Biochemical and Structural Assessment of the 1-N-Azasugar GalNAc-Isofagomine as a Potent Family 20 \(\beta\)-N-Acetylhexosaminidase Inhibitor*

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Azasugar inhibitors of the isofagomine class are potent competitive inhibitors of configuration-retaining \(\beta\)-glycosidases. This potency results from the formation of a strong electrostatic interaction between a protonated endocyclic nitrogen at the “anomeric” center of the inhibitor and the catalytic nucleophile of the enzyme. Although the majority of retaining \(\beta\)-glycosidases use a mechanism involving a carboxylate residue as a nucleophile, Streptomyces plicatus \(\beta\)-N-acetylhexosaminidase (SpHEX) and related family 20 glycosidases lack such a catalytic residue and use instead the carboxyl oxygen of the 2-acetamido group of the substrate as a nucleophile, Streptomyces plicatus \(\beta\)-N-acetylhexosaminidase (SpHEX) and related family 20 glycosidases lack such a catalytic residue and use instead the carboxyl oxygen of the 2-acetamido group of the substrate as a nucleophile, “attack” the anomeric center. Thus, a strong electrostatic interaction between the inhibitor and enzyme is not expected to occur; nonetheless, the 1-N-azasugar (2R,3R,4S,5R)-2-acetamido-3,4-dihydroxy-5-hydroxymethyl-piperidinium hydrochloride (GalNAc-isofagomine-HCl), which was synthesized and assayed for its ability to inhibit SpHEX, was found to be a potent competitive inhibitor of the enzyme \((K_i = 2.7 \mu M)\). A crystallographic complex of GalNAc-isofagomine bound to SpHEX was solved and refined to 1.75 \(\AA\) and revealed that the lack of a strong electrostatic interaction between the “anomeric” center of GalNAc-isofagomine and SpHEX is compensated for by a novel 2.8-Å hydrogen bond formed between the equatorial proton of the endocyclic nitrogen of the azasugar ring and the carboxylate of the general acid-base residue Glu-314 of SpHEX. This interaction appears to contribute to the unexpected potency of GalNAc-isofagomine toward SpHEX.

Natural and synthetic glycosidase inhibitors are useful biological tools for helping to understand the catalytic mechanism by which these ubiquitous enzymes process their natural substrates. Exploring the relationships between inhibitor structure, enzyme kinetics, and the molecular interactions that occur between an inhibitor and its enzyme target, not only provides insight into the catalytic mechanism of these enzymes but also gives an opportunity for knowledge-based design of potent and highly specific therapeutic agents (1). Indeed, glycosidases have been implicated in numerous carbohydrate-mediated processes related to disease, and much effort has been devoted to selectively controlling such glycosidase activity. Prominent examples include the use of sialidase inhibitors such as zanamivir (Relenza), oseltamivir (Tamiflu), and the experimental compound BCX-1812 for the treatment of influenza (2, 3) and intestinal \(\alpha\)-glycosidase inhibitors (e.g. acarbose and miglitol) for the treatment of non-insulin-dependent diabetes mellitus (4, 5). The recent successes of sugar-based therapeutics has encouraged the continuing effort to develop a more complete understanding of the catalytic mechanism of glycosidases through the synthesis and subsequent kinetic and structural analysis of novel inhibitors.

Glycosidases catalyze glycosidic bond hydrolysis via a nucleophilic substitution reaction that can result in two possible stereoechemical outcomes: net retention or inversion of anomeric configuration (6, 7). This distinction divides glycosidases into two broad families: the retaining and the inverting glycosidases. Interestingly, when glycosidases are grouped into families based on primary amino acid sequence similarity (8), this distinction holds; all enzymes within a family appear to catalyze reactions with the same stereoechemical outcome (9). There are currently more than 70 families of which at least 30 contain members whose three-dimensional structure has been determined (visit afmb.cnrs-mrs.fr/~pedro/CAZY/db.html on the Web) (10). Although glycosidases are a structurally diverse group of enzymes, two key active site carboxyl groups are conserved throughout the majority of glycosidase families and are intimately involved in glycosidic bond hydrolysis (7).

