Pyrimethamine as a Potential Pharmacological Chaperone for Late-onset Forms of GM2 Gangliosidosis*

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Late-onset GM2 gangliosidosis is composed of two related, autosomal recessive, neurodegenerative diseases, both resulting from deficiency of lysosomal, heterodimeric β-hexosaminidase A (Hex A, αβ). Pharmacological chaperones (PC) are small molecules that can stabilize the conformation of a mutant protein, allowing it to pass the quality control system of the endoplasmic reticulum. To date all successful PCs have also been competitive inhibitors. Screening for Hex A inhibitors in a library of 1040 Food Drug Administration-approved compounds identified pyrimethamine (PYR) (2,4-diaminopyrimidine)) as the most potent inhibitor. Cell lines from 10 late-onset Tay-Sachs (11 α-mutations, 2 novel) and 7 Sandhoff (9 β-mutations, 4 novel) disease patients, were cultured with PYR at concentrations corresponding to therapeutic doses. Cells carrying the most common late-onset mutation, αG269S, showed significant increases in residual Hex A activity, as did all 7 of the β-mutants tested. Cells responding to PC treatment included those carrying mutants resulting in reduced Hex heat stability and partial splice junction mutations of the inherently less stable α-subunit. PYR, which binds to the active site in domain II, was able to function as PC even to domain I mutants. We concluded that PYR functions as a mutation-specific PC, variably enhancing residual lysosomal Hex A levels in late-onset GM2 gangliosidosis patient cells.

GM2 gangliosidosis (GM2, OMIM 230700), is a clinically heterogeneous inherited neurodegenerative disorder charac-

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3 The abbreviations used are: GM2, Galα1,3Galβ1,4GlcNAc-ceramide; GM1, Galβ1,3Galα1,4GlcNAc-ceramide; GP1, Galβ1,4GlcNAc-ceramide; G2, GM2 tritiated GM2 ganglioside; Hex, hexosaminidase; IP, immunoprecipitation; Lamp-1, lysosomal associated membrane protein-1; LYSO, lysosomal fraction; PC, pharmacological chaperone; PNS, postnuclear supernatant; PYR, pyrimethamine; MU, methylumbelliferone; MUBGal, 4-methylumbelliferyl-β-D-galactopyranoside; MUG, 4-methylumbelliferyl(2-acetamido-2-deoxy)-β-D-glucopyranoside; MUGS, 4-methylumbelliferyl-7-(6-sulfo-2-acetamido-2-deoxy)-β-D-glucopyranoside. NTT, N-acetylglucosamine thiazoline; NINDS, National Institute of Neurological Disorders and Stroke; SD, Sandhoff disease; TGN, thio-guanine; TSG, Tay-Sachs disease; ER, endoplasmic reticulum.
or acute TSD and SD, associated with <0.5% of normal Hex A activity, resulting in rapid neurodegeneration, and culminating in death in infancy. At the other end of the spectrum are the late-onset forms, which are subdivided into juvenile or subacute and adult or chronic forms (6). These are usually associated with residual Hex A activities, ~1–10% of normal (7). Patients with juvenile GM2 gangliosidosis usually present with evidence of neurodeterioration starting after 1 year of age, experiencing a slower rate of progression than patients with the infantile forms (8). Patients with adult-onset forms may present with spinocerebellar, psychiatric, and/or peripheral neuropathies, which do not significantly decrease life expectancy in some cases (9). The rate of disease progression and severity has been found to correlate roughly with the level of residual Hex A activity. Generally, a clinical disease does not develop unless residual Hex A activity is <10% of normal (10). Thus, only a low level of residual Hex A activity is apparently needed to prevent or reverse substrate storage in this condition.

Pharmacological chaperones (PC) are low molecular weight compounds that stabilize the native conformation of a mutant enzyme in the ER, allowing it to escape aggregation and premature degradation by the ER-associated degradation pathway. The properly folded mutant enzyme, stabilized by the PC can then be transported to the lysosome, increasing the residual enzyme activity of the cells (11). Most PCs have also been competitive inhibitors of their target enzyme (12). Once the PC-enzyme complex reaches the lysosome, the large amounts of stored substrate(s) are believed to displace the PC and take over stabilization of the mutant enzyme (13). The PC approach has been shown to enhance the residual activity levels of five different lysosomal enzymes causing chronic forms of the lysosomal storage diseases, GM2 gangliosidosis (14), GM1 gangliosidosis (15), Fabry (16), Gaucher (11), and Morquio B diseases (17).

A major challenge to the exploitation of this phenomenon for the treatment of disease is identifying potential PCs from among the thousands of existing chemicals and drugs. High throughput screening of small molecule libraries has been used to identify specific enzyme inhibitors with PC potential (18, 19).

We report here the application of a small molecule library screening approach to search for novel inhibitors of lysosomal Hex A with the potential to treat late-onset variants of GM2 gangliosidosis by functioning as PCs. We undertook a manual screening for Hex inhibitors in a 1040-compound library of Food Drug Administration (FDA)-approved drugs obtained from NINDS (National Institutes of Health). Pyrithylamine (PYR) was discovered to have considerable PC potential. The evaluation of PYR as a PC was performed using fibroblast cell lines from 17 patients with juvenile and adult GM2 gangliosidosis. Ten of the 11 different HEXA mutations, and 5 of the 9 different HEB mutations that we identified in patients were reported previously. Each of the mutant cell lines from our patients responded differently to PYR, as well as to a carbohydrate-based PC, N-acetylcysteamine thiazoline (NGT), which we reported previously (14). In addition, the different responses of mutants to PYR brought interesting insights into the role of the 2 domains present in each subunit (α and β) in the folding and assembly of the Hex dimers.

