Received: 13 July 2020 | Revised: 28 September 2020 | Accepted: 8 October 2020

DOI: 10.1002/ajmg.c.31849

RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART C Seminars in Medical Genetics WILEY

Human recombinant lysosomal β-Hexosaminidases produced in *Pichia pastoris* efficiently reduced lipid accumulation in Tay-Sachs fibroblasts

Angela J. Espejo-Mojica[1] | Alexander Rodríguez-López[1] | Rong Li[2] | Wei Zheng[2] | Carlos J. Alméciga-Díaz[1] | Cindy Dulcey-Sepúlveda[3] | Germán Combariza[3] | Luis A. Barrera[1]

[1]Institute for the Study of Inborn Errors of Metabolism, Faculty of Science, Pontificia Universidad Javeriana, Bogotá, Colombia

[2]National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, Maryland

[3]Department of Mathematics, Faculty of Science, Pontificia Universidad Javeriana, Bogotá, Colombia

Correspondence
Angela J. Espejo-Mojica, Institute for the Study of Inborn Errors of Metabolism, Pontificia Universidad Javeriana, Cra 7 No. 43-82 Building 54, Room 305A. Bogotá, Colombia.
Email: aespejo@javeriana.edu.co

Funding information
Ministerio de Ciencia e Innovación, Grant/Award Numbers: 120352128575, 5174 - contract 120356933205, 5170 - contract 120356933427; Pontificia Universidad Javeriana, Grant/Award Numbers: 3637, 7204, 7520, 8275

Abstract

GM2 gangliosidosis, Tay-Sachs and Sandhoff diseases, are lysosomal storage disorders characterized by the lysosomal accumulation of GM2 gangliosides. This accumulation is due to deficiency in the activity of the β-hexosaminidases Hex-A or Hex-B, which are dimeric hydrolases formed by αβ or ββ subunits, respectively. These disorders show similar clinical manifestations that range from mild systemic symptoms to neurological damage and premature death. There is still no effective therapy for GM2 gangliosidoses, but some therapeutic alternatives, as enzyme replacement therapy, have being evaluated. Previously, we reported the production of active human recombinant β-hexosaminidases (rhHex-A and rhHex-B) in the methylotrophic yeast *Pichia pastoris*. In this study, we evaluated in vitro the cellular uptake, intracellular delivery to lysosome, and reduction of stored substrates. Both enzymes were taken-up via endocytic pathway mediated by mannose and mannose-6-phosphate receptors and delivered to lysosomes. Noteworthy, rhHex-A diminished the levels of stored lipids and lysosome mass in fibroblasts from Tay-Sachs patients. Overall, these results confirm the potential of *P. pastoris* as host to produce recombinant β-hexosaminidases intended to be used in the treatment of GM2 gangliosidosis.

KEYWORDS

enzyme replacement therapy, GM2 gangliosidosis, *Pichia pastoris*, recombinant hexosaminidases

1 | INTRODUCTION

Tay-Sachs (TSD, OMIM #272800) and Sandhoff (SD, OMIM #268800) diseases are two lysosomal storage disorders (LSDs) classified into the GM2 gangliosidoses group (Gravel, Kaback, Proia, Sandhoff, & Suzuki, 2001; Leal et al., 2020). TSD and SD are caused by mutations in *HEXA* or *HEXB* genes, respectively, which encoded for α or β subunits of the lysosomal β-hexosaminidases (Hex, EC 3.2.1.52) (Breiden & Sandhoff, 2019). Hexosaminidase A (Hex-A) and hexosaminidase B (Hex-B) are lysosomal enzymes formed as heterodimer (αβ subunits) or homodimer (ββ subunits), respectively. These enzymes are involved in the hydrolysis of N-acetylglucosamine or N-acetylgalactosamine residues of glycosphingolipids (Leal, Benincore-Florez, et al., 2020). TSD patients show decreased Hex-A activity levels while Hex-B remains normal. On the other hand, SD patients have decreased activity levels of both β-hexosaminidases. Clinical manifestations in TSD and SD are indistinguishable and include apathy, hyperacusia, motor weakness, hypotonia, poor head control, decreasing attentiveness, macular cherry-red spot in the retina, seizures, and progressive mental deterioration followed by blindness,

deafness, and spasticity. However, in SD the central nervous system (CNS) and visceral organs are affected, whereas in TSD only the CNS is impaired. Patients' lifespan varies from 1 to 5 years in the severe infantile form, to late teens for patients with the juvenile form; while it is still unclear if there is a reduction in the lifespan for the late-onset patients with the disease (Cachon-Gonzalez, Zaccariotto, & Cox, 2018; Gravel et al., 2001; Tropak & Mahuran, 2010).

Currently, the treatment of GM2 gangliosidoses is mainly focused on palliative interventions, and in the delay of disease progression in the late-onset form. However, several therapy approaches for TSD or SD are under development including enzyme replacement therapy (ERT), bone marrow or neural progenitor cell transplantation, gene therapy, and substrate reduction therapy (Leal, Benincore-Florez, et al., 2020; Solovyeva et al., 2018). Nowadays, ERT is available for 11 LSDs treatment including Gaucher, Fabry, Pompe, neuronal ceroid lipofuscinoses type II, α-mannosidosis, acid lipase deficiency, and mucopolysaccharidoses type I, II, IVA, VI, and VII (Leal et al., 2020). These recombinant proteins have been mainly produced in mammalian cells (Garbade et al., 2020). However, new eukaryotic platforms, as yeasts, have been also explored as platforms for the production of recombinant lysosomal enzymes (Espejo-Mojica et al., 2015). Among the different yeasts used for the development of novel ERT; *Pichia pastoris* has shown a great potential due to its well know genetics and metabolism and the about 70 recombinant proteins approved or in process to being approved for several conditions ([Karbalaei, Rezaee, & Farsiani, 2020] and www.pichia.com). In this sense, we have worked for several years in the production and characterization of recombinant lysosomal enzymes by using the yeast *P. pastoris* (Echeverri et al., 2018; Espejo-Mojica et al., 2015; Puentes-Tellez et al., 2020), which include iduronate-2-sulfatase (IDS) (Córdoba-Ruiz et al., 2009; Landázuri et al., 2009; Pimentel et al., 2018), N-acetylgalactosamine-6-sulfate sulfatase (GALNS) (Rodriguez-Lopez et al., 2016; Rodriguez-Lopez et al., 2019) and β-hexosaminidases (Espejo-Mojica et al., 2016; Vu et al., 2018). Noteworthy, these enzymes have shown to be biologically active and have the ability to be internalized and delivered to lysosomes, as well as to reduce the accumulated substrates in different cellular models. In addition, a single intravenous infusion of recombinant GALNS into wild-type mice showed that this enzyme was delivered to several organs and the immunogenicity caused in mice was slightly lower than to those obtained in other studies with GALNS produced in Chinese hamster ovary CHO cells (Rodriguez-Lopez et al., 2019). Nevertheless, no ERT, based on a *P. pastoris*-produced enzyme, has been approved yet, which could be associated to the differences in the N-glycans structures between proteins produced in mammalian cells and yeasts, which could be solved through glycoengineering approaches (Laukens, De Visscher, & Callewaert, 2015). In addition, we have also observed that some recombinant lysosomal enzymes, specially sulfatases, may have a significant lower activity compared with the enzyme produced in mammalian cells (Echeverri et al., 2018). Nevertheless, other enzymes have shown enzyme activities similar or higher than those observed for the enzymes produced in mammalian cells (Echeverri et al., 2018; Espejo-Mojica et al., 2015).

