Atsushi Kato, Shuichi Hirono et al.

In silico analyses of essential interactions of iminosugars with the Hex A active site and evaluation of their pharmacological chaperone effects for Tay–Sachs disease
**In silico** analyses of essential interactions of iminosugars with the Hex A active site and evaluation of their pharmacological chaperone effects for Tay–Sachs disease


The affinity of a series of iminosugar-based inhibitors exhibiting various ring sizes toward Hex A and their essential interactions with the enzyme active site were investigated. All the Hex A-inhibiting iminosugars tested formed hydrogen bonds with Arg178, Asp322, Tyr421 and Glu462 and had the favorable cation–π interaction with Trp460. Among them, DMDP amide (6) proved to be the most potent competitive inhibitor with a $K_i$ value of 0.041 μM. We analyzed the dynamic properties of both DMDP amide (6) and DNJNAc (1) in aqueous solution using molecular dynamics (MD) calculations; the distance of the interaction between Asp322 and 3-OH and Glu323 and 6-OH was important for stable interactions with Hex A, reducing fluctuations in the plasticity of the active site. DMDP amide (6) dose-dependently increased intracellular Hex A activity in the G269S mutant cells and restored Hex A activity up to approximately 43% of the wild type level; this effect clearly exceeded the border line treatment for Tay–Sachs disease, which is regarded as 10–15% of the wild type level. This is a significantly greater effect than that of pyrimethamine, which is currently in Phase 2 clinical trials. DMDP amide (6), therefore, represents a new promising pharmacological chaperone candidate for the treatment of Tay–Sachs disease.

**Introduction**

Lysosomal β-N-acetyhexosaminidase (Hex: [EC 3.2.1.52]) hydrolyzes terminal N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-galactosamine (GalNAc) residues of glycoproteins, glycolipids, and glycosaminoglycans. The human Hex are dimeric enzymes and their isozymes are produced through the combination of α and β subunits; they are classified into three subunits: Hex A (heterodimer of α- and β-subunits), Hex B (homodimer of α-subunits), and Hex S (homodimer of α-subunits). Among them, Hex A is the only isozyme that can hydrolyze the monosialic GM2 gangliosides in the brain, peripheral nervous system, and spinal cord. The α and β subunits of human Hex A are encoded by the evolutionarily related genes HEXA and HEXB, respectively. Many mutations in the HEXA gene have been reported which cause defects in transcription, translation, monomer folding and/or dimerization of Hex A.

Consequently, the amount and/or activity of Hex A decreases in the lysosomes leading to an accumulation of GM2 gangliosides to toxic levels, particularly in neurons in the brain and spinal cord that causes Tay–Sachs disease. Tay–Sachs disease is an inherited disease that is characterized by a progressive degeneration of the central nervous system, usually before 4 years of age, involving loss of motor skills, spasticity, seizures and progressive muscle weakness. There is also a later onset Tay–Sachs disease characterized by slower progression and more variable neurological signs including spinocerebellar degeneration, progressive dystonia and a bipolar form of psychosis.

