

**Structure-Based Discovery of Novel Amide-Containing Nicotinamide
Phosphoribosyltransferase (Nampt) Inhibitors**

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Expression, purification, and crystallization of human Nampt

The expression construct of NAMPT for structural studies was obtained from professor Yigong Shi's group (Tsinghua University, Beijing, China).

The C-terminal His-tagged NAMPT M1-H491 construct was transformed into BL21 codon optimized cells. Cells were grown at 37 °C to an absorbance of 0.5 (OD₆₀₀) in LB media containing 50 µg/mL of carbenicillin and then transferred to 22 °C prior to induction with 1 mM IPTG at an absorbance of 0.8 (OD₆₀₀). Cells were harvested 16 hours post induction and the pellet was lysed by passing through a microfluidizer in a buffer containing 20 mM TrisCl, pH 8.0, 200 mM NaCl, 5 mM imidazole, 1 mM TCEP and EDTA-free protease inhibitor tablets (Roche). Cell lysates were loaded onto a HisTrap HP column (GE Healthcare) in a buffer containing 20 mM TrisCl, pH 8.0, 500 mM NaCl, 5 mM imidazole and 1 mM TCEP and the bound Nampt was eluted with 250 mM imidazole in the same buffer. The protein was then loaded onto a 5 mL Q HP column (GE Healthcare) in a buffer containing 25 mM TrisCl, pH 8.0 and 1 mM TCEP and eluted at 300 mM NaCl with a linear gradient of the same buffer and 1.0 M NaCl. The protein was further purified on a size-exclusion Superdex 75 column (GE Healthcare) in a buffer containing 25 mM TrisCl, pH 8.0, 150 mM NaCl and 5 mM DTT. Fractions containing Nampt were pooled and concentrated to 6 mg/mL and confirmed by SDS-PAGE showing that the purity was greater than 95%.

Diffraction quality crystals were grown at 19 °C from 0.2 µL + 0.2 µL vapor diffusion drops containing 6 mg/mL Nampt, 0.1 M sodium phosphate, pH 8.6-9.0, 25-29% polyethylene glycol 3350, 0.2 M NaCl and 1 mM compound, which were then streak-seeded using Hampton's

seeding tool. Plate crystals appeared overnight and harvested for data collection with 15% ethylene glycol in the crystallization solution.

The diffraction data set of compound **7** was collected at ALS beamline 502 and processed with the HKL2000 package. The diffraction data set of compound **58** was collected at Shanghai Synchrotron Radiation Facility (SSRF) beam line BL17U1 and also processed with the HKL2000 package. Further processing was carried out with programs from the CCP4 suite. Data collection and structure refinement statistics are summarized in Table S1.

The structure was solved by molecular replacement (MR) with known Nampt structure (PDB code:3DHF) as the search model using the program Phaser. The structure was further refined using program REFMAC5 (Murshudov, G. N.; et al., *Acta Crystallogr. D Biol. Crystallogr.* **1997**, *53*, 240–255) and PHENIX (Adams, P. D.; et al., *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66*, 213–221) using the maximum likelihood target functions, anisotropic individual B-factor refinement method, and TLS refinement method, to achieve convergence. The data and refinement statistics are shown in Table S1. PDB nos.: 4KFN for **7** complexed with Nampt; 4KFO for **58** complexed with Nampt.

Table S1 X-ray crystallography data and refinement statistics

| Data collection | | |
|------------------------|-----------------|-----------------|
| | 58 | 7 |
| Space group | P2 ₁ | P2 ₁ |
| Unit cell | a=60.8Å, | a=60.5Å, |

| | | |
|--|---|---|
| | b=106.4Å,c=82.9Å, $\alpha=90^\circ \beta=96.6^\circ \gamma=90^\circ$ | b=106.6Å,c=83.0Å, $\alpha=90^\circ \beta=96.7^\circ \gamma=90^\circ$ |
| Resolution | 1.60Å | 1.60Å |
| Total number of reflections | 127910 (9917) ¹ | 135066(13199) ¹ |
| Completeness | 0.932 (0.727) | 0.977 (0.961) |
| Redundancy | 2.6 (1.8) | 3.8 (3.7) |
| I/ σ | 13.5 (2.3) | 14.8 (2.2) |
| Rsym ² | 0.067 (0.334) | 0.075 (0.613) |
| Refinement | | |
| Resolution range | 50-1.60Å | 50-1.60Å |
| Rcryst ³ / Rfree ⁴ | 0.156/0.181 | 0.156/0.183 |
| Non-hydrogen atoms | 8712 | 8874 |
| Water molecules | 1038 | 1165 |
| Average B, Overall | 19.74 | 14.11 |
| Protein | 17.90 | 11.89 |
| Ligand | 22.00 | 21.00 |
| Water | 32.80 | 27.73 |