In the case of GM2 gangliosidoses ERT, some of the recombinant enzymes have been modified to improve the cellular uptake and lysosome delivery (Leal, Benincore-Florez, et al., 2020). For instance, the use of a highly mannosylated recombinant Hex-A produced in the yeast *Ogataea minuta*, and treated with α-mannosidase to expose the mannose-6-phosphate (M6P) residues on the N-glycans, resulted in a GM2 storage reduction and an improvement in the motor function and lifespan in a SD mouse model (Akeboshi et al., 2007; Tsuji et al., 2011). Similarly, the use of a recombinant chimeric Hex-B produced in CHO cells allowed a reduction of GM2 ganglioside storage in SD patient cells, as well as in the parenchyma of SD mice intracerebroventricularly injected with the enzyme (Matsuoka et al., 2011). Current studies have combined the structural features of α-subunit active site and stable β-subunit interface into a single hybrid subunit (μ-subunit) forming a recombinant hybrid protein (Hex-M). This hybrid protein showed a high cellular uptake in TSD fibroblasts, which is important property for the development of an ERT. The therapeutic potential of this enzyme was showed through the reduction of brain ganglioside accumulation in TSD mouse models through administration of scAAV9 vectors containing *HEXM* gene (Tropak et al., 2016). Recently, it was proposed the use of a molecular Trojan horse through the fusing of Hex-A to either IgG-heavy or light chains (HIRMAb), which showed similar activity to that obtained by nonfused Hex-A (Boado, Lu, Hui, Lin, & Pardridge, 2019). Similarly, we reported the production and characterization of human recombinant Hex-A and Hex-B (rhHex-A and rhHex-B) in the yeast *P. pastoris* GS115 (Espejo-Mojica et al., 2016). These recombinant enzymes, without any modification on the N-glycans structure, showed between 25- and 50-fold higher enzyme activity than that observed in enzymes from normal human leukocytes, as well as a high stability at different acidic pHs and temperatures (Espejo-Mojica et al., 2016). Noteworthy, rhHex-A normalized the lipid storage in neural stem cells derived from induced pluripotent stem cells (iPSCs) from TSD patient fibroblasts (Vu et al., 2018). In this study, we continue the characterization of these enzymes showing that rhHex-A and rhHex-B are taken up through both mannose and M6P receptors and are delivered to lysosomes. rhHex-A demonstrated reduction of lysosomal mass and lipids storage in TSD patient fibroblasts.

2 | EXPERIMENTAL SECTION

2.1 | Editorial policies and ethical considerations

This study was approved by the Research and Ethics Committee from the Faculty of Science at Pontificia Universidad Javeriana, Bogotá D.C., Colombia.

2.2 | Recombinant β-hexosaminidases

Recombinant β-hexosaminidases (rhHex-A and rhHex-B) were produced in *Pichia pastoris* GS115 as previously reported (Espejo-Mojica

et al., 2016). Briefly, the cDNA of α and β subunits of human β-hexosaminidases were cloned into pPICK9K expression vector and transfected into the yeast *P. pastoris* GS115 (Invitrogen, Thermo Fisher Scientific, San Jose, CA). Production of both recombinant enzymes was carried out at 1.65 L scale using a modified FM22 saline medium under previously described conditions (Espejo-Mojica et al., 2016; Rodriguez-Lopez et al., 2016; Stratton, Chiruvolu, & Meagher, 1998). rhHex-A and rhHex-B were obtained from culture medium due an α-factor secretion signal present in vector and were purified by ion exchange chromatography (Espejo-Mojica et al., 2016).

2.3 | Enzymatic activity assays

β-hexosaminidase activity was assayed by using 4-methylumbelliferyl-β-D-acetyl-glucosaminide (MUG, Sigma-Aldrich, St. Louis, MO) or 4-methylumbelliferyl-β-D-acetyl-glucosaminide sulfate (MUGS, Calbiochem, San Diego, CA) substrates. One unit (U) was defined as the amount of enzyme hydrolyzing 1 nmol of substrate per hour. Specific hexosaminidase activity was expressed as U/mg of total protein determined by BCA assay (Shapira, Blitzer, Miller, & Africk, 1989).

2.4 | Cellular uptake of purified recombinant hexosaminidases

Cellular uptake assay was carried out as previously described (Mosquera et al., 2012; Pimentel et al., 2018; Rodriguez-Lopez et al., 2016). Briefly, 24 hr before the experiment, 1×10^5 cells/well of wild-type human skin fibroblasts or HEK293 (ATCC CRL1573) were cultured in 12-well plates with Dulbecco's modified medium (DMEM, Gibco, Carlsbad, CA), supplemented with fetal bovine serum 10%, penicillin 100 U/ml and streptomycin 100 U/ml, at 37°C in a CO_2 incubator. Two hours before the experiment, culture medium was replaced, and rhHex-A and rhHex-B were added to a final concentration of 10 or 50 nM. After 6 hours of incubation, culture medium was removed and stored at −20°C. Cells were washed with PBS 1x (composition per liter: 8 g NaCl, 0.2 g KCl, 1.44 g NaH_2PO_4, 0.24 g KH_2PO_4, pH 7.2) and were lysed by resuspension in sodium deoxycholate 1% (Sigma-Aldrich, St. Louis, MO) (Rodriguez-Lopez et al., 2016). To evaluate if the protein uptake was mediated by endocytosis, cellular uptake assay was carried out at 4 or 37°C with each recombinant protein (Rodriguez-Lopez et al., 2016). Enzyme activity was assayed in the culture medium and cell lysates. All assays were done in triplicate.

2.5 | Inhibition of mannose and mannose-6-phosphate receptors

To evaluate if rhHex-A and rhHex-B endocytosis process was mediated by membrane receptors, cellular uptake assays were carried out using 50 nM of recombinant proteins in presence or absence of 5 mM of mannose or mannose-6-phosphate (M6P) (Sigma-Aldrich, St. Louis, MO) (Pimentel et al., 2018; Rodriguez-Lopez et al., 2019). After 6 hr of incubation, the culture medium and cell lysate were treated as was described above. All assays were done in triplicate.

2.6 | Protein-Lysosome colocalization assay

To evaluate if the recombinant enzymes were targeted to lysosomes, rhHex-A and rhHex-B were labeled with AlexaFluor 568® following the manufacturer's protocol (Molecular Probes, Thermo Fisher Scientific, Eugene, OR). HEK293 cells were grown on coverslips treated with 0.01% (wt/vol) type II collagen (Sigma-Aldrich, St. Louis, MO) and placed onto 12-well plates. Cells were seeded at a density of 2×10^4 cells/well. Once the cells were confluent, the culture medium was exchanged for fresh medium 1 hr before adding the fluorescent-labeled enzymes to a final concentration of 50 nM (Pimentel et al., 2018; Rodriguez-Lopez et al., 2019). After 6 hr of incubation, the cells were stained with 375 nM LysoTracker® Green DND-26 following the manufacturer's protocol (Molecular Probes, Thermo Fisher Scientific, Eugene, OR). Cells were fixed using freshly prepared 4% paraformaldehyde in 1x PBS for 20 min at room temperature. Finally, the cellular nucleus was stained with 4′,6-Diamidino-2-phenylindole dichydrochloride (DAPI, Thermo Fisher Scientific, San Jose, CA). 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 and analyzed by using R software (R Core Team, 2018).

2.7 | Nile red and LysoTracker staining assays

Nile Red and LysoTracker assays were done as previously reported (Vu et al., 2018). Briefly, 24 hr before treatment, 4,000 cells/well of TSD fibroblasts (GM00221 and GM00515, Coriell Institute) were seeded in a 96-well black clear bottom plate (Greiner Bio-One, #655090, Monroe, NC). On the day of the experiment, fibroblasts were treated with 50 and 100 nM rhHex-A and cultured on complete DMEM media. TSD and wild-type human skin fibroblasts treated with PBS 1x were used as controls. Twenty four, 48, and 72 hr after treatment, the cells were treated with 50 nM LysoTracker Red DND-99 dye (Molecular Probes, Thermo Fisher Scientific, Eugene, OR) in complete DMEM media at 37°C for 1 hr or 1 μM Nile Red in complete DMEM media at 37°C for 10 min, followed by plate washing twice with DPBS. The plates were fixed and stained simultaneously in 3.2% paraformaldehyde solution with Hoechst dye at a 1:5,000 dilution for 30 min at room temperature. The plates were washed twice using DPBS and stored with 100 μl/well DPBS at 4°C until imaging. The images (9 images/well) were acquired using the IN-Cell Analyzer 2200

imaging system, using a ×20 objective lens (GE Healthcare Bio-Sciences, Pittsburgh, PA). The DAPI (excitation = 350 ± 50 nm, emission = 455 ± 50 nm), Texas Red (excitation = 545 ± 20 nm, emission = 593 ± 20 nm) and FITC (excitation = 490 ± 20 nm, emission = 525 ± 36 nm) filter sets were used to visualize Hoechst nuclear staining, LysoTracker Red DND-99, and Nile red staining, respectively. All assays were performed in triplicate.

