Supporting Information

Structure-based Identification of Ureas as Novel Nicotinamide Phosphoribosyltransferase (Nampt) Inhibitors

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Table of contents:

Title page	S1
Computational Methods	S2
Expression, purification, and crystallization of human Nampt	S5
LCMS Methods	S8
Microsomal stability and solubility assay	S19

<i>In vitro</i> Methods for the Determination of Nampt Inhibition and Cell Proliferation	S20
<i>In vivo</i> pharmacodynamics and efficacy studies	S23
References	S25

Computational Methods

Computational Solvent Mapping and Pharmacophore Definition

Solvent mapping was performed with CS-Map using PDB code 2GVJ with ligand and crystallographic waters removed. Each probe was distributed on 250 starting positions on the dimer surface and run in 30 minimizations from each position. All resulting consensus sites within the cavity of the protein were used as centers for pharmacophore points. Pharmacophore point were visually defined as a radius and a coordinate in relation to 2GVJ and translated to minimum/maximum distances and angles for use in the virtual screening.

Table 1: Ranks of the five consensus sites that were identified by CS-Map within the Nampt substrate and ligand binding regions. CS-Map clustered site BC as a single site because of strong overlap of the virtual fragments within the site. We subsequently separated the site into two distinct pharmacophore points B and C, based on the distinctive hydrogen donating and accepting nature of functional groups found in these areas.

Consensus Site	A	BC	D	E	F
Rank	2	1	24	9	13

Table 2: Pharmacophore point definitions. Coordinates are relative to 2GVJ.

Pharmacophore Point	A	B	C	D	E	F
Definition	Pyridine	Hydrogen Acceptor	Hydrogen Donor	Any Ring	Any polar atom	Any polar atom
Coordinate [x,y,z]	15.2, 9.5, 7.1	14.0, 8.6, 2.0	17.5, 8.2, 3.7	15.4, 3.2, -2.8	13.1, 16.3, 10.3	6.7, 5.4, 10.7
Radius [Å]	1.5	1.6	2.5	3.0	3.0	3.2

Virtual Screening

Our virtual screening approach used pharmacophore matching in combination with result filtering and docking. The pharmacophore search was applied to the emolecule screening compound library (~5.5 million compounds, version of May 2010). To account for conformational flexibility, we used the conformation generator balloon¹ to generate a variable number of conformers for each compound, employing 20 as the initial population size, 200 generations, and 0.8 Å as RMSD cutoff. Pharmacophore searching was performed using the pharmacophore matching algorithm of CDK.² Steric constraints in the Nampt tunnel region were too complex to be modeled via inclusion or exclusion spheres in the matching. Instead, we filtered all conformers which matched the pharmacophore by a grid based overlap computation. A regular grid with cell width 0.2 Å was created for 2GVJ with each grid point being 1 if inside the van der Waals sphere of a heavy atom and 0 if outside. The matched compounds were aligned into the protein pocket by minimizing the RMSD between the pharmacophore points and the center of the matched groups and the overlap with the grid was computed as the sum of the linearly interpolated grid value at each atom position times the van der Waals volume of the atom. Of all compounds for which at least one conformer matched each model's

pharmacophore, the 10,000 with the lowest overlap were docked. The 200 top scoring compounds were filtered further by visual inspection based on structural desirability and diversity.

Table 3: Number of compounds in consecutive screening steps

	Model ABCD	Model EABC	Model FABC
Pharmacophore Screening	~5.6 Million	~5.6 Million	~5.6 Million
Docking	10,000	10,000	10,000
Manual Inspection	200	200	200
Selected, in stock, and purchased	137	43	48
Biochem IC ₅₀ <1 μ M	3	0	0

Docking

For docking and ranking of results during virtual screening we used Glide SP³. For structural analysis of in vitro screening hits we used Glide XP. Grid Generation and Docking were run on PDB code 2GVJ with default parameters.

