Pharmacological Enhancement of β-Hexosaminidase Activity in Fibroblasts from Adult Tay-Sachs and Sandhoff Patients*

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Tay-Sachs and Sandhoff diseases are lysosomal storage disorders that result from an inherited deficiency of β-hexosaminidase A (αβ). Whereas the acute forms are associated with a total absence of hexosaminidase A and early death, the chronic adult forms exist with activity and protein levels of 5–30%, and unaffected individuals have been found with only 10% of normal levels. Surprisingly, almost all disease-associated missense mutations do not affect the active site of the enzyme but, rather, inhibit its ability to obtain and/or retain its native fold in the endoplasmic reticulum, resulting in its retention and accelerated degradation. By growing adult Tay-Sachs fibroblasts in culture medium containing known inhibitors of hexosaminidase we have raised the residual protein and activity levels of intralysosomal hexosaminidase A well above the critical 10% of normal levels. A similar effect was observed in fibroblasts from an adult Sandhoff patient. We propose that these hexosaminidase inhibitors function as pharmacological chaperones, enhancing the stability of the native conformation of the enzyme, increasing the amount of hexosaminidase A capable of exiting the endoplasmic reticulum for transport to the lysosome. Therefore, pharmacological chaperones could provide a novel approach to the treatment of adult Tay-Sachs and possibly Sandhoff diseases.

GM2 gangliosidosis, arising from the neuronal storage of GM2 ganglioside (GM2), occurs in three variants; Tay-Sachs disease (TSD), Sandhoff disease (SD) and the AB variant. The former two result from mutations in the evolutionarily related HEXA or HEXB genes, encoding the α or β subunits of heterodimeric β-N-acetylgalactosaminidase A (Hex A, αβ), respectively (reviewed in Ref. 1).

Two other homodimeric Hex isozymes exist, i.e. Hex B (αβ) and Hex S (αα). Each isozyme has its own characteristic pI and stability with Hex B being both the most stable and basic (pI = 6.9), followed by less stable Hex A (pI = 4.8), and unstable Hex S (pI = 3). In normal human tissue only Hex A and B are readily detectable (1). Although each subunit contains an active site, dimerization is required for enzymatic activity (2). However, in the lysosome only Hex A, along with its substrate-specific cofactor the GM2 activator protein, can hydrolyze the terminal, β-linked N-acetylgalactosamine (GalNAc) residue from GM2 ganglioside.

Whereas both the α- and/or β-active sites of dimeric Hex can hydrolyze synthetic N-acetylgalactosamine terminal substrates, only the α-sites of Hex A and S can efficiently utilize negatively charged substrates, e.g. 6-sulfated GlcNAc (2, 3). Therefore, total Hex activity is measured using 4-methylumbelliferyl-β-N-acetylglucosaminide (MUG), whereas, 4-methylumbelliferyl-β-N-acetylglucosaminide-6-sulfate (MUGS) is to measure Hex A and Hex S activity. At pH 4.2 the relative MUG/MUGS hydrolysis ratios of the three isozymes are: 150–300/1 for Hex B, 3–4/1 for Hex A, and 1–1.5/1 for Hex S (3).

The more common infantile TSD (ITSD) variant of GM2 gangliosidosis results from absent α subunits and elevated amounts of Hex B such that near normal levels of total Hex activity (MUG) are retained. Less common is acute infantile SD (ISD), resulting from an absence of β-subunits. Interestingly, despite normal levels of β-mRNA, only 2–4% residual Hex activity, from Hex S, can be detected in SD patient samples. In contrast to the infantile forms, adult TSD (ATSD) and SD (ASD) are chronic, slowly progressive, neurodegenerative diseases. In many cases these are associated with missense mutations, usually producing thermolabile Hex A with residual activity (MUGS) and protein levels that are <10% but >2% of normal. The majority of patients with ATSD possess a missense mutation in exon 7, αG268S (1). This and similar point mutations do not directly affect the active site of Hex (2) but are believed to result in increased amounts of misfolded protein in the endoplasmic reticulum (ER), which in turn are retained by its quality control system and degraded (4).

Only a low level of residual Hex A activity is needed to ameliorate the clinical phenotype in ITSD or ISD, and asymptomatic individuals have been identified with residual activities of >10% of normal (5, 6). Sandhoff and colleagues (7) have estimated that 5–10% of normal Hex A levels represent a “critical threshold” for disease.
Currently, there is no therapy for any form of GM2 gangliosidosis. Strategies involving bone marrow transplantation and enzyme replacement therapy are unlikely to be successful due to neuronal storage and the inaccessibility imposed by the blood-brain barrier. Substrate deprivation therapy, although feasible, is limited by specificity and toxicity issues.

