A sensitive fluorescence-based assay for monitoring GM2 ganglioside hydrolysis in live patient cells and their lysates

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Enzyme enhancement therapy, utilizing small molecules as pharmacological chaperones, is an attractive approach for the treatment of lysosomal storage diseases that are associated with protein misfolding. However, pharmacological chaperones are also inhibitors of their target enzyme. Thus, a major concern with this approach is that, despite enhancing protein folding within, and intracellular transport of the functional mutant enzyme out of the endoplasmic reticulum, the chaperone will continue to inhibit the enzyme in the lysosome, preventing substrate clearance. Here we demonstrate that the in vitro hydrolysis of a fluorescent derivative of lyso-GM2 ganglioside, like natural GM2 gangliosides, is specifically carried out by the β-hexosaminidase A isozyme, requires the GM2 activator protein as a co-factor, increases when the derivative is incorporated into anionic liposomes and follows similar Michaelis–Menten kinetics. This substrate can also be used to differentiate between lysates from normal and GM2 activator-deficient cells. When added to the growth medium of cells, the substrate is internalized and primarily incorporated into lysosomes. Utilizing adult Tay–Sachs fibroblasts that have been pre-treated with the pharmacological chaperone Pyrimethamine and subsequently loaded with this substrate, we demonstrate an increase in both the levels of mutant β-hexosaminidase A and substrate-hydrolysis as compared to mock-treated cells.

\textbf{Keywords:} AB-variant GM2 gangliosidosis/enzyme enhancement therapy/Pyrimethamine/Sandhoff disease/Tay–Sachs disease

\section*{Introduction}

Gangliosides are acidic, amphipathic molecules with a lipid portion, which is inserted into cell membranes, and a soluble accessible oligosaccharide moiety. Gangliosides are found at low levels in all animal tissues outside of the brain, where they can constitute as much as 6\% of total lipids. Catabolism of gangliosides occurs in the lysosome through a series of intermediate steps catalyzed by exoglycosidases that can eventually produce ceramide, the lipid backbone. When one of these glycosidases is deficient, further catabolism is blocked and the associated substrate molecule is stored, resulting in a lysosomal storage disease (LSD) (Jeyakumar et al. 2002).

GM2 ganglioside (GM2) is predominantly formed during the synthesis and breakdown of the higher (more complex oligosaccharide moieties) brain gangliosides, e.g., GM1 ganglioside. Its terminal, non-reducing, β-linked GalNAc residue is cleaved by lysosomal β-hexosaminidase A (Hex A). However, this reaction also requires a small sphingolipid activator protein, the GM2 activator protein (GM2AP), which acts as a substrate-specific cofactor (Meier et al. 1991). GM2AP removes GM2 from the lysosomal membrane, forming a soluble complex that can then be bound by Hex A (Wright et al. 2000; Mark et al. 2003). Thus the GM2-GM2AP complex is the true natural substrate for Hex A. Two major Hex isozymes exist in normal human tissue, heterodimeric Hex A (αβ) and homodimeric Hex B (ββ). The primary sequences of the α- and β-subunits are ∼60\% identical and both subunits have a similar active site. However, dimerization is necessary in order for either active site to become functional (Maier et al. 2003; Mark et al. 2003). Additionally, the β-active site lacks a positively charged pocket necessary to efficiently bind the negatively charged sialic acid residue of GM2 or the 6-sulfate group from the artificial substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside-6-sulfate (MUGS). Similarly, it also lacks a critical loop structure necessary to efficiently bind GM2AP (Sharma et al. 2001, 2003; Mark et al. 2003; Zarghooni et al. 2004; Lemieux et al. 2006). Thus only Hex A can bind the GM2–GM2AP complex (Mark et al. 2003). However both isozymes can hydrolyze the neutral artificial substrate, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG). The MUG/MUGS ratio for Hex A is 3.7:1 and ∼300:1 for Hex B (Hou et al. 1996). Thus the degradation of GM2 requires the correct synthesis, folding, assembly and intracellular transport of three gene products, the α- and β-subunits of Hex A, and GM2AP. A deficiency of any one of these proteins, below a surprisingly low critical threshold of ∼10\% of normal activity (Leinekugel et al. 1992), results in one of the three forms of GM2 gangliosidosis (OMIM 230700), i.e., Tay–Sachs, Sandhoff or the AB-variant, respectively (reviewed in Gravel et al. (1995) and Mahuran (1999)).

The late onset forms of GM2 gangliosidosis, which retain ∼4\% residual wild-type Hex A activity, primarily affect the ability of the mutant subunit to fold and the dimers to assemble (reviewed in Mahuran (1999)). The most common mutation resulting in adult Tay–Sachs disease (ATSD) is αG269S (Navon et al. 1990). As only a 2- to 3-fold increase in the residual activity of these patients would theoretically prevent and possibly reverse GM2 storage, small stabilizing molecules have recently...
been sought as potential agents for enzyme enhancement therapy (EET). To date, all the small molecules that have been able to enhance αG269S Hex A activity in patient cells have also been competitive inhibitors of the enzyme (Tropak et al. 2004, 2007; Maegawa et al. 2007), i.e., pharmacological chaperones (PCs). The theory is that the inhibitor will bind and stabilize the functional-fold of the mutant α-subunit in the endoplasmic reticulum (ER), preventing its degradation by the ER quality control system, allowing the subunits to assemble into Hex A, exit the ER, and be transported to the lysosome. Once the inhibitor-Hex A complex enters the lysosome, the inhibitor will be competed out of the complex by the stored substrate. However, a tighter binding of the PC to the enzyme at the neutral pH of the ER than at the acidic pH of the lysosome would represent an additional desirable characteristic (reviewed in Tropak and Mahuran (2007)).