Inverting glycosidases are understood to use a single-displacement mechanism in which the two carboxyl groups, acting as general base and general acid catalysts, are spaced ~10 Å apart, a distance large enough to allow the substrate and a water molecule to bind between them (6, 7, 11). Cleavage of the glycosidic linkage involves protonation of the glycosidic oxygen by the general acid-catalyst in concert with general base-catalyzed nucleophilic attack of a water at the anomeric center. The net result is a hemiacetal product with an anomeric configurat-
tion that is inverted relative to that of the substrate. In contrast, the analogous carboxyl groups in retaining glycosidases are typically spaced 5.5 Å apart and are involved in a double-displacement mechanism (6, 7, 11). The first carboxyl group promotes general acid-catalyzed cleavage of the glycosidic bond followed by nucleophilic attack at the anomeric center by the second carboxylate to form a covalent glycosyl-enzyme intermediate (see Fig. 1, lower pathway). General base-catalyzed nucleophilic attack of an incoming water molecule at the anomeric center of the intermediate yields a hemiacetal with the same anomeric configuration as the substrate.

Alternatively, the configuration-retaining β-N-acetylhexosaminidases from families 18 and 20, which lack an apparent enzymic nucleophile, distort the substrate such that the carboxyl oxygen atom of the adjacent 2-acetamido group becomes appropriately positioned to act in place of the missing nucleophilic carboxylate (see Fig. 1, upper pathway) (12–15). Indeed, it has been shown that the enzymic nucleophile of the family 7 β-retaining endoglucone I from Fusarium oxysporum overlaps the position of the 2-acetamido carboxyl oxygen of chitobiose bound to the family 20 chitobiose from Serratia marc- escens when the two structures are superimposed (16).

Anchimeric assistance by the neighboring 2-acetamido group of the substrate replaces the need for a second enzymic carboxylate and results in the formation of an enzyme-stabilized oxazolium ion intermediate (17, 18). The cyclic intermediate is hydroyzlyzed by general base-catalyzed nucleophilic attack of water at the anomeric center in a manner analogous to the "normal" double-displacement mechanism described above (18).

Transition state structures occurring along the reaction coordinates of both inverting and retaining glycosidases are known to have substantial oxocarbenium ion character (7, 19, 20). The reversible azasugar inhibitors of the deoxyxojirimycin and isofagomine classes are believed to mimic charge distributions found within oxocarbenium ion transition state structures (1, 21, 22). Assumed to be protonated and positively charged when bound in the enzyme active site, the endocyclic nitrogen atoms of azasugar inhibitors interact favorably with enzyme carboxylates (23). Interestingly, depending on whether the nitrogen is located at the position corresponding to endocyclic oxygen 05, as in the deoxyxojirimycin class, or located at the anomeric center, as for the isofagomine inhibitors, there appears to be distinct selectivity for α- and β-retaining glycosidases, respectively (21). This selectivity was suggested to result from the position of the enzyme nucleophile within the active sites of these enzymes. Indeed, it was demonstrated that, in cycloextrin glycosyltransferase, a retaining α-glyco- syltransferase/α-glycosidase, the nucleophile is positioned such that its carbonyl oxygen forms a syn interaction with the anomeric center and endocyclic oxygen of the substrate (24). It is conceivable that this interaction would favor binding of the deoxyxojirimycin class of azasugar inhibitors, because the protonated nitrogen (which replaces the endocyclic oxygen) could donate a stabilizing hydrogen bond to the carbonyl oxygen of the enzyme nucleophile (1, 22). For β-retaining glycosidases, however, the carbonyl oxygen of the nucleophile interact with the substrate from the opposite side and form instead a syn interaction with the anomeric center and 2-hydroxyl substituent of the substrate (24). Having no interactions with the endocyclic oxygen, the enzyme nucleophile of retaining β-glycosidases will preferentially interact with the isofagomine class of azasugars whose protonated nitrogen is located at the anomeric center and is poised to donate a hydrogen bond to the enzyme nucleophile (21). In support of this proposal is the recent crystallographic complex of a potent xylobiose-derived isofagomine inhibitor bound to a retaining family 10 xylanase. It showed a strong hydrogen-bonding interaction between the presumably protonated nitrogen of the inhibitor and the enzyme nucleophile (23). Recent results, however, with the neuromycins class of isofagomines, which contain a hydroxyl adjacent to the ring nitrogen at the position normally occupied by the 2-hydroxyl, cast some doubts on this conjecture. This class of inhibitor bound more tightly than the isofagomines and with approximately equal affinities to both α- and β-glycosidases (25).

