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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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Short Title: Recombinant GALNS in *Pichia pastoris*

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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 (KS) and chondroitin-6-sulfate. Currently, enzyme replacement therapy (ERT) 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 KS reduction, and *in-vivo* biodistribution and generation of anti-prGALNS antibodies. Taken together, these results represent an important step in the develop of a *P. pastoris*-based platform for production of a therapeutic GALNS for MPS IVA ERT.

Keywords: Morquio A, Mucopolysaccharidosis IV A, GALNS, *Pichia pastoris*, glycosylations

1. INTRODUCTION

Mucopolysaccharidosis type IVA (MPS IVA, Morquio A syndrome, OMIM 253000) is a genetic spondyloepipheseal 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 (GAGs) 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 administraton frequency (5 infusions of 10 mg/kg every other day) [7]. ERT with a recombinant enzyme on MPS IVA mice provided a limited imptact on bone pathology, while 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-minute walk 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: i) limited effect on skeletal, corneal, and heart valvular tissues [11, 12], ii) a short enzyme half-life and rapid clearance, iii) immune response [13], and iv) the need of weekly 4-5 hours infusions. This restricts the use of this therapy due to 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 above mentioned 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 since 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 since 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.

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 co-expressed 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.

2. MATERIALS AND METHODS

2.1 Bioreactor cultures

A *P. pastoris* GS115 (Invitrogen, Thermo Fisher Scientific, San Jose, CA, USA) 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, 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 done 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].

2.2 Crude protein extracts and enzyme purification

prGALNS was purified from culture medium following a previously reported protocol [28]. Briefly, culture medium (~1.7 L) was filtered sequentially through 0.45 and 0.22 μm using polyether sulphone membranes (Pall Corp, Port Washington, NY, USA). Permeate was ultra-filtrated through a 30 kDa cut-off membrane (Millipore, Billerica, MA, USA). Then, the retentate was dialyzed in acetate buffer 25mM, pH 5.0. Finally, prGALNS was purified by a two-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 SDS–PAGE and GALNS activity.

2.3 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 BMGY 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 BMMY medium (potassium phosphate 100 mM pH 6.0; yeast nitrogen base 1.34%; biotin 4×10^{-5}%; methanol 0.5%), and cultured for 48 hours at 28 °C and 200 RPM. N-glycans were labeled with 8-aminopyrene-1.3.6-trisulphonic acid (APTS, Sigma Aldrich, St. Louis, MO, USA) 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 (CE-LIF) 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.

2.4 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, USA).

2.5 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, USA), 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 mannose receptor, respectively, at a final concentration of 2 mM, according to a reported

159 study for recombinant GALNS produced in CHO cells [32]. After 6 h incubation, the
160 culture medium was removed and the cells were washed three times with cold 1x PBS.
161 Cells were lysed using 1% sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA).
162 The enzyme activity was determined in the cell lysate. All assays were performed in
163 triplicate.

164 **2.6 Intracellular trafficking**

165 HEK293 cells were cultured on coverslips at 2 x10^4 cells per well, previously treated with
166 0.01% (w/v) type II collagen (Sigma-Aldrich, St. Louis, MO, USA), with complete DMEM
167 medium, as described above. Culture medium was replaced with fresh medium 1 h before
168 the experiment and prGALNS, previously labeled with AlexaFluor 568 (Molecular Probes,
169 Thermo Fisher Scientific, San Jose, CA, USA) according to manufacturer's protocol, was
170 added at a final concentration of 50 nM. After 12 h of incubation with the labeled enzyme,
171 the lysosomes were stained with Lysotracker® Green DND-26 (Molecular Probes, Thermo
172 Fisher Scientific, San Jose, CA, USA) following the manufacturer's protocol. Cells were
173 then fixed using freshly prepared 4% paraformaldehyde in 1x PBS for 20 min at room
174 temperature. The cellular nucleus was stained with 4´,6-diamidino-2-phenylindole
175 dichydrochloride (DAPI, Thermo Fisher Scientific, San Jose, CA, USA). Cells were
176 imaged using an Olympus FV1000 confocal microscope equipped with 405, 473 and 559
177 nm laser lines using a 63×/1.49 NA oil objective. DAPI (excitation 382-393, emission 417-
178 477 nm), FITC (excitation 460-500 nm, emission 510-560 nm) and TRITC HyQ filter sets
179 (excitation 530-560 nm, emission 590-650 nm) were applied to collect DAPI,
180 Lysotracker® Green, and Alexa 568 signals, respectively. Images were processed by using
181 NIH Image J 1.8.0 [33].

2.7 *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 done by liquid chromatography coupled to a tandem mass spectrometry (LC-MS/MS), 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, NY, USA) 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., Okyo, Japan). After incubation, the disaccharides were collected by centrifugation for 15 min at 2500 ×g, and analyzed by LC-MS/MS 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, USA), under approved protocols.

2.8 Enzyme biodistribution

According to Tomatsu et al. [32], male C57BL/6 wild-type mice (7-8 weeks old) received a single intravenous administration of 1x PBS or 5 mg kg^{-1} of prGALNS labeled with AlexaFluor 568 (Molecular Probes, Thermo Fisher Scientific, San Jose, CA, USA). Mice were sacrificed at 12 and 24 h post-infusion. 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.

2.9 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 1x PBS weekly during 4 weeks (n=5 per group). The prGALNS was diluted in 1x PBS and injected intravenously through the lateral tail vein. Blood samples were taken at 0, 15, and 30 days post-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 1x PBS pH 7.4, 0.05% Tween 20 and 5% non-fat dry milk. The plates were washed two times with PBST (1x PBS pH7.4, 0.05% Tween 20). One hundred μL of serum dilutions, between 1:100 and 1:5000 in PBST and 5% non-fat dry milk, were added to the wells and incubated at 37 °C for 1 h. After three PBST washes, a peroxidase conjugated goat anti-mouse IgG (A9044 Sigma- Aldrich, St. Louis, MO, USA) 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 Sure Blue substrate (KPL, Milford. MA, USA) 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.

