Development of an Intrathecal Enzyme Replacement Therapy for Sanfilippo Syndrome Type D (Mucopolysaccharidosis IIID)

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Abstract

Sanfilippo syndrome (mucopolysaccharidosis type III; MPS III) is a devastating neurodegenerative disorder with no cure or effective treatment currently available. All subtypes of MPS III have similar clinical phenotypes with onset in infancy or early childhood: progressive and severe neurological deterioration, hearing loss, and visceral manifestations. Most patients die before reaching adulthood, but some may survive to the fourth decade with progressive dementia and retinitis pigmentosa. There are, however, many therapies in early development for MPS III, including genetic, stem cell, enzyme replacement (ERT), chaperone, and substrate reduction.

MPS IIID, the rarest of the MPS III subtypes, is an autosomal recessive storage disorder caused by genetic deficiency of N-acetylglucosamine-6-sulfatase (GNS), a lysosomal enzyme vital in the pathway of glycosaminoglycan (GAG) degradation. Over time, these GAG accumulate and eventually induce cellular death, with nervous tissue most severely affected. ERT has long been a strategy to treat MPS disorders, however, due to the difficulties in delivery of therapeutic agents across the blood brain barrier, treatment of the neurodegenerative pathology of the disease has proven difficult. Therefore, our strategy is to manufacture and deliver the enzyme directly to the affected tissue via an intrathecal route. Early studies of intrathecal ERT for other forms of MPS have already shown promise, with administration early in life preventing or reversing the visible signs of neurodegeneration in animal models.

Using a stably-transfected Chinese hamster ovarian (CHO) cell line, we have begun producing pre-clinical levels of recombinant human GNS (rhGNS) protein. rhGNS has been purified and enzymatically characterized, and we have optimized storage conditions for both longevity and safe administration. Using MPS IIID fibroblasts, we have evaluated its cellular uptake, mediated via the mannose-6-phosphate receptor, and further demonstrated localization in the lysosome and the ability to reduce GAG storage.

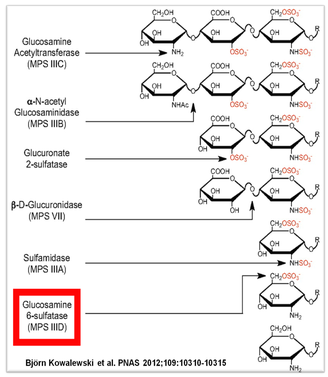
Introduction

Allison and Lacey have MPS IIID which is caused by genetic deficiency of N-acetylglucosamine-6-sulfatase (GNS):

a single gene defect.

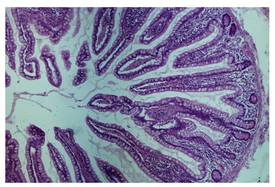
All subtypes of MPS III have similar clinical phenotypes with onset in infancy or early childhood: progressive and severe neurological deterioration, hearing loss, and visceral manifestations. Most patients become demented and die before adulthood, but some survive to the fourth decade with progressive dementia and retinitis pigmentosa.

Through our collaboration we are currently developing an intrathecal enzyme replacement therapy (ERT) for MPS IIID, a lysosomal enzyme vital in the breakdown of heparan sulfate.



In the absence of GNS, patient cells accumulate and store glycosaminoglycans (GAG). MPS brains have also been shown to accumulate beta amyloid (amyloidosis) as well exhibit hyper-phosphorylated tau (P-tau), both of which are common to other neurodegenerative diseases such as Alzheimer's disease. It is believed that the accumulation of these products along with observed tauopathy underlie the progressive neurodegeneration observed in MPS patients.

Despite considerable progress in recent years, there is currently no approved cure for any form of MPS III and no available treatment for MPS IIID.



NEED TO FIND SOURCE

Production and Purification of rhGNS

Table 1: Purification of secreted rhGNS from CHO cell line using c-myc affinity column.

