Enzyme Replacement Therapy for GM1 Gangliosidosis: Following a Proven Path to Clinical Success

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DISEASE BACKGROUND

GM1 gangliosidosis exhibits an autosomal recessive mode of inheritance and arises from mutations in the *GLB1* gene. *GLB1* encodes lysosomal β-gal, which catalyzes the stepwise lysosomal degradation of multiple galactose-containing substrates in the lysosome. A wide spectrum of clinical phenotypes have been described for GM1 gangliosidosis. In its most severe form, early infantile-onset GM1 gangliosidosis, patients exhibit developmental delay in the first year of life, which coincides with very low levels of residual mutant β-gal activity, accumulation of GM1 ganglioside predominantly in neurons of the brain, and widespread CNS degeneration. These patients rapidly lose all motor skills, with death occurring by 2 – 4.5 years of age. GM1 gangliosidosis patients with higher levels of residual mutant β-gal activity present with late-infantile, and juvenile forms of CNS-related disease progression, with incrementally longer survival. Similar to other LSDs, the range of clinical phenotypes for GM1 gangliosidosis are clustered within a narrow range of residual lysosomal β-gal activity, from 0 – 15%. Importantly, this suggests that therapeutic strategies for this disease do not necessarily need to normalize β-gal activity to mediate lysosomal degradation of stored glycan substrates and prevent disease progression.

The incidence of GM1 Gangliosidosis is estimated to be 1/200K live births, with a notably higher incidence in Brazil of 1/17K live births; GM1 is the most common lysosomal storage disorder in the UAE.

IN VITRO PROOF OF CONCEPT

Chen et al. published compelling proof of concept for ICV-ERT in the Journal of Biological Chemistry, 2019; the data contained in this poster is a summary of that publication. In GM1 gangliosidosis patient fibroblasts, very low nM doses of rhβ-gal cellular uptake over 24 hours are sufficient to normalize β-gal activity levels (Fig. 1A). K_{uptake}, defined as the concentration of enzyme at half-maximal cellular uptake, is 3.4 ± 1.1 nM, with a maximal uptake capacity (Vmax) corresponding to 7338 ± 1513 nmol/hr/mg (Fig. 1A). Following cellular uptake, rhβ-gal co-localizes with LysoTracker-Red, a marker of acidified lysosomes, suggestive of successful delivery of enzyme to lysosomes (Fig. 1B). Following cellular uptake of a single low dose of rhβ-gal (3 nM) for 18 hours, lysosome- delivered rhβ-gal activity decays slowly with a half-life of ~9 days in GM1 gangliosidosis patient fibroblasts, which coincides with rapid turnover of GM1 ganglioside substrate and glycan substrates within 1 week. Furthermore, a single low dose (3 nM) of rhβ-gal delivered to lysosomes of GM1 gangliosidosis patient fibroblasts is sufficient to prevent re- accumulation of GM1 ganglioside substrate and glycan substrates for up to six weeks (data available in Chen et al.).

FIGURE 1

Fig 1A. Kuptake

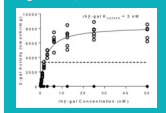

Fig 1B. PFA-fixed GM1 gangliosidosis patient fibroblasts

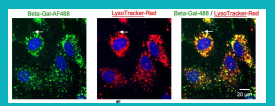

Fig 1C. Kdegradation

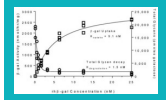

Fig 1. Purified rhβ-gal exhibits highly efficient cellular uptake in GM1 gangliosidosis patient fibroblasts, which coincides with substrate clearance for up to 6 weeks.

