Iminosugar-C-glycosides work as pharmacological chaperones of NAGLU, a glycosidase involved in MPS IIIB rare disease

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Abstract: Mucopolysaccharidosis type IIIB is a devastating neurological disease caused by a lack of the lysosomal enzyme, α-*N*-acetylglucosaminidase (NAGLU), leading to a toxic accumulation of heparan sulfate. Herein we explored a pharmacological chaperone approach to enhance the residual activity of NAGLU in patient fibroblasts. Capitalizing on the three-dimensional structures of two modest homoiminosugar-based NAGLU inhibitors in complex with bacterial homolog of NAGLU, CpGH89, we have synthesized a library of 17 six-membered iminosugar-*C*-glycosides mimicking *N*-acetyl-D-glucosamine and bearing various pseudo-anomeric substituents of both α- and β-configuration. Elaboration with the aglycon moiety results in low micromolar selective inhibitors of human recombinant NAGLU, but surprisingly it is the non-functionalized and wrongly configured β-homoiminosugar that was proved to act as the most promising pharmacological chaperone, promoting a 2.4 fold activity enhancement of mutant NAGLU at its optimal concentration.

Introduction

Mucopolysaccharidosis (MPS) is one group of autosomal recessive lysosomal storage disorders (LSD) caused by the accumulation of undegraded glycosaminoglycans in lysosomes. MPS III, or Sanfilippo syndrome, is characterized by the impaired function of one of four lysosomal enzymes involved in the sequential degradation of heparan sulfate.[1] MPS IIIA is caused by the reduced activity of heparan sulfate sulfamidase, MPS IIIB is caused by the reduced activity of an α-*N*-acetylglucosaminidase (NAGLU), MPS IIIC is caused by the reduced activity of acetyl-CoA: α-glucosaminide-*N*-acetyltransferase, and MPSIIID is caused by the reduced activity of *N*-acetyl-D-glucosamine 6-sulfatase.[2][3] In MPS IIIB, the resulting deficiency of NAGLU enzyme that cleaves *N*-acetyl-D-glucosamine from glycosaminoglycans (Figure 1a) causes lysosomal accumulation of heparan sulfate to a toxic level. It is a devastating and currently untreatable disease[4] that in the developed world concerns about 1000-2000 patients who generally only survive into early adulthood.

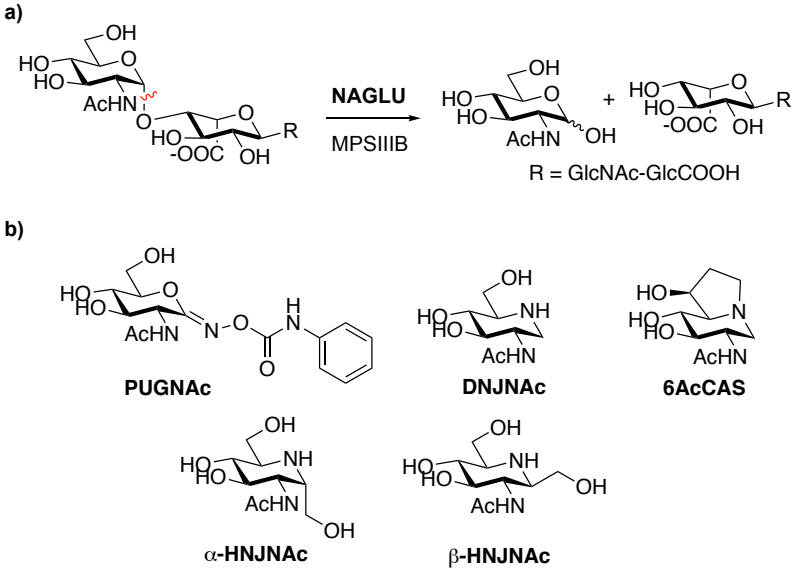

Figure 1. a) Enzymatic reaction performed by NAGLU; b) known inhibitors of NAGLU and scaffolds used in this study

