GAUCHER DISEASE: EX VIVO AND IN VIVO EFFECTS OF ISOFAGOMINE ON ACID β-GLUCOSIDASE VARIANTS AND SUBSTRATE LEVELS

Ying Sun1,3, Benjamin Liou1, You-Hai Xu1,3, Brian Quinn1, Wujuan Zhang2, Rick Hamler4
Kenneth D.R. Setchell2,3 and Gregory A. Grabowski1,3

From: The Division of Human Genetics1, The Division of Pathology and Laboratory Medicine2, Cincinnati Children’s Hospital Medical Center and the Department of Pediatrics3, University of Cincinnati College of Medicine, Cincinnati, OH 45229-3039. Amicus Therapeutics Inc.4, Cranbury, NJ 08512.

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Address correspondence to: Gregory A. Grabowski, M.D. Cincinnati Children’s Hospital Medical Center, Division of Human Genetics, 3333 Burnet Avenue, MLC 4006, Cincinnati, Ohio 45229-3039. Phone: (513) 636-7290. Fax: (513) 636-2261. E-mail address: greg.grabowski@cchmc.org

Capsule

“Chaperones” may enhance mutant enzyme activities, but therapeutic levels have not been shown in vivo.
A Chaperone, Isofagomine, stabilizes wild type and mutant acid β-glucosidases in tissues and sera, and reduces visceral substrates in vivo. These effects are enhanced pre- and post-synthetically.
The results are proof-of-principle for the potential therapeutic use in residual enzyme diseases.

Isofagomine (IFG) is an acid β-glucosidase (GCase) active site inhibitor that acts as a pharmacological chaperone. The effect of IFG on GCase function was investigated in GCase mutant fibroblasts and mouse models. IFG inhibits GCase with Kᵢ ~30 nM for wild-type and mutant enzymes (N370S and V394L). Fibroblasts treated with IFG at μM concentrations showed enhancement of WT and mutant GCase activities and protein levels. Administration of IFG (30 mg/kg/day) to the mice homozygous for GCase mutations (V394L, D409H, or D409V) led to increased GCase activity in visceral tissues and brain extracts. IFG effects on GCase stability and substrate levels were evaluated in a mouse model (hG4L/PS-NA) that has doxycycline (DOX) controlled human WT GCase (hGCase) expression driven by a liver specific promoter and is also homozygous for the IFG-responsive V394L GCase. Both human and mouse GCase activity and protein levels were increased in IFG-treated mice. The liver-secreted hGCase in serum was stabilized and its effect on the lung and spleen involvement was enhanced by IFG treatment. In 8-week IFG-treated mice, the accumulated glucosylceramide and glucosylsphingosine were reduced by 75 and 33%, respectively. Decreases of storage cells were correlated with >50% reductions in substrate levels. These results indicate that IFG stabilizes GCase in tissues and serum, and can reduce visceral substrates in vivo.

Abbreviations: WT, wild-type; GCase, acid β-glucosidase; hGCase, human WT GCase; GS, glucosylsphingosine; GC, glucosylceramide; IFG, isoformagomine; DOX, doxycycline.

Mutations of the acid β-glucosidase gene (GBA1) lead to a common inherited lysosomal storage disease, Gaucher disease, that results from defects in acid β-glucosidase’s (GCase) degradation of substrates, glucosylceramide (GC) and glucosylsphingosine (GS). Depending on the organ involvement, Gaucher disease is classified into three types: Type 1 is a visceral disease manifested by hepatosplenomegaly, and hematologic, and skeletal dysfunction (1). Type 2 is an acute neuronopathic disease, and Type 3 is a subacute neuronal/visceral disease. Over 350 mutations have been identified in affected patients (1,2). Some of the mutations are caused by recombination between GBA1 and pseudogene (3), which creates difficulties in assessments of genotype and phenotype correlation in Gaucher disease. The accumulation of the substrates leads...
to enlargement of the liver and spleen, bone lesions, and central nervous system manifestations (1,4,5). The macrophage is the primary cell displaying substrate accumulation (1).

Of the disease causing mutations in GBA1, many are rare or occur in single families (1). Homozygosity for N370S is associated with type 1, non-neuronopathic disease and variable visceral involvement (1,6,7). The N370S mutation has high frequency in the Ashkenazi Jewish population. The L444P recurrent mutation is highly associated with neuronopathic variants of Gaucher disease, and is the most common Gaucher disease allele world-wide (1). The D409H alleles also have significant frequency and D409H homozygotes manifest early onset of variable visceral and CNS involvement (8,9). Uniquely, calcific aortic root and valve disease occurs with D409H homozygosity (9). The V394L allele in humans has been reported only in the heteroallelic state and is associated with either types 1, 2 or 3 depending on the heteroallele and genetic background (10).

Mouse models having Gba1 mutations have been generated. In contrast to humans, N370S or L444P (as an insertional mutant) homozygosity in mice leads to death within 24-48 h (11,12). Homozygosity for V394L or D409H leads to defective GCase activity, but mice survive to ~24 mo. with only minor visceral abnormalities (11). More extensive involvement in such mouse models was developed by cross breeding of V394L or D409H with a hypomorphic prosaposin deficient mouse, termed 4L/PS-NA or 9H/PS-NA, respectively (13). Such mice show numerous engorged macrophages and large GC accumulations. GCase is not secreted out of cells, except when heavily over expressed (14-16). Conditional expression of human GCase (hGCase) in the liver of 4L/PS-NA mice (hG/4L/PS-NA) leads to secretion of the human enzyme into serum, reduction of GC tissue accumulation, and improvement in the visceral phenotype (14). The expression of hGCase in hG/4L/PS-NA mice is driven by liver-enriched activator promoter and controlled by the tetracycline transcriptional activation system. This model provides a system to evaluate endogenous enzyme therapy for Gaucher disease and assessment of substrate accumulation following periods off enzyme therapy.

