

Introduction

Gaucher disease is a lysosomal storage disorder caused by a deficiency in the activity of acid β -glucocerebrosidase (GCase). This disease has been broadly classified into two forms on the basis of central nervous system (CNS) involvement: a non-neuropathic form (Type II) and the more severe neuropathic form, with infantile or juvenile onset (Types II and III, respectively). Although several different GCase mutations have been reported, the N370S (Type I) and L444P (Type II) missense mutations are the most prevalent mutations in the Western hemisphere. Orally available small molecules, known as pharmacological chaperones, bind to and stabilize mutant forms of the enzyme, increase trafficking of the enzyme to the lysosomes and increase the cellular activity of less stable but still catalytically competent mutant proteins. Pharmacological chaperones provide a promising new therapeutic approach, in part because they have the potential to be orally available and to cross the blood-brain barrier.

To better understand the effects of the GCase-selective pharmacological chaperone isofagomine (IFG) *in vivo*, we have used a knock-in mouse model that expresses murine L444P GCase. Unlike human Gaucher disease, the L444P homozygous mice did not show the presence of Gaucher cells or gross hepatosplenomegaly, however they show somewhat an attenuated phenotype characterized by low tissue GCase activity (liver, spleen, lung and brain), moderate increases in liver and spleen weights, and elevated plasma levels of chitin III and IgG.

Our results show that IFG administered to L444P GCase mice increases the level of L444P GCase activity and lowers plasma chitin III and IgG levels, and decreases spleen and liver weights. In addition, IFG also increases GCase levels *in vitro* in Kupffer cells derived from L444P mouse liver and in fibroblasts derived from Gaucher patients homozygous for L444P mutation.

Materials and Methods

Animals. Mice homozygous for a L444P GCase mutation were obtained from Dr. Richard Proia (NIH, Bethesda, MD). Animal husbandry was conducted under AUCU protocol guidelines. Wild type (WT) C57BL/6 mice were purchased from Taconic Farms (Germantown, NY).

Cell Lines. Human fibroblasts derived from Gaucher patients homozygous for the L444P mutation were either purchased from Coriell Institute for Medical Research, Camden, NJ (GM10915 and GM07968), or were obtained from Mount Sinai Medical School, New York, NY (GC-1). Fibroblasts were maintained in T75 flasks containing Gibco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% penicillin-streptomycin at 37°C, 8% CO₂. All cell culture reagents were from Gibco (Grand Island, NY) unless otherwise noted. All chemicals were from Sigma (St. Louis, MO) unless noted otherwise.

In vivo GCase assay. L444P GCase mice were dosed with IFG in the drinking water as indicated in each figure legend. After treatment, mice were euthanized; liver, spleen, lung and brain tissues were harvested and stored. Tissue lysates were prepared by homogenizing ~50 mg tissue in M1 buffer (Molvaire buffer: 100 mM sodium citrate, 200 mM sodium phosphate dibasic, 0.25% sodium laurylsulfate, and 0.1% Triton X-100 (pH 5.2)). Lysates were treated at room temperature without and with 2.5 mM CBE for 30 min. Subsequently, 6 mM 4-methylumbelliferyl- β -D-glucuronide (4-MUG) substrate were added to the lysates and incubated at 37°C for 60 min. The reaction was stopped by addition of 0.4 M glycine, pH 10.8. Fluorescence was measured on a Victor 2 (PerkinElmer, Waltham, MA) for 1 sec/well using 365 nm excitation and 460 nm emission. GCase activity in the lysate was normalized for protein using the MicroBCA assay (Pierce, Rockford, IL). Data were analyzed using Microsoft Excel and Graph Pad Prism. For IFG selectivity experiments, specific substrates for α -glucosidase A and acid α -glucosidase (4-MU- α -D-galactopyranoside and 4-MU- α -D-glucopyranoside) were used according to the methodology described above.

