Supporting Information

Fragment-based Identification of Amides Derived From 2-(Pyridin-3-yl)cyclopropanecarboxylic Acid as Potent Inhibitors of Human Nicotinamide Phosphoribosyltransferase (NAMPT)

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LCMS Conditions

Method #1

Instrument Information: SHIMADZU LCMS-2020EV

LC Parameters:

Column: Shim-pack XR-ODS 50 mm*3.0 mm, 2.2 μ m

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5% to 100% B in 2.1 min, 100% B for 0.8 min, 100% to 5% B in 0.1 min, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Acetonitrile

Injection Volume: 1 μ L

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.05 kv

Method #2

Instrument Information: SHIMADZU LCMS-2010EV

LC Parameters:

Column: Shim-pack XR-ODS 2.2 μ m, 3.0*50 mm

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile/0.05% TFA

Gradient: 5% to 100% B in 2.0 min, 100% B for 1.1 min, 100% to 5% B in 0.2 min, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.5 kv

Method #3

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Shim-pack XR-ODS 3.0 x 50mm, 2.2 µm

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5% to 100% B in 2.0 min, 100% B for 1.2 min, 100% to 5% B in 0.2 min, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Acetonitrile

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 200 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.05 kv

Method #4

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Shim-pack XR-ODS 50*3.0 nm, 2.2 µm

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile/0.05% TFA

Gradient: 5% B to 100% B for 2.0 min, 100% B for 1.2 min, 100% B to 5% B in 0.1 min, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 70-900 (m/z)

Detector voltage: 1.05 kv

Method #5

Instrument Information: SHIMADZU LCMS-2010EV9

LC Parameters:

Column: Waters XBridge C18 3.0 x 50 mm, 3.5 µm

Mobile Phase A: Water / 5mM Ammonium Acetate

Mobile Phase B: Methanol

Gradient: 10% to 100% B in 1.8 min, 100% B for 1.3 min, 100% to 10% B in 0.1 min, then stop.

Flow Rate: 0.9 mL/min

Column Temperature: 40 °C

Detector: PDA and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive & Negative)

Interface Voltage: 4.0 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.5 kv

Method #6

Instrument Information: Agilent 1200/G6140

LC Parameters:

Column: Agilent ZORBAX SB-C18, 1.8 mm, 30 x 2.1 mm

Mobile Phase A: Water/0.05%TFA

Mobile Phase B: Acetonitrile/0.05%TFA

Gradient: 3% to 95% B over 7.0 min, 95% B for 1.5 mi, following equilibration for 1.5 min

Flow Rate: 0.4 mL/min

Column Temperature: 35 °C

Detector: UV 220 nm & 254 nm

MS Parameters:

Interface: ESI Positive

Interface Voltage: 4.0 kv

Heat Block: 350 °C

Nebulizing Gas: 13 L/min

Scan Range: 100-700 (m/z)

Method #7

Instrument Information: SHIMADZU LCMS-2010EV

LC Parameters:

Column: Shim-pack XR-ODS 2.2 µm, 3.0*50 mm

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile/0.05% TFA

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.1 minutes, 100% to 5% B in 0.2 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.5 kv

Method #8

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Shim-pack XR-ODS 2.2 µm, 3.0*50 mm

Mobile Phase A: Water / 0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5 % B to 100% B for 2.0 minutes, 100% B for 1.2 minutes, 100% B to 5% in 0.1minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: UV and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 70-900 (m/z)

Detector voltage: 1.1 kv

Method #9

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Gemini-NX C18 3.0*50 mm, 3 µm

Mobile Phase A: Water / 0.04% Ammonia

Mobile Phase B: Acetonitrile

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.1 minutes, 100% to 5% B in 0.1 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 35 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive & Negative)

Interface Voltage: 4.5 kv

Heat Block: 200 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 0.75 kv

Method #10

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Shim-pack XR-ODS 2.2 μm , 3.0*50 mm

Mobile Phase A: Water / 0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5 % B to 100% B for 2.0 minutes, 100% B for 1.2 minutes, 100% B to 5% in 0.1 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: UV and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 μL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 70-900 (m/z)

Detector voltage: 1.1 kv

Method #11

Instrument Information: SHIMADZU LCMS-2010EV

LC Parameters:

Column: Waters Xselect C18 3.0 x 50 mm, 3.5 μm

Mobile Phase A: Water / 0.1% TFA

Mobile Phase B: Acetonitrile /0.05% TFA

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.2 minutes, 100% to 5% B in 0.1 minutes, then stop.

