Glycosidase Inhibitors

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The Structural Basis of Glycosidase Inhibition by Five-Membered Iminocyclitols: The Clan A Glycoside Hydrolase Endoglycoceramidase as a Model System**

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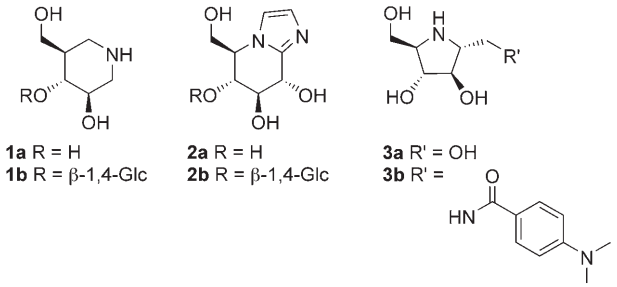
Glycosidase inhibition is an important pharmaceutical goal, reflected by current treatments for influenza (Tamiflu) and non-insulin-dependent diabetes (Miglitol/Glyset). Iminocyclitols (also often called azasugars or iminosugars) are particularly well-studied glycosidase inhibitors that have shown potential for the treatment of cancer,[1] glycosphingolipid storage disorders,[2] and viral diseases such as HIV[3] and hepatitis B[4] and C.[5] Their efficacy has been attributed to mimicry of the glycosidase "oxocarbenium-ion-like" transition state and serendipitous electrostatic binding interactions.[6] Interestingly, as well as the six-membered pyranoside isosteres, five-membered iminocyclitols have also been found to inhibit glycosidases. This has prompted a huge investment of research into synthesizing[7] and screening five-membered iminocyclitol libraries.[8,9] Despite the level of interest, the binding mode of the five-membered inhibitors has, as yet, eluded characterization. Here we report the first X-ray crystal structure of a five-membered iminocyclitol in complex with a retaining β-glycosidase. Further bound structures of classical six-membered iminocyclitol glycosidase inhibitors, an isofagomine and a glucoimidazole, are also presented to directly compare and contrast binding topologies.

Endoglycoceramidase II (EGC) is a family 5 glycosidase that hydrolyzes the β-glycosidic linkage between the sugar and ceramide moieties of gangliosides with net retention of configuration at the anomeric center.[10] The X-ray crystal structure of EGC has recently been reported, and complexes with a natural substrate, ganglioside G_{M3} (Svennerholm ganglioside nomenclature), and the trapped glycosyl–enzyme intermediate described.[11] To probe inhibitor binding in the −1 (donor) subsite, kinetic analyses of the classical six-membered glycosidase inhibitors isofagomine (**1a**) and glucoimidazole (**2a**), and a five-membered iminocyclitol, 2,5-imino-D-mannitol (**3a**), were carried out (Figure 1). Unfortunately, the K_i values for these "monosaccharide" inhibitors were greater than 0.75 mM, thereby rendering them unsuitable for crystallographic soaking experiments. In order to identify stronger binding derivatives of these inhibitors, a medium-sized (ca. 500 compound) "in-house" library was screened for EGC inhibition. Three hits were discovered: cellobiose-like isofagomine **1b**,[12] cellobiose-like imidazole **2b**,[13] and the five-membered iminocyclitol **3b**.[9] Kinetic analyses of these derivatives at the pH optimum of the enzyme (pH 5) showed them to be competitive inhibitors with K_i values of 5 μM, 0.5 μM, and 10 μM, respectively.



1a R = H
1b R = β-1,4-Glc
2a R = H
2b R = β-1,4-Glc
3a R' = OH
3b R' =

Figure 1. The inhibitors of EGC used in this study.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Crystals of EGC, obtained as described previously,[11] were soaked with 5–15 mM solutions of **1b**, **2b**, and **3b**, and the structures of the EGC–inhibitor complexes were determined to 1.50-Å, 1.80-Å, and 1.85-Å resolution, respectively. Both **1b** and **2b** bind in the −2 and −1 subsites, with the isofagomine (Figure 2a) and glucoimidazole (Figure 2b) moieties located in the catalytic −1 subsite (see the Supporting Information for additional figures). In agreement with previous structural studies on other enzymes,[14] the isofagomine assumes an undistorted $^4C_1$ (chair) conformation, as also observed in the −1 subsite of the trapped lactosyl–enzyme intermediate.[11] The ring nitrogen of the isofagomine superimposes with the anomeric carbon of the intermediate. The lack of distortion towards the "oxocarbenium-ion-like" half-chair may explain the order of magnitude difference in K_i values between **1b** and **2b**, the latter being distinguished by the imidazole-enforced, transition-state-mimicking, half-chair conformation. In addition to these conformational differences, inhibitor **2b** recruits multiple interactions at the 2-hydroxy group (Figure 2b) that are not possible in **1b** (Figure 2a) and which likely contribute to the difference in affinity. Interactions with the 2-hydroxy group have been shown to be particularly important in stabilizing the transition states of a range of retaining glycosidases by up to 45 kJ mol^{-1}.[15]

