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Fragment-based discovery of a potent NAMPT inhibitor

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ABSTRACT

NAMPT expression is elevated in many cancers, making this protein a potential target for anticancer therapy. We have carried out both NMR based and TR-FRET based fragment screens against human NAMPT and identified six novel binders with a range of potencies. Co-crystal structures were obtained for two of the fragments bound to NAMPT while for the other four fragments force-field driven docking was employed to generate a bound pose. Based on structural insights arising from comparison of the bound fragment poses to that of bound FK866 we were able to synthetically elaborate one of the fragments into a potent NAMPT inhibitor.

Nicotinamdie phosphoribosyltransferase, or NAMPT, is an enzyme found throughout the body and represents the rate-limiting step for salvage of the co-factor nicotinamide adenine dinucleotide (NAD+). It achieves this by catalyzing, in an ATP-dependent manner, the reaction of nicotinamide (NAM) with α-D-5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide (NMN), a NAD+ precursor (Fig. 1). Many tumor cells exhibit a very high rate of NAD+ turnover compared to normal cells and subsequently, NAMPT expression is elevated in many cancers.[1] This makes NAMPT a potential target for anticancer therapy. Several NAMPT inhibitors have been previously described most notable of which is FK866.[2] FK866 causes depletion of NAD+ in cancer cells which then triggers rapid apoptosis.

Here we describe the use of both fragment-based and structure-based drug discovery methods to design a novel, potent inhibitor of NAMPT. Fragment binders to NAMPT were discovered using saturation transfer difference nuclear magnetic resonance spectroscopy (STD-NMR) and a biochemical binding assay. The fragment binders we uncovered differ significantly in structure from the NAMPT fragment binders previously reported.[3] Crystal structures were obtained for two of the fragments bound to NAMPT while molecular modeling (docking) was used to obtain poses for the remaining fragments bound to the protein. Finally, overlay of these structures with that of NAMPT bound to FK866 led to the design and synthesis of a potent NAMPT inhibitor (Ki ~80 nM).

The gene for full-length human NAMPT (residues 1–491) with both a FLAG-tag (DYKDDDDK) and 6-His tag introduced at the C-terminus was synthesized and cloned into a pLVX-IRES-puro expression vector followed by introduction of the vector into HEK293-6E cells. Protein expression was carried out in a Wave Bioreactor on a 25 L scale. Cell lysis and clarification of the lysate via centrifugation was followed first by purification of the NAMPT protein on an anti-Flag M2 affinity resin and then by size-exclusion chromatography on HiPrep Sephacryl S200 resin. Purified protein was confirmed to be a dimer by SEC-MALS. About 40 mg of purified protein was typically obtained from the 25 L ferment.

Fragment-based screening was initially carried out against NAMPT using saturation transfer difference nuclear magnetic resonance spectroscopy (STD-NMR)[4] done in a competition mode with nicotinamide (NAM) serving as the STD-NMR probe (Supporting Information). NAM binds to NAMPT with an affinity of ~8 μM in the absence of ATP[5] and yields a good STD-NMR spectrum as shown in Fig. 2. The tightly-binding FK866 was used as a positive control and its addition resulted in complete disappearance of the STD-NMR signals arising from NAM binding to NAMPT. Subsequent screening of a 6000 member fragment library, initially screened in mixtures of 30 with each fragment at 400 μM, yielded four binders (compounds **1**–**4**, Table 1). All four of these fragments caused a decrease of at least an 80% in the NAM STD-NMR signal intensity when added at 400 μM. Affinity of these fragments for NAMPT was determined in a TR-FRET assay as described in

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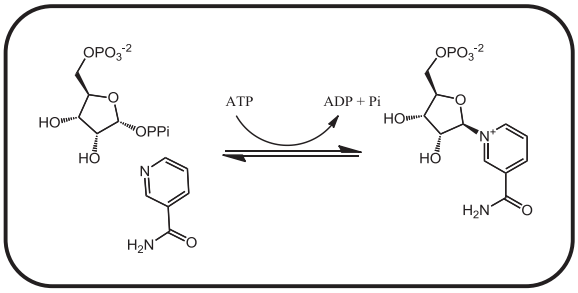


Fig. 1. Reaction catalyzed by NAMPT.