| | | |
|------------------------|-----------------|---------------------|
| r.m.s.d. bond lengths | 0.006Å | 0.008Å |
| r.m.s.d. angles | 1.061° | 1.184° |
| Ramachandran (C/A/G/D) | 0.914/0.086/0/0 | 0.980/0.019/0.001/0 |

¹Values in parentheses are of the highest resolution shell, which is 1.60-1.66 Å.

² $R_{\text{sym}} = \frac{\sum |I_{hi} - I_h|}{\sum I_{hi}}$, 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 $R_{\text{cryst}} = \frac{\sum_h |F_{\text{oh}} - F_{\text{ch}}|}{\sum_h F_{\text{oh}}}$, 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.

LCMS Methods:

LCMS1:

Instrument Information: HPLC-Agilent 1200

Column: Agilent Zorbax SB-C₁₈, 3.5 µm, 2.1 mm x 30 mm

Mobile Phase A: Water/0.1% Formic Acid

Mobile Phase B: Acetonitrile/0.1% Formic Acid

Gradient: 15 to 95% B in 2.5 minutes, hold 95% B to 3.5 minutes, 95% B to 15% in 0.2 minutes, 15% B for 0.2 minutes, then stop.

Flow Rate: 2.0 mL/min

Column Temperature: Ambient

Detector: 220 nm and 254 nm

MS Parameters: Agilent G1956B, MSD

Interface: ESI (Positive)

LCMS2:

Instrument Information: Waters Acquity UPLC

Column: Acquity UPLC BEH C₁₈ 1.7 µm, 2.1 mm x 50 mm

Mobile Phase A: 95/5/0.1%: 10 mM Ammonium Formate/Acetonitrile/Formic Acid

Mobile Phase B: 95/5/0.09%: Acetonitrile/Water/Formic Acid

Gradient: 5 to 100% B in 2.0 minutes, hold 100% B to 2.2 minutes, 100% B to 5% in 0.1 minutes, 5% B for 0.2 minutes, then stop.

Flow Rate: 0.6 mL/min

Column Temperature: Ambient

Detector: 220 nm and 254 nm

MS Parameters: Waters ZQ Mass Spectrometer

Interface: ESI (Positive)

LCMS3:

Instrument Information: HPLC: Waters HT2790 Alliance

Column: XBridge C18, 5 μ m, 4.6 mm x 150 mm

Mobile Phase A: 95% Water/5% Methanol with 0.1% Formic Acid

Mobile Phase B: 95% Methanol/5% Water with 0.1% Formic Acid

Gradient: Linear 5-95% B in 5.5 minutes, hold 95% B to 7.5 minutes

Flow Rate: 1.2 mL/min

Column Temperature: Ambient

Detector: 220 nm and 254 nm

MS Parameters: Waters ZQ Single Quad Mass Spectrometer

Interface: ESI (Positive)

LCMS4:

Instrument Information: SHIMADZU LCMS-2020

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

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

Interface: ESI (Positive)

LCMS5:

Instrument Information: SHIMADZU LCMS-2010EV

Column: Waters XBridge C18 3.5 μ m, 3.0 x 50 mm

Mobile Phase A: Water /5 mM Ammonium Acetate

Mobile Phase B: Methanol

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

Flow Rate: 0.9 mL/min

Column Temperature: 40 °C

Detector: PDA and ELSD

Interface: ESI (Positive & Negative)

LCMS6:

Instrument Information: SHIMADZU LCMS-2020

Column: Shim-pack XR-ODS 2.2 μ m, 3.0 mm x 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% B in 0.1 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: UV and ELSD

Interface: ESI (Positive)

LCMS7:

Instrument Information: SHIMADZU LCMS-2020

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

Mobile Phase A: Water/0.05% TFA

Mobile Phase B: Acetonitrile

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

Interface: ESI (Positive)

LCMS8:

Instrument Information: SHIMADZU LCMS-2020EV

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

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 °C

Detector: 254 nm and ELSD

Interface: ESI (Positive)

LCMS9:

Instrument Information: SHIMADZU LCMS-2010EV

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

Interface: ESI (Positive)

LCMS10:

Instrument Information: SHIMADZU LCMS-2020

Column: Shim-pack XR-ODS 2.2 µm, 3.0 mm x 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 1.2 minutes, 100% to 5% B in 0.1 minutes, then stop.