Flow Rate: 0.9 mL/min

Column Temperature: 35 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive & Negative)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.5 kv

Method #12

Instrument Information: SHIMADZU LCMS-2010EV

LC Parameters:

Column: Shim-pack XR-ODS 3.0 x 50 mm, 2.2 µm

Mobile Phase A: Water / 0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1 minute, 100% to 5% B in 0.3 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 μ L

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 $^{\circ}$ C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.3 kv

Method #13

Instrument Information: SHIMADZU LCMS-2020EV

LC Parameters:

Column: Shim-pack XR-ODS 50 mm * 3.0 mm, 2.2 μ m

Mobile Phase A: Water /0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5% to 100% B in 2.1 minutes, 100% B for 0.8 minutes, 100% to 5% B in 0.1 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 $^{\circ}$ C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Acetonitrile

Injection Volume: 1 μ L

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 $^{\circ}$ C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.05 kv

Method #14

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Shim-pack XR-ODS 50 * 3.0 nm, 2.2 µm

Mobile Phase A: Water / 0.05% TFA

Mobile Phase B: Acetonitrile / 0.05% TFA

Gradient: 5% B to 100% B for 2.0 minutes, 100% B for 1.2 minutes, 100% B to 5% in 0.1 minute, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 70-900 (m/z)

Detector voltage: 1.05 kv

Method #15

Instrument Information: SHIMADZU LCMS-2020EV

LC Parameters:

Column: Shim-pack XR-ODS 50 mm * 3.0 mm, 2.2 μ m

Mobile Phase A: Water /0.1% TFA

Mobile Phase B: Acetonitrile /0.05% TFA

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.1 minutes, 100% to 5% B in 0.1 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 $^{\circ}$ C

Detector: PDA and ELSD

Sample Preparation: 1 mg/mL in Acetonitrile

Injection Volume: 1 μ L

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: Tuning File

Heat Block: 250 $^{\circ}$ C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 0.9 kv

Method #16

Instrument Information: SHIMADZU UHPLCMS-2020EV

LC Parameters:

Column: shim-pack XR-ODS 50 * 2.0 mm, 1.6 μ m

Mobile Phase A: Water/0.1% TFA

Mobile Phase B: Acetonitrile /0.05% TFA

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.1 minutes, 100% to 5% B in 0.1 minutes, 5% B for 0.4 minutes, then stop

Flow Rate: 0.7 mL/min

Column Temperature: 40 °C

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive or Negative)

Interface Voltage: 4.0 kv

Heat Block: 200 °C

Nebulizing Gas: 1.5 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 0.9 kv

Method #17

Instrument Information: SHIMADZU LCMS-2010EV

LC Parameters:

Column: Shim-pack XR-ODS 2.2 µm, 3.0 * 50 mm

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile/0.05% TFA

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.1 minutes, 100% to 5% B in 0.2 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Methanol

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 250 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 1.5 kv

Method #18

Instrument Information: SHIMADZU LCMS-2020

LC Parameters:

Column: Shim-pack XR-ODS 3.0 x 50 mm, 2.2 µm

Mobile Phase A: Water/0.1% TFA

Mobile Phase B: Acetonitrile/0.05% TFA

Gradient: 5% to 100% B in 2.0 minutes, 100% B for 1.2 minutes, 100% to 5% B in 0.2 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Sample Preparation: 1 mg/mL in Acetonitrile

Injection Volume: 1 µL

Report: Area Normalized Purity

MS Parameters:

Interface: ESI (Positive)

Interface Voltage: 4.5 kv

Heat Block: 200 °C

Nebulizing Gas: 1.50 L/min

Scan Range: 90-900 (m/z)

Detector voltage: 0.95 kv

Chiral Analytical LCMS Conditions

Chiral Analytical Method #1

Instrument Information: Berger SFC-MS

Column: Phenomenex Cellulose-1 (4.6 x 50 mm, 3 μ m)