**EXPERIMENTAL PROCEDURES**

**Study Subject**—Fibroblast cell lines were obtained from 17 patients with late-onset forms of GM2 gangliosidosis. Patients were from two Genetic Metabolic centers, and informed consents and assents approved by relevant research ethic boards were obtained from each patient.

**Chemical Reagents and Antibodies**—The NINDS National Institutes of Health drug library was received from MicroSource Discovery Systems in a 96-well format; one drug per well at a concentration of 10 μM diluted in dimethyl sulfoxide (Me2SO), sealed under nitrogen. Plates were stored at −20 °C. The following fluorogenic substrates, all purchased from Sigma, 4-methylumbelliferyl-β-D-galactopyranoside (MUβGal) and 4-methylumbelliferyl phosphate, MUG and MUGS were used to assay the lysosomal enzymes β-galactosidase, acid phosphatase, total Hex, and Hex A/Hex S, respectively. Enzymatic reactions were stopped using 2-amino-2-methyl-1-propanol (at 0.1 M, pH 10.5). Purified Hex A and Hex B, which were used in kinetic characterization of PYR, were extracted from human placenta as described previously (21). Qiagen kits for DNA and RNA extraction from fibroblasts were used. Molecular kits for PCR and reverse transcriptase-PCRs were purchased from Invitrogen Inc. Specific oligonucleotides for sequencing of genomic and cDNA were synthesized by The Center of Applied Genomics from the Hospital for Sick Children. Rabbit polyclonal antibodies against human Hex A (Western blot) and sheep polyclonal IgG against human β-subunit (used for immunoprecipitation and cell immunofluorescence) were prepared as previously described (22). Mouse IgG monoclonal against Lamp-1 was purchased from the Iowa Hybridoma Bank. Secondary antibodies, donkey anti-sheep IgG, and chicken anti-mouse IgG were purchased from Molecular Probes Inc. Stock solutions of NGT, provided by Dr. S. Withers, University of British Columbia, were prepared by dissolving the compound in Me2SO, (4 mg/ml) or water (10 mg/ml). PYR, purchased from Sigma, was dissolved in Me2SO (stock solution of 4 mg/ml), or ethanol (ETOH) (stock solution of 0.1 mg/ml). For IP, Gamma beads were purchased from Amersham Biosciences (UK). Steel wool number 0000 (International Steel Wool, Mexico), FeCl2 and FeCl3 (Sigma), and dextran T4000 (Amersham Biosciences, UK) were used to prepare lysosomes by magnetic chromatography. Tritiated GM2 ganglioside, [3H]GM2 (10 mol %), cholesterol (20 mol %), phosphatidylinositol (20 mol %), phosphatidylcholine (50 mol %), polycarbonate Liposo-Fast filter (Avestin 100 nm), AG3X4 (acetic form) resin (Bio-Rad), concanavalin A spheroid beads (Amersham Biosciences), and recombinant Activator were used for the natural substrate assay. Chemical cross-linking was performed using dithio-bis(succinimidylpropionate) (DSP) as a cross-linker reagent. Stock solution of DSP was dissolved in Me2SO at 6 mm.

**Screening for Competitive Hex Inhibitors**—The NINDS National Institutes of Health library of 1040 FDA-approved compounds was screened to identify potential PCs for Hex A. The screening was performed using wild-type Hex B, and the common fluorescent MU-based artificial substrate, MUG. The library was screened in duplicate; with test compounds at a final concentration of 20 μM. From the original 96-well plates, com-
Pyrimethamine: Pharmacological Chaperone for GM2 Gangliosidosis

Pounds were initially diluted to 10 mM by addition of Me₂SO. Real-time Hex assays were performed in final volumes of 100 μl, containing 1 mM of each drug, 12 ng of purified Hex B diluted in citrate phosphate buffer (CP) (pH 4.1) containing 0.025% human serum albumin, and 25 μl of MUG (0.4 mM). Real-time fluorometric assays were performed using a Gemini EM Microplate Spectrofluorometer (Molecular Devices), with excitation at 345 nm and emission at 450 nm, every 2 s during the 20-min incubation period at 37 °C. Mean \( V_{\text{max}} \) was then obtained and expressed in relative fluorescence units/s using standardized SoftMax® Pro Software coupled to the spectrofluorometer. The values described on a replicate screen plot represent ratios related to mean of the \( V_{\text{max}} \) obtained from the control Me₂SO sample (Fig. 1).

Mutation Identification—Most mutations were identified and reported in previous studies from our group (20, 23, 24). For mutations of cell lines, 26649, 32429, and 36986, total genomic DNA and cDNA were isolated from fibroblasts by routine techniques (25). PCR amplification was performed using primers described previously (24, 26). Reverse transcriptase-PCR from extracted total RNA followed by specific PCR amplification from cDNA with oligonucleotides previously described were performed (26). Fragments were sequenced with the use of ABI 377 and 3700 sequence analyzers.