2.10 Statistical analysis

Differences between groups were tested for statistical significance by using two-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, California, USA). All results are shown as mean ± SD.

3. RESULTS AND DISCUSSION

3.1 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 ($\geq M_{10}$) (**Figure 1B**). In 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} (**Figure 1**C). The N-glycosylation pattern of the prGALNS-producing strain was characterized by the presence of signals that suggest either phospho-mannosyl phosphorylation (mono- or double-mannosyl phosphorylated N-glycans) or terminal phosphorylation (**Figure 1C**). A similar N-glycosylation pattern was observed for the strain co-expressing GALNS and SUMF1 (**Figure 1D**), suggesting that co-expression of SUMF1 does not affect the N-glycosylation as observed in CHO-produced GALNS due to the changes in cell uptake of SUM1-activated GALNS [6]. Maltodextrin was used as glucose units reference (**Figure 1A**) and RNAse B N-glycans were used as reference for high-mannose glycans (**Figure 1E**).

The $Man_{8-15}GlcNAc_2$ 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 phosphorylated glycans in prGALNS (**Figure 1 C and D**). However, phosphorylations reported in *P. pastoris* are characterized by capped mannosylphosphorylations 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, implications of the mentioned 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].

3.2 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 mannose receptor (MR) [7, 26, 32, 42, 44, 48, 49]. The results suggest that prGALNS is internalized using both M6PR and MR, since addition of inhibitors for these receptors significantly reduced the cell uptake of the recombinant enzyme (**Figure 2A**).

Based on the prGALNS N-glycosylation pattern described above, 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

291 importance, especially considering that we observed peaks suggesting capped non-terminal
292 phosphomannosyl glycans (D-mannose-α-1-phospho-6-D-mannose) in prGALNS (**Figure**
293 **1**), which is similar to that reported for α-galactosidase and α-glucosidase produced in
294 *Pichia pastoris* and *Yarrowia lipolytica*, respectively [41]. Nonetheless, further
295 characterization of M6PR interactions with these types of mannosyl phosphorylations are
296 required to better understand the internalization mechanism used by *P. pastoris*-produced
297 enzymes.

298 We observed that both M6PR and MR inhibitors did not abolish completely the prGALNS
299 internalization (**Figure 2A**). These findings suggest that the enzyme may also be
300 internalized through a M6PR/MR-independent pathway. Although other mechanisms have
301 been proposed for lysosomal enzymes internalization, these remain poorly understood [53,
302 54]. However, regardless of the mechanism, we observed that the internalized prGALNS
303 co-localized with the lysosomal staining (**Figure 2C,** Pearson correlation R = 0.56), similar
304 to previous reports for the recombinant GALNS produced in CHO cells [7, 55] and
305 iduronate-2-sulfatase produced in *P. pastoris* [20].

306 In the second stage, we explored the ability of prGALNS to be taken up by MPS IVA
307 patients skin fibroblasts. As shown in **Figure 2B**, the cellular uptake of prGALNS in the
308 fibroblasts showed a similar pattern to that reported for HEK293 cells [28], with clear
309 increment in the intracellular GALNS activity post-treatment in a dose-dependent manner.
310 However, this change was only statistically significant at the highest used dose of the
311 recombinant enzyme (200 nM) in contrast to HEK293 where statistically significant
312 differences were observed using 50 nM of prGALNS. Such behavior may reflect the
313 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 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.

3.3 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 post-treatment, it was observed that prGALNS was mainly localized in spleen, liver, and heart; while after 24 hours the enzyme was detected in spleen, heart, and kidney (**Figure 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 since 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].

3.4 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, since this is the main isotype found in other studies using exogenous lysosomal enzymes in different animal species [61-63]. The results showed the production of anti-prGALNS antibodies with an increasing trend along time (**Figure 5**), as would be expected by the difference in human and mouse GALNS, since these proteins share a 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]. Since 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 *Pichia pastoris*. The results showed that N-glycosylated proteins neither influenced 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 de-glycosylated protein; while 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 a clear correlation between antibody production and therapeutic efficacy of ERT [68]. In this sense, further studies are need to characterization antibody production against prGALNS produced, as well as studies addressing the effect of such antibodies on treatment response.

4. 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 mannosylphosphorylated 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 declare that they have no competing interests. Data available on request from the authors

AUTHORS' CONTRIBUTIONS

ARL, LNPV, AJEM performed the experiments. ARL, AVH, PT performed the N-glycan analysis. LNPV and ST performed the di-KS analysis. ARL, PT, ST, NC and CJAD conceived and designed the experiments. ARL, ST, NC and CJAD wrote the paper. All authors read and approved the final manuscript.