Flow Rate: 1.0 mL/min

Column Temperature: 40 °C

Detector: 254 nm and ELSD

Interface: ESI (Positive)

LCMS11:

Instrument Information: SHIMADZU LCMS-2020EV

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

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: 1.0mL/min

Column Temperature: 40 °C

Detector: PDA and ELSD

Interface: ESI (Positive)

Determination of phosphoribosylated adducts of compound 8, 20, 28, and 33

Compounds were incubated with the Nampt enzyme under the standard biochemical assay conditions, and the formation of the corresponding PRPP-derived phosphoribosylated adducts was assessed by LC-MS/MS

LC/MS/MS method and results for compound 8, 20, and 28

HPLC Separation

Column – Phenomenex Hydro-RP, 4 μm , (50 mm x 2.0 mm)

Mobile Phase A: H₂O, 1% Formic Acid

Mobile Phase B: Acetonitrile, 1% Formic Acid

Gradient Programs

| For compound 8 and 28 | | | For compound 20 | | |
|-----------------------|------------------|--|-----------------|------------------|--|
| Time (min) | % Mobile Phase B | Flow Rate ($\mu\text{L}/\text{min}$) | Time (min) | % Mobile Phase B | Flow Rate ($\mu\text{L}/\text{min}$) |
| 0.0 | 10 | 200 | 0.0 | 10 | 200 |
| 2.0 | 10 | 200 | 2.0 | 10 | 200 |
| 7.5 | 50 | 200 | 6.0 | 30 | 200 |
| 8.0 | 50 | 200 | 7.5 | 80 | 200 |
| 8.2 | 10 | 200 | 7.8 | 80 | 200 |
| 10.0 | 10 | 200 | 8.0 | 10 | 200 |
| | | | 10.0 | 10 | 200 |

