Design and Synthesis of Orally Bioavailable Aminopyrrolidinone Histone Deacetylase-6 Inhibitors

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Supporting Information

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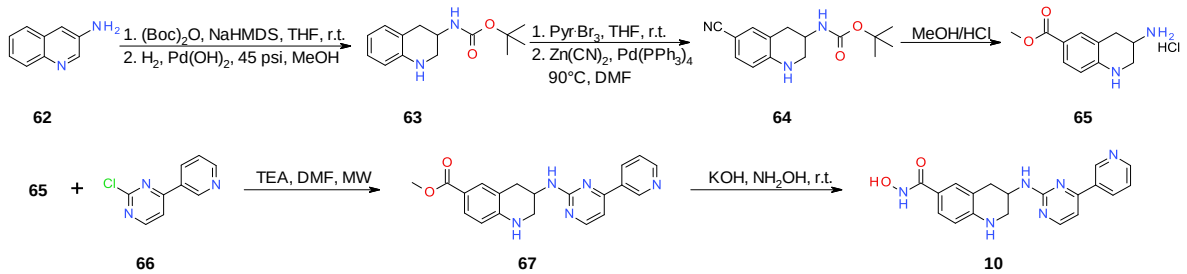
General Experimental Conditions Used in Synthesis and Purification

All of the starting materials were obtained commercially and were used without further purification. All of the reported yields are for isolated products and are not optimized. The microwave assisted reactions were carried out with a Biotage Initiator. All of the reactions involving air-sensitive reagents were performed under an argon atmosphere. Reagents were used as received from commercial suppliers without further purification unless otherwise

noted. The intermediates were purified by flash chromatography using one of the following instruments: i) Biotage SP1 system and the Quad 12/25 cartridge module. ii) ISCO CombiFlash chromatography instrument. Silica gel brand and pore size: i) KP-SIL 60 Å, particle size: 40−60 μM; ii) CAS registry NO: silica gel: 63231-67-4, particle size: 47−60 μM; iii) ZCX from Qingdao Haiyang Chemical Co., Ltd, pore size: 200−300 or 300−400 mesh. For SFC chiral separation, intermediates were separated using a chiral column (Daicel Chiralpak IC, 5 μm, 30 mm × 250 mm) column on a Mettler Teledo SFC-Multigram system: solvent system of 95% CO_2 and 5% IPA (0.5% TEA in IPA), backpressure of 100 bar, UV detection at 254 nm. All of the final compounds were purified by preparative HPLC on a reversed-phase column using Waters XBridge^{TM} OBD Phenyl (30 mm × 100 mm, 5 μm) or OBD RP18 (30 mm × 100 mm, 5 μm) column. Optical rotation was measured using a Rudolph Autopol V automatic polarimeter at a wavelength of 589 nm. LC-MS spectra were obtained using a MicroMass Platform LC (Waters Alliance 2795-ZQ2000). ^{1}H NMR spectra were obtained using a Bruker Avance 400 MHz NMR spectrometer. All of the final compounds had purities greater than 95% based upon LC-MS, and ^{1}H NMR analyses.

Synthesis of HDAC Inhibitor 10

Synthesis of 3-[[4-(3-Pyridyl)pyrimidin-2-yl]amino]-1,2,3,4-tetrahydroquinoline-6-carbohydroxamic Acid ***10****.*



To a solution of 3-aminoquinoline **62** (4.32 g, 30 mmol) in 100 mL of anhydrous THF was added dropwise 63 mL of sodium bis(trimethylsilyl)amide (1M solution in THF, 63 mmol) at rt and the mixture was stirred at rt for 30 min. Then an amount of di-*tert*-butyl

dicarbonate (7.2 g, 33 mmol) was added into the flask. The reaction was quenched 2 h later, with the addition of water (30 mL) and aqueous HCl (1N, 45 mL). The aqueous phase was separated and extracted with EtOAc. The combined organic phase was washed with saturated brine, dried over Na_2SO_4 and concentrated. After purification through silica gel column, the obtained product (6.0g, 24.6 mmol) was dissolved in 150 mL of MeOH and 18 mL of acetic acid was added in the solution. The mixture was bubbled with argon for 15 min, then palladium hydroxide (20% palladium on carbon, 1.2g) was added into the flask. The resulting suspension was subjected to hydrogenation under 45 psi for 16 h. After filtration, the filtrate was concentrated and the residue was dissolved in DCM. The organic solution was washed with saturated $NaHCO_3$, dried over Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography to give **63** (4.2 g, 56% for two steps). MS: calcd (MH^+) 249.1, exp (MH^+) 249.2.