REFERENCES

1. Tomatsu, S., et al., *Mucopolysaccharidosis type IVA (Morquio A disease): clinical review and current treatment.* Curr Pharm Biotechnol, 2011. **12**(6): p. 931-45.
2. Olarte-Avellaneda, S., et al., *Computational analysis of human N-acetylgalactosamine-6-sulfate sulfatase enzyme: an update in genotype-phenotype correlation for Morquio A.* Mol Biol Rep, 2014. **41**(11): p. 7073-88.
3. Sawamoto, K., et al., *Current therapies for Morquio A syndrome and their clinical outcomes.* Expert Opin Orphan Drugs, 2016. **4**(9): p. 941-951.
4. Tomatsu, S., et al., *Current and emerging treatments and surgical interventions for Morquio A syndrome: a review.* Res Rep Endocr Disord, 2012. **2012**(2): p. 65-77.
5. Sanford, M. and J.H. Lo, *Elosulfase alfa: First Global Approval.* Drugs, 2014. **74**(6): p. 713-8.
6. Tomatsu, S., et al., *Enzyme replacement therapy in a murine model of Morquio A syndrome.* Hum Mol Genet, 2008. **17**(6): p. 815-24.
7. Dvorak-Ewell, M., et al., *Enzyme replacement in a human model of mucopolysaccharidosis IVA in vitro and its biodistribution in the cartilage of wild type mice.* PLoS One, 2010. **5**(8): p. e12194.

8. Tomatsu, S., et al., *Enhancement of drug delivery: enzyme-replacement therapy for murine Morquio A syndrome.* Mol Ther, 2010. **18**(6): p. 1094-102.

9. Hendriksz, C.J., et al., *Efficacy and safety of enzyme replacement therapy with BMN 110 (elosulfase alfa) for Morquio A syndrome (mucopolysaccharidosis IVA): a phase 3 randomised placebo-controlled study.* J Inherit Metab Dis, 2014. **37**(6): p. 979-90.

10. Hendriksz, C.J., et al., *Multi-domain impact of elosufase alfa in Morquio A syndrome in the pivotal phase III trial.* Mol Genet Metab, 2015. **114**(2): p. 178-85.

11. Connock, M., et al., *A systematic review of the clinical effectiveness and cost-effectiveness of enzyme replacement therapies for Fabry's disease and mucopolysaccharidosis type 1.* Health Technol Assess, 2006. **10**(20): p. iii-iv, ix-113.

12. Rohrbach, M. and J.T. Clarke, *Treatment of lysosomal storage disorders : progress with enzyme replacement therapy.* Drugs, 2007. **67**(18): p. 2697-716.

13. Schweighardt, B., et al., *Immunogenicity of Elosulfase Alfa, an Enzyme Replacement Therapy in Patients With Morquio A Syndrome: Results From MOR-004, a Phase III Trial.* Clin Ther, 2015. **37**(5): p. 1012-1021.e6.

14. Sawamoto, K., et al., *Mucopolysaccharidosis Type IVA: Clinical Features, Biochemistry, Diagnosis, Genetics, and Treatment*, in *Mucopolysaccharidoses Update (2 Volume Set)*, S. Tomatsu, et al., Editors. 2018, Nova Science Publishers, Inc.: Hauppauge, NY. p. 235-272.

15. Tomatsu, S., et al., *Impact of enzyme replacement therapy and hematopoietic stem cell transplantation in patients with Morquio A syndrome.* Drug Des Devel Ther, 2015. **9**: p. 1937-53.

16. Espejo-Mojica, A., et al., *Production of human recombinant alpha-N-acetylglucosaminidase enzymes in two Pichia pastoris strains.* Mol Genet Metab, 2013. **108**(2): p. S38-S39.

17. Jung, J.W., et al., *Production and characterization of recombinant human acid alpha-glucosidase in transgenic rice cell suspension culture.* J Biotechnol, 2016. **226**: p. 44-53.

18. Shirley, M., *Sebelipase alfa: first global approval.* Drugs, 2015. **75**(16): p. 1935-40.

19. Espejo-Mojica, A.J., et al., *Human recombinant lysosomal enzymes produced in microorganisms.* Molecular Genetics and Metabolism, 2015. **116**(1-2): p. 13-23.

20. Pimentel, N., et al., *Production and characterization of a human lysosomal recombinant iduronate-2-sulfatase produced in Pichia pastoris.* Biotechnol Appl Biochem, 2018. **65**(5): p. 655-664.

21. Boer, E., et al., *Yeast expression platforms.* Appl Microbiol Biotechnol, 2007. **77**(3): p. 513-23.

22. Mosquera, A., et al., *Characterization of a recombinant N-acetylgalactosamine-6-sulfate sulfatase produced in E. coli for enzyme replacement therapy of Morquio A disease.* Process Biochemistry, 2012. **47**(12): p. 2097-2102.

23. Laukens, B., C. De Visscher, and N. Callewaert, *Engineering yeast for producing human glycoproteins: where are we now?* Future Microbiol, 2015. **10**(1): p. 21-34.

24. Espejo-Mojica, A.J., et al., *Human lysosomal β-N-acetylglucosaminidases produced in Pichia pastoris GS115: a feasible source for enzyme replacement therapy.* Mol Genet Metab, 2014. **111**(2): p. S42.

25. Chen, Y., et al., *Expression and characterization of glycosylated and catalytically active recombinant human alpha-galactosidase A produced in Pichia pastoris.* Protein Expr Purif, 2000. **20**(3): p. 472-84.

26. Du, H., et al., *The role of mannosylated enzyme and the mannose receptor in enzyme replacement therapy.* Am J Hum Genet, 2005. **77**(6): p. 1061-74.

27. Du, H., et al., *Enzyme therapy for lysosomal acid lipase deficiency in the mouse.* Hum Mol Genet, 2001. **10**(16): p. 1639-48.

28. Rodriguez-Lopez, A., et al., *Recombinant human N-acetylgalactosamine-6-sulfate sulfatase (GALNS) produced in the methylotrophic yeast Pichia pastoris.* Sci Rep, 2016. **6**: p. 29329.

29. Stratton, J., V. Chiruvolu, and M. Meagher, *High Cell-Density Fermentation*, in *Pichia Protocols*, D.R. Higgins and J.M. Cregg, Editors. 1998, Humana Press: Totowa, NJ. p. 107-120.