The iminocyclitol moiety of **3b** binds in the −1 subsite in an envelope conformation (Figure 3). The ring nitrogen superimposes with the anomeric carbon of the lactosyl–enzyme intermediate and the ring nitrogen of the isofagomine. This arrangement places the ring nitrogen of **3b** 3.0 Å from both of the catalytic glutamic acid residues, Glu^{233} and Glu^{351}, consistent with electrostatic interactions with the presumably protonated amine. This positioning potentially mimics charge development at the transition state of retaining β-glycosidases, where the *syn* interaction of the nucleophilic glutamate with the anomeric center and the 2-hydroxy group favors a large share of positive charge at the anomeric carbon.[15] The 2,5-imino-D-mannitol ring is flipped relative to the natural sugar substrate so that the 6-hydroxy group of **3b** mimics the 2-hydroxy groups of both the lactosyl–enzyme intermediate and **2b** (Figure 2d). Although this allows **3b** to capitalize on the important transition-state-stabilizing interactions with the substrate 2-hydroxy group, this orientation precludes any interactions between **3b** and residues Lys^{66},

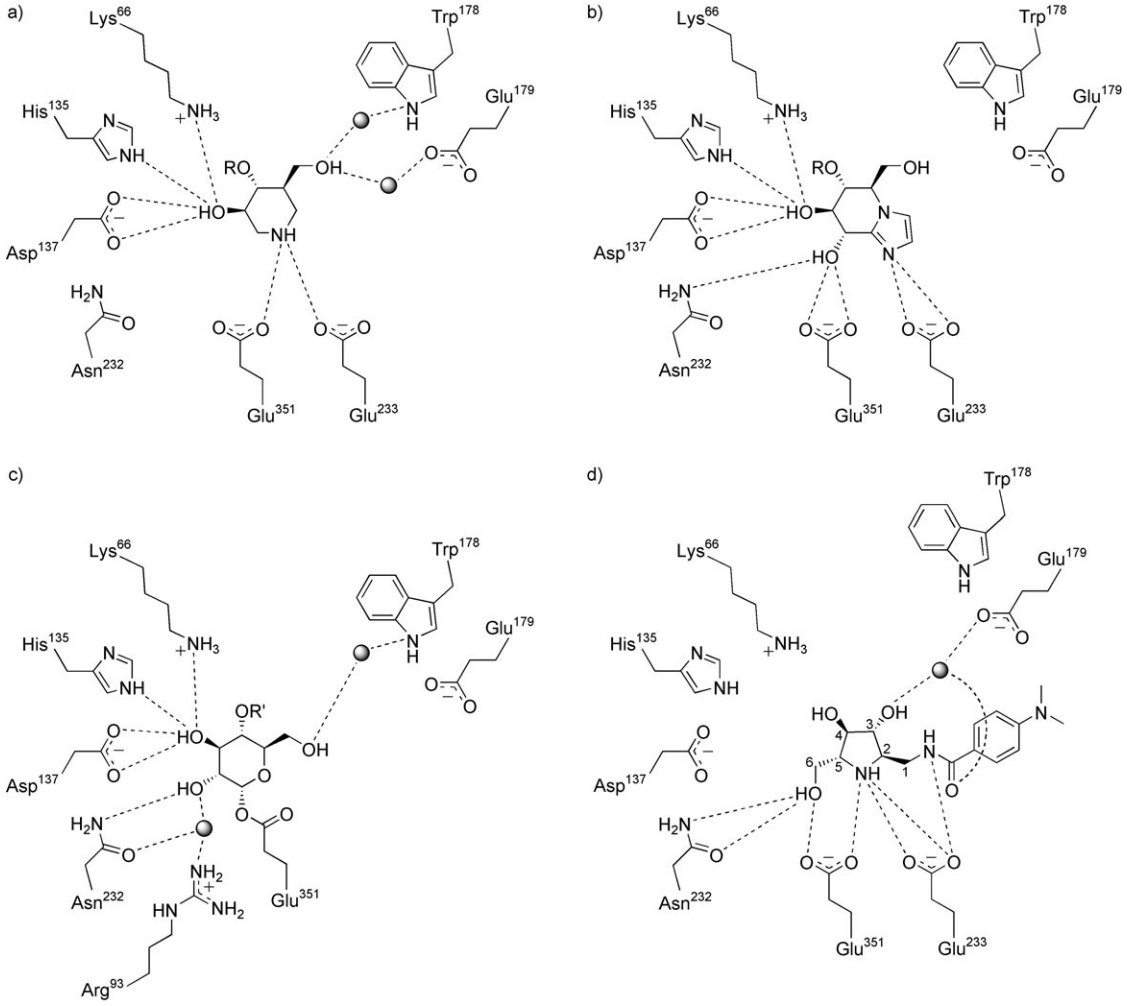

Figure 2. A schematic representation of direct polar close contacts and indirect interactions through water (≤3.2 Å) of: a) **1b**; b) **2b**; c) the lactosyl–enzyme intermediate,[11] which was trapped using the general acid/base mutant (E233A) of EGC; and d) **3b**. Only interactions in the −1 subsite are depicted, using molecule B of the asymmetric unit. Water molecules are represented by gray spheres.

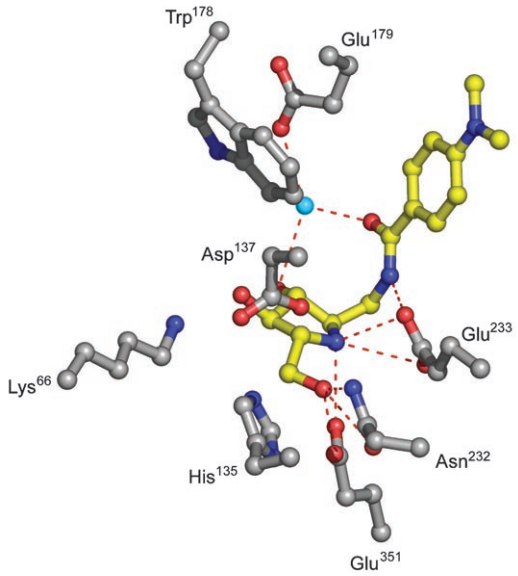

Figure 3. Enzyme–inhibitor interactions displayed by **3b**. The bound inhibitor is shown as a ball-and-stick representation in yellow. A water molecule is represented by a light-blue sphere.

