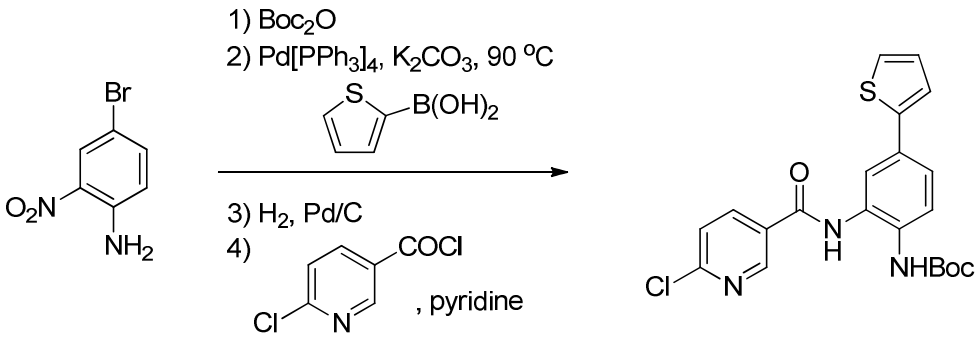
Delayed and Prolonged Histone Hyperacetylation with a Selective HDAC1/HDAC2 Inhibitor

Joey L. Methot,* Dawn Mampreian Hoffman, David J. Witter, Matthew G. Stanton, Paul Harrington, Christopher Hamblett, Phieng Siliphaivanh, Kevin Wilson, Jed Hubbs, Richard Heidebrecht, Astrid M. Kral, Nicole Ozerova, Judith C. Fleming, Hongmei Wang, Alexander A. Szewczak, Richard E. Middleton, Bethany Hughes, Jonathan C. Cruz, Brian B. Haines, Melissa Chenard, Candia M. Kenific, Andreas Harsch, J. Paul Secrist, Thomas A. Miller*

Supporting Information

I. Synthetic Chemistry Methods

Synthesis of Compound 2: *N*-(2-amino-5-(thiophen-2-yl)phenyl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide.



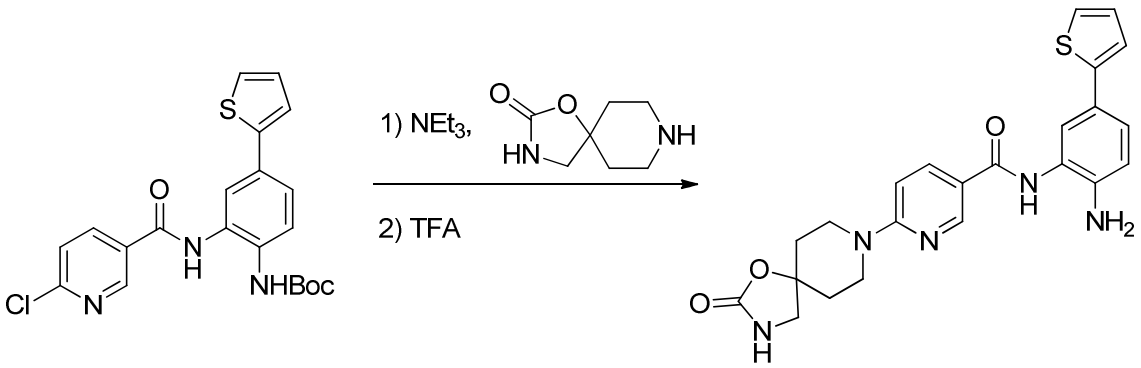
A solution of 4-bromo-2-nitroaniline (200 g, 0.922 mol) in 1,800 mL of dichloromethane was treated with Boc anhydride (221 g, 1.01 mol) and triethylamine (270 mL, 1.94 mol); then DMAP (5.62 g, 0.046 mol) was added in portions over 1 hour. The reddish orange solution was stirred overnight. By TLC, some SM remained (20% EtOAc/Hex). The volatiles were removed, and the crude brownish yellow solid was adsorbed onto 1 kg SiO_2 and was purified on 2 kg silica gel using a 0-2 % EtOAc/Hex gradient to afford 182 g (62%) of bright yellow solid *tert*-butyl (4-bromo-2-nitrophenyl)carbamate.

