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PAPER

Gaining insight into the inhibition of glycoside hydrolase family 20 *exo*-β-*N*-acetylhexosaminidases using a structural approach†.

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One useful methodology that has been used to give insight into how chemically synthesized inhibitors bind to enzymes and the reasons underlying their potency is crystallographic studies of inhibitor–enzyme complexes. Presented here is the X-ray structural analysis of a representative family 20 *exo*-β-*N*-acetylhexosaminidase in complex with various known classes of inhibitor of these types of enzymes, which highlights how different inhibitor classes can inhibit the same enzyme. This study will aid in the future development of inhibitors of not only *exo*-β-*N*-acetylhexosaminidases but also other types of glycoside hydrolases.

Introduction

The glycoside hydrolases are an important group of carbohydrate-processing enzymes found in nature as they have roles in the biosynthesis and degradation of glycoconjugates. A subgroup of these, the *exo*-β-*N*-acetylhexosaminidases (β-HEXs), have been shown to cleave both β-*N*-acetylgalactosamine (β-GalNAc) and β-*N*-acetylglucosamine (β-GlcNAc) linkages in oligosaccharides, glycoproteins and glycolipids. Due to the importance of these enzymes in the modeling of glycoconjugates in cells, various chemical tools and inhibitors have been developed to study them. One methodology that has been used to great effect in this regard is crystallographic studies of inhibitor–enzyme complexes, which have given insight into how chemically synthesized inhibitors bind to these types of enzymes and the reasons underlying their potency.[1–15] An excellent example of this methodology is the extensive work that has been conducted on the glycoside hydrolase family 84,[16–18] the most notable member of this family being the β-*N*-acetylglucosaminidase (OGA) whose function is to cleave β-GlcNAc residues from serine and threonine residues of nucleocytoplasmic proteins.[19] This modification, known more commonly as the '*O*-GlcNAc modification', has been linked to various disease states.[8,20–26] Using crystal structures of known inhibitors bound to bacterial homologues of OGA,[8,11] far more potent and selective inhibitors have been developed for the OGA[8,11] which now potentially have therapeutic benefits.[8]

On the other hand, the same rigorous analysis has not been conducted for the human family 20 β-HEXs, the dimeric isozymes, hexosaminidase A and B (HexA and HexB).[27] These two enzymes share high sequence similarity and are localized in the lysosome, where they hydrolyze β-GalNAc and β-GlcNAc residues of glycosphingolipids such as the ganglioside GM2.[28] Similarly to OGA, HexA and HexB have biological importance, with direct links to the neurodegenerative disorders known as Tay–Sachs and Sandhoff diseases,[29] whilst other effects have been shown to be mediated by the accumulation of GM2.[30,31] Although there have been efforts towards developing therapeutics to treat Tay–Sachs and Sandhoff diseases by using structural complexes of known inhibitors and the associated enzyme as a guide (most notably for pharmacological chaperone therapy[32,33]), a more rigorous structural analysis using known inhibitors of HexA and HexB would be very useful in developing tools to study and potentially treat these disease states.

HexA and HexB and other members of family 20 of the glycoside hydrolases use a two-step catalytic mechanism involving substrate-assisted catalysis to form a transient oxazoline intermediate (Fig. 1) that is broken down to liberate the free sugar hemiacetal, with an overall retention of anomeric configuration.[34–37] This overall catalytic mechanism is common to glycosidases from families 18, 56, 84, 85 and 123, all of which have evolved two carboxyl residues (for α-subunit (HexA), Glu323 and Asp322,[6] for the β-subunit (HexA and HexB) Glu355 and Asp354[2,3]) located within the active site that

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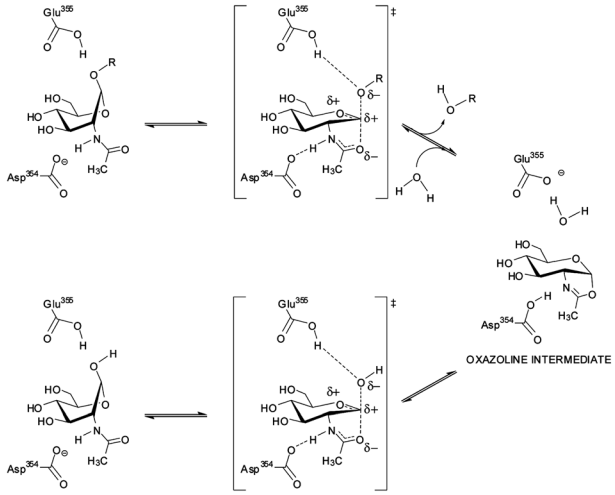