Mobile Phase: 30% Methanol w/ 0.1% NH_4OH , 70% Carbon Dioxide

Flow Rate: 4 mL/min

Run Time: 2.5 min

Detector: 254 nm

Pressure: 120 bars

Temperature: 40 $^{\circ}\text{C}$

Chiral Analytical Method #2

Instrument Information: Waters UPC2

Column: Chiralpak AD (4.6 x 50 mm, 3 μ m)

Mobile Phase: 20% Methanol w/ 0.1% NH_4OH , 80% Carbon Dioxide

Flow Rate: 4 mL/min

Run Time: 2.5 min

Detector: 220 nm

Pressure: 120 bars

Temperature: 40 $^{\circ}\text{C}$

Prep-HPLC Conditions

Method A

Instrument Name: Varian Prepstar

Column: Gemini-NX, 5 μ m, C18, 30 x 50 mm

Mobile Phase A: Water

Mobile Phase B: Acetonitrile/0.1% formic acid

Flow Rate: 60 mL/min

Gradient: 5% to 50% B over 8 min, then 50% B for 3.5 min, then stop.

Detector: 254 nm

Method B

First purification

Instrument Name: Varian Prepstar

Column: Gemini-NX, 5 μ m, C18, 30 x 50 mm

Mobile Phase A: Water

Mobile Phase B: Acetonitrile/0.1% ammonium hydroxide

Flow Rate: 60 mL/min

Gradient: 5% to 50% B over 8 min, then 50% B for 3.5 min, then stop.

Detector: 254 nm

Second purification

Instrument Name: Varian Prepstar

Column: Gemini-NX, 5 μ m, C18, 30 x 50 mm

Mobile Phase A: Water

Mobile Phase B: Acetonitrile/0.1% formic acid

Flow Rate: 60 mL/min

Gradient: 5% to 50% B over 8 min, then 50% B for 3.5 min, then stop.

Detector: 254 nm

Method C

Instrument Name: Waters

Column: Xbridge Shield RP 18, 5 μ m, 19*150 mm

Mobile Phase A: Water/50 mM NH_4HCO_3

Mobile Phase B: Acetonitrile

Flow Rate: 60 mL/min

Gradient: 10% to 28% B over 2 min, then to 46% B over 10 min, then, up to 100% B over 1 min, then down to 10% B over 1 min, then stop.

Detector: 254 nm

Method D

Instrument Name: Waters

Column: Xbridge Shield RP 18, 5 μ m, 19*150 mm

Mobile Phase A: Water/10 mM NH_4HCO_3

Mobile Phase B: Acetonitrile

Flow Rate: 60 mL/min

Gradient: 33% to 48% B over 10 min, then to 95% B over 1 min, hold at 95% B for 1 min, then down to 33% B over 2 min, then stop.

Detector: 254/220 nm

Chiral SFC Separation Conditions

Chiral Separation Method A

Instrument Name: Berger Multigram II

Column: Phenomenex Cellulose-1 (21.1 x 150 mm, 5 μ m)

Mobile Phase: 30% Methanol w/ 0.1% NH_4OH , 70% Carbon Dioxide (isocratic)

Flow Rate: 70 mL/min

Run Time: 6 min

Detector: 254 nm

Pressure: 100 bars

Temperature: 40 $^{\circ}\text{C}$

Chiral Separation Method B

Instrument Name: Berger Multigram II

Column: Chiralpak AD (21.1 x 150 mm, 5 μ m)

Mobile Phase: 20% Methanol w/ 0.1% NH_4OH , 80% Carbon Dioxide (isocratic)