At present, there are three established therapeutic strategies for LSDs: enzyme replacement therapy (ERT) consisting in the periodic infusion of recombinant enzymes,[5] substrate reduction therapy (SRT) based on the inhibition of the enzyme catalyzing the biosynthesis of the burden metabolite[6] and pharmacological chaperone therapy (PCT).[7a-c] To date, only a single LSD, Fabry disease, clinically benefits from this PCT strategy exploiting an iminosugar, 1-deoxygalactonijirimycin (DGJ, Galafold™, migalastat).[7d] Regarding MPSIIIB, impediments to ERT are the blood brain barrier and the absence of mannose 6-phosphate,[8] the signal for receptor-mediated endocytosis and targeting to lysosome,[9] on recombinant human NAGLU. These were recently overcome by delivering an enzyme-IGFII fusion protein of therapeutic potential or intra-cerebroventricularly to the brain.[10][11] An SRT approach through genistein supplementation, a molecule known to inhibit GAG synthesis in fibroblasts of patients, was also examined but failed to improve the patients disability.[12] Efforts have also been dedicated to the PCT approach that is based on the use of small molecules that bind and stabilize the correct conformation of mutant enzymes, thus facilitating their maturation and trafficking to the lysosome, when they would otherwise be retained in the endoplasmic reticulum by quality control mechanisms, a restoration of only 10-30% of normal catalytic activity being sufficient to prevent pathological manifestation.[13] A library of 1302 compounds was screened for their ability to enhance the activity of NAGLU in patient fibroblasts, but all failed,[14] leaving this disease essentially with no leads for a potential treatment.

Results and discussion

The family 89 glycoside hydrolase from *Clostridium perfringens* (CpGH89) is a bacterial homolog of human NAGLU that was used to model NAGLU and identify potent inhibitors of CpGH89, namely PUGNAc, DNJNAc and 6AcCAS (Figure 1b), three known N-acetylglucosaminidase inhibitors.[15] Indeed, the recent determination of the human NAGLU structure revealed the typical site of competitive inhibitor binding, the -1 sub-site, of CpGH89 and NAGLU to be nearly identical.[16] The structures of CpGH89 with these inhibitors suggested these small molecules as potential candidates for use as pharmacological chaperones. However, the use of these molecules should be impaired by their broad human hexosaminidase inhibition spectrum. This lack of selectivity can be attributed to the stereo undefined (PUGNAc)[17] or lack (DNJNAc,[18] 6AcCAS[19]) of anomeric substituent which makes these molecules also potent β-*N*-acetylglucosaminidases inhibitors.[20] Our groups[21][22] and colleagues[23] have recently reported the synthesis of α-HNJNAc and β-HNJNAc, two homoiminosugars derived from GlcNAc that constitute the first GlcNAc iminosugars mimics displaying a stereodefined and chemically stable pseudoanomeric substituent (Figure 1b). Here we report the glycosidase inhibition profile and crystal structure of α-HNJNAc and β-HNJNAc in complex with the bacterial homolog of human NAGLU. We capitalized on these data to synthesize a library of potential NAGLU inhibitors amongst which we identified several selective inhibitors. We then tested the whole family for chaperone

activity and proved that some of these inhibitors indeed act as pharmacological chaperones, although the most effective compound, that allows trafficking of the enzyme to the lysosome, did not possess the structural features we anticipated.

Structural investigations

Both α-HNJNAc and β-HNJNAc were first tested against a variety of glycosidases. It appears that both compounds inhibit hexosaminidases while not interfering with other glycosidases *in vitro* (Table 1 and SI). As expected for a α-*N*-acetylglucosaminidase, NAGLU is inhibited more efficiently by α-HNJNAc (67 μM) than by the uncorrectly configured β-HNJNAc (374 μM). In view of these modest inhibition values, we decided to design more potent inhibitors assuming that there was a correlation between inhibitory potency and chaperoning ability. To that purpose, the structures of α-HNJNAc and β-HNJNAc in complex with recombinant CpGH89 were solved at 2.13 Å and 2.0 Å resolution respectively (Protein Data Bank – PDB- entries 7MFK and 7MFL) (Figure 2A, 2B, 2D).
While both compounds show a very similar mode of binding, with a strong interaction between endocyclic amine and Glu^{601}, β-HNJNAc makes an additional hydrogen bond via its pseudoanomeric CH_2OH group with Gln^{664}. This corresponds to the interaction observed for DNJNAc between a water molecule and Gln^{664} residue (Fig 2C). In contrast, this CH_2OH group is too far in α-HNJNAc to allow such interaction. However, in β-HNJNAc, the pseudoanomeric methylene group is somewhat too close to the OH of Tyr^{825} leading to a potential steric clash. Given the identity between the -1 sub-site in CpGH89 and NAGLU, these observations likely explain the superior inhibition of NAGLU by α-HNJNAc compared to β-HNJNAc. Altogether, these data demonstrate the potential of this type of scaffold as MPS IIIB pharmacological chaperone. However, these two derivatives present poor NAGLU selectivity, a modest inhibitory activity, and a high hydrophilicity, three drawbacks that might preclude them from being pharmacological chaperone candidates.