To a solution of **63** (2.7 g, 10.9 mmol) in 50 mL of THF was added dropwise a solution of pyridinium tribromide (3.83, 0.41 mmol) in 50 mL of THF at rt. The reaction mixture was stirred for 15 min before 60 mL of water was added into the flask. The aqueous phase was extracted with EtOAc. The combined organic phase was washed with saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was purified by flash silica gel chromatography to afford 2.5 g of bromide intermediate as white solid (70%). Next, 1 g of the obtained bromide (3.04 mmol), $Pd(PPh_3)_4$ (300 mg) and $Zn(CN)_2$ (352 mg, 3 mmol) were dissolved in 5 mL of DMF. The reaction mixture was heated at 90 ºC overnight. When the mixture was cooled down and partitioned between EtOAc and water, the aqueous phase was separated and extracted with EtOAc. After workup, the crude product was purified by silica gel chromatography to give **64** (560 mg, 67%) as white solid. MS: calcd (MH^+) 274.1, exp (MH^+) 274.1.

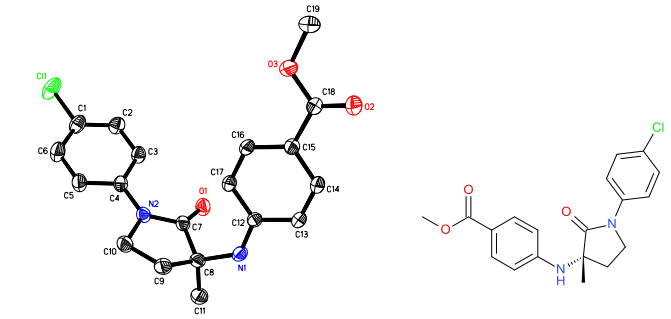
A mixture of **64** (560 mg, 2 mmol) in 10 mL of concentrated HCl was stirred at 80 ºC in a sealed tube overnight. Then the reaction mixture was cooled to rt and evaporated to dryness under reduced pressure. The residue was dissolved in MeOH and the solution was stirred at 60 ºC for 2 h. After removal of the solvent in vacuo, **65** was obtained as HCl salt in almost quantitative yield.

Then a mixture of **65** (242 mg, 1 mmol), 2-chloro-4-pyridin-3-pyrimidine **66** (191 mg, 1 mmol) and triethylamine (0.2 mL) in 3 mL of DMF was heated at 150 ºC for 1 h in a microwave reactor. The reaction mixture was diluted with EtOAc and washed with water. The combined organic phase was washed with saturated NaCl, dried over Na_2SO_4 and concentrated. The residue was purified through silica gel column to give intermediate **67** (172 mg, 48%) as white solid. MS: calcd (MH^+) 274.1, exp (MH^+) 274.1.

A mixture of **67** (172 mg), 50% aqueous NH_2OH (1 mL) and KOH (150 mg) in 1 mL of MeOH was stirred at rt for 1 h. Purification by the preparative HPLC afforded the title compound **10** as white powder. MS: calcd (MH^+) 363.1, exp (MH^+) 363.2. HRMS: calcd (MH^+) 363.1564, exp (MH^+) 363.1561. ^{1}H NMR (400 MHz, CD_3OD) δ 9.44 (s, 1H), 8.95 (d, *J* = 8.0 Hz, 1H), 8.50 (d, *J* = 4.8 Hz, 1H), 8.48 (d, *J* = 4.8 Hz, 1H), 7.92 (m, 1H), 7.42 (m, 1H), 6.59 (t, *J* = 5.2 Hz, 3H), 4.61 (m, 1H), 3.60 (m, 1H), 3.30 (m, 1H), 3.18 (m, 1H), 2.98 (m, 1H).