Fig. 1 The catalytic mechanism used by family 20 enzymes involves substrate-assisted catalysis from the 2-acetamido group of the substrate to form a transient oxazoline intermediate. The amino acids depicted are directly involved in catalysis.

perform this key catalytic role.[4–6,15,38–43] Based on the success of previous studies,[44–46] the three general features used in the development of glycosidase inhibitors,[47–50] (compounds that adopt a potential transition state-like conformation, or an sp^2-hybridized centre installed at C-1 of the pyranose ring to mimic the geometric requirements of the putative planar oxocarbenium ion-like transition state or compounds where a nitrogen replaces C-1 or O-5 in the pyranose ring to mimic the relative positive charge development at these centres within the transition state) have also been used in the development of inhibitors of enzymes that use substrate-assisted catalysis.

The best characterised inhibitor of HexA and HexB is NAG-thiazoline. NAG-thiazoline, first prepared by Knapp and co-workers[34] is a highly potent competitive inhibitor of both the family 20 β-HEXs[1,34,51] family 84[52] and family 85 enzymes.[42] The potential reason behind the potency of NAG-thiazoline lies in its geometric resemblance to the transient oxazoline intermediate.[12,53,54] Furthermore, elegant crystallographic studies have provided insight into the potency of NAG-thiazoline toward both HexA[6] and HexB[2,3] as well as bacterial homologues of these enzymes.[40,55] Recently, investigations into the binding mode of another inhibitor of HexA and HexB, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino *N*-phenylcarbamate, commonly known as PUGNAc,[56,57] were conducted on bacterial homologues.[58,59] Unfortunately, the same rigorous crystallographic analysis has not occurred for other known potent inhibitors of HexA and HexB. These molecules include: a molecule related to NAG-thiazoline, termed Gal-NAG-thiazoline,[60] a molecule related to PUGNAc, termed Gal-PUGNAc,[61] and the iminosugars 2-acetamido-2-deoxy-deoxynojirimycin (NHAc-DNJ)[62] and 6-acetamido-6-deoxy-castanospermine (NHAc-CAS)[63] (Fig. 2).

Due to all of these compounds being potent inhibitors of both HexA and HexB, either based on literature precedent or tested here (Table 1), gaining insight into the important binding interactions that these molecules make with these enzymes, or closely related bacterial homologues, could lead to the rational

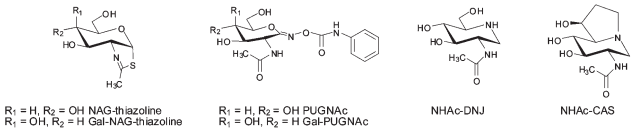

Fig. 2 The compounds used in this study.

Table 1 Inhibition constants of inhibitors of HexA, HexB and Hex1-ΔC

Compound	HexA K_i (μM)	HexB K_i (μM)	Hex1-ΔC K_i (μM)
NAG-thiazoline	0.27[51]	0.07[52]	70 ± 4
Gal-NAG-thiazoline	0.82[60]	0.32 ± 0.04	71 ± 5
Gal-PUGNAc	0.051[61]	0.018[61]	0.003 ± 0.002
PUGNAc	0.048 ± 0.006	0.036[52]	0.014 ± 0.002
NHAc-DNJ	0.067 ± 0.007	0.003[62]	28 ± 4
NHAc-CAS	0.38 ± 0.01	0.25[13]	20 ± 3

development of more potent inhibitors based on these structural scaffolds. Indeed, this has occurred for NHAc-DNJ, through the use of molecular docking, which has resulted in more potent and selective inhibitors for family 20 enzymes.[64]