Two therapeutic approaches are available for treating Gaucher disease patients. Enzyme reconstitution therapy is accomplished by infusing recombinant macrophage-targeted wild-type (WT) hGCase to supplement the residual activity of the endogenous mutant enzyme. Substrate synthesis inhibition therapy seeks to reduce substrate levels by decreasing glucosylceramide production through inhibition of glucosylceramide synthase. Additional pharmacological small molecule approaches have been proposed to treat Gaucher disease by enhancing mutant enzyme stability and trafficking to lysosomes (17,18). Isofagomine (IFG), a competitive inhibitor, binds to the active site of GCase with high affinity at neutral pH in endoplasmic reticulum, where GCase has decreased stability, and with low affinity in the acidic lysosomes, where IFG can be released from GCase. The binding of IFG to GCase enhances its folding, stability, and trafficking to lysosome (19,20). This effect has been called chaperoning; we prefer the term enzyme enhancement therapy (EET). Several mutant GCases show increases in enzymatic activity and protein in response to IFG (19-21). IFG distributes to brain, bone marrow, and visceral tissues following oral administration (21). The peak level of IFG in tissues reached within 1 h after administration is in the order: liver>plasma>spleen>brain with half-lives 2.6, 4.4, 4.6, and 9 h (21). However, the outcome of IFG treatment on substrate accumulation has not been reported.

Here, the effects of IFG on mutant GCases were evaluated with recombinant enzymes and GCases in D409H and D409V cells that displayed enzyme deficiencies (11). Assessments of IFG’s effects on substrate levels were evaluated in the hG/4L/PS-NA model that has doxycycline (DOX) controllable WT hGCase expression in the liver (14).

**EXPERIMENTAL PROCEDURES**

**Materials**- The following were from commercial sources: 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc; Biosynth AG, Switzerland). Sodium taurocholate (Calbiochem, La Jolla, CA). Mouse anti-β-actin monoclonal antibody (Sigma, St. Louis, MO). NuPAGE 4-12% Bis-Tris gel, NuPAGE MES SDS running buffer,
DMEM, (Invitrogen, Carlsbad, CA). Rat anti-mouse CD68 monoclonal antibody (Serotec, Oxford, UK). M-PER Mammalian Protein Extraction Reagent and BCA Protein Assay Reagent (Pierce, Rockford, IL). Hybond™-ECL™ nitrocellulose membrane and ECL detection reagent (Amersham Biosciences, Piscataway, NJ). ABC Vectastain and Alkaline Phosphatase Kit II (Black) (Vector Laboratory, Burlingame, CA). Isofagomine tartrate (IFG) was from Amicus Therapeutics Inc., Cranbury, NJ. Imiglucerase™ was from Genzyme Corp., Cambridge, MA.

Expression and purification of GCases—The mutant GBA1 constructs were generated from the human cDNA, cloned into pBluescript 4.5 (Invitrogen, CA), after site-directed mutagenesis (QuikChange, Stratagene, TX). The specific mutations and the entire clones were validated by complete DNA sequencing. The baculoviruses containing the requisite human GBA1 cDNAs were purified and then used to express hGCases that were subsequently purified from protein free medium (22).

Inhibitor interactions with mutant GCases—The GCase activities were determined fluorometrically as described (22). The IC_{50} values for IFG with each GCase were determined by using various concentrations of IFG that were incubated with homogeneous hGCase (imiglucerase) or the purified human mutant GCase (N370S or V394L) from insect cell medium. IFG was shown to be a competitive inhibitor of each GCase variant form (22). The experiments were performed in triplicate and repeated thrice. GraphPad Prism 5 software was used for data analysis.

Cultured skin fibroblasts—Human fibroblast lines that contained the WT, N370S/84GG (84GG is a null mutation), and L444P/L444P GCases were obtained from the Coriell Cell Repository. Mouse fibroblast cells with the designated genotypes were established from newborn pups (11,13). The cells were incubated for 5 days in the medium containing IFG at the indicated concentrations. The cells were washed thrice prior to harvest, and the cell pellets were re-suspended in 0.25% sodium taurocholate/0.25% Triton X-100 and lysated by cup sonication in an ice water bath for 3 x 1 min cycles. The cell lysates were used for enzymatic assays and cross-reacting immunological material (CRIM) determinations.

Co-localization of GCases and Lamp1 in the fibroblast—The fibroblasts were incubated with IFG for 5 days and re-seeded on the chamber slides at the density of 5000 cells/cm^2 in fresh medium containing IFG for additional 2 days. The cells were then fixed in 3% paraformaldehyde in PBS (pH 7.4) at room temperature for 40 min, washed twice with PBS, and quenched with 50 mM NH_4Cl for 10 min. Following two PBS washes, the cells were incubated with 0.5% saponin in PBS (pH 7.4) for 30 min at room temperature. After two washes with PBS, the cells were treated with 1.5% non-fat dry milk/1.5% BSA/0.5% gelatin in PBS and reacted with goat anti-hGCase (1:50) and rabbit anti-human Lamp1 for human fibroblasts, or rabbit anti-mouse GCase (1:100) and rat anti-mouse Lamp1 (1:50, RDI, MCD107A-D4B) for mouse fibroblasts. The following secondary antibodies were used for detection: Horse anti-goat biotin and streptavidin-Alex 610 (1:100, MP, S32359) for hGCase; goat anti-rabbit FITC (1:100, ICN, 55664) for human Lamp1; goat anti-rabbit FITC for mouse GCase and donkey anti-rat Texas red (1:100, abcam, ab6732-1) for mouse Lamp1. Images were captured with a Zeiss Apotome microscope (AxioV200) at excitation of 488 nm (for FITC) or 599 nm (for Alexa-610, Texas red).

Mouse models and IFG treatment—Mice homozygous for Gba1 encoding V394L (4L), D409V (9V) and D409H (9H) GCases (11) and 4L/PS-NA (PS-NA;V394L/V394L) mice were as described (13). The 4L/PS-NA mice contain a hypomorphic prosaposin cDNA against a prosaposin knockout (PS-/-) background that also had the Gba1 encoding homozygous V394L (13). hG/4L/PS-NA mice are generated by breeding 4L/PS-NA with two transgenic lines: one containing the tetracycline transactivator driven by liver-enriched activator promoter and one having the hGCase cDNA constructed into the vector containing a tetracycline-responsive promoter element (tet-off) (14). The strain backgrounds for hG/4L/PS-NA and 4L/PS-NA mice are 1/2:1/2, C57BL/6;129SvEvbrd. The mice were provided drinking water containing sufficient IFG to supply 30 mg/kg/day based on the assessed daily water consumption of 8 mL/day. The mice were maintained in a microisolator pathogen free
environment and in accordance with institutional guidelines under IACUC approval at Cincinnati Children’s Hospital Research Foundation.