A mixture of *tert*-butyl (4-bromo-2-nitrophenyl)carbamate (19.4 g, 61.2 mmol), thiophene-2-boronic acid (9.94 g, 77.7 mmol) and K_2CO_3 (22.2 g, 160 mmol) in 60 mL of dioxane and 60 mL of water was degassed by bubbling nitrogen through the mixture for 30 min. Next, $Pd[PPh_3]_4$ (5.25 g, 4.53 mmol) was added and the heterogeneous mixture was warmed to reflux for 20 h. The mixture was cooled and diluted with ethyl acetate, washed with water and brine, filtered through Celite, dried ($MgSO_4$), and concentrated. The solvents were removed under reduced pressure to yield the yellow-brown solid (18.0 g, 92%). The solid was triturated with methanol giving a bright yellow product, *tert*-butyl (2-nitro-4-(thiophen-2-yl)phenyl)carbamate: ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.65 (s, 9 H), 8.58 (d, J = 8.8 Hz, 1 H), 8.39 (d, J = 2.1 Hz, 1 H), 7.81 (dd, J = 8.8, 1.8 Hz, 1 H), 7.32 (m, 2 H), 7.09 (dd, J = 5.3, 3.8 Hz, 1 H), 1.54 (s, 9 H); MS (ESI+): cal'd $[M+Na]^+$ 343.1, obs'd 343.1.

A solution of *tert*-butyl (2-nitro-4-(thiophen-2-yl)phenyl)carbamate (18.0 g) in 350 mL of EtOAc was evacuated and refilled with nitrogen (2x). To the solution was added 10% Pd/C (4.46

g), and the reaction mixture was evacuated and refilled with hydrogen (2x). The black reaction mixture was stirred under an atmosphere of hydrogen overnight. The mixture was filtered through a pad of celite (with EtOAc then CH_2Cl_2 washes) and concentrated to provide a brownish-white solid. The solid was triturated with ether and filtered to provide the off-white *tert*-butyl [2-amino-4-(2-thienyl)phenyl]carbamate (12.4 g. 76%): ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.31 (br, 1 H), 7.41 (dd, J = 5.0, 0.9 Hz, 1 H), 7.26 (dd, J = 3.5, 1.2 Hz, 1 H), 7.23 (br d, J = 8.5 Hz, 1 H), 7.05 (dd, J = 5.0, 3.5 Hz, 1 H), 6.94 (d, J = 2.1 Hz, 1 H), 6.81 (dd, J = 8.2, 2.1 Hz, 1 H), 4.98 (s, 2 H), 1.43 (s, 9 H); MS (ESI+): cal'd $[M+H]^+$ 291.1, obs'd 291.1.

A mixture of *tert*-butyl [2-amino-4-(2-thienyl)phenyl]carbamate (20.0 g, 68.9 mmol) and 6-chloronicotinyl chloride (12.7 g, 72.3 mmol) in 80 mL of pyridine was stirred for 1.5 hours. The mixture was poured into water, filtered and the precipitate washed with water. The solid was triturated with DCM and placed under vacuum overnight giving 28.5 g (96%) of product, *tert*-butyl (2-(6-chloronicotinamido)-4-(thiophen-2-yl)phenyl)carbamate: ^{1}H NMR (600 MHz, CD_3OD): δ 8.95 (d, J = 2.3 Hz, 1 H), 8.35 (dd, J = 8.2 Hz, 2.3 Hz, 1 H), 7.85 (br s, 1 H), 7.62 (d, J = 8.5 Hz, 1 H), 7.55-7.51 (m, 2 H), 7.37-7.35 (m, 2 H), 7.07 (dd, J = 5.0 Hz, 3.5 Hz, 1 H), 4.59 (s, 1 H), 1.49 (s, 9 H); MS (ESI+): cal'd $[M+Na]^+$ 452.1, obs'd 452.1.



A solution of 1-oxa-3,8-diazaspiro[4.5]decan-2-one[1] (356 mg, 2.28 mmol), *i*Pr$_2$NEt (0.400 mL, 2.28 mmol), *tert*-butyl (2-(6-chloronicotinamido)-4-(thiophen-2-yl)phenyl)carbamate (491 mg, 1.14 mmol) in propanol was stirred at 97 °C for 25 hours. The mixture was cooled to RT and filtered. The solid was washed with propanol and air-dried. A mixture of the intermediate (168 mg, 0.31 mmol) in 2.5 mL of AcOH was treated with 12 N HCl (0.128 mL) and stirred for 2 h. The mixture was filtered and the precipitate washed with 1:1 AcOH/*i*-PrOH