We were interested in determining whether the isofagomine class of azasugars could inhibit a β-retaining glycosidase that uses anchimeric assistance and lacks an apparent enzymic nucleophile. The 1-N-azasugar inhibitor (2R,3R,4S,5R)-2-acetamido-3,4-dihydroxy-5-hydroxymethyl-piperidinium hydrochloride (GalNAc-isoafagomine·HCl) (3, Fig. 1) was synthesized and assayed for its ability to competitively inhibit Strepto- tyes plicatus β-N-acetylhexosaminidase (SpβHEX) (3). SpβHEX is a β-retaining family 20 exoglycosidase lacking an enzymic nucleophile (14, 18). It uses instead 2-acetamido group participation in a substrate-assisted catalytic mechanism as described above. The crystallographic structure of the complex of GalNAc-isoafagomine bound to SpβHEX demonstrated a novel binding mode for the inhibitor and provided insight into its unexpectedly potent inhibitory activity toward this family 20 glycosidase.

**EXPERIMENTAL PROCEDURES**

**Synthesis of** (2R,3R,4S,5R)-2-Acetamido-3,4-dihydroxy-5-hydroxymethyl-piperidinium Hydrochloride (GalNAc-isoafagomine·HCl)

The N-Boc acetamide (26) (2 Fig. 2) (26 mg, 0.075 mmol) was dissolved in 1.2 ml of trifluoroacetic acid at 0 °C. After 30 min, 0.2 ml of water was added dropwise, and the solution, still at 0 °C, was stirred for 20 min and then allowed to warm to room temperature over a 20-min period. The reaction mixture was concentrated to a residue, cooled to about 5 °C, dissolved in 1 ml of cold ethanol, and then treated with 0.1 ml of 12 n hydrochloric acid. The solution was allowed to warm to room temperature over a 10-min period then was concentrated and azetropically dried with toluene (3 × 2 ml). The residue was stirred with 0.5 ml of hexanes, whereupon a solid gradually formed. The solid was separated, washed with diethyl ether (2 × 1 ml), and then dried under vacuum to give 18 mg (98%) of GalNAc-isoafagomine (3, Fig. 2) as a white solid, m.p. 140–145 °C (dec) H NMR (300 MHz, CD3OD) δ 4.99 (δ, J = 9.9 Hz, H-2), 4.05 (br s, H-4), 3.78 (dd, J = 10.8, 6.3 Hz, H-7), 3.57 (dd, J = 10.8, 7.5 Hz, H-7), 3.05–3.21 (m, two H-6), 1.98–2.18 (m, H-5), 2.06 (s, NHCOCH3) 13C NMR (75 MHz, CD3OD) δ 175.2, 70.5, 68.9, 62.7, 61.9, 41.9, 41.8, 22.7, fast atom bombardment mass spectroscopy 205 (M+H+) for pyridinium ion of GalNAc-isoafagomine (3, Fig. 2)

**Protein Expression and Purification—**SpβHEX was overexpressed in Escherichia coli strain BL21(DE3) as an N-terminal His6-tagged fusion protein and affinity-purified using nickel-nitrilotriacetic acid Superflow resin (Qiagen) as described previously (18). Purified SpβHEX protein was stored at 4 °C elution buffer (20 mM Tris·Cl, pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 250 mM imidazole, pH 8.0) and was stable for several weeks.