Tissue Culture Conditions and Enzyme Assays—Fibroblast cell lines were cultured to confluency. The culture medium used, α-minimal essential medium with 10% of fetal calf serum and antibiotics, was then replaced by the same medium containing filter-sterilized (Millipore-0.45 μm) PYR or NGT at the described concentrations. For the initial experiments, 10-cm culture plates of each cell line were treated with PYR and NGT diluted in Me₂SO at final concentrations of 20, 10, and 5 μg/ml. In follow-up experiments, each cell line was treated with PYR at 3.0, 1.5, 0.5, and 0.1 μg/ml, and NGT, at 300, 150, and 75 μg/ml. Control plates containing only solvents, ETOH (used to dissolve PYR), and water (used to dissolve NGT) added to culture media were also established. After 5 days of incubation in drug-containing media, culture medium was removed; cells were washed twice with phosphate-buffered saline and harvested. Cell pellets were re-suspended in NaH₂PO₄ (10 mM, pH 6.0), containing 5% glycerol, and lysed by freezing-thawing on dry ice. Hex assays using MUG or MUGS were performed. Lysates were diluted 10-fold by addition of 20 mM CP buffer (pH 4.1).

For all lysosomal enzyme assays, stock solutions of the substrates dissolved in the same CP buffer of MUG (3.2 mM), MUGS (3.2 mM), 4-methylumbelliferyl phosphate (10 mM), and MUβGal (0.56 mM) were used. Assays were carried out by addition of 100 μl of substrate solution (final volume 200 μl), and incubation at 37 °C for 1 h for all artificial substrates used, except from MUG where the incubation time was 15 min. Reactions were stopped by addition of 1.5 ml of 0.1 M 2-amino-2-methyl-1-propanol (pH 10.5), and fluorescence was measured as described above (22). Enzyme activities were calculated in nanomoles of MU hydrolyzed/h/mg of protein. The relative activities of total Hex, Hex A, and Hex S were expressed as ratios of corresponding samples from treated versus control cells. Residual Hex A % activities were calculated based on those of the wild-type cell line lysate, assayed concomitantly with the mutant cell lines (range of Hex A activity 3,500–8,500 MU of nmol/h/mg of protein).

Immunoselection Assays for Hex A and Hex S—For measurement of residual Hex activities in SD cell lines (β-mutants), Hex A and Hex B (residual Hex B activity is generally undetectable in SD cells) were immunoprecipitated by solid-phase IP with polyclonal sheep anti-β-subunit IgG as previously described (22, 27, 28). Residual Hex A activity was determined by measurement of Hex activity from the immunoprecipitated phase (containing antibody bound to Hex A) with MUGS as substrate. Hex S activity was determined by measurement of enzyme activity in the IP supernatant, also using MUGS as substrate.

Western Blot Analysis—The total protein contained in clarified lysates was determined by the Lowry method (29). Aliquots of lysates containing 20 μg of total protein were diluted 1:1 with 1× standard Laemmli buffer containing 50 mM dithiothreitol and heating at 65 °C for 15 min. Each sample was then subjected to SDS-PAGE on a 10% bisacrylamide gel, and transferred to nitrocellulose. The nitrocellulose was then incubated with a rabbit anti-human Hex A antibody as previously described (23). Blots were developed using chemiluminescent substrate according to the manufacturer’s protocol (Amersham Biosciences). Bands were visualized, recorded, and their optical density quantitated using a high sensitivity documentation system (Fluorchem 8000) consisting of a cooled CCD camera coupled with software from Alpha Innotech Corp.

Purification of Iron-dextran-labeled Lysosomes by Magnetic Chromatography—Lysosomal fractions were prepared from fibroblasts grown for 5 days in media lacking or containing PYR, 3 μg/ml using a previously described procedure (14, 30). Solid-phase IP of the LYSO and postnuclear supernatant (PNS) fractions were performed as described above.

Immunofluorescence Labeling—Cells were grown on coverslips, fixed with 100% methanol for 15 min, and then blocked.
with 10% fetal calf serum for 30 min. After washing twice with phosphate-buffered saline, the cells were incubated in the presence of anti-β-subunit (diluted 1:400) and anti-Lamp-1 (diluted 1:500) antibodies at room temperature for 1 h. The cells were then immunostained with suitable secondary antibodies as cited earlier (Molecular Probes) at room temperature for 1 h. Confocal laser scanning microscopy on the Zeiss LSM 510 confocal system was performed. All images were taken with 100 × 1.4 numerical apertures (NA) and 63 × 1.4 NA Apochromat objective (Zeiss). All image processing was performed using the Zeiss LSM 5 image examiner software.

Natural Substrate Assay—Tritiated GM2 ganglioside, [3H]GM2, containing liposomes were prepared as previously described (4). To concentrate Hex A, as well as other soluble lysosomal enzymes, 3.5–3.8 mg of total cell lysate protein was incubated with concanavalin A beads (30 μl of drain solution) overnight at 4 °C. Beads were then washed with phosphate-buffered saline three times and assayed in a final volume of 100 μl, which contained CP (20 mM; pH 4.1), bovine serum albumin (50 μg/ml), 2.5 μg of recombinant Activator isolated from transformed Escherichia coli as described (31), and 20 nmol of [3H]GM2 contained in negatively charged liposomes. The incubation period of 18 h, as well as further procedures for stopping the reaction and preparation for liquid scintillation counting analysis, were performed as previously described (4).

Chemical Cross-linking with DSP—Total lysate protein from mutant cells grown in medium containing PYR (3.0 μg/ml) or solvent (ETOH) were adjusted to a concentration of 0.5 mg/ml with NaH2PO4 (10 mM, pH 6.0), containing sufficient DSP to give a final concentration of 0.9 μg/ml. The residual Hex A activity is apparently above the critical threshold, because this mutation was shown to specifically decrease the ability of the residual Hex A to bind and thus, hydrolyze the GM2 ganglioside-activator complex by ~3-fold (natural substrate assay).