MRM Scans

Declustering Potential: 120 V for all inhibitor analogues

Collision Energy: Optimized condition for each compound

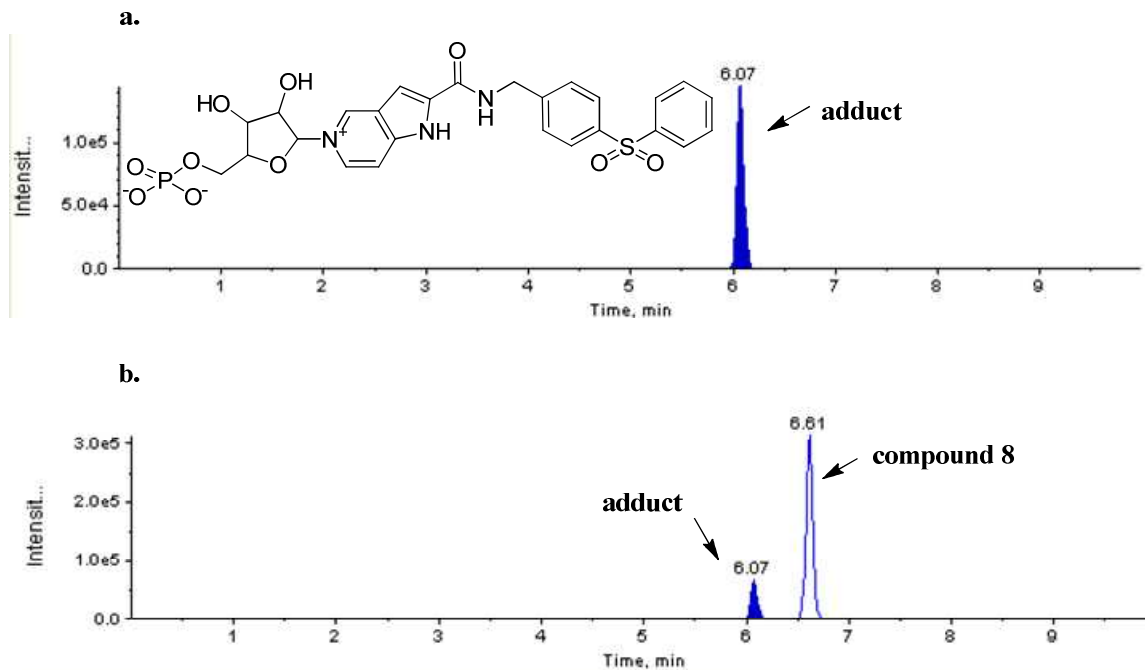


Figure 1. LC/MS/MS Chromatograms of compound **8** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 604.0 (adduct $M+H$ ion) to 392.0 (parent $M+H$ ion) indicating presence of phosphoribosylated adduct (b) MRM of transition from m/z 392.0 (parent $M+H$ ion) to 145.3 (representing fragmentation of the left hand side of the parent) indicating presence of both parent (**8**) and phosphoribosylated adduct.

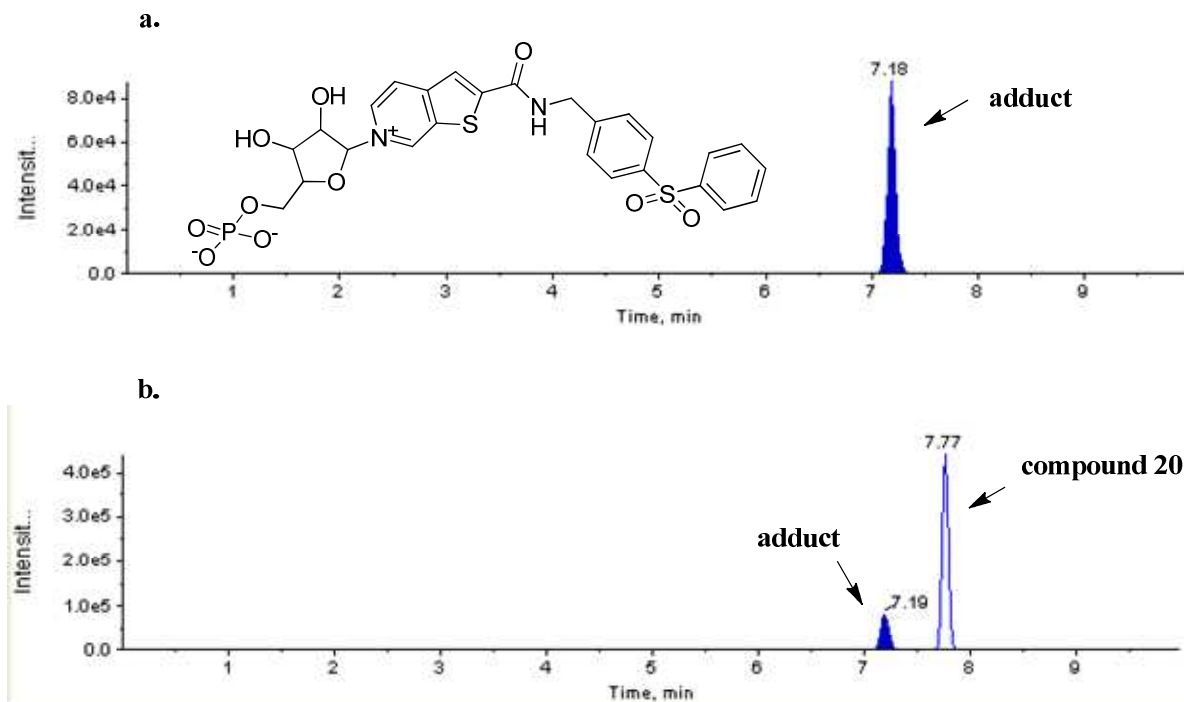


Figure 2. LC/MS/MS Chromatograms of compound **20** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 621.1 (adduct $M+H$ ion) to 409.1 (parent $M+H$ ion) indicating presence of phosphoribosylated adduct (b) MRM of transition from m/z 409.1 (parent $M+H$ ion) to 125.2 (representing fragmentation of the left hand side of the parent) indicating presence of both parent (**20**) and phosphoribosylated adduct.

