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Research paper

Antibodies that neutralize cellular uptake of elosulfase alfa are not associated with reduced efficacy or pharmacodynamic effect in individuals with Morquio A syndrome

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ABSTRACT

Many enzyme replacement therapies (ERTs) for lysosomal storage disorders use the cell-surface cation-independent mannose-6 phosphate receptor (CI-M6PR) to deliver ERTs to the lysosome. However, neutralizing antibodies (NAb) may interfere with this process. We previously reported that most individuals with Morquio A who received elosulfase alfa in the phase 3 MOR-004 trial tested positive for NAbs capable of interfering with binding to CI-M6PR ectodomain in an ELISA-based assay. However, no correlation was detected between NAb occurrence and clinical efficacy or pharmacodynamics. To quantify and better characterize the impact of NAbs, we developed a functional cell-based flow cytometry assay with a titer step that detects antibodies capable of interfering with elosulfase alfa uptake. Serum samples collected during the MOR-004 trial were tested and titers were determined. Consistent with earlier findings on NAb positivity, no correlations were observed between NAb titers and the clinical outcomes of elosulfase alfa-treated individuals with Morquio A.

1. Introduction

The mucopolysaccharidosis (MPS) diseases are a group of lysosomal storage disease caused by a deficiency of enzymes catalyzing the stepwise degradation of glycosaminoglycans (GAGs). Individuals with the autosomal recessive MPS disorder Morquio A syndrome (mucopolysaccharidosis IVA, MPS IVA; OMIM 253000) have mutations in the gene encoding the enzyme *N*-acetylgalactosamine-6-sulfatase (GALNS; EC 3.1.6.4), resulting in deficient GALNS enzyme activity and accumulation of the GAGs keratan sulfate and chondroitin-6-sulfate in the lysosomes of individuals with Morquio A syndrome. Although symptoms can vary, Morquio A syndrome generally manifests as a progressive disorder with multiple organ and tissue involvement that is characterized by restricted growth, severe skeletal malformations, corneal opacity, restricted hearing, and premature death (Harmatz et al., 2013; Hendriksz et al., 2013). Elosulfase alfa (Vimizim®; rhGALNS; BMN 110) is a US Food and Drug Administration-approved enzyme replacement therapy (ERT) for the treatment of Morquio A syndrome. The previously reported results of a pivotal phase 3 clinical trial, MOR-004, which demonstrated that elosulfase alfa administered either weekly (QW) or every other week (QOW) for 24 weeks to individuals with Morquio A syndrome had an acceptable safety profile, and QW dosing significantly improved 6-min walk test (6MWT) distance, 3-min stair climb test (3MSCT), and respiratory function (maximum voluntary ventilation [MVV]). In addition, the high urine levels of the pharmacodynamic marker keratan sulfate (uKS) characteristic of individuals with Morquio A syndrome were significantly reduced with elosulfase alfa treatment (Hendriksz et al., 2014).

A number of ERTs for the treatment of various lysosomal storage disorders, including elosulfase alfa, contain mannose-6-phosphate (M6P) moieties and depend on the CI-M6PR transmembrane receptor for internalization and trafficking to the lysosome. Biological therapies can elicit an immune response, resulting in the generation of antidrug antibodies capable of binding to the drug product and, in some cases, interfering with receptor binding on the target cell. Furthermore, these antibodies may negatively impact efficacy (Brooks et al., 2003; Desnick and Schuchman, 2012; Banugaria et al., 2011). The MOR-004 study showed that all individuals treated with elosulfase alfa developed drug-specific total antibodies (TAb). A subset of drug-specific antibodies

Abbreviations: TAb, total antibody; NAb, neutralizing antibody; LSD, lysosomal storage diseases; GAGs, glycosaminoglycans; ERTs, enzyme replacement therapies; rhGALNS, recombinant human *N*-acetylgalactosamine-6-sulfatase; 6MWT, six minute walk test; 3MSC, three minute stair climb; MVV, maximum voluntary ventilation; uKS, urine keratan sulfate; M6P, mannose-6-phosphate; CI-M6PR, cation-independent mannose-6-phosphate receptor; TQC, titer quality control; LQC, low quality control; HQC, high quality control; NPS, normal (human) pooled serum; SI, signal inhibition; AbPC, antibody positive control; MRD, minimum required dilution; NQC, negative quality control; MFI, median fluorescence intensity; CCP, confirmation cut point; SCP, screening cut point; TCP, titer cut point.

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may be elosulfase alfa-specific neutralizing antibodies (NAbs) capable of interfering with CI-M6PR binding, positivity for which was revealed using an ELISA-based in vitro assay (CI-M6PR binding assay). Although elosulfase alfa-specific antibody development was universal among treated individuals in the MOR-004 study, no relationship was detected between TAb titers or NAb positivity and the magnitude of physical improvements or reductions in uKS levels (Schweighardt et al., 2015).

In this study, we describe a novel, cell-based flow cytometry method for identifying and titering NAbs capable of inhibiting cellular uptake (functional NAb assay) to determine if this measurement might be more clinically meaningful than measuring inhibition of receptor binding, as was measured in the original study. Serum samples were co-incubated with Alexa488-labeled rhGALNS, and flow cytometry was used to detect interference of drug internalization by human Jurkat cells, which express transmembrane CI-M6PR on the cell surface. Assay sensitivity, precision, specificity, selectivity, as well as drug tolerance were determined, and cut points were calculated to set thresholds for assessing positive samples. In support of our previous findings that NAb positivity had no correlation with clinical outcomes or pharmacodynamic effects (Schweighardt et al., 2015), testing of MOR-004 samples with the functional NAb assay demonstrated that NAb titer is not correlated with efficacy outcomes or pharmacodynamic effects of elosulfase alfa in individuals with Morquio A syndrome.

2. Materials and methods

2.1. Clinical study design

MOR-004 (NCT01275066) was a phase 3, randomized, double-blind, placebo-controlled study designed to assess the safety and efficacy of elosulfase alfa administered at 2.0 mg/kg QW or QOW in individuals with Morquio A syndrome. The study protocol was approved at each participating clinical site by an institutional review board, independent ethics committee, or research ethics board. Prior to entering the study, each participant or his/her legally authorized representative provided written informed consent. The assigned treatment was blinded to investigators, site personnel, and patients until the completion of the final analysis. Of the 176 study participants, 59 received placebo, 58 received elosulfase alfa 2.0 mg/kg QW, and 59 received elosulfase alfa 2.0 mg/kg QOW. The MOR-004 study design, inclusion criteria, and endpoints were reported previously (Hendriksz et al., 2014).

2.2. Immunogenicity testing

Serum samples were collected from all individuals for immunogenicity testing prior to dose administration at baseline, weeks 2 and 4, and every 4 weeks thereafter (or within 1 week of the early-termination visit). Detailed methods for sample preparation and the CI-M6PR binding assay were reported previously (Schweighardt et al., 2015). In brief, TAb titers were determined by a validated bridging electrochemiluminescence (ECL) assay, and positivity for NAb was determined by a validated in vitro assay that assessed interference of elosulfase alfa binding to CI-M6PR immobilized on an ELISA plate.

2.3. Functional NAb assay

Alexa488 was conjugated to rhGALNS (BioMarin Pharmaceutical Inc.) using an Alexa Fluor 488 Protein Labeling Kit (Molecular Probes) according to the manufacturer's protocol. Alexa488-conjugated rhGALNS had an average labeling ratio of 1.75 mol of Alexa488 per mole of rhGALNS as determined by absorbance at 494 nm. Quality controls, serum samples, immunodepleted samples for confirmation, or 3-fold serially diluted serum samples for titration were diluted 1:2.5 in serum-free RPMI-1640 with high glucose, L-glutamine, and HEPES (ATCC) and incubated with 0.4 μg/mL Alexa488-conjugated rhGALNS in duplicate wells overnight at 4 °C. Samples were then added to Jurkat cells (clone E6-1, ATCC) plated at 7.5×10^4 cells per well in 96-well round bottom plates and incubated for 3 h at 37 °C and 5% CO_2. Cells were washed 3 times in PBS after centrifugation at 310 ×*g*, stained with 1:1000 LIVE/DEAD Fixable Red Dead Cell Stain Kit (Molecular Probes) for 15 min, centrifuged, and fixed with 1% paraformaldehyde for 10 to 15 min at room temperature or up to 72 h at 2 °C to 8 °C. Fixed cells were analyzed for fluorescence on a BD LSRII flow cytometer using BD FACSDiva software (BD Biosciences). Dead cells were excluded from the analysis, and approximately 10,000 events were recorded. Uptake of Alexa488-conjugated rhGALNS was reported as mean of duplicate median fluorescence intensity (MFI) measurements from adjacent wells.