### TABLE 1

| Fibroblast cell lines tested with their respective mutations in HEXA (TSD variant), HEXB (SD variant) genes, phenotype, and residual Hex A activities |
|--------------------------------------------------------------|
| Patient cell line number | Mutation 1 | Phenotype | Mutation 2 | Phenotype | Residual Hex A % |
|--------------------------|------------|-----------|------------|-----------|-----------------|
| TSD variants             |            |           |            |           |                 |
| 27991                    | R178H      | Juvenile  | c.1510delC | Infantile | 1.7             |
| 27986                    | R178H      | Juvenile  | R178H      | Juvenile  | 3.0             |
| 27985                    | R178H      | Juvenile  | Y277X      | Infantile | 3.4             |
| 28236                    | R178H      | Juvenile  | R499C      | Infantile | 2.2             |
| 27989                    | G269S      | Adult     | c.1278insTATC | Infantile | 5.3             |
| 32540                    | G269S      | Adult     | IVS6 + 1G>A | Juvenile  | 6.4             |
| 32664                    | R499H      | Adult     | IVS9 + 1G>A | Infantile | 2.4             |
| 7638                     | R499H      | Juvenile  | IVS11 + 1G>A | Infantile | 2.0             |
| 28237                    | R499H      | Juvenile  | IVS9 + 1G>A | Infantile | 2.0             |
| 26649                    | IVS9 + 1G>A | Infantile | IVS8 − 7G>A | Adult*    | 3.7             |
| SD variants              |            |           |            |           |                 |
| 1303                     | C137Y      | Juvenile  | C137Y      | Juvenile  | 1.3             |
| 32429                    | T150L      | Infantile | P417L      | Adult     | 2.5             |
| 30037                    | G533R      | Infantile | IVS12 − 26G>A | Juvenile | 3.5             |
| 3585                     | P417L      | Adult     | Δ16kb*     | Infantile | 3.6             |
| 2400                     | P504S      | Adult     | Δ16kb*     | Infantile | 12.7            |
| 32045                    | R505Q      | Adult     | IVS11 + 5G>A | Infantile | 4.2             |
| 360986                   | R505Q      | Adult     | Δ16kb*     | Infantile | 5.32            |

*Patient cell lines are labeled according to the storage number from our Tissue Culture Laboratory.

* Novel mutations.

* Predicted phenotype of novel mutations based on clinical data and residual Hex A activity levels.

* This specific mutation was originally identified by Dr. John O’Brien.

* Large deletion of promoter-exon5 previously described.

* The residual Hex A activity is apparently above the critical threshold, because this mutation was shown to specifically decrease the ability of the residual Hex A to bind and thus, hydrolyze the GM2 ganglioside-activator complex by ~3-fold (natural substrate assay).
mM. After 15 min incubation at 37 °C, the reaction was quenched by the addition of 2.2 volumes of Tris-HCl (pH 7.5) to bring the mixture to 100 mM. The protein samples (5 μg, normal control, 10 or 20 μg of mutant cell lines) were then mixed with the 2× Laemmli sample buffer (1:1, containing no reducing agent), heated at 65 °C for 15 min, and separated by SDS-PAGE (10% gel) for Western blot analysis and quantitation (see above).

Heat Denaturation Assays—The effect of PYR on the stability of the Hex A containing a mutant β-subunit (βR505Q) was determined by heat denaturation experiments. Lysates from mutant cell lines containing 100–150 μg of protein were added to preheated CP buffer (20 mM; pH 4.1) with 0.3% of heat-treated bovine serum albumin (Sigma) and PYR at a final concentration of 3.0 μg/ml. Aliquots of 100 μl were removed at fixed intervals of 0, 5, 10, and 30 min at 37 °C and put on ice. Aliquots from each fraction of heated and non-heated lysates were put in tubes with anti-anti-bodies bound to Gamma beads, and the IP procedure was followed (as described above). Mutant lysate samples containing only ETOH (PYR solvent) were also tested as a control for the PYR-exposed lysates.

Statistical Analysis—The statistical test Z’ factor was used to measure the quality of the assay and its applicability to screening (32). This single statistic takes into consideration both signal-to-noise and reproducibility. Assays with a Z’-statistic >0.5 are robust enough to identify enhancement of enzyme activity reliably (32). Where applicable, data are expressed as the mean ± S.E. Comparisons of parametric data were analyzed in the use of conventional parametric statistical methods as two-tailed Student’s t test.

RESULTS

Identification and Characterization of PYR as a Hexosaminidase Inhibitor—The results of screening each of the 1040 small molecules in the NINDS National Institutes of Health library, done in duplicate, for inhibition of Hex activity, are shown in Fig. 1. Each of the two replicates is defined by a X and Y pair of coordinates on the graph. Thus, consistent replicates fall on, or near the diagonal line (Z’ = 0.54) (32). Compounds within the box were considered “hits,” potentially effective as PCs, and were selected for secondary screening.

Two inhibitory compounds were identified as potential PCs, PYR and thioguanine (TGN) (Fig. 2). Secondary screening of
As expected, the PC candidates failed to show competitive inhibition properties at concentrations (20 μM) of PYR achievable in cerebrospinal fluid, thus available to neuronal cells of patients receiving therapeutic doses of the drug for malaria and toxoplasmosis, residual Hex A levels of two late-onset TSD cell lines, αG269S/IVS6 + 1G>A and αG269S/IVS6 + 1G>A, were enhanced (Fig. 6, A and B, respectively). The αG269S mutants showed lower relative increases (Fig. 6A) than those observed using higher concentrations of PYR (Fig. 3A). On the other hand, NGT, at 300 μg/ml, which is non-toxic in mice,4 showed up to an 8-fold relative increase (Fig. 6A). The residual Hex A from the αLVS9 + 1G>A/IVS8 − 7G>A cell line was also chaperoned by both PYR and NGT (Fig. 6B).