We have recently identified pyrimethamine (Pyr), a drug previously used to treat malaria, as a potential PC for ATSD (Maegawa et al. 2007). Pyr increased the residual activity of αG269S Hex A in patient fibroblasts by ~3-fold. However, these activity measurements were performed using total cell lysates and the artificial, GM2AP-independent, MUGS substrate. Additionally, the assay procedure involves the dilution of each cell lystate, which could reduce the inhibitory effect from any residual intra-lysosomal Pyr. Finally, since normal fibroblasts synthesize little of the higher gangliosides, ATSD fibroblasts do not store GM2 and no excess substrate would be present in these cells’ lysosomes to compete off the Pyr from the Hex A-Pyr complex. Thus a more sensitive method, based on first loading the lysosomes of patient cells with GM2 or a suitable derivative, followed by measuring its hydrolysis rate with or without pre-treatment of the cells with Pyr, is necessary to fully assess the efficacy of EET with Pyr or any other PC for ATSD.

Assaying Hex A enzymatic activity toward its natural substrate, even in vitro has generally been impractical outside of specialized laboratories, partially because of the high costs of obtaining sufficient amounts of purified GM2 and then radiolabeling it in order to perform the assay (Novak et al. 1979). Additionally, the original “natural substrate” assay required large amounts of Hex A activity, ~2500 Units (nmol h⁻¹) MUGS (purified placental Hex A ~2,700,000 U mg⁻¹), to generate a significant signal (Meier et al. 1991). Sandhoff and colleagues have made a major improvement to the natural substrate assay by demonstrating that the incorporation of radiolabeled GM2 into negatively charged liposomes, which more closely mimics the intra-luminal lysosomal membrane, increases the transfer rate of GM2 by GM2AP to Hex A for hydrolysis by 20- to 150-fold (Werth et al. 2001). In this report, we develop and validate a further improvement in the assay methodology by using a fluorogenic GM2 ganglioside analog, consisting of the fluorophore nitro-2,1,3-benzoxadiazol (NBD)-4-yl, covalently attached to a short (C6) sn2 acyl chain of lyso-GM2 ganglioside (NBD-GM2) as the substrate. We then demonstrate that NBD-GM2 is internalized by human fibroblasts into lysosomes and that there is an increased rate of in cellulo hydrolysis of this substrate after treatment of ATSD cells with Pyr.

Results

We initially tested the specificity of NBD-GM2 as a substrate for hydrolysis by using various amounts of purified placental Hex A or Hex B, in the presence or absence of recombinant GM2AP (rGM2AP, Mr 17,500). Since the hydrolysis rates of naturally occurring GM2 by Hex A and GM2AP is enhanced by its presence in liposomes containing other anionic lipids (Werth et al. 2001; Kolter and Sandhoff 2005), our initial assays utilized liposomes containing 20 mol% phosphatidyl inositol. After a 3 h incubation period, the residual substrate and hydrolysis products were separated by HPTLC. The resolved fluorescent bands were scanned and quantified using a Storm imaging system. Preliminary testing showed that the linear response of the Imager has a range of at least 2 orders of magnitude for this particular fluorophore (data not shown). The single major band seen in the substrate-only lane (Fig. 1, Ctrl) was unaffected by purified hexosaminidase Hex B levels up to 200 μg/mL (10 μg/assay mix; 333 U MUG or 100,000 U MUG), at which point a second product band appeared. This reaction was independent of the presence of rGM2AP (Fig. 1, Hex B lanes). In contrast the same product band was visible when incubating NBD-GM2 with as little as 0.2 μg/mL (0.01 μg/assay mix; 27 U MUGS or 100 U MUG) of purified Hex A. However, in this case the reaction was dependent on the presence of rGM2AP (Fig. 1, Hex A lanes). Thus in the presence of rGM2AP, Hex A is ~1000-fold more active toward NBD-GM2 than is Hex B.

