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Characterization of Human Recombinant N-Acetylgalactosamine-6-Sulfate Sulfatase Produced in *Pichia pastoris* as Potential Enzyme for Mucopolysaccharidosis IVA Treatment

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ABSTRACT

Mucopolysaccharidosis IVA (MPS IVA or Morquio A syndrome) is a lysosomal storage disease caused by the deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS), leading to lysosomal storage of keratan sulfate and chondroitin-6-sulfate. Currently, enzyme replacement therapy using an enzyme produced in CHO cells represents the main treatment option for MPS IVA patients. As an alternative, we reported the production of an active GALNS enzyme produced in the yeast *Pichia pastoris* (prGALNS), which showed internalization by cultured cells through a potential receptor-mediated process and similar post-translational processing as human enzyme. In this study, we further studied the therapeutic potential of prGALNS through the characterization of the N-glycosylation structure, *in vitro* cell uptake and keratan sulfate reduction, and *in vivo* biodistribution and generation of anti-prGALNS antibodies. Taken together, these results represent an important step in the development of a *P. pastoris*—based platform for production of a therapeutic GALNS for MPS IVA enzyme replacement therapy.

Introduction

Mucopolysaccharidosis type IVA (MPS IVA, Morquio A syndrome, OMIM 253000) is a genetic spondyloepiphyseal dysplasia caused by mutations in the gene encoding for the lysosomal enzyme N-acetylgalactosamine-6-sulfate sulfatase (GALNS, EC 3.1.6.4).[1,2] GALNS is involved in the intracellular degradation of the glycosaminoglycans chondroitin-6-sulfate (C6S) and keratan sulfate (KS). GALNS deficiency leads to lysosomal accumulation of C6S and KS.[1,2] Clinically, MPS IVA patients are characterized by short stature, corneal clouding, hypoplasia of the odontoid process, *pectus carinatum*, valvular heart disease, mild hepatomegaly, laxity of joints, kyphoscoliosis, and *genu valgum* without central nervous system impairment.[1,3] Treatment of these patients was only symptomatic and supportive, and patients often require surgical correction of the skeletal abnormalities to give them a better quality of life.[3,4]

The first specific therapy for MPS IVA patients was approved in 2014, consisting of the intravenous administration of a recombinant enzyme produced in CHO cells (elosulfase alfa) at 2 mg/kg weekly.[5] The cellular uptake of this recombinant enzyme occurs through mannose-6-phosphate receptors (M6PR).[6,7] *In vivo* studies on wild-type mice showed that the enzyme is detected in the growth plate, heart valve tissues, and hepatocytes with a high dose and high administration frequency (5 infusions of 10 mg/kg every other day).[7] Enzyme replacement therapy (ERT) with a recombinant enzyme on MPS IVA mice provided a limited impact on bone pathology, whereas the use of a bone-targeting GALNS enhanced the therapeutic efficacy in bone pathology.[6,8] Phase III studies showed that a weekly intravenous administration of 2.0 mg/kg during 24 weeks allowed a slight improvement in the 6-min walk

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test and the reduction of urinary KS,[9] as well as an improvement in the maximal voluntary ventilation, performance of daily life activities, and height/growth rate.[10] Despite of this, elosulfase alfa still has several constraints including (1) limited effect on skeletal, corneal, and heart valvular tissues,[11,12] (2) a short enzyme half-life and rapid clearance, (3) immune response,[13] and (4) the need of weekly 4-5 h infusions. This restricts the use of this therapy because of cost-effectiveness concerns of elosulfase alfa.[14] Limitations of current therapy indicate an unmet need for new therapeutic strategies to improve and expand the treatment options for MPS IVA patients.

To overcome some of aforementioned ERT issues, it has been alternatively proposed to produce recombinant GALNS in other hosts.[15,16] In fact, several human lysosomal enzymes have been produced in plant cells, transgenic animals, and microorganisms including *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica,* and *Ogataea minuta*.[17-20] Among these hosts, yeasts represent an important platform for the production of recombinant proteins because they can grow in low-cost culture media, are easily manipulated, secrete the recombinant protein to the medium, and produce heterologous proteins with similar post-translational modifications (e.g., disulfide bonds and N-glycosylations) to those observed in human proteins.[21] These characteristics are important because it has been demonstrated that GALNS N-glycosylations are not required to produce an active enzyme, but for protein cellular uptake.[22] Although yeast N-glycosylations have some differences in size and composition in comparison with those observed in human proteins, these N-glycosylations can be glyco-engineered to produce tailored or homogeneous structures.[23] Furthermore, recombinant human β-hexosaminidases,[16,24] α-glucosidase,[25] and lysosomal acid lipase[26,27] produced in the yeast *P. pastoris* have shown dose-dependent cell uptake without any additional processing of their N-glycosylations, although this is mostly true for macrophage-lineage cells that express mannose receptors (MRs).

Previously, we reported the characterization of a human recombinant GALNS produced in the methylotrophic yeast *P. pastoris* GS115 (prGALNS). This protein showed a high stability at pH 5.0 and low temperature (4°C), and its activity was enhanced when it was coexpressed with the sulfatase modifier factor 1 (SUMF1) cDNA. Furthermore, *in vitro* assays showed that prGALNS was taken up by HEK293 cells and human skin fibroblasts through a process potentially mediated by an endocytic pathway.[28] In this study, we further studied the therapeutic potential of prGALNS through the characterization of the N-glycosylation structure, *in vitro* cell uptake and KS reduction, and the evaluation of *in vivo* biodistribution and generation of anti-prGALNS antibodies.