**GCase activity assays and CRIM determinations**- Tissues and cell pellets were homogenized in 0.25% Na taurocholate and 0.25% Triton X-100. GCase activities were determined fluorometrically with 4MU-Glc as substrate (11).

CRIM for each GCase variant in fibroblasts was determined by immunoblot with rabbit anti-human or anti-mouse GCase polyclonal antisera assuming equal reactivity of the GCase in variants and wild-type. Equal GCase activity from each sample was applied to 10% SDS gels for immunoblot analysis. The density of the band corresponding to intact GCase CRIM was quantified in duplicate using ImageQuant 5.2 software. The known masses of homogeneous hGCase (2.5, 5 and 10 ng) were used as standard on each gel. Serum hGCase activity and protein were determined (14).

Mouse tissues were homogenized in M-PER Mammalian Protein Extraction Reagent. Protein concentrations were estimated using BCA Protein Assay Reagent. Tissue extracts were separated on NuPAGE 4-12% Bis-Tris gel with NuPAGE MES SDS running buffer, and electro-blotted on Hybond™-ECL™ nitrocellulose membranes. The membranes were blocked with 3% BSA in 1xPBS for 1 h, followed by incubation overnight with either rabbit anti-mouse GCase (1:2,000) or anti-hGCase (1:2,000) in 1.5% BSA/1xPBS. The goat anti-rabbit HRP (1:1500) conjugate was used to detect mouse or hGCase. The signals were developed using ECL detection reagent according to the manufacturer’s instructions. Mouse anti-β-actin monoclonal antibody (1/10,000 in 1.5% BSA) was applied to detect β-actin as a loading control. The density of the bands corresponding to GCase and β-actin were quantified in duplicate using ImageQuant 5.2 software.

**Histological Studies**- For immunohistochemistry, mouse tissues were perfused with saline followed by 4% Paraformaldehyde and collected. Frozen tissue sections fixed with 4% Paraformaldehyde were incubated with rat anti-mouse CD68 monoclonal antibody (1/200 in PBS with 5% BSA) overnight at 4°C. Detection was performed using ABC Vectastain and Alkaline Phosphatase Kit according to the manufacturer’s instruction. The slides were counterstained with hematoxylin. CD68-positive macrophages in liver and lung sections were counted manually in 10 images (305 μm x 228 μm/image) randomly selected from each mouse, n=3-5 mice.

**GCase stability in serum**- IFG effects on GCase stability in serum were determined using hGCase (imiglucerase). hGCase was added to mouse serum (pH 7.4) at 1 μg/mL with or without IFG (25 μM or 50 μM). The mixture was pre-incubated on ice for 10 min, then transferred to 37°C and incubated for indicated time (1-7 hrs). At each hour, 10 μL of each sample was removed to a new vial and diluted by 4x10^4-fold with reaction buffer (0.25% Na taurocholate, 0.25% Triton X-100, and 25 mM Citrate/50 mM Phosphate, pH 5.6) to achieve [IFG]< 0.65 or 1.25 nM, and then determined for GCase activity as described (22). hGCase incubated in the reaction buffer (pH 5.6) was assayed in parallel as a control. The assay is conducted with dual samples in triplicate.

**Glycosphingolipid analyses**- Mouse tissue samples (~60 mg wet weight) were homogenized with a PowerGen 35 (Fisher Scientific) in methanol/chloroform/water (3.6 mL, 2:1:0.6, v/v/v). Homogenates were shaken for 15 min and centrifuged (5 min @ 1,000 x g). Pellets were re-extracted with H2O (0.7 mL) and chloroform/methanol (3 mL, 1: 2 v/v). The combined extracts were centrifuged (10 min @ 7,000 x g). The supernatants were transferred to fresh tubes and the solvents evaporated under N2. Dried samples were then dissolved in chloroform/methanol/water (5 mL, 2:1:0.15, v/v/v) and subjected to alkaline methanolysis (23), followed by eluting from Sephadex G-25 fine columns to remove non-lipid contaminants.

The extracted samples were redissolved in methanol containing an internal standard. GC and GS analyses were carried out by ESI-LC-MS/MS using a Waters Quattro Micro API triple-quadrupole mass spectrometer (Milford, MA) interfaced with an Acquity UPLC system. Optimized parameters for GC and GS were determined with individual standard compounds. For quantification of GCs, the ESI-MS/MS was operated in the multiple reaction monitoring (MRM) modes in monitoring transition pair of the individual protonated parent ions and their common daughter ion m/z 264. GS was monitored by mass transition m/z 462.3 → 282.4. Calibration
curves were built for C16, C18 and C24:1 GCs using C12-GC as an internal standard (Avanti Polar Lipids, Inc., Alabaster, AL). The resolution of GC and galactosylceramide in brain samples was achieved using silica column (Supelco 2.1*250 mm) running under HILIC (hydrophobic interaction liquid chromatography) mode with a mobile phase of acetonitrile/methanol/acetic acid (97/2/1, v/v/v) with 5 mM ammonium acetate. Quantification of GCs with various chain lengths was realized by using the curve of the natural GC with the most similar fatty acid chain length. The quantification of GS was based on the curve using C8-GC as internal standard (Avanti Polar Lipids, Inc., Alabaster, AL). The linear responses for GCs and GS were in the range of 50 pg to 25 ng. Two to four samples for each cohort were included in the analysis. Data were analyzed by Student’s t-test using GraphPad Prism 5 software.

Quantitation of tissue and serum IFG levels

Frozen tissue samples were homogenized (7 µL of deionized water per 1 mg of tissue) using FastPrep-24 Instrument (MP Biomedical, Irvine, CA). The homogenization was processed on FastPrep at speed = 4.5 for three of 30 sec shakes (90 seconds total). Tissue homogenate (50 µL) or serum (50 µL) were diluted with 100 µL acetonitrile followed by mixing with 100 µL of 100 ng/mL IFG 13C2-15N (Internal Standard) dissolved in acetonitrile/methanol + 0.50% formic acid (70/30, v/v). The mixture was vortexed for 2 min and centrifuged at 15,294 x g for 5 min at room temperature. 20 µL supernatant was injected onto the column.