His[135], and Asp[137] (these residues present contacts to the 3-hydroxy group of **1b**, **2b**, and the lactosyl–enzyme intermediate). The amide side chain of **3b** binds to the enzyme through two polar contacts (Figure 2d) and hydrophobic interactions with the lipophilic channel that would normally bind the ganglioside ceramide chains. Although an indirect interaction, by means of water, between Glu[179] and the 3-hydroxy group of **3b** is observed, the minimal contacts between the enzyme and the 3- and 4-hydroxy groups suggest that a cellobiose-like five-membered iminocyclitol, branching from the 3-hydroxy group (analogous to the other cellobiose-like inhibitors discussed here) might be an improved inhibitor of EGC.

In conclusion, we have provided the long-sought structural basis of the potent inhibition of glycosidases by five-membered iminocyclitols, in which imitation of the substrate 2-hydroxy group by the inhibitor 6-hydroxy group, and electrostatic interactions between the ring nitrogen and catalytic glutamic acid residues play key roles. The structural details from this family 5 glycosidase can be extrapolated to all members of the structurally similar glycoside hydrolase clan A, permitting the structure–function relationships already identified from iminocyclitol library screening to be correlated with glycosidase structure. Combined, these studies will hopefully lead to the design of more potent and specific, therapeutically relevant glycosidase inhibitors.

Experimental Section

Recombinant EGC, lacking the 30 amino acid N-terminal signal sequence, was overexpressed in *E. coli* and purified by Ni^{II} affinity chromatography as described previously.[11] Methodology for the kinetic analysis and inhibitor-library screening, the conditions for crystal growth and inhibitor soaking, and the methods for data collection and structure refinement are reported in the Supporting Information. The atomic coordinates and structure factors have been deposited in the Protein Data Bank, http://www.rcsb.org (PDB ID codes 2OYK, 2OYL and 2OYM).

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