The subunits of Hex A are synthesized in the neutral pH environment of the endoplasmic reticulum (ER) and then translocated through the Golgi system to lysosomes. In order to progress through the ER quality control system and be transported to the lysosome, the subunits of Hex A have to both fold and dimerize. Pharmacological chaperones, such as sub-
strate-like reversible competitive inhibitors, appear to be able to act as a template that stabilizes the native folding state in the ER by occupying the active site of the mutant enzyme, thus allowing its maturation and trafficking to the lysosome.9,10 A large number of pharmacological chaperones have been identified from natural sources and synthetic analogues produced including the N-acetylgalactosamine thiazoline (NAG-thiazoline, NGT), an N-acetyl-β-hexosaminidase inhibitor.11 Two active sites are present in the Hex A dimer, one in the α-subunit and the other one in the β-subunit and NGT binds to both active sites. However, the hydrolysis of the GM2 gangliosides is carried out only by the α-subunit of Hex A. These active sites are located at the entrance of the TIM barrels at the interface between the α and β-subunits. Tyr456 contained with β-subunits is found in the α-subunit active site. These structural insights and the experimental data gained from Hex A suggest that dimerization is essential for activity. We have previously reported the inhibition of Hex A by iminosugars containing an acetamide (NHAc) group and displaying various ring sizes ranging from 4 to 7 atoms (Fig. 1). Previous studies suggested that the presence of an N-acyl moiety in the iminosugar is necessary for strong inhibition of Hex A. Piperidine iminosugars with structural similarity to GlcNAc, iminosugar is necessary for strong inhibition of Hex A. We have previously reported the inhibition of Hex A by iminosugars containing an acetamide (NHAc) group and displaying various ring sizes ranging from 4 to 7 atoms (Fig. 1). Previous studies suggested that the presence of an N-acyl moiety in the iminosugar is necessary for strong inhibition of Hex A. Piperidine iminosugars with structural similarity to GlcNAc, such as DNJNAc (1)12,13 and DGJNAc (2),14,15 provide typical examples. It is noteworthy that Hex A also tolerates five-membered scaffolds such as LABNAc (3)16 and XYLNAc (4)17 and also seven-membered NAc-azeapane (5).18,19 These compounds have a conformational flexibility comparable to the six-membered iminosugar inhibitors and may mimic the oxocarbonium ion-like transition state proposed for Hex A. Recently, iminosugar inhibitors containing a methyl amide (CONHMe) as a biosostere of the NHAc group have been identified. This class included the pyrrolidine DMDP amide (6), talo-DMDP amide (7) and the four-membered azetidine amide (8).20,21 We hypothesized that both folding and assembly of mutant Hex A might be assisted by all these acetamide or methyl amide containing iminosugars. However, from an enzymatic standpoint, little is known about the required essential interactions of the inhibitors with the Hex A active site.

The overall aim of this study was to provide general guidelines for the effective design of Hex A inhibitors and to show their applicability to human disease treatment. In the present study, we first determined the mode of inhibition and the inhibition constant (K_i) of all compounds. We then built the models of the complex structures of the Hex A–ligands (1–9) by a docking analysis and calculated the binding energy (∆E) from the complex models. Next, we revealed the interaction modes of each ligand (1–9) and clarified the difference by variation in ring size and stereochemistry. In particular, we focused on DMDP amide (6), which showed the highest affinity for Hex A, and identified the essential interactions of the inhibitors with the Hex A active site by using a molecular dynamics (MD) simulation. From a therapeutic point of view, we report for the first time that DMDP amide (6) is an excellent pharmacological chaperone, which effectively enhances intracellular Hex A activity in Tay–Sachs patient cells.

Results and discussion

Inhibition constant (K_i) of iminosugars with various ring sizes on human Hex A

To understand the structural basis of the interaction of iminosugars (1–8) with human Hex A, we first determined the mode of inhibition and the inhibition constant (K_i) of all compounds from Lineweaver–Burk plots; all the potent inhibitors have a trans-relationship between the NHAc moiety (or CONHMe moiety) and the adjacent OH group [shown in red in Fig. 1]. In many cases, the inhibition potency of the iminosugars for each glycosidase is dependent on the corresponding substrate similarity. As shown in Table 1, both DNJNAc (1) and DGJNAc (2) were micromolar inhibitors of Hex A, with K_i values of 2.2 and 1.7 μM, respectively.

Similarly, the 4-ring iminosugar 8, 5-ring iminosugars 3 and 4, and 7-ring iminosugar 5 gave very comparable levels of inhibition toward this enzyme, with K_i values of 1.1, 2.6, 1.9, and 1.8 μM, respectively. It is noteworthy that DMDP amide (6) and talo-DMDP amide (7) showed much stronger inhibition, with K_i values of 0.041 and 0.26 μM, respectively. LABNAc (3) and XYLNAc (4) have the same pyrrolidine ring but their affinities were about 50–60 fold weaker than DMDP amide (6). These results clearly suggested some plasticity of the active site of Hex A to tolerate these acetamide containing iminosugars exhibiting various ring sizes. Although the ring size of the