The instrument consisted of an AB SCIEX 4000 QTRAP® LC/MS/MS System (AB SCIEX, Foster City, CA), Shimadzu LC-10ADvp pumps, Shimadzu SIL HTc auto sampler and Shimadzu DGU-14A degasser. A Thermo Betasil Silica-100 (50×3 mm, 5 µm) was used for LC separation. Mobile phase was delivered at 0.6 mL/min with the following program: 100% A (5 mM ammonium formate and 0.05% formic acid in acetonitrile/H2O, 95/5) for 1.2 min, linear ramp to 80% B (5 mM ammonium formate and 0.05% formic acid in methanol/water/acetonitrile, 70/20/10) in 4.3 min, hold at 80%B for 0.5 min, then increased to 100% B in 0.01 min and hold 100% B for 0.49 min, followed by switching to 100% A and equilibrated for 2.2 min. The MS instrument was operated in the positive multiple reaction monitoring mode with detection of the transition pairs of m/z 148.1>112.1 and 151.1>115.1 for IFG and IFG 13C2-15N, respectively. Low limit of quantitation was set at 8 ng/g tissue and 1 ng/mL for serum.

RESULTS

IFG inhibition of GCases. Inhibition properties of IFG were tested with purified recombinant WT hGCase (imiglucerase™), and the recombinant human N370S and V394L mutant GCases in media from insect cells expressing these specific GCases (22). The respective IC50 and calculated Ki values were 88±9 and ~29 nM for WT and V394L, and 115±5 and ~38 nM for N370S at pH 5.5.

Ex vivo effects of IFG on GCases. Media concentrations of IFG between 6.25 to 50 µM enhanced GCase activities significantly in all cells tested as shown in concentration-response curves (Fig. 1A insert). The concentrations to achieve peak activity for each mutant cell are summarized in Figure 1A. Human and mouse N370S GCase had the same responses and were maximally increased by ~3.5-fold, or to ~60% of untreated WT levels. IFG did not increase human L444P activity significantly at any concentration, In comparison, the mouse GCase activities in V394L and 4L/PS-NA fibroblasts showed ~3-fold increases with 25 or 12.5 µM IFG, respectively; these enhanced activities were to ~50% of untreated WT levels. D409H and D409V cells showed smaller maximal enhancement, i.e., 1.6- and 1.3-fold, respectively.

GCase protein levels in WT and N370S fibroblasts were maximally increased 1.3- or 1.4-fold by IFG treatment (Fig. 1B). A 3-fold increase of GCase protein was observed in IFG-treated L444P cells. The CRIM specific activity (activity/nmol GCase protein) was used to estimate the change in catalytic rate constant for the various GCases following IFG treatment. No effect on WT was found (i.e., the GCase protein and activity increased concordantly), whereas with N370S the value was increased by 2.7-fold, but that for L444P decreased to 0.36. For the mouse fibroblasts, small decreases or increases in CRIM specific activity were observed, thereby indicating relatively concordant increases in activity and protein with IFG treatment (Fig. 1B). These data show that IFG’s major effects are to stabilize the
mutant GCase protein in fibroblasts suggesting that relatively minor conformational changes affect the catalytic rate constant as evidenced by the N370S structure (24). For the highly unstable L444P enzyme, IFG treatment did increase the amount of GCase protein, but had little-to-no effect on the enzyme’s catalytic properties.

To evaluate IFG’s effects on GCase intracellular trafficking, fibroblasts were treated with IFG (50 μM). Human and mouse WT cells with or without IFG showed colocalization of GCase and Lamp1, a lysosomal marker (Fig. 2). In IFG-treated L444P cells, the GCase signals were clearly increased and more robust than those in untreated cells, and IFG treatment resulted in partial lysosomal localization. For human N370S fibroblasts, the effect of IFG was similar to WT (data not shown). In mouse 4L/PS-NA cells, IFG increased V394L GCase protein trafficking into lysosome (Fig. 2B).

In vivo IFG effects. The mice were administrated IFG (30 mg/kg/day). For WT and homozygous V394L (4L) mice, this was started at postnatal day 10 and continued for 5 wks. Homozygous D409H (9H) and D409V (9V) mice began treatment at 3 wks of age and were continued for 4 or 8 wks. GCase activities in liver, spleen, lung, and brain were compared to the untreated mice (Fig. 3). IFG treatment had variable effects (<2–fold) on GCase activity in the visceral tissues (liver, lung, and spleen) of WT, 4L, 9H and 9V mice, and minor effects in the CNS of WT, 9H and 9V mice. No IFG effect was evident in the 4L brain.

IFG stabilized GCase in tissues and serum. The hG/4L/PS-NA mouse model contains the hGCase whose expression is under control of the tet-off system (14). In the absence of DOX, transgenic hGCase is expressed and secreted from liver in large amounts. GCase is rapidly denatured at neutral pH (25). Following exposure to pH 7.4 in the serum, only ~8% of the secreted enzyme remained active (14). To test if IFG could stabilize the secreted hGCase, the sera from IFG-treated (30 mg/kg/d; 4 weeks) hG/4L/PS-NA mice were analyzed for GCase activity and protein. Compared to untreated mice, serum GCase activity or protein in the treated mice increased 2.2-fold or 2.4-fold, respectively (Fig. 4 A and B). The results indicate a concordant stabilization of GCase activity and protein by IFG at neutral pH.

In vitro assays were performed to evaluate effect of IFG on GCase in the serum. hGCase (imiglucerase) was incubated in mouse serum (pH 7.4) at 1 μg/ml with or without IFG (25 μM) at 37°C for indicated time (1–7 hrs). hGCase in serum (pH 7.4) with IFG 25 μM had t1/2 ~ 2 hrs and the activity was decreased to 7% of initial activity by 7 hrs. In comparison, the t1/2 was <1 hr without IFG and the activity was declined to <5% by 3 hrs. As a control, the enzyme incubated in the reaction buffer alone at pH 5.6 (lysosomal pH) had a t1/2 ~ 6 hrs, remained at 80% of initial activity after 3 hrs incubation, and only reduced to 50% by 7 hrs. The result indicates that the enzyme was not stable at neutral pH 7.4 and IFG increased the stability of the liver hGCase secreted into the serum.