to give the HCl salt of the title compound: ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.6 (s, 1 H), 8.79 (d, J = 2.0 Hz, 1 H), 8.37 (dd, J = 9.4, 1.7 Hz, 1 H), 7.78 (d, J = 2.0 Hz, 1 H), 7.58 (m, 2 H), 7.55 (dd, J = 4.9, 0.8 Hz, 1 H), 7.48 (dd, J = 3.9, 0.9 Hz , 1 H), 7.42 (d, J = 8.5 Hz, 1 H), 7.27 (d, J = 9.1 Hz, 1 H), 7.12 (dd, J = 5.0, 3.4 Hz, 1 H), 4.04 (m, 2 H), 3.62 (m, 2 H), 3.28 (s, 2 H), 1.80-1.89 (m, 4 H); MS (ESI+): cal'd for $C_{23}H_{24}N_5O_3S$ $[M+H]^+$ 450.2, obs'd 450.1. Reverse phase LC purity using 10-100% MeCN/water gradient, 0.1% TFA additive, and a 10 min run time indicates ≥95% purity. Spectra are enclosed in the following pages.

Compounds 3-9 were prepared via the synthetic route outlined above for compound 2. Associated spectral data are tabulated below, purity assessments from LC/MS and NMR analyses are ≥95% unless otherwise noted.

***N*-(2-Amino-5-(thiophen-3-yl)phenyl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide (3):** MS (ESI+) cal'd for $C_{23}H_{24}N_5O_3S$ $[M+H]^+$ 450.2, obs'd 450.1.

***N*-(2-Amino-5-(4-methylthiophen-2-yl)phenyl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide (4):** Purity 90%; ^{1}H NMR (600 MHz, CD_3OD) δ 8.75 (br d, J = 1.9 Hz, 1 H), 8.08 (dd, J = 8.8, 2.3 Hz, 1 H), 7.42 (br d, J = 1.9 Hz, 1 H), 7.30 (dd, J = 8.2, 2.0 Hz, 1 H), 7.02 (d, J = 1.2 Hz, 1 H), 6.86-6.88 (m, 2 H), 6.79 (t, J = 1.2 Hz, 1 H), 4.08 (m, 2 H), 3.54 (m, 2 H), 3.37 (s, 2 H), 1.83 (m, 4 H); MS (ESI+) cal'd for $C_{24}H_{26}N_5O_3S$ $[M+H]^+$ 464.2, obs'd 464.1.

***N*-(4-Amino-[1,1'-biphenyl]-3-yl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide (5):** Purity 90%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.50 (s, 1 H), 8.82 (d, J = 2.3 Hz, 1 H), 8.30 (dd, J = 9.1, 2.1 Hz, 1 H), 7.78 (s, 1 H), 7.63 (m, 2 H), 7.58 (m, 2 H), 7.42-7.47 (m, 3 H), 7.36 (t, J = 7.3 Hz, 1 H), 7.17 (d, J = 9.1 Hz, 1 H), 4.01 (m, 2 H), 3.60 (m, 2 H), 3.54 (s, 2 H), 1.80-1.85 (m, 4 H); MS (ESI+) cal'd for $C_{25}H_{26}N_5O_3$ $[M+H]^+$ 444.2, obs'd 444.1.

***N*-(4-Amino-4'-fluoro-[1,1'-biphenyl]-3-yl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide (6):** ^{1}H NMR (600 MHz, CD_3OD) δ 8.76 (br d, J = 2.4 Hz, 1 H), 8.27 (dd, J = 9.4, 2.6 Hz, 1 H), 7.63-7.65 (m, 2 H), 7.57-7.60 (m, 2 H), 7.39 (br d, J = 8.2 Hz, 1 H), 7.16-7.19