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 50ug/mL of carbenicillin and then transferred to 22°C prior to induction with 1mM 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 20mM TrisCl, pH 8.0, 200mM NaCl, 5mM Imidazole, 1mM TCEP and EDTA-free protease inhibitor tablets (Roche). Cell lysates were loaded onto a HisTrap HP column (GE Healthcare) in a buffer containing 20mM TrisCl, pH 8.0, 500mM NaCl, 5mM Imidazole and 1mM TCEP and the bound Nampt was eluted with 250mM Imidazole in the same buffer. The protein was then loaded onto a 5mL Q HP column (GE Healthcare) in a buffer containing 25mM TrisCl, pH 8.0 and 1mM TCEP and eluted at 300mM NaCl with a linear gradient of the same buffer and 1.0M NaCl. The protein was further purified on a size-exclusion Superdex 75 column (GE Healthcare) in a buffer containing 25mM TrisCl, pH 8.0, 150mM NaCl and 5mM DTT. Fractions containing Nampt were pooled and concentrated to 6mg/mL and confirmed by SDS-PAGE showing that the purity was greater than 95%.

Diffraction quality crystals were grown at 19°C from 0.2uL + 0.2uL vapor diffusion drops containing 6mg/mL Nampt, 0.1M Sodium phosphate, pH 8.6-9.0, 25-29% polyethylene glycol 3350, 0.2M NaCl and 1mM 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 was collected at ALS beamline 502 and 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 Tables S1.

The structure was solved by molecular replacement (MR) with known Nampt structure (PDB code: 3DHF) as the search model using the 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 1. The structure is also deposited in the Protein Data Bank (PDB code: 4JR5).

Table 1 X-ray crystallography data and refinement statistics

Data collection	
Space group	P2 ₁
Unit cell	a=60.6Å, b=106.7Å, c=83.2Å, $\alpha=90^\circ$ $\beta=96.7^\circ$ $\gamma=90^\circ$
Resolution	1.91Å
Total number of reflections	78793(11548) ¹

Completeness	0.995 (0.999)
Redundancy	3.7 (3.7)
I/ σ	11.1 (2.9)
Rsym ²	0.110 (0.595)
Refinement	
Resolution range	50-1.91Å
Rcryst ³ / Rfree ⁴	0.162/0.210
Non-hydrogen atoms	7815
Water molecules	303
Average B, Overall	12.72
Protein	11.37
Water	23.46
r.m.s.d. bond lengths	0.006Å
r.m.s.d. angles	1.020°
Ramachandran (C/A/G/D)	0.912/0.088/0/0

¹Values in parentheses are of the highest resolution shell, which is 1.91-2.01Å.

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.

LCMS Methods:

LCMS1:

Instrument Information: HPLC-Agilent 1200

Column: Agilent Zorbax SB-C₁₈, 3.5 µm, 2.1 × 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 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: SHIMADZU LCMS-2020

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

Mobile Phase A: Water / 0.05% TFA

Mobile Phase B: Acetonitrile

Gradient: 5% 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 (254 nm) and ELS

Interface: ESI (Positive)

LCMS4:

Instrument Information: Waters Acquity UHPLC

Column: UPLC BEH C18, 1.7 mm, 2.1 x 30 mm

Mobile Phase A: Water/0.1% formic acid

Mobile Phase B: Acetonitrile/0.1% formic acid

Gradient: 5% to 95% B in 1.5 minutes, 95% B in 0.3 minutes, then stop.

Flow Rate: 0.7 mL/min

Column Temperature: 40 °C

Detector: 220 nm and 254 nm

MS Parameters: Waters SQD mass spectrometer

Interface: ESI (Positive)

LCMS5:

Instrument Information: HPLC-Agilent 1100

Column: ZORBAX SB-C18, 1.8 mm, 20 x 2.1 mm

Mobile Phase A: Water/0.05%TFA

Mobile Phase B: Acetonitrile/0.05%TFA

Gradient: 3% to 97% B in 7.0 minutes, 97% B for 1.5 minutes, then stop.