Feeding DOX to these mice turns off hGCase expression in liver and the remaining GCase activity in tissues is that of the mouse V394L enzyme. Neither the WT nor mutant mouse GCases are secreted from cells under non-overexpressed conditions (14,16). Consequently, the effects of IFG treatment on either the human WT or mouse V394L GCase activities and proteins can be determined by feeding mice DOX-free or –containing (DOX) food. At 4 wks of age, hG/4L/PS-NA mice were fed IFG in drinking water for 4 wks on either DOX or DOX-free food. DOX-free mice showed an increase of GCase (hGCase + V394L GCase) activity in the lung (7.6-fold) and spleen (2.1-fold) after IFG treatment (Fig. 5A). The GCase activity in IFG-treated livers showed a trend toward increases relative to the untreated mice; this may be due to the very large amounts of hGCase produced in the transgenic livers. In DOX-treated hG/4L/PS-NA mice, IFG did increase V394L GCase activity in the liver (3.8-fold), but not in the lung and spleen (Fig. 5A).

The specificity of our antibodies to human or mouse GCase allowed independent quantification of the human or mouse proteins under all experimental conditions. Compared to untreated mice, the hGCase protein level in IFG-treated liver increased by 2.6-fold and in the spleen by 1.3-fold. In the lung, hGCase protein was present in the IFG-treated mice, whereas it was undetectable in the untreated mice (Fig. 5B). In IFG-treated mice, the V394L GCase protein increased by 2.1-fold in the liver, 1.6-fold in the lung, and 1.4-fold in the spleen (Fig. 5C). These results indicated that IFG stabilizes hGCase derived from serum and the
endogenous mouse V394L mutant proteins, and enhanced their enzymatic activities to varying degrees in different organs. The delivery of hGCase present in the serum to the lung and spleen was enhanced by IFG. Importantly, the activity in the lung was increased more than the protein (Fig. 5A and 5B), thereby suggesting a reconformation of the enzyme by IFG during synthesis and secretion from the liver.

**IFG levels in mouse tissues and serum.** The samples from hG/4L/PS-NA mice treated with IFG in drinking water were collected at the end of 4-wks or 8-wks of treatment (GCase activity and lipids data shown in Figs 5, 6, 7 and 8). IFG levels in tissues and serum were determined by LC/MS. The liver had the highest IFG level. The tissue IFG level was in the order: liver>serum>lung>brain (Table 1). IFG levels were not significantly different between 4- and 8-wks of treatments. The mice without IFG treatment had no detectable IFG in tissues or serum. The relative IFG tissue levels were consistent with those reported in L444P mutant tissues (21).

**In vivo effects of IFG treatment on lipid storage.** In the absence of DOX (DOX-free), hGCase reduced substrate levels in 4L/PS-NA mouse tissues. Turning off hGCase expression with dietary DOX led to substrate accumulation (Fig. 6) (14). For these experiments, hG/4L/PS-NA mice started IFG treatment at 4 wks for either DOX or DOX-free groups. This treatment was continued for 4 (Fig. 6) or 8 wks (Fig. 7). No significant changes in substrate levels were observed in 4-wks IFG-treated mice, except that lung GS levels were reduced by 46% compared to those mice that received only DOX (Fig. 6). In 8-wks IFG-treated DOX mice, both GC and GS were reduced by 35-40% in liver and lung (Figs. 7A and 7B). However, decreases in GC (75%) and GS (62%) levels were observed in the lungs of DOX-free mice receiving IFG. The GC and GS levels in the liver of these DOX-free mice were very low, and changes could not be appreciated by the addition of IFG. The substrate reductions correlated with activity increases in IFG-treated mice (Fig. 7C). CD68 positive storage cells were quantified in liver and lung from each group of mice. Decreased numbers of storage cells were detected in the lungs only of DOX-free mice receiving IFG treatment (Fig. 7D). Consequently, the reduction of storage cells correlated with >50% decreases of substrate levels. In the brain, the transgenic hGCase activity (i.e., DOX-free) was slightly enhanced and V394L GCase activity (i.e., DOX-treated) was unchanged by IFG treatment (Fig. 7), but GC and GS levels in the brain were not significantly altered by IFG treatment (Fig. 7).

The ability to turn on and off hGCase synthesis in the liver permitted an evaluation of the extent to which IFG influenced V394L mouse GCase or newly synthesized hGCase. hG/4L/PS-NA mice were kept on DOX for 4 wks, i.e., between 3-7 wks of age, and then taken off DOX and treated with IFG for 4 wks. In these animals, IFG led to further reductions (50-54%) in GC levels in the liver and lung, whereas GS reductions were detected only in the lung (Fig. 8A and 8B). The lack of an effect on liver GS levels is likely attributable to concentrations of GS near the noise level in liver before IFG treatment, thereby obscuring any additional effects (Fig. 8B). There was inverse correlation of substrate reduction and enhancement of GCase activity in IFG treated liver and lung (Fig. 8C). The decrease in CD68 positive cells correlated with the changes in substrate levels (Fig. 8D). This result suggests that IFG has an effect on stabilizing the GCase conformation in the liver and lung during its synthesis. The minor increase of brain hGCase activity (Fig. 7) in IFG-treated mice likely resulted from hGCase in the blood due to incomplete perfusion. Such low level of activity had no effect on reduction of substrate levels in the brain (Fig. 8).

