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GlcNAcstatin: a Picomolar, Selective *O*-GlcNAcase Inhibitor That Modulates Intracellular *O*-GlcNAcylation Levels

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Many proteins in the eukaryotic cell are modified by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) on serines and threonines.[1] *O*-GlcNAcylation has been shown to be important for regulation of the cell cycle, DNA transcription and translation, insulin sensitivity, and protein degradation.[2,3] Misregulation of *O*-GlcNAcylation is associated with diabetes and Alzheimer's disease.[2,4,5] Two enzymes are involved in the dynamic cycling of this posttranslational modification, the *O*-GlcNAc transferase (OGT, classified as CAZY[6] family GT41) and *O*-GlcNAcase (OGA, GH84). PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate), a nanomolar inhibitor of OGA, has been extensively used to induce and study the effects of raised *O*-GlcNAc levels in the cell.[7,8] However, PUGNAc is also a potent inhibitor of the human lysosomal hexosaminidases HexA and HexB, inactivation of which has been associated with the Tay−Sachs and Sandhoff lysosomal storage disorders. While more selective derivatives of PUGNAc and other OGA inhibitors have recently been reported,[9−11] these are also associated with weaker (micromolar) inhibition of OGA. Here we report on a rationally designed glucoimidazole, GlcNAcstatin (**1**) (Figure 1), which inhibits a bacterial OGA (bOGA) with a K_i of 4.6 pM and 100000-fold selectivity over HexA/B, is active against human OGA (hOGA) in human cell lines, and is tethered in the active site as revealed by X-ray crystallography.

Recent structural data of bOGA in complex with PUGNAc has shown that this compound mimics the sp^2 configuration of the C1 atom in the transition state and binds with the acetamido group in a deep pocket that is significantly smaller in HexA/HexB than in OGA,[12] providing an inroad to engineering selectivity. The PUGNAc phenylcarbamate moiety extends out of the active site toward a solvent-exposed tryptophan. In an effort to increase affinity and selectivity in parallel, we decided to investigate the somewhat isosteric gluco-configured derivatives[13] of the naturally occurring hexosaminidase inhibitor nagstatin.[14] In 1995 Tatsuta reported the total synthesis of this compound[15] and a series of related glycoimidazole derivatives with variable configuration of the sugar ring, showing up to nanomolar inhibition against a panel of glycosidases.[16,17] Subsequent work by the Vasella group defined the structure−activity relationships of nagstatin analogues (tetrahydroimidazo[1,2-*a*]pyridines) as glycosidase inhibitors, making use of an original synthetic approach to the bicyclic core structure,[18] showing that the molecular architecture of these compounds in the ground state accurately mimics the assumed flattened half-chair/envelope conformation of the sugar ring in the transition state, while protonation of the imidazole ring effectively emulates the charge distribution in the oxocarbenium ion. Furthermore, critical evaluation of the nature of the C2 substituent showed that an aglycon-mimicking group in this position results in stronger inhibition.[19,20] Combining the current body of knowledge on the glycoimidazoles together with the structural data of the bOGA−PUGNAc complex, we designed a series of GlcNAc-configured nagstatin derivatives (GlcNAcstatins) that address specificity toward the OGA enzymes through elaborated N8 (tetrahydroimidazopyridine numbering) acyl derivatives while providing a source of increased affinity through the incorporation of suitable C2 substituents. Here we report the synthesis of GlcNAcstatin (**1**, Figure 1), a glucoimidazole with molecular architecture noticeably similar to that of PUGNAc, but bearing a larger isobutanamido group on N8 and a phenethyl group on C2.

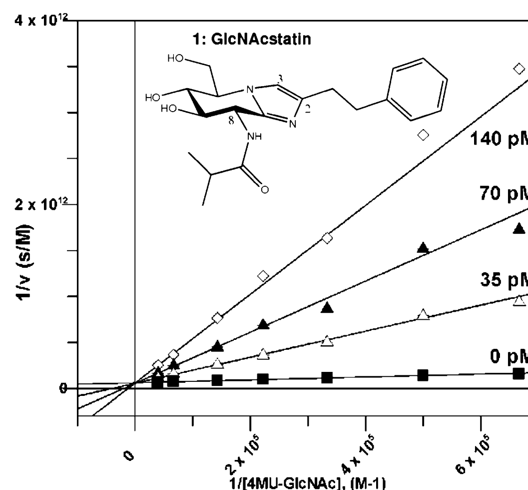

Figure 1. Structure of GlcNAcstatin and Lineweaver−Burk analysis of bOGA steady-state kinetics in the presence of inhibitor concentrations.

GlcNAcstatin was synthesized using a combination of the Tatsuta and Vasella approaches, as set out in the Supporting Information. Kinetic analysis using a previously published assay[12] (adapted for use with picomolar enzyme concentrations, see Supporting Information) shows that GlcNAcstatin is a very potent, competitive inhibitor of bOGA with a K_i of 4.6 ± 0.1 pM (Figure 1). Direct measurement of a binding K_d either by isothermal titration calorimetry or intrinsic tryptophan fluorescence was not possible due to complete depletion of free inhibitor concentration in the picomolar range by even the lowest experimentally possible concentration of enzyme (data not shown).