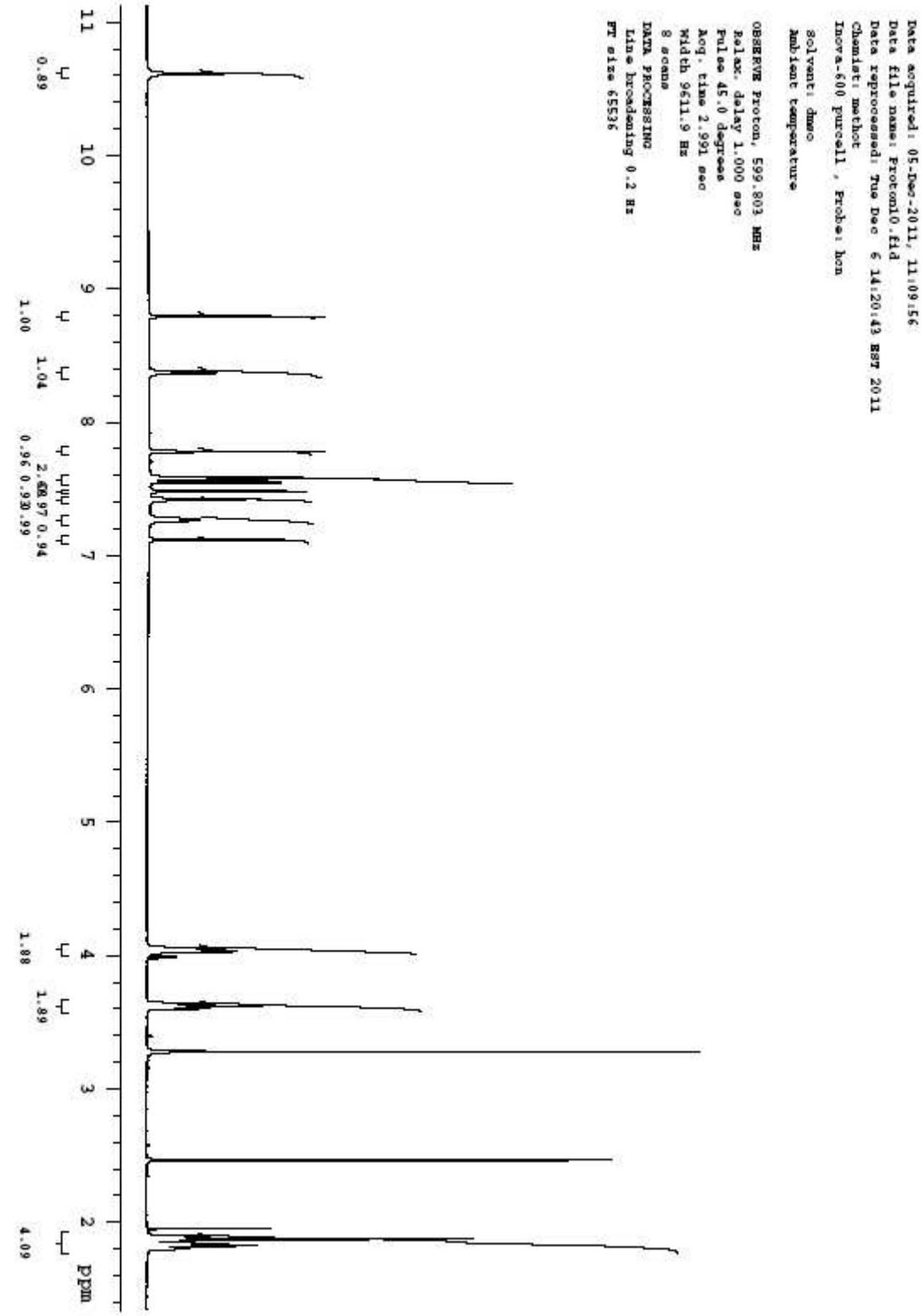
(m, 2 H), 7.13 (d, *J* = 9.1 Hz, 1 H), 4.14-4.17 (m, 2 H), 3.60-3.64 (m, 2 H), 3.42 (s, 2 H), 1.88-1.93 (m, 4 H); MS (ESI+) cal'd for $C_{25}H_{25}FN_5O_3$ $[M+H]^+$ 462.2, obs'd 462.1.

***N*-(2-Amino-5-(1-methyl-1H-pyrazol-4-yl)phenyl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide (7):** Purity 90%; ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.51 (s, 1 H), 8.73 (s, 1 H), 8.08 (dd, *J* = 9.1, 2.3 Hz, 1 H), 7.88 (s, 1 H), 7.63 (s, 1 H), 7.54 (s, 1 H), 7.30 (s, 1 H), 7.15 (dd, *J* = 8.2, 1.7 Hz, 1 H), 6.95 (d, *J* = 8.8 Hz, 1 H), 6.74 (d, *J* = 8.3 Hz, 2 H), 3.90-3.94 (m, 2 H), 3.79 (s, 2 H), 3.52-3.56 (m, 2 H), 3.14 (s, 3 H), 1.71-1.82 (m, 4 H); MS (ESI+) cal'd for $C_{23}H_{26}N_7O_3$ $[M+H]^+$ 448.2, obs'd 448.1.

