ORIGINAL RESEARCH ARTICLE

Development of a Biosimilar of Agalsidase Beta for the Treatment of Fabry Disease: Preclinical Evaluation

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Abstract

Background and Objective Fabry disease (FD) is a rare lysosomal storage disorder caused by a deficiency of the enzyme α-galactosidase A (aGal A). Since 2001, two different enzyme replacement therapies have been authorized, with agalsidase beta being used in most parts of the Western world. Currently, biosimilars of several expensive enzyme therapies are under development to improve their accessibility for patients. We present the preclinical results of the development of a biosimilar to agalsidase beta.

Methods Produced in a Chinese hamster ovary (CHO)-cell system, the biosimilar aGal A Biosidus (AGABIO), was compared with agalsidase beta with respect to amino acid sequence, glycosylation, specific α-galactosidase activity, stability in plasma, and effects on cultured human Fabry fibroblasts and Fabry mice.

Results AGABIO had the same amino acid composition and similar glycosylation, enzymatic activity, and stability as compared with agalsidase beta. After uptake in fibroblasts, α-galactosidase A activity increased in a dose-dependent manner, with maximum uptake observed after 24 h, which remained stable until at least 48 h. Both enzymes were localized to lysosomes. Reduction of accumulated globotriaosylceramide (Gb3) and lysoGb3 in cultured Fabry fibroblasts by AGABIO and agalsidase beta showed comparable dose–response curves. In Fabry knockout mice, after a single injection, both enzymes were rapidly cleared from the plasma and showed equal reductions in tissue and plasma sphingolipids. Repeated dose studies in rats did not raise any safety concerns. Anti-drug antibodies from patients with FD treated with agalsidase beta showed equal neutralization activity toward AGABIO.

Conclusion These findings support the biosimilarity of AGABIO in comparison with agalsidase beta. The clinical study phase is currently under development.

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Key Points

A recombinant α-galactosidase A (AGABIO) enzyme was developed, as a biosimilar to the approved agalsidase beta (Fabrazyme), for treatment of Fabry disease.

A high degree of similarity was observed in terms of all physicochemical and biological properties between AGABIO and the reference product.

AGABIO is a candidate for clinical trials in humans to provide an alternative treatment option for enzyme replacement in Fabry disease.

△ Adis

1 Introduction

Fabry disease (FD; MIM 301500) is one of the most prevalent lysosomal storage disorders, affecting around 1 in 40,000 people [1]. Deficiency of the enzyme α-galactosidase A (aGalA; EC 3.2.1.22) results in storage of glycosphingolipids, mainly globotriaosylceramide (Gb3) in multiple cell types [2]. The disorder is X-linked, resulting in differences in phenotypic expression: the most severe manifestations are present in male patients without any residual aGalA activity [1, 3]. The disease is much more variable in female carriers and in males with some residual aGalA activity. These latter patients show predominant involvement of the heart and an increased risk for central nervous system injury [3].

The pathophysiology of FD relates to storage of Gb3 and other lipids that are not or incompletely degraded due to the aGalA deficiency. Storage in vascular cells, podocytes, and cardiomyocytes may result in progressive brain, kidney, and heart injury [1]. However, the pathophysiology is only partially understood, and for example, abnormal cell signaling and impaired autophagy may play a role in irreversible manifestations of the disease [4].

Treatment consists of supportive care as well as timely intervention with a fixed dose of enzyme-replacement therapy, either with agalsidase alfa 0.2 mg/kg every other week (eow) (Replagal, Takeda Shire) or agalsidase beta 1.0 mg/kg eow (Fabrazyme, Sanofi Genzyme). In the pivotal trial that led to the registration of agalsidase beta it was shown that enzyme-replacement therapy (ERT) resulted in reduction of Gb3 in plasma, urine, and different cell types [5, 6]. In general, treatment with ERT can result in a stabilization of renal function and a reduction in cardiac mass and may delay the onset of new clinical events [7]. However, response differs depending on the timing of initiation of therapy, phenotype, and the presence of irreversible damage [3, 8]. In addition, dose matters: the biochemical response is clearly better with agalsidase beta compared with agalsidase alfa, the first being given at a five times higher dose [9]. Effects of anti-drug antibodies (ADA) are relevant in this aspect. In most classical males, development of ADA may partially neutralize the effectiveness of ERT [10]. Indeed, a higher dose of enzyme can partially overcome the inhibitory effect of circulating ADA [11].

Restrictions in reimbursement of this expensive therapy hamper further dose finding in patients with high antibody titers. A cheaper alternative would allow fine-tuning of dosing in classical males with ADA who do not benefit from the standard dose. Hence, development of biosimilars at lower costs is of interest. In addition, cheaper alternatives could improve access to treatment for patients in low- and middle-income countries that are unable to afford the high prices. Currently, new enzymes are being investigated [12], but so far biosimilars of agalsidase beta have become available only in Korea and Japan [13]. Biosidus is a pharmaceutical company based in Argentina aiming to provide high-quality biosimilar products at a fair price. In this study, we describe the development of a biosimilar of agalsidase beta and the most critical analytical characterization assays. For comparative preclinical studies, we designed a set of in vitro comparative analyses and Fabry patient cells were used to study intracellular agalsidase localization, cellular uptake, intracellular degradation of its specific substrate, and generation of metabolites. In addition, we tested the interaction of the molecule with specific antibodies raised in patients treated with agalsidase beta. The in vitro assays were complemented by, as few as possible, in vivo studies in rodents, focusing on pharmacokinetics, organ-specific pharmacodynamics, and toxicity.

2 Materials and Methods

2.1 Chemicals

Substrate 4-methylumbelliferyl-α-D-galactopyranoside (4-MU-αGal), D-mannose 6-phosphate (M6P), sodium hydroxide and ammonium formate, anthranilamide (2-AB), and globotriaosylsphingosine (lysoGb3) were purchased from Sigma (MO, USA). Trifluoroacetic acid (TFA), formic acid, butanol, and hydrochloric acid were obtained from Merck (MA, USA). Polypeptide *N*-glycosidase F (PNGase F) was obtained from Agilent (CA, USA). Culture medium, fetal bovine serum (FBS), BCA Protein Assay Kit, Alexa555, and Lysotracker were from Thermo Fisher Scientific (MA, USA).

N-dodecanoyl-NBD-ceramide trihexoside (NBD-Gb3) was purchased from Santa Cruz Biotechnology (TX, USA). Glucosylsphingosine (lysoGlcCer), lactosylsphingosine (lysoLacCer), and d5-glucosylsphingosine (d5-lysoGlcCer) (internal) standards were obtained from Avanti Polar Lipids Inc. (AL, USA), and all organic solvents were obtained from Biosolve (LC-MS/MS quality) (Dieuze, France). *N*-glycinated globotriaosylsphingosine (Gly-lysoGb3) internal standard was obtained from Matreya LCC (PA, USA).

Mouse-anti-human IgG and mouse-anti-human IgG4, labeled with horseradish peroxidase (HRP), were from Sanquin Reagents (Amsterdam, The Netherlands).