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**Fig. 1** Structures of potent four-, five-, six-, and seven-membered Hex A inhibitors. Highlighted in red 2S,3R trans-relationship present in potent inhibitors.
Iminosugar may vary, the configuration and/or conformation of the iminosugar is key for the inhibition of Hex A. We have reported the structure–activity relationship of the 16 stereoisomeric N-methyl proline amides of 6 and showed that the (2S,3R)-configuration is a requirement for achieving a strong affinity of this enzyme. All the stereoisomers of 6 with (2S,3R)-configuration [shown in red in Fig. 1] are potent inhibitors of Hex A. In our study, we also incorporated the non-iminosugar Hex A inhibitor pyrimethamine (9) that was originally approved as an anti-malarial/anti-toxoplasmosis agent but was discovered later to have considerable pharmacological chaperone potential and was identified by manual screening of a library of 1,040-FDA-approved drugs as Hex A inhibitors. Under the same assay conditions used here, the inhibition constant of pyrimethamine (9) was 8.8 μM. Interestingly, pyrimethamine, which is a 200-fold weaker Hex A inhibitor compared to DMDP amide (6), has nonetheless entered Phase II clinical trials for the treatment of Tay–Sachs disease.

In order to investigate the interaction between Hex A and the ligands, we modelled the three-dimensional structures of the complex structures by Hex A with nine ligands, iminosugars (1–8) and pyrimethamine (9), using the docking protocol.

As shown in Table 1 and Fig. 2, the binding energies (ΔE) correlated well with the pKᵢ values (r² = 0.78). This result suggested that the complex models of Hex A and these ligands were reliable. The interactions between Hex A and 6 (four-membered ring), 8 (five-membered ring), 1 (six-membered ring) and 5 (seven-membered ring) are illustrated in Fig. 3. As

### Table 1 Measured Kᵢ values and binding energy of ligands for human β-N-acetylhexosaminidase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ (μM)</th>
<th>pKᵢ</th>
<th>ΔE, binding energy (kcal mol⁻¹)</th>
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<tr>
<td><strong>Azetidine-type iminosugars</strong></td>
<td></td>
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<tr>
<td>Azetidine amide (8)</td>
<td>1.1</td>
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<td><strong>Pyrrolidine-type iminosugars</strong></td>
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<td>LABNAC (3)</td>
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<td>XYLNAc (4)</td>
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<td>5.72</td>
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<td>DMDP amide (6)</td>
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<td>DGJNAc (2)</td>
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<td><strong>Azepane-type iminosugars</strong></td>
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<tr>
<td>Pyrimethamine (9)</td>
<td>8.3</td>
<td>5.08</td>
<td>-37.85</td>
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</table>

Fig. 2 Graph of the binding energy (ΔE) versus the inhibition constant (pKᵢ) for each ligand (1–9) with Hex A (r² = 0.78).

Docking study of iminosugars (1, 4, 6, and 8) against human Hex A

![Fig. 3 Schematic diagram of hydrogen bonding interactions and cation–π interactions between Hex A and azetidine amide (A and E), DMDP amide (B and F), DNJNAc (C and G), and azepane NAc (D and H). Red dashed lines: hydrogen bonding interactions. Green dashed lines: cation–π interactions.](image-url)
shown in Fig. 3C and G, the positively charged nitrogen and the hydroxyl groups of DNJNAc (1) formed hydrogen bonds with Arg178, Asp322, Tyr421, Glu462 and Tyr 456 of the β subunit. Furthermore, the piperidine ring part of DNJNAc (1) had the favorable cation–π interaction with Trp460. Similarly, all iminosugars tested (8, 6, 1, and 5) formed hydrogen bonds with Arg178, Asp322, Tyr421 and Glu462. For DMDP amide (6) and azetidine amide (8), a hydrogen bond was additionally formed between Glu323 and the positive nitrogen. This hydrogen bond was found neither in DNJNAc (1) nor in azepane NAc (5). To clarify the ligand–Hex A interaction, we investigated the superimposition of these four ligands. As shown in Fig. 4, there are five common interaction moieties between Hex A amino acids and ligands, which are: (1) the hydrogen bond (HB) acceptor/donor for interaction with Arg178 and Glu462; (2) the HB acceptor/donor for interaction with Arg178 and Asp322; (3) positive nitrogen for cation–π interaction with Trp460 and ionic interaction with Asp322, Glu323, and Glu462; (4) the HB donor for interaction with Asp322; (5) HB acceptor for interaction with Tyr421.