Materials and Methods

Bioreactor Cultures

A *P. pastoris* GS115 (Invitrogen; Thermo Fisher Scientific, San Jose, CA) strain, previously transformed with the human GALNS and SUMF1 (EC 3.10.1.1) cDNAs,[28] was used to produce prGALNS. Cultures were performed at 1.65 L scale in a KLF 3.7 L reactor (Bioengineering AG, Wald, Zürich, Switzerland) using a modified fermentation medium FM22 (composition per liter: KH_2PO_4 25.74 g, $(NH_4)_2SO_4$ 3 g, K_2SO_4 8.58 g, $CaSO_4$ $2H_2O$ 0.6 g, glycerol 40 g, $MgSO_4$ $7H_2O$ 7.02 g, Biotin 4×10^{-5}% w/v, supplemented with Pichia trace minerals PTM4 1.0 mL).[29] Protein production was first carried out in a batch culture with glycerol followed by a fed-batch induction phase with methanol, as previously described. Cultures were done at 28°C and pH 5.0, under limited oxygen conditions, during 96 h.[28]

Crude Protein Extracts and Enzyme Purification

prGALNS was purified from culture medium after a previously reported protocol.[28] Briefly, culture medium (~1.7 L) was filtered sequentially through 0.45 and 0.22 μm using polyether sulfone membranes (Pall Corp, Port Washington, NY). Permeate was ultrafiltrated through a 30 kDa cutoff membrane (Millipore, Billerica, MA). Then, the retentate was dialyzed in acetate buffer 25 mM, pH 5.0. Finally, prGALNS was purified by a 2-step process using a cation exchange chromatography followed by size exclusion chromatography, as previously described.[22,28] Fractions with the highest GALNS activity were pooled, diafiltrated against 25 mM sodium acetate pH 5.0, and lyophilized. Protein purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis and GALNS activity.

N-glycans Analysis and Exoglycosidase Digestion

Samples for N-glycan analyses were obtained from the supernatant of *P. pastoris* cultures at 2 mL scale grown in buffered glycerol complex medium (yeast extract 1% p/v; peptone 2% p/v; potassium phosphate 100 mM pH 6.0; yeast nitrogen base 1.34%; biotin 4×10^{-5}%; glycerol 1%) and cultured for 24 h at 28°C and 200 RPM. Cells were recovered and resuspended in buffered methanol complex medium (potassium phosphate 100 mM pH 6.0; yeast nitrogen base 1.34%; biotin 4×10^{-5}%; methanol 0.5%), and cultured for 48 h at 28°C and 200 RPM. N-glycans were labeled with 8-aminopyrene-1.3.6-trisulfonic acid (APTS; Sigma-Aldrich, St. Louis, MO) according to a published method.[30] N-linked glycans were prepared by blotting on 96-well plate with PVDF membrane plates (Millipore, Bedford, UK), and analyzed by carbohydrate electrophoresis with laser-induced fluorescence detection using an ABI 3130 DNA sequencer as previously reported.[30] N-glycans structures are reported according to Jacobs et al.,[31] who used the same glycosylation analysis methodology used in the present study.

GALNS Activity

GALNS activity was assayed by using 4-methylumbelliferyl-β-D-galactopyranoside-6-sulfate (Toronto Chemicals Research, North York, ON, Canada) as substrate.[26] One unit (U) was defined as the amount of enzyme catalyzing the production of 1 nmol of 4-methylumbelliferone product per hour. Specific GALNS activity was expressed as U mg^{-1} of protein as determined by BCA assay (Pierce™ Thermo Scientific, Rockford, IL).

Cellular Uptake of Recombinant GALNS

The cellular uptake of prGALNS was assayed in HEK293 cells (ATCC CRL1573) and human skin MPS IVA fibroblasts, as previously described.[22] Cells were maintained in Dulbecco's modified medium (DMEM; Gibco, Carlsbad, CA) supplemented with fetal bovine serum 15% (Eurobio, Les Ulis, Francia), penicillin 100 U mL^{-1} and streptomycin 100 U mL^{-1} (Walkersville, MD), at 37°C in a CO_2 incubator. Twenty-four hours before the experiment, 1×10^5 cells per well were seeded in 12-well plates, and the culture medium was replaced with fresh medium 2 h before the experiment. The purified enzyme was added to a final concentration of 50 nM in HEK293 and 50, 100, and 200 nM in MPS IVA fibroblasts.[22,28] Assays with HEK293 cells were performed with and without mannose-6-phosphate (M6P) or methyl α-D-mannopyranoside as inhibitors of M6P and MR, respectively, at a final concentration of 2 mM, according to a reported study for recombinant GALNS produced in CHO cells.[32] After 6 h incubation, the culture medium was removed and the cells were washed 3 times with cold 1× PBS. Cells were lysed using 1% sodium deoxycholate (Sigma-Aldrich, St. Louis, MO).

The enzyme activity was determined in the cell lysate. All assays were performed in triplicate.