Pharmacological chaperones, small molecules that function either as rescue enzymes (receptor inhibitors) or as competitive inhibitors (enzymes), have been used to "rescue" several misfolded proteins (reviewed in Refs. 8 and 9) from ER-associated degradation. They apparently act by increasing the stability of the newly synthesized, mutant protein, allowing more of it to exit the ER for transport to its site of action, e.g. lysosome. Because only a modest increase in residual Hex A activity is needed for ATSD and ASD patients to achieve the ~10% critical threshold, these diseases are candidates for pharmacological chaperone-based therapy. In this report, we evaluate a panel of known Hex inhibitors (see Table I) as potential pharmacological chaperones. We show that the Hex inhibitors can increase levels of lysosomal Hex A activity to 35% of normal levels in fibroblasts from an αG689S homozygous ATSD patient. Additionally, the effects of these compounds on ASD, ISD, and ITSD fibroblasts were examined.

EXPERIMENTAL PROCEDURES

Reagents—The following fluorogenic substrates, all purchased from Sigma, methylumbelliferyl-β-D-glucuronide (MUbGlr), 4-methylumbelliferyl-β-D-galactopyrano- side (MUBgal), 4-methylumbelliferyl phosphate (MUP), MUG, and MUGS were used to assay the lysosomal enzymes β-glucuronidase, β-D-galactosidase, acid phosphatase, Hex A/B/S, and Hex A/S, respectively. Rabbit polyclonal antibodies against Human Hex A and β-glucosidase/gluco-cerebrosidase were prepared in our laboratory and have been previously described (10). Donkey polyclonal antibody developed against a C-terminal calnexin peptide was purchased from Santa Cruz Biotechnology. A monoclonal antibody developed against a C-terminal calnexin peptide was purchased from Santa Cruz Biotechnology. Castanospermine, deoxynojirimycin (DNJ), 6-acetamido-6-deoxycastanospermine (ACAS) (IRL, New Zealand), 2-acetamido-1,2-dideoxynojirimycin (AddNJ) (TRC, Canada), GalNac (Sigma) were commercially available; whereas 2-acetamido deoxynojirimycin (AdNJ) and N-acetylgalosamine thiazoline (NGT) were synthesized and purified according to Kappes and Legler (11) and Knapp et al. (12), respectively. All compounds were dissolved in water and used as 10–25 mg/ml solutions. Steel wool #0000 (International Steel Wool, Mexico), FeCl2 and FeCl3 (Sigma), and dextran T4000 (Amersham Biosciences, UK) were used to prepare lysis buffers by magnetic chromatography.

Cell Lines—The following fibroblast cell lines were used: 4212 (from hereon referred to as wild type (WT) or unaffected) was from an unaffected individual; 1766 (ATSD) was from a 40–45 year old female patient diagnosed with the chronic (adult) form of TSD (kindly provided by Dr. J. R. Donat, University of Saskatchewan, Kinsey Children’s Centre, Saskatoon, Saskatchewan, Canada) and found to be homozygous for the mutation 880G→A/880G→A (αG689S) in exon 7 of HEXA (Molecular Diagnostics Laboratory, Hospital For Sick Children, Toronto, Ontario, Canada); 2317 (ITSD) was from a female fetus with the acute (infantile) form of TSD (kindly provided by Dr. D. Legler (11) and Knapp et al. (12), respectively. All compounds were dissolved in water and used as 10–25 mg/ml solutions. Steel wool #0000 (International Steel Wool, Mexico), FeCl2 and FeCl3 (Sigma), and dextran T4000 (Amersham Biosciences, UK) were used to prepare lysis buffers by magnetic chromatography.

Enzyme Kinetics—Cells were grown in 96-well tissue culture plates (Falcon). Trypsinized cells were diluted to give 50% confluence when plated, and equal numbers of cells were aliquoted into each well of the plate. Cells grown for longer than 5 days were supplemented with fresh media. Compounds were diluted in media, filter-sterilized (Millipore), and evaluated in triplicate, i.e., three different wells.

Following 3–7 days of treatment, intracellular Hex A activities were determined. Media was removed, cells were washed twice with phosphate-buffered saline (PBS), and lysed using 60 μl of 10 mM citrate/phosphate (CP; pH 4.2) buffer (CP buffer), containing 0.5% human serum albumin and 0.5% Triton X-100. Aliquots of the lysates were transferred to a 96-well plate, and Hex A activity was measured using 25 μl of 3.2 mM MUGS in CP buffer, with incubation at 37 °C for 1 h. The reaction was stopped by the addition of 200 μl of 0.1 mM 2-amino-2methyl-1-propanol, pH 10.5. Fluorescence was measured using an excitation wavelength of 365 nm and emission wavelength of 450 nm as previously described (14). For experiments in Figs. 2 and 5–7, the relative increase in Hex activity was expressed as the average fluorescence reading from three wells of cells (n = 3) grown in the presence of compound, divided by the average fluorescence reading from three wells of cells (n = 3), grown for the same length of time, in the absence of any compound. For untreated cells, fluorescence readings for the individual wells varied by less than 20%. To measure total Hex activity, the activities of the other lysosomal enzymes MUbGlr (0.3 mM), MUG (3.2 mM), MUbGal (0.56 mM) and MUBGlr (2.33 mM) were dissolved in CP buffer and used as described for MUGS.