Inhibition of HexA/B was also evaluated, giving a K_i of 0.52 μM (see Supporting Information). Thus, GlcNAcstatin is 100000-fold selective for the *O*-GlcNAcase active site compared to the structurally most closely related enzymes in the human cell. To demonstrate the molecular basis of this selectivity, we characterized the binding mode of the inhibitor by determining the crystal structure of the bOGA−GlcNAcstatin complex to a resolution of 2.25 Å (Figure 2). Similar to the previously determined PUGNAc[12] and thiazoline[21] complexes, the sugar moiety of the inhibitor occupies a pocket in

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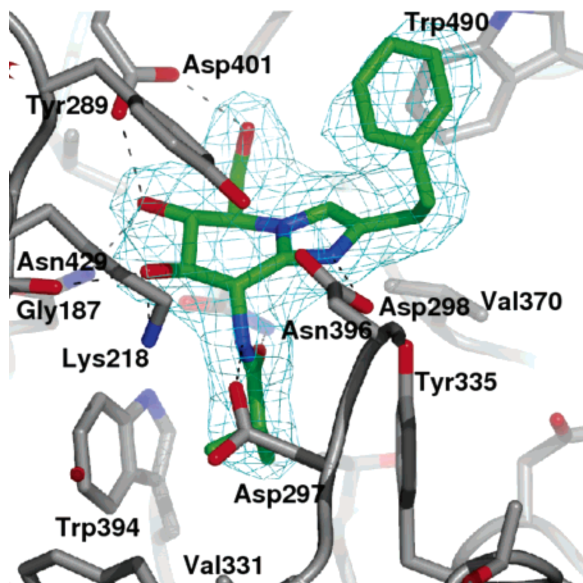

Figure 2. Crystal structure of GlcNAcstatin (sticks with green carbon atoms) complexed to bOGA. Unbiased 2.25 Å $|F_o| - |F_c|$, ϕ_{calc} (2.5σ) electron density for the inhibitor is shown in cyan.

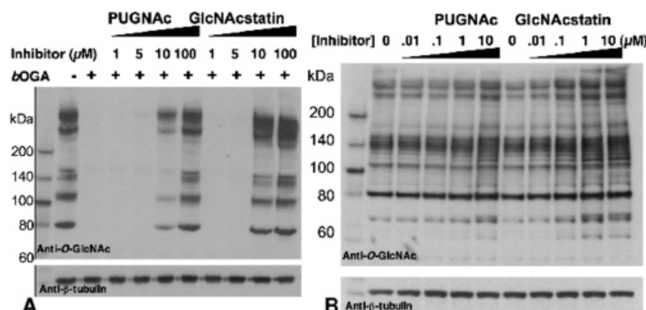

Figure 3. Immunoblot detection of O-GlcNAc modifications on cellular proteins, compared to molecular weight standards in the far left lanes. (A) bOGA (5 μM) activity on SH-SY5Y neuroblastoma cell lysates is inhibited by increasing amounts of PUGNAc/GlcNAcstatin, (B) HEK293 cells incubated for 12 h with increasing amounts of inhibitors.

the enzyme and interacts with four conserved hydrogen bond donors/acceptors. The pyranose ring adopts a ^{4}E conformation, similar to that observed in previously determined enzyme−glucoimidazole complexes[22,23] and the PUGNAc complex.[12] Interaction with Asp297 and Asn396 forces the isobutanamido group to adopt a conformation compatible with the proposed substrate-assisted catalysis mechanism,[9,12,21] with the carbonyl oxygen approaching the sp^2 configured "anomeric" carbon to within 3.4 Å (Figure 2). The terminal methyls of the isobutanamido group penetrate a pocket formed by the conserved Tyr335 and Trp394 (Figure 2). In HexA/B this pocket is significantly smaller, explaining why the larger acyl groups on the previously reported inhibitors[9−11] significantly increase the K_i against these enzymes. Asp298, the catalytic acid, is precisely positioned for the lateral protonation mechanism as proposed by Vasella.[18] The phenethyl group extends away from the active site, interacting with the solvent-exposed Trp490.

To evaluate the usefulness of GlcNAcstatin as a chemical tool to study the effects of inhibition of OGA in human cells, activity of the compound in human SH-SY5Y human neuroblastoma cell lysates and the HEK 293 cell line was evaluated qualitatively by Western blot analysis with an anti-*O*-GlcNAc antibody. Figure 3A shows that GlcNAcstatin appears more active than PUGNAc in inhibiting bOGA from removing *O*-GlcNAc from proteins in SH-SY5Y cell lysates. More importantly, when used to treat HEK 293 cells, GlcNAcstatin qualitatively appears more efficient at raising *O*-GlcNAc levels than PUGNAc (Figure 3B).

In conclusion, **1**, GlcNAcstatin reported here, represents a novel, potent, and highly selective tool to study the role of the *O*-GlcNAc modification in the human cell. Several other inhibitors such as the thiazolines[9] and PUGNAc derivatives[10,11] carrying larger N2 acyl groups have very recently been reported. While these compounds did improve the selectivity, showing weaker inhibition of HexA/B, they did so at the cost of also significantly reducing inhibition of OGA, with K_i's increasing to the micromolar range. The work described here shows that it is possible to achieve both picomolar inhibition and exquisite selectivity with rationally designed glucoimidazoles. The structural data of the GlcNAcstatin complex will allow for further fine-tuning of the inhibitory properties of GlcNAcstatin derivatives by elaboration of the N8 acyl group and the aromatic substituents off the imidazole ring.

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Supporting Information Available: Complete citation for ref 5; experimental details of synthesis of (**1**), enzyme inhibition, and X-ray crystallography. This material is available free of charge via the Internet at http://pubs.acs.org.

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