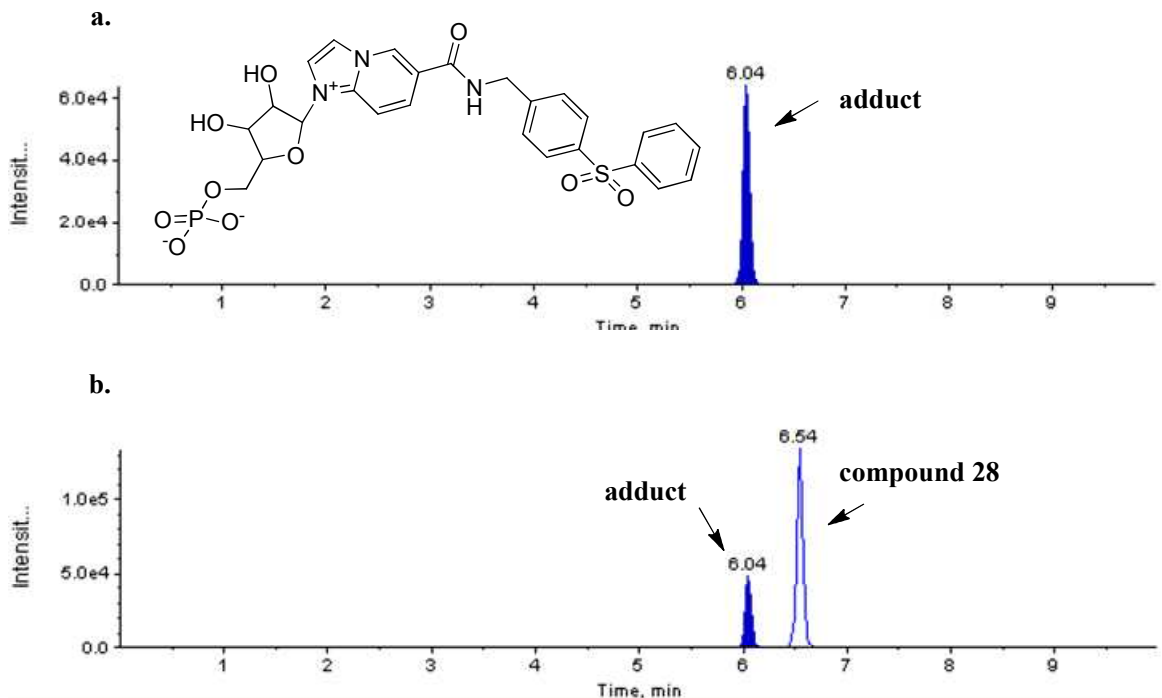


Figure 3. LC/MS/MS Chromatograms of compound **28** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 604.0 (adduct $M+H$ ion) to 392.3 (parent $M+H$ ion) indicating presence of phosphoribosylated adduct (b) MRM of transition from m/z 392.3 (parent $M+H$ ion) to 125.0 (representing fragmentation of the left hand side of the parent) indicating presence of both parent (**28**) and phosphoribosylated adduct.

LC/MS/MS method and results for compound 33

HPLC Separation

Column – ACE 3 C18, 3 μm , (50 mm x 2.1 mm)

Mobile Phase A: H_2O , 1% Formic Acid

Mobile Phase B: Acetonitrile, 1% Formic Acid

Gradient Programs

| Time (min) | % Mobile Phase B | Flow Rate ($\mu\text{L}/\text{min}$) |
|---------------|---------------------|---|
| 0.0 | 15 | 400 |
| 0.5 | 15 | 400 |
| 7.5 | 95 | 400 |
| 8.5 | 95 | 400 |
| 8.6 | 15 | 400 |
| 10 | 15 | 400 |

Injection Volume: 10 μL

Mass Spectrometry

API 4000 (Applied Biosystem /Sciex)