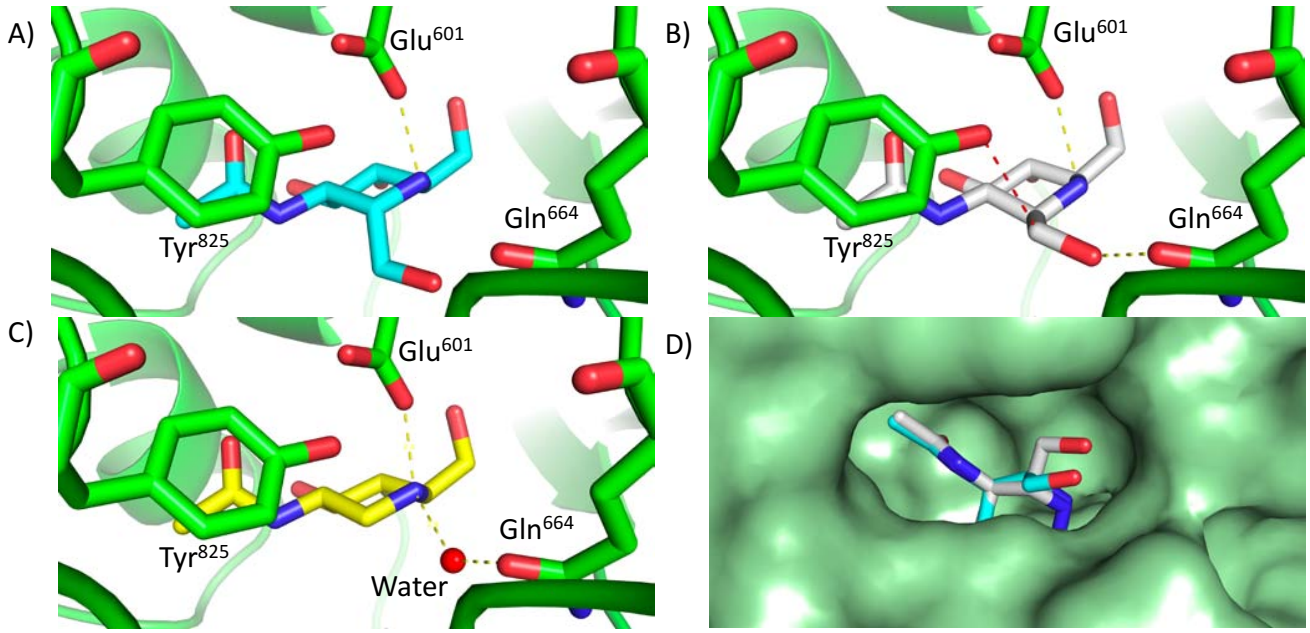

Figure 2. α-HNJNAc (A), β-HNJNAc (B) and DNJNAc (C) in complex with recombinant CpGH89 NAGLU. (D) View of the active site entry with α-HNJNAc (blue) and β-HNJNAc (grey) inside.

Synthesis of α-HNJNAc and β-HNJNAc derivatives

These drawbacks have been classically addressed by adding alkyl chains to the initial scaffold.[24] These alkyl chains have previously been added on the endocyclic nitrogen, the C1 or the C6 positions; however, as shown on figure 2D, only the pseudoanomeric CH_2OH of both anomers sits at the entrance of the rather deep enzymatic pocket. Therefore, introduction of alkyl/aryl groups at the pseudoanomeric position would be a reasonable way to enhance lipophilicity, potency and selectivity of these derivatives. To this end, *O*-alkyl derivatives **1a-c** of α-HNJNAc were prepared from known iminosugar-based azidoalcohol **3a**[20] via its *O*-alkylation to afford compounds **4a-c** followed by azide to acetamide conversion to yield derivatives **5a-c**, which upon hydrogenolysis, furnished the target iminosugars **1a-c** (Figure 3). Butyl and octyl chains were chosen as they are classically introduced in iminosugar *C*-glycosides to improve their potency.[24] The choice of the butylphenyl chain was dictated to mimic to some extend the phenyl carbamate moiety found in PUGNAc.[17] To further evaluate the importance of the pseudoanomeric oxygen for NAGLU binding, the corresponding imino-*C*-pentylphenyl glycoside **1d** was synthesized from azidoalcohol **3a** through primary alcohol oxidation and Wittig olefination to produce alkenes **6a** and **6a'** as a mixture of stereoisomers. Conversion of the azide moiety into the acetamide **7a** followed by hydrogenolysis yielded iminosugar *C*-

glycoside **1d** (Figure 3). Finally, in order to maintain the important pseudoanomeric OH group regarding water molecule binding observed with DNJNAc, while concomitantly introducing hydrophobic aglycon residues including butyl, octyl, propylphenyl and butylphenyl chains, the branched derivatives **1e-h** were synthesized as follows. Alcohol oxidation in **3a** followed by Grignard addition and conversion of the N_3 group into a NHAc function furnished esters **8a-d** as single stereoisomers (Figure 3). Their deacetylation yielded the corresponding alcohols **9a-d** that were uneventfully deprotected by hydrogenolysis to give iminosugars *C*-glycosides **1e-h**. The stereochemistry of the new pseudoanomeric stereocentre was proved to be *R* according to extensive NMR analysis of compound **1e** (see SI). As similar $J_{1,7}$ and $J_{1,2}$ coupling constants (see SI) were recorded for compounds **1e-h**, a *R* configuration was also assigned for the stereocentre at C-7 for all these molecules.