Intracellular Trafficking

HEK293 cells were cultured on coverslips at 2×10^4 cells per well, previously treated with 0.01% (w/v) type II collagen (Sigma-Aldrich, St. Louis, MO), with complete DMEM, as described previously. Culture medium was replaced with fresh medium 1 h before the experiment and prGALNS, previously labeled with Alexa Fluor 568 (Molecular Probes, Thermo Fisher Scientific) according to manufacturer's protocol, was added at a final concentration of 50 nM. After 12 h of incubation with the labeled enzyme, the lysosomes were stained with LysoTracker® Green DND-26 (Molecular Probes, Thermo Fisher Scientific) following the manufacturer's protocol. Cells were then fixed using freshly prepared 4% paraformaldehyde in 1× PBS for 20 min at room temperature. The cellular nucleus was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific). Cells were imaged using an Olympus FV1000 confocal microscope equipped with 405, 473, and 559 nm laser lines using a 63×/1.49 NA oil objective. DAPI (excitation 382-393, emission 417-477 nm), FITC (excitation 460-500 nm, emission 510-560 nm), and TRITC HyQ filter sets (excitation 530-560 nm, emission 590-650 nm) were applied to collect DAPI, LysoTracker® Green, and Alexa 568 signals, respectively. Images were processed by using NIH Image J 1.8.0.[33]

In Vitro *Keratan Sulfate Clearance Assay*

MPS IVA skin fibroblasts were cultured and treated with 50, 100, and 200 nM of purified prGALNS. Quantification of di-KS in the cell lysate was performed by liquid chromatography coupled to a tandem mass spectrometry, as previously described,[34,35] with some modifications. Briefly, 200 μL of cell extract were placed into a 96-well Omega 10 K filter plate (AcroPrep™, PALL Corporation, Port Washington, NY) and then centrifuged for 15 min at 2500 × *g*. All samples and standards were incubated at 37°C overnight with chondroitinase B, heparitinase, and keratanase II (Seikagaku Co., Tokyo, Japan). After incubation, the disaccharides were collected by centrifugation for 15 min at 2500 × *g*, and analyzed by liquid chromatography coupled to a tandem mass spectrometry following a standardized protocol.[35] All assays were performed in triplicate. Experiments were carried out at Nemours/Alfred I. duPont Hospital for Children (Wilmington, DE) under approved protocols.

Enzyme Biodistribution

According to Tomatsu et al.,[32] male C57BL/6 wild-type mice (7-8 weeks old) received a single intravenous administration of 1× PBS or 5 mg kg^{-1} of prGALNS labeled with Alexa Fluor 568 (Molecular Probes, Thermo Fisher Scientific). Mice were sacrificed at 12 and 24 h postinfusion. Brain, lung, heart, liver, spleen, kidney, and bone (femur) were collected and immersion-fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned (Laboratory of Pathology; Hospital Universitario San Ignacio, Bogotá D.C., Colombia). Tissues were studied by fluorescence microscopy for enzyme distribution. All procedures were carried out at the Biology Comparative Unit at Pontificia Universidad Javeriana, in accordance with the Institutional Animal Care and Use Committee guidelines under approved protocols.

Immunization of Mice and Detection of Anti-prGALNS Antibodies

To evaluate immunogenicity of prGALNS, male C57BL/6 wild-type mice (7-8 weeks old) received 5 mg kg^{-1} of prGALNS or 1× PBS weekly during 4 weeks ($n = 5$ per group). The prGALNS was diluted in 1× PBS and injected intravenously through the lateral tail vein. Blood samples were taken at 0, 15, and 30 days after infusion. All procedures were carried out at the Biology Comparative Unit at Pontificia Universidad Javeriana, in accordance with the Institutional Animal Care and Use Committee guidelines under approved protocols. An indirect ELISA was used to determine antibody generation in serum from mice immunized with prGALNS. For this purpose, 96-well plates were coated overnight at 37°C with 10 μg mL^{-1} of purified prGALNS in PBS pH 7.4. Wells were blocked for 2 h at 37°C with 1× PBS pH 7.4, 0.05% Tween 20 and 5% nonfat dry milk. The plates were washed 2 times with PBST (1× PBS pH 7.4, 0.05% Tween 20). One hundred microliter of serum dilutions, between 1:100 and 1:5000 in PBST and 5% nonfat dry milk, were added to the wells and incubated at 37°C for 1 h. After 3 PBST washes, a peroxidase conjugated goat anti-mouse IgG (A9044 Sigma-Aldrich, St. Louis, MO) was added to the wells in a 1:2000 dilution and plates were incubated at 37°C for 1 h. The reaction was developed with TMB SureBlue substrate (KPL; Milford, MA) and incubated at room temperature for 10 min. The reaction was stopped with 1N HCl and the plate was read at 450 nm on an Anthos 2020 ELISA plate reader.

Statistical Analysis

Differences between groups were tested for statistical significance by using 2-way ANOVA and Tukey's multiple comparison test. An error level of 5% ($p < 0.05$) was considered significant. All analyses were performed using GraphPad Prism v.7.0 (GraphPad Software, La Jolla, CA). All results are shown as mean ± SD.