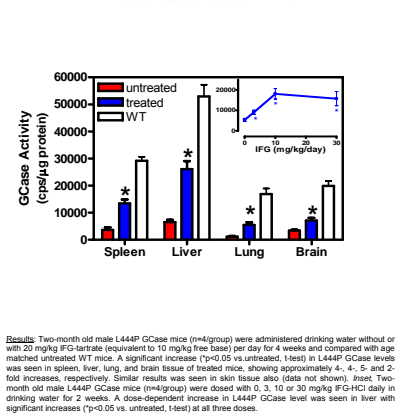
In vitro GCase assay. Cells were seeded in 96-well black plates and incubated at 37°C, 8% CO₂ for 3-6 hrs to allow cell attachment. Cells were then incubated without or with increasing concentrations of IFG for 5 days at 37°C. At the end of 5 days, cells were washed and incubated for 2 hrs at 37°C with 2.5 mM CBE in M1 buffer at room temperature. After washing, half the lysate in plate were treated with M1 buffer alone, while other half with 2.5 mM CBE in M1 buffer and incubated at room temperature for 30 min. Subsequently, 6 mM 4-MUG in M1 buffer were added and plates incubated at 37°C for 1-2 hrs. Reactions were stopped by addition of 0.4 M glycine, pH 10.8, and fluorescence was measured on a Victor2 as described above.

Generation of mouse liver Kupffer cells. Mice were euthanized with CO₂ and perfused through the inferior vena cava with PBS for 5 min, followed by digested media containing collagenase for 15 min. Liver was excised, minced and incubated for an additional 30 min at 37°C to ensure complete digestion. Liver was then washed through a 70 μ m cell strainer and centrifuged at 50g for 5 min at 4°C. The supernatant containing the nonparenchymal cells (endothelial and Kupffer) was layered onto a Histopaque solution and centrifuged at 450g for 5 min at 4°C. The pellet was suspended in 10 mL RPMI 1640 containing 15% FBS and 1% penicillin/streptomycin, and seeded in mouse collagen IV-coated 12-well plates. Nonadherent cells were removed after 1 hour and fresh media were added until reaching 50-60% confluence (~7 days). Varying concentrations of IFG were added to the culture and incubated for 5 days. GCase assay in cell lysates was conducted as described above.

Immunoprecipitation of GCase protein. Fibroblasts were seeded in 75 flasks and washed with varying concentrations of IFG for 5 days. Cells were washed twice with warm media and lysed with PBS containing 1% NP40. Protein concentrations in supernatants using the MicroBCA kit. Lysates (30-100 μ g) were transferred to fresh microcentrifuge tubes for immunoprecipitation with rabbit anti-human GCase antibody (kind gift of Dr. George Grabowski, University of Ohio, Columbus) and incubated for 2 hrs at 4°C. Finally, the antibody-protein complex was captured on Protein G beads (Pierce) by incubation for 1 hr at 4°C. Beads were washed twice with PBS containing 0.1% NP40 and suspended in M1 buffer. The GCase assay was conducted in 96-well plates as described above.

Plasma IgG and chitin III assays. Whole blood was drawn into lithium heparin tubes from the inferior vena cava after euthanization. Plasma was collected by spinning blood at 2700g for 10 min at 4°C. Mouse plasma chitin III assay was performed in a 96-well plate assay (Wada et al. (2002) PNAS 97:10954-9). Briefly, 5 μ L plasma was incubated with 95 μ L 0.1 M citric acid, 0.2 M sodium phosphate, pH 6.2, containing 22 μ M 3,3',4,4'-tetra-*o*-methyl-*o*-nitrobenzoyl- β -D-glucopyranoside (TMB) and 0.05% H₂O₂ for 120 min. The reaction was stopped by addition of 150 μ L 1 M glycine/NaOH, pH 10.6. Enzymatic activity was expressed as fluorescence released per microgram of protein per hour. Plasma IgG levels were quantitated using a mouse IgG ELISA kit (eBioscience Laboratories, Montgomery, TX) according to the manufacturer's instructions.

Figure 1. IFG Administration to L444P GCase Mice Increases Tissue GCase Levels *In Vivo*



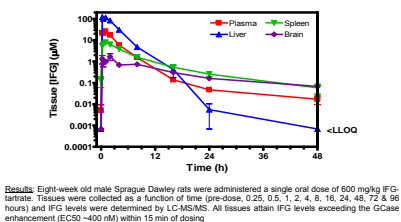
Results: Two-month old male L444P GCase mice (n=10/group) were administered drinking water without or with 20 mg/kg IFG-tartrate (equivalent to 10 mg/kg free base) per day for 4 weeks and compared with age matched untreated WT mice. A significant increase ($p < 0.05$ vs untreated, * test) in L444P GCase levels was seen in spleen, liver, lung, and brain tissue of treated mice, showing approximately 4-, 4-, 5- and 2-fold increases, respectively. Similar results were seen in skin tissue also (data not shown). Inset: Two-month old male L444P GCase mice (n=10/group) were dosed with 0, 3, 10 or 30 mg/kg IFG-HCl daily in drinking water for 2 weeks. A dose-dependent increase in L444P GCase level was seen in liver with significant increases ($p < 0.05$ vs untreated, * test) at all three doses.