MD simulations

Although the docking commonality was observed in the induced fit docking study, the reason why DMDP amide (6) showed much stronger inhibition than other iminosugars remained unclear. Hex A hydrolyses the terminal GlcNAc residue of the GM2 ganglioside. Therefore, DNJNAc (1) with a close structural similarity to GlcNAc should be bound stably and strongly in the active site of Hex A. Thus, we turned our attention to dynamic changes in the interaction between Hex A and the ligands. To analyze the dynamic properties of DMDP amide (6) and DNJNAc (1) in aqueous solution, we performed MD simulations. Snapshots were collected from trajectories where root mean squared deviation (RMSD) variation was stable (from 12 ns to 16 ns) (Fig. 5).

The MD study revealed that DMDP amide (6) binds stably and strongly in the active site of Hex A, while DNJNAc (1) moves dynamically in the binding pocket. In addition to ligand flexibility, fluctuations of amino acid residues in the binding pocket were observed in the complex structure of DNJNAc (1). The average and standard deviations of distances between two atoms forming hydrogen bonds were calculated (Fig. 6), (Table 2). From the comparison of DMDP amide (6) and DNJNAc (1) it was revealed that in the case of DNJNAc (1), the average distance between Asp322 and 3-OH and between Glu323 and 6-OH was clearly longer than that for DMDP amide (6). These differences in average distance not only directly influence the strength of the hydrogen bonds but also lead to a large fluctuation of amino acid residues in the binding pocket. Consequently, DNJNAc (1) could not form the hydrogen bonds between Asp322 and 3-OH and between Glu323 and 6-OH. On the other hand, DMDP amide (6) could form more rigid hydrogen bond interactions and reduce the fluctuations of amino acid residues in the binding pocket.

![Fig. 4 Alignment of binding poses for azetidine amide (1) (yellow), DMDP amide (6) (blue), DNJNAc (1) (light green) and azepane NAc (5) (green) in the binding pocket of Hex A (white surface). Functional groups common in the interactions between four ligands and Hex A were encircled by dashed lines.](image)

![Fig. 5 Snapshots of DMDP amide (6) and DNJNAc (1) in the binding pocket of Hex A obtained from MD simulation. (A) DMDP amide (6): blue; (B) DNJNAc (1): light green.](image)

![Fig. 6 The average and standard deviation of distances between two atoms forming the hydrogen bonds calculated from MD trajectory (12 ns–16 ns).](image)
Pharmacological chaperone effect of DMDP amide (6), DNJNAc (1), and pyrimethamine (9) against Tay–Sachs G269S mutant cells

The pharmacological chaperone theory, proposed for the first time by Fan et al.,24 is defined as "a small molecule whose function is to assist a protein to fold properly and enter the normal processing pathway smoothly". A pharmacological chaperone binds to the active site and acts as a template. Consequently, it is expected to reduce both the flexibility of folding in situ and the proportion that migrates to the endoplasmic-reticulum-associated protein degradation (ERAD) pathway. In Tay–Sachs patients a mutation in Hex A reduces the thermal stability at neutral pH; consequently it does not form the correct three-dimensional structure. In the present study we showed that DMDP amide (6) had a 54-fold higher affinity than the transition state mimic DNJNAc (1) toward Hex A (Table 1). Thus, we next evaluated the pharmacological chaperone effect of DMDP amide (6) in Tay–Sachs G269S patient cells. The cells were cultured in the presence of DMDP amide (6: 1–100 μM) and DNJNAc (1: 1–100 μM) for 5 days, and the intracellular enzyme activities were determined with 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside 6-sulphate (MUGS) (Fig. 7). Pyrimethamine (9; 0.03–3 μM), which is in Phase II clinical trials toward Tay–Sachs disease, was used as a positive control. Prior to the start of the pharmacological chaperone test, we measured the cytotoxic effect of each compound toward the Tay–Sachs G269S patient cells by the MTT assay. DMDP amide (6) and DNJNAc (1) exhibited no influence on cell growth even at concentrations as high as 100 μM. In contrast, pyrimethamine (9) showed strong cytotoxicity; 100 μM pyrimethamine (9) annihilated most of the cells. We further determined the concentration range of pyrimethamine (9), which did not influence the cell growth; it was preferable to use as a control at less than 3 μM (data not shown). Based on these results, we set up the dose range of