Results and Discussion

N-glycosylation Structure

N-glycans analysis performed in the culture supernatant of wild-type *P. pastoris* GS115 revealed a N-glycosylation profile consisting of a main peak of 9 mannose residues (M_9) and subsequent peaks representing additional mannose residues ($\geq M_{10}$) (Fig. 1b). By contrast, in the prGALNS-producing strain, a stronger hypermannosylation pattern was observed, characterized by an increase in the abundance of peaks corresponding to M_{10} and M_{11} (Fig. 1c). The N-glycosylation pattern of the prGALNS-producing strain was characterized by the presence of signals that suggest either phosphomannosyl phosphorylation (mono- or double-mannosyl phosphorylated N-glycans) or terminal phosphorylation (Fig. 1c). A similar N-glycosylation pattern was observed for the strain coexpressing GALNS and SUMF1 (Fig. 1d), suggesting that coexpression of SUMF1 does not affect the N-glycosylation as observed in CHO-produced GALNS because of the changes in cell uptake of SUM1-activated GALNS.[6] Maltodextrin was used as glucose units reference (Fig. 1a) and RNAse B N-glycans were used as reference for high-mannose glycans (Fig. 1e).

The $Man_{8\text{-}15}GlcNAc_2$ pattern observed in prGALNS agrees with previous reports for proteins produced in *P. pastoris*.[26,36] This profile is closer to the mammalian N-glycosylation than that observed in proteins produced in *Saccharomyces cerevisiae*, which is characterized by hypermannosylated glycan species ($>Man_{50}$).[37,38] Nevertheless, the N-glycosylation pattern observed in *P. pastoris* GS115 evidenced several differences in terms of complexity with the typical mammalian N-glycosylation. For instance, in *P. pastoris*, the N-glycan structure is mainly composed by mannose; while mammalian N-glycans display a variety of monosaccharide units in their structure, especially sialic acid, fucose, and galactose, among others.[26,39,40] As an additional finding, we observed the presence of