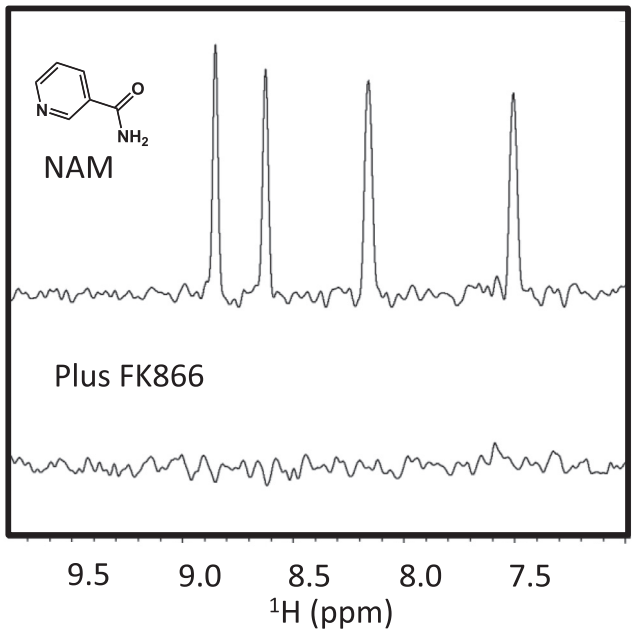


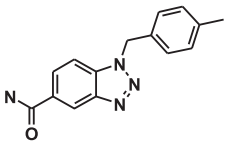
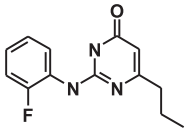
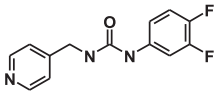
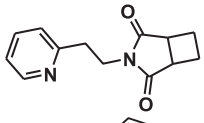
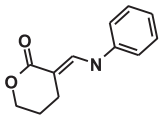
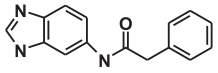
Fig. 2. STD-NMR assay used for fragment screening. Top spectrum is NAM plus NAMPT while bottom spectrum shows results upon addition of FK866. See Supporting Information for details.

Supporting Information. IC_{50} values are given in Table 1 along with the binding efficiency index (BEI)[6] for each fragment.

As a complement to this STD-NMR based fragment screen the TR-FRET based binding assay was used to screen a separate 9200-member library of fragments. This library was formatted specifically for high-throughput screening. In contrast to the NMR-based screen, fragments were tested initially as singles and at a lower concentration (80 μM). The two libraries had only 540 compounds in common and none of the fragment hits came from this common set. The TR-FRET assay used the full length Flag and His-tagged NAMPT protein, terbium labeled anti-His antibody, and an Oregon Green labeled probe based on FK866 (structure shown in Supporting Information). Unlabeled FK866 along with GMX-1778,[7] another reported NAMPT inhibitor, were used as positive controls. Structures for both are shown in Supporting Information. A competition curve for the binding of FK866 to NAMPT is shown in Fig. 3 from which a K_i of about 5 nM was obtained. After removing those hits that arose from fluorescent interference or lack of specificity, this TR-FRET based fragment screen yielded 8 hits, two of which were confirmed by NMR (compounds **5**–**6**, Table 1).

In order to drive synthetic elaboration of fragment hits most efficiently some structural hypothesis is typically necessary. Ideally, this is a crystal structure of the fragment bound to its protein target, but, in the absence of a crystal structure, this structural hypothesis could be based on an NMR-derived model, or simply on a model derived from force-field driven docking of the fragment into the protein binding site. Of the six fragment hits only two, 2 and 5, yielded co-crystals of sufficient quality such that a structure could be obtained (see Supporting Information for details). Bound poses for the other four fragments were obtained via force-field driven docking of the fragments into the binding site using the program Glide and the structure of the NAMPT/FK866 complex (PDB: 2GVJ)[8] as the target (Supporting Information). The co-crystal structure of 2 and 5 bound to NAMPT along with the docked poses for 1, 3, 4, and 6 are shown in Fig. 4, overlaid with the crystal structure of FK866 bound to NAMPT.

Table 1
NAMPT fragments.

No.	Structure	TR-FRET IC_{50} (μM)	BEI[1]
1		8	19
2		360	14
3		850	12
4		>1000	<13
5		10	25
6		90	16

[1] Binding Efficiency Index.

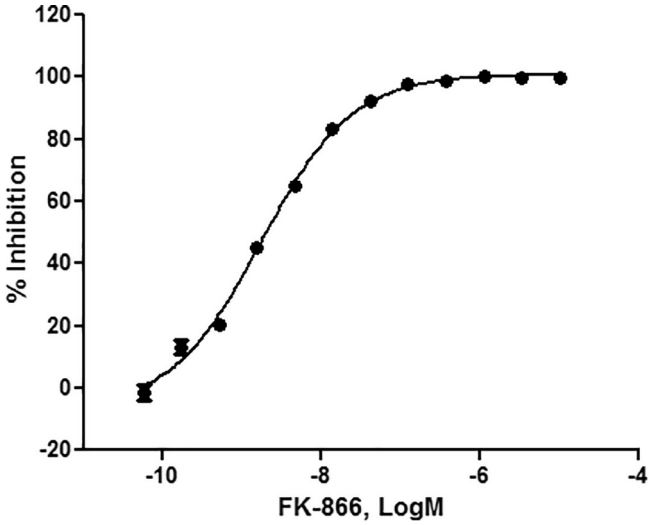


Fig. 3. TR-FRET competition binding curve for FK866.

