.



SI Figure 6. TSA inhibits HDAC5 in a reversible manner. HDAC5 was incubated with 10 µM or 1 mM TSA for 1 hour at room temperature. The solutions were diluted 100x and activity was measured; final concentrations of TSA after dilution were 0.1 µM and 10 µM.

Circular Dichroism of HDAC5 with BRD4354

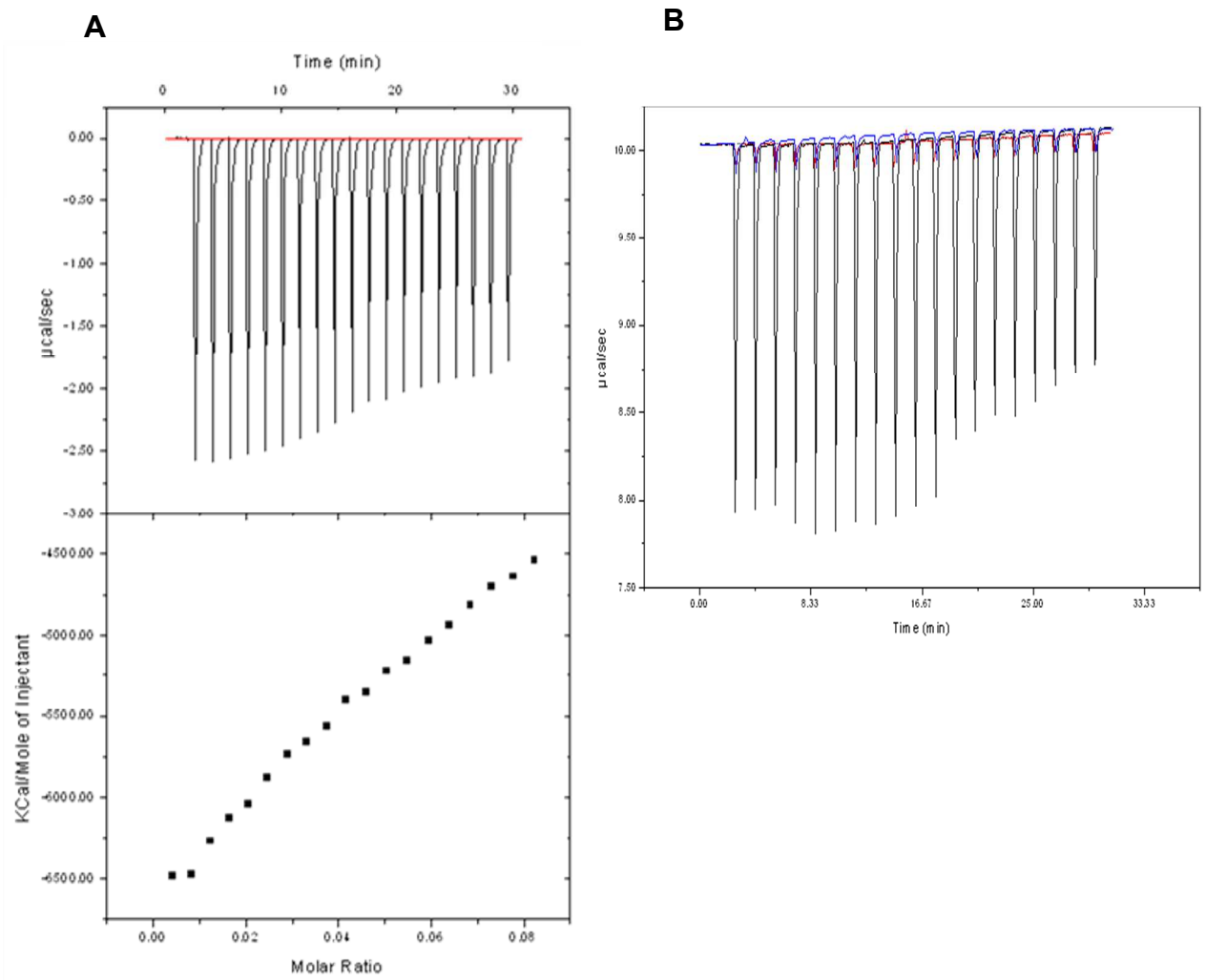
Circular dichroism (CD) was performed on a Jasco J-815 CD spectrometer to further evaluate the binding of BRD4354 with HDAC5 by observing a thermal stability melting assay. HDAC5 was used at a concentration of 6 μM, followed by the addition of BRD4354, for a final concentration of 100 μM, or DMSO control. The solution was added to 1 mm quartz cuvette, with a 0.1 data pitch, 0.5 nm/min scan speed, and 3 accumulations. The variable temperature was controlled by a Jasco programmable Peltier unit, starting from 20 °C to 110 °C at a rate of 100 nm/min. The CD signal at 222 nm, corresponding to α-helices, was continuously monitored with increasing temperatures and plotted as a function of time. BRD4354 has a stabilizing effect on HDAC5 with an estimated Tm of 85 °C, compared with HDAC5 without compound, which had an estimated Tm of 75 °C.