![Table 2](image)

<table>
<thead>
<tr>
<th>DMDP amide (6)</th>
<th>DNJNAc (1)</th>
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<tbody>
<tr>
<td>Hex A Distance (Å)a</td>
<td>Hex A Distance (Å)a</td>
</tr>
<tr>
<td>3-OH N’(Arg178)</td>
<td>3-OH N’(Arg178)</td>
</tr>
<tr>
<td>3-OH O’(Glu462)</td>
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</tr>
<tr>
<td>4-OH N’(Arg178)</td>
<td>4-OH N’(Arg178)</td>
</tr>
<tr>
<td>4-OH O’(Glu462)</td>
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</tr>
<tr>
<td>6-OH O’(Glu462)</td>
<td>6-OH O’(Glu462)</td>
</tr>
<tr>
<td>NH (CONHMe) O’(Asp322)</td>
<td>NH (NHAc) O’(Asp322)</td>
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<tr>
<td>C≡O O’(Tyr421)</td>
<td>C≡O O’(Tyr421)</td>
</tr>
<tr>
<td>N’ (pyrrolidine) O’(Asp322)</td>
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</tr>
<tr>
<td>N’ (pyrrolidine) O’(Glu462)</td>
<td>N’ (piperidine) O’(Glu462)</td>
</tr>
</tbody>
</table>

Table 2: The average distances and standard deviations between functional groups of ligands and important amino acids in binding to Hex A

a The distances between heavy atoms were calculated using structures sampled from MD trajectory.

![Fig. 7](image)

**Fig. 7** Effect of (A) DNJNAc (1), (B) DMDP amide (6), (C) pyrimethamine (9) on G269S Hex A in Tay–Sachs cells treated with increasing concentrations of 6, 1, or 9. Each value represents the mean ± SEM (n = 3). (D) Comparison of intracellular Hex A activity on wild type and Tay–Sachs G269S patient cells in the presence or absence of 100 μM DMDP amide (6) for 5 days.
pyrimethamine (9) from 0.03 to 3 μM. Relative to non-treated (NT), pyrimethamine (9) improved the intracellular Hex A activity in the Tay–Sachs G269S patient cells, with a 2.3-fold increase at 3 μM (Fig. 7C), an improvement similar to the one obtained with 1–3 μM of DNJNAc (1). However, DNJNAc (1) dose-dependently increased intracellular Hex A activity up to 6.9-fold at 100 μM (Fig. 7A), a cytotoxic dose for pyrimethamine (9). DMDP amide (6) was also assayed under the same conditions and was effective as a pharmacological chaperone at a significantly lower dose than either DNJNAc (1) or pyrimethamine (9). The effective doses under the experimental conditions used for Tay–Sachs G269S mutant cells were higher than their Ki values. As observed for other chaperones,25,26 compounds 1 and 6 did not show G269S Hex A-inhibitory activity even at high doses. They bind reversibly to the active site and act as a template in ER. In contrast, after reaching the lysosome, these compounds can be efficiently exchanged for the residual undecomposed substrate in the patient cells. Treatment with DMDP amide (6) for 5 days dose-dependently increased intracellular Hex A activity, with a maximal increase of 14.8-fold at 100 μM (Fig. 7B). Noteworthily, this corresponds to a restoration of the intracellular Hex A activity to approximately 43% of the wild type level (Fig. 7D), convincingly exceeding the border line treatment level for Tay–Sachs disease, which is regarded as 10–15% of the wild type level.27

In vitro normalization effect of DMDP amide (6) on the cellular trafficking of the G269S mutant