2.2 Alpha Galactosidase A and Fabry Fibroblasts

Different lots of the recombinant agalsidase originator, agalsidase beta (Fabrazyme, Sanofi Genzyme) were used for the analyses. The putative biosimilar product, agalsidase

beta BIOSIDUS (AGABIO), was manufactured by Biosidus SAU. Like agalsidase beta, AGABIO was produced from an established CHO cell line. The protein concentration for each product batch was determined using a high-performance liquid chromatography (HPLC)-based concentration assay calibrated with a control of a known concentration, determined by time-resolved amino acid analysis (Protagen, Germany). From this analysis, the extinction coefficient for agalsidase was determined using the Beer–Lambert Law, given a value of 2.55 l/(g × cm).

The Fabry fibroblasts cell lines used in this publication were GM00107 (Coriell Institute, NIGMS Human Genetic Cell Repository NJ, USA) and a Fabry patient-derived fibroblasts cell line.

2.3 Primary Sequence

Full amino acid sequence and disulfide linkage analysis of both agalsidase beta and AGABIO was performed by reversed-phase (RP)-HPLC/electrospray ionization (ESI)–mass spectrometry(MS)/MS. This study was performed at Protagen, Germany.

For amino acid sequence and *N*-glycosylation site identification, the samples were denatured, reduced, and proteolytically digested (trypsin, trypsin/chymotrypsin, GluC). To identify *N*-glycosylation sites, part of the trypsin digest was further deglycosylated enzymatically with PNGase F under enzyme-specific conditions. Asn-linked glycans can be cleaved by PNGase F, yielding a modified protein in which Asn residues, at the site of deglycosylation, are converted to Asp.

Liquid chromatography (LC)–ESI–MS and tandem mass spectrometry (MS/MS) mass spectra were obtained using the UltiMate 3000 system (Thermo Fisher Scientific) coupled to a Q Exactive Orbitrap Plus mass spectrometer (Thermo Fisher Scientific). The separation of the peptides was performed with RP chromatography on an Accucore RP–MS LC column (2.1 × 100 mm, 2.6 μm particle size, Thermo Fisher Scientific). The MS datasets were analyzed using the ProteinScape 2 bioinformatics platform for sequence confirmation (Bruker Daltonics, Protagen AG).

Cys-Cys-bridged residues were identified under nonreducing conditions, after alkylation of unlinked cysteines with *N*-ethylmaleimide. Samples were then proteolytically digested, and linked dipeptides were detected in MS/MS spectra.

2.4 Enzyme Activity Measurement

The enzymatic activity of agalsidase was measured using the fluorogenic substrate 4-MU-αGal at a final concentration of 3.0 mmol/l in 0.05 mol/l citrate–phosphate buffer (pH 4.6), containing 0.1% (w/v) BSA, as described previously [14].

Reactions were terminated after 15 min by the addition of glycine/NaOH buffer, pH 10.6, and fluorescent 4-methylumbelliferone was measured with a fluorimeter (Synergy H1 Multi-Mode Reader BioTek at 455 nm). Specific activity of each sample was measured using the calculated concentration.

2.5 Quantitation of Mannose-6-Phosphate Residues

Mannose-6-phosphate (M6P) residues were released from the enzymes by acid hydrolysis in TFA at 100 °C and then derivatized with 2AB. The labeled M6P was then separated by HPLC on a GlycanPac AXH column (3.0 × 150 mm, 1.3 μm particle size, Thermo Fisher Scientific), and its fluorescence was monitored by excitation at 330 nm and emission at 420 nm. M6P was quantified by comparing peak areas of the samples with M6P standard solutions. Values were normalized by protein content and expressed as mol M6P/mol of dimeric agalsidase.

2.6 *N*-Glycosylation Analysis

The glycan microheterogeneity was characterized by anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD). The study was performed at Alvotech (Germany). Briefly, 200 μg of each sample was reduced in 0.3% SDS/50 mM 2-mercaptoethanol for 30 min at 65 °C prior to enzymatic release of the *N*-linked carbohydrate chains from the protein in 50 mM Tris–HCl, pH 7.4 by adding PNGaseF. The purification and desalting of the released *N*-glycans was performed by using Hypercarb graphitic carbon cartridges.

Quantitative high-resolution HPAEC–PAD mapping of native *N*-glycans released from samples was performed on an ICS 5000+ ion chromatography system of Thermo Fisher Scientific (Waltham, MA, USA). Native *N*-glycans were applied to high-resolution CarboPac PA200 columns at a constant flow rate of 0.4 ml/min and at 30 °C column temperature and were eluted by using a concentration gradient consisting of 0.04 M sodium hydroxide, 0.4 M sodium hydroxide, and 0.04 M sodium hydroxide/1.2 M sodium acetate. *N*-glycans were detected via electrochemical detection, and the data were collected and processed using Chromeleon Chromatography Management System Version 7.2 SR5.

2.7 Stability of Agalsidase in Different Matrices and Temperatures

AGABIO and agalsidase beta were separately spiked into human plasma to a final concentration of 1 μg/ml, and incubated either on ice or at 37 °C for 0, 15, 30, 45, 60, 120, and 180 min. Following exposure to plasma, the samples were analyzed for residual enzymatic activity (compared with

$t = 0$). The same procedure was done in different matrices and temperatures: normal human serum, phosphate citrate buffer (pH 4.6), and culture medium supplemented with 10% FBS.

2.8 Agalsidase Cellular Uptake and Localization

Fabry fibroblasts were cultured at 37 °C in 35 mm CELL-view dishes from Greiner Bio-One (50% confluency) with 2 ml of DMEM culture medium containing 10% FBS, 21 mM HEPES, and fungizone/penicillin/streptomycin. After overnight adherence, medium was replaced with 1 ml DMEM medium containing Alexa-555-labeled AGABIO or Alexa-555-labeled agalsidase beta (2.5 μg enzyme/ml DMEM) and cells were cultured at 37 °C for 24 h. Forty-five minutes before analyzing the cells, LysoTracker Green DND-26 was added to the medium at a final concentration of 60 nM. Cells were analyzed using confocal microscopy with the use of a Leica TCS SP8 SMD mounted on a Leica DMI6000 inverted microscope.

2.9 Fabry Fibroblasts Agalsidase Activity Uptake

Fabry fibroblasts were cultured in 35 mm dishes (200,000 cells per well) with 1 ml of DMEM culture medium containing FBS at 37 °C. After overnight adherence and subsequent 24 h of culturing, AGABIO or agalsidase beta (5 μl) was added to 1 ml of fresh culture medium and the cells were cultured for up to 48 h. Agalsidase final concentrations evaluated were 0.1, 1, and 10 μg/ml. Untreated cells were used as control. Each sample was analyzed in six culture dishes.

Cells were trypsinized after extensive washing with phosphate-buffered saline (PBS). The cell pellet was disrupted by freeze–thawing in lysis buffer (27 mM citric acid, 46 mM sodium phosphate dibasic, 1% Triton X-100, pH 4.7). Debris was pelleted by centrifugation, and the soluble fractions were assayed for α-galactosidase activity using 4-MU-αGal as a substrate. Cell lysate total protein content was measured by BCA Protein Assay Kit.