SI Figure 7. Thermal stability of HDAC5 monitored by CD. Changes of CD signal at 222 nm for HDAC5 was monitored as a function of temperature, with BRD4354 (red) and without compound (blue). The change in Tm was approximately 10 °C in the presence of compound.

Isothermal Titration Calorimetry

Isothermal titration calorimetry experiments were carried out with ITC200 (Microcal, Inc. from GE Healthcare). Recombinant HDAC5 was added into the calorimeter cell at a concentration of 5 μM, with a 0.2% DMSO concentration. The compound was prepared at concentration of 2-50 μM with the same buffer conditions as the protein, and loaded into the titration syringe. Titrations were carried out at room temperature with 20 injections at 2 μl for each injection at 90 sec intervals. The cell was being stirred at a constant rate of 1000 rpm. A control of 100 μM of BRD4354 injected into 30 μM of BSA did not show any significant interaction, and was similar to injection into buffer.

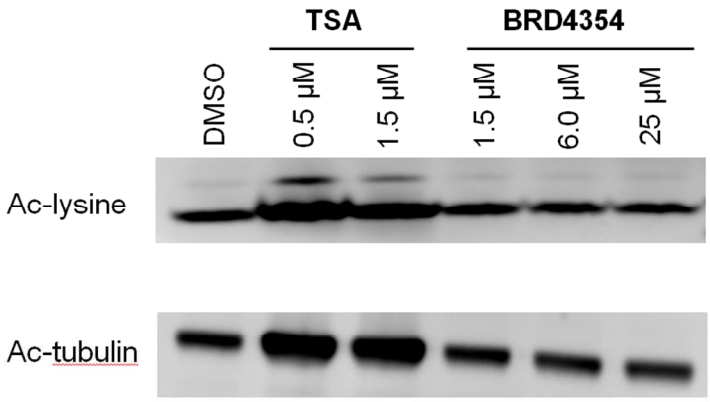


SI Figure 8. Isothermal calorimetry of BRD4354 and HDAC5 and controls. (A) 2 µM BRD4354 was injected into 5 µM HDAC5. (B) Overlay of injection of 2 µM BRD4354 and 5 µM HDAC5 (black), 30 µM BSA with 100 µM BRD4354 (blue), and 100 µM BRD4354 into Tris buffer (red).

Phenotypic experiments

Monitoring changes in bulk acetylation status via western blotting

HeLa cells were grown in DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum and 1% penicillin /streptomycin. HeLa were plated in a 6-well plate at a density of ~5x10^5 cells/well. The cells were cultured overnight a 37 °C and then treated with BRD4354 at 1.5, 6, and 25 μM, TSA at 0.5 and 1.5 μM, and DMSO control at 0.5% final concentration for 24 hours. The cells were then trypsinized by addition of 1 ml of 0.25% trypsin-EDTA solution and incubated at 37 °C for 5 minutes. The solution was collected and spun down at 500 xg for 5 minutes to collect the cells. The cells were lysed in RIPA buffer and spun down at ~14,000 xg to remove insoluble proteins. The proteins were separated on a 4-12% SDS-PAGE gel and transferred to a PVDF membrane and probed with acetylated histone lysine and acetylated tubulin antibodies. The western blots were visualized from secondary antibodies conjugated with horseradish peroxidase and developed with SuperSignal Chemiluminescent Substrate (Thermo Scientific).

