

Crystallization and Preliminary X-Ray Crystallographic Studies of *Rattus norvegicus* Visfatin/PBEF/Nampt in Complex with an FK866-Based Inhibitor

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Nicotinamide adenine dinucleotide (NAD⁺) plays a pivotal role in numerous biochemical and biological processes. Not only does it participate as a coenzyme during catalysis of redox reactions, it also serves as a substrate in several biochemical reactions, including mono- and poly-ADP-ribosylation, protein deacetylation and ADP-ribose cyclization.¹

Malignant tumor cells show a higher than normal rate of NAD⁺ turnover due to increased ADP-ribosylation.^{2,3} In these cells, expression of visfatin [pre-B-cell colony-enhancing factor (PBEF)/nicotinamide phosphoribosyltransferase (Nampt)], which is the rate-limiting enzyme in the salvage pathway for NAD⁺ synthesis, is upregulated so as to maintain adequate levels of NAD⁺.^{4,5} Consequently, visfatin is an attractive target for anticancer therapy.^{6,7} Indeed, FK866, a small potent inhibitor of visfatin activity, reduces NAD⁺ levels and induces apoptosis of tumor cells.⁸ A phase I clinical trial of FK866 has already been successfully completed,⁹ and a phase II trial of its efficacy against several forms of human cancer is currently ongoing.

The crystal structure of visfatin has been solved, with and without bound substrate, product and FK866.¹⁰⁻¹² The structure of the visfatin-FK866 complex reveals how the inhibitor interacts with the enzyme's active site residues. Moreover, comparison of the structures of the visfatin-FK866 and visfatin-nicotinamide mononucleotide (NMN) complexes shows how the pyridyl ring of FK866 is positioned at the nicotinamide ring of NMN. Given that the ribose ring and phosphate group of NMN make good contacts with the active site residues of visfatin, we endeavored to design a larger

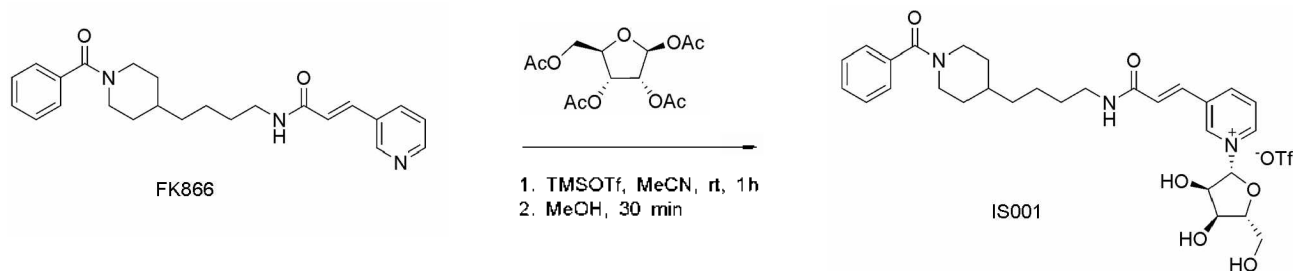
Table 1. Data collection statistics

	Visfatin-IS001 Complex
X-ray source	PAL-4A
Wavelength (Å)	1.0000
Space group	<i>P2₁2₁2₁</i>
Unit cell dimensions (Å)	<i>a</i> = 83.3, <i>b</i> = 107.4, <i>c</i> = 120.2
Resolution (Å)	50-3.0 (3.05-3.00)
Observed reflections	165,450
unique reflections	22,256
Completeness (%)	99.7 (100)
<i>R</i> _{sym} * (%)	14.0 (33.4)
<i>I</i> / σ (I)	24.4 (8.3)

* $R_{sym} = \frac{\sum_h \sum_i |I(h)_i - \langle I(h) \rangle|}{\sum_h \sum_i I(h)_i}$, where *h* is the intensity of reflection, $\langle I(h) \rangle$ is the sum over all reflections, and \sum_i is the sum over *i* measurements of reflection *h*.

FK866 derivative that would establish hydrogen bond interactions with residues in the NMN binding site. Toward that goal, we designed several hydrophilic moieties that we added to FK866 to manipulate its pharmacokinetic properties.¹³ Ultimately, we designed and synthesized IS001, which has an additional ribose ring on the FK866 pyridyl ring.