Flow Rate: 70 mL/min

Run Time: 6 min

Detector: 220 nm

Pressure: 100 bars

Temperature: 40 $^{\circ}\text{C}$

NAMPT Enzyme Assays

Full length human (XP_540386) NAMPT protein was expressed as C-terminal His6 fusion proteins using the pET system (Novagen) in *E. coli*. Human NAMPT expression was induced in Rosetta (DE3) cells at 260 rpm and 22 °C overnight using 1.0 mM IPTG. Cells were harvested by centrifugation at 4000 xg for 15 min. at 4 °C. The cell pellet was suspended in Lysis buffer A (50 mM Tris/HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM TCEP and 1X protease inhibitor cocktail Complete (Roche)). The cell suspension was disrupted by passing through a microfluidizer (Model 110F Microfluidizer Cooperator) three times. The whole cell lysate was centrifuged at 40,000 rpm (Beckman Ti45 rotor) for 1 hour at 4 °C. The supernatant was applied to a Ni-NTA SuperFlow column (Qiagen), and was washed extensively with wash buffer B (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.5 mM TCEP). NAMPT protein was eluted with elution buffer C (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 250 mM Imidazole, 0.5 mM TCEP). Fractions containing NAMPT were further purified with a Superdex 200 column (Amersham) pre-equilibrated with buffer D (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5 mM TCEP). The purified protein was greater than 95% pure as assessed by SDS-PAGE. The concentration of the purified protein was determined by a Bradford protein assay.

The NAMPT enzymatic reactions were carried out in Buffer A (50 mM Hepes pH 7.5, 50 mM NaCl, 5 mM MgCl₂, and 1 mM THP) in 96-well plates. The compound titrations were performed in a separate dilution plate in DMSO to make a 100x stock. NAMPT protein (XTAL Biostructures, MA) was added to 1 Buf 1 µL compound, incubated for 15 minutes at room temperature, and then 10x substrate and co-factors in were added to the test well to make a final concentration of 30 nM NAMPT, 1 µM NAM, 100 µM PRPP, and 2.5 mM ATP. The reaction

was allowed to proceed for 30 minutes at room temperature, and then was quenched with formic acid and L-cystathionine to make a final concentration of 1% formic acid and 10 μ M L-cystathionine. As the particular NAMPT enzyme preparation employed in the assay was estimated to be only 1-2% active, the effective protein concentration was approximately 0.50 nM (thus providing a theoretical inhibitor IC₅₀ measurement limit of 0.25 nM).

A2780 Cell Assays

A2780 cells were seeded in 96-well plates at 1×10^3 cells/well in 180 μ L of culture medium (10% FBS, 1% Pen/Strep Amphotericin B, RPMI-5 1640) with and without the addition of either nicotinamide mononucleotide (NMN) or nicotinamide (NAM). After overnight incubation at 37 °C and 5% CO₂, the compounds were added to cells with a final DMSO concentration of 0.1%. The plates were then incubated for 72 hours at 37 °C in a 5% CO₂ incubator. The number of viable cells was then assessed using sulforhodamine B (SRB) assay. Cells were fixed at 4 °C for 1 hour with the addition of TCA to make a final concentration of 6 % TCA. The plates were washed with H₂O and dried, whereupon 100 μ L of a 4% SRB in 1% acetic acid solution was added to each well and incubated at room temperature. The plates were then washed with 1% acetic acid, dried, and treated with 100 μ L of 10 mM Tris-Base solution. The plates were then read in a microplate reader at an absorbance of 570 nm.

Other Cell-Based Assays

All lines were obtained from ATCC. 1×10^3 cells were plated in Black-clear bottom 384 well plates (BD Falcon 353962) for 24 hours. Cells then were treated with a dose titration of compounds for 96 hours. Viability was measured with CyQuant-Direct (Invitrogen, C35011) according to manufactory instruction: 3x concentration of CyQuant reagent was added to cells at 1:2 ratio. Plates were incubated at 37 °C for 1 hour and read on Envision plate reader. The experiment was independently run three times with triplicate samples for each run. IC₅₀ curves were generated with GraphPad Prism. IC₅₀ values obtained from each run were then averaged in Excel.

SPR Assays (additional details)

The Genentech fragment library is largely consistent with the Astex Rule of Three. Fragment compounds generally contain fewer than 15 heavy atoms, possess an average molecular weight of ~200 Da, and are filtered to remove undesirable functional groups. The library is comprised of a mix of purchased compounds, in-house fragments, and synthetic intermediates. All compounds are tested for appropriate solubility of >1 mM in PBS before inclusion in the library. The 112 competitive hits were triaged with the chemistry team based on a variety of properties including but not limited to ligand efficiency, expected ability to impact the existing NAMPT inhibitor series, and chemical tractability. Some fragments, for example those with LE < 0.3, were deprioritized. No attempts were made to obtain crystal structures of fragments represented by blue squares in Figure 5. Full dose response plots for compounds **9**, **12**, and **13** are depicted in Figure SII below.