2.10 Agalsidase Treatment and Gb3 Levels

To examine intracellular Gb3 degradation activity in agalsidase-treated cells, Fabry fibroblasts were seeded on 24 mm dishes. NBD-Gb3, a green fluorescent Gb3 analog, was added (3 μM in serum-free culture medium) and incubated for 24 h [15, 16]. After cells were washed with PBS, fresh culture medium without (control) or with aGalA (agalsidase beta or AGABIO) was added for a further 24 h incubation. aGal A final concentration in medium was 0.1 μg/ml. After incubation, the culture medium was replaced with fresh medium and maintained for 48 h. Cells were washed with

PBS and fixed with 4% paraformaldehyde. Nuclei were visualized with DAPI (4′,6-diamidino-2-phenylindole). Intracellular fluorescence was visualized using microscopy (Nikon Eclipse TS100), and image was taken by the manufacturer's software (NIS Elements F version 4.0 Nikon).

2.11 Dose–Response Curve of NBD-Gb3 Degradation Through Flow Cytometry

NBD-Gb3 fluorescence was measured using a flow cytometry analyzer (Accuri C6 Plus BD Biosciences). Fibroblasts were cultured in 24-well plates (75,000 cells per well). After overnight adherence and subsequent 72 h of culturing in medium without FBS, cells were loaded with 0.75 μM NBD-Gb3 and incubated 24 h. Medium was replaced by 1 ml of fresh culture medium containing AGABIO or agalsidase beta (0.001–1 μg/ml) and incubated for 24 h. Cells were trypsinized and suspended in cytometry buffer. Cell fluorescence signal was quantified by fluorescence-activated cell sorting (FACS). Fibroblast population (P1) was selected by dot plot of forward scatter versus side scatter. P1 fluorescence histogram was acquired in FL1 channel (green). Results were expressed as the percentage of intracellular remaining Gb3; this is calculated as the sample mean fluorescence intensity (MFI) relative to the positive control MFI. Positive control is prepared using fibroblasts loaded with NBD-Gb3 without agalsidase treatment.

2.12 Dose–Response Curve of Gb3 Degradation Through MS/MS Analysis

Fibroblasts were cultured in six-well plates. After overnight adherence, the culture medium was replaced with culture medium containing AGABIO (0.5–5.0 μg/ml) or agalsidase beta (0.54–5.4 μg/ml). Subsequently, the cells were cultured for 24 h, washed with PBS, and sonicated. The cell suspension was used for analysis of glycosphingolipids and their sphingoid bases. The time-dependent degradation of Gb3 was studied using fibroblasts cultured in the presence of 1 μg/ml AGABIO or 1.08 μg/ml agalsidase beta for up to 6 days.

Gb3 was dissolved in culture medium using either sonication or supplementation with BSA. Loading of the fibroblasts with Gb3 was performed by incubating the cells with culture medium containing Gb3 for 5 days. Subsequently, the culture medium was replaced by fresh medium containing 5 μg/ml AGABIO or 5.4 μg/ml agalsidase beta, and the cells were cultured for another 24 h. Sphingolipid analysis was performed as described in 2.13. Cell lysate total protein content was measured by BCA Protein Assay Kit.

2.13 Sphingolipid Analysis

2.13.1 Extraction of Sphingolipids from Fibroblasts

The metabolites were extracted from fibroblast homogenates by a modification of the method of Bligh and Dyer [see Ref. [17] and online resource (Supplementary Fig. 1)]. Briefly, 600 µl methanol, 600 µl chloroform, and 450 µl water was added to 150 µl homogenate. Then, 500 µl of the lower phase, containing Gb3 and LacCer, was dried under a stream of nitrogen at 40 °C and the residue was dissolved in 500 µl 0.1 M sodium hydroxide in methanol. Samples were deacylated (microwave) for analysis of derived lysoGb3 and lysoLacCer as described earlier [17]. After hydrolysis, samples were neutralized by addition of 50 µl 0.1 M hydrochloric acid, and 25 µl 1 µM d5-lysoGlcCer in methanol (internal standard) was added. Next, samples were dried (N_2, 40 °C) and dissolved in butanol and water (1:1, v/v). After drying the butanol phase (N_2, 40 °C), samples were dissolved in 120 µl methanol and analyzed by LC–MS/MS.

For the upper phase (1 ml), containing lysoGb3, 25 µl 0.1 µM Gly-lysoGb3 in methanol (internal standard) was added and samples were dried (N_2, 40 °C), dissolved in 120 µl methanol, and analyzed by LC–MS/MS as described in Sect. 2.13.3.

2.13.2 Extraction of Sphingolipids from Mouse Tissues

Pieces of tissue were kept at –80 °C until use. After transferring the tissue pieces to 2 ml safe-lock tubes (Eppendorf), nine parts of water and a metal ball were added and tissues were homogenized using a MagNA Lyser Instrument (Roche Diagnostics GmbH) at a speed of 5000 rpm for 30 s. This was repeated until consistent homogenates were obtained.

LysoGb3 was extracted from plasma and tissue homogenates by a modification of the method of Bligh and Dyer (methanol:chloroform:water, 1:1:1, v/v/v [see Ref. [17] and online resource (Supplementary Fig. 1)]. Prior to protein precipitation, 25 µl 1 µM Gly-lysoGb3 in methanol was added to each sample. The upper phase, containing LysoGb3, was taken to dryness (N_2, 40 °C), dissolved in methanol, and analyzed by LC–MS/MS.

For extraction of Gb3 and LacCer, protein was precipitated and separation of phases was induced by addition of water (methanol:chloroform:water, 1:1:0.9, v/v/v) [17]. Next, the lower phase was transferred to a microwave tube and the upper phase was washed with chloroform. The lower phase was added to the first, and combined phases were dried under a stream of nitrogen at 40 °C. The residue was dissolved in 500 µl 0.1 M sodium hydroxide in methanol, and samples were deacylated [18]. After this, samples were neutralized by addition of 50 µl 0.1 M hydrochloric acid, 25 µl 1 µM d5-lysoGlcCer (internal standard) was added to each

sample, and samples were dried and dissolved in butanol and water (1:1, v/v). After drying the butanol phase, samples were dissolved in methanol and analyzed by LC–MS/MS as described in Sect. 2.13.3

2.13.3 LC–MS/MS Measurement of Sphingolipids

Metabolites were separated by RP-UPLC using an Acquity I-Class UPLC with BEH C18 column, 2.1 × 50 mm with 1.7-µm particle size (Waters Inc.) and detected by electron spray ionization in positive mode (ESI+) and MS/MS instrument (Xevo TQ MS, Waters Inc.) in multiple reaction monitoring (MRM) mode (see online resource Supplementary Table 1). In both upper and lower phases, metabolites were calculated using calibration lines within the appropriate concentration range, according to the internal standard ratio. For details on the used LC–MS/MS methods and settings, see online resource Supplementary Table 1 and Ref. [19].