We found that crystals of visfatin in complex with IS001 belong to the *P2₁2₁2₁* space group and have the unit-cell dimensions *a* = 83.3, *b* = 107.4, *c* = 120.2 Å, $\alpha = \beta = \gamma = 90^\circ$. Assuming one dimeric visfatin molecule is contained in an asymmetric unit, the Matthews coefficient was calculated to be 2.41 Å³/Da; the estimated solvent content was thus



Scheme 1. Reaction scheme for synthesis of IS001.

*These authors have equally contributed to this work.

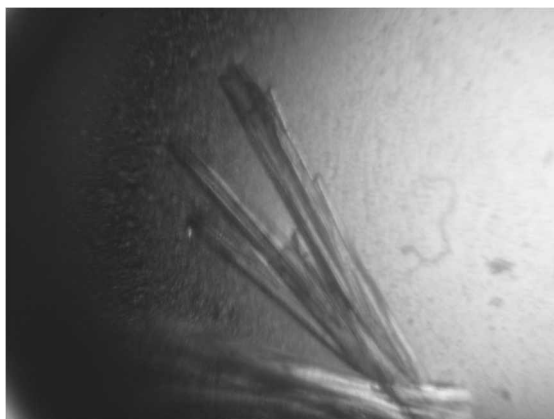


Figure 1. Crystals of visfatin-IS001 complex. Visfatin-IS001 complex was crystallized using the hanging drop vapor diffusion method at 294 K. Crystals of the complex were made by mixing 1 μ L of protein solution and the same volume of reservoir solution containing 0.2 M MgCl₂, 16% (w/v) PEG 3350 and 0.1 M HEPES-NaOH (pH 7.5).

48.9%,¹⁴ which is typical for protein crystals. Initial molecular replacement calculations were carried out with MOLREP¹⁵ using search models based on the structure of *Rattus norvegicus* visfatin (PDB code 2G95). At present, model building and refinements are ongoing. The statistics for data collection are summarized in Table 1.

Experimental Section

Protein expression and purification. Recombinant rat visfatin fused with a hexahistidine tag was expressed in *Escherichia coli* BL21(DE3) as described previously.⁹ For purification, the soluble protein was first applied to a nickel-agarose affinity chromatography column, after which the protein was eluted with buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl and 300 mM imidazole. The eluate was then concentrated by ultrafiltration using an YM30 membrane (Amicon), and TEV protease was added to remove the hexahistidine tag. The partially purified visfatin was then added to a Superdex 200 column (GE-Amersham-Pharmacia) equilibrated with buffer containing 20 mM HEPES-NaOH (pH 7.5) and 150 mM NaCl, and fractions containing the recombinant protein were pooled and concentrated to 15 mg/mL.

Chemical synthesis of the modified inhibitor IS001. We synthesized IS001 as summarized in Scheme 1. To a stirred solution of β -D-ribofuranose 1,2,3,4-tetraacetate (16.2 mg, 0.051 mol) and FK866 (20 mg, 0.051 mol) in dry acetonitrile (2 mL), we added TMSOTf (73 μ L, 0.41 mol) dropwise at room temperature and then stirred the mixture for an additional

1 h at the same temperature. Methanol (1 mL) was then added to the mixture and stirred for 30 min, after which the solvent was evaporated under vacuum, and the residue was chromatographed on activated charcoal (eluted with H₂O to 100% MeOH). After removing the solvent, the product was precipitated with ethyl acetate (12.7 mg, 18%).

Crystallization and data collection. Crystallization trials were carried out in a 24-well plate using the hanging-drop vapor diffusion method. Crystals of rat visfatin in complex with IS001 were grown at 294 K under 0.1 M HEPES-NaOH (pH 7.5), 0.2 M MgCl₂, 16% (w/v) polyethylene glycol 3350 (Figure 1). For cryogenic experiments, a suitable cryoprotectant was determined to be paratone oil. A complete data set was collected at 100 K at BL-4A of the Pohang Accelerator Laboratory, Korea. Data sets were indexed and processed using the HKL2000 package.¹⁶

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