Flow Rate: 0.4 mL/min

Column Temperature: 40 °C

Detector: 220 nm and 254 nm

MS Parameters: Agilent MSD

Interface: ESI (Positive)

LCMS6:

Instrument Information: SHIMADZU LCMS-2010EV

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: 254nm and ELSD

Interface: ESI (Positive)

LC/MS/MS method for compound 21

HPLC Separation

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

Mobile Phase A: H₂O, 1 % formic acid

Mobile Phase B: Acetonitrile, 1 % formic acid

Gradient Programs

Prgram C		
Time (min)	% Mobile Phase B	Flow Rate (µL/min)
0.0	10	200
2.0	10	200
7.5	90	200
8.0	90	200
8.2	10	200
10.0	10	200

Injection Volume: 10 µL

Mass Spectrometry

API 4000 (Applied Biosystem /Sciex)

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 **21** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 604.4 (adduct M+H ion) to 392.4 (parent M+H ion) indicating absence of phosphoribosylated adduct (b) MRM of transition from m/z 392.4 (parent M+H ion) to 156.0 (representing fragmentation of the left hand side of the parent) detecting parent (**21**) but no phosphoribosylated adduct.

LC/MS/MS method for compound 29, 30, and 32

HPLC Separation

Column – ACE3 C18, 3u (C18) 100A

Mobile Phase A: H₂O, 1 % formic acid

Mobile Phase B: Acetonitrile, 0.1 % formic acid

Flow Rate: 0.5 mL/min

Gradient 0 min, 15% MPB

0.5 min, 15% MPB

7.5 min, 95% MPB

8.5 min, 95% MPB

8.6 min, 15% MPB

10 min, 15% MPB

Injection Volume: 20 µL

Mass Spectrometry

API 4000 (Applied Biosystem /Sciex)

Figure 2. LC/MS/MS Chromatograms of compound **29** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 581.0 (adduct M+H ion) to 369.0 (parent M+H ion) indicating presence of phosphoribosylated adduct (b) MRM of transition from m/z 369.0 (parent M+H ion) to 110.0 (representing fragmentation of the left hand side of the parent) detecting phosphoribosylated adduct and parent (**29**). *BLQ: Below Limit of Quantification (< 1 nM).

Figure 3. LC/MS/MS Chromatograms of compound **30** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 581.0 (adduct M+H ion) to 369.0 (parent M+H ion) indicating presence of phosphoribosylated adduct (b) MRM of transition from m/z 369.0 (parent M+H ion) to 96.0 (representing fragmentation of the left hand side of the parent) detecting phosphoribosylated adduct and parent (**30**). *BLQ: Below Limit of Quantification (< 1 nM).

Figure 4. LC/MS/MS Chromatograms of compound **32** enzyme reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 579.0 (adduct $M+H$ ion) to 367.0 (parent $M+H$ ion) indicating presence of phosphoribosylated adduct (b) MRM of transition from m/z 367.0 (parent $M+H$ ion) to 134.0 (representing fragmentation of the left hand side of the parent) detecting phosphoribosylated adduct and parent (**32**). *BLQ: Below Limit of Quantification (< 1 nM).

LC/MS/MS method for compound 50

HPLC Separation

Column – Phenomenex Luna C18, 3 μ m, (50 x 2.0 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	10	200
2.0	10	200
6.0	30	200
7.5	80	200
7.8	80	200
8.0	10	200
10.0	10	200

Injection Volume: 10 μL

Mass Spectrometry

API 4000 (Applied Biosystem /Sciex)

Figure 5. LC/MS/MS Chromatograms of compound **50** with no Nampt enzyme present in reaction mixture: (a) Multiple Reaction Monitoring (MRM) of transition from m/z 615.2 (adduct $M+H$ ion) to 403.2 (parent $M+H$ ion) indicating absence of phosphoribosylated adduct (b) MRM of transition from m/z 403.2 (parent $M+H$ ion) to 135.1 (representing fragmentation of the left hand side of the parent) detecting parent (**50**) but no phosphoribosylated adduct.