2.8 | Statistical analysis

The results are shown as mean ± *SD* and were analyzed by a one-way analysis of variance (ANOVA), or by *t* test when appropriate. Differences between groups were considered significant when $p < .05$ on GraphPad PRISM 6.0.

3 | RESULTS AND DISCUSSION

3.1 | Cellular uptake

Conventional ERT for LSDs is based on the capacity of the recombinant lysosomal enzymes to be taken up and targeted to the lysosome, where they can catalyze the degradation of the stored substrates (Desnick & Schuchman, 2012). In this sense, we evaluated the ability of rhHex-A and rhHex-B to be internalized by HEK293 cells and human skin fibroblasts at 10 and 50 nM, as was previously reported for other recombinant enzymes produced in this host (Pimentel et al., 2018; Rodriguez-Lopez et al., 2016; Rodriguez-Lopez et al., 2019). Figure 1 shows that recombinant hexosaminidases were taken-up by HEK293 cells and fibroblasts, since the enzymatic activity in cell lysates was higher than that observed in nontreated cells. However, in HEK293 the highest increase in enzyme activity was observed with 10 nM, while a slight decrease on the enzyme activity was observed at 50 nM (Figure 1a,b). On the other hand, cellular uptake of the recombinant enzymes in fibroblasts was only observed with 50 nM (Figure 1c,d), while at a lower concentration (10 nM) no differences were observed compared to the control. These results of cellular uptake are in agreement with reports about other recombinant lysosomal enzymes produced in *P. pastoris* without any modification in the N-glycans structure, such as α-glucosidase (Chen et al., 2000), acid lipase (phLA) (Du et al., 2001; Du et al., 2005), GALNS (Rodriguez-Lopez et al., 2016; Rodriguez-Lopez et al., 2019) and IDS (Pimentel et al., 2018). The N-glycosylation pattern of recombinant acid lipase and GALNS produced in the same strain *P. pastoris* used in this study, showed that both capped- and uncapped-phosphorylated mannoses were present in the recombinant enzymes, which could suggest that presence of those uncapped-phosphorylated mannoses

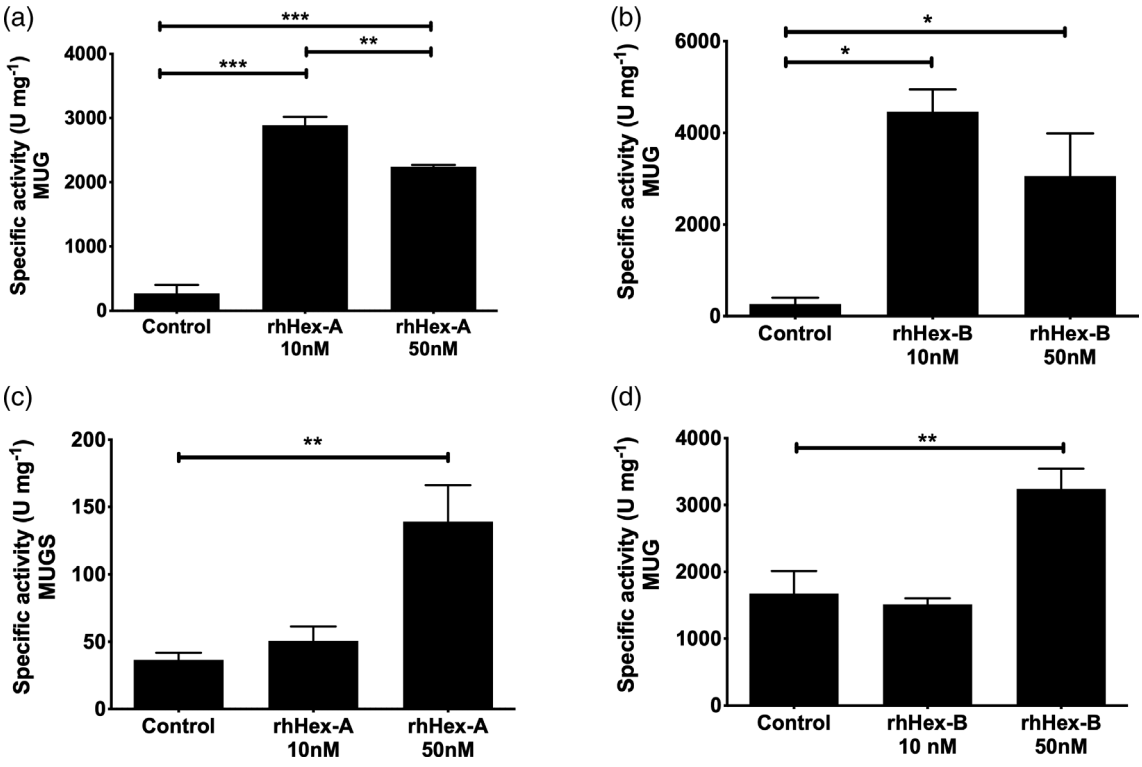


FIGURE 1 Cellular uptake of recombinant enzymes. The cellular uptake was assayed in HEK293 cells (a) rhHex-A and (b) rhHex-B and normal human skin fibroblasts (c) rhHex-A and (d) rhHex-B. The purified rhHex-A and rhHex-B were added to a final concentration of 10 and 50 nM. In all cases controls are cells without protein treatment. Results are expressed as mean ± *SD* Assays were done in triplicate (*$p < .05$, **$p < .01$, ***$p < .001$)

could be the cause of the internalization of these enzymes into the cells (Du et al., 2001; Du et al., 2005; Rodriguez-Lopez et al., 2019). The differences in cellular uptake between both HEK293 cells and human skin fibroblasts could be related to variation in the expression of receptors. In this sense, the type and number of receptors present on HEK293 cell surface could be different from those found in fibroblasts (Chen, Almo, & Wu, 2017; Sly, Kaplan, Achord, Brot, & Bell, 1978). Thus, at 50 nM receptors saturation may have occurred in HEK293 cells, causing the slight decreasing in protein internalization. It is expected that rhHex-A and rhHex-B may be also internalized by relevant cells for GM2 gangliosidoses, such as neurons, since the treatment with rhHex-A of neural stem cells derived from TSD iPSCs led to the normalization of lipid storage (Vu et al., 2018), suggesting that the enzyme was successfully internalized.

On the other hand, these results contrast with those of rhHex-A produced in the yeast *O. minuta*, which required the treatment with α-mannosidase to expose the M6P residues and allows the cellular uptake by GM2 gangliosidoses patient-derived fibroblasts (Tsuji et al., 2011). A similar approach was necessary for human α-glucosidase produced in *Y. lipolytica* (Tiels et al., 2012) and α-galactosidase A produced in *Saccharomyces cerevisiae* (Chiba et al., 2002) to allow the cellular uptake of the recombinant protein both in in-vitro and in-vivo assays.

3.2 | Endocytic pathway evaluation

It is well-known that cellular internalization processes occurs at physiological temperatures, and low temperatures can affect or even inhibit the endocytic pathway (Jiao et al., 2009). In order to confirm that cellular uptake of rhHex-A and rhHex-B was mediated by an endocytic pathway; we evaluated the cellular uptake of these proteins at 4 and 37°C. As is shown in Figure 2, the ratio of β-hexosaminidase activity between the intracellular (lysate) and extracellular (culture medium) fractions were higher at 37 than at 4°C, suggesting that the cellular capture of these enzymes is mediated by an endocytic pathway (Jiao et al., 2009; Rodriguez-Lopez et al., 2016).