**DISCUSSION**

Small molecule competitive inhibitors of lysosomal enzymes have been envisioned as potential “chaperones” for the improvement of residual mutant activity (EET) in cells of individuals afflicted with such diseases (18,26). To date several ex vivo and a few in vivo studies have assessed the effects of such compounds on normal and mutant enzyme activities when administered via culture media or orally (19-21,27,28). In particular, for lysosomal hydrolyses (e.g., α-galactosidase A or GCase), imino-sugars (e.g., IFG) are potent competitive inhibitors that could act as chaperones to improve the stability and/or activity of the residual mutant enzymes in specific cell types (19,20,27). In general, these studies are
based on relatively artificial assay systems in which cultured cells or removed tissues are homogenized with various buffers that lead to significant dilutions of the reversible inhibitor. In addition, synthetic substrates and/or detergents are used to assess the effects on specific enzymes (19-21,27). The dilution effects either on cells or lysates clearly removes the inhibitor from the environs of the enzyme and lead to essentially an uninhibited enzyme. Remarkably, many of these studies have shown substantial increases in residual enzyme activity as measured by these assays (19-21,27), suggesting that the mutant enzymes can retain different conformational states for some period after the dilution of the “chaperone” or reconforming molecule. In addition, imino sugars can be delivered to several locations throughout the body, including multiple intracellular and extracellular spaces with differential pH environments (20,21,29). The latter clearly affects the binding constants for these compounds to specific enzymes. For example, IFG has been used as a very potent inhibitor of GCase, the Gaucher disease enzyme, and this compound has a pK\textsubscript{a} $\approx$ 8.6 (30). The K\textsubscript{i} varies substantially with pH with IFG becoming a much more potent inhibitor at more neutral pH’s (31), i.e., K\textsubscript{i} $\approx$ 145, 30, 20, and 1 nM at pH 4.5, 5.6, 6.0, and 7.2, respectively, with purified hGCase (Unpublished data, Liou and Grabowski). Such ex vivo and in vitro studies indicate that this compound could have differential interactions and effects in several cellular, and in particular, extracellular environments. Such results have implications for the conformation of intracellular enzymes, specifically GCase, and extracellular enzymes for enzyme or gene therapy experiments in which the therapeutic enzyme is present in a hostile, potentially denaturing environment, e.g., plasma/serum. Because more neutral pHs denature GCase, such a “chaperone-like” agent might be highly useful in the treatment of Gaucher disease variants using approaches that require the enzyme’s exposure to non-lysosomal pH. Critical studies of the effects on actual in vivo substrate accumulation by the apparent increases assessed from in vitro enzyme assays must be shown for this approach to have viability in the lysosomal disease treatment paradigms.

Our data demonstrate that IFG is a highly potent inhibitor of human and murine WT and selected mutant GCases. These effects are comparable with mouse and hGCases, which differ by about 15% in amino acid sequence. Both the enhancement effects of IFG on WT and mutant GCase activity and their stabilities were similar within the murine or human fibroblast environments. In addition, each of the several different mutant GCases used in these experiments show differential effects of IFG on enzymatic activity, cell distribution, and the stability of the GCases within cells. Importantly, the V394L and N370S GCase activities and proteins were enhanced by IFG ex vivo to nearly heterozygous levels, which should be therapeutic in vivo.

In vivo IFG was shown to have variable effects on the WT and mutant GCase in different tissues with the effects in liver $\geq$ lung $\geq$ spleen $\geq$ brain vis-à-vis on GCase activity and protein levels. In these tissues, particularly liver and lung, a correspondence was observed between the degree of response of mutant GCases with that in cultured skin fibroblasts, suggesting a potential utility to predict in vivo effects. The differential tissue effects of IFG could have several etiologies, but the most likely would be the greater uptake into the various tissues with the amount delivered to liver $\geq$ lung $\geq$ spleen $\geq$ brain. However, some effects were observed in all tissues.

The above studies provide a backdrop for the evaluation of the in vivo effects of IFG on WT and mutant GCases and for determining effects on substrate accumulation. Our transgenic system that contains the doxycycline responsive element for expression of hGCase in liver (14) allowed us to examine IFG in conjunction with either enzyme or gene therapy approaches. The degree of IFG effects on the hepatically expressed hGCase was similar to that of the endogenous mouse WT GCase in the respective livers, spleens, and lungs. Importantly, the removed tissues had been perfused with saline so that hGCase-rich plasma was eliminated, thereby permitting assessments only of the enzyme that was taken up into the tissue. Consistent with the tissues IFG distribution levels, liver $\geq$ lung $\geq$ brain, the effects of IFG were specific to the tissue, with large effects on increasing WT hGCase activity in the liver (3 to 5 times) and also, 3- to 4-fold increases in mutant V394L GCase. In lung, very large effects were seen on the hGCase with little effect on the endogenous mutant enzyme V394L. In spleen and
brain, a lesser, but significant, effect occurred with
the hGCase, but no effect was detected on the
endogenous V394L GCases, indicating that these
tissues were exposed to lower concentrations of
IFG. As an example, there was the lack of major
effect from IFG treatment in the CNS of a V394L-
based model (28).

Importantly, together with the GCases activities,
the enzyme proteins were also increased by IFG
treatment in all tissues. However, disproportionate
increases were found in the GCases proteins
compared to the respective activities in all tissues,
except the liver. These results indicate that IFG
stabilized the WT human and mutant murine
GCases against proteolysis and that IFG had less
effect on conformations that restore or increase
enzymatic activities. Plasma that contained high
levels of hGCase in the mice that were off DOX
showed increases in hGCase activities when IFG
was also administered. This was further shown by
*in vitro* assays in which IFG diminished the rapid
inactivation of GCase activity in serum. This
indicates substantial protection afforded by IFG to
the hGCase from denaturation by plasma pH.

The ultimate aims of treatments are to decrease
storage of GC and GS in all tissues and improve
the health of patients with these diseases. In
particular, the tissue specific changes of GC and
GS were partially correlated with the GCases
activity effects. In the DOX-off transgenic mice
receiving IFG, there was little effect on the
substrate accumulation in the liver. Significant
decreases in GC levels were observed in the lung.
In the same mice, GS increased significantly in the
liver (Fig. 6B), whereas this substrate was
decreased in the lung, suggesting that greater
exposure of the liver to IFG was apparently at
inhibitory concentrations for this poor substrate,
i.e., GS is cleaved at ~100-200 times lesser rates
than GC (32). The paradoxical result in the lung
could have resulted from a lower level of IFG
being present in the tissue leading to enhanced
GCases activity without significant inhibitory
effects on either substrate. When these transgenic
mice were put on DOX, as well as IFG, only the
mutant V394L enzyme is present and some
decreases in GS concentrations were seen in
various tissues.

In comparison, 8 wks of IFG treatment resulted
in substantially different effects. These effects
were not dependent on IFG concentration, since
there was no significant difference of IFG levels in
each tissue between 4- and 8-weeks of treatment.
In the liver of DOX-off mice, IFG effects were
obscured since very low levels of GC and GS were
present, probably non-lysosomal, because of the
high level expression of human WT GCase. In the
lungs, the large amounts of GC and GS, particularly the latter, showed decreases relative to
the IFG-untreated mouse. However, the GS levels
obtained in the IFG-treated mice were nearly the
same as in the 4-wk treated mice. Reductions of
GC and GS levels were correlated with increase of
GCases activity in those treated tissues. This
suggests that the effect was either due to IFG
enhancing enzyme activity (e.g., $k_{cat}$) and/or
amounts of enzyme to prevent substrate
accumulation. Importantly, these changes in lung
glycolipid content were not observed in similar
mice treated with intravenous enzyme therapy
products in which no effect on lung lipid
accumulation was observed (33).