X-ray structure of 59

The absolute stereochemistry of intermedaite **59** was confirmed by X-ray.



59

Figure 1: *X-ray crystal structure of* ***59****.*

^{1}H NMR Spectra of 32 and 33

4-[[3-Methyl-2-oxo-1-(6-quinolyl)pyrrolidin-3-yl]amino]benzenecarbohydroxamic Acid (**32**).

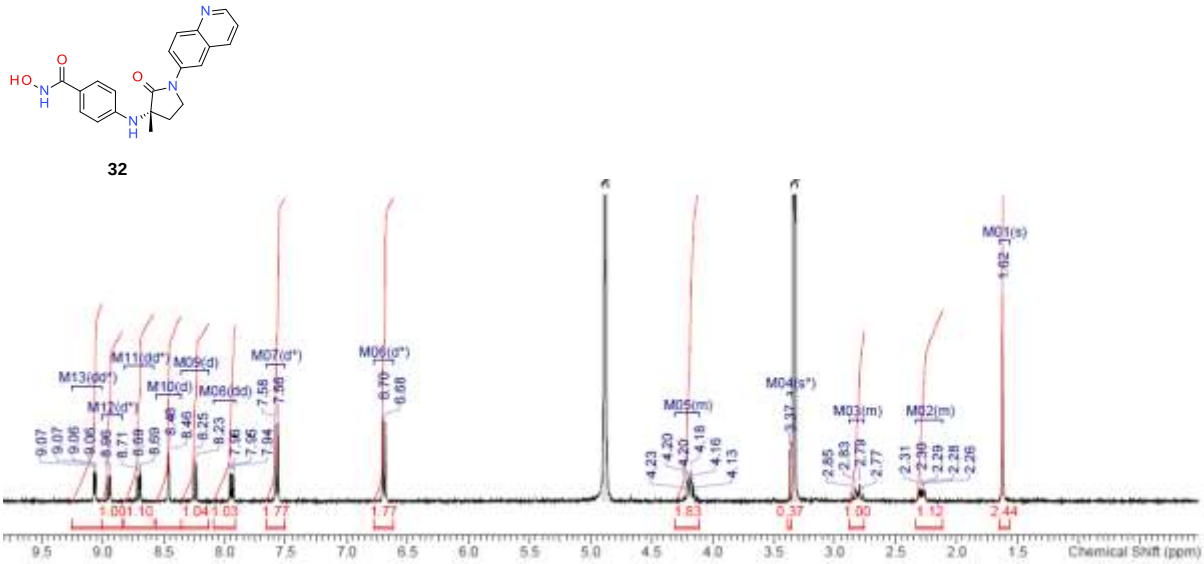


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Figure 2: *^{1}H-NMR spectrum of compound* ***32****.*

4-[[(3S)-1-(4-Chlorophenyl)-3-methyl-2-oxo-pyrrolidin-3-yl]amino]benzenecarbohydroxamic Acid (**33**).