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.

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.

***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.

Phosphoribosylated Adduct Formation Assay

The Nampt enzymatic reactions and control reactions without enzyme were carried out under the same conditions as the “**Biochemical Assay**” with the exception for these conditions: 1 or 0 μ M enzyme, 1 μ M compound, and an incubation time of 4 hours. Compounds **1** and **2** were utilized as controls and phosphoribosylated adducts were detected for both molecules (data not shown).

NAM Competition Assay

The assay was carried out under the same conditions as the “**Biochemical Assay**” with the exception of varying the final NAM concentrations. To determine substrate K_m and K_i values, the normalized data were analyzed using the substrate inhibition analysis module from GraphPad Prism.⁴ To determine the K_i , the slopes generated from plotting the IC_{50} values with different NAM concentrations were determined and then divided by the K_m .⁵ Substrate inhibition of Nampt was observed at high NAM concentrations, and this phenomenon was previously reported by others as well.⁶ This substrate inhibition complicated the use of traditional Michaelis-Menten type methods to visualize the associated biochemical data (i.e., $1/V$ vs $1/\text{Substrate}$ plots).

Figure 6. Nampt biochemical activity as a function of NAM concentration ($n = 3$). The assay was run in three separate experiments. The NAM K_m was determined to be $0.226 \pm 0.072 \mu\text{M}$ using the above data. The NAM K_i value was calculated to be $14.5 \pm 2.9 \mu\text{M}$ as described in the Supporting Information text.

***In vitro* Cell Proliferation Assay**

A2780 cells were seeded in 96-well plates at 1×10^3 cells/well in 180 μL 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% CO_2 , 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% CO_2 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 H_2O

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.

In vivo pharmacodynamics and efficacy studies

PK study method

Female BALB/c mice weighing 20 -22 grams (n=3) were administered a single 1 mg/kg dose as an IV bolus via the tail vein or a single 5 mg/kg PO dose by gavage. Animals were not fasted for dosing (?). Both the IV and PO dose was administered as a solution in 10% Ethanol/60% Poly Ethylene glycol-400 (PEG-400)/30% D5W (v/v/v) as vehicle at a dose volume of 5 mL/kg. Serial blood samples were collected from each mouse over a 24 h period after dosing. Plasma samples were analyzed for compound concentrations by LC/MS/MS (Column: Phenomenex Luna C18; Mobile phase: (10 mM ammonium formate with 0.1% formic acid: acetonitrile); ionization: electrospray positive ion mode; data acquisition: Analyst V 1.4). Pharmacokinetic parameters for compound were determined from the plasma concentration-time data using noncompartmental methods using Phoenix Winonlin 6.1 (Pharsight, Inc. CA)

In vivo Xenograft Studies:

C.B-17-*Igh-1b-Prkdc scid* mice (female) were injected s.c. with 5×10^6 A2780 cells (NCI) in the left flank. 10-12 days later when tumors reached 100–200 mm³ in size, mice were randomized into treatment groups of 8 mice per group including vehicle control and reference standard groups. The compounds were formulated in 60:30:10 PEG-400:D5W: 126 Ethanol (v/v/v) and administered PO, at the dose volume of 10 mL/kg BID for a duration of 5 or 10 days. The dose used for efficacy was selected from the MTD (Maximum Tolerated Dose) study. Mice were weighed and tumors measured using vernier calipers every alternate day. Tumor volume was measured thrice a week with a digital vernier caliper according to the formula $LXW^2/2$

where l and w are the length (mm) and width (mm) of the tumor. Tumor response endpoints such as Tumor growth delay (T-C), % T/C and % Tumor Growth Inhibition (% TGI) were also calculated. All animal work was approved by the Institutional Animal Care and Use Committee of Biological Resource Centre, Singapore.

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