For Western blot analysis and cellulose acetate electrophoresis (CAE), ATSD fibroblasts were grown for 5–6 days in 6-well plates (Falcon, 40 mm2) using 1.5 ml of medium with or without the compounds to be evaluated. Subsequently, media were removed, and cells were washed twice in PBS and finally scraped into 1 ml of PBS. Following pelleting by microcentrifugation, the cells were resuspended in 10 mM sodium phosphate buffer, pH 6.1, containing 5% glycerol and disrupted by sonication on ice. Cleared lysates were prepared by microcentrifugation for 15 min at 4 °C, and the total protein concentration was determined using BCA protein assay (Pierce) according to the manufacturer’s instructions. Hex A activities were determined using MUGS substrate at 37 °C for 1 h as described above and expressed as nanomoles of 4-methylumbelliferone (MU) released/h/mg of total protein.

Western Blotting—Lysates (5 μg of total protein) were subjected to SDS-PAGE on a 10% bissacrylamide gel, transferred to nitrocellulose, and processed as previously described (14). Blots were developed using chemiluminescent substrate according to the manufacturer’s protocol (Amersham Biosciences, UK) and recorded on BIORAD x-ray film (Kodak). Blots were probed multiple times following stripping of bound antibody following treatment with RESTORE buffer (Pierce).

Cellulose Acetate Electrophoresis—To separate and directly visualize the hex A isoforms, Blot (pI 4.8, αG, βG, and S (pI 3–6, αM)) was performed according to Ref. 17 with modifications. Briefly, 2 μg of lysates protein was spotted onto Sepharose (Gelman) strips (pre-wetted in 20 mM sodium phosphate buffer, pH 7.0), partially dried, and resolved electrophoretically at 10 mA for 30 min. Strips were overlaid with another cellulose acetate strip soaked in CP buffer containing 3.2 mM MUG, wrapped in plastic wrap, and incubated for 1 h at 37 °C. Subsequently, strips were briefly exposed to ammonium hydroxide vapor, and visualized bands were photographed under UV light (340 nm).

Heat Inactivation Assay—Heat inactivation experiments were performed using partially purified Hex A from unaffected or ATSD fibroblasts. Lysates from fibroblasts were prepared from fibroblasts in 10 mM sodium phosphate buffer, pH 6.1, 5% glycerol after sonication. DEAE columns were pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.1 (no salt). Lysates were applied, and the column was washed with 20 volumes of 10 mM sodium chloride in 10 mM sodium phosphate buffer, pH 6.1, to elute Hex A. Hex A was eluted using the same buffer containing 100 mM sodium chloride for 10 min. For heat inactivation experiments equal amounts of total protein (0.1–0.2 μg) from WT and mutant Hex A fractions were diluted in CP buffer, pH 4.2, containing 0.5% human serum albumin.

Prior to incubation at 42 °C, diluted samples of the mutant Hex A enzyme lacking or containing Hex inhibitors were left on ice for 15 min. Following incubation of the WT and mutant Hex A enzymes at 42 °C for the indicated time, the heated enzyme was then held on ice until completion of the time series. Time zero points correspond to samples that were not heat-treated and kept on ice for the duration of the heat inactivation experiment. The heat-treated samples were equilibrated to 37 °C for 10 min, followed by addition of MUGS substrate and incubation at 37 °C for a further 30 min.

Purification of Iron-dextran-labeled Lysosomes by Magnetic Chromatography—A lysosomal fraction was prepared from ATSD fibroblasts grown for 6 days in media lacking or containing 250 μg/ml (0.9 μM) of NGT, using a modification of a previously described protocol (14). Briefly, cells from one 150-mm tissue culture plate were incubated for 9 h at 37 °C in an iron-dextran solution (previously described) containing complete αMEM (with or without NGT). To chase the iron-dextran into lysosomes, cells were washed twice in PBS and incubated for a further 16 h in complete αMEM media lacking or containing NGT. Subsequently, cells were trypsinized, pelleted at 10,000 × g, and then sequentially washed in PBS followed by homogeni-
zation buffer (HB), consisting of 4 m M imidazole, pH 7.4, and 0.25 m M sucrose. To prepare a postnuclear supernatant (PNS) fraction, the cell pellet was resuspended in HB, homogenized with 10 strokes of a tight fitting Dounce homogenizer and finally centrifuged for 10 min. at 1000 × g. For the steel wool column, a 1-ml syringe fitted with a 23-gauge needle was packed with 60 mg of steel wool and held in place by a rare earth magnet. Prior to use, the column was washed with HB, loaded with the PNS, and then the column was washed with 10 column volumes of HB. To elute the iron-dextran-loaded lysosomes bound to the steel wool, the column was removed from the magnet and loaded with 10 m M sodium phosphate buffer, pH 6, containing 5 m M EDTA and 0.4% Triton X-100. Fractions were collected, and those containing increased MUG activity (“lysosomes”) were identified and stored frozen at −80 °C.