2.14 Patient Anti-αGAL A Immunoglobulin Cross-Reactivity Assay

Plasma samples from ten anti r-αGAL A antibody-positive patients were pooled and used to assess cross-reactivity of agalsidase beta versus AGABIO. Ninety-six-well microtiter plates (Nunc/Maxisorp) were coated overnight at 4 °C with 100 µl 2 µg/ml recombinant aGal A (agalsidase beta and AGABIO) in PBS (pH 7.4). Concentration of the AGABIO and agalsidase beta batches before dilutions was determined using BCA. After washing, the plates were incubated at 37 °C for 1.5 h with 200 µl blocking buffer composed of PBS containing 2% (w/v) BSA fraction V (Merck). Subsequently, plasma samples were diluted in PBS containing 0.1% (v/v) Tween-20 and 2% (w/v) BSA fraction V (dilution buffer). Plasma dilutions ranged from 1:20,000 to 1:2,560,000. Of each dilution, 100 µl was incubated on the plates at 37 °C for 1.5 h, after which plates were washed. Next, plates were incubated with the secondary antibodies—either mouse-anti-human IgG (no differentiation between subtypes) or mouse-anti-human IgG4, labeled with horseradish peroxidase (HRP). Secondary antibodies were diluted 1:2500 in dilution buffer. Plates were washed before 100 µl TMB substrate was added, plates were incubated approximately 3 min at room temperature, and the reaction was stopped using 1 M H_2SO_4. Absorbance was measured using a microtiter plate reader (Spectramax plus 384) at 450 nm using 540 nm as a reference for background absorption. A pool containing plasma of 35 healthy donors (Sanquin, Amsterdam, the Netherlands) was used as a negative control. All measurements were performed in duplicates. All wash steps consisted of five rinsing cycles with PBS 0.1% (v/v) Tween-20.

2.15 Substrate Reduction in GLA-Knockout Mice Organs

Animal experiments were carried out at the Jackson Laboratory In Vivo Services (Bar Harbor, ME, USA) This study complied with all applicable sections of the Guide for the Care and Use of Laboratory Animals from the National Research Council. The protocol and any amendments or procedures involving the care or use of animals in this study were approved by the Testing Facility Institutional Animal Care and Use Committee.

Fabry model mice (also known as *aGal A* KO) B6;129-*Gla^{tm1Kul}*/J mice, hemizygous) were used (JAX stock number 003535). Hemizygous mice were grouped into three dosing groups. Each group consisted of four mice. All animals used in this study were 12-week-old males. In one group AGABIO was administered at 1 mg/kg body weight, in a second group agalsidase beta was administered at 1 mg/kg body weight, and the third group received vehicle (50 mM phosphate buffer pH 6.8, 0.6 mg/ml mannitol) All substances were administered intravenously in the tail vein. Wild-type group mice (B6;129-*Gla^{tm1Kul}*/J) were administered vehicle and used as control.

Necropsies were performed 7 days after dosing. Mice were euthanized by CO_2 asphyxiation. Terminal blood was collected by cardiocentesis immediately after euthanasia. Plasma was separated by centrifugation at 10,000 rpm for 10 min at 4 °C and stored at –80 °C until analysis.

The following tissues were collected from each mouse: shaved skin sample, liver, spleen, kidneys, and heart. Each tissue was cut into five pieces. Each piece weight was recorded and snap frozen in dry ice in separate tubes. Frozen tissues were stored at –80 °C until sphingolipid analysis described in Sect. 2.13.

2.16 Pharmacokinetics

AGABIO or agalsidase beta was intravenously administered in mice, 8 weeks of age, five animals in each group, at a dose of 1.0 mg/kg body weight. Whole blood was collected by tail bleed at 1, 10, 30, and 60 min after injection. The enzyme activity of agalsidase beta and AGABIO in plasma were measured using the substrate fluorescent analog, 4MU-Gal.

2.17 Safety Evaluations

Safety evaluations was performed in a repeated-dose toxicity study in rats compliant with Good Laboratory Practice regulations. AGABIO or agalsidase beta was administered to Wistar rats (8–9 weeks of age, six males and six females per group) intravenously at 1.0 mg/kg, 2.2 mg/kg, and 5.0 mg/kg once every other week for 12 weeks. Recorded clinical observations included standard behavior, body weight, and food consumption. Necropsy was conducted on all animals. Weights and histopathology of the following organs and tissues were analyzed: site of injection (to evaluate local tolerance), brain, cerebellum, lung, heart, liver, spleen, thymus, bone marrow, stomach, duodenum, colon, kidney, and lymph nodes. Complementary analyses of hematology and serum biochemistry were done for all animals.

2.18 Statistical Analysis

Data are presented as mean ± standard deviation (SD). Statistical significance was determined by Student's *t*-test.

3 Results

3.1 Primary Sequence

Agalsidase beta and AGABIO were stably expressed in CHO cells, and peptide mapping showed that both products have the same amino acid sequence. The identification of the Asn residues that undergo *N*-linked glycosylation was performed by PNGase F treatment and amino acid sequence analysis. Identical to agalsidase beta, AGABIO contained three *N*-linked oligosaccharide binding sites (Asn108, Asn161, and Asn184). All five expected disulfide linkages in AGABIO were detected [20]. No additional linkages were detected in the originator Fabrazyme or AGABIO samples.

3.2 Specific Enzymatic Activity

Enzymatic analysis, carried out with an artificial substrate, did not reveal any significant differences in the specific activity between the two preparations (online resource Supplementary Fig. 2).

3.3 Quantitation of M6P

M6P content of AGABIO was similar to that of agalsidase beta. The M6P content (mean ± SD) of AGABIO (four batches) and agalsidase beta (three batches) were 3.7 ± 0.1 and 3.6 ± 0.1 mol/mol, respectively.

3.4 *N*-Glycosylation

The structure of the carbohydrate chains of aGal A is highly complex [21, 22]. Glycosylation profiling using normal-phase HPLC after glycosidase digestions showed similar peak patterns for AGABIO and agalsidase beta (online resource Supplementary Fig. 3). Similar content of total complex glycans was found in AGABIO and
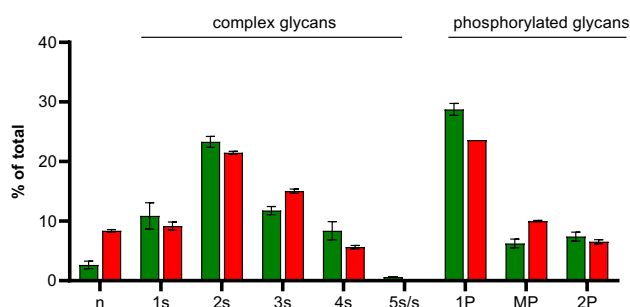
Fig. 1 Quantitative analysis of native *N*-glycans released from AGABIO (green bars, four separate batches) and agalsidase beta (red bars, three different batches) samples. Data are shown as mean ± standard deviation. Glycan references are *n* neutral, *1s* monosialylated, *2s* disialylated, *3s* trisialylated, *4s* tetrasialylated, *5s/s* pentasialylated and sulfated, *1P* phosphorylated oligomanosidic, *MP* phosphorylated hybrid type, *2P* diphosphorylated oligomannosidic, *AGABIO* α-galactosidase A Biosidus

agalsidase beta samples. Complex glycans constituted approximately 60% of the total glycans. In all samples, *N*-glycan structures with 0–4 terminal sialic acid residues (α2,3-linked Neu5Ac) were found. Sialylated isoforms are important for the half-life of circulating agalsidase. Subtle sialylated glycan distribution differences were observed among agalsidase beta and AGABIO samples (Fig. 1). Agalsidase beta showed higher amounts of trisialylated forms (15–16%) compared with AGABIO (11–12%) and lower levels of tetrasialylated glycans (5–6% and 6–10% for agalsidase beta and AGABIO, respectively).