Interestingly, with this cell line the relative fold-increases with PYR was higher than the one obtained with 100-fold higher concentrations of NGT (Fig. 6B). The increase in the Hex A activity was confirmed by the increased levels of the mature α-subunit (αm) in the respective Western blots shown of the Me2SO control for either PYR or NGT (Fig. 3B). Interestingly, Me2SO was found to be a weak competitive inhibitor of Hex (Ki of 170 ± 20 μM), and to have mild chaperone activity, complicating the initial interpretation of the data. For this reason, we used ETOH as a solvent of PYR in further experiments. The final ETOH concentration was 0.03% in culture media, and did not affect the Hex activity (“Experimental Procedures”). The PC effect of PYR was also specific for Hex; the activities of acid phosphatase and β-galactosidase were not enhanced in the αG269S/IVS6 TSD mutant cell line, whereas Hex A levels were enhanced at levels ≥0.1 mM (Fig. 4).

Responses of Different TSD and SD Mutants to PYR and NGT at Concentrations Known to Be Non-toxic—At concentrations of PYR achievable in cerebrospinal fluid, thus available to neuronal cells of patients receiving therapeutic doses of the drug for malaria and toxoplasmosis, residual Hex A levels of two late-onset TSD cell lines, αG269S/c.1278insTACT and αLVS9 + 1G>A/IVS8 − 7G>A, were enhanced (Fig. 6, A and B, respectively). The αG269S mutants showed lower relative increases (Fig. 6A) than those observed using higher concentrations of PYR (Fig. 3A). On the other hand, NGT, at 300 μg/ml, which is non-toxic in mice, showed up to an 8-fold relative increase (Fig. 6A). The residual Hex A from the αLVS9 + 1G>A/IVS8 − 7G>A cell line was also chaperoned by both PYR and NGT (Fig. 6B). Interestingly, with this cell line the relative fold-increases with PYR was higher than the one obtained with 100-fold higher concentrations of NGT (Fig. 6B). The increase in the Hex A activity was confirmed by the increased levels of the mature α-subunit (αm) in the respective Western blots shown.

PYR showed it had an IC50 of 5–13 μM for the Hex isozymes at pH 4.3 (Fig. 2A). On the other hand, TGN had a significantly higher IC50 of 170 μM (Fig. 2B). Unlike PYR, TGN was toxic to the fibroblast cell lines tested at the concentrations needed for it to act as a PC. Thus, PYR was chosen for further study. Kinetic examinations demonstrated that PYR behaved as a competitive inhibitor of Hex A with a Ki of 13 μM at pH 4.5 (Fig. 2C). Interestingly, PYR was found to have a pKaA of 6.5 with an IC50 ~ 2 μM at pH 6.5 (Fig. 2D). Thus, PYR would be least effective as an inhibitor in the acidic environment of the lysosome, but would bind maximally at the neutral pH of the ER, where optimal PC activity is desired. Other compounds considered to be possible candidates failed to show competitive inhibition properties in a secondary screening and were not studied further (Fig. 1).

PYR as a Pharmacological Chaperone for Hex A—Initially, two fibroblast cell lines from TSD patients were selected for testing of PYR, αG269S/IVS6 + 1G>A (number 32540) and αR178H/R178H (number 27986) (Table 1). The total relative Hex (A and B) activity (MUG) showed no significant increases in both treated cell lines (data not shown). However, when MUGS was used as the substrate, the αG269S/IVS6 + 1G>A cell line showed a statistically significant increase in Hex A activity, over 3-fold, with the highest concentration of PYR added to the culture media, whereas NGT at the same concentration (20 μg/ml) showed a smaller decrease (Fig. 3A). As expected, the αR178H/R178H cell mutant, which carries an active site mutation affecting substrate binding, also known as the B1 variant (33, 34), showed no effect above that

4 B. Rigat, M. Tropak, S. Withers, and D. J. Mahuran, unpublished data.
showed a significantly large increase in relative Hex A activity with PYR as compared with NGT (Table 2). This cell line also had the lowest starting residual Hex A activity (Table 1). On the other hand, a cell line with a mutation in the adjacent residue, βP504S/Δ16kb, showed higher relative Hex A increases with NGT than with PYR. The cell line βT150L/P417L showed a 2.7-fold relative increase in Hex A activity with PYR, which was a better response than the 1.6- and 1.5-fold relative increases observed for the βP417L/Δ16kb and βG353R/IVS12 – 26G>A cell lines, respectively (Table 2). Data from Western blots correlated with the increased Hex A activities as shown in the β-mutants from Table 2 (data not shown).

Hex A Is Increased in a Lysosomal Enriched Fraction of PYR-treated Cells—To confirm the cellular localization of Hex A in PYR-treated and untreated cell lines, we loaded lysosomes with ferrous-dextran colloid, and performed a magnetic fractionation from which we obtained a PNS, and an enriched lysosomal (LYSO) fraction, as previously described (14, 30). After separating Hex A from Hex S by IP, the LYSO as well PNS fractions of one

(35–37).