The ability to administer IFG after doxycycline
had been present for some period allowed, upon
DOX withdrawal, the new synthesis of hGCase in
the presence of IFG leading to some additional
enhancement of activity. This result suggests that
IFG may be useful during the synthesis of GCase
as well as post-synthetically, for conformational
retention/promotion effects. The effects of this
newly synthesized enzyme on substrate hydrolysis
was somewhat better than that observed at 8 wks
for hGCase that had been pre-formed prior to the
addition of IFG (Fig. 8).

These results support the contention that an
active site directed inhibitor can enhance
endogenous visceral wild type or mutant enzyme
activity, i.e., GCases, *in vivo*, but the effects are
tissue specific. These results could be due to the
differential exposure of tissue GCase to IFG, e.g.
liver>serum>lung>brain, and/or a differential
response of the GCase in various tissues or to
intrinsically different properties of the enzyme in
the tissue environments. Finally, the results
suggest that the effects of IFG are greater on
enzyme synthesized in the presence of IFG rather
than in a post-synthetic state. This implies that a
coformulation of such enzymes while the enzyme
is being made in various bioreactors might provide
specific enhancement that cannot be obtained after
the enzyme is fully formed. This would have
implications for the dosing and treatment of enzymes that are responsive to such agents.
REFERENCES


FOOTNOTES

The authors thank Lori Stanton, Huimin Ran, Venette Inskeep, Matt Zamzow, Rebecca Coyle and Rachel Reboulet for technical assistance, Lisa McMillin and Sabina Sylvest for skilled tissue preparation, and Michelle Cooley for clerical expertise. This work was supported by grants to GAG (HD 059823, DK 36729, and NS 36681)

FIGURE LEGENDS

Figure 1. IFG effects on fibroblast GCase activity (A) and protein (B) levels. (A) Mouse (WT, N370S, V394L, 4L/PS-NA, D409H, D409V) and human (WT, N370S, L444P) fibroblast cells were incubated with IFG. GCase activity at the indicated concentration to achieve peak effects (see insert) is presented as the percent of untreated WT activity. All treated mouse and human WT and mutant cells, except L444P, showed significant increases of activity. The activities in treated mouse V394L and 4L/PS-NA increased to ~50% of WT levels. GCase activity in IFG-treated human N370S cells was enhanced to 60% of WT levels. Fold changes relative to the untreated cells are indicated. The experiments were done in triplicate. Student’s t test, ***, p<0.001. (INSERT) IFG concentration-response curves for human and mouse WT and mutant GCase in fibroblast. The cells were treated with IFG at the indicated concentrations for 5 days. GCase activity changes after IFG treatment are presented as % untreated WT activity for mouse or human, respectively. (B) Quantitation of GCase protein in each variant was determined immunologically with mouse or human specific antibodies. All IFG-treated cells showed increases in GCase protein levels. These are presented as fold-change (indicated below the columns) relative to the untreated cells. The results represent the mean of three determinations.

Figure 2. Cellular localization of GCases in IFG-treated fibroblasts. Colocalization of (A) hGCase (red) with lysosomal marker, human Lamp 1 (green), and (B) mouse GCase (green) with mouse Lamp1 (red) was conducted by immunofluorescence staining. The fibroblast cells were treated with IFG (50 μM). Both untreated (-IFG) and treated (+IFG) human and mouse WT GCase colocalized with Lamp1 in
the lysosome. The untreated human L444P GCase was nearly undetectable. In contrast, the treated L444P cells had increased GCase and trafficking to the lysosome. In IFG-treated mouse 4L/PS-NA cells, the GCase signal was enhanced and localized in the lysosome. Scale bar is 10 μm.

Figure 3. IFG treatment enhanced GCase activity in mouse tissues. The mice were administered IFG (30 mg/kg/d) in drinking water. The treatments for WT and V394L mice were started at postnatal day 10 and continued for 5 wks; for D409H mice, at 21 days for 4 wks, and for D409V mice, at 23 days for 8 wks. GCase activities in each tissue were significantly increased in IFG-treated WT and mutant mice tissues, except V394L brain. Data were analyzed by Student’s t-test (n=3 mice). *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 4. IFG stabilized GCase protein in serum. The hGCase is expressed in and secreted from hG/4L/PS-NA mouse livers. (A) In IFG-treated mice (30 mg/kg/d, 4wks) hGCase activities secreted into the serum were significantly increased compared to untreated mice. (B) Immunoblot of GCase in serum determined by anti-hGCase antibody and quantitated relative to known amounts of imiglucerase as standard. Human GCase protein in the serum from IFG-treated mice was significantly increased compared to the untreated mice. Data were analyzed by Student’s t-test (n=3 mice, duplicate assays). **, p <0.01; ***, p<0.001.

Figure 5. GCase activity and protein in IFG-treated hG/4L/PS-NA mice. Human GCase was expressed in the liver of hG/4L/PS-NA mice when fed DOX-free food. In the mice on DOX food, hGCase expression was turned off, residual activity was from mouse V394L mutant GCase. (A) IFG treatment for 4 wks in drinking water enhanced both hGCase and mouse V394L mutant GCase activities in the liver, and hGCase activity in the lung and spleen (grey bars). (B) Immunoblot using anti-hGCase antibody demonstrated IFG treatment for 4 wks increased hGCase protein levels in the liver, lung and spleen of hG/4L/PS-NA mice. No protein was detected in untreated lung. (C) Immunoblot of mouse GCase detected by anti-mouse GCase antibody showed increases of mouse GCase protein in treated liver, lung and spleen. β-actin was the loading control. Data were analyzed by Student’s t-test (n=3 mice). ***, p<0.001.

Figure 6. Effects of IFG treatment for 4 wks in hG/4L/PS-NA mice. The mice were treated with IFG for 4 wks with DOX-free or DOX food. (A) No significant changes of GC levels were observed in IFG-treated liver and lung. (B) In liver of DOX-free IFG-treated mice, GS was increased. In lungs of DOX and IFG-treated mice GS was decreased. Liver GS level was not altered in these same mice. GC and GS levels were quantitated by LC/MS and normalized by mg wet tissues. Student’s t-test (n= 3 mice). *, p <0.05. GCase activities for those samples presented in Fig. 5.