***N*-(2-Amino-5-(1-methyl-1H-imidazol-4-yl)phenyl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]-decan-8-yl)nicotinamide (8):** ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.12 and 9.92 (2s, 1 H), 8.74 (2d, *J* = 2.4 Hz, 1 H), 8.11 (dt, *J* = 8.8, 2.4 Hz, 1 H), 8.08 and 8.05 (2s, 1 H), 7.80 and 7.76 (2s, 2 H), 7.56 (m, 1 H), 7.51 and 7.43 (2d, *J* = 2.1 Hz, 1 H), 7.23-7.26 (m, 1 H), 7.20 (d, *J* = 8.2 Hz, 1 H), 7.03 (dd, *J* = 9.1, 3.2 Hz, 1 H), 3.54-3.59 (m, 4 H), 3.28 (s, 2 H), 2.47 (s, 3 H), 1.73-1.83 (m, 4 H); MS (ESI+) cal'd for $C_{23}H_{26}N_7O_3$ $[M+H]^+$ 448.2, obs'd 448.1.

***N*-(2-Aminophenyl)-6-(2-oxo-1-oxa-3,8-diazaspiro[4.5]decan-8-yl)nicotinamide (9):** ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.43 (s, 1 H), 8.71 (d, *J* = 2.1 Hz, 1 H), 8.05 (dd, *J* = 8.8, 2.3 Hz, 1 H), 7.53 (s, 1 H), 7.11 (d, *J* = 7.6 Hz, 1 H), 6.91-6.95 (m, 2 H), 6.73 (dd, *J* = 8.0, 1.2 Hz, 1 H), 6.55 (dt, *J* = 7.7, 1.2 Hz, 1 H), 3.40-3.60 (m, 6 H), 1.73-1.81 (m, 4 H); MS (ESI+) cal'd for $C_{19}H_{22}N_5O_3$ $[M+H]^+$ 368.2, obs'd 368.1.

600 MHz NMR Data for Compound 2 (in DMSO-d_6).



LC/MS Data for Compound 2.

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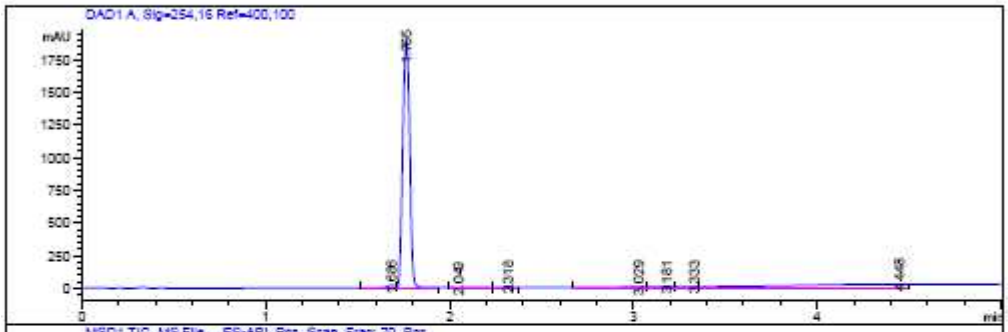