All seven of the late-onset SD cell lines showed some degree of relative increase in residual Hex A (and Hex S) activity with PYR or NGT (Table 2 and Fig. 8). IP was used to separate the residual Hex A from Hex S in lysates obtained from treated β-mutants (“Experimental Procedures”). The highest relative increase of Hex A with PYR was seen in cells with the βR505Q/Δ16kb mutant. The βR505Q/Δ16kb mutant showed the best response to PYR of all 7 SD cell lines (Fig. 8). The response to PYR was even greater than the response to NGT (Fig. 8). Interestingly, the βR505Q/IVS11 + 5G>A cell line showed a smaller, although significant increase (up to 4-fold) of residual Hex A with PYR (Table 2). The cell line βC137Y/C137Y also

FIGURE 6. TSD or α-mutant cell lines that responded to PYR and NGT. The αG269S/IVS12insTACT (A) and αIVS9 + 1G>A/IVS8 – 7G>A (B) are shown with their relative Hex A activity and α-subunit protein levels at different treatment regimens of PYR and NGT. The fold-increase in activities was calculated based on the activity measured in control cell lines treated only with the dissolvent, i.e. ETOH for PYR-treated and H2O, for NGT-treated cells (under “Experimental Procedures”). αm indicates the α-subunit precursor; αm, the mature, lysosomal α-subunit. *, p < 0.01; **, p < 0.001.

PYR-treated cell line (βC137Y/C137Y) showed significantly higher absolute Hex A and Hex S activities than the untreated controls (Table 3).

Mutant Hex β-Subunits of Hex A Are Present in the Lysosomes of PYR-treated SD Cells—To further confirm that enhanced Hex A levels in SD cells was lysosomal, we localized Hex A by virtue of its mutant β-subunit in one of our high responding SD cell lines, βR505Q/IVS11 + 5G>A, treated with PYR (3.0 μg/ml). Immunofluorescence microscopy, using sheep anti-β-subunit IgG and mouse anti-Lamp-1 IgG, co-localization the β-subunit (green) of Hex A with Lamp1 (red) only in treated cells (Fig. 9, A–F). In control cells lines treated only with solvent (Fig. 9, G–L), β-subunit staining was difficult to detect even in the ER.

PYR Enhances Natural Substrate Hydrolysis—PYR treatment was confirmed to enhance both natural substrate (Activator-[3H]GM2 complex) and MUGS hydrolysis in a αG269S chronic TSD cell line (Fig. 10).

PYR Mechanism of Function on Hex—To investigate the mechanism in which PYR promotes the observed increases in Hex A activity, two experiments were conducted. First, lysates from two mutants, αG269S, a mutation that is located well away from the subunit-subunit interface, and βR505Q, located at the
Pyrimethamine: Pharmacological Chaperone for GM2 Gangliosidosis

This study demonstrated that PYR functions as a PC for several α- and β-mutants affecting Hex A (Figs. 6 and 8, Table 2). The αG269S mutant, the most prevalent mutation encountered in late-onset, adult GM2 gangliosidosis (TSD) (40, 41), showed significant response to PYR. An early pulse-chase study demonstrated that the majority of mutant αG269S precursors remain primarily as monomers in patients’ cells and are degraded. Only low level mature α-subunits were found in the lysosome where they were always associated with β-subunits (35), confirming that dimerization is necessary for transport from the ER to lysosomes. It was later demonstrated that this apparent defect in association could be substantially overcome by overexpressing the mutant α-subunit along with the normal human β-subunit in co-transfected COS cells, but the resulting Hex A was unstable at 37 °C. Additionally, when the same mutation was made in the aligned Gly of the more stable β-subunit, there was little effect on the levels of expression of Hex B (22). Taken along with the new crystal structure of Hex A (2), these data indicate that the mutation destabilizes the folded α-monomer, accelerating its clearance by the ER-associated degradation pathway, which results in a diminished pool of α-monomers available for heterodimer formation. We also noted that, at high PYR concentrations, >0.1 mM, the inhibitory effects of the drug on Hex became evident, whereas at the same time, there was no effect on the activities of two other lysosomal enzymes (Fig. 4). This indicates that, when applied to patients, dosages must be carefully evaluated to maximize the PC activity of PYR while minimizing its inhibitory effect.

Another α-mutant, αIVS9 + 1G>A/IVS8 − 7G>A, showed a small, but significant increase in Hex A levels with PYR (Fig. 6B). Interestingly, in these cells, PYR treatment produced a higher relative level of Hex A activity than NGT, an effect that was not observed with αG269S mutants (Fig. 6A). This observation suggests that, in addition to missense mutations, some splice mutants may be treatable by PC therapy. Moreover, the αIVS9 + 1G>A mutation has been shown to produce no normal mRNA (42, 43), so all the residual Hex A activity in this cell line originates from the novel αIVS8 − 7G>A mutation we have identified. Because properly spliced mRNA from this allele would have the wild-type sequence, we concluded that stabilizing the pool of normal α-monomer in the ER, i.e. increasing the half-life and thus the concentration of α-monomers, leads to an increase in heterodimer formation. This model is also consistent with the previously published biochemical data on the in vivo effects of the αG269S mutant (see above) and the cross-linking studies we now report (Fig. 11A).