Lysosomal enzymes undergo several posttranslational modifications during their trafficking from endoplasmic reticulum to the lysosome or extracellular compartments (Kornfeld, 1987). One of the main modifications is the addition of M6P-ended N-glycosylations in Golgi apparatus, which allows the delivery of the enzyme to the endosome/lysosome system through the interaction with M6P-receptors (M6PR) in lysosome or cell membrane (Braulke & Bonifacino, 2009). It is well-known that the percentage of oligosaccharides added to lysosomal enzymes depends on the source and subcellular location of the enzyme (von Figura & Hasilik, 1986). In this sense, some oligomannose residues remain uncovered and exposed to be recognized by other receptors like mannose

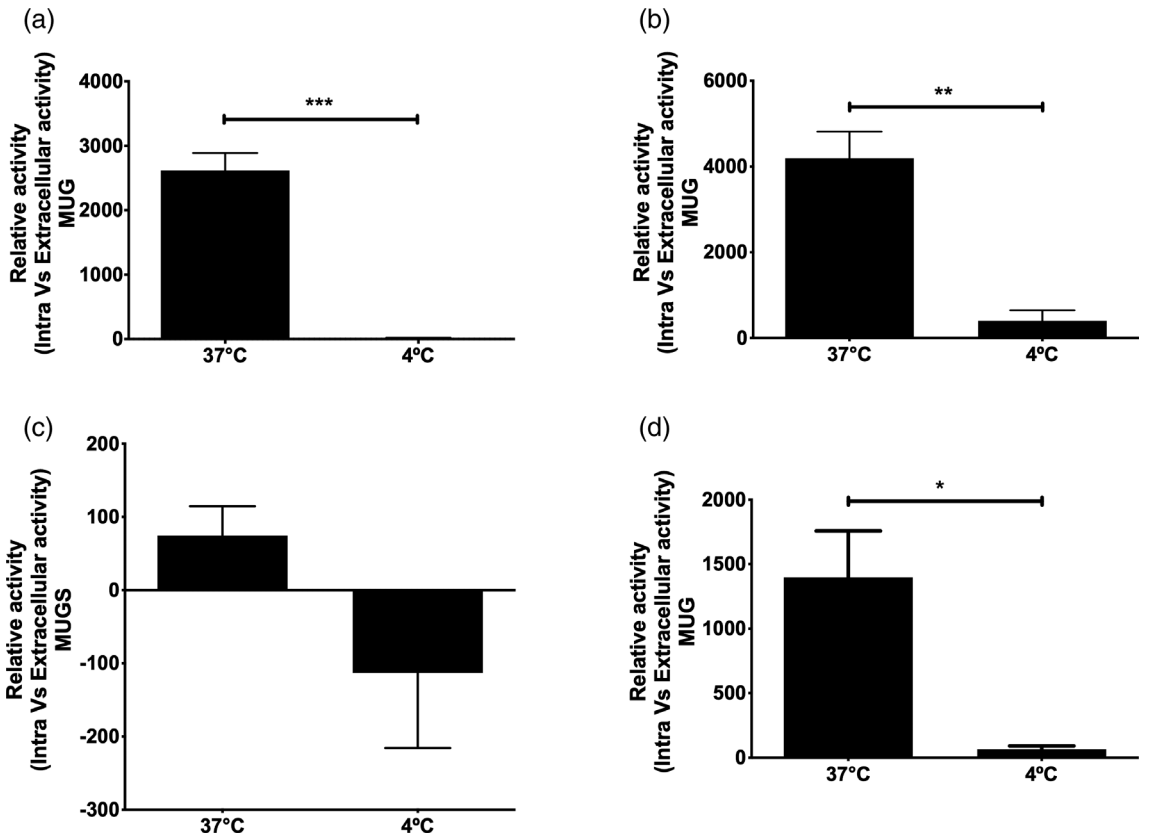


FIGURE 2 Endocytic pathway evaluation—Temperature evaluation. Cellular uptake evaluation was carried out at 4 or 37°C in HEK293 cells (a) rhHex-A and (b) rhHex-B and normal human skin fibroblasts (c) rhHex-A and (d) rhHex-B. Results are reported as the relation of intracellular vs. extracellular activity. Assays were done in triplicate $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

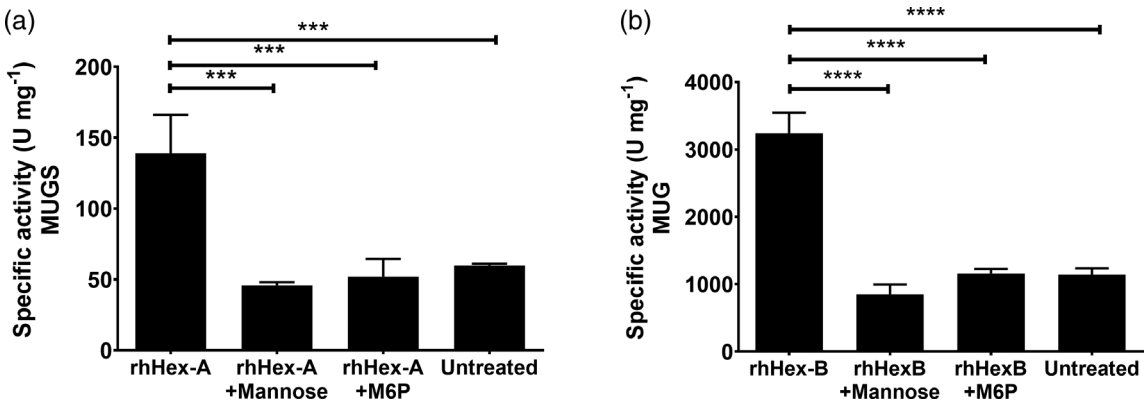


FIGURE 3 Endocytic pathway evaluation—Receptor inhibition. Cellular uptake assayed in normal human skin fibroblasts after treatment with 50 nM rhHex-A (a) or 50 nM rhHex-B (b), with and without addition of mannose or mannose-6-phosphate (M6P). Results are expressed as mean ± *SD*

receptors (MR), which would favor the internalization of lysosomal enzymes (Martinez-Pomares, 2012). To evaluate the receptors used by rhHex-A and rhHex-B during internalization, we added the recombinant enzymes to cells in the presence or absence of mannose and M6P. Results showed that the cellular uptake of both rhHex-A and rhHex-B was inhibited in the presence of both mannose and M6P, suggesting that MR and M6PR mediate the uptake of these enzymes in wild-type fibroblasts (Figure 3). These results agreed with those reported for rhHex-A and rhHex-B produced in CHO cells, as well as for other recombinant lysosomal proteins produced in *P. pastoris*, such as IDS and GALNS (Pimentel et al., 2018; Rodriguez-Lopez et al., 2016; Rodriguez-Lopez et al., 2019), and even hexosaminidase produced in other yeasts, which showed an internalization via cation M6P independent manner (Akeboshi et al., 2007; Matsuoka et al., 2011; Ohsawa et al., 2005; Tsuji et al., 2011). The cellular uptake of rhHex-A and rhHex-B through both MR and M6PR could be associated to the presence of N-glycans with high-mannose chains and mannosyl phosphorylated derivatives thereof, as was described for the recombinant GALNS produced in *P. pastoris* under the same conditions used in the present study (Rodriguez-Lopez et al., 2019). Although the cellular uptake was demonstrated in HEK293 cells, the internalization pathway could not be demonstrated through the inhibition of MR and M6PR receptors, since the results showed no significant differences between treatments and controls (data not shown).

