Co-expression of S1S3 phosphotransferase in production cell line improves mannose 6-phosphorylation and cellular uptake of alpha-N-acetylglucosaminidase (Sanfilippo B syndrome)

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Sanfilippo B syndrome is a currently untreatable neurodegenerative disorder of childhood. The cause is deficiency of the soluble lysosomal enzyme NAGLU (alpha-N-acetylglucosaminidase), which cleaves terminal N-acetylglucosamine residues from the non-reducing ends of heparan sulfate glycosaminoglycans in the lysosome (**Figure 1**).

Other lysosomal diseases are amendable to enzyme replacement therapy (ERT), including intra-cerebroventricular ERT to circumvent the blood-brain barrier. ERT generally relies on mannose 6-phosphate receptor-mediated uptake and trafficking to the lysosome. NAGLU has poor mannose 6-phosphate content when produced recombinantly. One solution was to generate a fusion protein with NAGLU and the mannose 6-phosphate receptor binding portion of insulin-like growth factor-II. This ICV-delivered ERT reached clinical trials, but due to difficulty raising funds for further trials, the company (Allievex) is undergoing liquidation (press release National MPS Society, July 24, 2024).

A more natural solution is to increase the mannose 6-phosphate content of NAGLU. GlcNAc-1-phospho-transferase is a transmembrane enzyme that is responsible for mannose-6-phosphorylation. S1S3 is a modified form of GlcNAc-1-phosphotransferase that is more efficient than wild-type and does not require the gamma subunit for activation (**Figure 2**; Liu et al 2017). Co-expression of S1S3 in the production cell line should increase the mannose 6-phosphate content of NAGLU in the media, improving ERT for Sanfilippo B syndrome, and because the S1S3 is not secreted, the resulting recombinant NAGLU^{M6P} would be a more natural, effective enzyme for ERT.

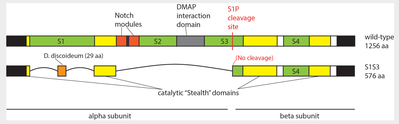

Figure 2. Wild-type and S1S3 GlcNAc-1-phosphotransferase. The wild-type has several spacer domains (S1, S2, S3, and S4), some of which are removed in S1S3. From Liu et al 2017.

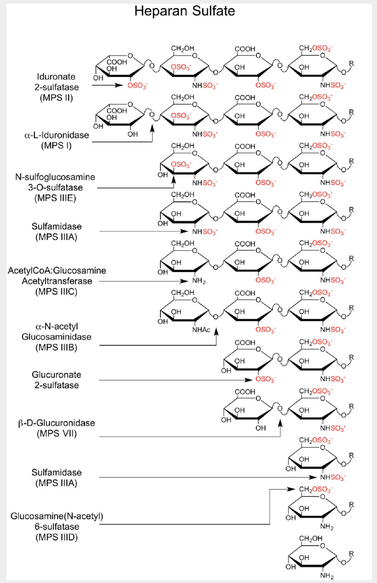

Figure 1. Steps in heparan sulfate catabolism. MPS: mucopolysaccharidosis. Sanfilippo B syndrome is MPS IIIB. From Lawrence et al 2014.

S1S3 co-expression improved mannose 6-phosphate receptor binding of recombinant NAGLU: We transfected Hek293 cells with a bicistronic vector bearing S1S3 and the cDNA for human NAGLU (**Figure 3**). Media was harvested, assayed for activity, and tested for binding to the cation-independent mannose 6-phosphate receptor as described (Liu et al 2017).

Figure 3. A 2229 bp hNAGLU gene was inserted into pcDNA3.1(+) for NAGLU single expression or a bicistronic vector including IRES and S1S3

NAGLU secreted by this cell line ("NAGLU^{M6P}") showed up to 40% binding to the cation-independent M6P receptor, compared with no appreciable binding by NAGLU produced without S1S3 and that depends on endogenous GlcNAc-1-phosphotransferase to acquire the M6P moiety ("NAGLU"). NAGLU^{M6P} displayed similar kinetics, thermostability, and pH optimum as recombinant NAGLU and NAGLU purified from human urine.

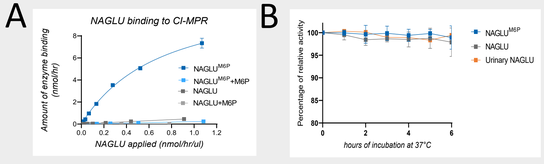

Figure 3. A) Binding to the CI-MPR (cation-independent mannose 6-phosphate receptor) in the presence or absence of 5 mM M6P. B) Properties such as thermostability of NAGLU and NAGLU^{M6P} were similar.

We performed cellular uptake experiments in Sanfilippo B patient fibroblasts and neuronal cells. Uptake was measured after incubation with each enzyme for 4h, after which time cells were lysed and intracellular NAGLU activity measured by 4-methylumbelliferyl assay, which showed robust uptake of NAGLU^{M6P} into cells that was nearly completely eliminated by addition of 5 mM M6P.

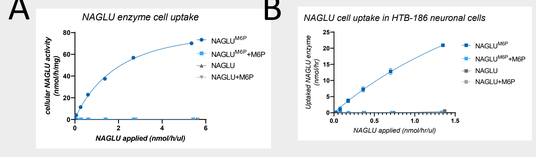

Figure 4. Cellular uptake experiments in Sanfilippo B patient fibroblasts GM1462 (A) or HTB-186 neuronal cells (B) in the presence or absence of 5 mM M6P.