On other hand, the Hex A levels of other α-mutant cell lines from our collection, αR178H, αR499H (Fig. 7), and αR499C
Pyrimethamine: Pharmacological Chaperone for GM2 Gangliosidosis

TABLE 2
Six of seven of the SD or β-mutant cell lines with their relative activities of Hex A and Hex S after treatment with PYR or NGT

| β-Mutant | β-C137Y/C137Y | β-T150P/P417L | β-G353R/ IVS12 = 26G>A | β-P417L/Δ16kb | β-R505Q/ IVS11 + 5G>A | β-P504S/Δ16kb |
|----------|----------------|----------------|--------------------------|----------------|--------------------------|----------------|
|          | Hex A | Hex S | Hex A | Hex S | Hex A | Hex S | Hex A | Hex S | Hex A | Hex S |
| PYR*     | 4.7 ± 0.1  | 6.7 ± 0.2  | 2.7 ± 0.9  | 10.8 ± 0.9  | 1.5 ± 0.1  | 3.2 ± 0.1  | 4.0 ± 0.2  | 3.7 ± 0.5  | 3.9 ± 0.4  | 1.9 ± 0.1  | 2.2 ± 0.1  |
| NGT†     | 4.8 ± 1.5  | 4.7 ± 0.1  | 2.1 ± 0.1  | 10.3 ± 1.9  | 1.6 ± 0.1  | 1.0 ± 0.1  | 3.4 ± 0.1  | 4.6 ± 0.1  | 5.4 ± 0.1  | 4.7 ± 1.0  | 3.1 ± 0.4  |

* 3.0 µg/ml.
† p < 0.01.
†† p < 0.05.
‡ 300 mg/ml.

Pyrimethamine increases the enzyme activity and protein level of the α- and β-subunits of Hex A in the βR505Q/Δ16kb cell line. Histograms show relative activity enhancement of the lysate (total Hex A and S), as well as Hex A and S separated by IP (Experimental Procedures). Western blots of the samples treated with PYR and NGT also document an increase in protein levels of the mature, lysosomal α- and β-subunits in the total lysate of treated cells. αβm indicates the α- and β-subunit precursors; αm and βm indicate the mature (lysosomal) α- and β-subunits, respectively. * p < 0.05; ** p < 0.01.

TABLE 3
Increased Hex A and S activities in the enriched lysosomal fraction, LYSO, in relation to postnuclear supernatant (PNS) fraction in a domain I β-mutant, βC137Y/C137Y, cell line treated with PYR

| Regimen | PNS | LYSO | Control (ETOH only) |
|---------|-----|------|---------------------|
|          | PNS | LYSO | PNS | LYSO |
| Hex A‡  | 125 | 205 | 80.5 | 97.4 |
| Hex S‡  | 122 | 237 | 75.5 | 117 |
| Lysate‡ | 409 | 1140 | 173 | 385 |

* 3.0 mg/ml.
† The difference in Hex A activity between the PNS and LYSO fractions of PYR-treated cells was significantly higher than the difference observed in control cells (p < 0.01).
‡ Units: hydrolyzed MU nmol/mg total protein.
IVS12/Δ16kb, cell line treated with PYR, was seen (Fig. 8). However, the βR505Q/IVS11 + 5G>A mutant (number 32045) showed a smaller increase in both Hex A and S activities (Table 2). In addition, the fibroblast cell line from an affected sibling of this patient produced the same levels of enhancement (data not shown). Thus, the observed differences in response to PC treatment may not be solely due to the identity of the β-mutations, but may also be influenced by other factors, e.g., components of the ER quality control system.

Treatment of the βP504S/Δ16kb cell line with PYR resulted in a ~2-fold increase of Hex A activity. However, NGT produced an even higher level of enhancement in this cell line, 4.7-fold. The βP504S mutation is unique in its effect on Hex A. It was shown to reduce the specific activity of the mutant Hex A toward its natural substrate by ~3-fold as compared with MUGS; thus explaining its apparently high residual MUGS activity (23). Interestingly, βP504S is adjacent to βR505Q, which is enhanced better by PYR than NGT. Both residues are near the subunit-subunit interface contained in domain II, which buries a surface area of 2694 Å² in each monomer and forms a large groove into which the Activator-GM2 ganglioside complex can be docked. Pre406 introduces a kink into helix α8, which is required for proper packing of the helix against two loops that interact directly with the docked Activator (3). Arg505 forms a salt bridge and some hydrogen bonds, which are needed to stabilize the surfaces of the dimer interface once they are buried (2). Like the αG269S substitution, βP504S and also βR505Q generate a more heat-labile form of mutant Hex A (46), which indicates that monomer stability also affects the stability of the dimer. Such mutations appear to respond well to PYR treatment.

The βG353R mutation found in one of the β-mutant cell lines (number 30037) is located in a very well conserved amino acid sequence, GGDE, found in all members of family 20 glycoside hydrolases (47). The Glu residue in this sequence is the catalytic acid group, and the Asp residue is involved in stabilizing the reaction intermediate (47). This cell line had βIVS12 − 26G>A as the second allele, which was shown to result in inefficient splicing in another juvenile SD cell line, reported
to produce ~3% of normal Hex A activity (48, 49). Thus, it is likely that the 3.5% of normal Hex A activity that we found in cells (Table 1) of the patient is the result of the splice junction mutation and not the missense mutation.

One interesting observation was the different responses of Hex A and Hex S to PC treatment (Table 2). In general, cells treated with PYR showed higher relative increases of Hex S, as compared with Hex A, again suggesting that PYR is more effective than NGT in stabilizing the wild type α-subunit. As a consequence, the α-subunit could then act as a chaperone for the mutant β-subunit, rather than the other way around as with the wild-type subunits (35, 37).