To confirm the identities of the two major bands produced after HPTLC as NBD-GM2 and NBD-GM3, we extracted the corresponding material generated after overnight hydrolysis and analyzed it by mass spectrometry (Figure 2). The bands produced peaks (NBD-GM2 1393.6 and NBD-GM3 1189.6) matching the predicted masses of the two derivatives (1393.65 and 1189.56). Furthermore, fragmentation of both parent ions yielded a diagnostic ion of neutral mass 290.1 that is consistent with the collision-induced release of sialic acid (Tsui et al. 2005). The ion fragments at 1102.5 and 898.5 matched the predicted masses of the NBD-asialo-ganglioside derivatives. Thus, the lower band and the slightly more rapidly migrating band denoted by the arrows (Figure 1) correspond respectively to the substrate, NBD-GM2, and product, NBD-GM3, of the Hex A and rGM2AP catalyzed reaction.
Fig. 2. MS/MS profile of the extracted bands from a HPTLC separation of the substrate (NBD-GM2) and product (NBD-GM3) generated by incubating overnight NBD-GM2 with Hex A and rGM2AP. In NBD-GM2, the fatty acid of the naturally occurring ganglioside has been replaced with a fluorescent short acyl chain NBD fatty acid, the C6-NBD group. In the presence of Hex A and rGM2AP, the terminal GalNAc (dashed oval) is released resulting in the product NBD-GM3. The predicted and experimentally determined sizes of the derivatives are shown alongside the product ion spectra of the precursor ions 1393.6 (698.8 − 2) and 1189.6, which correspond to NBD-GM2 (top) and NBD-GM3 (bottom), respectively. The loss of the terminal sialic acid moiety, 290.1, from either compound results in peaks of 1102.5 NBD-GM2 (top) and 898.5 NBD-GM3 (bottom). The product ion spectrum of NBD-GM2 has been reconstructed in the form of singly charged ions. Both NBD-GM2 and NBD-asialo-GM2 were present as double charged ions in the original spectrum (698.8 and 550.3, respectively). To aid in comparison with NBD-GM3, the doubly charged peaks produced from NBD-GM2 are also shown reconstructed as singly charged ions.

Fig. 3. Kinetic characterization of NBD-GM2 hydrolysis by Hex A with rGM2AP acting as a co-factor. (A) Comparison of the hydrolysis rate of NBD-GM2 when presented as simple micelles (open squares), in neutral (filled circle) or acidic (open upward triangles) liposomes, or acidic liposomes that had been extruded through a 100 nm filter (filled downward triangle). (B) Michaelis–Menten plot of the hydrolysis rates of NBD-GM2 incorporated into acidic liposomes in the presence of increasing concentrations of rGM2AP. (C) Michaelis–Menten plot of the hydrolysis rates of increasing amounts of the NBD-GM2 substrate contained in acidic liposomes in the presence of saturating levels of rGM2AP.

The kinetics of the NBD-GM2 assay using purified Hex A and rGM2AP were examined in greater detail. First, the effects on hydrolysis rates of presenting NBD-GM2 to the rGM2AP and Hex A in neutral versus anionic liposomes were determined. The rate of hydrolysis of NBD-GM2 incorporated into anionic liposomes, 16 ± 1 (standard error, S.E.) pmoles NBD-GM2 h⁻¹ U⁻¹ (MUGS), was 26-fold greater than when it was incorporated into neutral liposomes (Figure 3A). The rate was not significantly affected by extruding the anionic liposomes through a polycarbonate filter (100 nm), nor was the rate of hydrolysis from neutral liposomes found to be significantly
different from that obtained when the substrate was presented in a simple micellar form (Figure 3A).

The dependence of Hex A on rGM2AP to act as a substrate-specific cofactor for the hydrolysis of NBD-GM2 was examined kinetically. Hydrolysis levels with various amounts of rGM2AP were determined and the data fitted to the Michaelis–Menten equation. This resulted in an apparent $K_m$ of 0.16 ± 0.06 μM (2.2 ± 0.8 μg mL$^{-1}$ rGM2AP) and $V_{max}$ of 18 ± 2 pmole NBD-GM3 h$^{-1}$ U$^{-1}$ (MUGS) (Figure 3B). A similar kinetic analysis utilizing saturating levels of rGM2AP and various amounts of NBD-GM2 provided an apparent $K_m$ of 80 ± 40 μM, and $V_{max}$ of 24 ± 6 pmole NBD-GM3 h$^{-1}$ U$^{-1}$ (Figure 3C).

Given the increased sensitivity gained by the use of fluorescent NBD-GM2 incorporated into anionic liposomes as a substrate, and the specificity of this substrate toward both GM2AP and the Hex A isozyme, we tested the new assay protocol as a potential tool to identify AB-variant fibroblasts utilizing cell lysates as a source for both Hex A and GM2AP. Since cell lysates also contain all the other necessary hydrolases for the total turnover of NBD-GM2, we included conduritol B epoxide (CBE), a non-reversible inhibitor of glucocerebrosidase, in the assay mix. CBE arrests the degradation pathway of NBD-GM2 at the NBD-glucosyl-ceramide (NBD-GlcCer) step. Lysate from normal fibroblasts produced a weak, but easily detectable NBD-GM2 band (Figure 4, lanes 2 and 3) that was not present in the substrate blank (lane 1). When 1 μg (20 μg mL$^{-1}$) of rGM2AP was present in the reaction, much more intense GM3 bands were produced (Figure 4, lanes 4 and 5), consistent with the low level of GM2AP found in fibroblasts (Xie et al. 1991). With this increased level of NBD-GM2 hydrolysis, the effect of adding CBE became apparent. When compared to the mix without CBE (lane 4), a more intense, faster migrating band (NBD-GlcCer) was detectable in the CBE-containing assay mix (lane 5). The band migrating between NBD-GM3 and NBD-GlcCer, seen in both lanes, but not the substrate blank, likely corresponds to the asialo derivative of NBD-GM3, NBD-lactosyl-ceramide (NBD-LacCer). In comparison with the normal fibroblasts, lysates containing the same level of MUGS activity (60 U) from AB-variant cells produced a pattern (Figure 4, lanes 6 and 7) indistinguishable from the substrate blank (lane 1). The addition of rGM2AP to the assay mix along with the lysate from AB-variant cells produced a pattern identical to that seen in lane 4 (Figure 4 lane 8). Thus, this assay could be used in a clinical laboratory to diagnose the AB-variant form of GM2 gangliosidosis from patient fibroblasts.