2.4. Confirmation assay sample preparation

Samples that met the threshold for NAb positivity in the screening assay were verified using a confirmation assay. Briefly, tosyl-activated magnetic beads (Dynabeads®; ThermoFisher Scientific) conjugated with rhGALNS were washed 3 times in coupling buffer (1× DPBS, 0.01% Tween) and added to wells, and the buffer was removed. Samples diluted 1:2.5 in serum-free RPMI-1640 were added to the wells with the beads and incubated with shaking at 800 rpm for 1 h. The beads and samples were separated, and the eluent was tested in the functional NAb assay (described in Section 2.3).

2.5. Calculations and statistics

Percentage signal inhibition (%SI) was calculated using the equation $\%SI = [1 - (\textit{mean MFI of sample} / \textit{mean MFI of cut-point control})] \times 100$. Confirmatory assay data were analyzed as recovery ratio, defined as *mean confirmatory assay MFI of sample / mean screening assay MFI of*

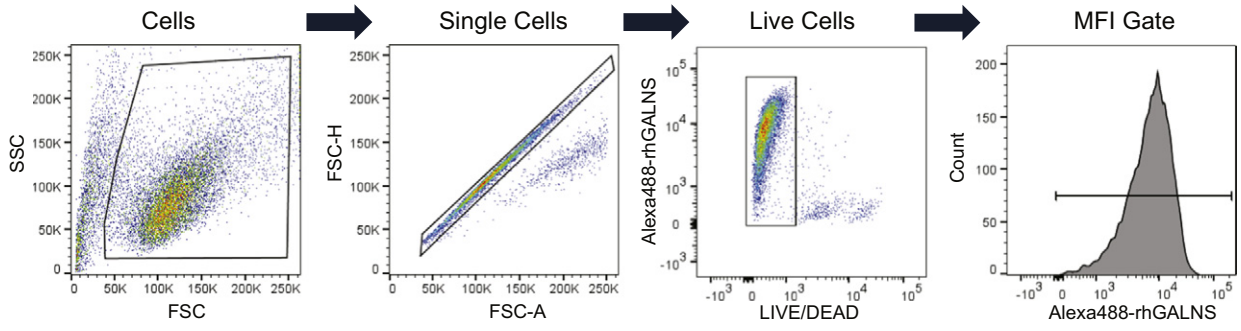

Fig. 1. Assay platform flow cytometry gating strategy. Jurkat cells were separated from all events captured by the instrument detector using the forward scatter (FSC) and side scatter (SSC) channels. Cell doublets were then removed by gating on single cells using the FSC area (FSC-A) and FSC height (FSC-H) parameters. Dead cells were removed from analysis by gating on cells not labeled with LIVE/DEAD cell stain. Median fluorescence intensity (MFI) of Alexa488-rhGALNS was calculated using live cells plotted as a histogram. Cells incubated with or without Alexa488-rhGALNS (red and blue histograms, respectively) are shown.

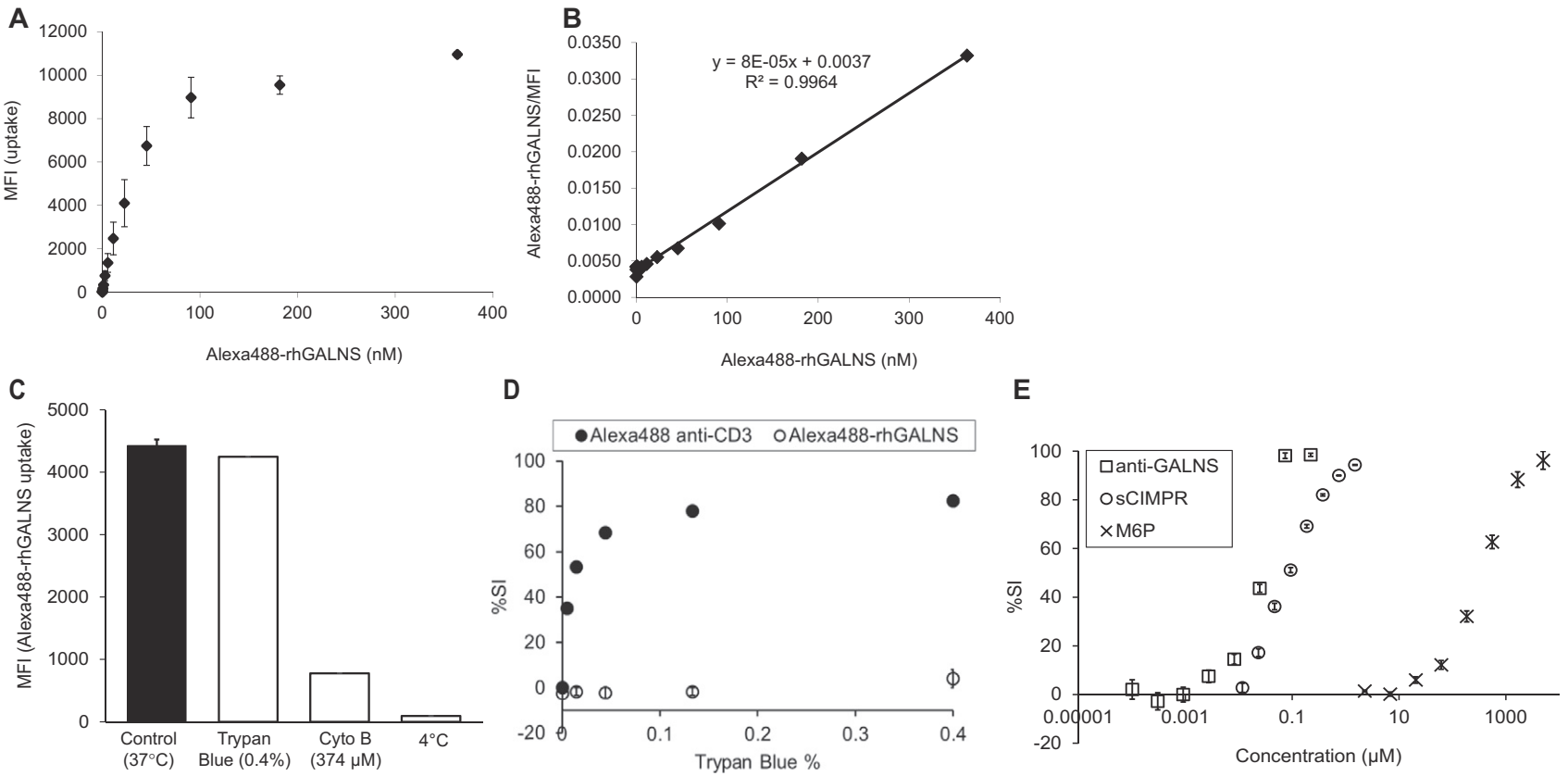

Fig. 2. Uptake of Alexa488-rhGALNS by Jurkat cells measured by flow cytometry. (A) Dose-response curve showing uptake (MFI) of Alexa488-rhGALNS (0–363.6 nM). (B) Hanes-Woolf plot between MFI and concentration of Alexa488-rhGALNS. (C) Uptake of Alexa488-rhGALNS (MFI) following no treatment or treatment of cells with trypan blue, cytochalasin B, or incubation at 4 °C. Error bars (±standard deviation) for each condition are included but may be indistinguishable from the top of histogram bar. (D) Uptake of Alexa488-rhGALNS (MFI) and Alexa488-anti-CD3 following treatment of cells with 0.005%–0.4% trypan blue. Error bars represent the means ± SDs. (E) Percent signal inhibition (%SI; Alexa488-rhGALNS uptake MFI relative to non-treated control) following treatment of cells with increasing concentrations of sCI-M6PR, M6P, or goat anti-GALNS antibody.

sample. The titer was calculated as the interpolated dilution factor at which the sample crossed the titer cut point determined by linear regression analysis (Microsoft Excel).