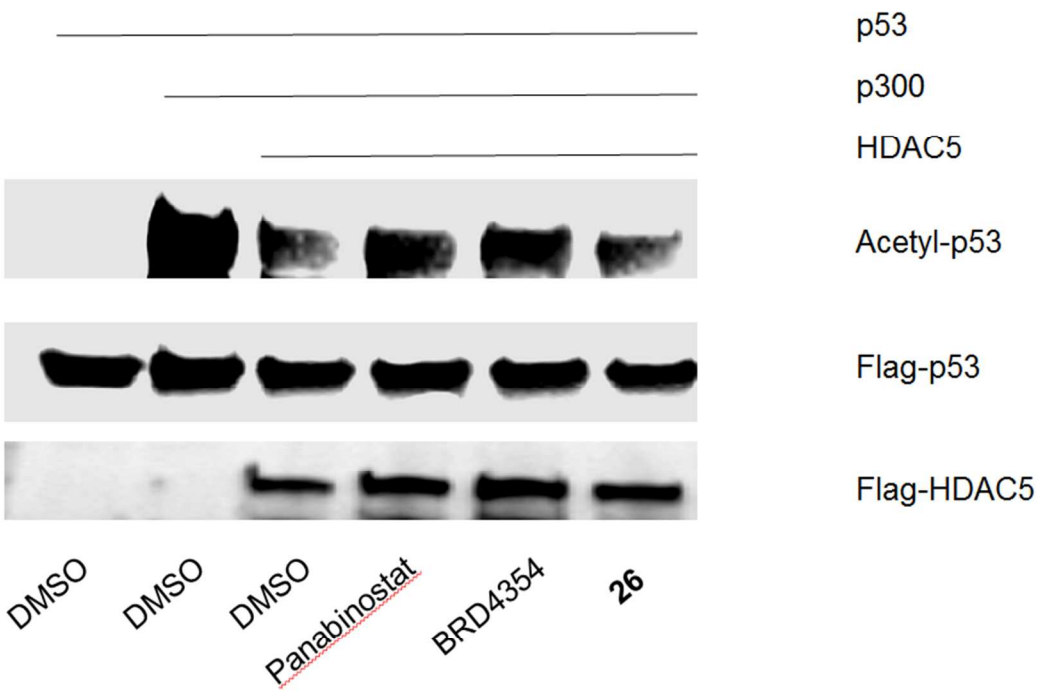


SI Figure 9. Changes in bulk acetylation of histones and tubulin in response to treatment with TSA and BRD4354.

Monitoring changes in acetylation status for p53 via western blotting

HEK293 cells were co-transfected with FLAG-p53 and p300. Separately, cells were transfected with FLAG-HDAC5. After 48 hours, cells were harvested and resuspended in RIPA lysis buffer supplemented with protease inhibitors and subjected to three freeze/thaw cycles and sonication (30% power, 50% cycle, 15 pulses). Crude lysates were centrifuged for 15 minutes at 13000 rpm/4°C. Cleared lysate were incubated with anti-FLAG M2-conjugated agarose beads overnight at 4°C. The bead complexes were washed twice in a mild lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40) and twice in HDAC assay buffer (25 mM HEPES, pH 7.4, 137 mM NaCl, 2.7 mM

KCl). HDAC5 beads were equally divided and pre-incubated for 1 hour at room temperature with 8 µM compound or with 0.1 µM Panabinostat or 0.5% DMSO. p53-captured beads were equally divided and mixed with HDAC5 beads and incubated for 1 hour at room temperature with continuous mixing. Bead mixtures were centrifuged briefly and resuspended in 1x sample buffer and subjected to SDS-PAGE followed by western blotting using a pan-Acetyl antibody and M2 anti-FLAG antibody. Westerns were visualized as described above.