The above data indicate that NBD-GM2 is a valid substitute for naturally occurring GM2 for the evaluation GM2AP-Hex A activity in vitro. We next determined if it could be used in cellulo to further demonstrate the efficacy of Pyr as a PC for the enhancement of mutant Hex A in G269S/G269S ATSD fibroblasts (Maegawa et al. 2007). Since Hex B levels are normal in Tay–Sachs cells, in order to fully evaluate the effects of Pyr in these cells, we generated an α-subunit of Hex A-specific antibody by absorbing our rabbit anti-Hex A IgG with excess placental Hex B to remove the anti-β-subunit components. The specificity of the antibody was first evaluated by indirect immunofluorescence staining and confocal microscopy imaging. When infantile Tay–Sachs fibroblasts carrying two null α-alleles were examined, only a faint and diffuse staining of non-specific background was observed (Figure 5, compare intensity and staining pattern of 5α, ITSD, with 5d, WT, in green). Importantly, none of the background signal co-localized with the punctate staining pattern of the lysosomal marker Lamp-1 (Figure 5, ITSD, red) as shown by the absence of yellow in the merge panel (Figure 5C). In contrast wild-type fibroblasts produced an intense Hex A-staining pattern, much of which strongly co-localized with the Lamp-1 signal (Figure 5D–F, WT). However, a significant amount of the green signal (α-subunit) did not co-localize with the red (Lamp-1). This likely represents a pool of monomeric α-subunits, which is believed to be retained in the ER to facilitate heterodimer formation with newly synthesized β-subunits (Proia et al. 1984; Mahuran 1991). A similar examination of ATSD fibroblast produced the expected staining pattern of intermediate intensity, with much less co-localization of the Hex A (α-subunit) and Lamp-1 signals (Figure 5G–I, ATSD, Merge).

Next, we used confocal microscopy imaging to examine the fate of NBD-GM2 added overnight to the medium of wild-type fibroblasts using its intrinsic fluorescence properties. Lysosomes of the wild-type fibroblasts were visualized with lysotracker red DND 99 (Figure 6, WT, red). In wild-type cells, the vast majority of NBD-GM2 co-localized with lysotracker (Figure 6, WT, Merge). Thus we were able to use NBD-GM2 as a marker for lysosomes and determine the effect of Pyr-treatment on the Hex A signal in ATSD cells (Figure 6, ATSD, red). Using the same confocal imaging settings, the intensity of the Hex A signal in untreated cells was observably less than that in treated ATSD cells (compare the red in Figure 6E and H). These observations correlated well with the ~3-fold increase in MUGS-activity determined from parallel treated versus untreated plates of ATSD cells (Table I). Additionally the degree of co-localization of the NBD-GM2 and Hex A signals was greatly increased in Pyr-treated ATSD fibroblast (Figure 6F and I ATSD versus ATSD + Pyr, Merge). These data indicate that, as previously demonstrated, when measuring MUGS activity after subcellular fractionation of ATSD cells (Maegawa et al. 2007), Pyr-treatment increases the steady-state level of lysosomal Hex A in ATSD cells. Additionally since NBD-GM2 appears to be primarily internalized by cells through pathways that end in the lysosome, it offers itself as an ideal artificial substrate for assessing the in cellulo efficacy of EET-agents, e.g., Pyr, for ATSD.
Fig. 5. Validation of the specificity of an anti-human Hex A isozyme (α-subunit) IgG obtained after absorption of a polyclonal rabbit anti-Hex IgG with purified Hex B. Fibroblasts from a patient with infantile TSD (ITSD, A–C), unaffected (WT, D–F) or ATSD (G–I) were incubated with the prepared Hex α-subunit-specific rabbit IgG (A, D, G) and a mouse Lamp-1 IgG (B, E, H), followed by fluorescently labeled secondary antibodies against rabbit (green) or mouse (red) IgG. Merged images are shown in the right most panels with co-localization of the signals appearing as yellow-orange (C, F, I).

Finally, we evaluated the levels of hydrolysis of NBD-GM2 in Pyr-treated versus untreated ATSD fibroblasts from three independent pairs of plates. After 11 days of treatment, NBD-GM2 and CBE were added to FBS-free media, incubated overnight, washed and chased for 2 h with fresh FBS-containing media, and the cells from each of the six plates were subjected to a Folch extraction (Folch et al. 1957). Both the upper (acidic glycolipid-enriched, Figure 7A) and lower (neutral glycolipid-enriched, Figure 7B) phases were analyzed by HPTLC. The upper phase extracts from each of the three plates of Pyr-treated cells produced more intense NBD-GM3 bands (Figure 7A, lanes Pyr1–Pyr3), as compared to mock-treated ATSD control cells (Figure 7B, Ctrl1–Ctrl3). Additionally, because of the inclusion of CBE in the growth media, the NBD-GlcCer, as well as the NBD-LacCer bands present in the lower-phase separation could be used as downstream markers of Hex A activity. Again extracts from each of the three plates of Pyr-treated cells produced more intense NBD-GlcCer and NBD-LacCer bands