30. Laroy, W., R. Contreras, and N. Callewaert, *Glycome mapping on DNA sequencing equipment.* Nat Protoc, 2006. **1**(1): p. 397-405.

31. Jacobs, P.P., et al., *Engineering complex-type N-glycosylation in Pichia pastoris using GlycoSwitch technology.* Nat Protoc, 2009. **4**(1): p. 58-70.

32. Tomatsu, S., et al., *Characterization and pharmacokinetic study of recombinant human N-acetylgalactosamine-6-sulfate sulfatase.* Mol Genet Metab, 2007. **91**(1): p. 69-78.

33. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis.* Nature Methods, 2012. **9**: p. 671.

34. Kubaski, F., et al., *Newborn screening for mucopolysaccharidoses: a pilot study of measurement of glycosaminoglycans by tandem mass spectrometry.* J Inherit Metab Dis, 2017. **40**(1): p. 151-158.

35. Kubaski, F., et al., *Glycosaminoglycans detection methods: Applications of mass spectrometry.* Mol Genet Metab, 2017. **120**(1-2): p. 67-77.

36. Teh, S.H., M.Y. Fong, and Z. Mohamed, *Expression and analysis of the glycosylation properties of recombinant human erythropoietin expressed in Pichia pastoris.* Genet Mol Biol, 2011. **34**(3): p. 464-70.

37. Tanner, W. and L. Lehle, *Protein glycosylation in yeast.* Biochim Biophys Acta, 1987. **906**(1): p. 81-99.

38. Lehle, L., S. Strahl, and W. Tanner, *Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases.* Angew Chem Int Ed Engl, 2006. **45**(41): p. 6802-18.

39. Lalonde, M.E. and Y. Durocher, *Therapeutic glycoprotein production in mammalian cells.* J Biotechnol, 2017. **251**: p. 128-140.

40. Croset, A., et al., *Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells.* J Biotechnol, 2012. **161**(3): p. 336-48.

41. Tiels, P., et al., *A bacterial glycosidase enables mannose-6-phosphate modification and improved cellular uptake of yeast-produced recombinant human lysosomal enzymes.* Nat Biotechnol, 2012. **30**(12): p. 1225-31.

42. Friedman, B., et al., *A comparison of the pharmacological properties of carbohydrate remodeled recombinant and placental-derived beta-glucocerebrosidase: implications for clinical efficacy in treatment of Gaucher disease.* Blood, 1999. **93**(9): p. 2807-16.

43. Ben Turkia, H., et al., *Velaglucerase alfa enzyme replacement therapy compared with imiglucerase in patients with Gaucher disease.* Am J Hematol, 2013. **88**(3): p. 179-84.

44. McVie-Wylie, A.J., et al., *Biochemical and pharmacological characterization of different recombinant acid alpha-glucosidase preparations evaluated for the treatment of Pompe disease.* Mol Genet Metab, 2008. **94**(4): p. 448-55.

45. Clerc, F., et al., *Human plasma protein N-glycosylation.* Glycoconj J, 2016. **33**(3): p. 309-43.

46. Tekoah, Y., et al., *Glycosylation and functionality of recombinant beta-glucocerebrosidase from various production systems.* Biosci Rep, 2013. **33**(5).

47. Liu, Y., et al., *Liver-directed neonatal gene therapy prevents cardiac, bone, ear, and eye disease in mucopolysaccharidosis I mice.* Mol Ther, 2005. **11**: p. 35-47.

48. Dahms, N.M., P. Lobel, and S. Kornfeld, *Mannose 6-phosphate receptors and lysosomal enzyme targeting.* J Biol Chem, 1989. **264**(21): p. 12115-8.

49. Zhao, Y., et al., *Lysosome sorting of beta-glucocerebrosidase by LIMP-2 is targeted by the mannose 6-phosphate receptor.* Nat Commun, 2014. **5**: p. 4321.

50. Shen, J.S., et al., *Mannose receptor-mediated delivery of moss-made alpha-galactosidase A efficiently corrects enzyme deficiency in Fabry mice.* J Inherit Metab Dis, 2016. **39**(2): p. 293-303.

51. Limkul, J., et al., *The production of human glucocerebrosidase in glyco-engineered Nicotiana benthamiana plants.* Plant Biotechnol J, 2016. **14**(8): p. 1682-94.

52. Hagymasi, K., et al., *[Investigation of redox homeostasis of liver in experimental and human studies].* Acta Pharm Hung, 2004. **74**(1): p. 51-63.

53. Coutinho, M.F., M.J. Prata, and S. Alves, *A shortcut to the lysosome: the mannose-6-phosphate-independent pathway.* Mol Genet Metab, 2012. **107**(3): p. 257-66.

54. Braulke, T. and J.S. Bonifacino, *Sorting of lysosomal proteins.* Biochim Biophys Acta, 2009. **1793**(4): p. 605-14.

55. Grubb, J.H., C. Vogler, and W.S. Sly, *New strategies for enzyme replacement therapy for lysosomal storage diseases.* Rejuvenation Res, 2010. **13**(2-3): p. 229-36.

56. Dittmer, F., et al., *Alternative mechanisms for trafficking of lysosomal enzymes in mannose 6-phosphate receptor-deficient mice are cell type-specific.* J Cell Sci, 1999. **112 (Pt 10)**: p. 1591-7.

57. von Figura, K. and A. Hasilik, *Lysosomal enzymes and their receptors.* Annu Rev Biochem, 1986. **55**: p. 167-93.

58. Fuller, M., J.J. Hopwood, and D.S. Anson, *Receptor mediated binding of two glycosylation forms of N-acetylgalactosamine-4-sulphatase.* Biochim Biophys Acta, 1998. **1406**(3): p. 283-90.