The present study has demonstrated that treatment with DMDP amide (6) dramatically increased intracellular Hex A activity in the G269S mutant cells. However, for the mutant enzyme to function properly, the rescued Hex A with correct folding assisted by the pharmacological chaperone must be transported to the lysosome. Thus, we next focused on the subcellular localization of the mutant Hex A. The subcellular location of Hex A was determined by double immunofluorescence analysis (Fig. 8). Tay–Sachs G269S patient cells were treated with rabbit polyclonal anti-Hex A as a primary antibody for the detection of Hex A, and mouse monoclonal anti-LAMP-1 for the detection of lysosomes. Hilyte Fluor™ 488-conjugated goat anti-rabbit IgG serum and Fluor™ 594-conjugated goat anti-mouse IgG serum were used as secondary antibodies. Compared with the wild type, untreated G269S patient cells clearly showed a decrease in the amount of Hex A (Fig. 8A and D, green) and co-localization of Hex A and the lysosomal marker LAMP-1, which showed the overlap (artificially colored yellow, Fig. 8C and F). In contrast, treatment with 100 μM DMDP amide (6) resulted in an increase in the intensity of Hex A fluorescence throughout the cells (Fig. 8G) and Hex A staining pattern showed substantial overlap with that of the lysosomal marker LAMP-1 (Fig. 8I). These results clearly indicated that DMDP amide (6) restored the subcellular trafficking of the G269S mutant enzyme to lysosomes.

![Fig. 8 Immunocytochemistry of Hex A in Tay–Sachs G269S fibroblasts treated with DMDP amide (6). Tay–Sachs G269S fibroblasts were cultured with or without 100 μM DMDP amide (6) for 5 days. Immunocytochemistry was performed in which cells were double labeled with Hex A antibody (green), and a lysosome marker (LAMP-1, red).](image)

Conclusion

In summary, we have studied the structural requirements of iminosugars for strong binding to the active site of Hex A. We modelled the three-dimensional structure of the Hex A–ligand complex using a docking protocol, which showed good correlation with both the binding energy (ΔE) and the pKi. The present work revealed the following features: (a) DMDP amide (6: Ki = 0.041 μM) had a 54-fold higher affinity than the transition state mimic DNJNAc (1) toward Hex A; (b) all the high affinity ligands, azetidine amide (8), DMDP amide (6), DNJNAc (1), and azepane NAc (5) established hydrogen bonds with Arg178, Asp322, Tyr421 and Glu462 and had the favorable cation–π interaction with Trp460; (c) the interatomic distances between Asp322 and 3-OH and Glu323 and 6-OH were shorter for DMDP amide (6) than for DNJNAc (1) leading to stronger hydrogen bond interactions compared to other Hex A-inhibiting iminosugars; (d) DMDP amide (6) dramatically increased intracellular Hex A activity in the G269S mutant cells dose-dependently, up to approximately 43% of the wild type level at 100 μM concentration. DMDP amide (6), therefore, represents a promising pharmacological chaperone candidate for the treatment of Tay–Sachs disease.

Experimental section

Molecular docking calculations and binding energy estimation

A crystal structure of the Hex A heterodimer consisting of α and β subunits complexed with two N-acetylglucosamine-thiazolines was available (PDB code: 2GK1). This structure includes four Hex A heterodimers in the asymmetric unit.
which we used for each biological assembly (chain: AB, CD, EF, GH). These structures were prepared with the Protein Preparation Wizard in Maestro. Hydrogen atoms were added under the acidic conditions of the lysosome interior (around pH 5.5). 2D structures of iminosugars (1–8) and pyrimethamine (9) were prepared by LigPrep and multiple conformers for docking inputs were generated by ConfGen. The compounds were docked into the active site of α subunits of Hex A using Glide with a standard precision mode. Docking poses were evaluated in terms of both docking scores and the interaction energies. A docking pose that exhibited a preferable docking score and a stable interaction energy was selected as the most suitable complex structure for each ligand. This strategy was verified by the analyses of N-acetylglucosamine-thiazoline that reproduced both the binding conformation and hydrogen bonds of N-acetylglucosamine-thiazoline in a co-crystallized structure. Then the complex structures were energy-minimized using the distance-dependent dielectric constant ($\varepsilon = 4\varepsilon_0$) by MacroModel and interaction energies between Hex A and ligands were calculated. For these analyses, we used the Schrödinger Suite v2015-3 (Schrödinger, LLC.).