Neutral glycan content was less than 10% in all the samples. However, agalsidase beta showed higher amounts of neutral *N*-glycans (8–9%) compared with the AGABIO preparations (2–4%). The Man5GlcNAc2 structure was identified as the main glycan component in the neutral *N*-glycan group. Phosphorylated hybrid-type *N*-glycans and phosphorylated oligomannosidic *N*-glycan chains bearing one and two phosphate residues, respectively, were present in each AGABIO and agalsidase beta sample. *N*-glycan phosphorylated structures detected in the three agalsidase beta samples represent about 40% of the total *N*-glycans. All samples contained 6–8% of biphosphorylated glycans (Fig. 1).

3.5 Stability of Agalsidase in Different Matrices and Temperatures

A time course of changes in enzyme activity of AGABIO and agalsidase beta in plasma at different temperatures and other matrices revealed that there was no difference in stability between AGABIO and agalsidase beta under these conditions (online resource Supplementary Fig. 4).

3.6 Subcellular Localization

The subcellular localization of AGABIO and agalsidase beta was determined by confocal microscopy. Intracellular accumulation of Alexa-555-labeled AGABIO or Alexa-555-labeled agalsidase beta was observed as a punctuated form in cytoplasmic area. To verify lysosomal localization, cells were exposed to LysoTracker Green. When merged, red fluorescence from Alexa-555-labeled agalsidase and green fluorescence from LysoTracker Green showed apparent colocalization and presented as yellow dots, showing the same localization of AGABIO and agalsidase beta in lysosomes (Fig. 2).

3.7 Agalsidase Activity in Cultured Human Fabry Fibroblasts

The intracellular enzymatic activity in cultured Fabry fibroblasts increased in a dose-dependent manner after 24 h. Dose–response curves were similar for AGABIO and agalsidase beta (Fig. 3A). The maximum α-galactosidase A activity uptake was observed after 24 h and remained stable until at least 48 h (Fig. 3B). Incubation of culture medium, containing agalsidase beta or AGABIO, at 37 °C for 3 h resulted in an almost complete loss of agalsidase A activity (Fig. 4). However, the addition of preincubated culture medium (37 °C, 3 h) containing agalsidase beta or
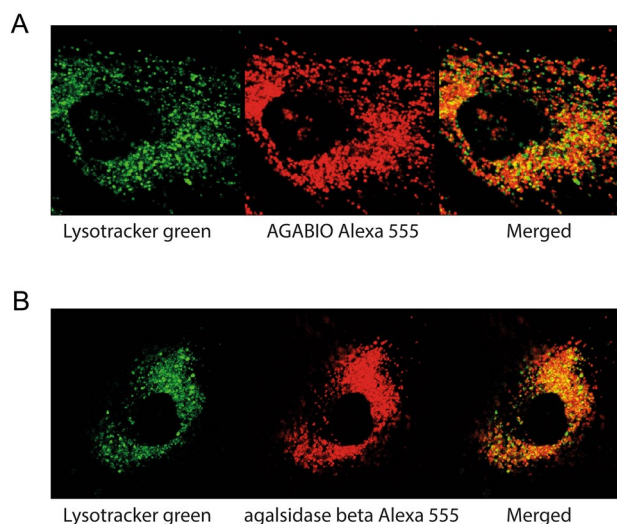

Fig. 2 Internalization and lysosomal localization of Alexa-555-labeled agalsidase into Fabry fibroblasts. Cells were grown overnight on glass coverslips and incubated for 24 h in the presence of Alexa-555-labeled AGABIO (red color in panel **A**) or Alexa-555-labeled agalsidase beta (red color in panel **B**). Lysosome labeling was achieved using LysoTracker Green DND-26. The apparent colocalization of the lysosomal staining (green) and the agalsidase containing granules (red) is represented in yellow when the images are superimposed (Merged)
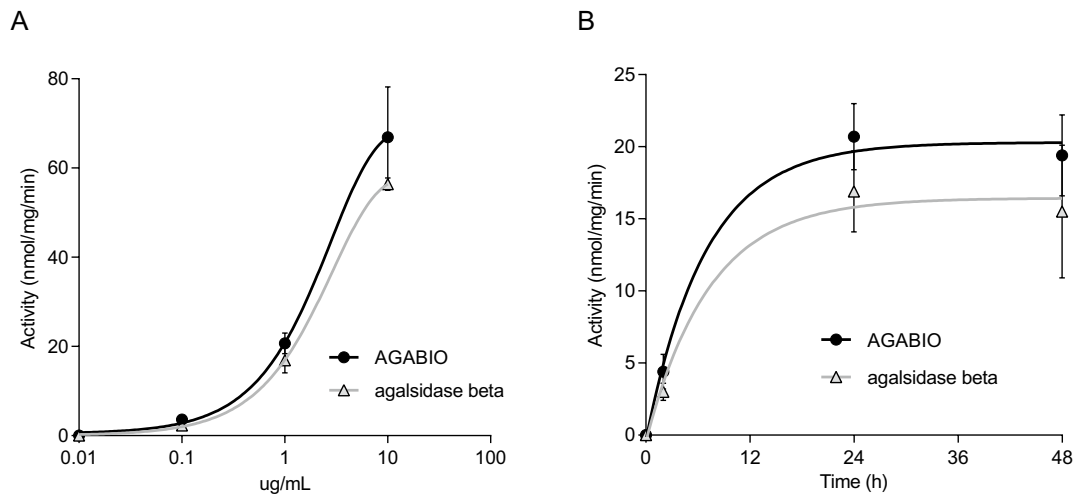
A