Previously, we reported the molecular cloning and characterization of two novel β-HEXs, Hex1 and Hex2, from *Paenibacillus* sp. TS12, which are members of family 20 of the glycoside hydrolases, and solved the crystal structure of a C-terminal-truncated TS12 β-HEX (Hex1-ΔC; position 1–502 amino acid), at 1.8 Å resolution, as well as in complex with β-GlcNAc and β-GalNAc.[65] *Paenibacillus* sp. TS12 β-HEX1 is the first enzyme found capable of hydrolyzing glycosphingolipids in prokaryotes.[65] Structurally, Hex1-ΔC consists of two domains, an N-terminal domain which consists of two long α-helices and seven β-sheets, and a central catalytic $(\beta/\alpha)_8$-barrel domain. In the $(\beta/\alpha)_8$-barrel is located the substrate-binding pocket with the active site having amino acid conservation *versus* HexA and HexB (see ESI Fig. 1†) and is therefore a potentially suitable representative for these enzymes. Here, we report the crystal structures of Hex1-ΔC complexed with six known potent inhibitors of HexA and HexB, covering the three general features of inhibitor design, which gives the first rigorous structural insight into these inhibitors' binding mode and potential reasons for their potency, using one representative enzyme of family 20 β-HEXs.

Results and discussion

First, we examined whether the six compounds in question were inhibitors of Hex1-ΔC. We found in all cases that the compounds were potent competitive inhibitors of Hex1-ΔC (Table 1 and ESI Fig. 2†) and were in good agreement with what has been determined for these compounds against not only HexA and HexB but also for other bacterial homologues that have been used for comparison to the human enzymes.[1,60]

To elucidate the inhibitor recognition of Hex1-ΔC, we determined the crystal structure of the Hex1-ΔC complexed with the six compounds at high resolution of 1.6–1.9 Å (refinement statistics in ESI Table 1†) to reveal an unambiguous density for each of the six compounds within −1 subsite of the active site (Fig. 3A–F). The root-mean-square deviation values between the known β-GlcNAc-bound form[65] and the six inhibitor-bound