RESULTS

Competitive Inhibitors of Hex Attenuate Heat Inactivation of WT and ATSD Mutant Hex A—To assess the chaperoning potential of the inhibitors listed in Table I, the compounds were tested initially for their ability to attenuate heat inactivation of the ATSD mutant Hex A at 42 °C. Greater than 50% of the activity of partially purified Hex A from ATSD fibroblasts (Fig. 1a) was lost after 30 min, as compared with WT fibroblast Hex A whose activity was not significantly reduced. Attenuation of heat inactivation was observed when mutant Hex A was incubated at 42 °C in the presence of GalNAc, AddNJ, AdNJ, ACAS, or NGT, increased hydrolysis of MUGS was observed (Fig. 2). This effect was dose-dependent, but was limited by the toxicity of GalNAc and ACAS, both of which reduced cell viability above concentrations of 200 and 0.1 m M, respectively. The decline in effectiveness with decreasing concentration of inhibitors was greatest for GalNAc and least for ACAS, which was still effective in enhancing MUGS activity even at concentrations of 5 m M. These results also demonstrated that Hex inhibitors, which attenuated heat inactivation of mutant enzyme, also enhanced Hex A activity in ATSD fibroblasts. Furthermore, because the effective concentration at which the inhibitors enhance MUGS activity correlates with their Ki values (Table I), the observed increases are most likely due to the specific binding of the compounds to the α-active site of Hex A. Two compounds, DNJ and castanospermine, which lacked the 2-acetamido group, did not increase MUGS hydrolysis and were used as negative controls.

Treatment of ATSD Fibroblasts Results in Increased Levels of the Lysosomally Processed (Mature) α-Subunit and the Hex A Heterodimer—To directly show that treatment of ATSD fibroblasts with the inhibitory compounds results in increased amounts of the α-subunit in the lysosome, cell lysates were subjected to Western blotting with an anti-Hex A antibody (Fig. 3a). In comparison to untreated cells, increased amounts of a 56-kDa band corresponding to lysosomally processed α-subunit (αm) were seen in cells treated with AddNJ, GalNAc, NGT, or ACAS. The corresponding band was also seen in WT fibroblasts, but not in TTS fibroblast cells (which do not express the α-subunit). With the exception of cells treated with DNJ, the bands at 28 kDa, corresponding to lysosomally processed β-subunit of Hex (βm), remained largely unaffected by the treatments. As judged from densitometry, the increased levels of these bands (Fig. 3c) closely paralleled the increase in specific MUGS activity from lysates of inhibitor-treated ATSD cells. Of these, ATSD cells treated with 0.9 m M NGT showed the greatest increase in specific MUGS activity (−3-fold) and levels of mature α-subunit.

To rule out the unlikely possibility that the observed increased MUGS hydrolysis in treated cells was solely due to Hex S, the different Hex isoforms were resolved using cellulose acetate electrophoresis combined with MUG zymography (Fig. 3b). Increased amounts of a band that co-migrated with purified placental Hex A, were observed in inhibitor treated cell lysates, but could not be detected in untreated ATSD cells. Hex S (pI = 3.5), which migrates faster than Hex A (pI = 4.8), could not be detected. The increase in α-subunits (Fig. 3a) and the Hex A isoform (Fig. 3b) paralleled the increase in MUGS hydrolysis (Fig. 3c) indicating that the increased MUGS activity was a direct measure of increased Hex A in ATSD cells.