59. Dooley, K.C. and D.A. Applegarth, *Uptake of lysosomal enzymes by human fibroblasts: lack of uptake of fungal or plant glycosidases in comparison with a mammalian enzyme.* J Inherit Metab Dis, 1980. **3**(4): p. 145-7.

60. Puckett, Y., H. Mulinder, and A.M. Montaño, *Enzyme Replacement Therapy with Elosulfase alfa for Mucopolysaccharidosis IVA (Morquio A Syndrome): Milestones and Challenges.* Expert Opin Orphan Drugs, 2017. **5**(9): p. 741-752.

61. Richards, S.M., T.A. Olson, and J.M. McPherson, *Antibody response in patients with Gaucher disease after repeated infusion with macrophage-targeted glucocerebrosidase.* Blood, 1993. **82**(5): p. 1402-9.

62. Wang, J., et al., *Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment.* Nat Biotechnol, 2008. **26**(8): p. 901-8.

568 63. Vera, M., et al., *Immune response to intrathecal enzyme replacement therapy in*
569 *mucopolysaccharidosis I patients.* Pediatr Res, 2013. **74**(6): p. 712-20.

570 64. Tomatsu, S., et al., *Development of MPS IVA mouse (Galnstm(hC79S.mC76S)slu)*
571 *tolerant to human N-acetylgalactosamine-6-sulfate sulfatase.* Hum Mol Genet,
572 2005. **14**(22): p. 3321-35.

573 65. Du, H., et al., *Wolman disease/cholesteryl ester storage disease: efficacy of plant-*
574 *produced human lysosomal acid lipase in mice.* J Lipid Res, 2008. **49**(8): p. 1646-
575 57.

576 66. Ou, L., et al., *RTB lectin-mediated delivery of lysosomal α-l-iduronidase mitigates*
577 *disease manifestations systemically including the central nervous system.* Mol
578 Genet Metab, 2018. **123**(2): p. 105-111.

579 67. Kreer, C., et al., *N-glycosylation converts non-glycoproteins into mannose receptor*
580 *ligands and reveals antigen-specific T cell responses in vivo.* Oncotarget, 2017.
581 **8**(4): p. 6857-6872.

582 68. Melton, A.C., et al., *Antibodies that neutralize cellular uptake of elosulfase alfa are*
583 *not associated with reduced efficacy or pharmacodynamic effect in individuals with*
584 *Morquio A syndrome.* J Immunol Methods, 2017. **440**: p. 41-51.

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Figure legends

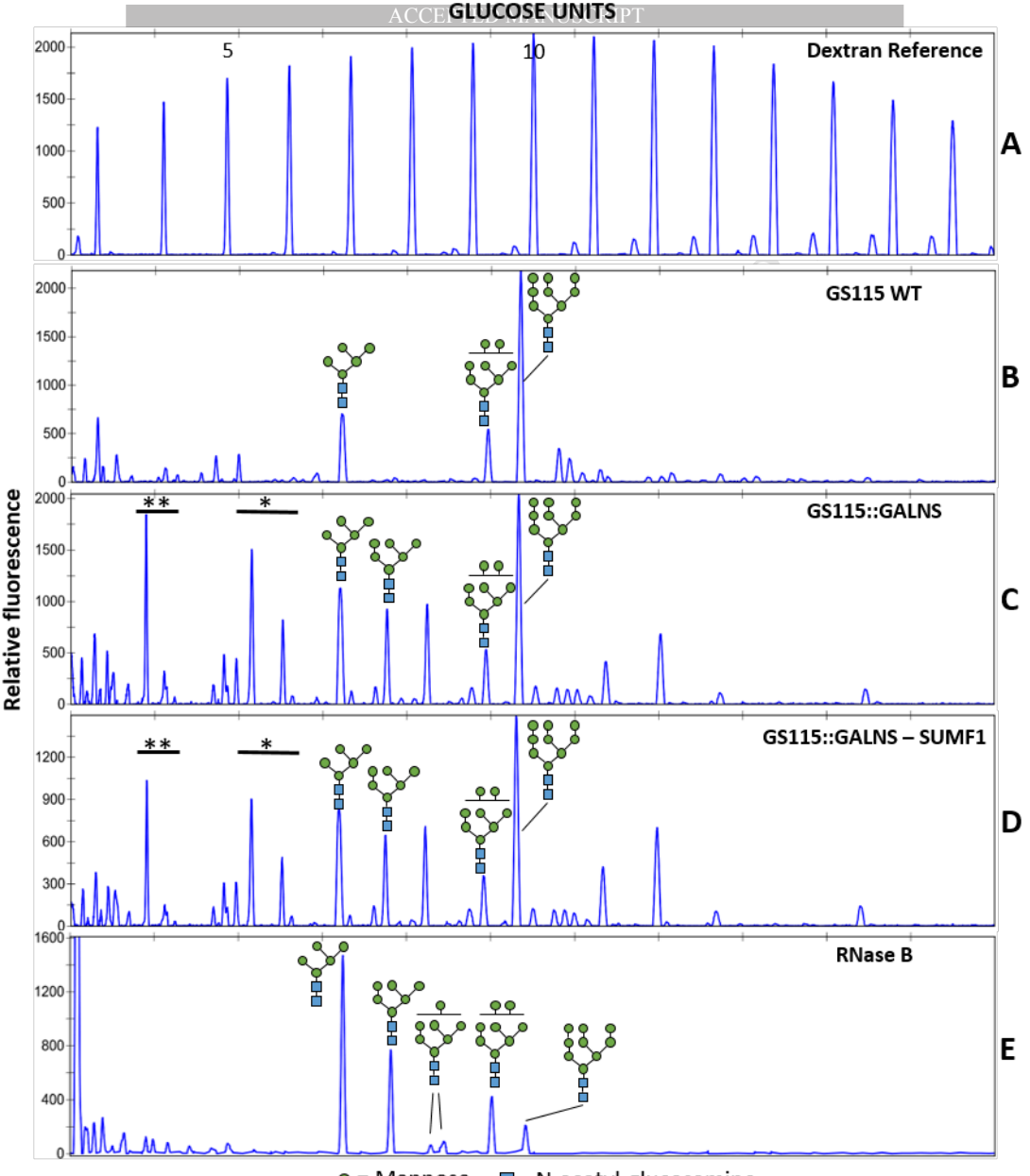
Figure 1. N-glycan profile of *P. pastoris* strains. A. Maltodextrin reference. B-D. N-glycan profile of the proteins present in growth medium of *P. pastoris* GS115 (B) *P. pastoris* GS115::GALNS (C) and *P. pastoris* GS115::GALNS-SUMF1 (D). * monomannosyl phosphorylated N-glycans; ** double-mannosyl phosphorylated N-glycans. E. Reference N-glycans from bovine RNase B.