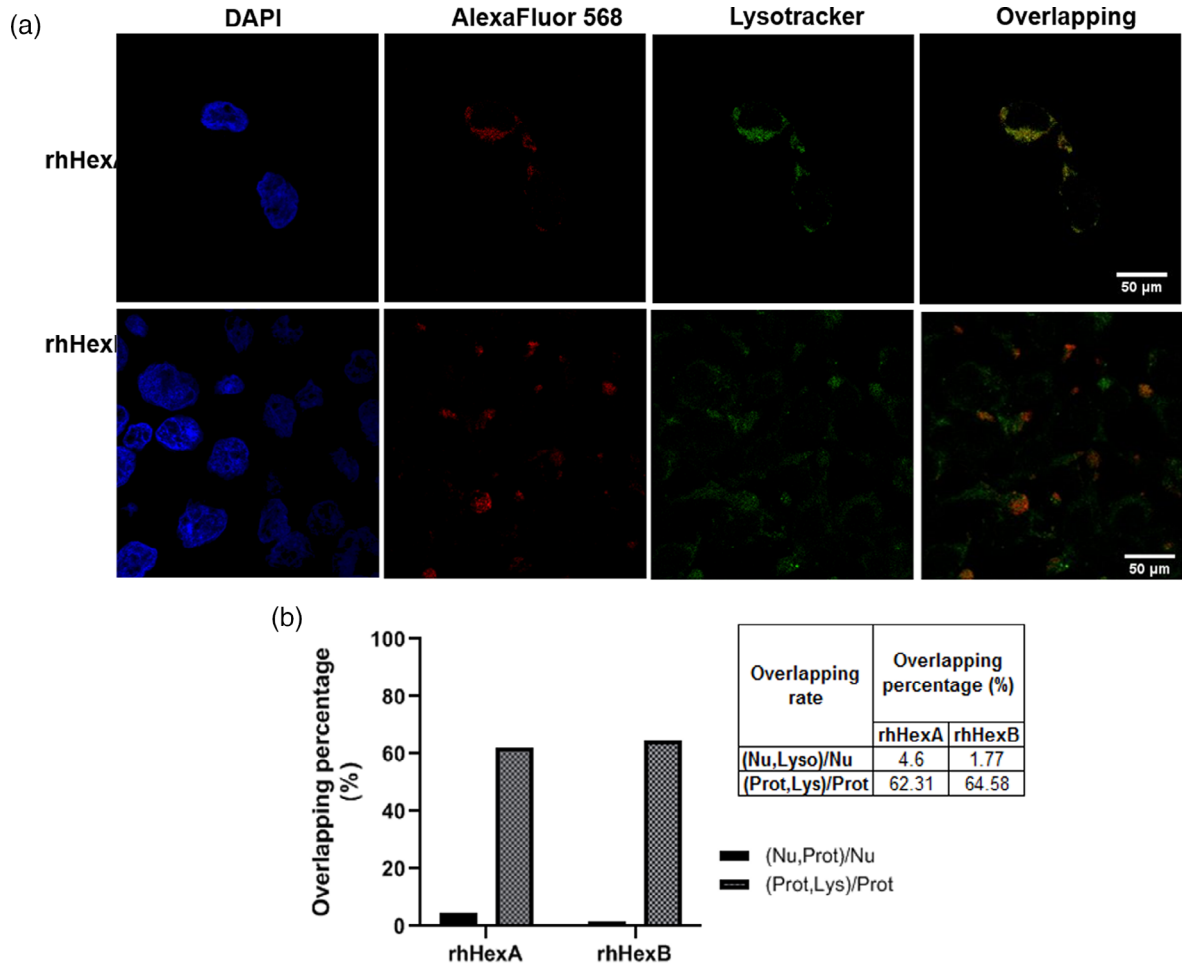
These results may be related to recent findings about the identification of other receptors, as clathrin or caveolae, involved in the internalization of α-galactosidase in HEK293, HUVEC, primary fibroblasts, and UKEC cells (Ivanova et al., 2020), and the fact that apparently M6PR gene is low-expressed in HEK293 cell line. In this sense, other receptors or mechanisms could be involved in the cellular uptake of rhHex. However, these mechanisms have not been well described and remain poorly understood.

3.3 | Intracellular delivery of recombinant enzymes to lysosome

The intracellular location of rhHex-A and rhHex-B was carried out by treating the cells with fluorescent labeled recombinant enzymes. As is showed in Figure 4 the labeled proteins were observed inside the cell and co-localized with the LysoTracker staining, a phenotypic assay that detects acidic organelles (Lloyd-Evans et al., 2008; Xu et al., 2014). In this sense, these results confirm the internalization of the recombinant enzymes and their targeting to lysosomes (Figure 4a). Analyzed images showed that ~62.3 and 64.58% of rhHexA and rhHexB, respectively, overlapped with LysoTracker staining (acidic compartments / lysosomes) (Figure 4b). In addition, it was observed that a fraction of the internalized enzymes appears to be localized in the cytoplasm or possibly in earlier endosomal compartments. Similar results were reported for the recombinant GALNS (Rodriguez-Lopez et al., 2019) and IDS (Pimentel et al., 2018) produced in *P. pastoris*, as well as α-glucosidase produced in *Y. lypolitica* (Tiels et al., 2012), which support the use of *P. pastoris* in the development of an ERT for GM2 gangliosidoses. These results also agree with those of rhHex-A-treated neural stem cells (Vu et al., 2018), since the normalization of the stored substrates (GM2 gangliosides) in those cells suggest that the enzyme was efficiently delivered to the lysosomes.

3.4 | Ganglioside and lysosomal mass reduction

Once confirmed the internalization and cellular location of recombinant enzymes, we evaluated the effect of rhHex-A on the phenotype of TSD human skin-fibroblasts. First, the effect of rhHex-A on the lysosomal mass was evaluated by using the LysoTracker Red staining. This fluorescent dye accumulates in acidic organelles and fluoresces at low pH environment. Since an increase in LysoTracker staining, compared to normal cells, has been observed in LSDs patients cells accumulating different substrates (e.g., GAGs, lipids, or oligosaccharides), (Lloyd-Evans et al., 2008; Xu et al., 2014); it has been proposed its use in drug screening for LSDs (Xu et al., 2014). As observed in Figure 5a, both GM00221 and GM00515 TSD fibroblasts showed a 1.5-fold increase in the LysoTracker fluorescent intensity compared with the wild-type fibroblasts, consistent with previous findings in TSD fibroblasts (Xu et al., 2014) and neural stem cells (Vu et al., 2018).



Overlapping rate	Overlapping percentage (%)	
	rhHexA	rhHexB
(Nu,Lyso)/Nu	4.6	1.77
(Prot,Lys)/Prot	62.31	64.58

FIGURE 4 Intracellular delivery of recombinant hexosaminidases to lysosome. (a). Fluorescent labeled-enzymes were added to HEK293 cells and colocalization with fluorescent labeled-target organelles were visualized by confocal microscopy. Upper panel: rhHex-A. Down-panel: rhHex-B. Scale bar 50 µm. DAPI (nuclei); AlexaFluor 568 (recombinant enzymes); Lysotracker (acidic organelles/lysosomes); Overlapping (red/green signal overlapping). (b) Images were analyzed by R software. Each image was normalized adjusting the threshold at 15% for DAPI and 5% for both AlexaFluor 568 and Lysotracker. Percentage of overlapping was established through the pixels counting by one filter over other using the R software. Nu, nuclei; Prot, protein; Lyso, lysosome

After 72 hr of rhHex-A treatment, a reduction in the fluorescent intensity was observed for both TSD fibroblasts and leading to normalization of the intensity in GM00515 fibroblasts. We did not observe significant difference between 50 and 100 nM rhHex-A. In this sense, these results show that the TSD fibroblasts have an increased lysosomal mass that can be reduced, and even normalized, after treatment with rhHex-A.

Since GM2 ganglioside buildup promotes the accumulation of others lipids such as phospholipids, cerebrosides, sphingomyelin, and cholesterol (Leal, Benincore-Florez, et al., 2020), the staining of these lipids could be used as an assay for drug development. In this sense, it was reported that Nile Red staining offers a valid method for evaluating drug efficacy and compound screening in TSD patient neural stem cells (Vu et al., 2018). As observed in Figure 5b, the Nile Red staining intensity increased 1.7- and 2.1-fold in TSD fibroblasts compared with wild-type fibroblasts, consistent with previous findings in TSD neural stem cells (Vu et al., 2018). After treatment with rhHex-A a reduction in the fluorescent intensity was observed for both TSD fibroblasts, even during the first 24 hr posttreatment. Noteworthy, wild-type levels of fluorescent intensity were observed for both TSD fibroblasts after 72 hr treatment with 100 nM rhHex-A. Overall, these results show that rhHex-A allows the normalization of lipids storage in TSD fibroblasts with a subsequent reduction in the lysosomal mass. These results agree with those obtained after the treatment of TSD neural stem cells with the same rhHex-A, produced in *P. pastoris*, using neural stem cells derived from TSD iPSC (Vu et al., 2018). Nevertheless, normalization of lipids storage in the neural stem cells was observed after only 4 hr treatment with 100 nM rhHex-A. In this sense, these results confirm the fact that there are differences in recombinant enzyme uptake depending on the cellular model, and suggest that neurons may be easy to treat with rhHexA. All together, these results show that rhHex-A produced in *P. pastoris* may catalyze the reduction