Hex A Activity in NGT-treated ATSD Fibroblasts Is Enriched in Lysosomes—To demonstrate directly that the increased Hex A (MUGS) activity is found in lysosomes, an ER-free lysosomal enriched fraction was prepared by magnetic chromatography of the crude lysates from untreated and NGT-treated ATSD fibroblasts loaded with iron-dextran colloid (18, 19). An ~10-fold enrichment in Hex A-specific activity was observed in the lysosomal fraction relative to the postnuclear supernatant (Fig. 4a). NGT treatment resulted in a greater than 6-fold increase in intracellular, lysosomal Hex A-specific activity relative to untreated cells. A similar increase in mature α-subunit protein levels was seen in the PNS and lysosomal fractions of NGT-treated cells (Fig. 4b). Although a Hex α-subunit-specific band is observed in the PNS from untreated cells, it corresponds to the α-chain precursor, because it is not found in the lysosomal fraction (Fig. 4b). Both glucocerebrosidase (another lysosomal enzyme) and the β-subunit of Hex are enriched in the lysosomal fraction relative to PNS, confirming the enrichment of lysosomal organelles in the “lysosome” fraction from both treated and untreated cells. Furthermore, these lysosomal-enriched fractions did not contain significant ER contamination as judged by Western blots probed with an antibody against calnexin, a resident ER protein (Fig. 4b). The combined results presented in Figs. 3 and 4 confirm that, in NGT-treated ATSD fibroblasts, there are increased amounts of functional mutant Hex A protein in lysosomes.

Kinetics of MUGS Activity Enhancement in ATSD Fibroblasts Grown in the Presence of NGT—Because NGT (Table I) resembles the reaction intermediate formed during catalysis by Hex (2), it is most likely to act as a specific Hex inhibitor. Thus, we further examined its potential as a chemical chaperone (Fig. 5a). Acid phosphatase activity (MUP hydrolysis) was used as a normalization factor, because the assay methodology is similar to Hex and very sensitive. Importantly, we also determined that MUP hydrolysis remains largely unchanged in NGT-treated versus untreated cells (Fig. 5b). When normalized to MUP hydrolysis, MUGS hydrolysis increased in a time- and concentration-dependent manner in NGT-treated ATSD fibroblasts. However, Hex A activity began to plateau after 6 days in cells treated with 0.9 m M NGT and after 4 days in cells treated with lower concentrations of NGT (0.036 and 0.18 m M). Thus, growth of cells for 6 days in the presence of NGT appeared to be optimal for induction of MUGS (Hex A) activity (Fig. 5a).

Enhanced Hex A Activity Persists in Treated Cells following Removal of ACAS and NGT from the Growth Media—MUGS activity continued to increase in ATSD fibroblasts initially grown in media containing NGT or ACAS (Fig. 6, a and b, filled symbols) for 6 days followed by culture in fresh drug-containing media for an additional 3 days. However, in ATSD fibroblasts...
### Table I

**List of compounds evaluated for enhancing Hex A activity**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>$K_i$ / $IC_{50}$</th>
<th>Reference(s)</th>
</tr>
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<td><img src="image1.png" alt="Structure 1" /></td>
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<td>(11)</td>
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<tr>
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<td>Castanospermine (CAS)</td>
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<td>(40)</td>
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<td>6-Acetamido-6-deoxycastanospermine (ACAS)</td>
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<td>(20)</td>
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<tr>
<td><img src="image7.png" alt="Structure 7" /></td>
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<td>$K_i = 280$ nM$^2$ \ $K_i = 300$ nM$^1$</td>
<td>(12)</td>
</tr>
</tbody>
</table>

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1. Value determined using human placental HEX.
2. Value determined using Jack Bean Hex.
initially grown in drug-containing media for 6 days and subsequently cultured in drug-free media, relative MUGS activities declined (Fig. 6, a and b, open symbols) but persisted at levels above those of untreated cells. After 2 days of growth in drug-free media, MUGS activity in NGT-treated ATSD cells was reduced to levels found in untreated cells. In contrast, even after 3 days growth in drug-free media MUGS activity was still increased 2-fold in ATSD cells previously treated with ACAS.

Thus, although relative to untreated cells MUGS (Hex A) activity in treated cells remained at elevated levels for 2–3 days following removal of the inhibitors, it eventually declined, demonstrating that the enhancing effects of ACAS and NGT are reversible. The decay kinetics of MUGS activity in drug-treated cells following removal of the inhibitors may reflect differences...
in the pharmacodynamics of NGT and ACAS, as well as the reduced half-life of the mutant enzyme in the lysosome.

**NGT Also Enhances Hex A in WT and Sandhoff Fibroblasts**

We next addressed the question of whether NGT can enhance the levels of activity of WT Hex A, S, or B, or a thermolabile mutant of Hex B (βP504S) found in some ASDs. We observed increased MUGS activity in treated ATSD fibroblasts. This increase in activity parallels the increase in amount of mature α-subunit from lysates of treated ATSD fibroblasts. The specific MUGS activity (nanomoles/mg total cell protein/h) from lysates used to produce the Western blot and zymogram in a and b is shown plotted (open bars) adjacent to the optical density of the αm band (filled bars) from the Western blot in panel c. The optical density was standardized relative to the density of the bands corresponding to βm.