One SD cell line was homozygous for a novel βC137Y mutation in domain I (Fig. 5). This cell line responded to PYR treatment with a relatively high increase in residual Hex A activity. Because protein domains are believed to fold independently of each other (50–52), and domain II contains both the active site and the subunit-subunit interface, it is possible that the heterodimer can form without domain I. If this is the case, the increased stability of the dimer resulting from PC treatment might allow domain I additional time to achieve its proper conformation before being directed to ER-associated degradation pathway. Regardless of the mechanism, this is the first example of a PC appearing to enhance the folding of a domain adjacent to the domain containing its site of binding. This phenomenon was not observed in the βT150P cell line that also had a novel domain I mutation in heterozygosity with the previously reported βP417L (24, 26) (Fig. 5 and Table 2). In previous reports (24, 26), the βP417L allele was present with the Δ16kb null allele and was shown to affect mRNA splicing. Thus, it conferred the residual Hex A activity we observed (24, 26).
PYRIMETHAMINE: PHARMACOLOGICAL CHAPERONE FOR GM2 GANGLIOSIDOSIS

TABLE 4
Fibroblast cell lines with relative increases of residual Hex A activity in the presence of PYR and NGT

| Patient cell line number | Mutation 1          | Mutation 2          | Residual Hex A | Hex A with PYR | Hex A with NGT |
|--------------------------|----------------------|----------------------|----------------|----------------|----------------|
| TSD or α-mutants         |                      |                      |                |                |                |
| 27989                    | G269S                | c.1278insTAC         | 5.3            | 9.5            | 41.9           |
| 32540                    | G269S                | IV6 + 1G>A           | 6.5            | 10.4           | 46.8           |
| 26649                    | IV59 + 1G>A          | IV58 + 7G>Av         | 3.7            | 5.5            | –d            |
| SD or β-mutants          |                      |                      |                |                |                |
| 32045                    | R505Q                | IVS11 + 5G>Aa        | 4.2            | 15.5           | 19.3           |
| 36986                    | R505Q                | Δ16kb                | 5.3            | 41.9           | 19.0           |
| 1303                     | C137Yb               | C137Yb               | 1.3            | 6.1            | 6.2            |
| 30037                    | G353Rb               | IVS12 – 26G>A        | 3.5            | 5.2            | 5.6            |
| 32429                    | T1500bc              | P417L                | 2.6            | 7.0            | 5.4            |
| 3585                     | Δ16kb                | P417L                | 3.6            | 5.8            | 5.4            |
| 2400                     | Δ9048                | Δ16kb                | 12.7c           | 23.1           | 59.7           |

a Patient cell lines are labeled according to the storage number from our Tissue Culture laboratory.
b Novel mutations.
c NGT failed to show any significant increases in Hex A activity or α-mutant protein level (Fig. 6b).
d bP504S mutation reduces the ability of residual Hex A to bind the GM2 ganglioside-Activator complex by 3-fold as previously described.

Testing the βP417L/Δ16kb cell line with PYR or NGT resulted in similar enhancements (Table 2) as were achieved in the βT150P/P417L cells. Thus, as for domain II mutants, not all domain I mutants can benefit from PC treatment.

PYR was originally developed as a dihydrofolate reductase inhibitor, which is used for treatment of parasitic diseases, including chloroquine-resistant malaria and toxoplasmosis (53, 54). PYR is an orally administered drug, with a well studied pharmacokinetic profile (55). Studies have shown that 12–26% of serum levels cross the blood-brain barrier (39). Thus to test PYR as a PC for mutant forms of Hex A, we used PYR concentrations corresponding to levels achieved in the nervous system of normal individuals by screening for the clinical phenotype associated with a specific mutation.

The potential clinical impact of the PYR acting as a PC may be estimated from the level of enhancement of the residual Hex A activity in tissues from the patients compared with the critical threshold approximation of 5–10% of normal (10). In Table 4, we calculate the % of residual Hex A based on the maximum enhancements that were achieved with either PYR or NGT treatment. Five mutant cell lines, αG269S/c.1278insTAC, αG269S/IV6 + 1G>A, βP504S/Δ16kb, βR505Q/IVS11 + 5G>A, and βR504S/Δ16kb, showed increases in residual Hex A activity over the 10% critical threshold. Similar treatment of other cell lines significantly increased residual Hex A levels, but failed to produce levels over 10% of normal. Given the wide range of clinical phenotypes associated with a very narrow range of residual enzyme activities, even these smaller enhancements might be expected to be clinically relevant and slow the rate of progression of neurodegeneration in GM2 gangliosidosis. NGT, in general, performed better as a PC, but at much higher concentrations than PYR. However, whereas PYR is an FDA-approved drug with extensive data about its pharmacokinetics and safety, studies on the toxicity of NGT in animals are still in progress.

In conclusion, PYR was identified, and shown to function as a PC for mutant cell lines from patients presenting with a late-onset form, i.e., juvenile or adult, of GM2 gangliosidosis. Our studies on the effects of PYR treatment on α- and β-mutants have provided interesting insights into the independence of protein domains during the folding and subunit assembly stages of Hex in the ER. We have shown that screening of a library of FDA-approved drugs for competitive inhibitors of lysosomal enzymes is a feasible and practical approach to find potential PC for mutants causing devastating inherited metabolic disorders, such as GM2 gangliosidosis. The drugs represented in the library are already in current use for other medical purposes, indicating that they already passed extensive testing for toxicity, both in animals and humans. This considerably decreases the time and expense associated with conventional drug development, bringing potentially effective therapies to patients more rapidly. Clinical trials using such drugs could also provide a proof-of-principle for the concept of PC therapy, which would encourage investments into development and testing more optimized PC molecules.

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