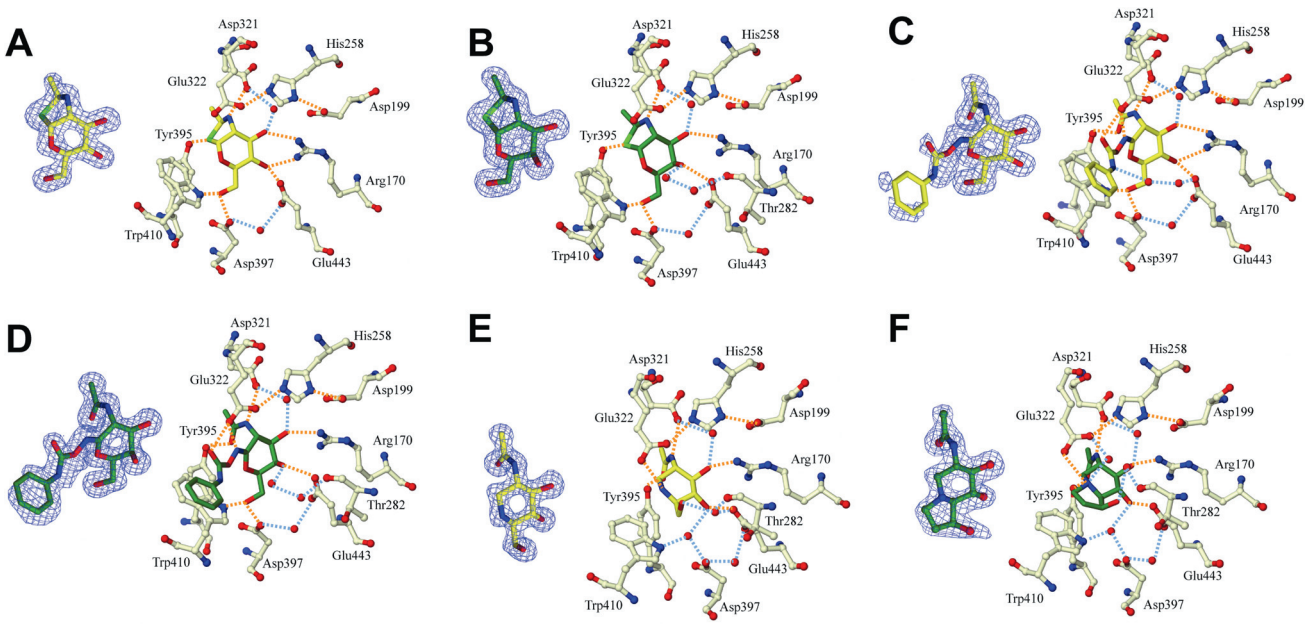

Fig. 3 The recognition of the inhibitors by Hex1-ΔC. *Fo–Fc* omit maps for (A) NAG-thiazoline, (B) Gal-NAG-thiazoline (C) PUGNAc (D) Gal-PUGNAc (E) NHAc-DNJ and (F) NHAc-CAS all at the 4.0 σ contour level. The hydrogen bonds recognizing the inhibitors are indicated by broken orange lines. The water molecules around the inhibitors are shown by red balls and the hydrogen bonds formed with water molecules are indicated by broken light blue lines.

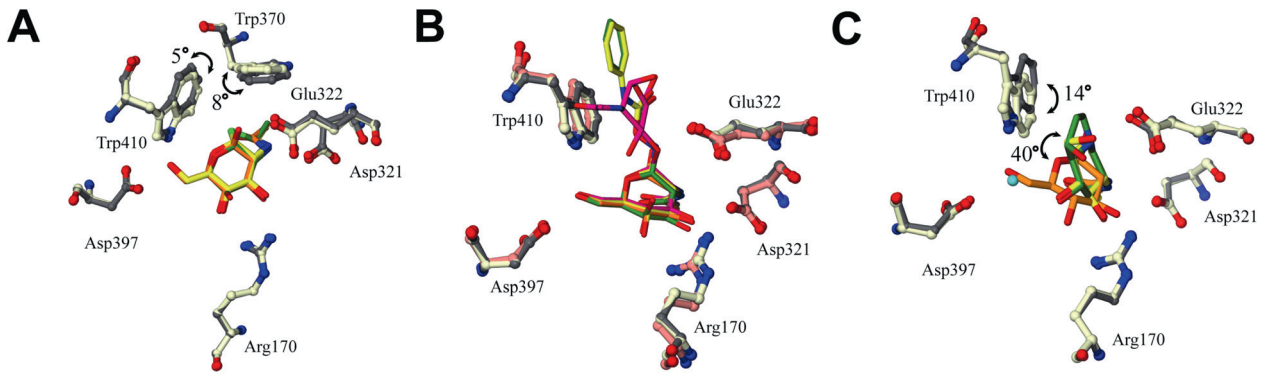

Fig. 4 (A) Superimposed β-GlcNAc (orange and grey), NAG-thiazoline (yellow and white), and Gal-NAG-thiazoline (green) in the catalytic site of Hex1-ΔC. (B) Superimposed β-GlcNAc (orange and grey), PUGNAc (yellow and white), Gal-PUGNAc (green) and chitobiose (magenta and pink). (C) Superposed β-GlcNAc (orange and grey), NHAc-DNJ (yellow and white), NHAc-CAS (green). Water molecules are depicted by light blue balls.

forms were 0.12–0.14 Å for 504 C_α atoms, indicating the seven forms share very similar overall structures. The arrangement of the active site residues is almost identical, with the most important being the three Trp residues (Trp352, Trp370 and Trp441), which are thought to be important for positioning of the 2-acetamido group of the substrate in the active site (see ESI Fig. 3†).[2,3,6,39,40] The other amino acid residues (Arg170, Asp321, Glu322, Tyr395, Asp397 and Glu443), which form the active site, also directly interact with the six inhibitors.