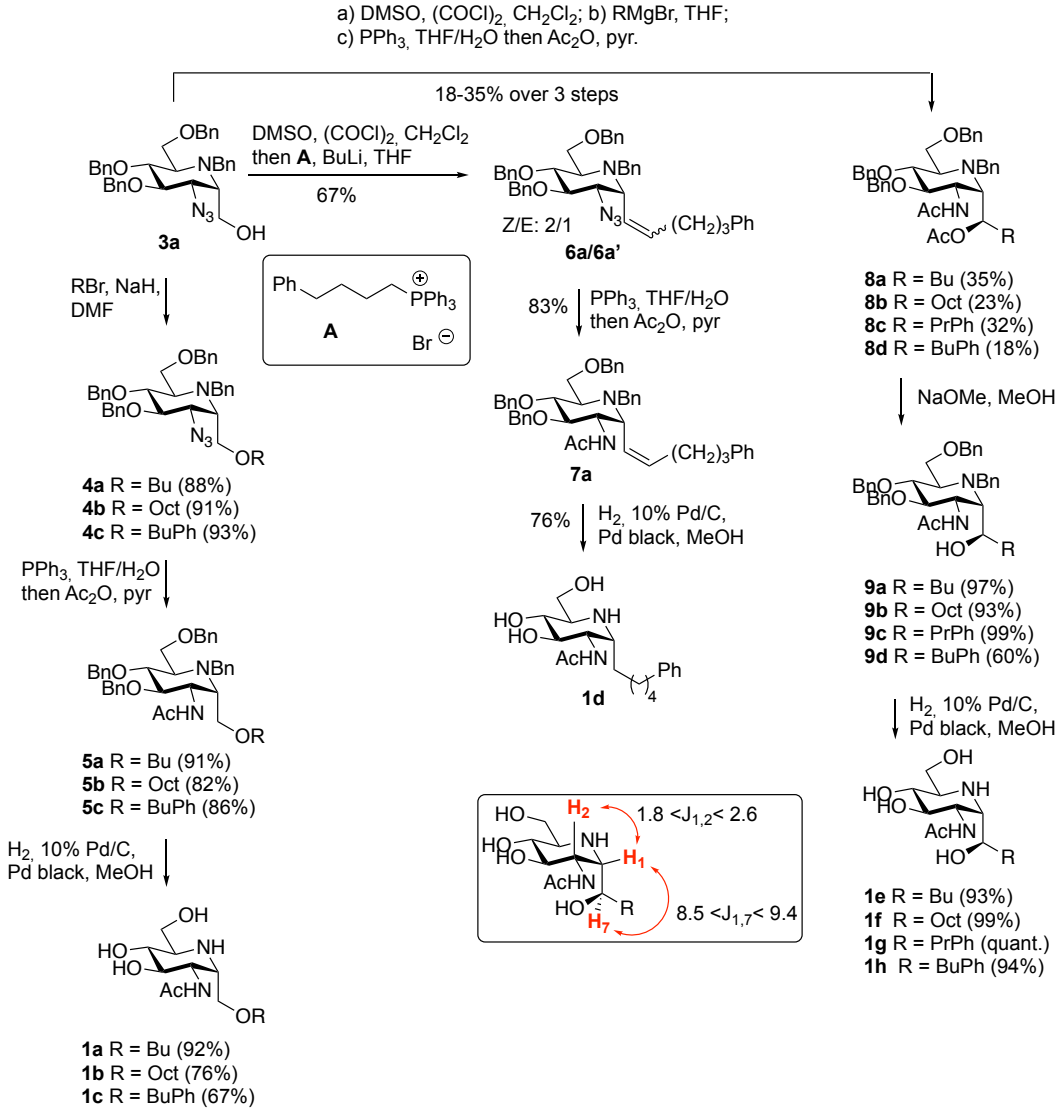

Figure 3. Synthesis of α-configured iminosugar *C*-glycosides **1a-h** from azidoalcohol precursor **3a** and structural assignment.