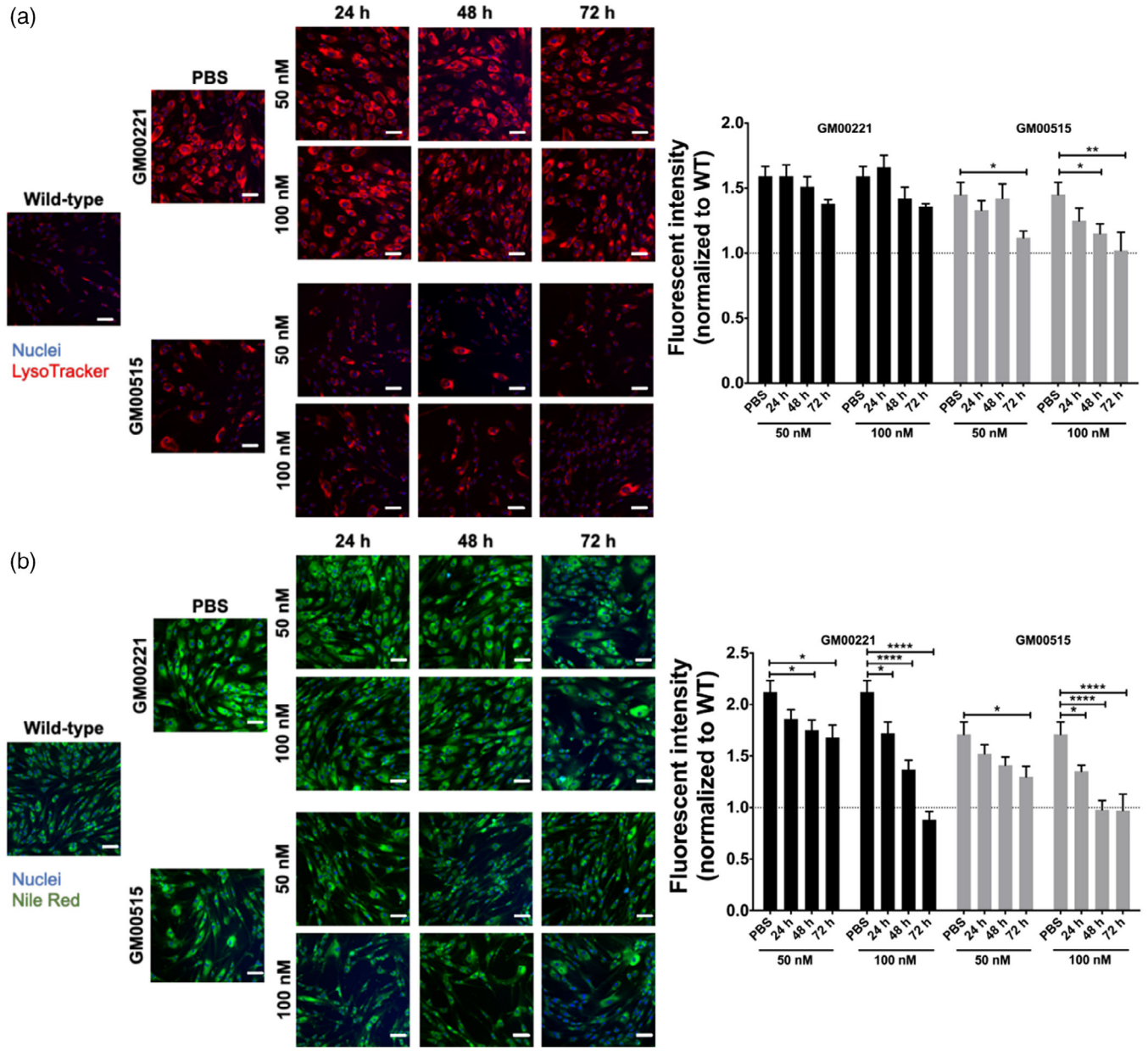


FIGURE 5 Effect of rhHex-A on reduction of lysosomal mass and lipid accumulation in TSD fibroblasts. TSD fibroblasts (GM00221 and GM00515) were treated with rhHex-A (50 and 100 nM). Cells were either stained with LysoTracker Red DND-99 dye (a) or Nile Red (b). The images (9 images/well) were acquired using the IN-Cell Analyzer 2200 imaging system (left). Results, on right figure, are presented as cell intensity normalized to WT fluorescence level (dotted lines). All assays were performed in triplicate (ANOVA Sidak *t* test $^{*}p < .05$, $^{**}p < .01$, $^{****}p < .0001$). Scale bar: 100 μm

accumulated GM2 ganglioside in different cellular models, which confirm the potential of this enzyme toward the development of an ERT for TSD.

Gangliosides reduction in patient fibroblasts has been also observed by other recombinant hexosaminidases, although using different assays. For instance, Akeboshi et al., 2007, demonstrated that recombinant M6P-HexA produced in *O. minuta* diminished GM2 gangliosides in TSD fibroblasts using up to 1,800 nmol/h/well of recombinant M6P-HexA (Akeboshi et al., 2007). This enzyme was then improved by increasing level of a high M6P-type-N-glycan content (Om4HexA), producing and improvement in some motor functions and an increase in the survival rate in a SD mouse model after intracerebroventricularly administration (Tsuji et al., 2011). Similarly, Matsuoka et al., 2011, used a chimeric Hex-B to treat TSD patients fibroblasts, showing a reduction of GM2 gangliosides (Matsuoka et al., 2011). A recent report using this chimeric Hex-B showed that stored GM2 was only significant reduced in TSD fibroblasts when the protein was co-administered with a recombinant GM2 ganglioside activator (GM2A) (Kitakaze et al., 2016).

4 | CONCLUSIONS

We have extended the characterization of rhHex-A and rhHex-B produced in *P. pastoris*, showing that uncapping of the N-glycosylations ends was not necessary to facilitate the cellular uptake, and that these enzymes are highly active without the need of GM2A co-administration, as reported for other recombinant β-hexosamindases. Thus, rhHex-A and rhHex-B may have an advantage over other recombinant β-hexosamindases produced in different host, such as the yeast *O. minuta*, for which further modification on N-glycans are necessary. The results showed that the cellular uptake of rhHex-A and rhHex-B is mediated by MR and M6PR, which allows the delivery of those enzymes to the lysosome and the reduction of the stored lipids and lysosomal mass. Overall, these results continue offering valuable information toward the importance of *P. pastoris* as host to produce recombinant β-hexosaminidases for the design of an ERT for GM2 gangliosidoses. Further studies should be focused on the in vivo evaluation of these recombinant enzymes on GM2 gangliosides levels, as well as the effect on the disease phenotype and the delivery of the enzyme to the CNS.