Table I. Pyr-treatment of ATSD cells increases both Hex A activity and NBD-GM2 hydrolysis in cellulo

<table>
<thead>
<tr>
<th>Assay</th>
<th>Pyr – treated cells</th>
<th>Ctrl – untreated cells</th>
<th>Fold increase over Ctrl</th>
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<tbody>
<tr>
<td>Hex A (MUGS)a</td>
<td>46,000 ± 1,700e</td>
<td>14,000 ± 1,600</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>β-Galactosidaseb</td>
<td>12,000 ± 500</td>
<td>9,200 ± 1,300</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>NBD-GM3c</td>
<td>2,000,000 ± 410,000</td>
<td>520,000 ± 48,000</td>
<td>3.9 ± 0.9</td>
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<tr>
<td>NBD-LacCer</td>
<td>1,900,000 ± 530,000</td>
<td>510,000 ± 87,000</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>NBD-GlcCer</td>
<td>5,700,000 ± 680,000</td>
<td>1,900,000 ± 290,000</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>NBD-totald</td>
<td>9,600,000 ± 600,000</td>
<td>2,900,000 ± 300,000</td>
<td>3.3 ± 0.2</td>
</tr>
</tbody>
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aActivity in relative fluorescence units.
bUsed as a control lysosomal enzyme.
cAverage intensity, in relative fluorescence units, of each of the NBD-species summed across the corresponding sets of sample lanes.
dAverage intensities, in relative fluorescence units, of the NBD-GM3, -LacCer, and -GlcCer bands summed downward in each set of sample lane.
eS.E. (n = 3).
Monitoring GM2 ganglioside hydrolysis in live patient cells

**Fig. 6.** Increased colocalization of αG269S Hex A with lysosomal NBD-GM2 in Pyr-treated ATSD fibroblasts. Wild-type fibroblasts were treated with NBD-GM2 (WT, A) and Lysotracker (WT, B). The merged images for the two fluorescent molecules show virtually total overlap of the NBD-GM2 signal (green) with that of the lysosomal marker (Lysotracker, red), as denoted by the yellow-orange punctate pattern (WT, C). Fibroblasts from mock-treated (ATSD; D–F) and Pyr-treated (ATSD + Pyr; G–I) ATSD patient were loaded with NBD-GM2 (visualized in green in D and G) and incubated with our Hex A-specific primary IgG (visualized in red in E and H). Merged images of the two fluorophores (F, I) show an increased degree of overlap of the two probes (denoted by the yellow-orange color) in the treated fibroblasts (ATSD + Pyr).

than did any of the three extracts from untreated ATSD control plates. Comparison of the fluorescence intensities of the bands corresponding to the different NBD derivatives from Pyr-versus mock-treated ATSD cells revealed an ~3-fold increase in the levels of NBD-GM3 and downstream products NBD-LacCer and NBD-GlcCer, resulting from the initial hydrolysis of NBD-GM2 by Hex A (Table I). This increase is not significantly different from the ~3-fold increase in the residual Hex A activity (Figure 7B, Table I) obtained after Pyr-treatment of ATSD cells.

**Discussion**

EET utilizing small molecules, usually acting as PCs, is a promising new approach for treating LSD variants that are associated with a severely reduced, but not absent acidic glycohydrolase activity (Tropak and Mahuran 2007). The major roadblock in validating the efficacy of EET is the lack of suitable animal models to test novel PCs in vivo. Most mouse models of LSDs, described thus far, are knockouts without any residual enzymatic activity that can be targeted for enhancement by EET agents. Additionally, as is the case with the Tay–Sachs mouse model (Phaneuf et al. 1996), mice often possess unexpected alternate metabolic pathways for substrate-clearance not found in humans. Other than individual compound-specific toxicity issues, the primary concern surrounding EET is that the PC, which is an inhibitor of its target enzyme, will continue to inhibit the enzyme once it enters the lysosome, off-setting its ability to increase the amount of enzyme able to reach its properly folded, transportable-form in the ER. From the standpoint of assessing the potentially inhibitory intracellular concentrations of the PC, in vitro assays for increased enzymatic activity are problematic because the cell lysates are substantially diluted before being assayed and saturating concentrations of artificial substrate are used. Additionally, as is the case for GM2 gangliosidosis (Callahan et al. 1970), cultured patient cells may contain little of the affected enzyme’s substrate, making it impossible to evaluate EET efficacy in cellulo by monitoring the clearance of natural substrate.

In the present report, we demonstrate the efficacy of EET utilizing Pyr for treating the most common mutant HEXA allele associated with ATSD (αG269S), by loading treated and untreated patient fibroblasts with the fluorescent GM2...
derivative NBD-GM2. This is not the first time that NBD-GM2 has been used as a substrate for Hex A and GM2AP. Sandhoff and colleagues in 1991 used it to evaluate the relationship between the hydrophobicity of various derivatives introduced into the ceramide moiety and/or the size of the oligosaccharide moiety (all containing the terminal β-GalNAc residue) and the need for the GM2AP to act as a co-factor in the reaction (Meier et al. 1991). In that report it was concluded that unlike GM2, a significant amount of NBD-GM2 could be hydrolyzed when presented to Hex A in a micellar form without GM2AP, but like GM2, its hydrolysis was dependent on the presence of GM2AP when incorporated into liposomes. In liposomes, the extent of NBD-GM2 hydrolysis was reported to be similar to that of natural GM2, in the presence of the same levels of Hex A and GM2AP. GM2 hydrolysis was reported to be similar to that of natural GM2, in the presence of the same levels of Hex A and GM2AP. Thus, the evaluation in liposomes greatly increased the rate of GM2AP-dependent group discovered that the inclusion of a negatively charged lipid early report was that the article was published before the same 