A. Representative Kuptake determination experiment for rhβ-gal cellular uptake in GM1 gangliosidosis patient fibroblasts. Six independent cultures of cells were incubated with increasing concentrations of rhβ-gal for 24 hours in the absence (open circles) or presence (closed circles) of 8 mM mannose-6-phosphate (M6P). Dashed line represents the level of β-gal activity detected in normal fibroblasts. Average K_{uptake} = 3.4±1.1 nM, N=13; Average Vmax=7388±1513 nmol/hr/mg, N=13. **B.** Representative images of PFA-fixed GM1 gangliosidosis patient fibroblasts following a 24 incubation with 25 nM Alexa-fluor 488 β-gal (green channel). Prior to fixation cells were incubated with LysoTracker- Red (red channel). An example of β-gal co-localization with a LysoTracker-Red$^+$ acidified organelle is indicated with an arrow. **C.** $K_{degradation}$ determination for rhβ-gal-mediated clearance of the three major glycan substrates that accumulate in GM1 gangliosidosis patient fibroblasts. Triplicate cultures of cells were incubated for 4 hours with increasing doses of rhb-gal. The uptake medium was removed and cells were washed and chased for a further six hours in the absence of enzyme. Cells lysates were prepared and then assayed for β-gal activity (squares) or total glycans (circles). N=2 repeats.

ICV-ERT Augments β-gal activity in the brain of the GLB1 mouse

When rhβ-gal is delivered to the acidified lumen of the lysosome, proteolytic cleavage generates mature β-gal, resulting in a ~20-kDa reduction in mass, which can be monitored by Western blotting as an indicator of successful β-gal delivery to lysosomes. X-gal substrate, which is specifically cleaved by β-gal under acidic conditions, can also be used to detect β-gal activity *in situ*. Chen initially tested both of these methods in GM1 gangliosidosis patient fibroblasts to monitor the delivery of rhβ-gal to lysosomes. β-gal activity toward X-gal substrate in normal human fibroblasts is readily detected *in situ* under acidic conditions but not neutral pH conditions (Fig. 2A). Furthermore, as expected, β-gal protein is predominantly detected as the mature, lysosomal enzyme in normal fibroblasts by Western blotting (Fig. 2B). β-gal activity toward X-gal substrate is not detected *in situ* in GM1 gangliosidosis patient fibroblasts but is readily detected following a short 3-h exposure to rhβ-gal (Fig. 2A), which coincides with the appearance of mature, lysosomal rhβ-gal as well as the precursor nonlysosomal enzyme by Western blotting (Fig. 2B), suggestive of partial delivery of rhβ-gal to lysosomes. Following an ICV administered dose (100 µg) of rhβ-gal, β-gal activity toward X-gal substrate in hippocampal neurons becomes apparent after 48 hours (Fig. 2C). Four ICV- ERT injections with rhβ-gal over 2 weeks results in the detection of mature, lysosomal β-gal in brain homogenates of GLB1 KO mice (quantified in Fig. 2D).

FIGURE 2

Fig 2A.

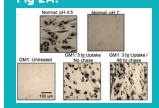

Fig 2B.

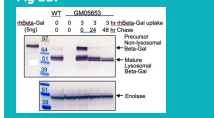

Fig 2C.

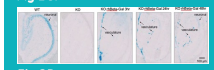

Fig 2D.

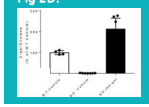

Fig 2. ICV-ERT with rhβ-gal augments lysosomal β-gal activity in patient fibroblasts and hippocampal neurons of the GM1 gangliosidosis mouse brain.

A. *In situ* detection at 40X magnification of β-gal activity observed in normal fibroblasts at pH 4.5 and pH 7 after 18 hours using X-gal substrate. X-gal staining was also performed in control GM1 gangliosidosis untreated fibroblasts or following 6 hours of cellular uptake with 100 nM rh**b**-gal. Alternatively, after cellular uptake, cells were washed and chased in growth medium without enzyme for a further 48 hours. **B.** β-gal western blot of cells described in A. Enolase was used as a loading control. **C.** *In situ* detection, at 20X magnification, of β-gal activity at acidic pH using X-gal substrate in cryo-frozen sagittal brain sections from a 16 week old control WT mice, a control GLB1 KO mouse or GLB1 KO mice following a single ICV dose (100 µg) of rhβ-gal. rhβ-gal ICV-ERT-treated mice were taken down after 3 hours, 24 hours or 48 hours post-ICV administration. Blue signal indicates areas of **b**-gal activity. Black arrows indicate development of signal in the CA3 region of the hippocampus (neuronal) and in vascular structures. **D.** Quantification of mature β-gal protein levels, standardized to the enolase loading control and expressed as individual values, with the standard deviation indicated.