ACKNOWLEDGMENTS

A. E. M. and A. R. L. received a doctoral scholarship from the Ministry of Science, Technology and Innovation and the Pontificia Universidad Javeriana, respectively. C. J. A. D. received a scholarship from Fulbright Colombia. A. E. M. and L. A. B. were supported by Pontificia Universidad Javeriana (Grant ID 3637) and the Ministry of Science, Technology and Innovation (Grant contract No. 120352128575). A. E. M. was supported by Pontificia Universidad Javeriana (Grant ID 8275). C. J. A. D. was supported by Pontificia Universidad Javeriana (Grant ID 7204 and 7520) and the Ministry of Science, Technology and Innovation [Grant ID 5174 - contract No. 120356933205, and Grant ID 5170 - contract No. 120356933427]. RL and WZ were supported by the Intramural Research Program of the Therapeutics for Rare and Neglected Diseases, National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD, USA.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Angela J. Espejo-Mojica, Alexander Rodríguez-López, Rong Li, and Carlos J. Alméciga-Díaz performed the experiments. Angela J. Espejo-Mojica, Rong Li, Wei Zheng, Carlos J. Alméciga-Díaz, and Luis A. Barrera conceived and designed the experiments. Cindy Dulcey-Sepúlveda and Germán Combariza analyzed the colocalization of the confocal images through R software algorithms. Angela J. Espejo-Mojica and Carlos J. Alméciga-Díaz wrote the paper. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Angela J. Espejo-Mojica https://orcid.org/0000-0001-9670-6435
Alexander Rodríguez-López https://orcid.org/0000-0001-5409-3466
Wei Zheng https://orcid.org/0000-0003-1034-0757
Carlos J. Alméciga-Díaz https://orcid.org/0000-0001-6484-1173
Germán Combariza https://orcid.org/0000-0002-1878-665X
Luis A. Barrera https://orcid.org/0000-0001-8287-0197

REFERENCES

Akeboshi, H., Chiba, Y., Kasahara, Y., Takashiba, M., Takaoka, Y., Ohsawa, M., … Jigami, Y. (2007). Production of recombinant beta-hexosaminidase A, a potential enzyme for replacement therapy for Tay-Sachs and Sandhoff diseases, in the methylotrophic yeast Ogataea minuta. *Applied and Environmental Microbiology*, *73*(15), 4805–4812. https://doi.org/10.1128/AEM.00463-07

Boado, R. J., Lu, J. Z., Hui, E. K., Lin, H., & Pardridge, W. M. (2019). Bifunctional IgG-lysosomal enzyme fusion proteins for brain drug delivery. *Scientific Reports*, *9*(1), 18632. https://doi.org/10.1038/s41598-019-55136-4

Braulke, T., & Bonifacino, J. S. (2009). Sorting of lysosomal proteins. *Biochimica et Biophysica Acta*, *1793*(4), 605–614. https://doi.org/10.1016/j.bbamcr.2008.10.016

Breiden, B., & Sandhoff, K. (2019). Lysosomal glycosphingolipid storage diseases. *Annual Review of Biochemistry*, *88*, 461–492. https://doi.org/10.1146/annurev-biochem-013118-111518

Cachon-Gonzalez, M. B., Zaccariotto, E., & Cox, T. M. (2018). Genetics and therapies for GM2 Gangliosidosis. *Current Gene Therapy*, *18*(2), 68–89. https://doi.org/10.2174/1566523218666180404162622

Chen, J., Almo, S. C., & Wu, Y. (2017). General principles of binding between cell surface receptors and multi-specific ligands: A computational study. *PLoS Computational Biology*, *13*(10), e1005805. https://doi.org/10.1371/journal.pcbi.1005805

Chen, Y., Jin, M., Egborge, T., Coppola, G., Andre, J., & Calhoun, D. H. (2000). Expression and characterization of glycosylated and catalytically active recombinant human alpha-galactosidase A produced in *Pichia pastoris*. *Protein Expression and Purification*, *20*(3), 472–484. https://doi.org/10.1006/prep.2000.1325

Chiba, Y., Sakuraba, H., Kotani, M., Kase, R., Kobayashi, K., Takeuchi, M., … Jigami, Y. (2002). Production in yeast of alpha-galactosidase A, a lysosomal enzyme applicable to enzyme replacement therapy for Fabry disease. *Glycobiology*, *12*(12), 821–828. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/12499404

Córdoba-Ruiz, H. A., Poutou-Piñales, R. A., Echeverri-Peña, O. Y., Algecira-Enciso, N. A., Landázuri, P., Sáenz, H., & Barrera-Avellaneda, L. A. (2009). Laboratory scale production of the human recombinant iduronate 2-sulfate sulfatase-like from Pichia pastoris. *African Journal of Biotechnology*, *8*(9), 1786–1792.

Core Team, R. (2018). R: A language and environment for statistical computing. In *R foundation for statistical computing*. Available from. https://www.R-project.org/

Desnick, R., & Schuchman, E. (2012). Enzyme replacement therapy for lysosomal diseases: Lessons from 20 years of experience and remaining challenges. *Annual Review of Genomics and Human Genetics*, *13*, 307–335.

Du, H., Levine, M., Ganesa, C., Witte, D. P., Cole, E. S., & Grabowski, G. A. (2005). The role of mannosylated enzyme and the mannose receptor in enzyme replacement therapy. *American Journal of Human Genetics*, *77*(6), 1061–1074. https://doi.org/10.1086/498652

Du, H., Schiavi, S., Levine, M., Mishra, J., Heur, M., & Grabowski, G. A. (2001). Enzyme therapy for lysosomal acid lipase deficiency in the

mouse. *Human Molecular Genetics*, *10*(16), 1639–1648. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/11487567

Echeverri, O. Y., Guevara, J. M., Espejo-Mojica, A. J., Ardila, A., Pulido, N., Reyes, M., … Barrera, L. A. (2018). Research, diagnosis and education in inborn errors of metabolism in Colombia: 20 years' experience from a reference center. *Orphanet Journal of Rare Diseases*, *13*(1), 141. https://doi.org/10.1186/s13023-018-0879-2

Espejo-Mojica, A. J., Almeciga-Diaz, C. J., Rodriguez, A., Mosquera, A., Diaz, D., Beltran, L., … Barrera, L. A. (2015). Human recombinant lysosomal enzymes produced in microorganisms. *Molecular Genetics and Metabolism*, *116*(1–2), 13–23. https://doi.org/10.1016/j.ymgme.2015.06.001

Espejo-Mojica, A. J., Mosquera, A., Rodrfguez-Lopez, A., Diaz, D., Beltran, L., Hernandez, F. L., … Barrera, L. A. (2016). Characterization of recombinant human lysosomal beta-hexosaminidases produced in the methylotrophic yeast Pichia pastoris. *Universitas Scientiarum*, *21*, 195–217. Retrieved from. http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0122-74832016000300195&nrm=iso

Garbade, S. F., Zielonka, M., Mechler, K., Kolker, S., Hoffmann, G. F., Staufner, C., … Ries, M. (2020). FDA orphan drug designations for lysosomal storage disorders - A cross-sectional analysis. *PLoS One*, *15*(4), e0230898. https://doi.org/10.1371/journal.pone.0230898

Gravel, R. A., Kaback, M. M., Proia, R. L., Sandhoff, K., & Suzuki, K. (2001). The GM2 gangliosidoses. In C. R. Scriver, A. L. Beaudet, W. S. Sly, & D. Valle (Eds.), *The metabolic and molecular bases of inherited diseases* (Vol. 8, pp. 3827–3877). New York: McGraw-Hill.

Ivanova, M. M., Dao, J., Kasaci, N., Adewale, B., Fikry, J., & Goker-Alpan, O. (2020). Rapid Clathrin-mediated uptake of recombinant α-gal-A to lysosome activates autophagy. *Biomolecules*, *10*(6), 837–857. https://doi.org/10.3390/biom10060837

Jiao, C.-Y., Delaroche, D., Burlina, F., Alves, I. D., Chassaing, G. r., & Sagan, S. (2009). Translocation and endocytosis for cell-penetrating peptide internalization. *The Journal of Biological Chemistry*, *284*, 33957–33965.

Karbalaei, M., Rezaee, S. A., & Farsiani, H. (2020). Pichia pastoris: A highly successful expression system for optimal synthesis of heterologous proteins. *Journal of Cellular Physiology*, *235*(9), 5867–5881. https://doi.org/10.1002/jcp.29583

Kitakaze, K., Tasaki, C., Tajima, Y., Hirokawa, T., Tsuji, D., Sakuraba, H., & Itoh, K. (2016). Combined replacement effects of human modified β-hexosaminidase B and GM2 activator protein on GM2 gangliosidoses fibroblasts. *Biochemistry and Biophysics Reports*, *7*, 157–163. https://doi.org/10.1016/j.bbrep.2016.04.012

Kornfeld, S. (1987). Trafficking of lysosomal enzymes. *The FASEB Journal*, *1*(6), 462–468. Retrieved from. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3315809

Landázuri, P., Poutou-Piñales, R. A., Acero-Godoy, J., Córdoba-Ruiz, H., Echeverri-Peña, O. Y., Sáenz, H., … Barrera-Avellaneda, L. A. (2009). Cloning and shake flask expression of hrIDS-like in Pichia pastoris. *African Journal of Biotechnology*, *8*(12), 2871–2877.