Figure S11. SPR data for compounds **9**, **12**, **13**, and associated measurements of the control compound **7**. Compound **7** was tested at the beginning and end of the experiment to verify surface integrity. Equilibrium points measured 2 seconds before the end of the association phase are plotted versus their concentration to yield dose-response curves. Response levels were normalized to the saturation level of the control, and K_D values were determined through global analysis linked to the binding level of the control as previously described (*Methods in Enzymology* **2011**, 493, 169).

Mouse Xenograft Efficacy Studies

All *in vivo* studies were approved by Genentech's Institutional Animal Care and Use Committee (IACUC) and adhere to the NIH Guidelines for the Care and Use of Laboratory Animals. Human HT-1080 fibrosarcoma tumor xenografts were established by subcutaneous injection of 10 million cells suspended in a 1:1 ratio of HBSS and phenol red-free matrigel (Becton Dickinson Bioscience, San Jose, CA) into female NCR nude mice from Taconic (Cambridge City, IN). Animals were distributed into treatment groups (n = 10/group) when tumors reached a mean volume of approximately 200-300 mm³. Compound **39** was administered twice daily (BID) at 10 mg/kg by oral gavage (PO) in vehicle (60% PEG400 / 10% EtOH / 30% H₂O). Tumor volumes were determined using digital calipers (Fred V. Fowler Company, Inc., Newton, MA) using the formula (L x W x W)/2. Percentage tumor growth (% TGI) was calculated based on the following formula: % TGI = 100 × (day 21 mean volume of tumors in animals given vehicle – day 21 mean volume of tumors in animals given the test article)/day 21 mean volume of tumors in animals given vehicle. Tumor sizes and body weights were recorded twice weekly over the course of the study. Mice with tumor volumes ≥ 2000 mm³ or with losses in body weight ≥ 20% from their weight at the start of treatment were euthanized per IACUC guidelines. Statistical significance was defined as p < 0.05. To compare two groups, an unpaired t-test assuming unequal variances was used. Body weight changes are depicted in Figure SI2 below.

Figure S12. Body weight changes observed for compound **39** dosed orally and twice daily (BID) at 10 mg/kg for 5 consecutive days in the HT-1080 human fibrosarcoma xenograft model. Ten (10) mice were employed per group. Vehicle = 60% PEG400 / 10% EtOH / 30% H₂O. Data reflect the % change in body weights during the course of the study. Rx = treatment period.

Aqueous Solubility Determinations

Compounds were dissolved in DMSO to a concentration of 10 mM. These solutions were diluted into PBS buffer (pH 7.2, composed with NaCl, KCl, Na₂HPO₄, and KH₂PO₄) to a final compound concentration of 100 µM, DMSO concentration of 2%, at pH 7.4. The samples were shaken for 24 hours at room temperature followed by filtration. LC/CLND was used to determine compound concentration in the filtrate, with the concentration calculated by a caffeine calibration curve and the sample's nitrogen content. An internal standard compound was spiked into each sample for accurate quantification.

Metabolic Stability Studies in Human Liver Microsomes and Mouse Liver Microsomes

The metabolic stability of tested compounds was evaluated in pooled mixed gender human (150 donor pool) or female pooled CD-1 mouse (240 donor pool) liver microsomes (BD Biosciences, San Jose, CA). The incubation mixture (final incubation volume = 75 µL) was prepared in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mg/mL microsomal protein, 1 mM NADPH, and 1 µM of the tested compounds. The liver microsomes were pre-warmed at 37 °C for 5 minutes before the compound was added. Reactions were initiated with the addition of the NADPH and test compound. The mixtures were incubated at 37 °C for 0, 20, 40, and 60 min. At each timepoint, a 15 µL aliquot of reaction was quenched with 30 µL of acetonitrile with internal standard. Samples were centrifuged at 1000 g for 10 min. Supernatant (30 µL) was diluted in water (60 µL) before LC–MS/MS analysis.