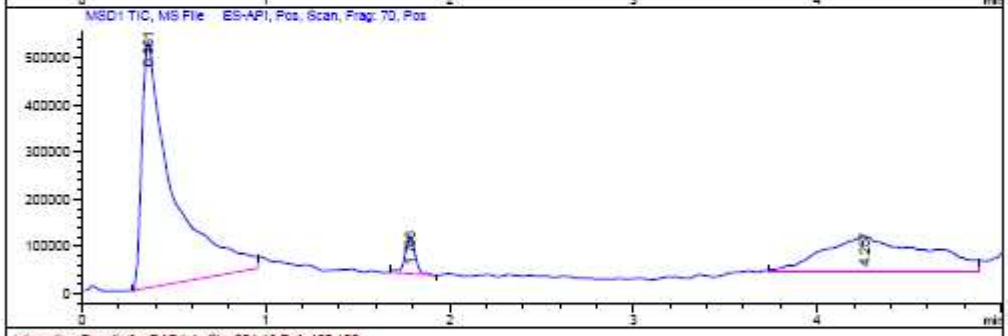

Integration Results for DAD1 A, Sig=254,16 Ref=400,100

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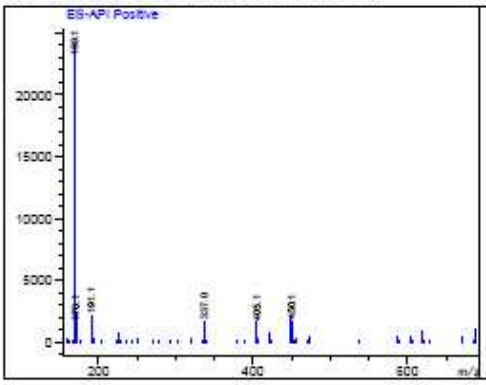
1.77	0.04	5008.80	1899.54	70.14	169
2.05	0.17	92.83	7.03	1.30	169
2.32	0.07	75.94	16.50	1.06	169
3.03	0.25	217.40	10.74	3.04	169
3.18	0.10	107.28	13.76	1.50	169
3.33	0.10	90.58	12.29	1.27	169
4.45	0.56	1459.02	30.88	20.43	169

Ret. Time: 1.69 <<<< POSITIVE SPECTRA >>>>



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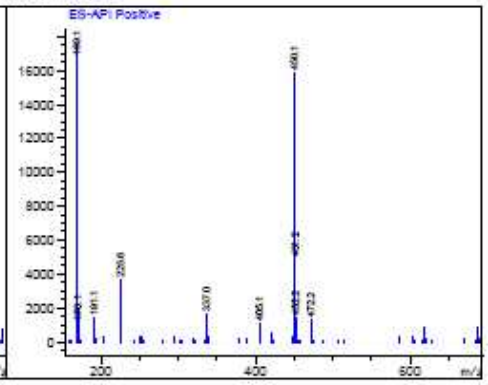

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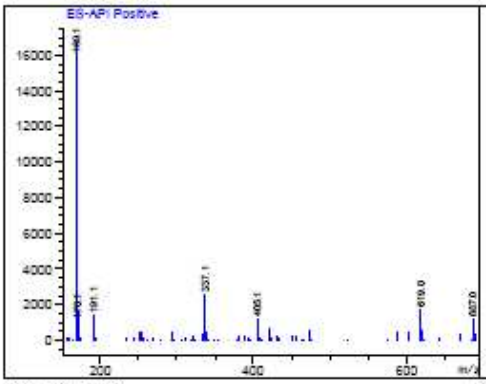

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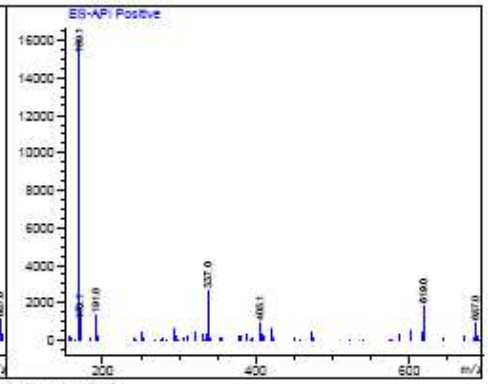

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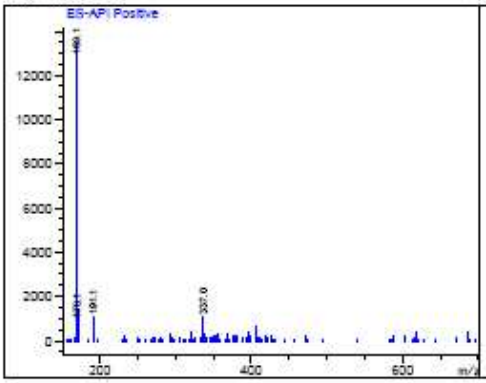

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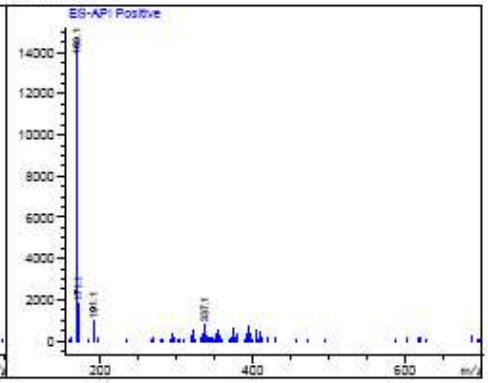