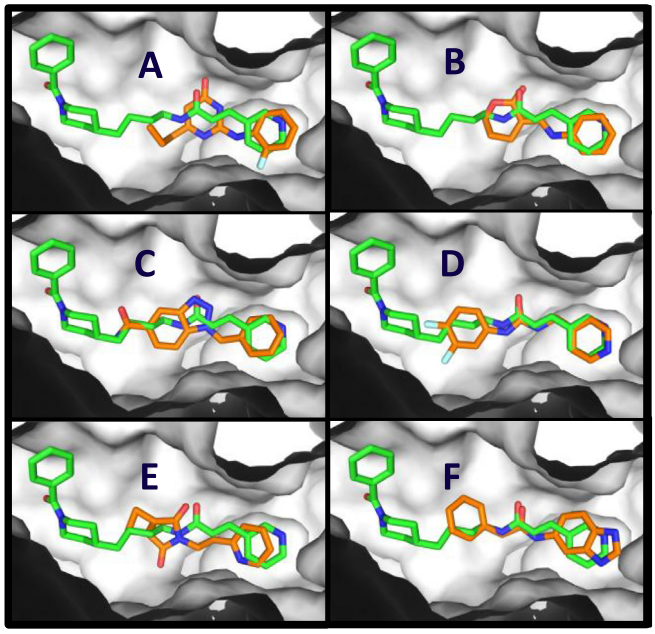


Fig. 4. A–B) Co-crystal structure of **2** and **5** (respectively) bound to NAMPT overlaid with bound FK866 in green (PDB: 2GVJ). C–F) Docked poses for 1, 3, 4 and 6 (respectively) overlaid with bound FK866.

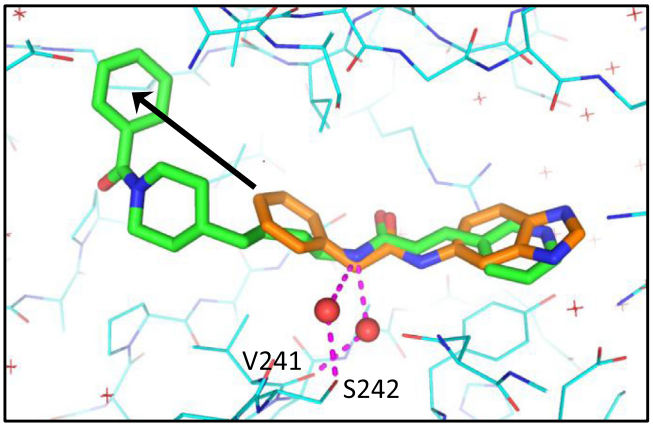
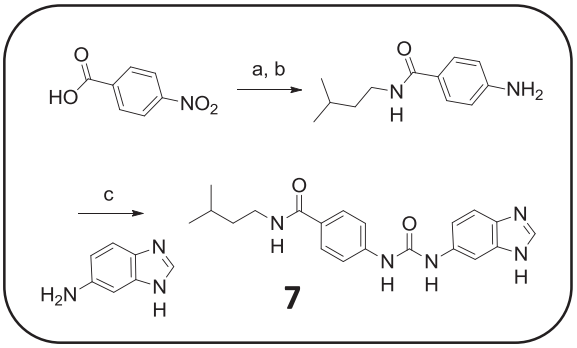


Fig. 5. Overlay of **6** with FK866 (PDB: 2GVJ) highlighting water-mediated H-bonds from FK866 to protein and hydrophobic space for fragment elaboration (arrow).

As can be seen in Fig. 4, all six fragments occupy the "back" of the binding pocket filling the space which would normally be occupied by the nicotinamide substrate. This leaves only one direction for potential fragment elaboration as is clear from the overlay of each fragment with bound FK866. While fragments 1 and 5 are the most efficient binders to NAMPT, fragment 6 proved to be a better choice based on ease of synthetic elaboration. The overlay of this fragment, docked into the binding pocket of NAMPT, with FK866 shows that the methylene of the fragment overlaps the amide nitrogen of FK866. This amide nitrogen makes two water-mediated hydrogen bonds to the protein. One is to the sidechain hydroxyl of S241 while the other is to the backbone carbonyl of V242 (Fig. 5). This observation suggested that substitution of the methylene amide of the fragment with a urea moiety could be one way to gain some potency. In addition, from the overlay of 6 with FK866 it was obvious that there was likely additional hydrophobic space to be filled in the "tunnel" portion of the binding site as exemplified by the terminal phenyl group of FK866



Scheme 1. Reagents and conditions: (a) i-pentyl amine, EDC·HOBt, *N*-methylmorpholine, DMF, rt, 88%; (b) H_2, 10% Pd-C, MeOH, rt, 98%; (c) *N*,*N*′-Disuccinimidyl carbonate, pyridine, *N*,*N*-diisopropylethylamine, MeCN-NMP, rt, 28%.

(arrow in Fig. 5). The observations outlined above led to the design of compound 7, synthesis of which is outlined in Scheme 1 below.

Compound **7** exhibits a TR-FRET affinity (Ki) of ~80 nM and is poised for further elaboration into an even more potent NAMPT inhibitor.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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A. Supplementary data

Supplementary data (protein preparation, NMR-based fragment screening, high-throughput fragment screening, X-ray crystallography, and force-field driven docking, along with the structures of FK866, GMX-1778, and the TR-FRET probe) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.12.023.

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