The pyranose rings of NAG-thiazoline and Gal-NAG-thiazoline, adopt a $^4C_1$ conformation which is similar to what is observed when β-GlcNAc is complexed with Hex1-ΔC (PDB ID 3GH5)[65] and is also consistent with what has been observed for other members of family 20 and other glycoside hydrolase families that use substrate-assisted catalysis, complexed with these compounds.[2,4,40,42,55] An overlay of the NAG-thiazoline·Hex1-ΔC and Gal-NAG-thiazoline·Hex1-ΔC structures, against the known β-GlcNAc parent structure (Fig. 4A) reveals the pyranose rings of both compounds are in good agreement with the conformation of the β-GlcNAc with all critical amino acid interactions remaining in place. Minor conformational changes were observed at Trp370 and Trp410. The side chain of Trp370 and Trp410 was rotated by 8° and 5°, respectively, in the inhibitor-bound forms compared to the β-GlcNAc-bound form. Tyr395 also makes a hydrogen bond with the sulfur atom of the thiazoline ring instead of the O7 atom of the 2-acetamido group of β-GlcNAc (Fig. 3A and B). Glu322, which is the general acid/base residue of Hex1-ΔC, makes no hydrogen bond with NAG- or Gal-NAG-thiazoline which is consistent with what is observed for other family 20 enzymes.

For the binding modes of PUGNAc and Gal-PUGNAc on the other hand, the pyranose ring of these compounds appear to be in a ^4E conformation, similar to what is observed for the binding of PUGNAc with family 20[58,59] and 84 β-*N*-acetylhexosaminidases[5,9] and this conformation is close to what is believed to be part of the conformational itinerary of the pyranose ring in the putative transition state for enzymes that use substrate-assisted catalysis.[12,43,53,54] In the PUGNAc·Hex1-ΔC and Gal-PUGNAc·Hex1-ΔC structure, the electron density of the phenyl moiety of PUGNAc is slightly weaker than that of Gal-PUGNAc (Fig. 3C and D) and this was attributed to slight structural disorder in the crystal lattice. PUGNAc and Gal-PUGNAc bind with the *N*-acetyl group nestled in the acyl binding pocket that is lined by tryptophan residues. The extended phenyl *N*-carbamate moiety in PUGNAc and Gal-PUGNAc is located in a similar orientation to that of the +1 subsite that is observed in the *Serratia marcescens* chitobiose·chitobiase structure[39] and a recent structure of PUGNAc complexed with a mutant of *Of*Hex1.[58] Furthermore, the moiety is also involved in a stacking-type interaction with Trp410 (Fig. 4B). In contrast to the thiazolines, which only interact with residues found directly within the active site, the binding of PUGNAc/Gal-PUGNAc is enhanced by adventitious interactions of its extended *N*-phenylcarbamate moiety. This gives a direct insight into why these two compounds exhibit a more potent inhibition profile compared to these inhibitors. In addition to the interaction of the phenyl *N*-carbamate moiety with Trp410, the carboxyl group of Glu322 makes two hydrogen bonds with the oxime nitrogen N1 and carbonyl oxygen O9 atom of the phenyl *N*-carbamate moiety instead of the O1 atom of β-GlcNAc (Fig. 3C and D). These interactions likely contribute significantly to the binding of PUGNAc and Gal-PUGNAc as LOGNAc, an analogue of PUGNAc lacking the phenyl carbamoyl moiety, is a poorer inhibitor of family 20 β-HEXs.[66] The nitrogen N3 atom of the phenyl *N*-carbamate moiety of PUGNAc makes a water-mediated hydrogen bonding interaction with the Glu443 (Fig. 3C). In Gal-PUGNAc, the O4 atom makes a water-mediated hydrogen bond with the Thr282 (Fig. 3D).

Overall, the potent inhibition by PUGNAc and Gal-PUGNAc, therefore, stems from two key features. First, it has a trigonal sp^2 center at the pseudoanomeric position (C1) that enhances binding through partial mimicry of the transition states of *exo*-β-*N*-acetylhexosaminidases. PUGNAc and Gal-PUGNAc also gain significant binding energy through adventitious interactions between enzyme and the pendant *N*-phenyl carbamate group.