The synthesis of the β-configured counterparts was achieved in parallel using the same protocols applied to known azidoalcohol **3b**[21] yielding the *O*-alkyl derivatives **2a-b**, the *C*-alkyl derivative **2d** and the branched iminosugars **2e-h** (Figure 4a). For these latter, an X-ray structure was solved for compound **2g** in agreement with a *S* configuration for the stereogenic centre at C-7 (Figure 4b). A 7*S* configuration was also deduced for compounds **2e-f** and **2h** according to their very similar $J_{1,7}$ and $J_{1,2}$ coupling constants with the ones observed for compound **2g** (Figure 4c).

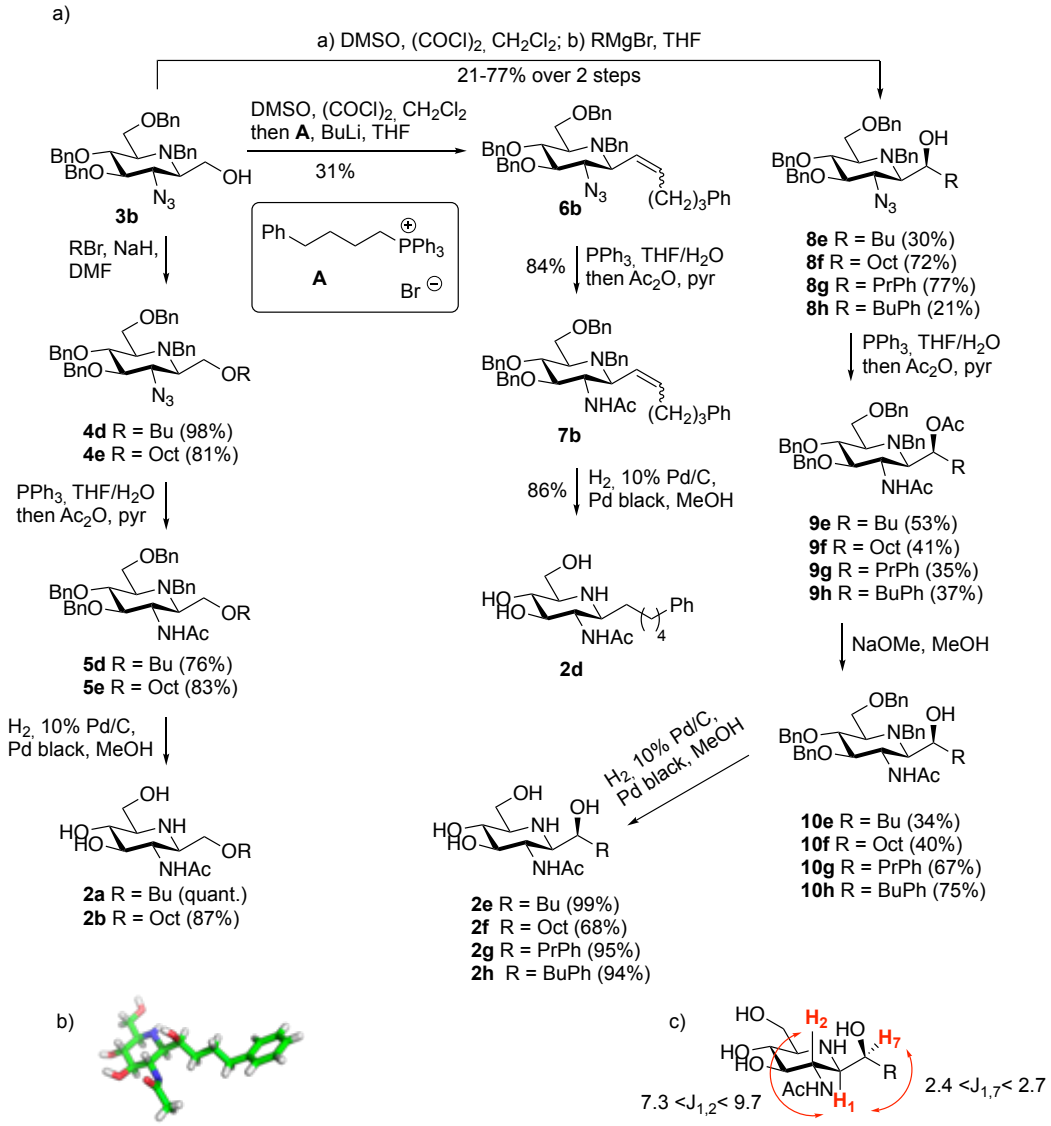

Figure 4. a) Synthesis of β-configured iminosugar *C*-glycosides **2a-h** from azidoalcohol precursor **3b;** b) X-ray structure of compound **2g**; c) key coupling constants to assign the 7S stereochemistry of compounds **2a-h.**