ICV-ERT broadly distributes throughout the brain and clears glycan substrates

A single 100-µg ICV dose of rhβ-gal into WT mice coincides with broad bilateral distribution of the enzyme throughout the brain 24 h after administration, as determined using a MS-based assay (Fig. 3A). Two short-term proof-of-concept (PoC) ICV-ERT dosing experiments were evaluated, commencing at 12 weeks of age, well after secondary neuroinflammation has commenced in this mouse model of GM1 gangliosidosis. Weekly ICV dosing for 8 weeks results in the detection of mature lysosomal rhβ-gal protein by Western blotting in brain homogenates, which coincides with normalization of β-gal activity to varying extents in individual mice (Fig. 3B). Weekly ICV dosing with rhβ-gal for 8 weeks in GLB1 KO mice also coincides with near-to-complete clearance of two classes of substrates in GM1 gangliosidosis brain tissue, with the GM1 and GA1 gangliosidosis substrates requiring a longer duration of ERT for maximal clearance, when compared with glycan substrates.

FIGURE 3

Fig 3A.

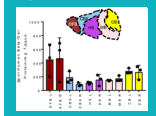

Fig 3B.

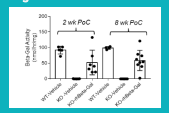

Fig 3C.

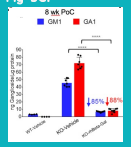

Fig 3. ICV administered rhβ-gal exhibits broad bilateral bio-distribution throughout the brain, which coincides with clearance of multiple substrates in GM1 gangliosidosis mice.

A. Detection of rhβ-gal in WT mouse brain tissue (n = 3 mice) 3 h after a single, unilateral injection (100-µg dose) into the lateral ventricle utilizing MS. *Colored bars* represent detection of enzyme activity in each macro-dissected area. Mouse left and right brain hemispheres were each dissected into the following regions: OFB, CBX, HS, CB, and MB (containing pons and medulla) and analyzed individually. Each *pair* of *bars* represents signal in the left (*L*) or right (*R*) sagittal hemispheres. **B.** Corresponding β-gal activity levels detected in brain homogenates prepared from mice, with results expressed as individual values with the standard deviation indicated. 2 Wk PoC: WT vehicle, N=5; KO vehicle, N=9; KO rhβ-gal, N=7. 8 Wk PoC: WT vehicle, N=4; KO vehicle, N=6; KO rhβ-gal, N=9. **C.** GM1 and GA1 ganglioside levels detected in brain homogenates from the 2 wk PoC (WT vehicle, N=5; KO vehicle, N=9; KO-rhβ-gal, N=7) and 8 wk PoC mice (WT vehicle, N=4; KO vehicle N=7; KO rhβ-gal, N=9). **** indicates P<0.0001.

ICV-ERT reverses secondary neuropathology in the GLB1 mouse

GLB1 KO mice contain elevated levels of LAMP2 protein, a marker of lysosomal storage pathology, throughout the brain, as detected by immunohistochemistry (IHC) using a polyclonal LAMP2 antibody (Fig. 4A); (see Fig. 4B for quantification) and by Western blotting (Fig. 4C; see Fig. 4D for quantification), when compared with vehicle-treated WT mice. ICV dosing with rhβ-gal for 8 weeks in GLB1 KO mice coincides with normalization of LAMP2 levels, as detected by IHC (Fig. 4A; see Fig. 4B for quantification) and by Western blotting (Fig. 4C; see Fig. 4D for quantification). GFAP, a marker for astrogliosis (Fig. 4E; see Fig. 4F for quantification), as well as IBA1, a marker of microgliosis (Fig. 4H; see Fig. 4H for quantification), were significantly elevated in the cortex from vehicle-treated KO mice at 3 months of age, when compared to vehicle-treated WT mice by IHC. Both GFAP levels (Fig. 4E; see Fig. 4F for quantification) and IBA1 levels (Fig. 4G; see Fig. 4H for quantification) were normalized in the cortex of KO mice following eight weekly ICV doses of rhβ-gal, when compared to vehicle-treated KO mice. Collectively, the data in Figs. 2-4 suggest that an intermittent ICV-ERT dosing approach with rhβ-gal results in broad, bilateral biodistribution of enzyme, which coincides with near-to-complete clearance of multiple substrates in the brain and reversal of well-entrenched secondary neuropathology in a mouse model of GM1 gangliosidosis.