The hepatic clearance (CL_{hep}, or predicted hepatic clearance) was calculated using the well-stirred model and is scaled from the CL_{int}. Scaling factors are listed in Table SI1 below. The fraction unbound in the microsomal incubation and in blood was assumed to be 1.

Table SI1.

Parameters	Species	
	Human	Mouse
mg microsomal protein/g liver weight ^a	48.8	50
g liver weight/kg body weight ^b	25.7	87.5
liver blood flow (mL/min/kg) ^b	20.7	90
^a Naritomi et al. (2001), <i>DMD</i> 29:1316-1324		
^b Davies B. & Morris T. (1993), <i>Pharm. Res.</i> 10:1093-1095		

Metabolic Stability Studies in Mouse Hepatocytes

The metabolic stability of tested compounds (1 μ M) was evaluated in female pooled CD-1 mouse cryopreserved primary hepatocytes (60 donor pool, 0.5 million cells/mL; Celsis, Baltimore, MD). Incubations (final incubation volume = 250 μ L) were performed at 37 °C under an atmosphere of 5% CO₂ in DMEM. Aliquots (50 μ L) of the reaction mixture were added to 100 μ L ice-cold acetonitrile with internal standard at 0, 1, 2 and 3 h and the samples were centrifuged at 3000 g for 10 min. Supernatant (80 μ L) was diluted in water (160 μ L) before LC-MS/MS analysis.

The hepatic clearance (CL_{hep}, or predicted hepatic clearance) is calculated using the well-stirred model and is scaled from the CL_{int}. Scaling factors are listed in Table SI2 below. The fraction unbound in the hepatocyte incubation and in blood was assumed to be 1.

Table SI2.

Parameters	Species
	Mouse
number of hepatocytes/g liver weight	1.20 x 10 ⁸
g liver weight/kg body weight ^a	87.5
liver blood flow (mL/min/kg) ^a	90
^a Davies B. & Morris T. (1993), <i>Pharm. Res.</i> 10:1093-1095	

Madin-Darby Canine Kidney I (MDCK) Permeability

The Madin-Darby canine kidney (MDCK) cell line was acquired from the American Type Culture Collection (Manassas, VA). The cells were seeded at an initial concentration of 2.5×10^5 cells/mL in 24 well plates and allowed to grow for 4 days at 37 °C, 95% humidity, and 5% CO₂ in culture with Eagle's Minimum Essential Medium Earle's BSS (0.1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate) supplemented with 10% fetal bovine serum. The MDCKI monolayers were equilibrated for 30 minutes in transport buffer (HBSS with 10 mM HEPES, pH 7.4) at 37 °C with 5% CO₂ and 95% humidity prior to the experiment. The permeability of test compounds was examined at 10 μM in the apical to basolateral (A-B) direction. The dose solutions also contained the monolayer integrity marker LY (100 μM) and a final DMSO percentage of 1%. The test compounds were analyzed by LC-MS/MS. The apparent permeability ($P_{app, A-B}$) was calculated using the following equation:

$$P_{app, A-B} = \frac{dQ}{dt} \cdot \frac{1}{C_0} \cdot \frac{1}{A}$$

Where dQ/dt is the rate of compound appearance in the receiver compartment (Q is the quantity of compound), C_0 is the concentration in the donor compartment and A is the surface area of the insert.

Reversible Plasma Protein Binding

The extent of protein binding ($n = 2$) was determined *in vitro* in pooled mouse plasma (Bioreclamation, Inc., Hicksville, NY) by equilibrium dialysis using a Rapid Equilibrium Dialysis (RED) device (Pierce Biotechnology / Thermo Fisher Scientific; Rockford, IL) in 96-well format with a molecular weight cut-off of 8000 Daltons. Test compounds were dissolved in dimethyl sulfoxide (DMSO), and added to plasma, for a final concentration of 5 μM in the plasma. The final DMSO percentage was 1%. 300 μL plasma containing compound were dialyzed against 500 μL PBS on a shaking platform in a humidified incubator for 4 hours at 37 $^{\circ}\text{C}$. Post-equilibrium buffer and plasma samples were transferred to a 96-well plate and opposing matrix was added. Plasma proteins were precipitated using 100% acetonitrile containing internal standard (100 nM propranolol). Concentration of compounds in plasma and PBS were measured by LC-MS/MS and the percent unbound fraction (f_u) was calculated using the equation:

$$f_u = \left(\frac{\text{PA in Buffer/IS PA}}{\text{PA in Plasma/IS PA}} \right)$$

where PA represents analyte peak area and IS PA represents internal standard peak area.