B

Fig. 3 Quantitative cellular uptake—dose–response curve (**A**) and time-dependent cellular uptake (**B**). Data are means with standard deviation bars from four replicates of one representative lot of AGABIO and agalsidase beta, respectively. *AGABIO* α-galactosidase A Biosidus
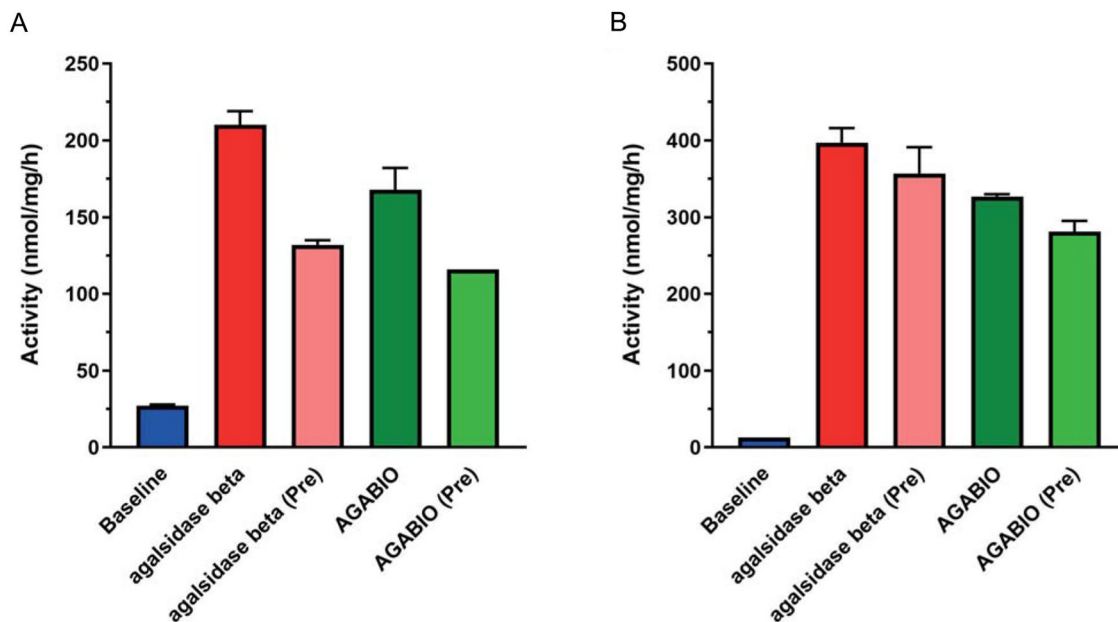
A

B

Fig. 4 Quantitative cellular uptake of agalsidase beta and AGABIO enzymatic activity after preincubation (Pre) of the culture medium containing agalsidase beta or AGABIO at 37 °C for 3 h, followed by culturing the fibroblasts with the preincubated medium for 3 h (panel **A**) or 24 h (panel **B**). *AGABIO* α-galactosidase A Biosidus

AGABIO, to Fabry fibroblasts, restored the agalsidase A activity (Fig. 4).

3.8 Degradation of Accumulated Gb3 in Cultured Fabry Fibroblasts

The uptake of agalsidase beta and AGABIO resulted in a marked reduction in Gb3 content. Gb3 uptake was identified by treating Fabry fibroblasts with a medium containing a fluorescently labeled Gb3 (NBD-Gb3). Intracellular

NBD-Gb3 was detected within 4 h after treatment, and persisted up to 96 h. In the presence of agalsidase, the fluorescence of the substrate completely disappeared after 48 h (Fig. 5). In addition, NBD-Gb3 degradation at different agalsidase beta and AGABIO doses was quantified using FACS. The incorporated enzymes degraded Gb3 and lowered accumulation of Gb3 in cultured Fabry fibroblasts in a dose-dependent manner. Dose–response curves were similar for AGABIO and agalsidase beta. After 24 h, almost all initial NBD-Gb3 was degraded with either

Fig. 5 Intracellular NBD-Gb3 fluorescence. Fluorescent uptake analysis of NBD-Gb3 (green) after incubation in Fabry cells (**A**). After 48 h treatment with media (control), green fluorescence remained unchanged (**B**). After 48 h treatment either with AGA-BIO (**C**) or agalsidase beta (**D**), all accumulated NBD-Gb3 was degraded. Nuclei are stained with DAPI (blue). *AGABIO* α-galactosidase A Biosidus, *DAPI* 4′,6-diamidino-2-phenylindole, *NBD-Gb3* N-dodecanoyl-NBD-ceramide trihexoside
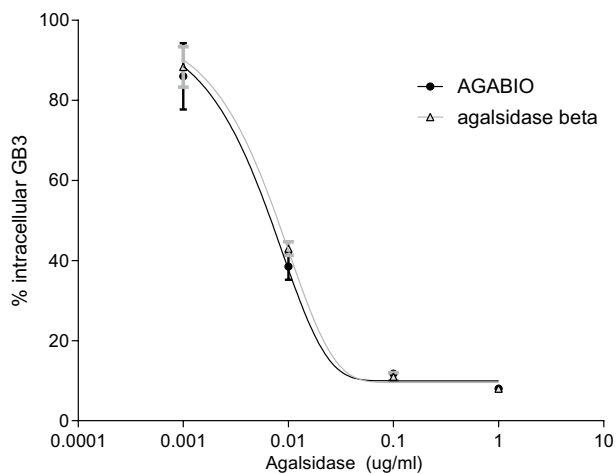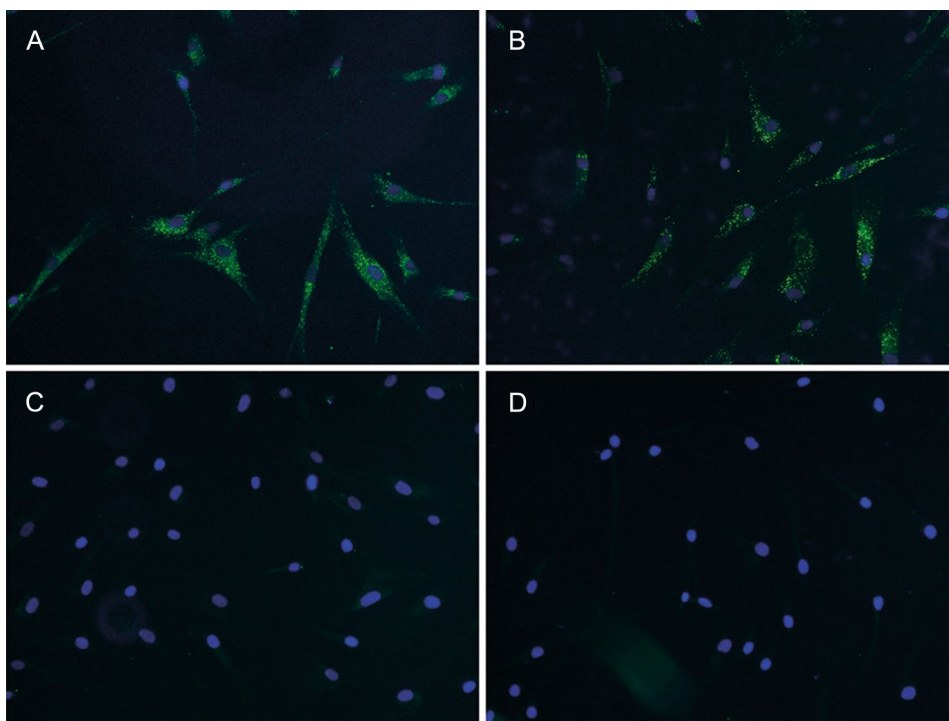

Fig. 6 Intracellular NBD-Gb3 degradation by agalsidase treatment dose–response curve. Data are means with standard deviation bars from four and three lots of AGABIO and agalsidase beta, respectively. *AGABIO* α-galactosidase A Biosidus, *NBD-Gb3 N*-dodecanoyl-NBD-ceramide trihexoside

product at 0.1 µg/ml concentration (Fig. 6). Fibroblasts loaded with Gb3 showed increased intracellular levels of Gb3 and lysoGb3 when compared with that observed in the nonloaded cells. Treatment of the nonloaded and Gb3-loaded fibroblasts with AGABIO or agalsidase beta resulted in decreased intracellular Gb3 and lysoGb3 levels and increased lactosylceramide levels (online resource Supplementary Figs. 5–7).