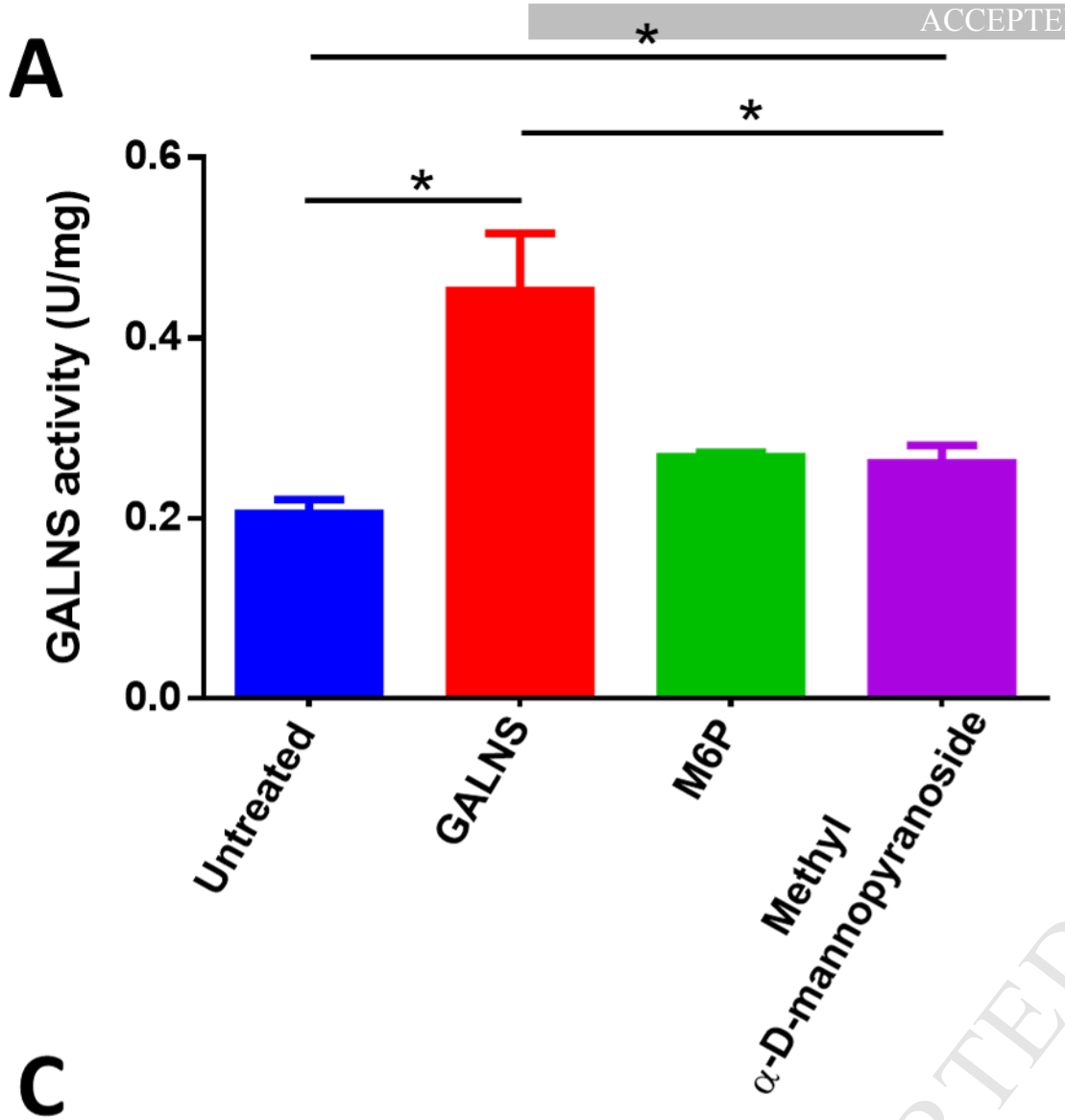
Figure 2. Internalization and trafficking of prGALNS in mammalian cells. A. Cellular uptake assayed in cultured HEK293 after treatment with 50 nM prGALNS with and without inhibitors. B. Cellular uptake assayed in MPS IVA patients fibroblasts after treatment with different concentrations of prGALNS. Assays were performed in by triplicate. * $p < 0.05$. C. Intracellular trafficking in HEK293 cells treated with prGALNS labeled with Alexa Fluor 568. Scale bar 50µm. 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.