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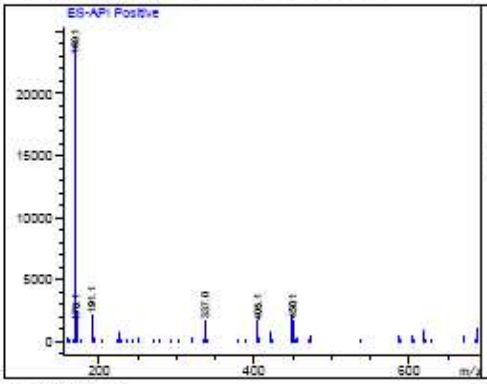

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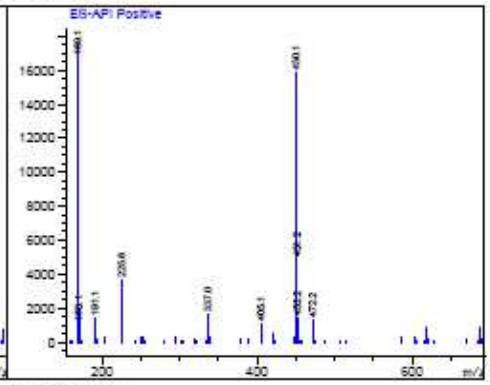

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Ret. Time: 2.32

II. HDAC Inhibition Assays

FLAG-tagged full length recombinant human HDAC1 and HDAC2 were used for these studies. Human HDAC1 was stably expressed in HEK293F cells and HDAC2 was expressed using a baculovirus-expression system in Sf9 insect cells. Both enzymes were affinity purified using an anti-Flag antibody resin. The HDAC2 protein was further purified over an HDAC1-affinity column to remove co-precipitating HDAC1. HDAC-catalyzed deacetylation of a synthetic acetyl-lysine containing peptide substrate was measured by fluorescence intensity using the commercially available Fluor-de-Lys Assay system (BioMol Research Laboratories, Plymouth Meeting, PA). The deacetylase reactions were conducted using 1-2 nM HDAC enzyme and 30 μM peptide substrate (previously determined to approximate the K_m) in 96 well plates in the presence of 20 mM Hepes, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.1 mg/ml bovine serum albumin. Inhibitor was added as a ten-point, 3-fold dilution series in DMSO. The final concentrations of inhibitor ranged from 0.5 nM to 10 μM with a constant DMSO concentration of 0.5%. In the standard assay, enzyme was pretreated with inhibitor for 10 minutes prior to initiation of the reaction. For the kinetic experiments, pretreatment times ranged from 10 min – 4 hours. Following pretreatment, the deacetylase reactions were initiated by addition of substrate peptide and allowed to proceed at 37ºC for 60 minutes. Reactions were stopped by addition of Fluor-de-Lys Developer Solution containing excess HDAC inhibitor (10 μM vorinostat). After 1 h at room temperature, fluorescence (Ex 360 nm/Em 460 nm) was quantified in a VictorV plate reader (Perkin Elmer, Wellesley, MA). Sample fluorescence was corrected for background (defined as the mean signal of samples containing no HDAC enzyme) and normalized to the positive control (defined as the mean signal of samples containing enzyme but no inhibitor). IC_{50} and inflection point (IP) values were obtained by fitting the normalized data with a 4-parameter logistic equation. Reported values represent averages of multiple ($n \geq 6$) experiments.

The selectivity of **2** was evaluated against a panel of eight HDAC subtypes (in addition to HDAC1 and 2) and one histone acetyltransferase. The HDAC enzymes were purified as follows. Full-length HDAC3 (co-expressed with the corepressor SMRT), HDAC6, and HDAC11 were FLAG-tagged, transiently expressed in HEK293F cells, and affinity purified using an anti-Flag antibody resin. The HDAC3 protein was further purified over an HDAC1-affinity column to remove co-precipitated HDAC1. HDACs 4, 5, and 7 were prepared as His-tagged catalytic

domains (HDAC4_CD, HDAC5_CD, and HDAC7_CD) and expressed in *E. Coli*. Full length HDAC8 was also His-tagged and expressed in *E.Coli*. The full length class III HDAC, SirT1, and the histone acetyltransferase, P/CAF, were purchased from BIOMOL.