We measured intracellular heparan sulfate by MS/MS (UCSD GlycoAnalytics Core) in Sanfilippo B human fibroblasts following treatment with NAGLU or NAGLU^{M6P} (**Figure 5**). The mean heparan sulfate of untreated Sanfilippo B cells was 184 ± 28.3 ng/mg protein. Incubation for 30 min with NAGLU^{M6P} resulted in intracellular HS of mean 66 ± 18.6 ng/mg protein (64% reduction, p<0.001). Incubation for 4h with NAGLU^{M6P} resulted in intracellular HS of mean 42 ± 3.08 ng/mg protein (77% reduction, p<0.001); p=NS compared to WT (19.4 ± 5.31 ng/mg protein).

Glycan analysis of NAGLU^{M6P} showed >50% of the N-linked oligosaccharides on the protein are phosphorylated. There was a combination of monophosphorylated mannose residues and bis-phosphorylated mannose residues. On average, NAGLU^{M6P} contains greater than 3 mols of phosphorylated glycan per mole of NAGLU, and the total number of M6P per mol NAGLU^{M6P} was estimated from the analysis as 5.3-6.7.

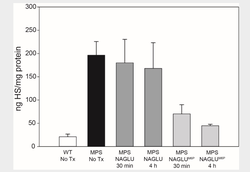

Figure 5. Heparan sulfate (HS) glycosaminoglycans in wild-type (WT) and Sanfilippo B patient fibroblasts and following incubation with NAGLU or NAGLU^{M6P}.

In vivo studies in Sanfilippo B mice: We performed a pilot in vivo experiment in Sanfilippo B mice. Adult mice (n=3/group) received NAGLU or NAGLU^{M6P} in a single dose into a lateral ventricle and were sacrificed at 24h. NAGLU activity was detected in brain (**Figure 6**), as well as liver (56-66% of carrier levels) and serum (7-26% of carrier levels) of NAGLU^{M6P} treated mice.

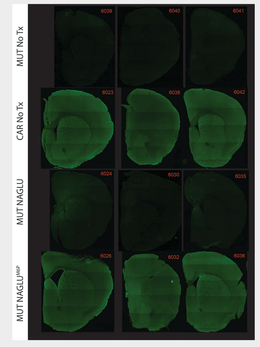

Figure 6. Uptake of NAGLU^{M6P} into mouse brain in vivo. Sanfilippo B (MUT) mice received 560 units/~4.5 µg rhNAGLU or rhNAGLU^{M6P} ICV x 1. MUT and normal carrier (CAR) controls received no treatment. Anti-NAGLU immunofluorescence staining in sections at 24h (green). N=3/group.

Next, we performed a repeat-dose experiment in which mice received two doses of ERT weekly by ICV injection. Mice were dosed at 3.22 µg NAGLU^{M6P} (743 activity units; n=4) or an equivalent activity units of NAGLU (742 units; this came out to 4.88 µg; n=4) or NAGLU-IGF2 (746 units; this came to 5.68 µg; n=3). These doses are considered low and were intentionally selected to prevent "floor effects" and maximize the potential to detect intergroup differences between treatments. Vehicle-treated Sanfilipo B mice (n=3) and heterozygous carrier mice (n=4) were used as controls. Interim results are shown in **Figure 7**.

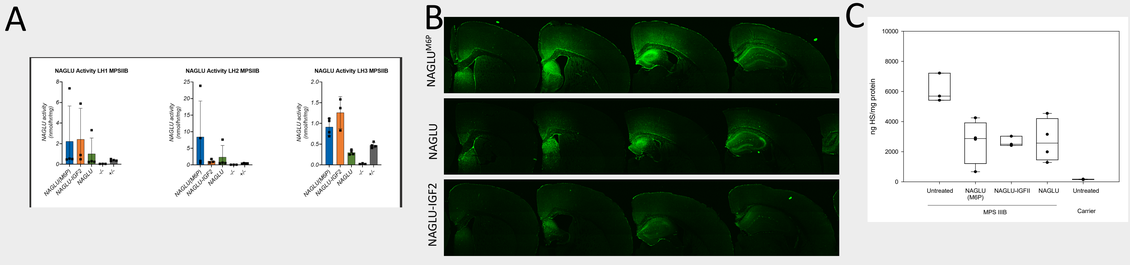

Figure 7. Repeat dosing experiment in adult Sanfilippo B mice. A) NAGLU enzymatic activity in hemicoronal left hemisphere (LH) sections of treated mice and controls. B) Immunofluorescence for NAGLU in hemicoronal sections of treated mice. C) Heparan sulfate (HS) glycosaminoglycans in brain of treated mice and controls. Mice were sacrificed 24h after the final ICV dose.

Conclusions:

Recombinant NAGLUM6P may be effective as ERT for Sanfilippo B syndrome. We are currently evaluating whether there is evidence of superiority to NAGLU or NAGLU-IGF2. Additionally, we are conducting gene therapy experiments with AAV-S1S3 and NAGLU (two-vector or bicistronic) in vivo in Sanfilippo B mice.

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