2.6. Confocal imaging

Internalization of Alexa488-rhGALNS and trafficking to lysosomes was confirmed by confocal microscopy by incubating cells from the functional NAb assay described in Section 2.3 with 50 nM LysoTracker Red (Molecular Probes) at 37 °C for 1 h. Cells were then washed with serum-free RPMI-1640 and mounted with Prolong Gold Antifade Mountant with DAPI (Molecular Probes), sealed, and visualized using a Leica SP8 confocal microscope (Leica Microsystems). Confocal *Z*-stacks were acquired using a 63× Plan-Apo objective with 4× zoom and LASX software (Leica Microsystems). Z-stacks were rendered in 3-D and exported using Volocity software version 6.3 (PerkinElmer).

3. Results

3.1. Functional NAb assay development

3.1.1. Mechanism of Alexa488-rhGALNS uptake by Jurkat cells

The flow cytometry gating strategy restricted MFI analysis to single, live Jurkat cells for measurement of Alexa488-rhGALNS uptake (Fig. 1). To assess optimal rhGALNS uptake conditions by Jurkat cells in vitro, an Alexa488-rhGALNS dose-response curve was generated (0 to 363.6 nM or 0 to 40 μg/mL). A dose-dependent increase in MFI was observed in Jurkat cells incubated with Alexa488-rhGALNS, with a background of untreated cells detected at an MFI of 43.7 ± 4.7 (K_{uptake} = 46.3 nM by Hanes-Woolf plot linear regression analysis; Fig. 2A and B).

To determine if the MFI signal detected after incubation of cells with Alexa488-rhGALNS resulted from internalized drug or drug bound to the cell surface, a series of experiments was conducted whereby cells incubated with Alexa488-rhGALNS (0.4 μg/mL) in the presence of 20% pooled human serum were either placed at 4 °C, quenched with trypan blue, or treated with cytochalasin B. Cells incubated at 4 °C, which allowed for drug binding but not uptake, produced an MFI of 92.9 ± 1.50 compared with an MFI of 4425 ± 98.6 when cells were incubated for the same length of time at 37 °C (Fig. 2C), suggesting that MFI predominantly measured internalization of Alexa488-rhGALNS. Next, cells post-incubation with Alexa488-rhGALNS were treated with trypan blue, which is impermeable to vital cells, quenches Alexa488 fluorescence (Sahlin et al., 1983), and was expected to reduce MFI signals if Alexa488-rhGALNS was bound to the cell surface. As a control to ensure that trypan blue quenched Alexa488 on the surface of Jurkat cells, cells were labeled with an Alexa488-conjugated mouse monoclonal antibody (Clone SP34-2) that binds CD3 expressed on the cell surface of T cells (Osman et al., 1992). Increasing concentrations of trypan blue reduced the fluorescence of cells treated with Alexa488-labeled anti-CD3 (Fig. 2D). However, concentrations of trypan blue up to 0.4% had no impact on Alexa488-rhGALNS MFI values, suggesting that almost all of the MFI signal emitted from Alexa488-rhGALNS-treated cells was the result of drug internalization within cells (Fig. 2C and D). To determine whether drug internalization was a result of endocytosis, cells were pretreated with cytochalasin B for 1 h prior to incubation with Alexa488-rhGALNS. MFI signal emitted from Alexa488-rhGALNS-treated cells was reduced to 777 ± 4.2 MFI in the presence of 374 μM cytochalasin B (Fig. 2C). To confirm the uptake mechanism of Alexa488-rhGALNS via CI-M6PR, cells were incubated with Alexa488-rhGALNS in the presence of excess soluble CI-M6PR (sCI-M6PR) or M6P to compete for CI-M6PR binding sites, or an affinity-purified goat anti-rhGALNS polyclonal antibody to neutralize rhGALNS binding. Increasing concentrations of sCI-M6PR, M6P, and anti-rhGALNS antibody dose dependently abrogated Alexa488-rhGALNS uptake (IC_{50} [μM] = 0.085 for sCI-M6PR, 452.9 for M6P, and 0.027 for anti-rhGALNS; Fig. 2E). IC_{50} values for M6P were higher than those for sCI-M6PR or anti-rhGALNS, likely due to competition with multiple M6P moieties on rhGALNS and additional proteins containing M6P binding sites. Furthermore, confocal imaging of Alexa488-rhGALNS-treated cells revealed that Alexa488-rhGALNS co-localized with lysosomes, confirming that labeled rhGALNS is efficiently internalized and trafficked to the lysosomes (Fig. 3). Collectively, these data indicate that internalization of Alexa488-rhGALNS occurs through M6P-mediated receptor binding to CI-M6PR followed by endocytosis and trafficking to the lysosome.

3.1.2. Determination of optimal Alexa488-rhGALNS concentration and serum dilution for detection of inhibitory antibodies

A series of dose-response curves was generated to determine the optimal drug dose that would provide detection of approximately 1 μg/mL affinity-purified goat anti-rhGALNS polyclonal antibody positive control (AbPC) in the presence of 20% pooled human serum. Alexa488-rhGALNS was added to cell media at a final concentration of 0.156 μg/mL to 2.50 μg/mL in the presence of increasing concentrations of AbPC ranging from 0.046 to 100 μg/mL. AbPC interfered with uptake of Alexa488-rhGALNS in a dose-dependent manner (Fig. 4A). These data were then transformed and plotted as the relative fold change over the maximum concentration of AbPC tested (100 μg/mL; Fig. 4B) to determine the dose of Alexa488-rhGALNS that would provide a sensitivity of approximately 1 μg/mL for AbPC. A concentration of 0.313 μg/mL Alexa488-rhGALNS gave the highest signal-to-noise ratio, and an approximate maximal SI of 92% was observed in the presence of 11.1 μg/mL AbPC. Together, these data led to the selection of 0.4 μg/mL Alexa488-rhGALNS for further assay development, including the assay sensitivity analyses described in Section 3.1.4.

To characterize the potential effect of interference from serum components (matrix interference) on detection of NAbs, NPS samples spiked with AbPC at concentrations ranging from 0.046 to 100 μg/mL were diluted 2.5-, 5-, 10-, and 20-fold in serum-free media and mixed

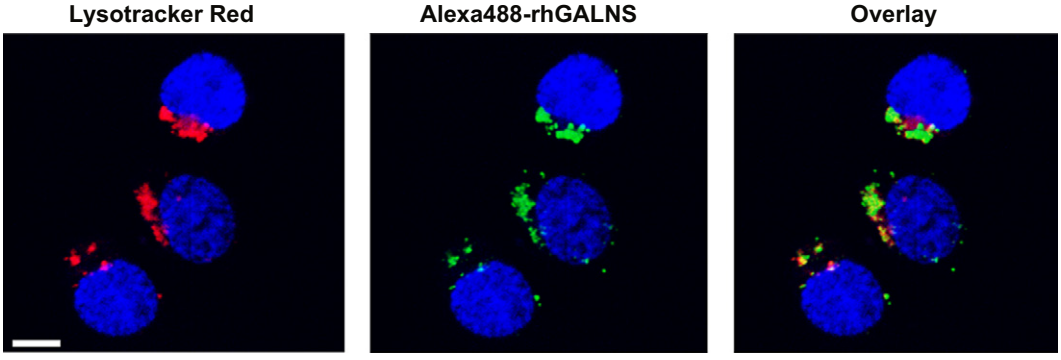

Fig. 3. Alexa488-rhGALNS traffics to cell lysosomes. Confocal images of fixed Jurkat cells incubated with LysoTracker Red and Alexa488-rhGALNS (green). Cells were counterstained with DAPI nuclear stain (blue) just prior to imaging. Scale bar = 5 μm.