MD analysis

MD simulations for DMDP amide (6) and DNJNAc (1) were performed using the OPLS3 force field in the Desmond program (Schrödinger Suite 2015-3, Schrödinger, LLC.). Each system was solvated with SPC water in an orthorhombic simulation box under periodic boundary conditions. Sodium or chloride counterions were added to neutralize the overall charge of the system for the particle mesh Ewald method. A minimization was performed with a convergence threshold of 0.05 kcal mol$^{-1}$ Å$^{-1}$ up to 20,000 cycles. MD simulations were performed for 16 ns at 1 atm and 310 K using 1 fs timesteps in the NPT ensemble. For each system, the snapshots were sampled with intervals of 3 ps. Root mean squared deviation (RMSD) calculations were performed using the Simulation Interactions Diagram in the Desmond program. Snapshots were collected from the trajectory in which the RMSD value was stable (from 12 ns to 16 ns).

Enzyme inhibition assays

The iminosugars 1–8 were prepared as previously described. Inhibition activity of human β-N-acetylhexosaminidase was performed according to our previous method. Briefly, β-N-acetylhexosaminidase was used in combination with 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside 6-sulphate (MUGS; Carbosynth Ltd; Berkshire, UK) as a substrate. The reaction mixture consisted of 100 mM McIlvaine buffer (pH 4.2) with 0.025% HSA and the appropriate amount of enzyme. The reaction mixture was pre-incubated at 0 °C for 30 min, and the reaction was started by adding 5 mM substrate solution, followed by incubation at 37 °C for 60 min. The reaction was stopped by the addition of 400 mM glycine–NaOH solution (pH 10.6). The released 4-methylumbelliferone was measured (excitation 362 nm, emission 450 nm) with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Kinetic parameters were determined by the double-reciprocal plot method of Lineweaver-Burk plots at increasing substrate concentrations.

Pharmacological chaperone activity

The Tay-Sachs cell line with Hex A mutation of G269S (GM003461) was obtained from the Coriell Cell Repositories (Camden, NJ). The Tay-Sachs G269S lymphoblasts were cultured in 2 mM l-glutamine containing MEM (Minimum Essential Media; Sigma-Aldrich Co.) supplemented with 10% FCS. The cells were cultured in a water-jacket incubator at 37 °C under 5% CO$_2$ in the presence or absence of samples for 5 days. After washing twice with PBS, the cell pellets were dissolved in cell lysis buffer and freezing and thawing treatment was performed. After sonication for 5 min, the whole cell lysate was subjected to enzyme assays and protein determination. Intercellular Hex A activities were determined with MUGS as the substrate at pH 4.2.

Immunofluorescence staining and detection of subcellular localization

The Tay-Sachs cell line with Hex A mutation of G269S (GM13204) was obtained from the Coriell Cell Repositories (Camden, NJ). The Tay-Sachs G269S fibroblasts were cultured in 2 mM l-glutamine containing DMEM (Dulbecco’s Modified Eagle’s medium; Sigma-Aldrich Co.) supplemented with 10% FCS. The anti-Hex A (206–220) antibody was produced in a rabbit as a primary antibody for the detection of Hex A (Sigma-Aldrich Co.), and we used the mouse monoclonal anti-LAMP1 [H4A3] antibody (Abcam Plc., Cambridge, UK) for the detection of lysosomes. Hilyte Fluor™ 488-conjugated goat anti-rabbit IgG serum and Fluor™ 594-conjugated goat anti-mouse IgG serum (both from AnaSpec, Inc., Fremont, CA) were used as secondary antibodies. Tay-Sachs fibroblasts (2.5 × 10$^4$ cells) were grown on sterile coverslips in the presence or absence of 100 μM iminosugar (6) and cultured for 5 days in DMEM. All immunocytochemistry procedures were performed at room temperature. After the cells had been washed three times with PBS, they were fixed with 5 mM substrate solution for 10 min, and then washed three times with PBS. The cells were permeated with 0.01% Triton X-100 in PBS for 10 min, and then washed three times with PBS. After a 20 min treatment with blocking solution with 5% skim milk in PBS, the cells were incubated with the primary antibody diluted in 1% skim milk in PBS for 20 min, and this was followed by three washes with PBS. Coverslips were then incubated in the dark with the secondary antibody diluted in 1% skim milk in PBS for 20 min, and this was followed by three washes with PBS. The coverslips were mounted with a drop of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA), and fluorescence was visualized using an Olympus fluorescence microscope (Olympus, Tokyo, Japan).
Conflicts of interest

There are no conflicts to declare.

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