Before using NBD-GM2 in cellulo to evaluate Pyr-treatment of ATSD cells, we confirmed that NBD-GM2 could be efficiently internalized by fibroblasts and incorporated into their lysosomes (Figure 6, top set of panels), and that Pyr-treatment increased the protein levels of αGal269S Hex A, which also co-localized with loaded NBD-GM2 in ATSD cells (Figure 6D–I). Finally we analyzed NBD-GM2-loaded, Pyr-treated, and untreated cells by HPTLC after differential extraction of their acidic and neutral glycolipids. All three plates of treated cells exhibited an ∼3-fold increase (Table I) in the fluorescent bands corresponding to NBD-GM3 (Figure 7A), NBD-GlCer, and NBD-LacCer (Figure 7B) indicating an increased clearance of NBD-GM2 by the ATSD cells treated with Pyr. Importantly, this level of increased substrate clearance was virtually identical to the level of enhancement of residual Hex A activity achieved after ATSD cells were treated with Pyr (Table I). Pyr is a drug with a long history of use in humans as a treatment for malaria and is known to readily cross the blood brain barrier. Thus it would be expected to reach neurons of the CNS, the primary sites of GM2 storage in Tay–Sachs disease. These characteristics combined with the new data presented in this report suggest that Pyr may be an effective treatment for ATSD. An investigator initiated, phase I/II clinical trial of Pyr for this purpose is presently underway at centers in Canada and the USA.

Material and methods

Chemical reagents

The following synthetic fluorogenic substrates, 4-methylumbelliferyl-β-D-galactopyranoside and MUG purchased from Sigma-Aldrich (Canada), and MUGS from Toronto Research Chemicals (Canada), were used to assay β-galactosidase, total Hex and Hex A, respectively. CBE was from Toronto Research Chemicals (Canada). Cholesterol was purchased from Sigma-Aldrich (Canada), phosphatidyl choline (egg) and phosphatidyl inositol (bovine liver) from Avanti Polar Lipids (USA), and polycarbonate 100 nm filters from Avestin, Inc. (Canada). Hex A and Hex B were extracted and purified from human placenta as described previously (Muirhead and Lowden 1980). Recombinant wild-type human His6-tagged GM2AP (rGM2AP) was expressed in Escherichia coli then purified and re-folded (Smiljanic-Georgijev et al. 1997). Primary antibodies used were a Hex A (α-subunit)-specific antibody prepared by absorbing a rabbit polyclonal IgG against purified human Hex A with excess human Hex B, and a mouse monoclonal IgG1 anti-human Lamp-1 (DHSB, USA). Secondary antibodies were Alexa Fluor 488 chicken anti-rabbit
IgG, Alexa Fluor 594 chicken anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, USA). Nuclear staining was done with DAPI and lysosome labeling with LysoTracker red DND 99, a fluorescent acidotropic probe, both from Molecular Probes (USA). All other reagents were of research or molecular biology grade, as appropriate.

Cell lines and tissue culture
Primary skin fibroblast cultures derived from an unaffected individual referred to as wild type (WT) and from a female fetus with the acute (infantile) form of TSD homozygous for a 4 bp insertion, 1278insTATC, in exon 11 of HEXA, referred to as ATSD, were provided by The Hospital For Sick Children’s tissue culture service. Fibroblasts from a 40 year old female patient diagnosed with the chronic (adult) form of TSD with the mutation 805G>A/805G>A (αG269S), referred to as ATSD, was as previously reported (Tropak et al. 2004). The AB-variant fibroblasts, homozygous for a nonsense mutation in exon 2 of GM2A, was from our own cell culture collection (Chen et al. 1999). The fibroblasts were maintained in α-MEM media (Wisent Inc., Canada) supplemented with antibiotics (ampicillin and streptomycin from Gibco BRL, Canada) and 10% FCS (Wisent Inc., Canada, v/v) in humidified atmosphere at 37°C in the presence of 5% CO2.

Synthesis of NBD-C6-labeled lyso-GM2 (NBD-GM2)
Synthesis was performed essentially as described by Schwartzman and Sandhoff (1987). Succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoate (NBD-XSE) (Molecular Probes, Canada), 6.6 mg (17.2 μmol) was dissolved in 1.5 mL of CH3OH:CH2Cl2 (1:1) with 3 μL of disopropylamine. Then 9.4 mg (6.6 μmol) of lyso-GM2 (a kind gift from NEOSE Technologies, USA) was added to the NBD-XSE solution and the reaction was incubated in the dark for 16 h at room temperature. The reaction mixture was evaporated to dryness in a speed-vac and re-dissolved in 200 μL of CH3OH:CH2Cl2 (1:1). This mixture was spotted on a 0.5 mm thick, 20 cm×20 cm silica plate (Whatman Silica-60 PK6F, Canada) and developed with CHCl3:CH3OH:2.5 M NH4OH (65:35:8), and evaporated through a C8 Sep-pak cartridge to remove silica particles. The concentration was measured using the molar extinction coefficient of 22,000 L·mol⁻¹·cm⁻¹ at 466 nm. The yield of the isolated NBD-GM2 was 4.4 μmoles (66% based on input of lyso-GM2).