Reversible CYP and Time-dependent CYP Inhibition Studies in Human Liver Microsomes

Reversible CYP inhibition was evaluated for each test compound. Compounds were incubated with human liver microsomes (150 donor pool, BD Biosciences, San Jose, CA) fortified with NADPH and isoform-specific probe substrates. Reactions were stopped by the addition of cold acetonitrile/formic acid (94:6 v/v) containing internal standard. Five concentrations of each compound were tested (10, 5, 1, and 0.1 μM as well as a solvent control)

to generate IC₅₀ values. The assay was performed according to methodology described in: Halladay, J. S.; et al. *Drug Met. Lett.* **2011**, 5, 220. Time-dependent inhibition studies were performed as described in Mukadam, S. et al. *Drug Met. Lett.* **2012**, 6, 43.

Mouse PK Experiments

Female NCR nude mice were obtained from Taconic (Cambridge City, IN, US). The animals were given two PO doses of 10 mg/kg compound **39** with the second dose occurring 8 h after the first. The dose solution was 60% polyethylene glycol (PEG 400)/10% ethanol/30% H₂O (v/v/v), and it was administered at a dosing volume of 10 mL/kg. Mice weighed approximately 20-30 g at the start of the study, and they were not fasted for dosing. After oral administration of compound **39** (n = 3 per time point), each animal was bled twice via retro orbital (150 µL of blood was collected) and cardiac puncture (200 µL of blood was collected). PK time points for PO doses were 0.083, 0.25, 0.5, 1, 4, 8, 8.08, 8.25, 8.5, 9, 12, and 24 hours post the first dose. Blood samples were collected into 1.5% EDTA-containing syringes, transferred to microcentrifuge tubes and spun to harvest plasma. Plasma was collected, transferred to cluster tubes, and stored at approximately -80 °C until analysis. PK data analysis was performed on mean plasma concentration–time data and PK parameters were determined by non-compartmental methods using Phoenix® WinNonlin® Enterprise, version 6.3 (Pharsight Corporation; Mountain View, CA).

Crystallography

Structure Determinations of Compounds 9, 11, 12, 13, 15, and 17 in Complex with NAMPT

The NAMPT protein was expressed, purified and crystallized as previously described. (DOI: 10.1021/jm400186h). Compounds **9**, **12**, and **13** were soaked into apo-NAMPT crystals in the mother liquor with compound concentrations of 30 mM, 25 mM, and 38 mM respectively.

The diffraction data sets were collected at various synchrotron beamlines as indicated in table SI3. The data sets for compound **9**, **11**, **13**, **15**, and **17** were processed with the HKL2000 package (Otwinowski, Z.; Minor W. *Methods Enzymol.* **1997**, 276, 307). The data set for **12** was processed with program autoPROC (Global Phasing Ltd.). Further processing was carried out with programs from the CCP4 suite (Winn, et al. *Acta. Cryst.* **2011**, D67, 235). Data collection and structure refinement statistics are summarized in Table SI3.

The structures were solved by molecular replacement (MR) with a known NAMPT structure (PDB code:3DHF) as the search model. The MR solution was further refined with the program program REFMAC5 (Murshudov, G. N.; et al., *Acta Crystallogr. D Biol. Crystallogr.* **1997**, 53, 240) and PHENIX (Adams, P. D.; et al., *Acta Crystallogr. D Biol. Crystallogr.* **2010**, 66, 213) using the maximum likelihood target functions, anisotropic individual B-factor refinement method, and TLS refinement method, to achieve convergence. The data and refinement statistics for all structures are shown in Table SI3.

Table SI3. X-ray crystallography data and refinement statistics.