**Enzyme Kinetics—**All inhibition constants for GalNAc-isoafagomine were measured at 25 °C using p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate in a reaction buffer composed of 15 mM sodium phosphate/15 mM sodium citrate buffer, 100 mM sodium chloride, pH 4.00, and 0.1% bovine serum albumin. Measurements were initiated by addition of SpβHEX previously dialyzed against the reaction buffer. Rates were determined by following the increase in absorption at 380 nm arising from the release of p-nitrophenolate in a continuous assay. K values were determined by a direct fit of the experimental data using the program GraFit (27).

1 The abbreviations used are: SpβHEX, Streptomyces plicatus β-N-acetylhexosaminidase; β-HEX, β-hexosaminidase; β-N-acetylhexosaminidase; SmChNB, Serratia marcescens chitobase; Nag-thiagoline, N-acetyl-glucosamine-thiogalactosamine; GalNAc, N-acetyl-galactosamine; GalNAc-isoafagomine, (2R,3R,4S,5R)-2-acetamido-3,4-dihydroxy-5-hydroxymethyl-piperidinium hydrochloride; pNPGLcNAc, p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside.
RESULTS AND DISCUSSION

The potency of isofagomine inhibitors for retaining β-glycosidases has been attributed to the formation of a strong electrostatic interaction between a protonated endocyclic nitrogen at the “anomeric” center of the inhibitor and the catalytic nucleophile of the enzyme (23). Because family 20 glycosidases lack the enzymic nucleophile required to form this strong enzyme-inhibitor interaction, it was hypothesized that isofagomines would be poor inhibitors of family 20 glycosidases. Contrary to this hypothesis, however, GalNAc-isoagomine (3, Fig. 2) was found to act as a potent competitive inhibitor of the family 20 glycosidase SpHEX, exhibiting a $K_i$ of 2.7 μM when using pNPβGlcNAc as substrate (Fig. 3).

Insight into the mechanism by which GalNAc-isoagomine inhibited SpHEX has been provided by the x-ray crystal structure of the enzyme-inhibitor complex (Fig. 4A). Crystals of the complex diffracted to 1.75-Å resolution, and the structure refined to an $R_{work}$ of 17.6% and $R_{free}$ of 19.2% (Table I). The excellent crystallographic data allowed for the calculation of an easily interpretable $|F_o| - |F_c|$, $\alpha_e$ electron density map for GalNAc-isoagomine (Fig. 4B). The inhibitor was located in the −1 subsite of the SpHEX active site pocket (Fig. 4). The azasugar ring of the inhibitor adopts a conformation approaching that of a half-chair, where the endocyclic nitrogen N1 is displaced 0.4 Å from a least-squares plane formed by atoms C2, C3, C5, and C9 (atom C9 replaces the atom O5 found in pyranose rings); whereas atom C4 is displaced 0.7 Å on the opposite side of this plane. Torsion angle measurements within the azasugar ring also demonstrate the flattened nature of the ring about N1 as compared with the x-ray structure of N-acetyl-galactosamine (32) (Table I). The flattened ring conformation of GalNAc-isoagomine differs from the relaxed $^4C_1$ chair conformation observed for the proximal azasugar ring of xylobiose-derived isofagomine bound to the family 10 glycosidase Cex from Cellulomonas fimii (23), indicating that the enzyme-bound conformation of GalNAc-
isofagomine better approximates the planar conformation believed to occur for atoms C1, C2, O5, and C5 during the oxocarbenium ion transition state of the natural substrate. The oxocarbenium ion transition state is thought to be a half-chair or skew-boat with atoms C1, C2, O5, and C5 forming a plane (6). The co-planarity of these atoms during the transition state is required for effective overlap of the non-bonding lone pair electrons of O5 and the anti-bonding orbital at the electron-deficient anomeric center of the oxocarbenium ion (however, the conformation of GalNAc-isofagomine is not truly planar about atoms C1, C2, C9, and C5). Taken together with the observations for xylobiose-derived isofagomine bound to Cex, it appears that, although the charge distribution in the isofagomine class of aza-sugars may mimic the oxocarbenium ion transition state, the enzyme-bound conformation of the azasugar ring better reflects that of the pyranose ring of the enzyme-bound intermediate that occurs during reactions catalyzed by β-retaining glycosidases (18, 33).