Step	Volume (ml)	Protein (µg)	Total activity (units)*	Specific Activity (units/mg protein)
PF CHO Media	1,450	330,165	108,998	330
Concentrated Media	110	304,920	307,826	1,010
Flow through	110	302,878	273,667	904
myc Elution	3	186	26,408	142,263
Elution Concentration	0.14	119	28,593	241,058

The coding sequence (**cDNA**) of *GNS* was codon-optimized (GenScript) and inserted into a mammalian expression plasmid using restriction enzyme digestion and ligation. The construct contains a C-terminal TEV protease cleavable c-myc moiety (EQKLISEED) for ease of purification, and expression is driven by a CMV promoter. Chinese hamster ovary (CHO) cells were stably transfected and screened for high expressing clones. Cells were grown in roller bottles and media harvested to obtain the secreted rhGNS. Following concentration, media was loaded into a c-myc affinity column, washed, and then eluted using soluble c-myc peptide (EQKLISEED) in artificial cerebrospinal fluid (Elliotts B Solution USP). Eluted rhGNS was then concentrated to a final concentration of 1 mg/ml. We achieved a yield of >100 µg per 1500 mL media, reaching a specific activity >200,000 units/mg (**Table 1**).

Purified rhGNS is Heavily Glycosylated and Enzymatically Active

Western Blot analysis and glycosidase digestion revealed that rhGNS was highly glycosylated Fig. 1A). Using the fluorogenic substrate 4-Methylumbelliferyl 6-Sulfo-2-acetamido-2-deoxy-α-D-glucopyranoside (4-MU-GNS), purified rhGNS was shown to be both enzymatically active and stable after storage in artificial cerebral spinal fluid (Elliotts B Solution USP). Further biochemical characterization of the enzyme showed optimal reaction conditions within the lysosomal pH range (4-5.6), with 10-fold lower activity at neutral pH (**Fig. 1C**).

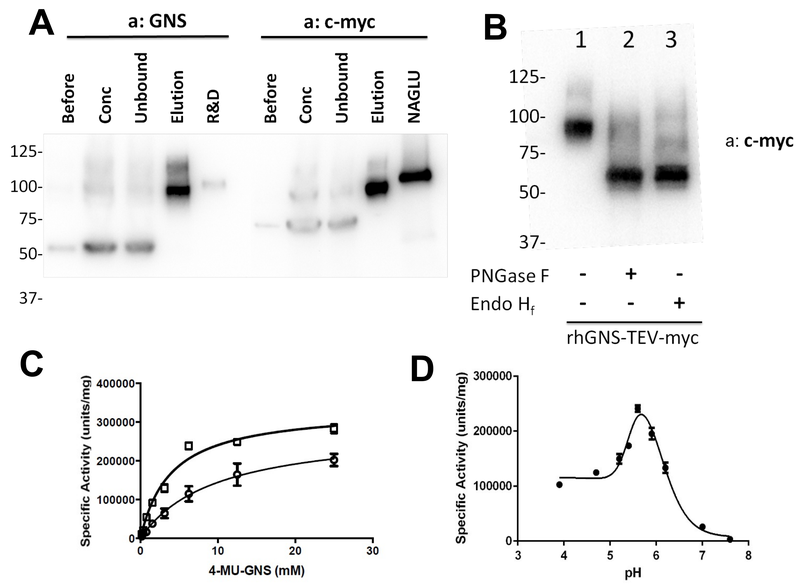


Fig. 1: Purification and glycosylation of rhGNS. **A**) Western blot of rhGNS purification using antibodies against GNS and against our purification tag, myc. Non-clinical-grade rhGNS purchased from R&D systems and alpha-N-acetylglucosaminidase (NAGLU) produced by the Dickson lab were used as positive controls. **B**) PNGase F and Endo H treatment of purified rhGNS results in a shift in molecular weight, demonstrating that the protein is glycosylated. **C**) Michaelis-Menten Curves of rhGNS. Enzymatic activity of rhGNS was assayed using a fluorogenic substrate (4-MU-GNS) with a 4h second step (squares) vs. 24h second step (circles). K_m was 3.97 mM and V_{max} was 336,359 units/mg with the shorter assay. **D**) pH profile of rhGNS activity. Optimal assay conditions occurred within acidic pH range (4-6). Means and S.D. of triplicate experiments.