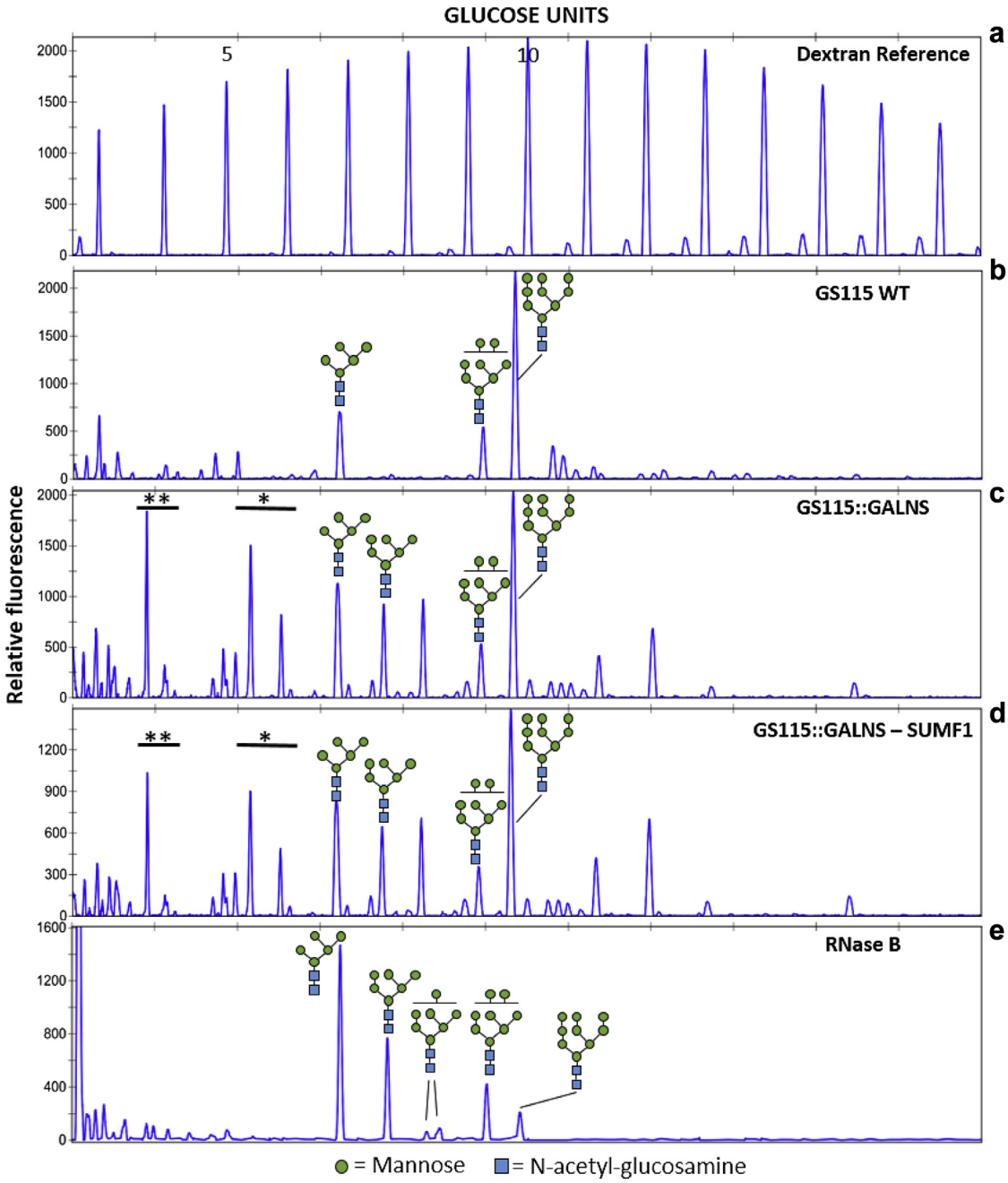

Figure 1. N-glycan profile of *Pichia pastoris* strains. (a) Maltodextrin reference. (b-d) N-glycan profile of the proteins present in growth medium of *P. pastoris* GS115 (b), *P. pastoris* GS115:GALNS (c), and *P. pastoris* GS115:GALNS-SUMF1 (d). * monomannosyl phosphorylated N-glycans; ** double-mannosyl phosphorylated N-glycans. (e) Reference N-glycans from bovine RNase B.

phosphorylated glycans in prGALNS (Figs. 1c and 1d). However, phosphorylations reported in *P. pastoris* are characterized by capped mannosyl phosphorylations contrasting to the typical mammalian terminal mannose-6-phosphate residues.[41] For this reason, it has been proposed that *Pichia*-derived proteins for therapeutic uses may need further enzymatic treatment to uncap and expose the M6P residues.[41]

N-glycosylation has been associated with stability, immunogenicity, internalization, efficacy, and biodistribution of proteins.[42-44] However, the consequences of the differences in N-glycosylation patterns between mammalian and *P. pastoris* GS115 are still not well understood.[36] Although yeast hypermannosylated N-glycans have been suggested to trigger immune response, no detailed study has been performed to evaluate such association.[36] Conversely, low amounts of oligomannose structures (Man_{5-9}) have been reported in human plasma proteins,[45] as well as in approved recombinant proteins for different lysosomal storage disorders.[42,44,46] The N-glycosylation profile of elosulfase alfa revealed that the dominant N-glycan species are BisP-Man_6, BisP-Man_7, and non-phosphorylated Man_8 and Man_9. In addition, no complex, hybrid glycans or other sialic acid—containing glycans were also detected in elosulfase alfa.[47] Taken together, the N-glycans analysis of prGALNS confirm the potential of *P. pastoris* as expression system for the development of an alternative ERT for MPS IVA. These potential could be enhanced by tailoring the N-glycans,[23] which could improve cell stability, cell uptake, and therapeutic efficacy.[41]

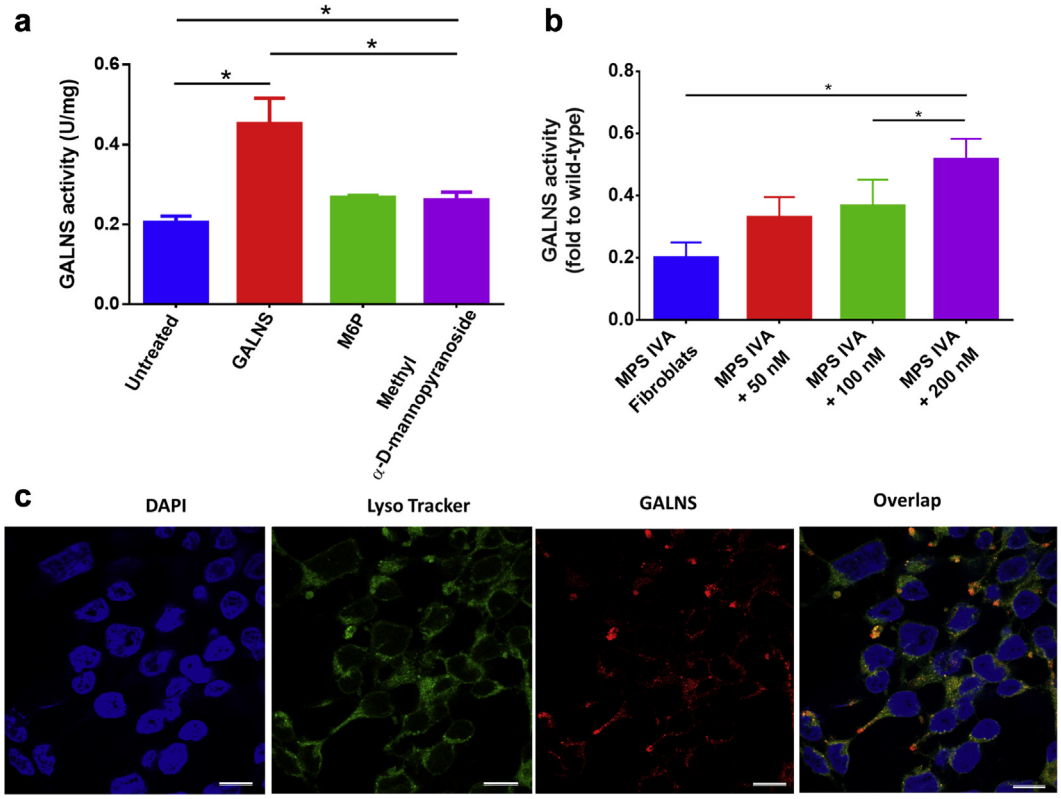

Figure 2. Internalization and trafficking of prGALNS in mammalian cells. (a) Cellular uptake assayed in cultured HEK293 after treatment with 50 nM prGALNS with and without inhibitors. (b) Cellular uptake assayed in MPS IVA patients fibroblasts after treatment with different concentrations of prGALNS. Assays were performed in by triplicate. $^{*}p < 0.05$. (c) Intracellular trafficking in HEK293 cells treated with prGALNS labeled with Alexa Fluor 568. Scale bar 50 μm. Pearson correlation value was calculated by Fiji (Image J) with R = 0.56 (above threshold) and thresholded Mander's coefficients tM1 and tM2 were 0.975 and 0.533, respectively.