Preparation of NBD-GM2 containing liposomes
Neutral and anionic liposomes containing NBD-GM2 ganglioside plus other lipid components were prepared essentially as previously described (Meier et al. 1991). Briefly, liposomes with different lipid composition were obtained by mixing NBD-GM2 (10 mol%), cholesterol (20 mol%), phosphatidyl inositol, 0 (neutral) or 20 (anionic) mol%, and phosphatidyl choline, 70 (neutral) or 50 (anionic) mol% in CH3OH:CH2Cl2 (1:1), and then drying the mixture under high vacuum so that upon rehydration a final lipid concentration of either 2 or 4 mM could be achieved. The lipid mixture thus obtained was rehydrated in Mc Ilvaine’s citrate phosphate buffer (pH 4.1) and then freeze-thawed 10 times using a dry-ice/ethanol bath to ensure solute equilibration between bulk and trapped solutions. Unilamellar vesicles were prepared by successive passage of the rehydrated lipid suspension through polycarbonate filters (100 nm) mounted in a mini-extruder (Liposo-Fast, Avestin, Inc. USA), with 21 passes as recommended by the manufacturer.

MUG/MUGS/Protein assay
Total hexosaminidase and Hex A enzymatic activity were measured as previously reported (Tropak et al. 2004; Maegawa et al. 2007). Protein assays were performed using the Bio-Rad Protein Assay Kit.

In vitro hydrolysis of NBD-GM2 assay
NBD-GM2 containing liposomes were prepared as described above. Assay reactions were set-up into a final volume of 50 μL, containing Mc Ilvaine’s citrate phosphate buffer (20 mM; pH 4.1), BSA (0.5% w/v) plus variable amount of rGM2AP (0–1.5 μg) and NBD-GM2 (0–10 nmol) incorporated into a fixed amount of liposomes (described above). The hydrolysis rates were determined using various sources of enzyme. These included purified human placental Hex A (25–100 U MUGS, i.e. 92–370 U MUG) or Hex B (1000–100,000 U MUG) and cell lysates from cultured normal or AB-variant fibroblasts (60 U MUGS). Each reaction was performed in a well of a 96-well PCR-plate and incubated with the enzyme for 1–3 h at 37°C. The reaction was stopped by the addition of 100 μL ice-cold C2H3OH, dried using a rotary speed-vac, and then stored at −20°C until HPTLC analysis. Reaction mixtures were re-dissolved in 25 μL CHCl3:CH3OH (2:1), by repeated pipetting. Typically 5–20 μL of the resuspended mixture was spotted on a HPTLC plate (as above, Whatman Silica-60 PK6F, Canada), developed with CHCl3:CH3OH:2.5 M NH4OH (65:35:8), and evaporated to complete dryness before scanning using a Storm Imager (Molecular Devices, USA; blue fluorescence, 1000 volts). In some cases the images thus obtained were further analyzed using densitometry software (ImageQuant v5.0, Molecular Devices, USA). The % GM3 obtained in each of the in vitro kinetic assays with purified Hex A was calculated from the NBD-GM3 band intensity and the total intensity of both the starting substrate (NBD-GM2) and product (NBD-GM3) bands. Actual quantity of product generated was determined by taking into account the amount of substrate placed in the assay. When cell lysates were used as an enzyme source or in cellulo assays were done, where substrate concentrations were very high and multiple products were generated, due to the presence of other hydrodases, only the bands associated with products of the reaction were analyzed.

In cellulo hydrolysis of NBD-GM2 after uptake by ATSD cells
ATSD fibroblasts (grown in 10 cm tissue culture plates, n = 3) were treated with Pyr (dissolved in DMSO, final concentration of 12.1 μM) or mock treated (DMSO 1%) for 11 days, in complete media changed every 3 days. Both set of cells, DMSO and Pyr treated, were then grown for an additional 18 h in FBS-free, α-MEM media containing NBD-GM2 (4.7 μg mL⁻¹) and CBE (50 μM). After, media removal, cells were rinsed with PBS and incubated with Pyr-free media containing 5% FBS for an additional 2 h before harvesting. Following centrifugation and PBS
washes, the cell pellet was resuspended in 1 mL PBS. One tenth of the cell suspension was removed for monitoring lysosomal glycosidase activities. The remaining cell suspension was used for the differential extraction of gangliosides and neutral glycolipids according to Folch (Folch et al. 1957). After the addition of 1.5 mL of CHCl₃:CH₃OH (2:1), the mixture was vortexed, sonicated for 1 min, and 300 μL of water added. This mixture was vortexed again and separated into organic (containing neutral glycolipids) and aqueous (containing gangliosides) phases by low speed centrifugation (2000 rpm/5 min). The aqueous phase (upper layer) was dried by speed-vac, resuspended in 25 μL of water, and applied to a C18 Zip Tip. The matrix was rinsed with 50 μL of water and the bound gangliosides eluted with 20 μL of 100% CH₃OH. Half of this material was loaded onto a HPTLC plate (Silica gel 60). The organic phase (lower layer), was also dried, but then re-dissolved in 10 μL of 2:1 CHCl₃:CH₃OH and applied immediately to a HPTLC plate. Glycolipids extracted from the aqueous and organic layers were resolved with CHCl₃:CH₂OH:2.5 M NH₄OH (60:35:8) as the mobile phase. Bands corresponding to NBD glycolipid derivatives were visualized and quantified using the Storm Imager as above.