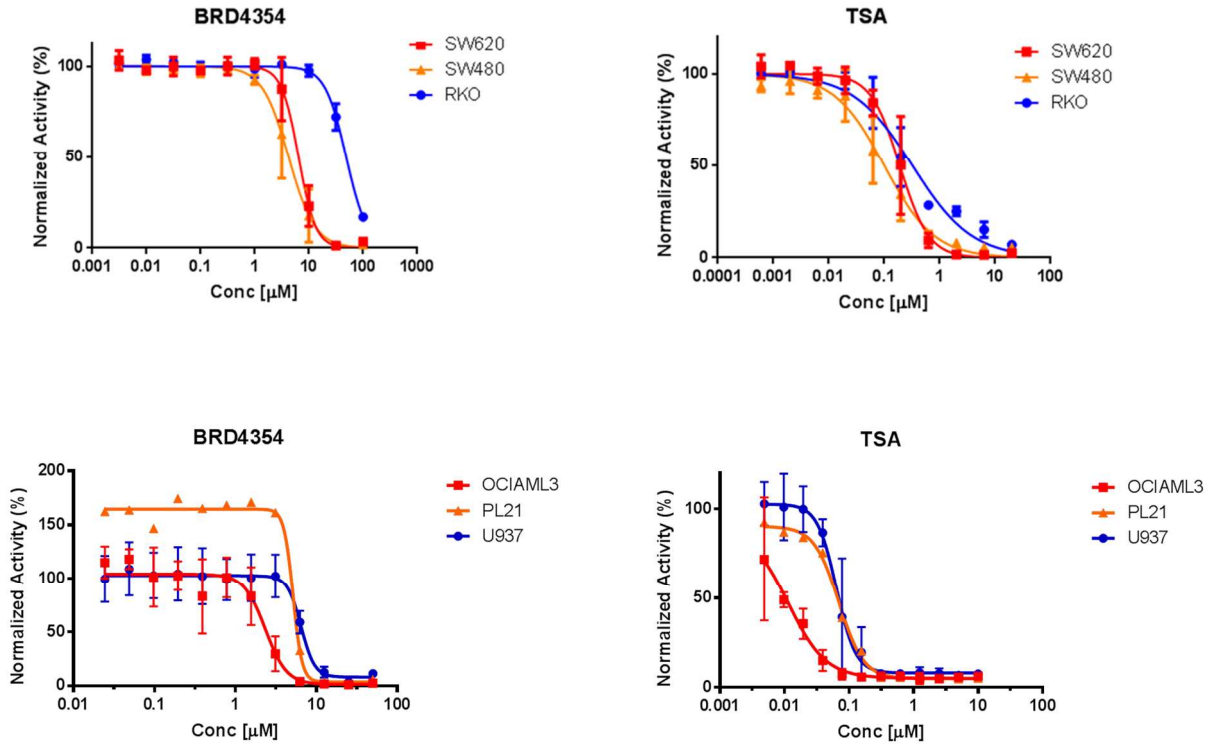
SI Figure 10. Changes in acetylation of p53 (target of acetyltransferase domain of p300 and deacetylase HDAC5) in response to panabinostat (known HDACi), BRD4354, and inactive analog **26**.

Cell Viability Profiling

BRD4354 was previously profiled in a larger study to assess the effects of compounds on cell viability using a panel of different cell lines.[5] To confirm the activity of the compound a subset of cancer cell lines from large intestine or haematopoietic and lymphoid lineages were retested. The large intestinal lines were colorectal cancer cells that were sensitive to the compound, SW620 and SW480, or unresponsive RKO. SW620 and SW480 were grown in L-15 media and RKO was grown in RPMI, both supplemented with 10% FBS and 1% penicillin and streptomycin. From the haematopoietic and lymphoid lineage, PL21 (myeloid leukemia) and U937 (lymphoma) were chosen as unresponsive lines, while OCIAML3 (acute myeloid leukemia) was a

line that was sensitive to treatment with BRD4354. The two unresponsive lines were grown with RPMI and OCIAML3 was grown with AMEM media, both supplemented with 10% FBS and 1% penicillin and streptomycin.

The assay was repeated using conditions that are similar to what was previously reported by Basu *et al*.[4] Briefly, cells were plated in 384-well plate at a density of 1000 cells/well and allowed to grow overnight at 37 °C. The cells were treated with BRD4354 or TSA in dose, and allowed to incubate for 72 hours. The cells were then treated with Cell Titer-Glo to measure the levels of ATP, an indirect correlation to viability. Fluorescence was measured using a plate reader and plate stacker (Envision, Perkin-Elmer). The signal was normalized against DMSO and plotted using Prism GraphPad. For PL21 one of the replicates was out of range and removed, only the average of two reps was used. For the other cell lines these were plotted in triplicate.