Biological investigations

All new compounds were evaluated as inhibitors of human recombinant α-*N*-acetylglucosaminidase, as well as inhibitors of β-*N*-acetylglucosaminidase (Jack bean, HL60 and Bovine kidney) to determine their potency and selectivity. We also evaluated whether these compounds were endowed with pharmacological chaperone activity in cells from patients of MPSIIIB. To this end, the residual NAGLU activity in GM01426 fibroblasts cultured for three days in the presence of inhibitors was measured after lysis using p-nitrophenyl-α-D-GlcNAc fluorogenic substrate.[25] All results are listed in Table 1. DNJNAc, α-HNJNAc and β-HNJNAc were also included in the assays for comparison. The following observations were made: 1) The pseudoanomeric configuration of these iminosugar *C*-glycosides is important for α-*N*-acetylglucosaminidase recognition as almost all α-configured iminosugars proved more potent than their β-counterparts. Nevertheless, two β-configured iminosugars, the *O*-butyl **2a** (IC_{50} 19.9 μM) and *O*-octyl **2b** (IC_{50} 34.4 μM) derivatives exhibit significant human recombinant NAGLU inhibition. 2) Replacement of the pseudoanomeric CH_2O linkage by a CH_2CH_2 linkage is detrimental to NAGLU inhibition as the α–*C*-pentylphenyl iminosugar **1d** (IC_{50} 37.5 μM) is approximately ten times less potent than its *O*-analog **1c** (IC_{50} 2.6 μM). 3) For the α-configured derivatives, *O*-alkyl derivatives **1a-c** proved superior to branched derivatives **1e-h** demonstrating that a free OH group on the aglycon moiety is not compulsory for strong NAGLU inhibition. 4) Regarding the nature of the aglycon moiety, the butylphenyl group furnished the best NAGLU inhibitors either in linear **1c** (IC_{50} 2.6 μM) or in branched **1h** (IC_{50} 8.6 μM). More importantly, while such degree of inhibition is also attained by DNJNAc (IC_{50} 4 μM), compounds **1c** and **1h** are much more selective for NAGLU, which opens the door to further investigation. 5) Introduction of an aglycon moiety in the β-configured iminosugar *C*-glycosides **2a-h** is not well tolerated by β-*N*-acetyl glucosaminidases from Jack beans, HL60 and

Bovine kidney as all these compounds are weak inhibitors unlike DNJNAc that is devoid of aglycon moiety. To further gain insight into the inhibition developed by these iminosugars, the Ki of α-HNJNAc, β-HNJNAc and compounds **1a-c** toward human recombinant NAGLU were measured and the mode of inhibition determined. All five iminosugar *C*-glycosides act as competitive inhibitors (see Table 1 and SI). Satisfyingly, some of these derivatives demonstrated a pharmacological chaperone effect on GM01426 patient fibroblasts, but, much to our surprise, the highest level of enzyme activity enhancement was not reached by the best NAGLU inhibitors. Thus, at 100 μM concentration, a similar 1.5-1.6 fold enzyme activity enhancement was obtained with α-configured derivatives *O*-octyl **1b** and *O*-butylphenyl **1c** and also with the β-configured *O*-butyl **2a** and *O*-octyl **2b** compounds. Counter-intuitively, the best effect was obtained with wrongly configured β-HNJNAc, a poor NAGLU competitive inhibitor, with a 2.1 fold increase of NAGLU residual activity at 10 μM concentration, while α-HNJNAc had no effect at 100 μM (Table 1), emphasizing the difficult prediction of a chaperone effect only based on inhibitory data.[26] Importantly, there have been some cases reported in the literature where wrongly configured glycomimetics including L-configured derivatives, targeting D-glycosidases, proved to be potent pharmacological chaperones.[27]

Compound	Structure of the aglycon	α-N-acetyl glucosaminidase human recombinant	β-N-acetyl glucosaminidase Jack beans	β-N-acetyl glucosaminidase HL60	β-N-acetyl glucosaminidase Bovine kidney	Inhibitory Selectivity[b] IC_{50} β-Glu / IC_{50} NAGLU	Enzyme activity fold enhancement at 100 μM[a]
DNJNAc	H	4	3	5.8	14.7	2	1.4
α–HNJNAc	α-CH_2OH	67 (11.4) [d]	58	48	96	1	1.1
β-HNJNAc	β-CH_2OH	374 (130) [d]	23	42	ND	0,1	2.1[c]
1a	α-CH_2OButyl	4.8 (1.7) [d]	331	307	444	75	1.1
1b	α-CH_2OOctyl	6 (2.2) [d]	359	362	NI	60	1.5
1c	α-CH_2OButylPh	2.6 (0.4) [d]	186	276	320	100	1.5
1d	α-CH_2PentylPh	37.5	285	297	299	8	ND
1e	α-$CH_2CH(OH)$Butyl	80	545	NI	NI	7	ND
1f	α-$CH_2CH(OH)$Octyl	19	NI	NI	NI	>100	1.2
1g	α-$CH_2CH(OH)$PropylPh	12	NI	NI	ND	>100	1
1h	α-$CH_2CH(OH)$ButylPh	8.6	NI	NI	ND	>100	1.3
2a	β-CH_2OButyl	19.9	NI	722	NI	84	1.5
2b	β-CH_2OOctyl	34.4	583	410	NI	14	1.6
2d	β-CH_2PentylPh	346	NI	NI	NI	3	ND
2e	β-$CH_2CH(OH)$Butyl	NI	NI	NI	NI	ND	ND
2f	β-$CH_2CH(OH)$Octyl	838	NI	NI	NI	ND	ND
2g	β-$CH_2CH(OH)$PropylPh	NI	NI	1000	ND	ND	ND
2h	β-$CH_2CH(OH)$ButylPh	NI	659	NI	ND	ND	ND