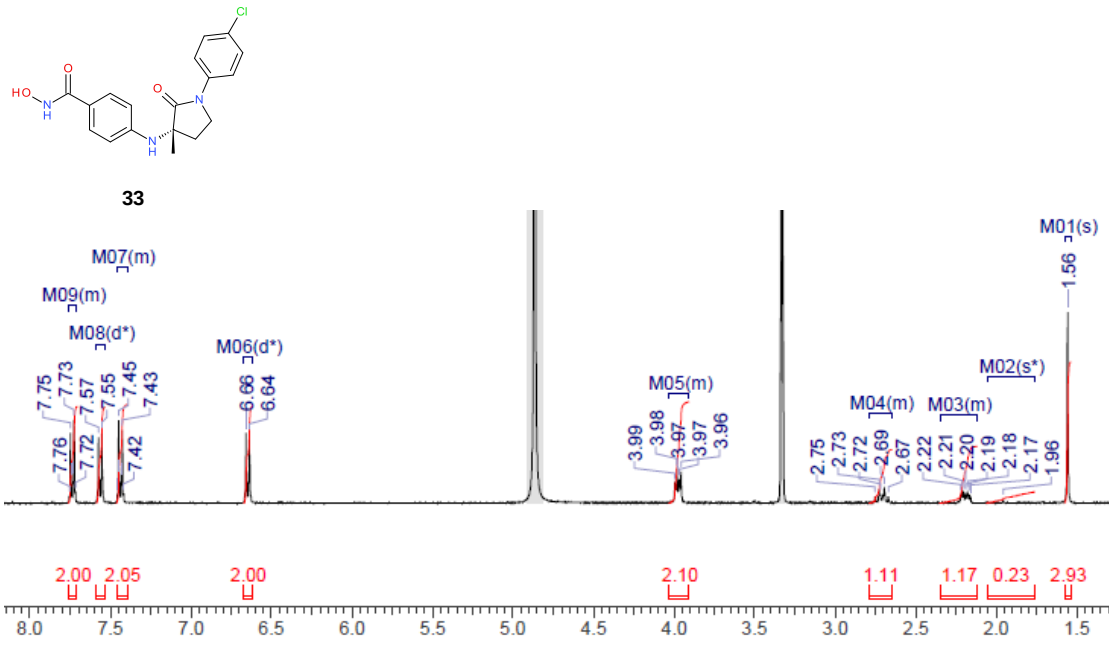

Figure 3: *^{1}H-NMR spectrum of compound **33**.*

Enzymatic Inhibitory Activities of 33 and 34 in the Panel of 11 Zn^{2+}-dependent HDACs

HDAC enzyme inhibition assays were conducted by Reaction Biology Corporation (Malvern, PA, USA) using a ten point dose response curve with half-log serial dilutions, fluorogenic peptides at 50 µM as enzymatic substrates. Substrate for HDAC1,2,3,6,10 and 11: fluorogenic peptide from p53 residues 379-382 (RHKK(Ac)AMC). Substrate for HDAC 4,5,7,9: fluorogenic HDAC class II substrate (Boc-Lys(TFA)-AMC). Substrate for HDAC-8: fluorogenic peptide from p53 residues 379-382 (RHK(Ac)K(Ac)AMC). Compound **33** inhibited HDAC6 and HDAC8 selectively, on the basis of the enzymatic inhibitory study in a panel of 11 Zn^{2+}-dependent HDACs.

Table 1*. Enzymatic Inhibition of **33** and **34** against 11 HDAC Isoforms*

Compd ID	HDAC isoform IC_{50} [a]										
	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9	HDAC10	HDAC11

33	74.1	> 100	> 100	> 100	> 100	0.017	30.6	0.18	35.0	> 100	> 100
34	24.5	31.2	26.4	> 100	> 100	0.26	> 100	0.59	> 100	44.2	39.8

[a] Trichostatin A was used as a positive control unless otherwise indicated (www.reactionbiology.com). TMP-269 was used as positive control for the testing of **33** and **34** against class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9). Data are reported in μM.

CYP Inhibition, Plasma Protein Binding and Permeability Tests

The aminopyrrolidinone analogues **11**, **18**, **32**–**34** were evaluated for their CYP inhibition activity, plasma protein binding and cell permeability with a TECAN Freedom EVO automation system (Tecan Group US, Inc.). A SpectraMax Plus UV plate reader was used to collect the UV spectra. The LC-MS/MS was carried out with an Agilent 1200 series HPLC system (Applied Biosystems API 4000 triple quadrupole with TurboIonSpray interface). The mobile phase contains 0.1% acetic acid in Milli-Q water and 0.1% acetic acid in methanol.

CYP Inhibition Assay. All incubations were carried out under conditions shown to be linear with respect to time, protein concentration and substrate concentration (all at the literature *Km* values). Each sample contained 0.125 mg/ml of human liver microsome (protein content), 5 mM of $MgCl_2$, 100 mM of potassium phosphate buffer (pH 7.4), cocktail substrates, test compound and 2 mM of NADPH in a final volume of 200 μL. And the DMSO concentration was 0.75% v/v. Samples (except NADPH) were pre-warmed at 37 ℃ for at least 10 min before the addition of NADPH to initiate the reaction. Incubation was terminated 30 min later by addition of 100 μL of methanol and dextrorphan (3 μM) was used as an internal standard. Samples were centrifuged at 4000 rpm for 10 min at 4 ℃. The supernatant from each sample was then analyzed by LC-MS/MS. Analogues **32**, **33** showed weak inhibitions to all five tested CYP enzymes (3A4, 2D6, 2C9, 2C19, 1A2) with IC_{50} values above 50.0 μM (Table 1).