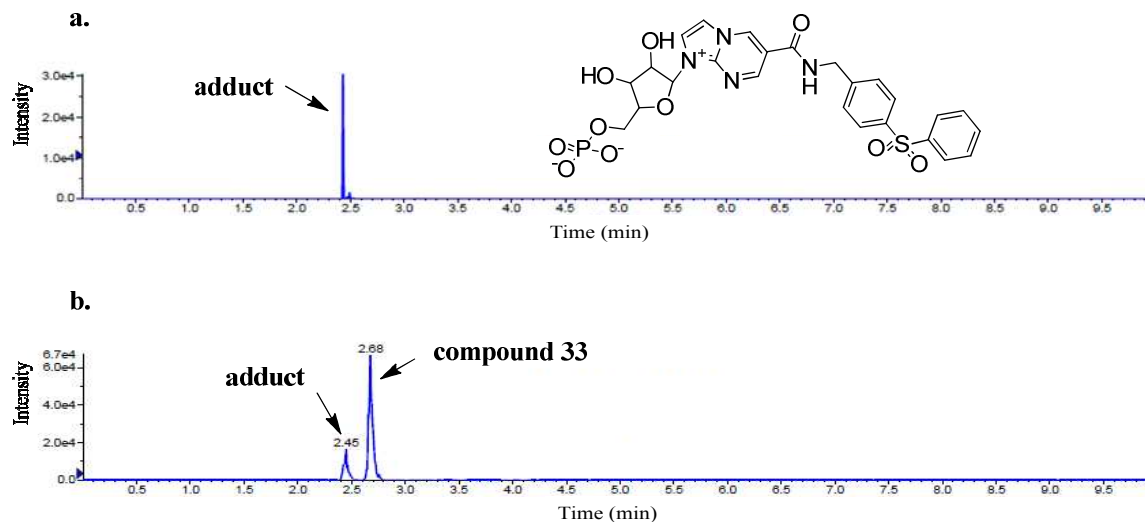


Figure 4. LC/MS/MS Chromatograms of compound **33** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 605 (adduct $M+H$ ion) to 393 (parent $M+H$ ion) indicating absence of phosphoribosylated adduct (b) MRM of transition from m/z 393 (parent $M+H$ ion) to 148 (representing fragmentation of the left hand side of the parent) indicating presence of both parent (**33**) and phosphoribosylated adduct.

***In vitro* ADME assays**

Microsomal stability assay

Test compounds (1 μM) were incubated in 0.1 M phosphate buffer (pH 7.4 at 37 °C) containing human or mouse microsomes (0.5 mg/mL) and an NADPH-generating system. Stock solutions (100 μM) of compounds were made in acetonitrile/water (50/50, v/v) along with reference compounds that showed high, medium, and low % remaining at 30 minutes. The reaction was initiated by the addition of the NADPH-generating system. Thereafter, the 0, 10, and 30 minute time points were taken and added to appropriate volumes of cold acetonitrile containing an internal standard. The signal was measured by LCMS and the % remaining of test compound relative to the 0 minute time point was calculated.

Hepatocytes stability assay

The metabolic stability of tested compounds (1 μM) was evaluated in female pooled CD-1 mouse cryopreserved primary hepatocytes or pooled human cryopreserved primary hepatocytes (0.5 million cells mL^{-1}) (Celsis, Baltimore, MD). Incubations (final incubation volume = 250 μL) were performed at 37 °C under an atmosphere of 5% CO_2 in DMEM. Aliquots (50 μL) of the reaction mixture were added to 100 μL 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 μL) was diluted in water (160 μL) 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 the table below. The fraction unbound in the hepatocyte incubation and in blood was assumed to be 1.

| Parameters | Species | |
|---|------------------------|------------------------|
| | Human | Mouse |
| number of hepatocytes/g liver weight ^a | 1.35 x 10 ⁸ | 1.20 x 10 ⁸ |
| g liver weight/kg body weight ^b | 25.7 | 87.5 |
| liver blood flow (mL/min/kg) ^b | 20.7 | 90 |

^aHouston, J. (1994), *Biochem. Pharm.* 47:1469-1479

Solubility assay

Stocks of 10 mM drug solutions in DMSO were diluted into universal phosphate buffer to a maximum concentration of 100 μM . The 1% DMSO buffered drug solutions were incubated over a period of 24 hours and then were filtered through a 96 well Millipore Multi-Screen plate. Standards were prepared at 100 μM in neat DMSO. Analyses were carried out by LCMS and compound solubility was determined by the ratio of the UV signal of the filtrate peak versus to that of the corresponding 100 μM standard.