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FIGURE 4

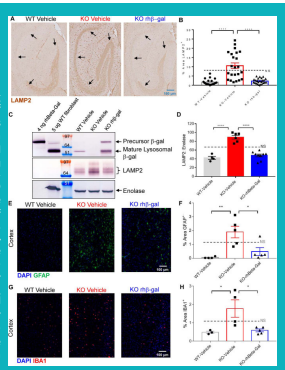


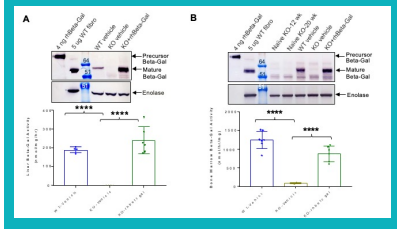
Fig 4. ICV administered rhβ-gal reverses secondary neuropathology in GM1 gangliosidosis mice.

A. Representative LAMP2 IHC images (20× magnification) of sagittal hippocampus sections prepared from the left brain hemisphere from mice dosed weekly for 8 weeks (dosing regimen indicated in Fig. 3B). Black arrows, presence (KO vehicle) or absence (WT vehicle and KO rhβ-gal-treated) of LAMP2 signal in the CA1–CA3 and hippocampal neurons. **B.** Quantitative analysis of the percentage of area of LAMP2-positive signal measured over the cortex, hippocampus, thalamus, brain stem and cerebellum. n = 4–6 mice for each group. **C.** Representative β-gal, LAMP2, and enolase Western blots of pooled brain homogenates prepared from the left hemispheres from WT or GLB1 KO mice treated with eight weekly doses of vehicle or rhβ-gal: WT vehicle, n = 4; KO vehicle, n = 6; KO rhβ-gal, n = 9. **D.** Quantification of LAMP-2 protein levels from Western blots of individual samples, standardized to an enolase loading control, with individual values indicated error bars represent the standard deviation (S.D.). WT vehicle, n = 4; KO vehicle, n = 6; KO rhβ-gal, n = 9. **E–H.** Representative GFAP IF (E) or IBA1 IF (G) images of cortical mouse brain tissue. Corresponding percentage area analyses for cortical GFAP (F) or IBA1 (H) are indicated. All quantitative data are represented as individual values error bars represent the standard error of the mean (SEM). GFAP IF, WT vehicle, n = 4; KO vehicle, n = 5; KO rhβ-gal, n = 5. IBA1 IF, WT vehicle, n = 3; KO vehicle, n = 3, KO rhβ-gal, n = 5. ****, p < 0.0001. **, p < 0.01; *, p < 0.05

ICV-ERT normalizes β-gal activity in systemic tissues of the GLB1 mouse

β-gal activity toward X-gal substrate was detected *in situ* in GLB1 KO mouse brain tissue immediately following ICV-ERT in areas that appeared vascular-like suggesting that ICV delivered enzyme may be gaining access to the systemic circulation. In support of this, eight weekly ICV doses of rhβ-gal in GLB1 KO mice coincides with near-to-complete normalization of β-gal activity levels and β-gal protein levels in the liver (Fig. 5A) and bone marrow (Fig. 5B) of the majority of animals. Whereas substrate levels in liver and bone marrow of these mice were not measured, the degree of β-gal augmentation in liver and bone marrow is well above the critical threshold of ~15% of normal residual lysosomal enzyme activity that is needed to mediate substrate turnover and prevent lysosomal storage disease progression in GM1 gangliosidosis patients. It is therefore possible that an ICV route of rhβ-gal administration may also result in exposure of enzyme to systemic sites of disease progression in sufficient amounts to mediate substrate clearance.