The SpHEX active site contains a tight hydrophobic pocket composed of Trp-344, Trp-361, Trp-442, Tyr-393, and Asp-313 that is responsible for orienting the 2-acetamido group in po-

The conformational changes observed for GalNAc-isofagomine upon binding SpHEX are consistent with the conformational changes observed for the 2-acetamido group of the non-reducing sugar of chitobiose upon binding to the family 20 chitobiose from S. marcescens (SmCHB). The small molecule crystal structure of chitobiose reveals the carbonyl oxygen atom of the 2-acetamido group of the non-reducing sugar to be +anticlinal to C1, with the torsion angle C1–C2–N2–C7 being 105.5° (34). However, the crystallographically observed Michaelis complex between chitobiose and the SmCHB shows the 2-acetamido group of the non-reducing GlcNAc residue (bound in the –1 subsite) to be rotated 158° about its C2–N2 bond with a final C1–C2–N2–C7 torsion angle of 102.2°. This bond rotation, in combination with a distortion of the pyranose ring from a chair toward a sofa conformation, brings the carbonyl oxygen of the 2-acetamido group of chitobiose to within 3.0 Å of the anomeric center, positioning it for attack at C1 and formation of a cyclic GlcNAc-oxazolinium ion intermediate (12).
isoformigenome is not normally expected to bear a negative charge; however, considering that SpHEX has evolved to stabilize a protonated and positively charged 2-acetamido group nitrogen (N2), it is very possible that the enzyme may polarize the amide of GalNAc-isoformigenome beyond its naturally occurring dipole moment, resulting in a further increase of charge density on the carbonyl oxygen. The polarized amide could favorably interact with the formal positive charge on the protonated endocyclic nitrogen (as indicated by the 2.7-Å hydrogen bond in Fig. 5); whereas, the electron deficient 2-acetamido nitrogen (N2) would be stabilized through donation of a hydrogen bond to the carbonyl of Asp-313 in a manner similar to the stabilization of the oxazolinium ion intermediate (18). The hydrogen bond donated from the side-chain hydroxyl group of Tyr-393 could also help stabilize the increased charge density of the carbonyl oxygen of GalNAc-isoformigenome. Such polarization of the amide of the natural substrate would greatly enhance the nucleophilicity of the 2-acetamido carbonyl oxygen atom and increase the efficiency of catalysis by this family of enzymes. Indeed, in the transition states leading to the formation and breakdown of the oxazolinium intermediate, it is very likely that these favorable interactions are enhanced.

This structure of GalNAc-isoformigenome bound to SpHEX is the first heterocycle with a galacto configuration to be solved in complex with a family 20 hexosaminidase. For substrates of gluco configuration, it has been observed, in both the Michaelis complex (12) and cyclic enzyme intermediate (18), that family 20 glycosidases form a bidentate hydrogen-bonding interaction between O3 and O4 of the substrate and the two nitrogens of the guanidinium group of a conserved Arg residue (Arg-162 SpHEX, Arg-349 SmCHB). This bidentate hydrogen-bonding interaction appears to be crucial for substrate binding, for the mutation of this conserved Arg residue (R162H) in SpHEX results in a 40-fold increase in Km and a 5-fold decrease in Vmax relative to wild type (18). The resultant 200-fold decrease in Vmax/Km confirms that this residue is intimately involved in stabilization of the transition states occurring along the reaction coordinate. However, the crystallographic complex between SpHEX and GalNAc-isoformigenome demonstrates that the enzyme cannot accommodate a bidentate hydrogen-bonding interaction between Arg-162 and the galacto configuration of GalNAc-isoformigenome (Fig. 6). Nevertheless, SpHEX and the related family 20 glycosidases catalyze the removal of terminal N-acetylhexosamine residues of both gluco and galacto configuration. A comparison of the structures of SpHEX in complex with either the intermediate analogue NAG-thiazoline or