**FIG. 3.** Increased MUGS activity in treated ATSD fibroblasts is associated with an increase in the mature α-subunit protein and levels of the active Hex A isozyme. a, Western blot using a rabbit polyclonal anti-human Hex A antibody, showing increased amounts of lysosomally processed, mature α-subunits (αm) in inhibitor-treated fibroblasts. Bands corresponding to the mature β-subunits (βm) are shown in the lower strip. Position of two relevant M, markers are shown to the left of the blot. b, resolution of the Hex isozymes from treated and untreated cell lysates by cellulose acetate electrophoresis shows increased amounts of Hex A activity (using MUG) in inhibitor treated fibroblasts (MU visualized under UV light). Bands corresponding to Hex A and Hex B are denoted by arrows to the right of the zymogram. For both a and b, the concentrations (mM) of inhibitor used are shown below the inhibitor name. c, the increase in MUGS activity parallels the increase in amount of mature α-subunit from lysates of treated ATSD fibroblasts. The specific MUGS activity (nanomoles/mg total cell protein/h) from lysates used to produce the Western blot and zymogram in a and b is shown plotted (open bars) adjacent to the optical density of the αm band (filled bars) from the Western blot in panel c. The optical density was standardized relative to the density of the bands corresponding to βm.

**FIG. 4.** Increased MUGS (Hex A) activity is found in the lysosomal fraction from NGT treated ATSD fibroblasts. a, comparison of MUGS activity (nanomoles/mg of total cell protein/h) in the postnuclear supernatant (PNS) and lysosome-enriched (Lyso.) fraction from untreated (open bar) and NGT (0.9 mM) treated (filled bar) ATSD fibroblasts. b, Western blots comparing the levels of α-subunits of Hex (α Hex), mature β-subunits of Hex (β Hex), glucocerebrosidase (Gcase), and calnexin in the PNS and lysosomal fractions (Lyso.) from untreated and NGT-treated ATSD cells. Position of relevant M, markers (in kDa) are shown to the left of the blots. One microgram of total protein from each of the PNS and lysosomal fractions were analyzed.
patients. Hex A/S activity and total Hex A/B/S activity were assessed based on their established MUG/MUGS values of ~4, 150, and 1, respectively. These ratios were used to obtain an estimation of the relative levels of each Hex isozyme. With increasing concentrations of NGT, MUGS activity in ATSD cells increased (Fig. 7d), whereas the MUG/MUGS ratio declined. The initial high MUG/MUGS ratio is due to the predominance of Hex B isozyme. However, as the relative levels of Hex A increased the MUG/MUGS ratio declined, approaching that found in unaffected fibroblasts (Fig. 7a).

In fibroblasts from a patient with ISD (homozygous for a 16-kb HEXB 5′ deletion mutation), all detectable Hex activity is due to Hex S, as indicated by the MUG/MUGS of ~1 in untreated cells (Fig. 7c). A parallel and coincident rise in MUG and MUGS activity with increasing NGT concentration was observed, consistent with an increase in Hex S activity, presumably in the lysosome. At the highest concentration of NGT (0.9 mM), there was a >5-fold increase in MUG and MUGS activities.

In contrast to the enhancement of Hex S levels in ISD fibroblasts, only a modest (~25%), but consistent increase in MUGS activity was seen in unaffected fibroblasts (Fig. 7a) treated with NGT. This was coupled with decreased MUG activity, resulting in a more than 2-fold decrease in the MUG/MUGS ratio. These data suggest that NGT treatment of normal fibroblasts promotes the formation of heterodimeric Hex A at the expense of homodimeric Hex B assembly in the ER.

To determine whether NGT can increase the formation of WT β-dimers in the ER, ultimately increasing lysosomal WT Hex B levels, ITSD cells (Fig. 7b) were treated with NGT. Because these ITSD cells possess a 4-bp insertion in the coding region of their HEXA genes, which results in a frameshift mutation, they have no Hex A or S isozymes and any increase in MUG activity can be attributed to Hex B. No significant changes in either MUG or MUGS activity were observed in the ITSD line (Fig. 7b). Thus, NGT does not appear to significantly enhance the folding and dimerization of WT β in the ER. The ITSD cell line can thus be viewed as a negative control showing that the increase in MUG and/or MUGS activity is due to lysosomal Hex A or S and not some other unrelated enzyme, which would also be present in the ITSD cells.