Table 1. IFG Levels in Various Tissues

Species	IFG (mg/kg)	Tissue	[IFG] (ng/g or ng/mL)		LOQ (ng/g or ng/mL)
			Day 0	Day 2	
L444P	20	Plasma	71 ± 7	<LOQ	5
		Liver	177 ± 17	<LOQ	100
		Spleen	70 ± 3	<LOQ	40
		Lung	68 ± 10	<LOQ	40
Monkey	1000	Brain	<LOQ	<LOQ	50
		CSF	673 ± 128	ND	100

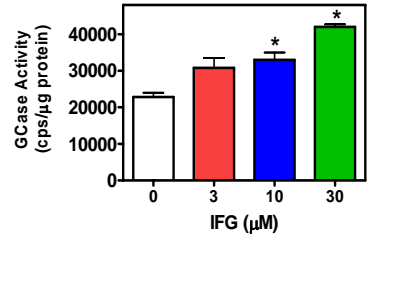
Results: Four-month old L444P (n=6) mice were administered IFG-tartrate in drinking water for 4 weeks; animals were sacrificed on the last day of dosing (Day 0) or two days after drug removal (Day 2). For monkey (n=10), a single dose of IFG-tartrate was administered by oral gavage. Tissues were collected 2 hours later and IFG levels were quantitated by LC-MS/MS. Tissue levels are expressed as ng/ml (plasma and CSF) or ng/g (liver, spleen, lung, brain). LOQ, limit of quantitation and ND, not determined.

Figure 4. IFG Tissue Distribution/PK in Rats



Results: Eight-week old male Sprague-Dawley rats were administered a single oral dose of 600 mg/kg IFG-tartrate. Tissues were collected as a function of time (pre-dose, 0.25, 0.5, 1, 2, 4, 8, 16, 24, 48, 72 & 96 hours) and IFG levels were determined by LC-MS/MS. All tissues attain IFG levels exceeding the GCase enhancement (EC50 ~400 nM) within 15 min of dosing.

Figure 7. IFG Increases L444P GCase Levels *In Vitro* in Mouse Liver Kupffer Cells



Results: IFG increases L444P GCase levels *in vitro* in mouse liver Kupffer cells isolated from L444P GCase mice. Primary cultures of liver Kupffer cells were derived from 2 month old untreated male mice. Cells were grown in 12-well plates and treated with 0, 3, 10 or 30 μ M IFG-tartrate for 5 days, after which GCase levels were measured. The data shown are representative of three independent experiments. Each bar represents the mean \pm SEM of two wells/concentration of drug analyzed in triplicate. A significant increase ($p < 0.05$, * test) in L444P GCase levels was observed after IFG treatment at 10 and 30 μ M.

Figure 2. IFG Selectively Increases L444P GCase Levels *In Vivo*

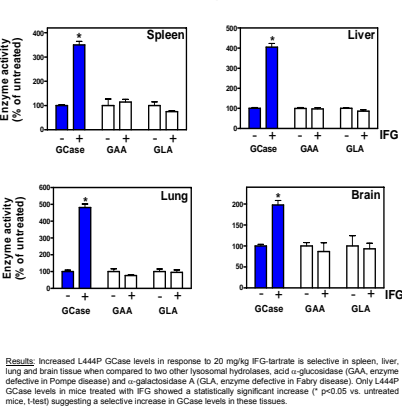
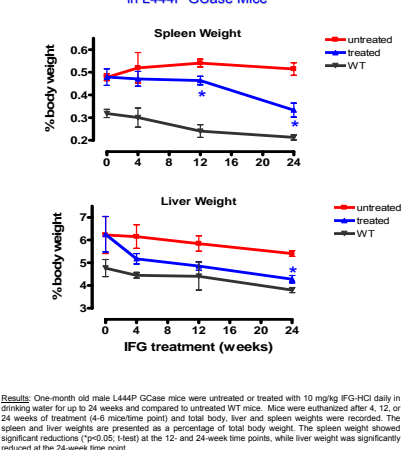
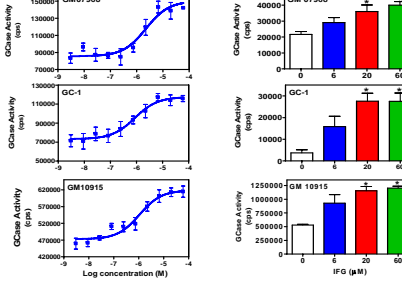