Figure 7. Effects of IFG treatment for 8 wks in hG/4L/PS-NA mice. IFG was administered to the mice by drinking water for 8 wks with the mice either on DOX or DOX-free food. (A) Liver GC level was low in mice on DOX-free food and not significantly changed by IFG treatment. Liver GC in the mice fed with DOX food was reduced by 38% with IFG treatment. In comparison, lung GC levels were significantly reduced in IFG-treated mice on either DOX or DOX-free food. Brain GC levels were not significantly changed by IFG treatment. (B) Liver GS level was very low in the mice on DOX-free food and not significantly altered by IFG, whereas mice fed DOX food GS was reduced by 39% with IFG treatment. Lung GS levels were significantly reduced in IFG-treated mice on DOX or DOX-free food. Brain GS levels were not significantly changed by IFG treatment. (C) GCase activity. IFG treatment for 8 wks in drinking water enhanced both hGCase (hatched bar) and mouse V394L mutant GCase (square bar) activities in the liver and lung. IFG treatment enhanced hGCase activity (hatched bar) by 1.6-fold in brain, but did not alter mouse V394L GCase activity (square bar). (D) The CD68 positive macrophages were counted in liver and lung sections of 8-wks IFG-treated mice. Decreased cell numbers were observed in IFG-treated lung from the mice on DOX-free food. The CD68 positive cells were counted
from 10 images (305μm x 228μm for each image) per mouse. GC and GS levels were quantitated by LC/MS and normalized by mg wet tissues. Student’s t-test (n= 3 mice). *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

Figure 8. Reduced substrates and storage cells in hG/4L/PS-NA mice treated for 4-wks IFG during hGCase synthesis in the liver. hG/4L/PS-NA mice placed on DOX food from 3 to 7 wks of age to turn off hGCase expression. The mice were then withdrawn from DOX to permit hGCase synthesis and fed with IFG containing drinking water from 7-11 wks of age. (A) GC levels in IFG-treated liver and lung were reduced. Brain GC levels were not significantly changed by IFG treatment. (B) GS levels were decreased in IFG-treated mouse lungs. (C) GCase activities were increased in IFG-treated liver, lung and brain. (D) CD68 positive cell numbers in IFG-treated mice were decreased in both liver (upper panel) and lung (lower panel). Images of liver and lung were stained with anti-CD68 antibody on macrophage cells (brown) count-stained with hematoxylin (blue). The cell counts, lipids level determination and data analyses are as described in Figure. 7.
Figure 1

A

![Graph showing % Untreated WT activity vs. Isofagomine (yl)](image)

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Fold-change ± S.E.

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Mouse Human

B

![Graph showing Fold increase (C-R/M) vs. IFG (μM)](image)

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Fold-change ± S.E.

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Mouse Human
Figure 2

A

WT
-IFG

WT
+IFG

L444P
-IFG

L444P
+IFG

hGCase

hLamp1

Merged
Figure 2

B

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| **IFG**  | ![Image](image2)
|          | ![Image](image3)
| **WT**   | ![Image](image4)
| **+IFG** | ![Image](image5)
|          | ![Image](image6)
| **4L/PS-NA** | ![Image](image7)
| **IFG**  | ![Image](image8)
|          | ![Image](image9)
| **4L/PS-NA** | ![Image](image10)
| **+IFG** | ![Image](image11)
Figure 4

A  GCase activity

B  GCase protein

Immunoblot

- - + - IFG

hGCase
Figure 5

A

Liver

Lung

Spleen

DOX

IFG

WT

hG/4L;PS-NA

DOX

IFG

WT

hG/4L;PS-NA

DOX

IFG

WT

hG/4L;PS-NA

B

Liver

Lung

Spleen

hGCase

β-actin

IFG

C

Liver

Lung

Spleen

mGCase

β-actin

IFG
Figure 6

A  Glucosylceramide

Liver

Lung

B  Glucosylsphingosine

Liver

Lung
Figure 7

A  Glucosylceramide

Liver

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Lung

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Brain

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B  Glucosylsphingosine

Liver

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Lung

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C  GCase activity

Liver

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Lung

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Brain

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D  CD68 positive cells

Liver

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Lung

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Figure 8

A  Glucosylceramide

Liver

- 46%

pmol Gb/mL tissue

FG

Brain

pmol Gb/mL tissue

DOX

B  Glucosylsphingosine

Liver

pmol GS/mL tissue

IFG

Lung

pmol GS/mL tissue

IFG

Brain

pmol GS/mL tissue

IFG

C  GCase activity

Liver

150

100

50

0

3.6-fold

pmol CE/mg tissue

IFG

Lung

1.2-fold

pmol CE/mg tissue

IFG

Brain

1.16-fold

pmol CE/mg tissue

IFG

D  CD68 positive cells

Liver

# cells/mg

IFG

Lung

IFG

-IFG  +IFG

-IFG  +IFG
Table 1: IFG levels in tissues and serum

<table>
<thead>
<tr>
<th>IFG treatment (wks)</th>
<th>IFG (ng/g tissues)</th>
<th>IFG (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Lung</td>
</tr>
<tr>
<td>4</td>
<td>206.00±20.61 (7)</td>
<td>29.23±9.02 (3)</td>
</tr>
<tr>
<td>8</td>
<td>268.50±38.40 (12)</td>
<td>32.47±11.43 (12)</td>
</tr>
</tbody>
</table>

Tissues and sera were collected at end of 4 or 8 wks of treatment of IFG at 30 mg/kg/day in drinking water. There was no significant difference (Student’s t test) in IFG levels in individual tissues at 4 or 8 wks of IFG treatment. Tissue GCas e activities and lipid results at 4 wks treatment are presented in Figures 5 and 6, and at 8 wks of treatment are in Figures 7 and 8. Numbers in parentheses indicates the number of mice.
Gaucher disease: Ex vivo and in vivo effects of isofagomine on acid β-glucosidase variants and substrate levels
Ying Sun, Benjamin Liou, You-Hai Xu, Brian Quinn, Wujuan Zhang, Rick Hamler, Kenneth D.R. Setchell and Gregory A. Grabowski

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