FIGURE 5



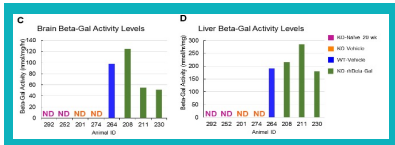


FIG 5. ICV-ERT with rhβ-gal for 8 weeks normalizes β-gal activity in systemic tissues, which coincides with a partial reduction in urinary A2G2' substrate. A and B, β-gal activity **detected in liver tissue.**

A. or bone marrow **B.** from WT vehicle (n = 4–7), KO vehicle (n = 6–8), and KO rhβ-gal–treated mice (n = 6), with results expressed as individual values *error bars* represent the standard deviation (S.D.). ****, $p < 0.0001$. Also indicated *above* each *graph* is a representative Western blot of β-gal protein levels in pooled liver homogenates (A) or bone marrow lysates (B) prepared from WT or GLB1 KO mice treated with vehicle or rhβ-gal. For comparison, 4 ng of purified rhβ-gal was also included on gels as an indicator of the precursor, nonlysosomal enzyme. Also included was 5 µg of cell lysate prepared from WT human fibroblasts (WT fibro) as an indicator of mature β-gal successfully delivered to lysosomes. Enolase was used as loading control. **C–D,** correlation between β-gal activity levels in brain (C) and liver (D).

24-wk dose-ranging behavioral study in the GLB1 mouse is ongoing

The Cure GM1 Foundation provided funding to Tega Therapeutics to conduct a multi-dose study in a second GLB-1 KO mouse (different strain from the one used in Chen et al.). We dosed the GLB1-KO mouse with escalating doses of ICV-delivered β-gal of 10 µg weekly, 30 µg weekly, and 100 µg biweekly. Mice were evaluated over a 24-week period. In addition to biochemical and histological endpoints, we also collected neurobehavioral outcomes via the rotarod assay. Data analysis is currently underway.

A Path to GMP Manufacture of rhβ-Galactosidase

Chen et al demonstrated that rhβ-gal can be formulated to high concentrations (20 mg/ml) in artificial CSF and at 1 mg/ml in a neutral pH test buffer, with loss of stability only being observed under neutral pH conditions over a period of several days at low concentrations (0.1 mg/ml). In contrast, rhβ-gal appears to be a stable dimer under acidic pH conditions at low concentrations (0.1 mg/ml). These biophysical properties of rhβ-gal become important following ICV administration, when the enzyme becomes rapidly diluted as it diffuses further from the injection site in extracellular fluids, which are presumably at neutral pH.

The Cure GM1 Foundation has recently partnered with a contract manufacturing organization to conduct cell line development and small-scale manufacture of rhβ-gal; work is ongoing.

Summary

The collective data from the Chen publication suggest that a single 100-µg dose of ICV administered rhβ-gal exhibits broad bilateral biodistribution throughout the brain of GM1 gangliosidosis mice as determined by utilizing an MS-based assay (Fig. 3A). Detection of β-gal activity *in situ* suggests that whereas the majority of lysosome-delivered enzyme activity is detected in perivascular regions at early time points (3 and 24 h post-ICV-ERT; Fig. 2C), lysosome-delivered β-gal activity in hippocampal neurons only becomes noticeable at the latest time point analyzed (48 h post-ICV-ERT; Fig. 2C), presumably by axonal transport. These observations help to explain how rhβ-gal bio distributes to lysosomes throughout the brain as well as the systemic circulation following ICV administration. Strikingly, weekly ICV-ERT dosing for 8 weeks is sufficient to promote near-to-complete substrate clearance in the brain (Fig. 3) and reversal of well-entrenched secondary neuropathology in GM1 gangliosidosis mice (Fig. 4). Intermittent ICV-ERT dosing is a tunable therapeutic option that can safely and precisely deliver rhβ-gal to lysosomes to clear pathological lysosomal substrates and reverse neuropathology associated with the disease.

Next Steps

The Foundation intends to hold a pre-IND meeting with FDA in early 2026 to discuss our proposed clinical development path forward.

Acknowledgements

The data included in this poster is a summary of Chen et al, Journal of Biological Chemistry, "Intracerebroventricular enzyme replacement therapy with β-galactosidase reverses brain pathologies due to GM1 gangliosidosis in mice," 2019.

CURE GM1 FOUNDATION