## Table I

| Crystallographic statistics | Refinement |
|-----------------------------|------------|
| Space group                 | P6,2       | Resolution Å |
| Unit cell                   | a = b = 132.8, c = 177.0 | 100–1.75 |
| Detector                    | Mar Research 345 | Rwork = 17.6% |
| Wavelength                  | 0.979 Å   | Rfree = 19.2% |
| Resolution                  | 100–1.75 Å | Number of atoms |
| Total observations          | 1,013,193 | Protein |
| Unique reflections          | 92,286 (4,366) | GalNAc-isoformigenome |
| ⟨I/o⟩                       | 36.2 (10.9) | Water |
| Completeness                | 99.6 (95.7%) | Average B (Å²) |
| R_sym                       | 0.043 (0.15) | Root mean square deviation from ideal geometry |

### Table II

| GalNAc-isoformigenome | N-Acetyl-α-galactosamine |
|-----------------------|--------------------------|
| Endocyclic* (C8/O5)   | (N1/C1)–C2–C3            |
| (N1/C1)–C2–C3–C4     | 30.0*                    |
| C2–C3–C4–C5          | 52.0*                    |
| C3–C4–C5–C9/O5       | 58.6*                    |
| C4–C5–C9/O5–(N1/C1)  | 51.3*                    |
| C5–C9/O5–(N1/C1)–C2  | 36.3*                    |
| N-Acetyl group* (N1/C1)–C2–N2–C7 | 51.5* |
| C3–C2–N2–C7          | 76.0*                    |
| C2–N2–C7–O7          | 2.5*                     |
| C2–N2–C7–C8          | 178.3*                   |

### Table III

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2-acetamido group of GalNAc-isoformigenome (2.7 Å) as compared with the 3.0-Å distance observed between the analogous atoms in chitobiose bound to SmCHB. If the conformation of GalNAc-isoformigenome bound to SmCHB is considered equivalent to the pyranose ring conformation of the enzyme-bound intermediate during a normal catalytic cycle, then the difference in distance between the nucleophile and anomeric centers for GalNAc-isoformigenome and chitobiose can be attributed to conformational changes that occur in the pyranose ring as the reaction proceeds from the enzyme-substrate complex to the enzyme-bound intermediate. The pyranose ring changes from a sofa, as seen in the Michaelis complex between chitobiose and SmCHB, to a chair conformation as observed for the cyclic thiazolinium ion intermediate analogue (18) and GalNAc-isoformigenome bound to SpHEX.