**Fig. 5. Kinetics of Hex A activity enhancement.** a, MUGS activity in ATSD fibroblasts grown in the presence of three different concentrations of NGT over a 9-day period. Shown is the relative increase in MU fluorescence from MUGS hydrolysis (MU from treated/MU from untreated) in lysates from ATSD fibroblasts grown in different concentrations of NGT (corresponding symbols shown in the left-hand corner of the graph) for increasing periods of time (days). Each MUGS assay was further normalized using the acid phosphatase activity (MUP) of the same lysate. b, demonstrates that MUP hydrolysis is a valid means of normalization, because there were no changes in relative acid phosphatase activity (MUP) in another set of paired treated/untreated cell lysates over the same 9-day period. For each of the graphs, standard deviations are shown above the data points (average MU fluorescence from three wells of treated cells/three wells of untreated cells grown for the same period of time). Dashed lines denote the position at which there is no change in MU fluorescence, i.e. relative increase = 1. The **tities** to the left and bottom of all panels describe the y- and x-axis of all graphs in the figure.
To determine whether mutant β-subunits could be stabilized and thus rescued, ASD patient fibroblasts were treated with NGT. An increase in both MUG and MUGS activity with increasing levels of NGT was accompanied by a general decline in the MUG/MUGS ratio (Fig. 7e), indicating a relative increase in the level of Hex A/S after treatment. However, in cells treated with 0.03 mM NGT the MUG/MUGS ratio was still significantly above that of untreated cells. These observations combined with the increased MUG activity at the lowest NGT concentrations support the conclusion that Hex A levels, as well as Hex S, are likely being increased, i.e. both wild type α- and mutant β-subunits appear to be stabilized by NGT.

Other Lysosomal Enzymes Are Not Significantly Affected by NGT Treatment—To confirm that in vivo NGT is specifically affecting Hex activity, the activities of β-glucuronidase and β-galactosidase as well as acid phosphatase (see also Fig. 5b) were also examined. Unlike Hex, in all NGT-treated cell lines, the activity of these other three lysosomal enzymes remained largely unaffected (Fig. 8).

DISCUSSION

We have shown that a panel of Hex inhibitors can increase Hex A activity in fibroblasts from both unaffected individuals and patients with chronic GM2 gangliosidosis. These compounds contain an 2-acetamido group, which in substrates for Family 20 enzymes (12) like Hex, acts as the catalytic nucleophile. We have focused on NGT, because it is a stable mimic of the reaction intermediate of Family 20 enzymes, a cyclized oxazolinium ion (12) (Table I; thus, it is the most likely to act as a specific Hex inhibitor. For example, although ACAS and NGT inhibit Hex, ACAS also inhibits α-N-acetylgalcosaminidase (21), whereas NGT does not. Furthermore, NGT did not appear to reduce cell numbers even at the highest concentrations used, and it is easily synthesized in gram quantities. Thus, in this report, the chaperoning properties of NGT were examined more thoroughly.

Four independent approaches support the conclusion that Hex inhibitors added to culture media resulted in increased amounts of active mutant Hex A in the lysosomes from ATSD patient fibroblasts. First, treatment of fibroblasts resulted in increased intracellular MUGS hydrolysis (the preferential indicator of Hex A/S activity) relative to total cell protein or other lysosomal enzymes (Figs. 3–5, 8). Second, Western blots documented an increased level in mature (lysosomal) α-subunit in treated cells (Fig. 3a). Third, subcellular fractionation of NGT-treated cells into an ER-depleted lysosomal-enriched fraction, resulted in a further 10-fold increase in MUGS-specific activity (Fig. 4). Lastly, separation of the Hex isozymes in treated cells by CAE demonstrated that their increased MUGS activity and α-protein were associated with an increase in the active Hex A

isoenzyme (Fig. 3b). These results document that inhibitors of Hex can rescue the mutant enzyme.

A seeming paradox that arises from these experiments is “how can inhibitors of Hex A result in its increased activity”? First, the in vivo substrates of Hex A, e.g. GM2, are not present in the ER (22) where the inhibitors function as pharmacological chaperones. Second, even in cases of ATSD or ASD, the in vivo substrates of Hex A (GM2) are highly concentrated, well above the $K_m$ of Hex A, in patients’ lysosomes (7). This large differential in endogenous substrate versus inhibitor (NGT or ACAS) concentrations in the two compartments, would favor inhibitor binding by the enzyme in the ER (locking Hex into a conformation compatible with exit from the ER), but favor inhibitor release once the enzyme-inhibitor complex reached the substrate-rich lysosome. Once released by Hex A the inhibitor would likely then be treated like any other small molecule that was a product of lysosomal degradation and be actively transported out of the lysosome (23). Because TSD and SD fibroblasts contain much lower levels of stored substrate that do neuronal cells, the enzyme enhancement we have documented in this report would be the minimum expected if such an approach were to be adopted for patient treatment. As well, if patients were to be treated by this approach, the dose of a compound such as NGT could be carefully regulated during the course of the treatment to minimize any potential inhibitory effect on lysosomal Hex.