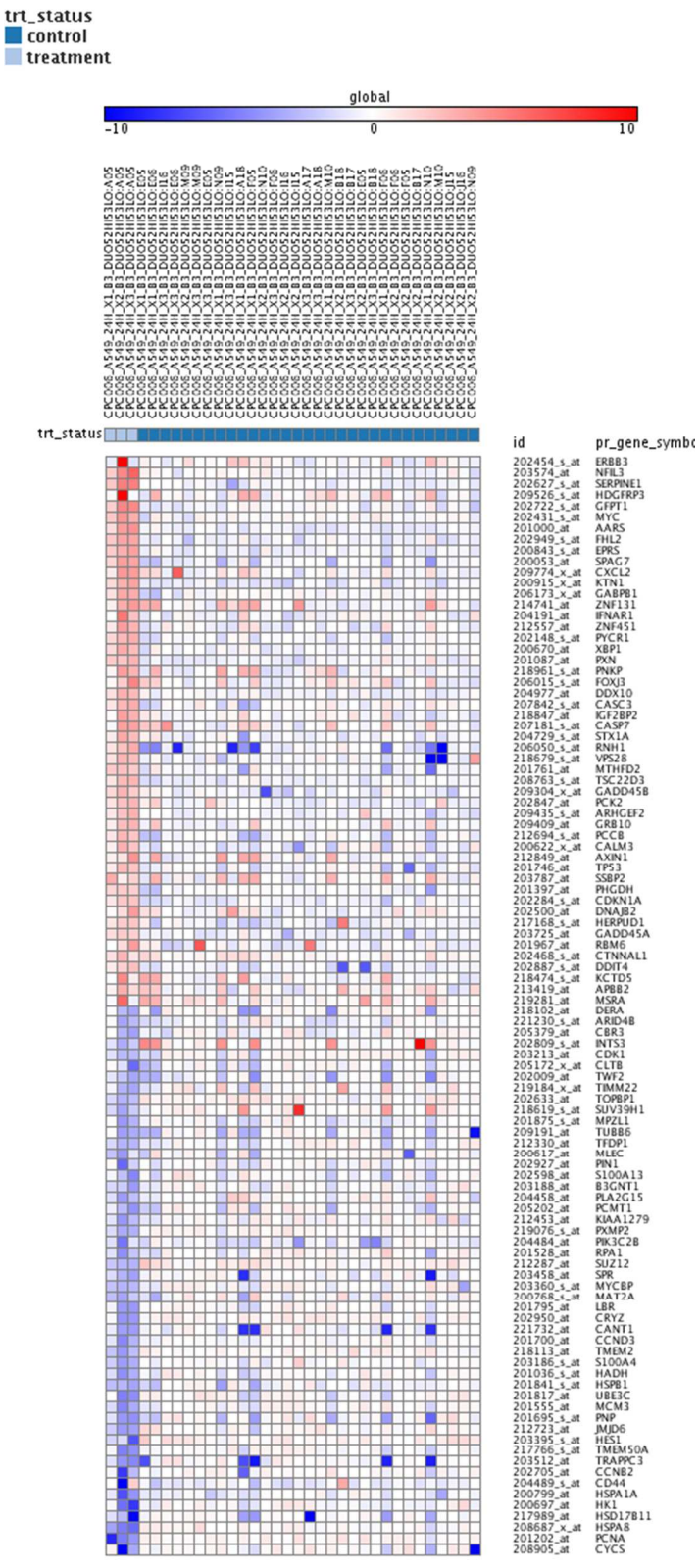


SI Figure 11. Curves corresponding to repeated viability experiments for a subset of large intestine and haematopoietic and lymphoid lineages for cellular viability profiling. BRD4354 and TSA were incubated with each cell line in dose. The viability was measured using Cell Titer Glo and the fluorescence signal was plotted versus concentration.

Change in expression of 1,000 landmark genes in response to treatment with BRD4354

L1000 (http://www.lincscloud.org/l1000/ and http://www.broadinstitute.org/LINCS/) is a high-throughput, bead-based gene expression assay in which mRNA is extracted from cultured human cells treated with various chemical or genomic perturbagens (small molecules, gene knockdowns, or gene over-expression constructs). This mRNA is reverse-transcribed into first-strand cDNA. Gene specific probes containing barcodes and universal primer sites are annealed to the first strand cDNA. The probes are ligated to form a template for PCR. The template is PCR amplified with biotinylated-universal primers. The end products are biotinylated, fixed length, barcoded amplicons. The amplicons can then be mixed with Luminex beads that contain complementary barcodes to those encoded in each of the 1000 amplified landmark genes. These beads are then stained with fluorescent streptavidin-phycoerythrin (SAPE) and detected in 384 well plate format on a Luminex FlexMap flow cytometry-based scanner. The resulting readout is a measure of mean fluorescent intensity (MFI) for each landmark gene. The raw expression data are log2-scaled, quantile normalized, and z-scored, such that a differential expression value is achieved for each gene in each well. In the standard L1000 protocol, each well corresponds to a different perturbagen and these differential expression values are collapsed across replicate wells to yield a differential expression signature for each perturbagen. The signatures of different perturbagens can then be compared to identify those that result in similar or dissimilar transcriptional responses.[6]

Changes in gene expression of BRD4354-treated cells compared to DMSO-treated cells were determined by using L1000 platform. Computed, most highly upregulated and most highly down-regulated genes are presented as a heatmap in SI Figure 12.



SI Figure 12. Top 50 upregulated and top 50 down-regulated genes from landmark 1000 genes (L1000) in response to treatment of A549 cells with BRD4354 (10 μM) for 24 hours. Three leftmost columns correspond to compound treatment.

Supplemental References

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