**Mass spectrometry**

After an extended overnight hydrolysis of NBD-GM2 by Hex A in the presence of GM2AP, rectangular zones containing the bands assumed to correspond to NBD-GM2 and NBD-GM3 were scraped from HPTLC plates into eppendorf tubes. Gangliosides were released from the matrix by the addition of CH₃Cl:CH₃OH (2:1) combined with vortexing. Prior to mass spectrometry analysis, ammonium acetate (10 mM) was added to the supernatant after centrifugation to remove the matrix. Samples were applied to an ABI Q Star (Applied Biosystems Inc., USA) by direct injection in negative ion mode. Ions of interest were fragmented by collision-induced dissociation (CID).

**Kinetic analysis**

Enzymatic hydrolysis reactions utilizing NBD-GM2 as a substrate were carried out as described above. Each kinetic analysis was carried out at least three times to optimize the assay conditions, e.g. ensure that less than 15% of total input substrate was hydrolyzed during the reaction period. The optimized experiments, to establish if the reaction followed Michaelis–Menten kinetics with respect to the NBD-GM2 liposome substrate and GM2AP co-factor, utilized 50 or 100 U MUGS (nmoles h⁻¹) of purified Hex A and were performed by varying either the substrate or co-factor concentration while keeping the other constant. Kinetic constants were extracted via nonlinear regression analysis of the measured residual enzyme activity using Prism 5.0 (Graph Pad Software, Inc., USA). Standard error was calculated from the best-fit curve generated by the computer program from each of the optimized sets of assays shown in Figure 3.

**Indirect immunofluorescence, uptake of a specific lysosomal marker, and confocal microscopy imaging**

Primary fibroblasts, Pyr treated or mock treated for 11 days, were loaded with NBD-GM2 (as indicated above). Cells were harvested by trypsinization and diluted in fresh culture medium. An aliquot of each cell suspension was transferred onto several cover slips for overnight attachment to generate a low-density cell culture for further processing.

Indirect immunofluorescence staining was performed as previously described (Martin et al. 2008). Generally 5–10 cells were photographed at high magnification and a representative cell selected for display in each of the panels comprising Figures 5 and 6. Primary and secondary antibodies were as described above in chemical reagents, while nuclear staining was as previously reported (Tropak et al. 2008).

Direct labeling of the lysosomal compartment was performed using a specific organelle marker. After overnight attachment, the cells were washed twice with PBS containing Ca²⁺ and Mg²⁺ (PBS 2+); fresh media containing lysotracker red DND 99 (Molecular probe, USA) at a final concentration suggested by the manufacturer was added and the cells incubated for ~30 min. Then, the cells were rinsed three times with PBS 2+ and fresh media added before checking for lysosomal staining with a standard fluorescence microscope. Finally cell fixation was performed using a 4% paraformaldehyde solution followed by nuclear staining with DAPI, and then the coverslips were mounted onto glass slides and confocal images recorded (as indicated below). NBD-GM2 possesses intrinsic fluorescence properties that can be recorded using a confocal laser microscope.

Samples were analyzed using a Zeiss Axiowert confocal laser microscope equipped with a 63 x 1.4 numerical aperture APOCHROMAT objective (Zeiss) and LSM 510 software; DAPI-stained nuclei were detected on the same system with a Chameleon two-photon laser. Confocal images were imported and contrast/brightness adjusted using Velocity 5 program (Improvision Inc., USA).

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**Abbreviations**

ATSD, adult Tay–Sachs disease; BSA, bovine serum albumin; CBE, conduritol B epoxide; DMSO, dimethylsulfoxide; EET, enzyme enhancement therapy; ER, endoplasmic reticulum; FBS, fetal bovine serum; GM2, GM2 ganglioside; GM2AP, GM2 activator protein; Hex, β-hexosaminidase; HPTLC, high-performance thin-layer chromatography; LSDs, lysosomal storage diseases; Lamp-1, lysosomal-associated membrane protein-1; MUGS, 4-methylumbelliferon-(2-acetamido-2-deoxy)-β-D-glucopyranoside; MUGS, 4-methylumbelliferon-7-(6-sulfo-2-acetamido-2-deoxy)-β-D-glucopyranoside; NBD, nitro-2,1,3-benoxadiazol; NBD-GM2, NBD-C6 derivative.
of lyso-GM2 ganglioside; NBD-GM3, NBD-C6 derivative of lyso-GM3 ganglioside; NBD-GlcCer, NBD-C6 derivative of lyso-glucosyl-ceramide; NBD-LacCer, NBD-C6 derivative of lyso-lactosyl-ceramide; PBS, phosphate buffered saline; PC, pharmacological chaperone; Pyr, pyrimethamine; rGM2AP, recombinant wild-type human C-terminal His6-tagged GM2AP; TSD, Tay–Sachs disease; WT, wild-type.

References
TSD, Tay–Sachs disease; WT, wild-type.