Table 1. IC_{50} values (μM) of iminosugars on α- and β-*N*-acetyl-D-glucosaminidases and enhancement of residual NAGLU activity in GM01426 cell lines at 100 mM
[a] on GM01426 cell lines; [b] IC_{50} β-Glu is the average of the IC_{50} obtained for the three β-N-acetyl glucosaminidases; [c] Enzyme activity fold enhancement at 10 μM; [d] Ki values (μM) for selected inhibitors are indicated in brackets
NI: no inhibition (less than 50% inhibition at 1000 μM); ND: not determined.

As more than 100 mutations, randomly scattered throughout the protein with only four occurring in the active site,[15a] have been identified in the NAGLU gene of MPS IIIB patients,[1] in addition to GM01426 cell lines, β-HNJNAc was probed at different concentrations following a 3 days exposure on two other mutant cell lines, namely GM02931, and GM00737,[28], using α-HNJNAc as a control (Table 2). α-HNJNAc showed no effect on GM00737 and GM01426 cell lines at 10μM and 100μM concentration, but some effect on GM02931 cell line with a significant 1.8 fold activity increase. β-HNJNAc had a different activity on cell lines, either showing a concentration dependent increase up to 1 μM in GM01426 cell line with the best activation to date (2.4 fold increase), or activation at lower concentrations in GM02931 and GM00737 cell lines. The fact that β-HNJNAc, a poor human recombinant NAGLU inhibitor, is the best chaperone of the series, demonstrating enzyme activity enhancement even at 0.1 μM concentration, is puzzling. It is difficult to rationalize this result as we do not know the characteristics of the active site of mutant enzyme. The weak competitive inhibition of β-HNJNAc cannot explain the significant chaperone effect on its own. We therefore tentatively hypothesize that β-HNJNAc also exert a non-inhibitory

pharmacological chaperone effect on E153K mutated NAGLU, a mutation expected to disrupt the overall fold of the protein.[16] Such allosteric ligands, including L-iminosugars,[27c, 29] have been already identified for several LSDs[30] including Fabry[31], Pompe[32] and Krabbe[33] diseases. They act by targeting previously unknown binding pockets, thus limiting the risk of adverse enzymatic inhibition. Demonstrating such behavior is challenging as it needs identification of the pharmacophore, requiring a massive screening-based approach outside the remit of this work. Interestingly, regarding their preliminary safety profile, both β-HNJNAc and α-HNJNAc had no toxicity on human HL60 cell proliferation when treated for three days at the highest 100μM concentration. They were also evaluated for their inhibition of α-glucosidases, α-mannosidases and β-hexosaminidases in cells by analyzing potential oligosaccharide storage products accumulating in the ER, cytosol and lysosome (see SI). SR1, a known β-hexosaminidase inhibitor,[34] was used as a control and showed the expected accumulation of GlcNAc-terminating oligosaccharides as a result of lysosomal enzyme inhibition. Upon administration of β-HNJNAc and α-HNJNAc for 30 days at 50μM concentration, HPLC analysis of isolated oligosaccharides labeled with a fluorescence tag showed no difference to control, untreated cells, thus indicating that both compounds have no effect on cellular glycosidases. This is not the case with DNJNAc that has been shown to inhibit lysosomal β-hexosaminidases.[20]

Table 2. Enhancement of residual NAGLU activity by β-HNJNAc in MPS IIIB

Compd	Conc. [μM]	Enzyme activity fold enhancement		
		GM02931	GM01426	GM00737
α-HNJNAc	10	1.5	1.1	1.1
α-HNJNAc	100	1.8	1.1	ND
β-HNJNAc	0.01	1.6	1.3	1.4
β-HNJNAc	0.1	1.5	2.1	1.5
β-HNJNAc	1	1.2	2.4	1.3
β-HNJNAc	10	1.2	2.1	1.1