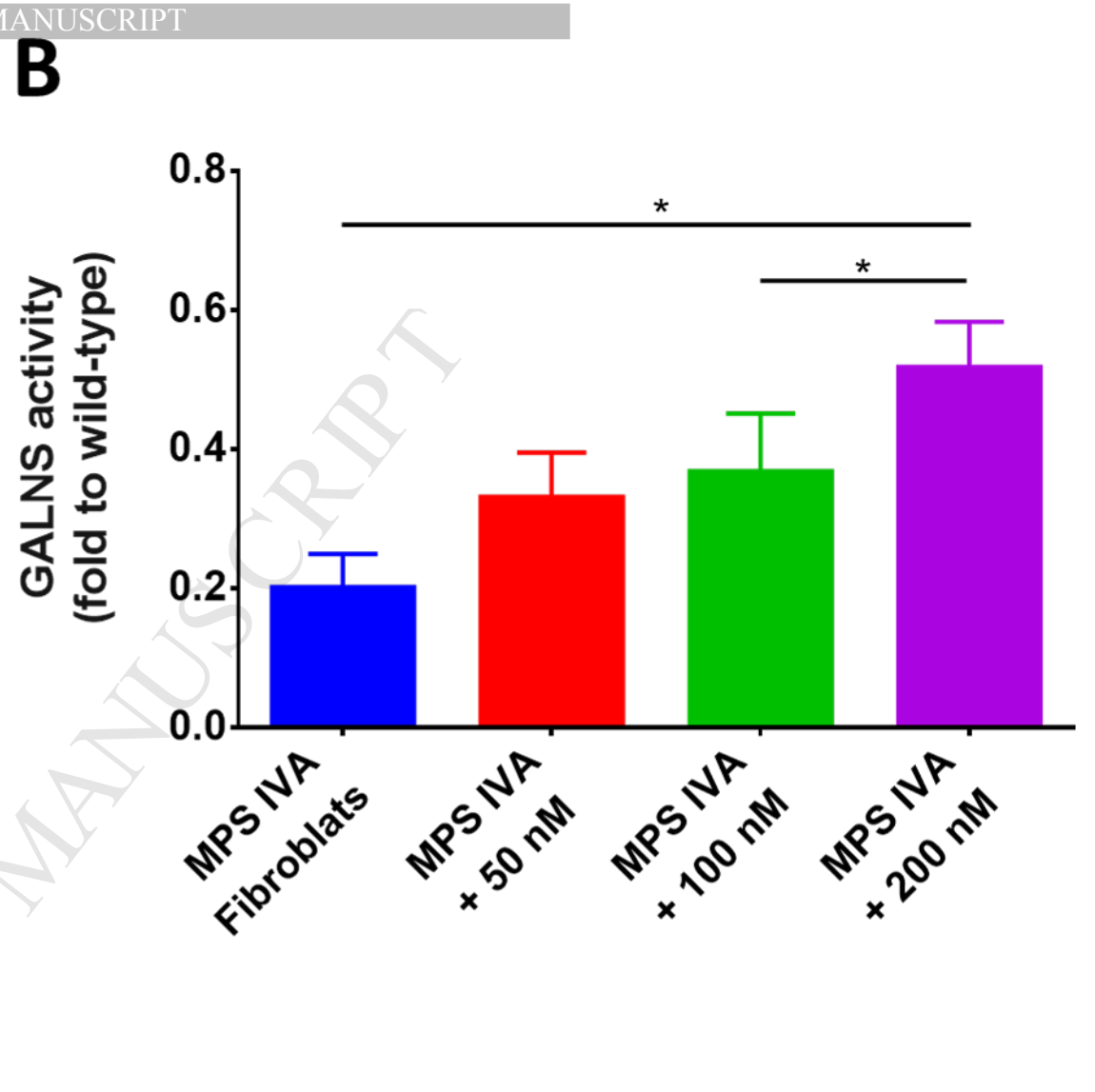
Figure 3. Quantitation of KS in MPS IVA fibroblasts treated with prGALNS. MPS IVA patient skin fibroblast were treated with 50, 100, and 200 nM of prGALNS and the KS levels were quantified by LC-MS/MS. Results are reported as fold to untreated MPS IVA fibroblast (blue). * $p < 0.05$.

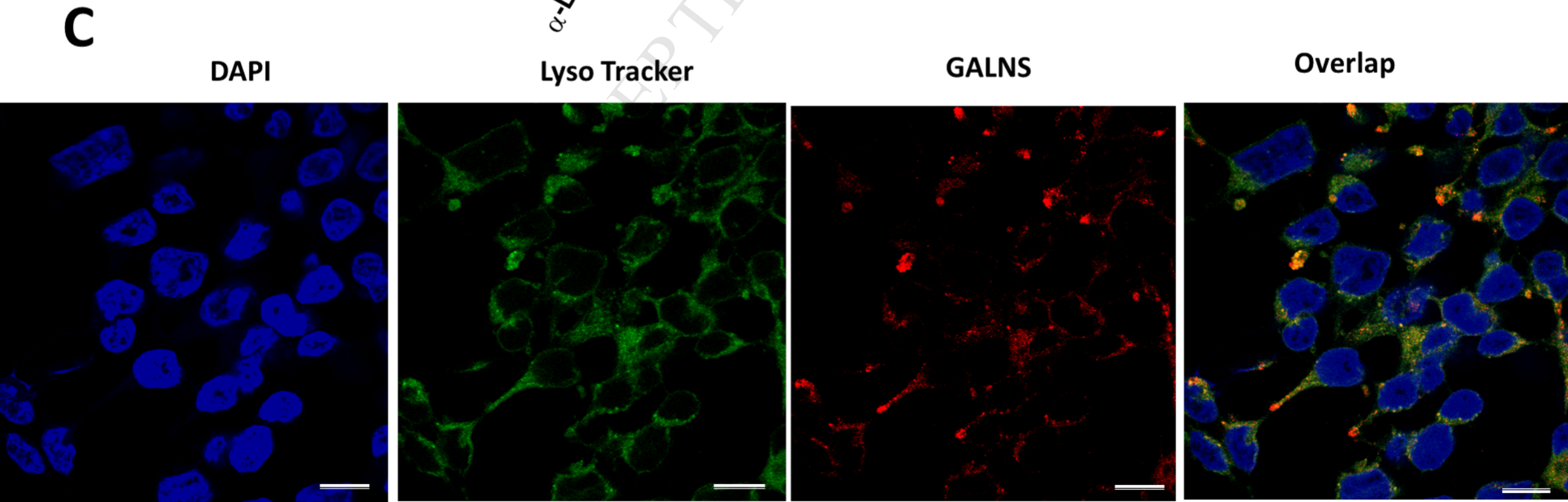
Figure 4. Biodistribution of prGALNS. Male C57BL/6 mice (7-8 weeks old) received a single intravenous administration of 1x PBS (control) or 5 mg kg^{-1} of prGALNS labeled with AlexaFluor 568 Histological sections of spleen, liver, heart and kidney were taken at 12 and 24 h post-treatment. Scale bar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