	NAMPT + 9	NAMPT+ 11	NAMPT+ 12
PDB accession code	4LV9	4LVA	4LVB
Beamline	SSRF BL17U	CLS-08ID-1	SSRL-111
Space group	P2 ₁	P2 ₁	P2 ₁
Unit cell	a=60.9Å, b=106.5Å, c=83.1Å $\alpha=90^\circ$ $\beta=96.4^\circ$ $\gamma=90^\circ$	a=60.5Å, b=106.8Å, c=83.0Å $\alpha=90^\circ$ $\beta=96.6^\circ$ $\gamma=90^\circ$	a=60.6Å, b=106.7Å, c=83.1Å $\alpha=90^\circ$, $\beta=96.4^\circ$, $\gamma=90^\circ$

Resolution	50.0 - 1.80 Å	50.0 - 1.55 Å	50.0 - 1.84 Å
Total number of reflections	95731 (9544) ¹	151480 (15053) ¹	90042 (800) ¹
Completeness (%)	100 (100)	100 (100)	98.3 (98.4)
Redundancy	4.4 (4.5)	3.8 (3.8)	3.4 (3.4)
I/σ	10.4 (2.2)	15.7 (2.3)	9.8 (2.4)
Rsym ²	0.140 (0.722)	0.084 (0.685)	0.085 (0.510)
Resolution range	50 - 1.80 Å	50 - 1.55 Å	50.0 - 1.84 Å
Rcryst ³ / Rfree ⁴	0.173/0.213	0.183/0.215	0.175/0.215
Non-hydrogen atoms	8585	8669	8544
Water molecules	948	1007	966
Average B, Overall	20.47	17.17	13.88
Protein	18.94	15.57	12.54
Water	30.85	28.76	23.84
r.m.s.d. bond lengths	0.007 Å	0.016 Å	0.007 Å
r.m.s.d. angles	1.015°	1.616°	1.039°
Ramachandran (C/A/G/D)	0.905/0.095/0/0	0.909/0.091/0/0	0.905/0.095/0/0

	NAMPT+ 13	NAMPT+ 15	NAMPT+ 17
PDB accession code	4LVD	4LVF	4LVG
Beamline	SSRF BL17U	SSRF BL17U	SSRF BL17U
Space group	P2 ₁	P2 ₁	P2 ₁
Unit cell	a=60.4Å, b=106.0Å, c=83.1Å α=90° β=96.3° γ=90°	a=60.5Å, b=107.0Å, c=83.0Å α=90°, β=96.6°, γ=90°	a=60.8Å, b=106.9Å, c=83.2Å α=90° β=96.8° γ=90°
Resolution	50.0 - 1.75 Å	50.0 - 1.50 Å	50.0 - 1.70 Å
Total number of reflections	104834 (10387) ¹	162957 (6445) ¹	110655 (4107) ¹
Completeness (%)	99.9 (100)	97.5 (77.6)	96.2 (71.7)
Redundancy	5.5(5.0)	3.3 (2.1)	3.6 (2.5)
I/σ	11.4 (2.6)	14.9 (2.0)	19.3 (2.0)
Rsym ²	0.156 (0.789)	0.090 (0.471)	0.059 (0.446)
Resolution range	50 - 1.75 Å	50.0 - 1.50 Å	5.00 - 1.70 Å
Rcryst ³ / Rfree ⁴	0.178/0.213	0.135/0.172	0.122/0.171
Non-hydrogen atoms	8562	8708	8549
Water molecules	989	1074	906
Average B, Overall	18.78	17.52	17.58
Protein	17.17	15.14	15.63
Water	30.36	33.41	33.34
r.m.s.d. bond lengths	0.007 Å	0.006 Å	0.005 Å
r.m.s.d. angles	1.047°	1.023°	0.960°
Ramachandran (C/A/G/D)	0.898/0.102/0/0	0.906/0.094/0/0	0.911/0.089/0/0

¹Values in parentheses for are of the highest resolution shell

²Rsym = $\sum |I_{hi} - I_h| / \sum I_{hi}$, where I_{hi} is the scaled intensity of the i th symmetry-related observation of reflection h and I_h is the mean value.

³ $R_{\text{cryst}} = \sum_h |F_{\text{oh}} - F_{\text{ch}}| / \sum_h F_{\text{oh}}$, where F_{oh} and F_{ch} are the observed and calculated structure factor amplitudes for reflection h .

⁴Value of R_{free} is calculated for 5% randomly chosen reflections not included in the refinement.