Plasma Protein Binding Assay. The plasma protein binding assay was based on equilibrium dialysis in 96-well plates (HTDialysis, Gales Ferry, CT) with warfarin and phenobarbital as positive controls. Pooled human, mouse or rat plasma were purchased from Innovative Research, Inc., Southfield, MI. Sorensen's phosphate buffer (0.15M, pH 7.4) was ordered from Electron Microscopy Sciences, Hatfield, PA. At first, the dialysis membrane was activated in Milli-Q water for 1 h and then in 20% ethanol for 20 min, and washed twice with Sorensen's phosphate buffer. A 2 μL aliquot of test compound stock solution (2 mM in DMSO) was spiked into 400 μL of plasma to make a final concentration of 10 μM. Then 150 μL of aliquots were loaded into the dialysis wells (donor) and dialyzed against 150 μL of Sorensen's phosphate buffer (receiver) in a 37 °C water bath for 5 h. At the end of incubation, aliquots of plasma and buffer sample were transferred into a 96-well plate, vortex-mixed with blank and tolbutamide solution (internal control) and centrifuged before LC-MS/MS analysis. The aminopyrrolidinone HDAC inhibitors generally had low plasma protein binding. For example, the unbound fraction of **33** accounted for 27%, 46% and 30% in human, rat and mouse plasma, respectively.

Transcellular Permeability or PAMPA Assay. The parallel artificial membrane permeability assay (PAMPA) was used to predict the human intestinal permeability of the aminopyrrolidinone analogues, with dapsone as quality control. A sample stock solution in DMSO was diluted to 150 μM (DMSO < 2%) with donor buffer (0.05 M MOPSO buffer at pH 6.5 + 0.5% (w/v) glyco cholic acid), and after the filtration the solution was added into donor plate. An acceptor plate coated with phospholipid membrane (10% (w/v) egg lecithin + 0.5% (w/v) cholesterol in dodecane) was placed onto the donor plate containing the compound solution. Finally the upper plate was filled with acceptor buffer (0.05 M MOPSO buffer at pH 6.5), and the compound distribution in acceptor, membrane and donor buffer was determined after 18 h incubation. Data calculation and analysis was performed on

PAMPA Evolution Version 3.3 (pION INC). The tested compounds, except analogues **32**, demonstrated high permeability in the PAMPA assay.

Table 2. *CYP Inhibition, Plasma Protein Binding and Permeability Profiles of* ***11***, ***18*** *and* ***32–34***.

	CYP isoform IC_{50} [a]					Plasma protein binding ($f_{u,p}$, unbound %) [b]			PAMPA [c]
Compound	3A4	2D6	2C9	2C19	1A2	Human	Rat	Mouse	(10^{-6} cm/s)
SAHA [d]	46.2	15.4	50.0	2.2	42.9	29	68	69	1.4
11	7.81	> 50	> 50	> 50	> 50	38	68	64	4.9
18	0.94	> 50	> 50	43.4	> 50	13	30	13	7.1
32	> 50	> 50	> 50	> 50	> 50	32	53	27	1.0
33	> 50	> 50	> 50	> 50	> 50	27	46	30	5.7
34	17.7	> 50	> 50	48.3	> 50	17	32	25	6.1

[a] The IC_{50} values of CYP inhibition are reported in μM.

[b] The mean values of unbound fraction ($f_{u,p}$) of tested compounds are reported. Experiments were run in duplicate.

[c] The PAMPA experiments were run in triplicate, SD < 10%.

[d] The unbound fraction ($f_{u,p}$) of SAHA (vorinostat) in human, rat and mouse plasma is cited from http://www.accessdata.fda.gov/drugsatfda_docs/nda/.