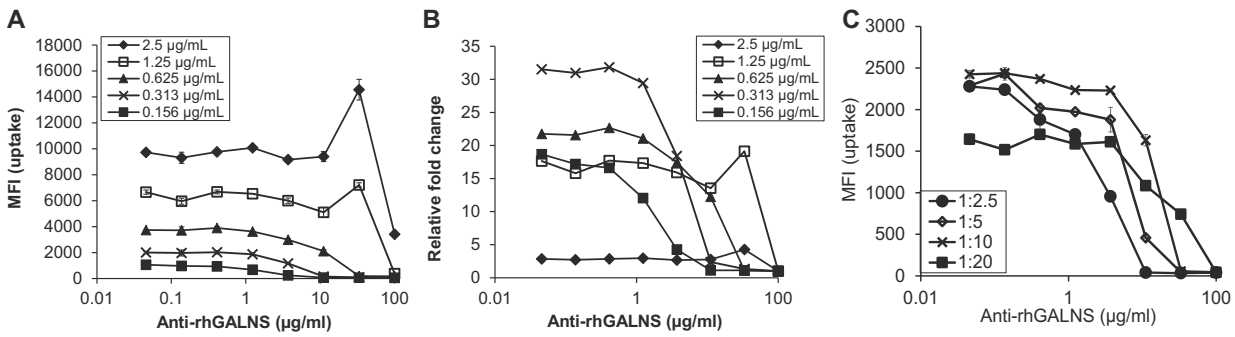

Fig. 4. Determination of optimal Alexa488-rhGALNS concentration for detection of inhibitory antibodies. (A) Jurkat cells treated with varying concentrations of Alexa488-rhGALNS (0.156–2.5 μg/mL) premixed with the indicated concentrations of AbPC (anti-rhGALNS). (B) Data from (A) presented as the relative MFI fold change over the MFI from the maximum concentration of AbPC tested (100 μg/ml). (C) Alexa488-rhGALNS uptake at the indicated concentrations of AbPC in the presence of different serum dilutions (1:2.5–1:20).

1:1 with 0.4 μg/mL Alexa488-rhGALNS. A dilution of 1:2.5 (equivalent to 20% pooled human serum after mixing with Alexa488-rhGALNS) showed inhibition of Alexa488-rhGALNS uptake at <1 μg/mL and inhibited uptake at the lowest AbPC concentration relative to the other dilutions (Fig. 4C). The 1:20 dilution reduced detection of Alexa488-rhGALNS, even in the absence of inhibition by AbPC (Fig. 4C). This result indicated that low NPS concentrations can independently reduce drug uptake in this assay, and suggests that factors present in human serum may stimulate uptake of Alexa488-rhGALNS by CI-M6PR in Jurkat cells. Therefore, a minimum required dilution (MRD) of 1:2.5 was chosen to provide optimal sensitivity.

3.1.3. Assay cut points for the cellular uptake NAb assay

A tiered clinical testing strategy was adopted to screen, confirm, and titer NAb samples that were confirmed positive for binding anti-elosulfase antibodies (also referred to as total anti-elosulfase antibody, TAb) (Mire-Sluis et al., 2004). To define a NAb assay positive/negative cut point, serum was collected from drug-naïve individuals with Morquio A syndrome ($n = 48$) and analyzed in duplicate in 6 separate experiments performed by 2 analysts over 2 days. At the time of assay development, serum was unavailable from individuals with Morquio A syndrome; thus, we used pooled serum from healthy donors without Morquio A syndrome. The sample %SI data distribution for each run was analyzed for normality using the Shapiro-Wilk test (JMP software, SAS Institute Inc.). Outliers, identified using box plot analysis (JMP), were excluded conservatively by removing the minimum number required to achieve a normal distribution for each run (Shankar et al., 2008). This approach resulted in normal distributions being established after exclusion of 2 outliers (0.69%) from the total screening data set. Pooled variance was then used to calculate the screening cut point (SCP) based on the 95th percentile to avoid false-negatives responses, resulting in an SCP of 14.02% SI. Thus, individual serum samples that reduced signal by ≥14.02% compared with control serum in the screening step were considered reactive (potentially positive) during sample testing.

For samples that screened as reactive (potentially positive), a confirmation step assessed whether the sample signal was increased by depletion of rhGALNS-specific antibodies, as would be expected of true-positive samples. Drug-conjugated beads were used to deplete rhGALNS-specific antibodies from serum prior to retesting in the cell-based assay. A confirmation cut point (CCP) was established by depleting the same drug-naïve serum samples described above and comparing each with its corresponding non-depleted sample tested on the same plate. A fixed CCP (recovery ratio of immunodepleted sample signal/non-depleted sample signal) was determined by first evaluating results from each run for normality and outliers. Lack of normality was observed in 2 of 6 runs based on the Shapiro-Wilk test ($p \leq 0.05$), but the removal of the minimum number of outliers from the data set ($n = 4$ [1.41% of data set]) resulted in a normal distribution for all runs ($p \geq 0.05$). Pooled variance was then used to calculate the fixed CCP based on the 99th percentile of the normal distribution model of the data, resulting in a recovery ratio CCP of 1.24. Thus, samples that were reactive in the screening step and had a recovery ratio ≥ 1.24 in the confirmatory step were considered positive during sample testing.

NAb-positive samples were subsequently 3-fold serially diluted (1:2.5, 1:7.5, 1:22.5, etc.) in a titer experiment to determine relative NAb levels. A titer cut-point (TCP) of 25.76% SI was calculated based on the 99.9th percentile of the normal distribution data model described for the screening step. To extrapolate a titer value, at least 1 dilution of a positive sample had to drop below the TCP.

Once samples from drug-naïve individuals with Morquio A syndrome became available from the MOR-004 clinical trial, the screening and confirmation cut points established during assay validation were verified using raw MFI data from 115 baseline and placebo samples collected from 60 drug-naïve individuals with Morquio A syndrome and tested over 4 months by 4 analysts. Using a similar statistical approach to the one described above for donors without Morquio A syndrome, the target treatment population cut points were comparable to the original cellular uptake NAb assay cut points derived from non-affected individuals (Table 1). For the purpose of a concordance assessment (described in Section 3.2), the cut points of the ELISA-based CI-M6PR binding assay previously used to detect anti-elosulfase alfa NAb (Schweighardt et al., 2015) were also confirmed in the 115 baseline and placebo samples (Table 1). NAb assay concordance using the cut points from Morquio A individuals was 90% (see Section 3.2), supporting the use of cut points established with non-affected individuals in the validated assay.

3.1.4. Sensitivity and precision

For detection of neutralizing antibodies elicited by biologic therapies, industry guidelines recommend an assay sensitivity of ≤1 μg/mL positive control antibody (Gupta et al., 2007). To determine the lowest concentration of antibody that can be detected in the assay (i.e. sensitivity or limit of detection [LOD]), AbPC was spiked into NPS at 20 μg/mL and serially diluted 3-fold to generate an 8-point series ($n = 18$). For each dilution series, values crossed the screening cut point with the %SI signals from at least 2 dilutions ≥ SCP and at least 1 dilution < SCP, and the mean neat concentration of AbPC was calculated from the

Table 1
Receptor binding and cellular uptake NAb assay cut points based on drug-naïve healthy or target-treatment population samples.

Cut point type	Normal serum		Morquio A serum	
	Cell-based assay	Ligand-binding assay	Cell-based assay	Ligand-binding assay
SCP (%SI)	14.0	14.8	19.6	22.8
CCP (recovery ratio)	1.24	1.05	1.15	1.09
TCP (%SI)	25.8	N/A	30.0	N/A

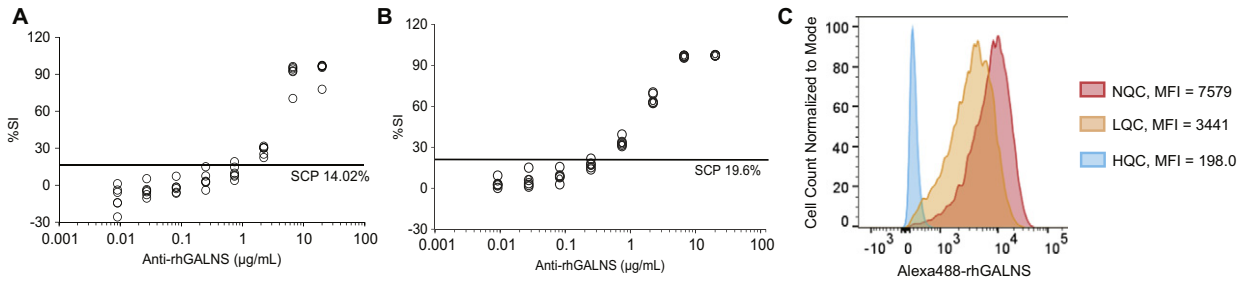

Fig. 5. Assay sensitivity and example quality control data. Individual %SI means for 1:3 serial dilutions, starting at 20 μg/mL, from 6 independent validation runs to establish the assay sensitivity in NPS (A) or in pooled serum from individuals with Morquio A syndrome (B). The assay screening cut point (SCP) of 14.02% in (A) or 19.6% in (B) is shown to demonstrate where serial dilutions crossed the cut point. (C) Representative flow cytometry histograms and Alexa488-rhGALNS MFI for NQC (0 μg/mL), LQC (3 μg/mL), and HQC (20 μg/mL) samples.

interpolated concentration at the SCP using linear regression analysis and correction for the MRD. The sensitivity and LOD of the assay was determined to be 0.97 μg/mL (Fig. 5A).