Laukens, B., De Visscher, C., & Callewaert, N. (2015). Engineering yeast for producing human glycoproteins: Where are we now? *Future Microbiology*, *10*(1), 21–34. https://doi.org/10.2217/fmb.14.104

Leal, A. F., Benincore-Florez, E., Solano-Galarza, D., Garzon, J. R. G., Echeverri-Pena, O. Y., Suarez, D. A., … Espejo-Mojica, A. J. (2020). GM2 gangliosidoses: Clinical features, pathophysiological aspects, and current therapies. *International Journal of Molecular Sciences*, *21*(17), 6213–6240. https://doi.org/10.3390/ijms21176213

Leal, A. F., Espejo-Mojica, A. J., Sanchez, O. F., Ramirez, C. M., Reyes, L. H., Cruz, J. C., & Almeciga-Diaz, C. J. (2020). Lysosomal storage diseases: Current therapies and future alternatives. *Journal of Molecular Medicine*, *98*(7), 931–946. https://doi.org/10.1007/s00109-020-01935-6

Lloyd-Evans, E., Morgan, A. J., He, X., Smith, D. A., Elliot-Smith, E., Sillence, D. J., … Platt, F. M. (2008). Niemann-pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nature Medicine*, *14*(11), 1247–1255. https://doi.org/10.1038/nm.1876

Martinez-Pomares, L. (2012). The mannose receptor. *Journal of Leukocyte Biology*, *92*(6), 1177–1186. https://doi.org/10.1189/jlb.0512231

Matsuoka, K., Tamura, T., Tsuji, D., Dohzono, Y., Kitakaze, K., Ohno, K., … Itoh, K. (2011). Therapeutic potential of intracerebroventricular replacement of modified human β-hexosaminidase B for GM2 gangliosidosis. *Molecular Therapy*, *19*(6), 1017–1024. https://doi.org/10.1038/mt.2011.27

Mosquera, A., Rodriguez, A., Soto, C., Leonardi, F., Espejo, A., Sanchez, O. F., … Barrera, L. A. (2012). Characterization of a recombinant N-acetylgalactosamine-6-sulfate sulfatase produced in *E. coli* for enzyme replacement therapy of Morquio A disease. *Process Biochemistry*, *47*(12), 2097–2102. https://doi.org/10.1016/j.procbio.2012.07.028

Ohsawa, M., Kotani, M., Tajima, Y., Tsuji, D., Ishibashi, Y., Kuroki, A., … Sakuraba, H. (2005). Establishment of immortalized Schwann cells from Sandhoff mice and corrective effect of recombinant human b-hexosaminidase a on the accumulated GM2 ganglioside. *Journal of Human Genetics*, *50*, 460–467.

Pimentel, N., Rodríguez-Lopez, A., Díaz, S., Losada, J. C., Díaz-Rincón, D. J., Cardona, C., … Barrera-Avellaneda, L. A. (2018). Production and characterization of a human lysosomal recombinant iduronate-2-sulfatase produced in Pichia pastoris. *Biotechnology and Applied Biochemistry*, *65*(5), 655–664. https://doi.org/10.1002/bab.1660

Puentes-Tellez, M. A., Lerma-Barbosa, P. A., Garzon-Jaramillo, R. G., Suarez, D. A., Espejo-Mojica, A. J., Guevara, J. M., … Almeciga-Diaz, C. J. (2020). A perspective on research, diagnosis, and management of lysosomal storage disorders in Colombia. *Heliyon*, *6*(3), e03635. https://doi.org/10.1016/j.heliyon.2020.e03635

Rodriguez-Lopez, A., Almeciga-Diaz, C. J., Sanchez, J., Moreno, J., Beltran, L., Diaz, D., … Barrera, L. A. (2016). Recombinant human N-acetylgalactosamine-6-sulfate sulfatase (GALNS) produced in the methylotrophic yeast *Pichia pastoris*. *Scientific Reports*, *6*, 29329. https://doi.org/10.1038/srep29329

Rodriguez-Lopez, A., Pimentel-Vera, L. N., Espejo-Mojica, A. J., Van, H. A., Tiels, P., Tomatsu, S., … Almeciga-Diaz, C. J. (2019). Characterization of human recombinant N-acetylgalactosamine-6-sulfate sulfatase produced in *Pichia pastoris* as potential enzyme for mucopolysaccharidosis IVA treatment. *Journal of Pharmaceutical Sciences*, *108*(8), 2534–2541. https://doi.org/10.1016/j.xphs.2019.03.034

Shapira, E., Blitzer, M. G., Miller, J. B., & Africk, D. K. (1989). *Biochemical genetics: A Laboratory Manual*. New York: Oxford University Press.

Sly, W. S., Kaplan, A., Achord, D. T., Brot, F. E., & Bell, C. E. (1978). Receptor-mediated uptake of lysosomal enzymes. *Progress in Clinical and Biological Research*, *23*, 547–551. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/662919

Solovyeva, V. V., Shaimardanova, A. A., Chulpanova, D. S., Kitaeva, K. V., Chakrabarti, L., & Rizvanov, A. A. (2018). New approaches to Tay-Sachs disease therapy. *Frontiers in Physiology*, *9*, 1663. https://doi.org/10.3389/fphys.2018.01663

Stratton, J., Chiruvolu, V., & Meagher, M. (1998). High cell-density fermentation. In D. R. Higgins & J. M. Cregg (Eds.), *Pichia protocols* (pp. 107–120). Totowa, NJ: Humana Press.

Tiels, P., Baranova, E., Piens, K., De Visscher, C., Pynaert, G., Nerinckx, W., … Callewaert, N. (2012). A bacterial glycosidase enables mannose-6-phosphate modification and improved cellular uptake of yeast-produced recombinant human lysosomal enzymes. *Nature Biotechnology*, *30*(12), 1225–1231. https://doi.org/10.1038/nbt.2427

Tropak, M., & Mahuran, D. J. (2010). Tay-Sachs disease. In *Encyclopedia of Life Sciences (eLS)*, (Ed.). https://doi.org/10.1002/9780470015902.a0006019.pub2.

Tropak, M. B., Yonekawa, S., Karumuthil-Melethil, S., Thompson, P., Wakarchuk, W., Gray, S. J., … Mahuran, D. (2016). Construction of a

hybrid β-hexosaminidase subunit capable of forming stable homodimers that hydrolyze GM2 ganglioside in vivo. *Molecular Therapy - Methods & Clinical Development*, *3*, 15057. https://doi.org/10.1038/mtm.2015.57

Tsuji, D., Akeboshi, H., Matsuoka, K., Yasuoka, H., Miyasaki, E., Kasahara, Y., … Itoh, K. (2011). Highly Phosphomannosylated enzyme replacement therapy for GM2 Gangliosidosis. *Annals of Neurology*, *69*(4), 691–701.

von Figura, K., & Hasilik, A. (1986). Lysosomal enzymes and their receptors. *Annual Review of Biochemistry*, *55*, 167–193. https://doi.org/10.1146/annurev.bi.55.070186.001123

Vu, M., Li, R., Baskfield, A., Lu, B., Farkhondeh, A., Gorshkov, K., … Zheng, W. (2018). Neural stem cells for disease modeling and evaluation of therapeutics for Tay-Sachs disease. *Orphanet Journal of Rare Diseases*, *13*(1), 152–152. https://doi.org/10.1186/s13023-018-0886-3

Xu, M., Liu, K., Swaroop, M., Sun, W., Dehdashti, S. J., McKew, J. C., & Zheng, W. (2014). A phenotypic compound screening assay for lysosomal storage diseases. *Journal of Biomolecular Screening*, *19*(1), 168–175. https://doi.org/10.1177/1087057113501197

How to cite this article: Espejo-Mojica AJ, Rodríguez-López A, Li R, et al. Human recombinant lysosomal β-Hexosaminidases produced in *Pichia pastoris* efficiently reduced lipid accumulation in Tay-Sachs fibroblasts. *Am J Med Genet Part C*. 2020;1–11. https://doi.org/10.1002/ajmg.c.31849