Cell Uptake and Intracellular Trafficking

Previously, we reported that prGALNS can be internalized by human skin fibroblasts from unaffected individuals and HEK293 cells through a potential endocytic pathway.[28] Here, we further explored the mechanism for prGALNS internalization. Cell capture assays were performed in the presence of selective inhibitors of the main receptors associated with the internalization of lysosomal enzymes: M6PR or MR.[7,26,32,42,44,48,49] The results suggest that prGALNS is internalized using both M6PR and MR because addition of inhibitors for these receptors significantly reduced the cell uptake of the recombinant enzyme (Fig. 2a).

Based on the prGALNS N-glycosylation pattern described previously, it was expected that the high content of oligomannose glycans favor interaction with the MR, which is in good agreement with the results observed for other lysosomal proteins produced in *P. pastoris*, plants, and moss.[26,50-52] The role of the M6PR in the internalization of yeast produced enzymes, to the best of our knowledge, has not been directly assessed. This is of great importance, especially considering that we observed peaks suggesting capped nonterminal phosphomannosyl glycans (D-mannose-α-1-phospho-6-D-mannose) in prGALNS (Fig. 1), which is similar to that reported for α-galactosidase and α-glucosidase produced in *P. pastoris* and *Yarrowia lipolytica*, respectively.[41] Nonetheless, further characterization of M6PR interactions with these types of mannosyl phosphorylations are required to better understand the internalization mechanism used by *P. pastoris*—produced enzymes.

We observed that both M6PR and MR inhibitors did not abolish completely the prGALNS internalization (Fig. 2a). These findings suggest that the enzyme may also be internalized through a M6PR- or MR-independent pathway. Although other mechanisms have been proposed for lysosomal enzymes internalization, these remain poorly understood.[53,54] However, regardless of the mechanism, we observed that the internalized prGALNS colocalized with the lysosomal staining (Fig. 2c, Pearson correlation R = 0.56), similar to previous reports for the recombinant GALNS produced in CHO cells[7,55] and iduronate-2-sulfatase produced in *P. pastoris*.[20]

In the second stage, we explored the ability of prGALNS to be taken up by MPS IVA patients' skin fibroblasts. As shown in Figure 2b, the cellular uptake of prGALNS in the fibroblasts showed a similar pattern to that reported for HEK293 cells,[28] with clear increment in the intracellular GALNS activity post-treatment in a dose-dependent manner. However, this change was only statistically significant at the highest used dose of the recombinant enzyme (200 nM) in contrast to HEK293 where statistically significant differences were observed using 50 nM of prGALNS. Such behavior may reflect the differences that occur in the internalization ability of different cell types for the same protein.[56-59] These differences have been associated with changes in expression level of receptors and the effect of N-glycosylation variants on the cellular uptake process.[56-59]

To evaluate the therapeutic potential of prGALNS, we measured the di-KS levels in MPS IVA skin fibroblasts treated with 50, 100, and 200 nM of prGALNS (equivalent to 6 to 24 μg). Similar amounts of recombinant GALNS produced in CHO cells were used both *in vitro* (0.78 to 200 nM)[7,32] and *in vivo* (0.24 to 24 μg).[6,7] As observed in Figure 3, there was a significant reduction in the intracellular di-KS levels after treatment with prGALNS for all of the evaluated doses. A reduction up to 81% of accumulated di-KS was obtained using 100 nM of prGALNS. Similar behavior was observed

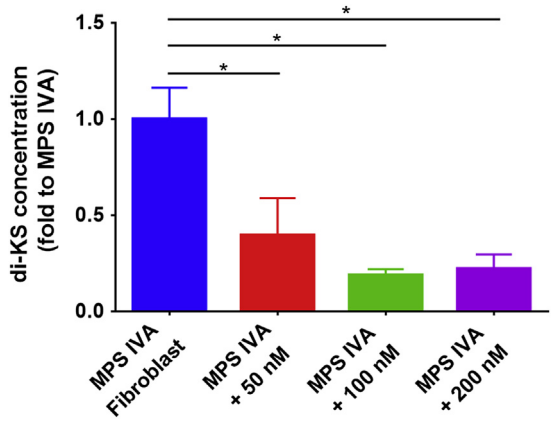

Figure 3. Quantitation of KS in MPS IVA fibroblasts treated with prGALNS. MPS IVA patient skin fibroblast were treated with 50, 100, and 200 nM of prGALNS and the KS levels were quantified by LC-MS/MS. Results are reported as fold to untreated MPS IVA fibroblast (blue). $^{*}p < 0.05$. LC-MS/MS, liquid chromatography coupled to a tandem mass spectrometry.

in MPS IVA chondrocytes with the enzyme produced in CHO cells (reduction of KS accumulation 80%-100%), using a lower enzyme concentration (1 and 10 nM), which might be related to the higher specific enzyme activity levels reported for that enzyme (120,000 U mg^{-1}).[7] Nonetheless, our results suggest that the *P. pastoris*–produced enzyme is not only structurally but also functionally similar to the human enzyme, which makes it a potential option for therapeutic purposes.