In order to confirm that β-HNJNAc enhances mutant NAGLU trafficking from the ER to the lysosome, immunolabeling experiments were performed using patient fibroblasts (GM01426) and the lysosomal marker (LAMP-1). Patient fibroblasts were incubated in the presence or absence of 100 μM α-HNJNAc or β-HNJNAc for 5 days. The subcellular localization of the mutant NAGLU treated with α-HNJNAc and β-HNJNAc was determined by double immunofluorescence analysis (Figure 5). Compared with non-treated cells, treatment of α-HNJNAc increased the NAGLU staining levels and their pattern showed substantial overlap with that for lysosomal marker LAMP-1, indicating the presence of NAGLU in the lysosome. More importantly, the NAGLU staining levels of β-HNJNAc-treated cells was much brighter, strongly suggesting that β-HNJNAc improves the subcellular trafficking and distribution of the mutant NAGLU enzyme. While this result does not support the ability of β-HNJNAc to enhance residual activity of intralysosomal NAGLU, we expect a similar behavior as for Tay-Sachs LSD, where closely related GlcNAc-like iminosugars showed significant enhancement in protein activity.[35]

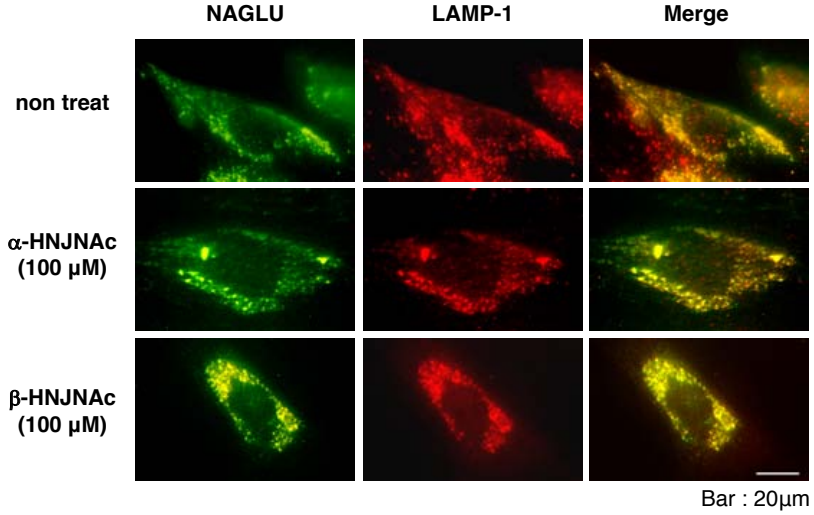

Figure 5. Immunocytochemistry of NAGLU in MPS IIIB GM01426 fibroblasts treated with α-HNJNAc and β-HNJNAc respectively

Conclusion

The mucopolysaccharidosis type IIIB, characterized by profound intellectual disability, dementia and a lifespan of about two decades, has been investigated through enzyme replacement therapy and substrate reduction therapy. Here we have explored the design, synthesis and evaluation of small iminosugar-based molecules as pharmacological chaperones, allowing

identification of potent and selective NAGLU inhibitors. Unpredictably, β-HNJNAc, a weak competitive inhibitor of NAGLU, demonstrated a significant pharmacological chaperone activity on mutant NAGLU for the first time to the best of our knowledge. However, its mode of action is still unclear and will be investigated in due course.

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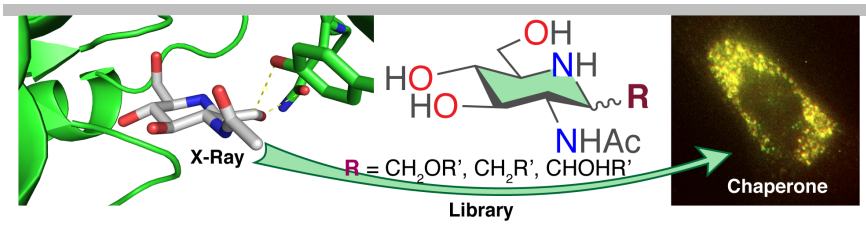

Capitalizing on the X-ray structure of two NAGLU-homoiminosugar complexes, the synthesis and evaluation as glycosidase inhibitors of a library of rationally designed iminosugar *C*-glycosides led to the discovery of several selective NAGLU inhibitors and allowed the identification of a pharmacological chaperone candidate to treat mucopolysaccharidosis type IIIB, a rare and devastating disease.

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Iminosugar-C-glycosides work as pharmacological chaperones of NAGLU, a glycosidase involved in MPS IIIB rare disease