Once study samples became available, the sensitivity of the method was confirmed using serum from individuals with Morquio A syndrome prior to treatment with elosulfase alfa. From 6 dilution curves assayed over 3 days by a single analyst, a mean value of 0.30 μg/mL was interpolated at the SCP for samples from individuals with Morquio A (Fig. 5B), a value similar to the 0.97 μg/mL value obtained during assay validation using NPS (Fig. 5A).

Assay precision describes the closeness of replicate measurements of the same sample on the same plate (intra-assay) or between plates (inter-assay). QC concentrations were selected to assess assay precision and monitor the assay performance in the presence of high and low concentrations of NAb. AbPC spiked into NPS at 20 μg/mL and 3 μg/mL were chosen as high quality control [HQC] and low quality control [LQC] concentrations, respectively, based on AbPC performance when serially diluted (Fig. 5A). To evaluate assay precision, AbPC was spiked into NPS at 20 μg/mL (HQC and titer quality control [TQC] concentrations), 3 μg/mL (LQC concentration) or unspiked (negative quality control [NQC] concentration) and the intra-assay and inter-assay coefficient of variation (CV) was assessed. An example of the raw histogram data for each of these QC samples is presented in Fig. 5C. The intra- and inter-assay precision was 0.60% and 0.65% CV for the HQC, 12.02% and 17.62% CV for the LQC, 14.88% and 16.50% CV for the NQC, and 12.41% and 28.04% CV for LQC samples that were confirmed (Table 2). Intra-batch n values vary slightly between QCs due to differences in the number of QCs tested on each plate and exclusion of several samples due to instrument malfunction. Titration of TQC samples showed that 14 of 18 results were within ±one 3-fold dilution of the median interpolated dilution of 28.54 (data not shown).

3.1.5. Specificity and selectivity

To evaluate the specificity of rhGALNS neutralization by NAbs in the uptake assay, samples were prepared by spiking both NPS and NPS + LQC AbPC with a non-specific goat IgG—negative control at a concentration of 3 μg/mL. NPS spiked with only non-specific goat IgG antibody tested negative in the screening and confirmatory assays. NPS + LQC AbPC, both with and without non-specific goat IgG antibody, screened and confirmed positive (Table 3), suggesting that the assay is specific for anti-rhGALNS antibodies.

To assess effects of serum components on the detection of anti-rhGALNS in this assay, serum from 10 (5 male/5 female) individual donors as well as 4 lipemic and 4 hemolyzed sera from drug-naïve, healthy individuals (without Morquio A syndrome) were tested using the screening assay. To each of these samples, 0 μg/mL (NQC concentration) or 3 μg/mL (LQC concentration), of AbPC was added to evaluate assay selectivity. All normal serum samples (10/10) spiked with LQC were potentially positive/reactive in the screening step (Fig. 6A). Of the 4 lipemic samples spiked with LQC concentration of AbPC, 1 of 4 (25%) screened positive, suggesting that there may be interference from serum lipids in the detection of lower levels of anti-rhGALNS. Conversely, all of the hemolytic samples spiked with LQC screened positive/reactive, suggesting that hemolysis does not interfere with the detection of anti-rhGALNS.

Assay selectivity was confirmed in drug-naïve baseline samples from the MOR-004 study. Selectivity was performed in 10 individual samples by adding AbPC to the LQC concentration in an aliquot of each sample. These spiked MOR-004 baseline samples, as well as the corresponding unspiked serum, were assayed together on a single plate. No matrix interference was observed using either the healthy or target treatment population-based SCP. All 10 unspiked samples (100%) screened negative and all 10 samples (100%) containing LQC AbPC screened positive using the target population-derived SCP (Fig. 6B).

3.1.6. Drug tolerance

The susceptibility of the assay to interference from circulating levels of drug was evaluated using NPS spiked with or without LQC or HQC concentrations of the AbPC in the presence of 0 to 2 μg/mL unlabeled rhGALNS. As expected, LQC and HQC AbPC screened positive in the absence of unlabeled rhGALNS (Table 4). When low concentrations of unlabeled rhGALNS (≤0.074 μg/mL) were added to LQC or HQC AbPC, the %SIs were comparable to those measured in the absence of unlabeled rhGALNS (Table 4), with slight differences likely due inherent assay

Table 2
Intra-batch and inter-batch precision of the functional NAb assay. Note the intra-batch "N" is an average from runs containing 2 or 3 sets of QCs.

	Intra-batch (within run) statistics (Pooled)				Inter-batch (between run) statistics (ANOVA)			
	N (samples)	Average	SD	%CV	N (samples)	Average	SD	%CV
		% SI				% SI		
HQC	2.22	96.80	0.58	0.6	67	96.80	0.63	0.65
LQC	2.26	60.28	7.24	12.02	68	60.74	10.71	17.62
		Median MFI				Median MFI		
NQC	2.27	8654.6	1287.6	14.88	66	8691.7	1434.0	16.5
		Recovery ratio				Recovery ratio		
LQC-C	2.33	3.02	0.37	12.41	54	3.05	0.85	28.04

Table 3
Evaluation of non-specific IgG in the functional NAb assay.

	LQC	Non-specific IgG	%SI	%CV	Recovery ratio	Above SCP	Above CCP	Result
Screening assay	+	+	25.54	4.38	N/A	Yes	N/A	Positive
	+	−	31.86	9.28	N/A	Yes	N/A	Positive
	−	+	−6.32	9.67	N/A	No	N/A	Negative
	−	−	−1.02	7.18	N/A	No	N/A	Negative
Confirmatory assay	+	+	N/A	2.04	1.42	N/A	Yes	Positive
	+	−	N/A	3.46	1.55	N/A	Yes	Positive
	−	+	N/A	3.87	1.05	N/A	No	Negative
	−	−	N/A	7.24	1.13	N/A	No	Negative

variability. All LQC and HQC AbPC-spiked samples screened positive in the presence of 2 μg/mL unlabeled rhGALNS. However, concentrations ≥0.22 μg/mL of unlabeled rhGALNS reduced Alexa488-rhGALNS uptake in NPS without AbPC. These data suggest that unlabeled rhGALNS inhibited uptake of Alexa488-rhGALNS and indicate that the assay can tolerate up to 0.22 μg/mL rhGALNS introduced via patient serum. Since NAb samples were collected from individuals with Morquio A syndrome just prior to dosing, when elosulfase alfa has been completely cleared (Qi et al., 2014), it is unlikely that elosulfase alfa would be present in samples at concentrations sufficient to interfere with the assay.

3.2. NAb titers and clinical outcomes

3.2.1. High concordance between functional NAb assay and CI-M6PR binding assay

To compare the functional NAb assay with the previously reported ELISA-based CI-M6PR binding assay for detecting NAb in elosulfase alfa-treated individuals with Morquio A syndrome (Schweighardt et al., 2015), serum samples from the MOR-004 trial were reanalyzed with the cell-based assay. Sufficient serum-sample volumes were available for the majority (842/847 [99%]) of MOR-004 serum sample collection time points to obtain confirmed positive/negative results. The overall concordance was high (89%) between NAb positivity in the receptor binding assay and the cell-based assay. Furthermore, the concordance was consistent over the 24-week treatment period (Fig. 7A). Concordance between the assays at each study visit week is presented in Table 5.