Biodistribution

We evaluated tissue distribution of prGALNS after a single IV of 5 mg kg^{-1} (83.5 U kg^{-1}), in wild-type C57BL/6 mice. Twelve hours after treatment, it was observed that prGALNS was mainly localized in spleen, liver, and heart; whereas after 24 h, the enzyme was detected in spleen, heart, and kidney (Fig. 4). There was no presence of the recombinant enzyme in brain, growth plate, or bone tissue. These results suggest a rapid clearance of the enzyme by highly perfused tissues, which is in agreement with a previous report of intravenous administration of recombinant GALNS produced in CHO cells.[32] In MPS IVA, the main affected tissues include cardiac valves, lung, and cartilage (growth plate), making these tissues the main target for any therapeutic approach.[7,8,60] Our results show that prGALNS preferentially reaches visceral organs. However, the therapeutic implications of such behavior might not be properly assessed by single infusion because other studies suggest that higher doses (10 mg/kg) and repeated administration may saturate the enzyme in liver and spleen and allow the distribution to other tissues.[7]

Generation of Anti-prGALNS Antibodies

To analyze the generation of anti-prGALNS antibodies, wild-type C57BL/6 mice were weekly infused with 5 mg kg^{-1} of prGALNS and the anti-prGALNS antibodies were assayed after 15 and 30 days into the immunization regimen. Anti-prGALNS IgG antibodies were only evaluated because this is the main isotype found in other studies using exogenous lysosomal enzymes in

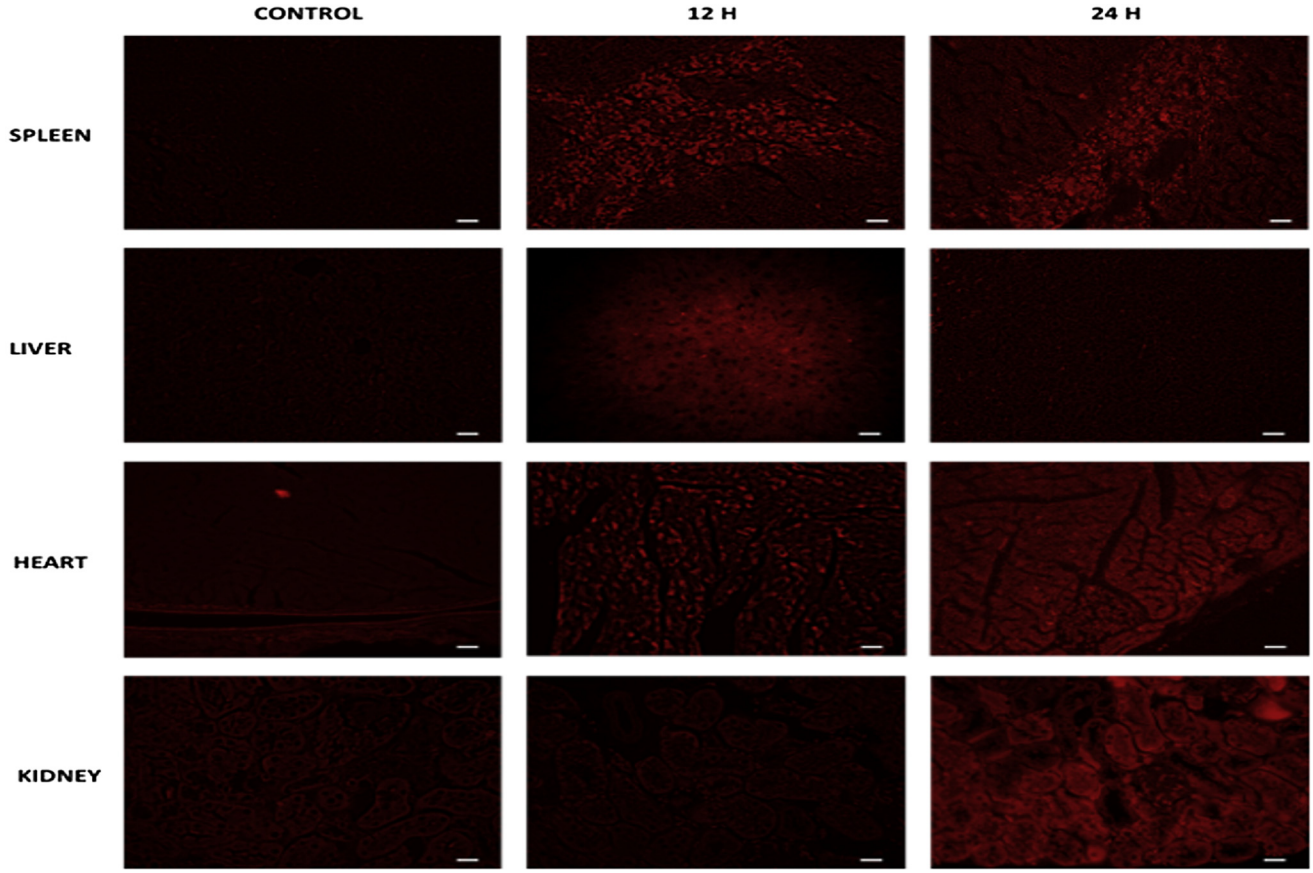

Figure 4. Biodistribution of prGALNS. Male C57BL/6 mice (7-8 wk old) received a single intravenous administration of 1× PBS (control) or 5 mg kg^{-1} of prGALNS labeled with Alexa Fluor 568 histological sections of spleen, liver, heart, and kidney were taken at 12 and 24 h after treatment. Scale bar 100 μm.