The iminosugars NHAc-DNJ and NHAc-CAS both adopt a $^4C_1$ conformation when bound, which is similar to what was observed when β-GlcNAc is complexed with Hex1-ΔC (PDB ID 3GH5)[65] but, quite strikingly, is not consistent with what has been observed for family 84 glycoside hydrolases, which use substrate-assisted catalysis, complexed with these compounds.[13] In the co-crystal structure of NHAc-CAS with *Bt*GH84, a bacterial homologue of OGA, the pyranose ring of the iminosugar adopts a $^{1,4}B/^4E$ conformation, which is similar to what was observed when castanospermine (a compound structurally similar to NHAc-CAS) is bound to the family 1 β-glucosidase from *Thermotoga maritima*.[67] With regards to the important 2-acetamido moiety needed for substrate-assisted catalysis, the structures for both NHAc-DNJ and NHAc-CAS show the group is located in a similar position to that of the β-GlcNAc 2-acetamido moiety which is nestled in with the three Trp residues (Trp 352, Trp370 and Trp441) forming interactions with Tyr395 and Asp321. Despite the similarity in the positioning of the 2-acetamido moiety, the piperidine ring of NHAc-DNJ and NHAc-CAS in both these structures is rotated by 40° as compared to the pyranose ring of β-GlcNAc, and the 2-acetamido carbonyl oxygen does not make any significant hydrogen bonding interaction with the amine moiety which is in stark contrast to what is observed in the *Bt*GH84·NHAc-CAS structure where the 2-acetamido carbonyl oxygen is suitably positioned below the amine moiety of castanospermine to make an important hydrogen bonding interaction.[13]

When comparing the NHAc-DNJ and NHAc-CAS structures with the β-GlcNAc structure (Fig. 4C), the amine nitrogen of both inhibitors is found in the position corresponding to exocyclic oxygen (O1) of β-GlcNAc. This drastic change presumably may be in an effort to make a hydrogen bond with general acid–base residue, Glu322. As a result of this movement, the position of the O6 atom of the NHAc-DNJ and NHAc-CAS is quite different to that seen with β-GlcNAc, with this atom making a water-mediated hydrogen bond with the Thr282. In terms of changes to the enzyme active site to compensate for the changes in the position of the inhibitor, the indole side chain of Trp410 is rotated by 14° in the NHAc-DNJ- and NHAc-CAS-bound forms (Fig. 4C). This then allows the five-membered ring of NHAc-CAS to make a stacking interaction with Trp410. Additionally, presumably in an effort to compensate for these structural changes, a water molecule (indicated by a light blue ball) is found in the same position as the O6 atom of β-GlcNAc which allows Asp397 and Trp410 to make hydrogen bonding interactions with the O4 of NHAc-DNJ and NHAc-CAS.

Comparison of the known family 84 NHAc-CAS·*Bt*GH84 structure with the NHAc-CAS·Hex1-ΔC structure reveals that the amine nitrogen is farther away from the general acid–base residue than that found for family 20.[13] Indeed, in case of family 84, the amine nitrogen of NHAc-CAS is located in the same position as that of the ring oxygen of NAG-thiazoline and is not positioned within hydrogen bonding distance to any active site residues. On the other hand, in the family 20 structure, the amine nitrogen of NHAc-CAS is in close proximity to the general acid–base residue, potentially allowing a critical hydrogen bond to be made.

Based on these observations we thought we would compare our results with the structural complex of a related nitrogen containing heterocycle GalNAc-isofagomine, which is an inhibitor of *Streptomyces plicatus* SpHEX, a bacterial homologue of HexA and HexB.[1] In both the NHAc-DNJ and NHAc-CAS structures, the position of the pyranose ring is quite different from the GalNAc-isofagomine–SpHEX complex (PDB ID, 1JAK).[1] In the GalNAc-isofagomine–SpHEX complex the piperidine ring of GalNAc-isofagomine is in an identical position when compared to the analogous β-GlcNAc (PDB ID, 1M01) and NAG-thiazoline (PDB ID, 1HP5) structures.[1,40] The ring nitrogen atom of GalNAc-isofagomine makes a hydrogen bonding interaction with the general acid–base residue of *Sp*HEX (Glu314)[1] and in the case of this molecule the nitrogen atom is located at the position corresponding to C1 atom of β-GlcNAc and the location of Glu314 and the C1 atom of

β-GlcNAc is close enough to form a hydrogen bond abrogating the need for the piperidine ring to bind in a different orientation. Overall, it seems for piperidine-based inhibitors of family 20 β-HEXs, that binding of the compound in the active site so that the ring nitrogen is able to hydrogen bond with the general acid–base residue may be critical to the overall potency of these molecules.