TAb titers detected using the CI-M6PR binding assay reported previously (Schweighardt et al., 2015) were compared to the time course for NAb titer development detected with the functional NAb assay (Fig. 7B). Titers of both TAbs and NAbs increased rapidly during the first 8 weeks of treatment then plateaued from week 8 to week 24 in both the QOW and QW treatment groups (Fig. 7B). Although the frequency of drug administration had minimal effect on TAb and NAb titers at week 24, the QW treatment group appeared to have higher titers of both TAbs and NAbs in the initial 4 weeks of treatment. Subjects in both the QOW and QW treatment groups developed different NAb titer courses over 24 weeks (Fig. 7C and D). Titers either initially developed and then remained relatively constant or gradually declined, or showed an intermittent pattern (Fig. 7C and D).

3.2.2. NAb titers and efficacy responses at week 24

The primary efficacy measure in the MOR-004 study was the distance walked in a 6MWT, which provides a measure of endurance. Keratan sulfate, an rhGALNS substrate, was also measured in individuals' urine (uKS) to track the pharmacodynamic effect of treatment with elosulfase alfa. NAb titers detected with the functional NAb assay were plotted against the change from baseline to week 24 in 6MWT distance, and the percentage change in uKS from baseline. No association was observed between NAb titer and 6MWT (Fig. 8A) or NAb titer and uKS (Fig. 8B) in individuals dosed weekly or every 2 weeks. These data indicate that NAb titers had no detrimental impact on elosulfase alfa efficacy during the 24-week treatment period.

3.2.3. Effect of NAb titer on elosulfase alfa pharmacokinetics

In order to further explore the association between NAbs and pharmacokinetics, NAb titers from the functional NAb assay were compared to total clearance (CL) of drug after intravenous administration and elimination half-life ($t_{1/2}$). Increased NAb titers were associated with prolonged $t_{1/2}$ in both the QOW and QW cohorts (Fig. 9A). Both cohorts also displayed a trend toward a correlation between increased NAb

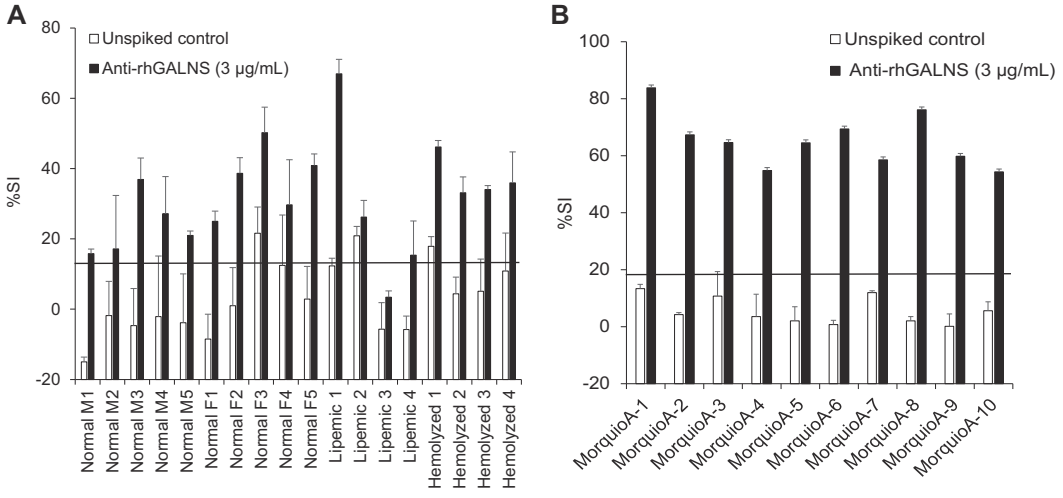

Fig. 6. Interference of matrix on the detection of anti-elosulfase alfa antibodies. %SI data from 5 male and 5 female healthy individual serum samples, as well as 4 lipemic and 4 hemolyzed serum samples, spiked with 3 μg/ml anti-rhGALNS (LQC) or unspiked controls. (B) Drug-naïve serum samples from 5 male and 5 female individuals with Morquio A syndrome spiked with 3 μg/mL AbPC (LQC) or unspiked controls. The assay screening cut point of 14.02% in (A) or 19.6% in (B) is shown as a line to demonstrate where samples cross the cut point. Bars represent the means (±standard deviation) of samples tested in 2 independent experiments over 2 days.

Table 4
Drug tolerance evaluated using NPS-, LQC-, and HQC-spiked samples in the presence of increasing elosulfase alfa concentrations. Entries in bold are interpreted as sample results that test negative to indicate the concentration of free drug that interferes with detection of the LQC or HQC samples. Sample results with an asterisk indicate where free elosulfase alfa directly competes with Alexa488-rhGALNS.

Elosulfase alfa (μg/mL)	NHPS (%SI)	LQC (%SI)	HQC (%SI)
2.00	55.42*	53.63*	**22.90**
0.67	26.94*	**16.72**	95.08
0.22	**8.00**	**23.21**	95.35
0.074	**−5.58**	50.70	95.76
0.025	**−7.21**	66.34	95.47
0.008	**3.79**	69.97	95.26
0.0027	**−10.84**	61.71	95.26
0	**−13.25**	61.34	95.02

titers and decreased CL (Fig. 9B). Together these results suggest that NAbs may slow the clearance of elosulfase alfa from plasma.

4. Discussion

Many approved and investigational ERTs for lysosomal storage diseases traffic drugs from the extracellular space to lysosomes by using CI-M6PR, a transmembrane receptor with binding sites for M6P and IGF-2. Immunogenicity is a concern with biologic therapies, and NAb generated against an ERT may influence therapeutic outcomes (Brooks et al., 2003; Desnick & Schuchman, 2012; Banugaria et al., 2011; Dvorak-Ewell et al., 2010). NAbs that block enzyme activity may also develop, although many ERTs, including elosulfase alfa, are not enzymatically active in the neutral pH of blood. Therefore, generation of NAbs that directly inhibit ERT activity has not been a primary concern in studies of ERTs (Brooks et al., 2003; Desnick & Schuchman, 2012) because the low pH and proteolytic environment of the lysosome is likely to dissociate and/or degrade antibodies. Instead, the focus of our surveillance was assessing the capacity of antibodies to interfere with cellular uptake of elosulfase alfa.

In this study, we analyzed MOR-004 serum samples using a validated cell-based NAb flow cytometry assay with a titer step. Compared with the previously used CI-M6PR binding assay, the format of this cell-based assay measures interference with cellular uptake of elosulfase alfa. This assay format was anticipated to demonstrate greater physiological relevance to the in situ environment due to the interaction of NAb-drug complexes with functional CI-M6PR on living human cells. In contrast, our previously reported in vitro CI-M6PR binding assay measures the interaction of biotin-labeled rhGALNS with bovine-derived CI-M6PR immobilized on an ELISA plate (Schweighardt et al., 2015). For the functional NAb assay, human T-cell lymphoma Jurkat cells were selected for their native expression of CI-M6PR, which is also expressed on primary human T cells (Wood & Hulett, 2008; Motyka et al., 2000).

Several experiments in the present study support the in vivo relevance of this assay format. Experiments with cytochalasin B, trypan blue, and incubation of cells at 4 °C indicate that nearly all Alexa488-rhGALNS fluorescence signal resulted from internalized drug (i.e. drug