3.9 Patient Anti-αGAL A Immunoglobulin Cross-Reactivity

Anti-drug antibodies that developed in ten male patients with classical FD upon treatment with agalsidase beta showed complete cross-reactivity in vitro with AGABIO compared with agalsidase beta. Both the assay measuring total IgGs (Fig. 7a) and the assay measuring IgG4 (Fig. 7b) showed no differences between agalsidase beta and AGABIO.

3.10 Pharmacodynamics in Fabry Mice

Preclinical studies of enzyme-replacement therapy for FD were performed in agalsidase-deficient mice. The Gb3 and lysoGb3 content in the organs of untreated Fabry mice was markedly higher than that in those of wild-type mice. Following a single injection (1 mg/kg) of either AGABIO or agalsidase beta into the blood vessels of Fabry mice, the degradation of substrates in the kidneys, heart, skin, spleen, and liver was comparable between wild-type and knockout mice. Tissue and plasma sphingolipids decreased with both products in a similar manner (Fig. 8).

3.11 Pharmacokinetics

Plasma activity of AGABIO and agalsidase beta (three batches each) showed no marked difference throughout the study period (online resource Supplementary Fig. 8). After a single intravenous dose of 1 mg/kg, product was rapidly
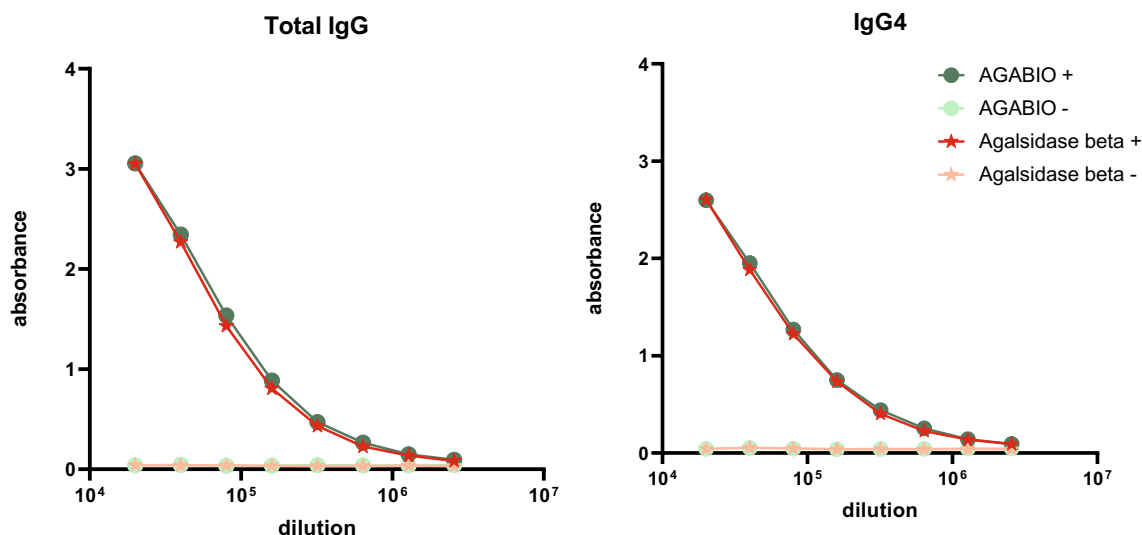
Total IgG

IgG4

Fig. 7 In vitro cross reactivity of anti-aGal A antibodies (anti-drug antibodies, ADA) from a pool of ten ADA-positive male patients with FD. ELISAs were performed using HRP-labeled mouse-anti human antibodies directed at all IgGs (**A**) as well as IgG4 specifically (**B**). Darker-colored lines (+) represent the cross-reactivity of various dilutions of the Fabry serum pool, consisting of ten ADA-positive patients with FD, with either Agalsidase beta (red) or AGABIO (green). Lighter colors represent negative controls using a serum pool derived from 35 healthy donors. Dilutions of serum started at 1:20,000 up to 1:2,560,000. All measurements were performed in duplicate on the same plate. *ADA* anti-drug antibodies, *AGABIO* α-galactosidase A Biosidus

cleared from the circulation with a *T½* of 12–13 min. These results indicate that both AGABIO and agalsidase beta have similar pharmacokinetic profiles.

3.12 Repeated-Dose Toxicity Study in Rats

AGABIO or agalsidase beta was administered to Wistar rats (8–9 weeks of age, six males and six females per group) intravenously at 1.0 mg/kg, 2.2 mg/kg, and 5.0 mg/kg once every other week for 12 weeks. During the experimental period, mortality and body mass were monitored, and necropsy was undertaken at the end of the experimental period. No deaths or clinical signs were observed. No abnormalities were found during necropsy. There were no significant effects of the treatment on body weight, food consumption, biochemistry, necropsy, organ weight, or histopathology in either males or females in all test groups. No pathological signs were observed. There were no notable differences in safety profile between AGABIO and agalsidase beta.

4 Discussion

In this study, AGABIO was characterized preclinically as a biosimilar to agalsidase beta. At this stage of AGABIO development, a first set analytical characterization assays was performed. A high degree of similarity was observed for all physicochemical and biological properties tested. However, subtle differences in the distribution of sialylated

glycans were observed between agalsidase beta and AGABIO samples. It has been previously reported that sialylated tri- and tetra-antennary *N*-glycans may correlate well with longer half-lives of glycoproteins [23]. The observation that agalsidase beta and AGABIO contained similar amounts of total highly sialylated glycans (tri-plus tetrasialylated glycans) is in line with the comparable pharmacokinetic profile of AGABIO and agalsidase beta in mice.

The pharmacodynamic studies performed in situ in Fabry fibroblasts and in vivo in Fabry knockout mice, showed that the properties of AGABIO are similar to those of agalsidase beta. Despite having a normal lifespan without organ failure, Fabry knockout mice have been shown to biochemically reproduce abnormalities found in patients with Fabry disease, including significant Gb3 [24] and lyso-Gb3 [25] accumulation in plasma and lysosomes of most tissues (particularly liver, spleen, heart, skin, and kidney).