During a normal catalytic cycle SpHEX donates a hydrogen bond from the side-chain hydroxyl group of Tyr-393 to the 2-acetamido carbonyl oxygen, helping to align the carbonyl oxygen with the anomeric center of the substrate (18). Cyclization to form the oxazolinium intermediate results in a formal positive charge developing on the protonated 2-acetamido nitrogen (N2). SpHEX stabilizes this positive charge by accepting a hydrogen bond from N2 to the carbonyl of Asp-313 (18). Both of these important hydrogen-bonding interactions with Tyr-393 and Asp-313 occur between SpHEX and the 2-acetamido group of GalNAc-isoformigenome, and they essentially lock the 2-acetamido group into position within the hydrophobic pocket (Figs. 5 and 6). The 2-acetamido group of GalNAc-
GalNAc-isofagomine demonstrate that O3 forms a hydrogen bond with the guanidino group of Arg-162 regardless of whether the sugar ring is of a gluco or galacto configuration; however, when O4 is in an axial position as observed for the galacto azasugar ring GalNAc-isofagomine, it is too far away (3.7 Å) and too poorly positioned to form a hydrogen bond with the guanidinium group of Arg-162 (Fig. 6). Instead, the SpHEX-GalNAc-isofagomine complex shows the axial O4 atom of GalNAc-isofagomine forming a short 2.5-Å hydrogen-bonding interaction with the carboxyl group of Glu-444 and not with Arg-162 (Figs. 5 and 6). SpHEX Glu-444 is conserved in family 20 glycosidases, suggesting that this residue is critical for binding substrates of galacto configuration. The carboxylate of this conserved Glu residue has also been shown to form a 2.6-Å hydrogen bond with O4 of substrates and intermediates of gluco configuration as observed in the crystal structure of SpHEX in complex with the intermediate analogue NAG-thiazoline (18) and SmCHB in complex with chitobiose (12).

Perhaps the most surprising result from the crystallographic analysis of the complex between SpHEX and GalNAc-isofagomine is the 2.8-Å hydrogen-bonding interaction between the general acid-base residue Glu-314 and the equatorial proton of the endocyclic nitrogen of the isofagomine inhibitor (Figs. 5 and 6). Such a hydrogen-bonding interaction between the protonated endocyclic nitrogen of an isofagomine inhibitor and the acid/base catalytic residue has not been observed in the other known complex of an isofagomine and retaining β-glycosidase (23). The ability of GalNAc-isofagomine to form such a hydrogen bond with the general acid-base residue of a family 20 glycosidase appears to result from the ability of the catalytic nucleophile to move in concert with the azasugar ring within the active site pocket of SpHEX (Fig. 6). Relative to the position of the cyclic intermediate analogue NAG-thiazoline bound within the active site of SpHEX (18), the azasugar ring of GalNAc-isofagomine is rotated about an axis best defined by atoms C2 and C5 such that the endocyclic nitrogen is brought closer to Glu-314 while maintaining a 2.7-Å hydrogen bond.
with the carbonyl oxygen atom of the attached 2-acetamido group (Figs. 5 and 6). Furthermore, compared with the wild type SpHEX structure, Glu-314 responds to the binding of GalNAc-isofagomine with a 26° rotation about χ2 such that Oε2 is brought to within hydrogen bonding distance of the endocyclic nitrogen of GalNAc-isofagomine. The conformational change in the side chain of Glu-314, in combination with the binding mode of GalNAc-isofagomine, reduces the distance between the endocyclic nitrogen of GalNAc-isofagomine and Oε2 of Glu-314 by 1.2 Å as compared with the 4.0-Å distance observed between the analogous atoms in the SpHEX-NAG-thiazoline complex (18). Considering the potent inhibition of GalNAc-isofagomine toward SpHEX, this hydrogen-bonding interaction appears to compensate for the missing electrostatic interaction that usually occurs between the protonated endocyclic nitrogen of iso-fagomine and Oε2.

The unexpected potency of GalNAc-isofagomine toward SpHEX highlights the need for detailed molecular analysis of protein-inhibitor interactions to understand clearly the mechanism by which a small molecule inhibits the catalytic activity of its target enzyme. Prior biochemical and structural information suggested that the isofagomine class of inhibitors would act as a poor competitive inhibitor of family 20 glycosidases. However, kinetic analysis of the inhibitory activity of GalNAc-isofagomine toward SpHEX proved otherwise, and it was through the analysis of the crystallographic complex formed between these two molecules that detailed insight into the mechanism of this unexpected inhibitory activity was obtained.

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Biochemical and Structural Assessment of the 1-N-Azasugar GalNAc-isofagomine as a Potent Family 20 \(\beta\)-N-Acetylhexosaminidase Inhibitor

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