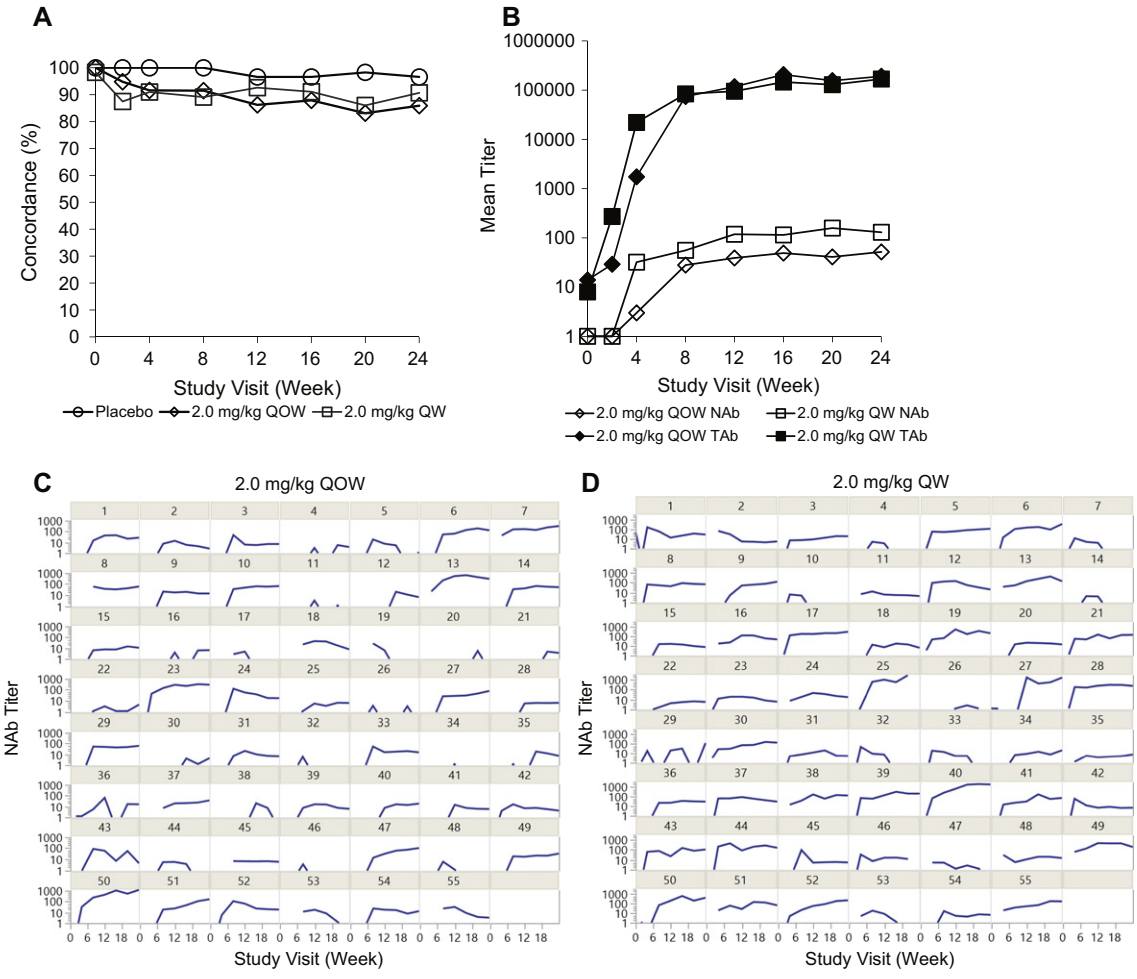

Fig. 7. NAb assay concordance and titers in individuals treated with elosulfase alfa in the MOR-004 study. (A) Concordance between NAb positivity detected with the in vitro CI-M6PR binding assay and the cell-based functional NAb assay in samples from individuals treated with placebo, elosulfase alfa 2.0 mg/kg QOW, or elosulfase alfa 2.0 mg/kg QW. (B) Mean TAb and NAb titer (from cell-based functional NAb assay) over 24 weeks in samples from individuals treated with placebo, elosulfase alfa 2.0 mg/kg QOW, or elosulfase alfa 2.0 mg/kg QW. Individual NAb titers from subjects in the 2.0 mg/kg QOW (C) or 2.0 mg/kg QW (D) elosulfase alfa treatment groups over 24 weeks.

Table 5
Concordance of NAb assay results by study week. Ligand receptor binding NAb and cell-based uptake NAb assay positive (+) and negative (−) values are shown with percentages in parentheses.

Treatment group	Study week	Receptor binding (−) Cellular uptake (−)	Receptor binding (+) Cellular uptake (+)	Receptor binding (−) Cellular uptake (+)	Receptor binding (+) Cellular uptake (−)
Placebo	Baseline	59(100.0)			
	2	59(100.0)			
	4	58(100.0)			
	8	57(100.0)			
	12	56(94.9)	1(1.7)		2(3.4)
	16	57(96.6)			2(3.4)
	20	58(98.3)			1(1.7)
	24	57(96.6)			2(3.4)
2.0 mg/kg/QOW	Baseline	59(100.0)			
	2	54(93.1)	1(1.7)		3(5.2)
	4	47(79.7)	7(11.9)		5(8.5)
	8	14(23.7)	40(67.8)	3(5.1)	2(3.4)
	12	7(12.1)	43(74.1)	3(5.2)	5(8.6)
	16	9(15.5)	42(72.4)		7(12.1)
	20	5(8.5)	44(74.6)	3(5.1)	7(11.9)
	24	8(14.0)	41(71.9)	2(3.5)	6(10.5)
2.0 mg/kg/QW	Baseline	57(98.3)		1(1.7)	
	2	45(80.4)	4(7.1)	4(7.1)	3(5.4)
	4	12(21.4)	39(69.6)	1(1.8)	4(7.1)
	8	2(3.6)	47(85.5)	3(5.5)	3(5.5)
	12	3(5.6)	47(87.0)	4(7.4)	
	16	2(3.6)	49(87.5)		5(8.9)
	20	3(5.3)	46(80.7)	1(1.8)	7(12.3)
	24	6(11.1)	43(79.6)	1(1.9)	4(7.4)

not bound to the cell surface). Confocal imaging of cells incubated with Alexa488-rhGALNS and LysoTracker Red, a lysosome-specific pH-sensitive dye, demonstrated that the drug was trafficked to the lysosome within the timeframe of the assay. Internalization by CI-M6PR appears to be the sole mechanism of Alexa488-rhGALNS entry in Jurkat cells, because competition with sCI-M6PR or M6P completely blocked Alexa488-rhGALNS uptake. Because most ERTs traffic to lysosomes via CI-M6PR, this assay platform has the potential for broad application in the study of NAbs to ERTs.

Serum samples collected from individuals with Morquio A syndrome who received elosulfase alfa intravenously at 2.0 mg/kg QOW or QW in the MOR-004 phase 3 clinical study were previously assessed as positive or negative for neutralizing antibodies, without titers, using an in vitro CI-M6PR binding ELISA assay (Schweighardt et al., 2015). Despite the differences between this in vitro assay format and the cell-based format presented here, results were highly concordant with >89% testing either positive or negative in both assays. Preliminary analysis of the minority of samples that differed between the assays showed that the results of ≥90% of discordant samples were within 30% of the SCP, CCP, or both. Therefore, at least some of the discordant data may be attributable to analytical variability associated with testing samples with relatively low positivity.

An analysis of the potential impacts of NAb on efficacy was performed using the percentage of study visits with a positive NAb result. Confirming previous results (Schweighardt et al., 2015), no associations were found between the number of occasions an individual tested positive for receptor binding NAb and changes in efficacy responses from baseline to week 24 in 6MWT distance or changes in the pharmacodynamic marker uKS. NAb titers derived from the new cell-based assay were also not correlated with efficacy outcomes in either dose group, measured by changes in 6MWT or uKS from baseline to week 24. We previously reported that NAb positivity, but not TAb titer, was associated with a decreased rate of CL from serum and prolonged $t_{1/2}$ in individuals receiving a weekly dose of elosulfase alfa (Qi et al., 2014). In this study, we found that increased NAb titers also correlated with decreased plasma clearance rates and increased $t_{1/2}$ compared with

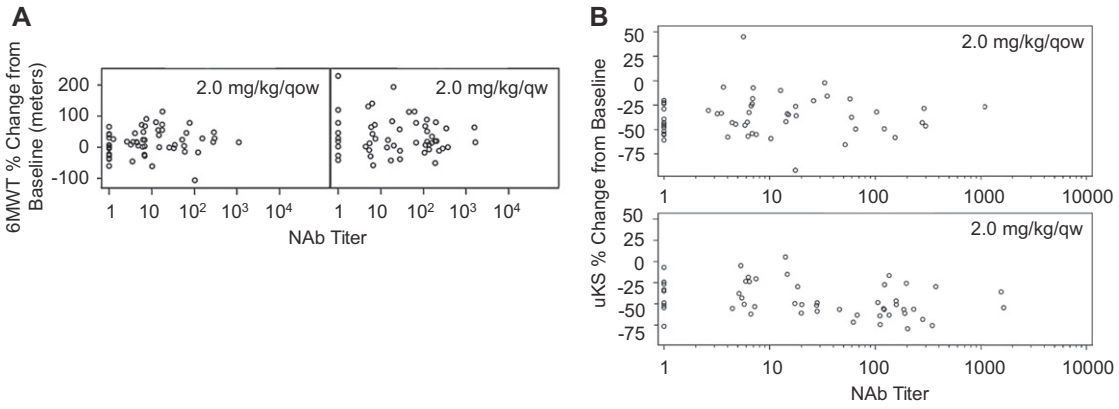

Fig. 8. Efficacy and NAb titers for individuals receiving elosulfase alfa 2.0 mg/kg QOW or QW in the MOR-004 study. (A) Change in 6MWT distance from baseline to week 24 plotted against NAb titer in the QOW (left) and QW (right) dose cohorts. (B) Percentage change from baseline to week 24 in uKS levels plotted against NAb titer in the QOW (top) and QW (bottom) dose cohorts.