Figure 5. IFG Reduces Elevated Spleen and Liver Weights in L444P GCase Mice



Results: One-month old male L444P GCase mice were untreated or treated with 10 mg/kg IFG-HCl daily in drinking water for up to 24 weeks and compared to untreated WT mice. Mice were euthanized after 4, 12, or 24 weeks of treatment (4 is midline point) and total body, liver and spleen weights were recorded. The spleen and liver weights are presented as a percentage of total body weight. The spleen weight showed significant reductions ($p < 0.05$, * test) at the 12- and 24-week time points, while liver weight was significantly reduced at the 24-week time point.

Figure 8. IFG Increases L444P GCase Levels in Gaucher Patient Fibroblasts *In Vitro*



Results: IFG-tartrate treated in a concentration-dependent increase in L444P GCase levels in patient fibroblasts. The assay was conducted in 96-well plates using 50K cellwell for GM 07968 (n=3) and GM-1 and 30K cellwell for GM10915 (n=15). EC₅₀ values for GM07968, GC-1 and GM10915 were 1.6E-07, 1.0E-09 and 1.2E-011 μ M, respectively. B. Immunoprecipitation of L444P GCase from patient fibroblasts with human GCase antibody resulted in significant increases (p < 0.05, ANOVA) in human L444P GCase levels. IFG (10 μ M) increased GCase levels in GM07968 (n=3), GC-1 (n=3) and GM10915 (n=4), by 3.1, 3.0, 3.8, 3.1 and 3.2, 3.1 fold, respectively.

Figure 3. L444P GCase Levels Returns to Pre-dose Levels Upon IFG Withdrawal

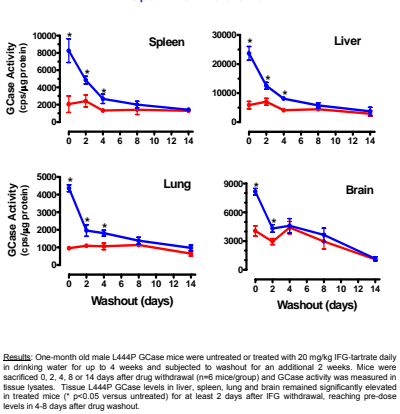
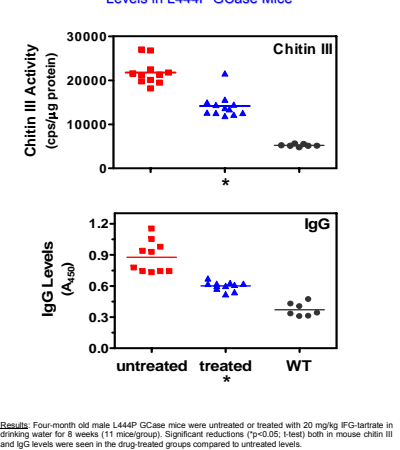


Figure 6. IFG Reduces Elevated Plasma Chitin III and IgG Levels in L444P GCase Mice



Results: Four-month old male L444P GCase mice were untreated or treated with 20 mg/kg IFG-tartrate in drinking water for 8 weeks (11 mice/group). Significant reductions ($p < 0.05$, * test) both in mouse chitin III and IgG levels were seen in the drug-treated groups compared to untreated levels.

Summary and Conclusion

- Oral administration of IFG to L444P GCase mice resulted in significant increases in GCase levels (2- to 5-fold) in spleen, liver, lung, and importantly brain, without altering the levels of two other lysosomal hydrolases, acid α -glucosidase and α -galactosidase A in the same tissues.
- Concomitant with increased GCase levels, IFG also improved Gaucher disease like phenotype by lowering mouse chitin III and IgG levels as well as spleen and liver weights in L444P GCase mice.
- In vitro* IFG increased mouse L444P GCase levels in Kupffer cells and human L444P GCase levels in Gaucher patient fibroblasts.
- Collectively, these data indicate that IFG has the potential to increase L444P GCase levels *in vivo* in mice and *in vitro* in Gaucher patient fibroblasts, and thus merits further evaluation for the treatment of Gaucher disease.