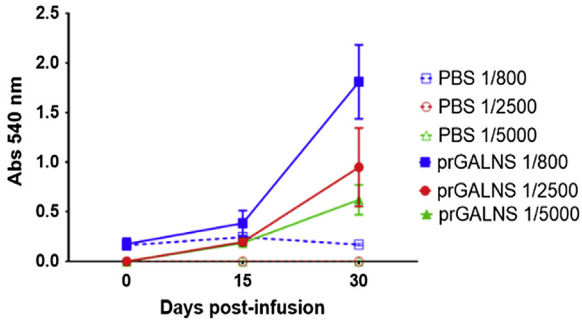

Figure 5. Generation of anti-prGALNS antibodies. Wild-type C57BL/6 mice were weekly infused 1× PBS or 5 mg kg^{-1} of prGALNS and the anti-prGALNS antibodies were assayed in serum samples after 15 and 30 d into the immunization regimen (n = 5 per group).

different animal species.[61-63] The results showed the production of anti-prGALNS antibodies with an increasing trend along time (Fig. 5), as would be expected by the difference in human and mouse GALNS because these proteins share an 85% identity.[64] The results are about one order of magnitude lower than those reported in C57Bl/6 MPS IVA knock-out mice infused with a recombinant GALNS produced in CHO cells.[64] Because a higher amount of protein was infused in the present study (5 mg kg^{-1}) than in recombinant GALNS produced in CHO cells (about 1.5 mg kg^{-1}), we can speculate that prGALNS could have a similar immunogenicity compared to CHO cell-produced enzyme. In addition, similar results were observed in mice treated with a human recombinant lysosomal acid lipase (hLAL) produced in *Nicotiana benthamiana*.[65] The authors showed that anti-hLAL antibodies were produced against both glycosylated and deglycosylated recombinant hLAL, suggesting that that the antibodies are mainly produced against the protein backbone rather than to the N-glycans. A recent report showed that, in mice, similar antibodies titers are produced against plant- or mammalian-derived lysosomal α-L-iduronidase.[66] In the same way, Kreer et. al.,[67] developed a direct comparison of immunogenicity between N-glycosylated and de-glycosylated proteins produced in *P. pastoris*. The results showed that N-glycosylated proteins influenced neither dendritic cells maturation nor their general capacity to activate T cells, pointing out that enforced N-glycosylation does not increase the immunogenicity of an antigen *per se*. They also observed a strong IgG response after injection of deglycosylated protein; whereas protein-specific IgGs were hardly detectable after immunization with the N-glycosylated protein, suggesting that N-glycosylations may prevent MHC II—restricted presentation of the recombinant protein. Taken together, we consider that anti-prGALNS antibodies are mainly produced against protein backbone rather than to the N-glycans. Nevertheless, anti-GALNS antibodies have been reported in 100% of the patients treated with elosulfase alfa,[13] suggesting that other factors could be involved in the immune response against the recombinant protein. However, up to now, there is no clear correlation between antibody production and therapeutic efficacy of ERT.[68] In this sense, further studies are needed to characterize antibody production against prGALNS produced, as well as studies addressing the effect of such antibodies on treatment response.

Conclusions

In this study, we have characterized the N-glycosylation structure of a recombinant GALNS produced in the yeast *P. pastoris* GS115 and evaluated, *in vitro* and *in vivo*, some therapeutic characteristics of this recombinant enzyme. *In vitro*, it was observed that prGALNS presented mainly yeast-type high-mannose chains and mannosyl phosphorylated derivatives thereof. This recombinant protein was successfully internalized by mammalian cells reaching the lysosome. The internalized enzyme was able to clear the stored intracellular di-KS in MPS IVA patients' fibroblasts. In addition, the *in vivo* results suggested that prGALNS is rapidly cleared from the organism. In summary, these findings show the potential of *P. pastoris* as a yeast platform for the production of a therapeutic human recombinant enzyme for MPS IVA. Future works should aim to improve the enzyme activity, as well as biodistribution and targeting of prGALNS, to main affected tissues in MPS IV A. The rise of some level of anti-drug antibodies over time for this human protein in the mouse experimental model needs to be taken into account for long-term treatment model studies.

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Authors' contributions: A.R.L., L.N.P.V., and A.J.E.M. performed the experiments. A.R.L., A.V.H., and P.T. performed the N-glycan analysis. L.N.P.V. and S.T. performed the di-KS analysis. A.R.L., P.T., S.T., N.C., and C.J.A.D. conceived and designed the experiments. A.R.L., S.T., N.C., and C.J.A.D. wrote the article. All authors read and approved the final manuscript.

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