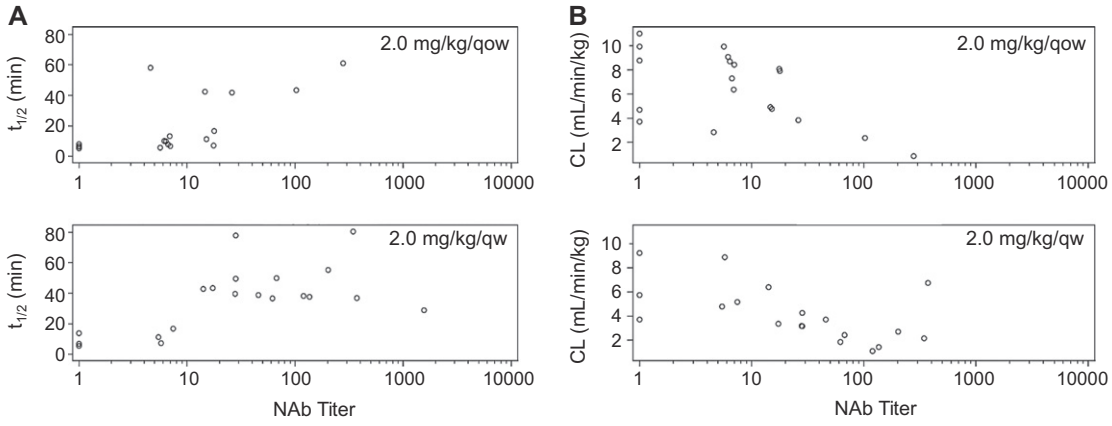

Fig. 9. NAb titer correlation with elosulfase alfa half-life and clearance in individuals in the MOR-004 study. (A) Serum half-life ($t_{1/2}$; min) of elosulfase alfa at week 22 is plotted against week 24 NAb titer (log scale) in the 2.0 mg/kg QOW (top panel) and QW (bottom panel) dose groups. (B) Elosulfase alfa clearance (CL; mL/min/kg) from serum at week 22 is plotted against week 24 NAb titer (log scale) in the 2.0 mg/kg QOW (top) and QW (bottom) dose groups.

lower NAb titers. However, higher NAb titers were not associated with a less robust pharmacodynamic effect, as measured by change in uKS levels from baseline to week 24. Results from a neutralizing antibody assay would ideally correlate with an effect on drug efficacy that could inform physicians of a developing immune response that would impact the patient's ability to benefit from the drug. While this study was designed to monitor antibodies that neutralize cellular uptake of drug, it is possible that an immune response to elosulfase alfa may develop in some individuals that exerts biological effects not measured in this assay. Future work on neutralizing antibody assays to ERTs should continue to be refined so that all circumstances are monitored for identifying antibodies capable of neutralizing the clinical effect of the drug.

Our finding that NAb titers had no correlation with elosulfase alfa efficacy contributes to an emerging body of work on the relationship between ERT immunogenicity and drug activity (Banugaria et al., 2011; Benichou et al., 2009; Brands et al., 2013; Jameson et al., 2013). The bulk of publications on this topic come from studies on the immunogenicity of patients with infantile-onset Pompe disease following treatment with alglucosidase alfa. Patients with Pompe disease may be classified as either positive or negative for cross-reactive immunological material (CRIM), depending on whether they express immunologically detectable levels of acid alpha-glucosidase protein. A negative CRIM status has been reported to correlate with higher antibody titers and worse therapeutic outcomes (Kishnani et al., 2010); however, one recent study of 11 patients showed no correlation between antidrug antibody titers and CRIM status (van Gelder et al., 2013). Another study of patients with MPS II (Hunter syndrome) treated with idursulfase found that those with nonsense or frameshift mutations were more likely to develop antibodies to idursulfase, but antibody positivity had no association with improvements in the 6MWT distance, percent predicted forced vital capacity, or liver and spleen volume (Barbier et al., 2013). Although the CRIM status of individuals with Morquio A syndrome was not assessed in the MOR-004 study, the lack of any relationship between antibody positivity, antibody type (total antibodies vs neutralizing), or antibody titers with efficacy outcomes indicates that elosulfase alfa maintains therapeutic activity in the presence of an antibody response. Subjects may also develop immunologic tolerance to repeated dosing of elosulfase alfa. Indeed, for subjects participating in the MOR-005 long-term extension study, the proportion of patients with NAb positivity decreased between week 36 and week 120 (Long et al., submitted for publication.).

Antibodies that neutralize elosulfase alfa uptake through CI-M6PR may not exist at concentrations sufficient to prevent effective amounts of elosulfase alfa from reaching target tissues. Although NAbs that block uptake of drug are measureable in our cell-based NAb assay, this amount of NAbs may be insignificant relative to elosulfase alfa dose. This cell-based assay was designed to detect the lowest possible NAb concentrations, ideally prior to observed clinical effects. Of note, the plasma Cmax for elosulfase alfa (4.0 μg/mL for 2.0 mg/kg QW and 2.6 μg/mL for 2.0 mg/kg QOW groups after 22 weeks) is much higher than concentrations used in the assay (0.4 μg/mL Alexa488-labeled elosulfase alfa) (Qi et al., 2014). While the titer and affinity of the assay positive control antibody may not be comparable to the antibody response in study subjects, the low and stable titers (mean titer $= 118 \pm 271$ at 12 weeks) support the hypothesis that NAbs may not be present in sufficient quantities to impact elosulfase alfa efficacy. Moreover, elosulfase alfa is rapidly taken into cells and is cleared from plasma with a half-life ($t_{1/2}$) of 19 or 36 min for the 2.0 mg/kg QOW or 2.0 mg/kg QW groups after 22 weeks, respectively (Qi et al., 2014). Although further investigation is needed, one aspect to the lack of neutralizing antibody effect on efficacy may be that rapid clearance of drug from plasma diminishes the rate of neutralizing antibody-elosulfase alfa complex formation. For other biologic therapies where NAb have been shown to impact efficacy, such as interferon-β for treatment of multiple sclerosis, administration of higher concentrations of drug has been shown to overcome NAb and restore efficacy (Millonig et al., 2009).

Another possible explanation for the lack of correlation between NAbs and efficacy is that elosulfase alfa may be taken up as NAb-drug complexes through binding of the Fc portion of NAbs by Fc receptor-expressing cells independently of CI-M6PR. The role of Fc receptor-expressing cells, such as monocytes and macrophages, in Morquio A syndrome is poorly understood, although it is possible these cells participate in the clearance or regulation of GAG (Dvorak-Ewell et al., 2010). Uptake of elosulfase alfa through NAb complexes could result in GAG clearance within these cell types or otherwise improve the function of these cells previously impaired by accumulated GAG. Further investigation into the role of Fc receptor-expressing cells in Morquio A syndrome will shed light on this possibility.

5. Conclusion

The development of a novel cell-based functional assay for analyzing the inhibition of elosulfase alfa receptor binding and internalization confirmed the presence of low NAb titers in the serum of 98% of individuals with Morquio A syndrome treated with elosulfase alfa. The presence of NAbs at these titers was insufficient to negatively impact efficacy or pharmacodynamic effects observed in the MOR-004 study.

Conflicts of interest

All authors are employees and shareholders of BioMarin Pharmaceutical Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2016.10.006.

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