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-to-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 bound fraction (f_b) was calculated using the equation:

$$f_b = \left(1 - \frac{\text{PA in Buffer/IS PA}}{\text{PA in Plasma/IS PA}}\right) \cdot 100\%$$

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

***In vitro* Methods for the Determination of Nampt Inhibition and Cell Proliferation**

Biochemical Assay

Part 1: The Nampt enzymatic reaction

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) in 89 μ L Buffer A was added to 1 μ L compound, incubated for 15 minutes at room temperature, and then 10x substrate and co-factors were added to the test well to make a final concentration of 30 nM Nampt, 1 μ M NAM, 100 μ M 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.

Part 2: Quantification of NMN

A mass spectrometry-based assay was used to measure the Nampt reaction product (NMN) and the internal control (L-Cystathionine). NMN and L-Cystathionine were detected using the services of Agilent with the RapidFire system. In short, the NMN and L-Cystathionine were bound to a graphitic carbon cartridge in 0.1% formic acid, eluted in 30% acetonitrile buffer, and injected into a Sciex 4000 mass spectrometer. The components of the sample were ionized with electrospray ionization and the positive ions were detected. The Q1 (parent ion) and Q3 (fragment ion) masses of NMN were 334.2 and 123.2, respectively. The Q1 and Q3 for L-Cystathionine were 223.1 and 134.1, respectively.

***In vitro* Cell Proliferation Assays**

A2780 Cell-Based 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). 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 10mM Tris-Base solution. The plates were then read in a microplate reader at an absorbance of 570 nm.

Other Cell-Based Assays

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 manufacturer's 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 time. IC₅₀ curves were generated with GraphPad Prism. IC₅₀ obtained from each run were then averaged in Excel and STDEV were calculated and shown.

***In vivo* pharmacodynamics and efficacy studies**

PK study method

Female NCR nude mice were obtained from Taconic (Cambridge City, IN, US). 12 animals were given a single intravenous dose of 5 mg/kg of compound **58** via the tail vein, and 12 animals were given a single oral dose of 10 mg/kg of compound **58**. The dose solutions for IV and PO were in 35% polyethylene glycol (PEG 400)/10% ethanol/15% PG/40% H₂O (v/v/v) and 60% polyethylene glycol (PEG 400)/10% ethanol/30% H₂O (v/v/v), respectively. Mice weighed approximately 25–30 g at the start of the study, and they were not fasted for dosing. After intravenous and oral administration of compound **58** (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) under isoflurane anesthesia. PK time points were 0.033 (IV administration only), 0.083 (PO administration only), 0.17 (IV administration only), 0.25 (oral administration only), 0.5, 1, 3, 6, 8 and 24 hours post-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 thawed for LC-MS/MS analysis (Column: Kinetex 50 x 2.1 mm C18 2.6 µ 100A; Mobile phase: (water with 0.1% formic acid and MeCN with 0.1% formic acid); ionization: electrospray positive ion mode; data acquisition: Analyst version 1.5.2. PK data analysis was performed on mean plasma concentration–time data and PK parameters were determined by non-compartmental methods using WinNonlin[®] Enterprise, version 5.2.1 (Pharsight Corporation; Mountain View, CA).

In vivo Xenograft Studies:

The efficacy study was carried out as per the guidelines established by the internal Institutional Animal Care and Use Committee. Prior to the implantation, the U251 cells were harvested, washed twice with PBS and resuspended in serum free medium containing Matrigel (BD Biosciences) in the ratio 1: 1. To determine the efficacy of the test compounds, female athymic nude mice were inoculated with 5×10^6 U251 cells subcutaneously in right flank. The animals were then monitored for their tumor growth. Tumors were allowed to grow to a predetermined size (100-200 mm³) and were then evenly randomized into control and treatment groups (n = 8). Animals in the treatment group were administered the test substance (compound **58**; 25 mg/kg BID) according to their individual body weight for the described period of time and observed for treatment related toxicity. Tumor volume was measured thrice per week with a digital vernier caliper according to the formula $L \times W^2/2$ where L and W are the length (mm) and width (mm) of the tumor, respectively. Tumor response endpoints such as % Tumor Growth Inhibition (% TGI) were also calculated.