The same animal model and similar fibroblast uptake studies were used in the preclinical studies of JR-051, a biosimilar of agalsidase beta made by Morimoto and coworkers [26]. However, in contrast to the results obtained with the Japanese biosimilar JR-051, AGABIO showed a similar uptake to agalsidase beta in Fabry fibroblasts. This is consistent with the fact that the AGABIO samples showed similar levels of M6P and phosphorylated *N*-glycans (mono- and bi-phosphorylated) compared with agalsidase beta. The content of M6P and glycans containing phosphorylated residues are important for the efficacy of agalsidase. When therapeutic lysosomal enzymes carrying M6P glycans are
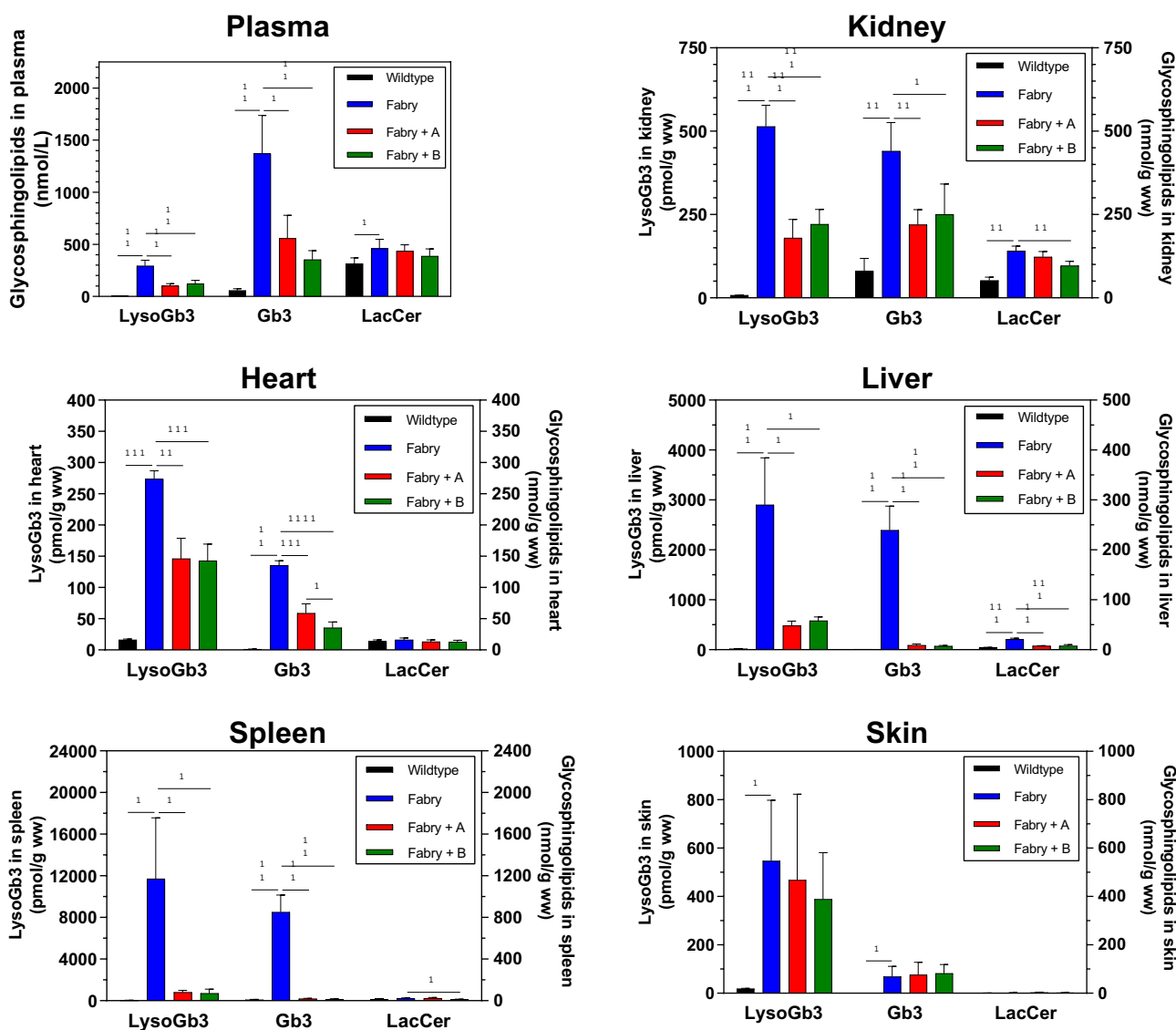
Fig. 8 Glycosphingolipids in plasma and tissues of wild-type mice. Fabry mice and Fabry mice treated with (agalsidase beta) (**A**) or (AGABIO) (**B**). The bars represent the mean ± SD values. Statistical analysis was performed using the Welch's *t*-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. *AGABIO* α-galactosidase A Biosidus, *Gb3* globotriaosylceramide, *LAcCer* lactosylsphingosine, *LysoGb3* globotriaosylsphingosine

administrated to the body, they can be recognized and internalized to cells by M6P receptor at the plasma membranes of target organs. Importantly, the M6P receptor has a higher affinity for biphosphorylated glycans than for monophosphorylated glycans [27–29].

Immunoreactivity to specific agalsidasa beta antibodies in sera from patients with Fabry disease was similar for both products, indicating that there are no significant differences in antigenic determinants. Furthermore, no notable differences in safety profile between AGABIO and agalsidase beta were observed in rats.

The results presented in this manuscript support the biosimilarity of AGABIO to Fabrazyme in terms of quality attributes as well as preclinical pharmacodynamics, pharmacokinetics, and safety profile. As a result, the clinical development program for AGABIO has commenced and clinical trials are ongoing. To date, the comparative phase I study in healthy volunteers has been completed. Preliminary results showed pharmacokinetic with biosimilarity to agalsidase beta, and no safety concerns for AGABIO were observed. A study in patients with Fabry disease to confirm biosimilarity in terms of efficacy, safety, and immunogenicity is already underway. Further clinical analysis of individualized dosing in patients with neutralizing antibodies may lead to improved benefit in these patients.

5 Summary and Conclusions

Our preliminary characterization and preclinical results support the biosimilarity of AGABIO and agalsidase beta. We think that AGABIO may improve the access of enzyme therapy to patients in need at a lower price.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40268-023-00421-x.

Declarations

Conflicts of interest Ana Travella, Melisa Jacobs, Lucas D. Gentillini, Marcelo Criscuolo, and Mariana Papouchado are employed by Biosidus, which manufactures AGABIO. The remaining authors declare no conflict of interest.

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval Written informed consent was obtained to use patients fibroblasts for the study as part of our biobanking protocol, approved by the Ethics Committee.

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

Author contributions André B. P. van Kuilenburg, Carla E. M. Hollak, Marcelo Criscuolo and Mariana Papouchado designed the study. Ana Travella, Melisa Jacobs, Lucas D. Gentillini, René Leen, Karen M. M. Ghauharali-van der Vlugt, Femke S. Beers Stet, Sanne van der Veen, and Mariana Papouchado gathered the data. André B. P. van Kuilenburg, René Leen, Karen M. M. Ghauharali-van der Vlugt, Femke S. Beers Stet, Susan. M. I. Goorden, Sanne van der Veen, and Mariana Papouchado analyzed the data. André B. P. van Kuilenburg, Carla E. M. Hollak, René Leen, Karen M. M. Ghauharali-van der Vlugt, Susan. M. I. Goorden, Sanne van der Veen, Marcelo Criscuolo, and Mariana Papouchado wrote the publication. All authors read and approved the final version

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