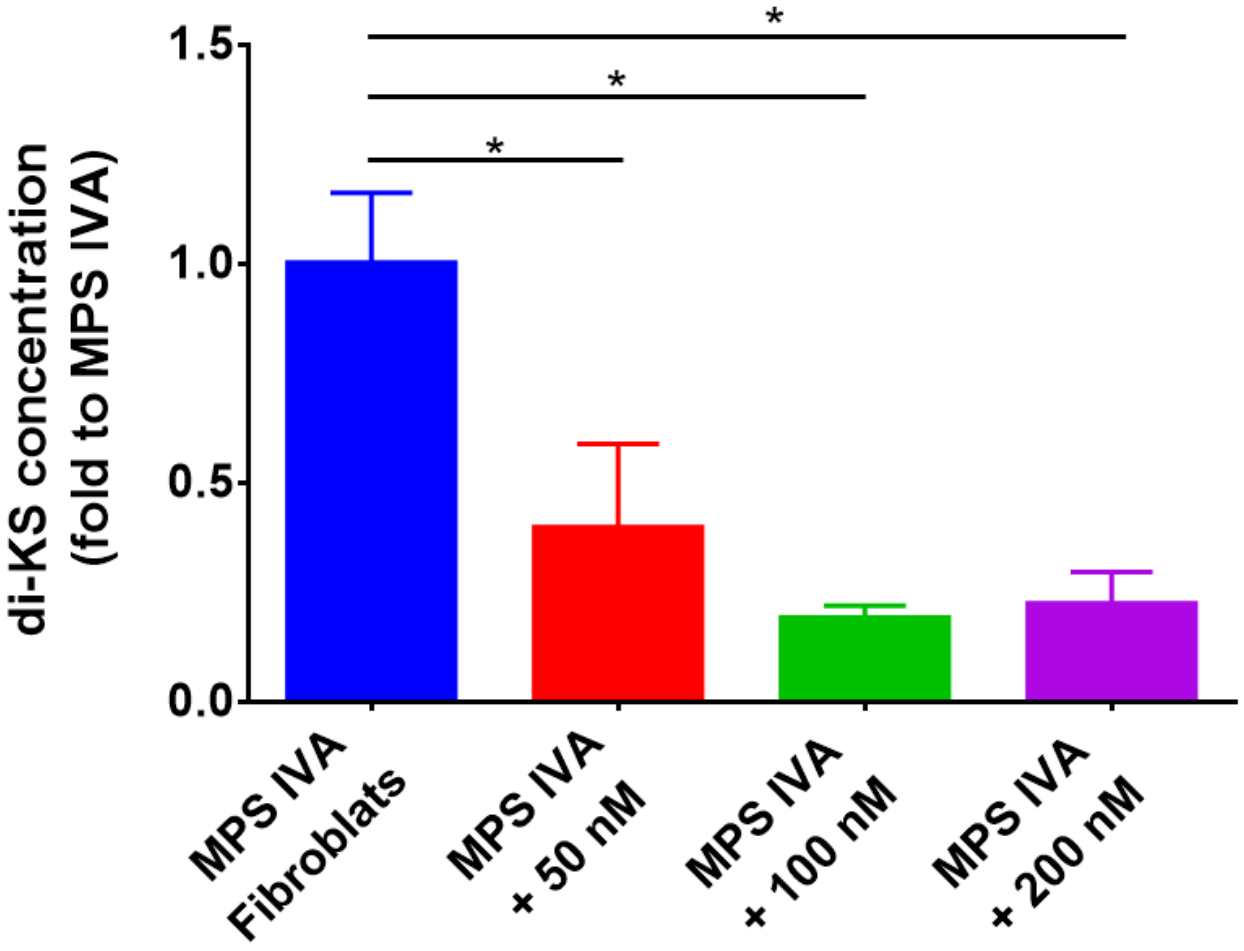
Figure 5. Generation of anti-prGALNS antibodies. Wild-type C57BL/6 mice were weekly infused 1x PBS or 5 mg kg^{-1} of prGALNS and the anti-prGALNS antibodies were assayed in serum samples after 15 and 30 days into the immunization regimen (n=5 per group).










di-KS concentration
(fold to MPS IVA)
1.5
1.0
0.5
0.0
*
*
*
MPS IVA
Fibroblats
MPS IVA
+ 50 nM
MPS IVA
+ 100 nM
MPS IVA
+ 200 nM