Conclusions

In conclusion, we have successfully crystallized Hex1-ΔC in complex with six known inhibitors of HexA and HexB. These structures reveal novel binding modes for the inhibitors and give structural insight into the reasons behind their potency. The binding modes of NHAc-DNJ and NHAc-CAS are of interest as they demonstrate that for Hex1-ΔC, and potentially for family 20 β-HEXs in general, that imino- and aza sugars potentially change conformation in a way to make a hydrogen bond interaction with the general acid–base residue. This observation has been observed with the parent compounds of NHAc-DNJ and NHAc-CAS, with β-glucosidases, 1-deoxynojirimycin and castanospermine respectively.[67,68] This study also highlights the importance of designing inhibitors that take advantage of the three general features used in the development of glycosidase inhibitors and that using either one or a combination of these features could result in highly potent compounds. Using these X-ray structures and by optimization of the synthetic routes to analogues of the compounds presented, more potent and highly selective inhibitors of HexA and HexB as well as other family 20 β-HEXs will be able to be realized, which could also translate to new inhibitors of glycoside hydrolases in general.

Experimental

Enzymatic assays

Activity against β-hexosaminidase A and B. All assays were carried out in triplicate at 37 °C for 30 minutes by using a stopped assay procedure in which the enzymatic reactions (50 μl) were quenched by the addition of a 4-fold excess (200 μl) of quenching buffer (200 mM glycine, pH 10.75). Assays were initiated by the careful addition, *via* pipette, of enzyme (5 μl), and in all cases the final pH of the resulting quenched solution was greater than 10. Time-dependent assay of β-hexosaminidases revealed that the enzymes were stable in the buffer over the period of the assay: 50 mM citrate, 50 mM NaCl, 0.1% BSA, pH 4.25. The progress of the reaction at the end of 30 minutes was determined by measuring the extent of 4-nitrophenol liberated as determined by UV measurements at 400 nm using a 96-well plate (Sarstedt) and 96-well plate reader (Molecular Devices/BMG Labtech). Hexosaminidase A from porcine placenta and hexosaminidase B from human placenta (Sigma) were used in the assays at a concentration (μg μl^{-1}) of 0.0004 and 0.0006, respectively, using the substrate 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside at a concentration of 0.5 mM (K_m of both enzymes ≈0.5 mM). The compounds were tested at six concentrations with K_i values determined by linear regression of data from Dixon plot.[69]

Activity against Hex1-ΔC. The activity of Hex1-ΔC was measured using pNP-β-GlcNAc as a substrate (K_m 0.117 mM[65]). The reaction mixture contained 100 nmol of pNP-β-GlcNAc and an appropriate amount of the enzyme in 100 μl of 25 mM sodium acetate buffer, pH 6.0. Following incubation at 37 °C for a specified period, the reaction was stopped by adding 100 μl of 1 M NaOH, and absorbance was measured at 405 nm. One unit of the enzyme was defined as the amount which catalyzes the release of 1 μmol of *p*-nitrophenol per min from pNP-β-GlcNAc under the conditions used. K_i values of Hex1-ΔC for inhibitors were determined from Dixon plots.[69]

Data collection and structure determination

Purification, crystallization and preparation of the protein crystals for data collection were described previously.[65] The apo crystals of Hex1-ΔC were soaked in a solution (2.5 mM) of the inhibitor for 1 h. Before measurement, the crystals were soaked in a cryo-protection solution containing 15% glycerol and the inhibitor (2.5 mM). The data sets were collected on the BL26B2 beam line (SPring-8). Phasing of the Hex1-ΔC structures in a complex with various inhibitors was carried out by molecular replacement using MOLREP and the coordinates of the apo enzyme (ligand free form, Protein Data Bank ID, 3GH4) as a search model. The atomic model was built manually using CueMol (http://www.cuemol.org/en/) and Coot.[70] The refinement was carried out using CNS[71] and Refmac.[72] After the ligands were built, further rounds of refinement were performed. Ramachandran plots analyzed using the program PROCHECK[73] showed that all of the residues in the six structures were in the most favored or additionally allowed regions. The data collection and refinement statistics are summarized in ESI Table 1†.

Data bank accession codes

The atomic coordinates and structure factors of the Hex1-ΔC in complex with the six inhibitors have been deposited in the Protein Data Bank (http://www.rcsb.org) under accession codes 3SUR, 3SUS, 3SUT, 3SUU, 3SUV and 3SUW.

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