rhGNS Enters Human MPS IIID Cells, Targets to Lysosomes, and Reduces GAG Storage

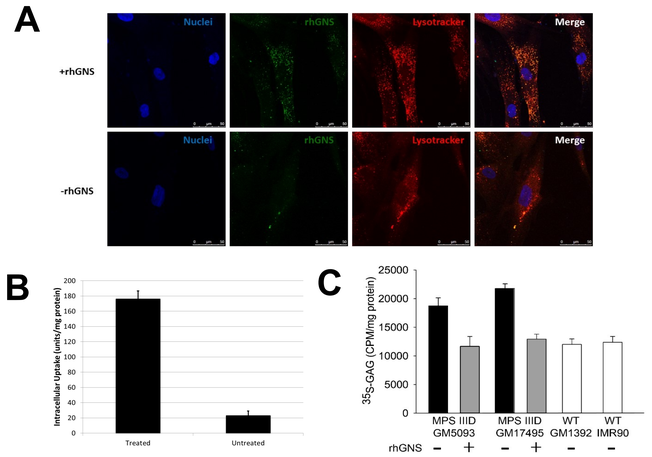


Fig. 2: **A**) Confocal microscopy of rhGNS uptake into MPS IIID human fibroblasts. Blue: DAPI, Green: rhGNS (anti-myc), Red: Lysotracker. Top row: treated with rhGNS. Bottom row, no rhGNS applied. **B**) rhGNS intracellular uptake assay. Cell lysates from MPS IIID human fibroblasts were assayed for GNS activity following 4 hour treatment with rhGNS. **C**) Heparan sulfate GAG reduction in MPS IIID human fibroblasts treated with 150 ng/ml rhGNS for 72h at 37˚C or untreated. Shown is a representative experiment from triplicate experiments. Means and S.D. of triplicate assays. Two wild-type (WT) fibroblast lines are shown as controls.

Enzymatically-active rhGNS was taken up by MPSIIID fibroblasts (**Fig. 2**) and colocalized with lysosomal markers. Both uptake and lysosomal targeting were shown to be decrease significantly in the presence of free mannose-6-phosphate (M6P), suggesting that our rhGNS is rich in mannose-6-phosphate glycosylation and uptake is M6P receptor dependent. We demonstrated a minimum of 33 and a maximum of 65% reduction in heparan sulfate in three independent experiments in two human MPS IIID cell lines treated with rhGNS, reaching wild-type levels of heparan sulfate (**Fig. 2C**). This result indicates that rhGNS produced by our group is able to catabolize the primary substrate that is responsible for MPS IIID neuropathology.

Conclusion & Future Studies

Rare childhood neurodegenerative disorders like MPS IIID are some of the most heartbreaking and tragic diseases imaginable. To date, there is no treatment or cure in development for MPS IIID. However, recent progress made in enzyme replacement therapies for other MPS diseases has provided a pathway for developing a treatment for MPS IIID patients.

We have demonstrated that, using a CHO stable cell line, we are able to produce and purify enzymatically active and highly-glycosylated rhGNS. Our product is mannose-6-phosphorylated, enters into human MPS IIID cells, targets to the lysosomal compartment, and is stable in the ideal vehicle for intrathecal delivery. Furthermore, we have shown that it is able to decrease accumulation of GAG in MPS IIID patient fibroblasts to WT levels, thereby correcting the primary physiological effects of the disease.

Now that we have characterized and shown *in vitro* efficacy, our next phase in development of rhGNS for treatment of MPS IIID patients will involve a short-term, *in vivo* proof-of-principle study in mice. We have previously shown that intravenous delivery of GNS is unable to reach the blood-brain barrier. Therefore, we will be using an intrathecal route of administration to circumvent this obstacle and deliver the therapeutic agent directly to the most-affected tissue. Following these initial studies, we will then begin process development for scale-up and manufacturing of rhGNS for preclinical development, funded by a phase II STTR grant. If successful this could grow the company, and ultimately we may need to partner to fund clinical trials.

Acknowledgments & References

Dr. Alexey Pshezhetsky, Dr. Brian Bigger, Dr. Matt Ellinwood, Allison, Lacey and family and many others. **Funding** NIH NINDS 1R41NS89061-01

National Organization for Rare Diseases Database https://www.rarediseases.org/rare-disease-information/rare-diseases

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