III. HCT116 Cell H2BK5 Assay

HCT116 human colon carcinoma cells were plated in 6-well plates, allowed to attach overnight, and then incubated at 37°C in the presence of vehicle (0.2% DMSO) or various concentrations of inhibitor. At appropriate timepoints, media was removed, and whole cell lysates were prepared by disrupting cell membranes with 2% deoxycholate followed by sonication. Histone H2BK5 acetylation levels were quantified from these lysates using an indirect ELISA and normalized to total histone H2B levels obtained using a second indirect ELISA. Primary antibodies used were rabbit anti-acetylated histone H2BK5 (Cell Signaling) and sheep anti-Histone H2B (Abcam). Goat anti-rabbit IgG HRP (Bio-Rad) and rabbit anti-sheep-HRP (Jackson ImmunoResearch) were used as secondary antibodies, respectively. TMB substrate (Pierce) was used for detection with absorbance read at 450nM.

IV. Cell Proliferation Assays

Cells were plated in 96-well plates, allowed to attach overnight, and then incubated at 37°C in the presence of vehicle (0.2% DMSO) or various concentrations of inhibitor. After 96 h, viable cells were quantified using either Vialight Plus (Cambrex) or Alamar Blue (Invitrogen) assays according to manufacturer's instructions. In the bioluminescent Vialight Assay, cellular ATP levels are measured as a means of quantifying cellular proliferation. In the presence of ATP, luciferase converts luciferin to oxyluciferin and light. The amount of light produced is proportional to the number of viable cells in the sample and hence provides a measure of proliferation. The colorimetric Alamar Blue assay measures the reductive capacity of cells and in doing so also provides a measurement of viable, proliferating cells. GI_{50} values for inhibition of cell proliferation were calculated from dose response curves.

V. Effects on Expression of Biomarker Genes

HCT116 cells were treated with GI_{25} (50 nM), GI_{50} (97 nM), GI_{75} (188 nM) and GI_{90} (367 nM) concentrations of inhibitor for 24, 48, or 72 h and the cells were washed with PBS, lysed using Qiashredder (Qiagen), and RNA extracted using RNAeasy mini kit (Qiagen). First strand cDNA was synthesized using High Capacity cDNA Archive (API). Beta-actin mRNA (ABI# 4310881E) was used as an internal control. cDNA was amplified using TaqMan Gene Expression Assays (ABI) and an ABI Prism Sequence Detection instrument 7900HT.

VI. Mouse HCT116 Xenograft H2BK5 Acetylation

For xenograft tumor studies, HCT116 cells (5 x 10^6 per mouse) were injected sc into the right flank of CD1 *nu/nu* mice. When the tumors reached a size of approximately 325 mm^3, mice were randomized into groups of 12 animals each. Mice received one of the following dosing regimens: 1. a single dose of inhibitor (200 mg/kg); 2. two doses of inhibitor (100 mg/kg) spaced 12 h apart (bid); 3. three doses of inhibitor (67 mg/kg), spaced 8 h apart (tid). At 24, 48, and 72 h after the final dose, tumors were harvested and flash frozen in liquid nitrogen. Tumor histones were isolated by sulfuric acid extraction from tumor homogenates followed by acetone precipitation and histone H2BK5 hyperacetylation was determined by indirect ELISA.

VII. Mouse HCT116 Xenograft Growth Inhibition

HCT116 sc xenografts in CD1 nu/nu mice were prepared as in Section 5.2. Mice were randomized into groups of 12 animals each and orally treated daily with inhibitor or vehicle. Other groups were orally administered inhibitor twice weekly, with doses spaced alternating 3 or 4 days apart. Animals were weighed and tumor volumes estimated by caliper 2-3 times per week during the course of the 21-day dosing study. At the completion of dosing, tumors from 4 mice in each group were harvested at 24, 48, and 72 hours, respectively. Tumors were weighed, cut into pieces, and flash frozen in liquid nitrogen. Tumor histone acetylation levels were determined by indirect ELISA. Serum was also taken at various time points post-final dose to evaluate serum drug exposure.

[1] 1-Oxa-3,8-diazaspiro[4.5]decan-2-one (CAS 5052-95-9) is commercially available from Chembridge Corporation as well as several other suppliers. Alternatively, it can be synthesized via the route described in the following patent: Altman, M.; Christopher, M.; Grimm, J. B.; Haidle, A.; Konrad, K.; Lim, J.; Maccoss, R. N.; Machacek, M.; Osimboni, E.; Otte, R. D.; Siu, T.; Spencer, K.; Taoka, B.; Tempest, P.; Wilson, K.; Woo, H. C.; Young, J.; Zabierek, A. *2-Aminothiophene-3-carboxamide derivatives as inhibitors of janus kinases and their preparation and use in the treatment of myeloproliferative disorders and cancers.* PCT Int. Appl. (2008), WO 2008156726.