Although the Hex inhibitory compounds differ in their $K_i$ values and, thus, their effective concentration ranges, all were able to enhance mutant Hex A activity in ATSD cells. The fact the effective concentrations were above the $K_i$ values of the compounds most likely reflects the bioavailability of the compounds in terms of membrane permeability. Although we only demonstrated the ability of NGTs to stabilize mutant β-subunits and, thus, enhance residual Hex A activity in fibroblasts from an ASD patient, it is expected that most Hex inhibitors will also function as pharmacological chaperones in ASD.

Because NGT, as well as the other inhibitory compounds, affect the ratio of the Hex isozymes even in normal cells, they appear to exert their effect on Hex early during its biosynthesis and assembly, i.e. in the ER. However, it is not clear whether these inhibitors act by stabilizing the monomers, the dimers, or both. Although the α- and β-monomers are catalytically inactive, each subunit does possess an active site that in the dimer is stabilized by residues supplied by its companion subunit. Thus, the monomers may be capable of binding the inhibitors (2).

The fact that NGT treatment of fibroblasts can augment the levels of WT α-subunit, but not WT β-subunits, is not surprising. Previous data on the biosynthesis and assembly of Hex (24) suggests that a pool of monomeric α-subunits are retained in the ER until they either assemble with newly synthesized β subunits to form heterodimeric Hex A or are degraded. This pool would exist because of the low affinity of the α-subunits for each other. However, a small amount of α-homodimers, Hex S, may be able to form when a sufficiently high concentration of properly folded α-monomer is reached, e.g. due to a lack of β-subunits in Sandhoff disease. Because homodimers of β-subunits are more stable than the Hex A heterodimers, some Hex α-subunits are retained in the ER until they either assemble with newly synthesized β-subunits or are degraded. This suggests that a pool of monomeric α-subunits would likely then be treated like any other small molecule that was a product of lysosomal degradation and be actively transported out of the lysosome (23). Because TSD and SD fibroblasts contain much lower levels of stored substrate that do neuronal cells, the enzyme enhancement we have documented in this report would be the minimum expected if such an approach were to be adopted for patient treatment. As well, if patients were to be treated by this approach, the dose of a compound such as NGT could be carefully regulated during the course of the treatment to minimize any potential inhibitory effect on lysosomal Hex.

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Because NGT was able to rescue the Hex A activity in both
ATSD fibroblasts homozygous for the α2G69S mutation and ASD fibroblasts heterozygous for the βF504S mutation and the 16-kb 5′ HEXB deletion, other Hex mutants should also be rescued using this approach. Candidates would be mutations that are associated with detectable levels of residual Hex A activity and protein. Additionally, if this residual activity is thermostable, the patient with the mutation would be an even better candidate for treatment. To date, like the α2G69S and βF504S Hex mutants, the P-glycoprotein (25), β-galactosidase (26), α-galactosidase (27, 28), and glucocerebrosidase (29) mutants, which have also been rescued by this approach, all were associated with some residual thermostable enzyme activity (27, 29). Thus, Hex mutants, such as αY180H (30), αV391 M (31), αK197T (32), βF505N (33), or βA543T (33, 34) that have residual enzyme activity and are associated with ATSD/ASD, would be candidates for this therapeutic approach. Finally, because we also found enhanced Hex A activity in treated normal cells, TSD patients with partial splice junction defects (35), may also be candidates.

Patients with mutations in their HEXA or HEXB genes display symptoms when their Hex A activity is ≤10% of WT levels. In ATSD, no Hex S can be detected, but normal levels of Hex B (MUG/MUGS ratio is ~150/1) are present. Based on the MUG/MUGS ratio of pure Hex A and Hex B and assuming no Hex S, the residual Hex A activity in untreated cells can be calculated to be 6.8% of WT fibroblasts increasing to 37% in NGT-treated cells, significantly above the critical threshold of activity required for a patient to become asymptomatic. Currently the only potential therapeutic approach for ATSD or ASD patients is the use of α-n-butyl-DNJ (NB-DNJ) to inhibit the synthesis of GM2 and other higher gangliosides, i.e., substrate deprivation therapy (36, 37). The goal of this approach is to reduce the incoming GM2 levels to a point below the maximum turnover rate of the patient’s residual Hex A activity. If successful, this should reverse the progressive nature of ASTD and ASD. Three problems are associated with this approach 1) the toxic effects of the NB-DNJ on liver and spleen observed in treated humans (38) and mice (36), 2) the continued accumulation of oligosaccharides from glycoprotein degradation in the lysosomes of patients with ASD (37), and 3) its general lack of specificity, as which affect other ganglioside biosynthesis pathways. Although our data indicate that the pharmacological chaperone approach, which may affect other ganglioside biosynthesis pathways. Therefore, we also included the substrate deprivation therapy. This would allow lower concentrations of NB-DNJ to be used, due to the synergistic effects of lowering GM2 synthesis and raising residual Hex A levels.

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